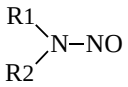
Overview information for

NNN (Emission)

DIALKYLNITROSAMINES

CAS Registry Number: See Table 2

Molecular Formula: See Table 2



N-Nitrosamines
R_1 and R_2 = organic groups

Dialkylnitrosamines include a group of chemicals of the form R_1R_2NNO, where R_1 and R_2 are alkyl groups such as methane, ethane, alcohols, or other organic groups. Table 1 provides a partial list of dialkylnitrosamines. Many of these compounds occur as yellow liquids or crystals. For specific information on either n-nitrosodimethylamine, N-nitroso-N-methylurea or N-nitrosomorpholine, please refer to their corresponding summary sheets.

Table 1
Partial List of Dialkylnitrosamines

Synonyms: N-Nitrosamines; N-Nitroso compounds

Examples:	
N-Nitrosodiethylamine	N-Nitrososarcosine
N-Nitrosodiethanolamine	N-Nitrosodimethylamine
N-Nitrosodiphenylamine	N-Nitrosodi-n-propylamine
N-Nitroso-N-ethylurea	N-Nitroso-N-methylurea
N-Nitroso-N-methylurethane	N-Nitrosomethylvinylamine
N-Nitrosomorpholine	N-Nitrosonornicotine
N-Nitrosopiperidine	N-Nitrosopyrrolidine
3-(N-Nitrosomethylamino)propionitrile	N-Nitrosomethylethylamine

(CCR, 1994; IARC, 1987; U.S. EPA, 1994a)

SOURCES AND EMISSIONS

A. Sources

Many dialkylnitrosamines are used in research, while only a few have commercial uses. N-nitrosodimethylamine is used as an industrial solvent, a rubber accelerator, in the production of rocket fuel, and as a nematicide. N-nitrosodiethylamine is used as a gasoline and lubricant additive, an antioxidant, and a stabilizer (HSDB, 1993). A number of N-nitroso compounds have

been found in tobacco smoke (NTP, 1994a).

The primary stationary sources that has reported emissions of dialkylnitrosamines in California are manufacturers of guided missiles and space vehicles and parts (ARB, 1997b).

B. Emissions

Only trace emissions of N-nitrosodiethylamine and the general category of dialkylnitrosamines were reported from stationary sources in California. The total emissions of N-nitrosodimethylamine from stationary sources in California are estimated to be less than 1 pound per year, based on data reported under the Air Toxics "Hot Spots" Program (AB 2588) (ARB, 1997b).

C. Natural Occurrence

Dialkylnitrosamines have been found in a variety of vegetables, fruits, cheeses, meats, and alcoholic beverages (NTP, 1994a). Additionally, dialkylnitrosamines may be formed in the gastrointestinal tract of humans and animals by ingestion of nitrite and nitrate containing foods (IARC, 1980).

AMBIENT CONCENTRATIONS

No Air Resources Board data exist for ambient measurements of dialkylnitrosamines. However, the United States Environmental Protection Agency (U.S. EPA) has compiled ambient data for n-nitrosodimethylamine during 1975 from several locations in the United States. The overall mean concentration was 2.4 micrograms per cubic meter (U.S. EPA, 1993a).

INDOOR SOURCES AND CONCENTRATIONS

A major source of N-nitroso compounds in indoor air is environmental tobacco smoke (ETS). ETS has been found to contain N-nitrosodimethylamine, N-nitrosodiethylamine, N-nitrosopyrrolidine, N-nitrosonornicotine, N-nitrosodiethanolamine and other N-nitroso compounds (Guerin et al., 1992). ETS emissions of four N-nitroso compounds were measured from six cigarette brands popular in California and one reference cigarette. N-nitrosodimethylamine had an average emission of 0.57 micrograms per cigarette (μg/cigarette). N-nitrosopyrrolidine had an average emission of 0.10 μg/cigarette. Emissions for N-nitrosodiethylamine and N-nitrosomorpholine were below the detection limit of 0.02 μg/cigarette (Daisey et al., 1994). Concentrations of N-nitroso compounds have not been measured in any statistically selected sample of residences in California or the United States (Hodgson and Wooley, 1991).

ATMOSPHERIC PERSISTENCE

The atmospheric persistence of N-nitrosodimethylamine and N-nitrosomorpholine are discussed under the summary sheets for these compounds. In general, this class of compounds exist in the gas phase and their dominant tropospheric loss process is by photolysis to form NO plus the dialkylamino radical (Atkinson, 1995).

AB 2588 RISK ASSESSMENT INFORMATION

Although dialkylnitrosamines are reported as being emitted in California from stationary sources, no health values (cancer or non-cancer) are listed in the California Air Pollution Control Officers Association Air Toxics "Hot Spots" Program Revised 1992 Risk Assessment Guidelines for use in risk assessments (CAPCOA, 1993). For information on either N-nitrosodimethylamine, N-nitroso-n-methylurea, or N-nitrosomorpholine, please refer to their corresponding summary sheets.

HEALTH EFFECTS

Probable routes of human exposure to dialkylnitrosamines are inhalation, ingestion, and dermal contact (NTP, 1994a).

Non-Cancer: Little information is available on the acute and chronic effects of dialkylnitrosamines. Large doses of dimethylnitrosamine are known to cause hepatotoxicity in humans (Amdur, 1993).

Cancer: Many dialkynitrosamines are potent carcinogens. Members of the class such as N-nitrosodimethylamine, N-nitrosodiethylamine and dibutylnitrosamine have been found to cause cancer in rats and mice. The alkyl substituents appear to determine in part the site of the cancer, at least in rats. The diethyl and dimethyl derivatives cause liver cancer in rats. In mice, liver neoplasms are seen with these compounds. Dibutylnitrosamine causes cancer of the urinary bladder in rats (Amdur, 1993).

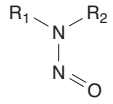
The most recent cancer rankings and potency values available for specific dialkylnitrosamines are listed in Table 2.

Table 2 - Dialkylnitrosamines Cancer Information

Compound	CAS Number	Proposition 65	OEHHA Potency Value	U.S. EPA Ranking	IARC Ranking
N-Nitrosodi-n-butylamine	924-16-3	Carcinogen	3.1×10^{-3}		2B: Possible
N-Nitrosodiethanolamine	1116-54-7	Carcinogen	8.0×10^{-4}		2B: Possible
N-Nitrosodiethylamine	55-18-5	Carcinogen	1.0×10^{-2}		2A: Probable
N-Nitrosodimethylamine	62-75-9	Carcinogen	4.6×10^{-3}	B2: Probable	2A: Probable
N-Nitrosodiphenylamine	86-30-6	Carcinogen	2.6×10^{-6}		3: Not Classifiable
N-Nitrosodi-n-propylamine	621-64-7	Carcinogen	2.0×10^{-3}		2B: Possible
N-Nitroso-N-ethylurea	759-73-9	Carcinogen	7.7×10^{-3}		2A: Probable
3-(N-Nitrosomethylamino)propionitrile	60153-49-3	Carcinogen			2B: Possible
4-(N-Nitrosomethylamino)-1-(3-pyridyl)1-butanone	64091-91-4	Carcinogen			2B: Possible
N-Nitrosomethylethylamine	10595-95-6	Carcinogen	6.3×10^{-3}		2B: Possible
N-Nitroso-N-methylurea	684-93-5	Carcinogen	3.4×10^{-2}	B2: Probable	2A: Probable
N-Nitroso-N-methylurethane	615-53-2	Carcinogen	3.1×10^{-2}		2B: Possible
N-Nitrosomethylvinylamine	4549-40-0	Carcinogen			2B: Possible
N-Nitrosomorpholine	59-89-2	Carcinogen	1.9×10^{-3}		2B: Possible
N-Nitrosonornicotine	16543-55-8	Carcinogen	4.0×10^{-4}		2B: Possible
N-Nitrosopiperidine	100-75-4	Carcinogen	2.7×10^{-3}		2B: Possible
N-Nitrosopyrrolidine	930-55-2	Carcinogen	6.0×10^{-4}		2B: Possible
N-Nitrososarcosine	13256-22-9	Carcinogen			2B: Possible

References: CCR, 1994; IARC, 1987; U.S. EPA, 1994a

N-Nitrosamines: 15 Listings



Generic *N*-nitrosamine structure

N-Nitrosamines are a class of chemical compounds with the general structure shown above. The essential feature of *N*-nitroso compounds is the N–N=O structure; the R_1 and R_2 groups attached to the amine nitrogen may range from a simple hydrogen (H) atom to more complex chemical substituents (including ring structures that incorporate the nitrogen atom), as shown in the structures of the individual nitrosamines listed below.

Human exposure to nitrosamines can result from formation of *N*-nitroso compounds either in food during storage or preparation or *in vivo*, usually in the stomach (Mirvish 1975). Individual nitrosamines are not found in isolation, but occur in mixtures of various nitrosamines. Nitrosamines or their precursors occur in a wide variety of foods and manufactured and natural products, such as agricultural chemicals, tobacco, detergents, rust inhibitors, cutting fluids, rubber additives, solvents, drugs, plastics, tanned leather products, textiles, and cosmetics (ATSDR 1989). Nitrosamines generally are not intentionally added to foods or consumer products, but are formed from constituents of the foods or products that are either naturally present, such as the amines that are part of the structure of proteins in meat, or added during production (e.g., nitrates or nitrites added to meats as preservatives). Nitrosamines are formed when nitrites, which can be formed from nitrates, react with a secondary or tertiary amine. The concentration of nitrosamines tends to increase over time, and their formation is enhanced by high temperatures, such as occur while frying food, and high acidity, such as in stomach acid. Ascorbic acid or its isomers inhibit the formation of nitrosamines and often are added to food preparations to prevent nitrosamine formation.

Although food and tobacco products are important sources of external exposure to *N*-nitrosamines, exposure also occurs from nitrosamines produced internally in the digestive tract (Hotchkiss 1989). About 5% of ingested nitrates are reduced to nitrites in saliva (NRC 1995). These nitrites can subsequently react in solution with secondary and tertiary amines, as well as N-substituted amides, carbamates, and other related compounds, to form *N*-nitroso compounds within the gastrointestinal tract (Mirvish 1975, Hotchkiss 1989). This internal formation is a major source of human exposure to *N*-nitrosamines.

The listings below are for the individual *N*-nitrosamines and do not constitute a listing for *N*-nitrosamines compounds as a class.

References

ATSDR. 1989. *Toxicological Profile for* N-*Nitrosodimethylamine (Final Report)*. Atlanta, GA: Agency for Toxic Substances and Disease Registry. 132 pp.

Hotchkiss JH. 1989. Preformed *N*-nitroso compounds in foods and beverages. *Cancer Surv* 8(2): 295-321.

Mirvish SS. 1975. Formation of *N*-nitroso compounds: chemistry, kinetics, and *in vivo* occurrence. *Toxicol Appl Pharmacol* 31:325-351.

NRC. 1995. *Nitrate and Nitrite in the Drinking Water*. National Research Council of the National Academies of Science. Washington, DC: National Academies Press. 64 pp.

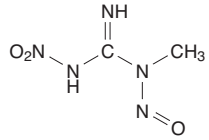
N-Methyl-*N′*-nitro-*N*-nitrosoguanidine

CAS No. 70-25-7

Reasonably anticipated to be a human carcinogen

First listed in the *Sixth Annual Report on Carcinogens* (1991)

Also known as MNNG or 1-methyl-3-nitro-1-nitrosoguanidine



Carcinogenicity

N-Methyl-*N′*-nitro-*N*-nitrosoguanidine (MNNG) is *reasonably anticipated to be a human carcinogen* based on sufficient evidence of carcinogenicity from studies in experimental animals.

Cancer Studies in Experimental Animals

MNNG caused tumors in several species of experimental animals, at several different tissue sites, and by several different routes of exposure. It caused tumors primarily at the site of administration, mostly in the gastrointestinal tract, including tumors of the forestomach (papilloma or carcinoma), glandular stomach (adenoma, adenocarcinoma, carcinoma, or sarcoma), small intestine (papilloma, carcinoma, adenocarcinoma, or sarcoma), and large intestine (adenomatous polyps or polypoid carcinoma). Tumors of the forestomach or glandular stomach were observed in rats exposed to MNNG in the drinking water, by stomach tube, and by intraperitoneal injection; in mice exposed by stomach tube; and in male hamsters and dogs exposed via the drinking water. MNNG also caused tumors of the large intestine in rats exposed by intrarectal instillation. It caused tumors of the small intestine in rats exposed via the drinking water, subcutaneous injection, or intraperitoneal injection and in mice exposed by intraperitoneal injection (IARC 1974, 1987).

In addition, MNNG caused tumors of the liver and peritoneum in rats exposed orally (by stomach tube or drinking water) and injection-site tumors (fibrosarcoma and rhabdomyosarcoma) in rats exposed by subcutaneous injection. In mice, MNNG administered by subcutaneous injection caused benign tumors of the liver, lung, and blood vessels (hemangoendothelioma) and by dermal application caused benign and malignant skin tumors (papilloma and carcinoma) (IARC 1974, 1987).

Cancer Studies in Humans

The data available from epidemiological studies are inadequate to evaluate the relationship between human cancer and exposure specifically to MNNG. Three deaths from brain tumors (glioma) and one death from colon cancer were reported among workers in a genetics laboratory over a 13-year period. All of the subjects had probably been exposed to MNNG for 6 to 15 years prior to death, but other carcinogens had also been used in the laboratory (IARC 1974, 1987).

Properties

MNNG is an *N*-nitrosamine alkylating agent that exists as a yellow crystal at room temperature and is soluble in water, dimethyl sulfoxide, and polar organic solvents. It reacts violently with water and can explode on heating or high impact. MNNG reacts with various nucleophiles, especially amines (Akron 2009, HSDB 2009). At low pH, it slowly releases nitrous acid, and at high pH in the presence of hydroxyl alkali, it produces the highly toxic gas diazomethane. When MNNG is heated to decomposition, it emits highly toxic fumes of

nitrogen oxides (IARC 1974, HSDB 2009). Physical and chemical properties of MNNG are listed in the following table.

Property	Information
Molecular weight	147.1[a]
Melting point	118°C to 123.5°C (with decomposition)[a]
Log K_{ow}	–0.92[b]
Water solubility	267 g/L at 25°C[b]
Vapor pressure	0.00012 mm Hg at 25°C[b]

Sources: [a]HSDB 2009, [b]ChemIDplus 2009.

Use

In the 1940s and 1950s, MNNG was used to prepare diazomethane. It currently is used as a research chemical and has no known commercial use (IARC 1974, HSDB 2009).

Production

MNNG is not produced commercially. In 2009, it was available in small quantities for research purposes from seven suppliers worldwide, including five U.S. suppliers (ChemSources 2009). The United States International Trade Commission does not provide specific information for imports and exports of MNNG. Import and export data are reported for U.S. Census Bureau Schedule B category 29.29, which includes MNNG and related compounds (USCB 2018).

Exposure

The extent of exposure to MNNG is unknown, but it is probably limited to scientists using it as a research chemical (IARC 1974, HSDB 2009). The National Occupational Exposure Survey (conducted from 1981 to 1983) estimated that 523 workers potentially were exposed to MNNG (NIOSH 1990).

Regulations

Consumer Product Safety Commission (CPSC)

A voluntary standard provides that rubber pacifiers shall not contain more than 10 ppb of any single nitrosamine or more than 20 ppb of total nitrosamines.

Environmental Protection Agency (EPA)

Clean Water Act

Effluent Guidelines: Nitrosamines are listed as a toxic pollutant.

Water Quality Criteria: Based on fish or shellfish and water consumption = 0.0008 µg/L for nitrosamines; based on fish or shellfish consumption only = 1.24 µg/L for nitrosamines.

Comprehensive Environmental Response, Compensation, and Liability Act

Reportable quantity (RQ) = 10 lb.

Resource Conservation and Recovery Act

Listed Hazardous Waste: Waste code for which the listing is based wholly or partly on the presence of MNNG = U163.

Listed as a hazardous constituent of waste.

Toxic Substances Control Act

Nitrosating agents distributed in commerce require warning labels and instructions on use.

Food and Drug Administration (FDA, an HHS agency)

The action level for *N*-nitrosamines in rubber baby-bottle nipples is 10 ppb.

In order to use nitrites and/or nitrates as food additives in curing premixes, a petition must be filed supported by data demonstrating that nitrosamines are not formed.

References

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ChemIDplus. 2009. *ChemIDplus Advanced*. National Library of Medicine. http://chem.sis.nlm.nih.gov/chemidplus and select Registry Number and search on CAS number. Last accessed: 6/4/09.

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IARC. 1974. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine. In *Some Aromatic Amines, Hydrazine and Related Substances,* N-*Nitroso Compounds and Miscellaneous Alkylating Agents*. IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Humans, vol. 4. Lyon, France: International Agency for Research on Cancer. pp. 183-195.

IARC. 1987. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG). In *Overall Evaluations of Carcinogenicity*. IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Humans, suppl. 7. Lyon, France: International Agency for Research on Cancer. pp. 248-250.

NIOSH. 1990. *National Occupational Exposure Survey (1981-83)*. National Institute for Occupational Safety and Health. Last updated: 7/1/90. http://www.cdc.gov/noes/noes1/b0070sic.html.

USCB. 2018. Chapter 29 - Organic Chemicals. In *U.S. Census Bureau - Foreign Trade Schedule B (2018)*. https://uscensus.prod.3ceonline.com and search on CAS number or substance name. Last accessed: 10/10/18.

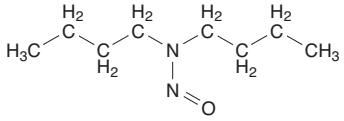
N-Nitrosodi-*n*-butylamine

CAS No. 924-16-3

Reasonably anticipated to be a human carcinogen

First listed in the *Second Annual Report on Carcinogens* (1981)

Also known as *N*-dibutylnitrosamine



Carcinogenicity

N-Nitrosodi-*n*-butylamine is reasonably anticipated to be a human carcinogen based on sufficient evidence of carcinogenicity from studies in experimental animals.

Cancer Studies in Experimental Animals

N-Nitrosodi-*n*-butylamine caused tumors in several species of experimental animals, at several different tissue sites, and by several different routes of exposure. It was carcinogenic after a single dose, and was particularly effective as a urinary-bladder carcinogen, causing benign and/or malignant urinary-bladder tumors (papilloma or squamous- or transitional-cell carcinoma) in mice, rats, hamsters, and guinea pigs exposed orally and in mice, rats, hamsters, and rabbits exposed by subcutaneous injection (IARC 1974, 1978).

N-Nitrosodi-*n*-butylamine also caused tumors of the respiratory tract following oral or prenatal exposure in hamsters; subcutaneous injection in rats, hamsters, and adult and newborn mice; and intraperitoneal injection in hamsters of both sexes. Benign or malignant liver tumors were observed in mice, rats, and guinea pigs exposed orally and in newborn mice exposed by subcutaneous injection. Tumors of the upper digestive tract (pharynx, esophagus, or forestomach) occurred following oral exposure in mice, rats, and hamsters and subcutaneous injection in rats (esophagus) and hamsters (forestomach). Intravenous injection of *N*-nitrosodi-*n*-butylamine caused leukemia in mice of both sexes (IARC 1974, 1978).

Since *N*-nitrosodi-*n*-butylamine was listed in the *Second Annual Report on Carcinogens,* an additional study in rats has been identified. Administration of *N*-nitrosodi-*n*-butylamine to male rats by stomach tube caused cancer of the forestomach (carcinoma), in addition to cancer of the liver (carcinoma) and urinary bladder (transitional-cell carcinoma) (Lijinsky and Reuber 1983).

Cancer Studies in Humans

No epidemiological studies were identified that evaluated the relationship between human cancer and exposure specifically to *N*-nitrosodi-*n*-butylamine.

Properties

N-Nitrosodi-*n*-butylamine is a nitrosamine compound that is a yellow oil at room temperature (HSDB 2009). It is slightly soluble in water and soluble in vegetable oils and organic solvents. It is stable in the dark in neutral or alkaline solution for at least 14 days, but is less stable in more acidic solutions or in light, especially ultraviolet light (IARC 1978). Physical and chemical properties of *N*-nitrosodi-*n*-butylamine are listed in the following table.

Property	Information
Molecular weight	158.2[a]
Specific gravity	0.9009 at 20°C/4°C[a]
Melting point	< 25°C[b]
Boiling point	116°C at 14 mm Hg[a]
Log K_{ow}	2.63[a]
Water solubility	1.27 g/L at 24°C[b]
Vapor pressure	0.05 mm Hg at 25°C[b]

Sources: [a]HSDB 2009, [b]ChemIDplus 2009.

Use

N-Nitrosodi-*n*-butylamine is used primarily as a research chemical (IARC 1974). It has also been used as an intermediate in the synthesis of di-*n*-butylhydrazine.

Production

N-Nitrosodi-*n*-butylamine is not produced commercially in the United States (HSDB 2009). In 2009, it was available in small quantities for research purposes from seven U.S. suppliers (ChemSources 2009).

Exposure

The routes of potential human exposure to *N*-nitrosodi-*n*-butylamine are ingestion, inhalation, and dermal contact (HSDB 2009). *N*-Nitrosodi-*n*-butylamine has been detected in a variety of products as a result of the nitrosation of amines present in these products. *N*-Nitrosodi-*n*-butylamine may be formed from secondary or tertiary *n*-butylamines and quaternary ammonium salts by reaction with nitrosating agents, such as nitrite, in the stomach or during cooking processes. The degree of this potential exposure is unknown. *N*-Nitrosodi-*n*-butylamine has been measured in soybean oil at a concentration of 290 µg/kg, in cheese at 20 to 30 µg/kg, and in smoked or cured meats at up to 3.9 µg/kg. It has also been detected in tobacco smoke at a concentration of 3 ng per cigarette. *N*-Nitrosamines frequently are produced during rubber processing and may be present as contaminants in the final rubber product. Potential exposure depends on the ability of the nitrosamine to migrate from the product into the body. Nitrosamines present in pacifiers and baby-bottle nipples can migrate into saliva, which could result in ingestion of nitrosamines (IARC 1974, 1978).

According to the U.S. Environmental Protection Agency's Toxics Release Inventory, all environmental releases of *N*-nitrosodi-*n*-butylamine since 1998 have been to landfills. Annual releases did not exceed 15 lb from 1998 through 2000 or in 2004, but were 4,510 lb in 2001. In 2007, one facility released 500 lb of *N*-nitrosodi-*n*-butylamine to an off-site hazardous-waste landfill (TRI 2009). The estimated half-life of *N*-nitrosodi-*n*-butylamine in the vapor phase is 2.8 days. *N*-Nitrosodi-*n*-butylamine was detected in the effluent water from a coke facility at a concentration of 0.82 µg/L (IARC 1978).

Occupational exposure potentially could occur among researchers studying the biological effects of *N*-nitrosodi-*n*-butylamine.

Regulations

Consumer Product Safety Commission (CPSC)

A voluntary standard provides that rubber pacifiers shall not contain more than 10 ppb of any single nitrosamine or more than 20 ppb of total nitrosamines.

Environmental Protection Agency (EPA)

Clean Water Act

Effluent Guidelines: Nitrosamines are listed as a toxic pollutant.
Water Quality Criteria: Based on fish or shellfish and water consumption = 0.0063 µg/L; based on fish or shellfish consumption only = 0.22 µg/L.

Comprehensive Environmental Response, Compensation, and Liability Act

Reportable quantity (RQ) = 10 lb.

Emergency Planning and Community Right-To-Know Act

Toxics Release Inventory: Listed substance subject to reporting requirements.

Resource Conservation and Recovery Act

Listed Hazardous Waste: Waste code for which listing is based wholly or partly on the presence of *N*-nitrosodi-*n*-butylamine = U172.
Listed as a hazardous constituent of waste.

Toxic Substances Control Act

Nitrosating agents distributed in commerce require warning labels and instructions on use.

Food and Drug Administration (FDA, an HHS agency)

The action level for *N*-nitrosamines in rubber baby-bottle nipples is 10 ppb.
In order to use nitrites and/or nitrates as food additives in curing premixes, a petition must be filed supported by data demonstrating that nitrosamines are not formed.

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IARC. 1974. *N*-Nitrosodi-*n*-butylamine. In *Some Aromatic Amines, Hydrazine and Related Substances, N-Nitroso Compounds and Miscellaneous Alkylating Agents*. IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Humans, vol. 4. Lyon, France: International Agency for Research on Cancer. pp. 197-210.

IARC. 1978. *N*-Nitrosodi-*n*-butylamine. In *Some N-Nitroso Compounds*. IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans, vol. 17. Lyon, France: International Agency for Research on Cancer. pp. 51-75.

Lijinsky W, Reuber MD. 1983. Carcinogenicity of hydroxylated alkylnitrosoureas and of nitrosooxazolidones by mouse skin painting and by gavage in rats. *Cancer Res* 43(1): 214-221.

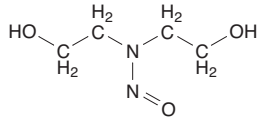
TRI. 2009. *TRI Explorer Chemical Report.* U.S. Environmental Protection Agency. Last updated: 3/19/09. http://www.epa.gov/triexplorer and select *N*-Nitrosodi-*n*-Butylamine.

N-Nitrosodiethanolamine

CAS No. 1116-54-7

Reasonably anticipated to be a human carcinogen

First listed in the *Second Annual Report on Carcinogens* (1981)



Carcinogenicity

N-Nitrosodiethanolamine is *reasonably anticipated to be a human carcinogen* based on sufficient evidence of carcinogenicity from studies in experimental animals.

Cancer Studies in Experimental Animals

N-Nitrosodiethanolamine caused tumors in two rodent species, at several different tissue sites, and by two different routes of exposure. Administration of *N*-nitrosodiethanolamine in the drinking water

caused liver cancer (hepatocellular carcinoma) and benign kidney tumors (adenoma) in rats of unspecified sex (IARC 1978). Subcutaneous injection of *N*-nitrosodiethanolamine caused cancer of the nasal cavity (adenocarcinoma) and at the injection site (fibrosarcoma) and benign tumors of the trachea (papilloma) and liver (hepatocellular adenoma) in hamsters of both sexes.

Since *N*-nitrosodiethanolamine was listed in the *Second Annual Report on Carcinogens,* additional studies in rodents have been identified. Studies in several strains of rats consistently reported that exposure to *N*-nitrosodiethanolamine in the drinking water caused liver cancer (primarily hepatocellular carcinoma, but also cholangiocellular carcinoma) in both sexes; some studies also found increased incidences of nasal-cavity cancer (adenocarcinoma and squamous-cell carcinoma). In female strain A/J mice (a strain with a high spontaneous incidence of lung tumors), administration of *N*-nitrosodiethanolamine in the drinking water increased the incidence of benign lung tumors and the number of tumors per animal. Tumors of the nasal cavity were observed in hamsters of both sexes exposed to *N*-nitrosodiethanolamine in several studies by subcutaneous injection and in one study by swabbing of the oral cavity (IARC 2000).

Cancer Studies in Humans

No epidemiological studies evaluating the relationship between human cancer and exposure specifically to *N*-nitrosodiethanolamine were available when it was listed in the *Second Annual Report on Carcinogens.* Since then, the International Agency for Research on Cancer (IARC 2000) concluded that there was inadequate evidence of the carcinogenicity of *N*-nitrosodiethanolamine from studies in humans. *N*-Nitrosodiethanolamine can be formed from ethanolamines and sodium nitrates, which are additives to soluble and synthetic metalworking fluids. In a review of studies of workers exposed to metalworking fluids, IARC noted increased cancer mortality or incidence among workers using fluids containing ethanolamines and sodium nitrates. One study found that esophageal cancer increased with increasing duration of exposure to nitrosamines as assessed by co-exposure to ethanolamines and sodium nitrate; however, the same workers were also exposed to biocides.

Properties

N-Nitrosodiethanolamine is a nitrosamine compound that exists at room temperature as a yellow oil with no distinctive odor (HSDB 2009). It is miscible in water and soluble in polar organic solvents, but insoluble in nonpolar organic solvents. It is stable in the dark in neutral or alkaline solution for at least 14 days, but is less stable in more acidic solutions or in light, especially ultraviolet light (IARC 1978, Akron 2009). Physical and chemical properties of *N*-nitrosodiethanolamine are listed in the following table.

Property	Information
Molecular weight	134.1[a]
Boiling point	114°C at 1.4 mm Hg[a]
Log K_{ow}	–1.28[b]
Water solubility	1,000 g/L[b]
Vapor pressure	0.0005 mm Hg at 20°C[b]

Sources: [a]HSDB 2009, [b]ChemIDplus 2009.

Use

N-Nitrosodiethanolamine is used primarily as a research chemical and has no known commercial uses (HSDB 2009).

Production

N-Nitrosodiethanolamine is not produced commercially in the United States (HSDB 2009). In 2009, it was available in small quantities for research purposes from 11 suppliers worldwide, including 8 U.S. suppliers (ChemSources 2009).

Exposure

The routes of potential human exposure to *N*-nitrosodiethanolamine are dermal contact, ingestion, and inhalation (HSDB 2009). *N*-Nitrosodiethanolamine is widespread in the environment. It is a known contaminant of cosmetics, lotions, shampoos, cutting fluids, certain pesticides, antifreeze, and tobacco at concentrations of up to 130 ppm (130,000 ppb) (IARC 2000). Nitrosamines are formed within these products by reactions of precursors (nitrosating agents and primary or secondary amines) or are introduced through the use of contaminated raw materials (Schothorst and Somers 2005).

As of 1980, the U.S. Food and Drug Administration had analyzed over 300 cosmetic products and found that over 40% were contaminated with *N*-nitrosodiethanolamine. It was detected in facial cosmetics at concentrations of 42 to 49,000 µg/kg, in lotions at up to 140 µg/kg, and in shampoos at up to 260 mg/kg (IARC 1978). Cosmetics at least five years old had higher concentrations of *N*-nitrosodiethanolamine than new samples of the same products, indicating that the formation of *N*-nitrosodiethanolamine limits the shelf-life cosmetic products (Matyska *et al.* 2000). *N*-Nitrosodiethanolamine was also measured in 35 of 140 soap and shampoo products at concentrations of 23 to 992 µg/kg (Schothorst and Somers 2005). *N*-Nitrosodiethanolamine was detected in cigarette smoke at concentrations of 24 to 36 ng per cigarette and in smokeless tobacco products at up to 6.8 µg/g (Brunnemann and Hoffmann 1981, Brunnemann *et al.* 1982). The presence of *N*-nitrosodiethanolamine in tobacco is attributed to the use of an herbicide, maleic hydrazide diethanolamine, commonly applied to tobacco (IARC 2000).

Occupational exposure to *N*-nitrosodiethanolamine could occur during the use of synthetic cutting fluids to reduce the temperature of the metal-tool interface during metal cutting or grinding. *N*-Nitrosodiethanolamine is present in most cutting fluids containing triethanolamine and sodium nitrite, at concentrations ranging from 0.02% to 3% (IARC 1978). In addition, an atrazine pesticide formulation emulsified with triethanolamine was reported to contain *N*-nitrosodiethanolamine at a concentration of 0.5 mg/kg (IARC 1978). In 1976, the National Institute for Occupational Safety and Health estimated that 780,000 workers potentially were exposed to cutting fluids during their manufacture and use (NIOSH 1976). In a study of factory workers directly exposed to metalworking fluids, the post-shift concentration of *N*-nitrosodiethanolamine in the urine of workers using the cutting fluids was highly correlated with the concentration of *N*-nitrosodiethanolamine in the cutting fluids; urinary concentrations were up to 277 µg/L in workers using "nitrate-formulated" fluids, compared with 2.7 µg/L in workers using "nitrate-free" fluids. When nitrite concentrations in cutting fluids were less than 20 mg/L, *N*-nitrosodiethanolamine levels in the fluids remained below 5 mg/L (Ducos *et al.* 1999, Ducos and Gaudin 2003).

Regulations

Consumer Product Safety Commission (CPSC)

A voluntary standard provides that rubber pacifiers shall not contain more than 10 ppb of any single nitrosamine or more than 20 ppb of total nitrosamines.

Environmental Protection Agency (EPA)

Clean Water Act

Effluent Guidelines: Nitrosamines are listed as a toxic pollutant.

Water Quality Criteria: Based on fish or shellfish and water consumption = 0.0008 µg/L for nitrosamines; based on fish or shellfish consumption only = 1.24 µg/L for nitrosamines.

Comprehensive Environmental Response, Compensation, and Liability Act

Reportable quantity (RQ) = 1 lb.

Resource Conservation and Recovery Act

Listed Hazardous Waste: Waste code for which the listing is based wholly or partly on the presence of *N*-nitrosodiethanolamine = U173.

Listed as a hazardous constituent of waste.

Toxic Substances Control Act

Nitrosating agents distributed in commerce require warning labels and instructions on use.

Food and Drug Administration (FDA, an HHS agency)

The action level for *N*-nitrosamines in rubber baby-bottle nipples is 10 ppb.

In order to use nitrites and/or nitrates as food additives in curing premixes, a petition must be filed supported by data demonstrating that nitrosamines are not formed.

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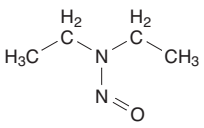
N-Nitrosodiethylamine

CAS No. 55-18-5

Reasonably anticipated to be a human carcinogen

First listed in the *Second Annual Report on Carcinogens* (1981)

Also known as diethylnitrosamine



Carcinogenicity

N-Nitrosodiethylamine is *reasonably anticipated to be a human carcinogen* based on sufficient evidence of carcinogenicity from studies in experimental animals.

Cancer Studies in Experimental Animals

N-Nitrosodiethylamine caused tumors in several species of experimental animals, at several different tissue sites, and by several different routes of exposure. It was carcinogenic in animals exposed perinatally and as adults, causing tumors mainly in the liver, respiratory tract, kidney, and upper digestive tract (IARC 1972, 1978).

Benign and malignant liver tumors occurred in mice, rats, hamsters, guinea pigs, rabbits, dogs, and pigs orally exposed to *N*-nitrosodiethylamine. Liver tumors also occurred in rats following inhalation exposure or rectal administration; in mice, rats, and hamsters following intraperitoneal injection; in hamsters, guinea pigs, gerbils, and hedgehogs following subcutaneous injection; in mice following prenatal exposure; in birds following intramuscular injection; and in fish and frogs exposed to *N*-nitrosodiethylamine in the tank water. In dogs, exposure to *N*-nitrosodiethylamine by stomach tube followed by subcutaneous injection caused cancer of the liver and nasal cavity (squamous-cell carcinoma).

Tumors of the lung and upper respiratory tract occurred in mice, rats, hamsters, dogs, and pigs following oral administration of *N*-nitrosodiethylamine. Inhalation exposure caused tumors of the trachea, bronchi, and lungs in hamsters, and dermal exposure caused tumors of the nasal cavity in mice and hamsters. Subcutaneous injection caused respiratory-tract tumors in adult and newborn mice and hamsters, in pregnant hamsters (benign tracheal tumors), and in adult guinea pigs, gerbils, and hedgehogs. Intraperitoneal injection caused lung tumors in mice and respiratory-tract tumors in hamsters and monkeys, and intravenous injection caused tumors of the nasal cavity in gerbils. Prenatal exposure caused benign lung tumors (adenoma) in mice and hamsters.

Tumors of the kidney occurred in rats following oral, intravenous, or prenatal administration of *N*-nitrosodiethylamine. Oral administration also caused kidney tumors in pigs and tumors of the upper digestive tract in mice, rats, and hamsters. Prenatal exposure caused benign and malignant tumors of the upper digestive tract in mice and tumors of the thymus (thymoma) and benign mammary-gland tumors (adenoma) in rats. One study reported hematopoietic-system tumors in frogs exposed to *N*-nitrosodiethylamine in the tank water.

Since *N*-nitrosodiethylamine was listed in the *Second Annual Report on Carcinogens,* additional studies in experimental animals have been identified. As in earlier studies, the most common tumor sites were the liver, kidney, digestive tract, and respiratory tract. However, some of these studies reported that *N*-nitrosodiethylamine caused tumors by additional routes of exposure, in additional species, or at additional tissue sites. Liver tumors were also observed in (1) chickens after intramuscular administration, (2) cats after oral administration (dietary or by stomach tube) (Schmahl *et al.* 1978), and (3) newborn mice after intraperitoneal injection (Lai and Arcos 1980, Vesselinovitch *et al.* 1984). Tumors of the lung or trachea were also observed in (1) hamsters of both sexes after intratracheal administration (Yamamoto *et al.* 1985, Ishinishi *et al.* 1988, Tanaka *et al.* 1988), (2) rabbits after subcutaneous injection (Huntrakoon *et al.* 1989), (3) newborn mice after intraperitoneal injection (Vesselinovitch *et al.* 1984), and (4) snakes after oral exposure (Schmahl and Scherf 1983, 1984). Kidney tumors also were observed in orally exposed snakes. Addition of *N*-nitrosodiethylamine to the tank water increased the incidence of benign or malignant pancreatic tumors (adenoma, cystadenoma, or adenocarcinoma) in larval or juvenile fish (Thiyagarajah and Grizzle 1986) and tumors of the digestive gland and hematopoietic system in mollusks (Khudoley and Syrenko 1978). Benign laryngotracheal tumors (papilloma) were observed in pregnant hamsters exposed by intraperitoneal injection and in the prenatally exposed offspring, and laryngotracheal tumors (neuroendocrine-cell tumors) were observed in the second generation of offspring (Mohr *et al.* 1995).

Cancer Studies in Humans

No epidemiological studies were identified that evaluated the relationship between human cancer and exposure specifically to *N*-nitrosodiethylamine.

Properties

N-Nitrosodiethylamine is a nitrosamine compound that is a slightly yellow, volatile liquid at room temperature (HSDB 2009). It is soluble in water, ethanol, ether, organic solvents, and lipids. It is stable in the dark in neutral or alkaline solution for at least 14 days, but is less stable in more acidic solutions or in light, especially ultraviolet light. (IARC 1978). Physical and chemical properties of *N*-nitrosodiethylamine are listed in the following table.

Property	Information
Molecular weight	102.1[a]
Specific gravity	0.9422 at 20°C/4°C[a]
Melting point	< 25°C[b]
Boiling point	175°C to 177°C[a]
Log K_{ow}	0.48[a]
Water solubility	106 g/L at 24°C[b]
Vapor pressure	0.86 mm Hg at 20°C[b]

Sources: [a]HSDB 2009, [b]ChemIDplus 2009.

Use

N-Nitrosodiethylamine is used primarily as a research chemical (HSDB 2009). Previously, it was used as a gasoline and lubricant additive, antioxidant, stabilizer in plastics, fiber-industry solvent, and copolymer softener, and in the synthesis of 1,1-diethylhydrazine. It was also used to increase dielectric constants in condensers (IARC 1972, HSDB 2009).

Production

No commercial producers of *N*-nitrosodiethylamine were identified. In 2009, it was available from 11 U.S. suppliers (ChemSources 2009). The United States International Trade Commission does not provide specific information on imports and exports of *N*-nitrosodiethylamine. Import and export data are reported for U.S. Census Bureau Schedule B category 29.29, which includes *N*-nitrosodiethylamine and related compounds (USCB 2018).

Exposure

The routes of potential human exposure to *N*-nitrosodiethylamine are ingestion, inhalation, and dermal contact. The general population may be exposed to unknown quantities of *N*-nitrosodiethylamine present in foods, beverages, tobacco smoke, drinking water, and industrial pollution (HSDB 2009). Intake from exposure via air, diet, and smoking has been estimated at a few micrograms per day. *N*-Nitrosodiethylamine has been measured in a variety of foods, including cheese at concentrations of 0.5 to 30 µg/kg, soybeans at 0.2 µg/kg, soybean oil at 4 µg/kg, various fish at up to 147 µg/kg, salt-dried fish at 1.2 to 21 mg/kg, cured meats at up to 40 µg/kg, and alcoholic beverages at 0.1 µg/kg. *N*-Nitrosodiethylamine was detected in tobacco-smoke condensate at concentrations of 1.0 to 28 ng per cigarette (IARC 1978). Up to 8.3 ng per cigarette was found in mainstream smoke and 8 to 73 ng in sidestream smoke. *N*-Nitrosodiethylamine was found at concentrations of up to 0.2 ng/L in indoor air polluted with tobacco smoke and at 10 ng/m^3 in the smoking compartment of a train (Brunnemann *et al.* 1977, Brunnemann and Hoffmann 1978).

Nitrosamines frequently are produced during rubber processing and may be present as contaminants in the final rubber product (HSDB 2009). Potential exposure depends on the ability of the nitrosamines to migrate from the product into the body. Nitrosamines present in pacifiers and baby-bottle nipples can migrate into saliva, which could result in ingestion of nitrosamines (IARC 1974, 1978).

According to the U.S. Environmental Protection Agency's Toxics Release Inventory, 11,795 lb of waste containing *N*-nitrosodiethylamine was released by three facilities in 1999; 99.6% was released to land. In 2007, one facility released 500 lb of *N*-nitrosodiethylamine to a hazardous-waste landfill (TRI 2009). *N*-Nitrosodiethylamine is widespread in the environment, but is rapidly decomposed by sunlight and does not usually persist in ambient air or water exposed to sunlight. It was found at concentrations of 0.07 and 0.24 µg/L in wastewater from two chemical plants, 0.010 µg/L in high-nitrate well water for drinking, and 0.33 to 0.83 µg/L in deionized water (HSDB 2009). *N*-Nitrosodiethylamine and other nitrosamines were found at very low concentrations in ion-exchange resins (Gough *et al.* 1977).

There is some potential for occupational exposure of laboratory, copolymer, and lubricant workers to *N*-nitrosodiethylamine (IARC 1972, 1978). No data were found on the numbers of workers potentially exposed.

Regulations

Consumer Product Safety Commission (CPSC)

A voluntary standard provides that rubber pacifiers shall not contain more than 10 ppb of any single nitrosamine or more than 20 ppb of total nitrosamines.

Environmental Protection Agency (EPA)

Clean Water Act

Effluent Guidelines: Nitrosamines are listed as a toxic pollutant.
Water Quality Criteria: Based on fish or shellfish and water consumption = 0.0008 µg/L; based on fish or shellfish consumption only = 1.24 µg/L.

Comprehensive Environmental Response, Compensation, and Liability Act

Reportable quantity (RQ) = 1 lb.

Emergency Planning and Community Right-To-Know Act

Toxics Release Inventory: Listed substance subject to reporting requirements.

Resource Conservation and Recovery Act

Listed Hazardous Waste: Waste code for which the listing is based wholly or partly on the presence of *N*-nitrosodiethylamine = U174.
Listed as a hazardous constituent of waste.

Toxic Substances Control Act

Nitrosating agents distributed in commerce require warning labels and instructions on use.

Food and Drug Administration (FDA, an HHS agency)

The action level for *N*-nitrosamines in rubber baby-bottle nipples is 10 ppb.
In order to use nitrites and/or nitrates as food additives in curing premixes, a petition must be filed supported by data demonstrating that nitrosamines are not formed.

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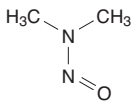
N-Nitrosodimethylamine

CAS No. 62-75-9

Reasonably anticipated to be a human carcinogen

First listed in the *Second Annual Report on Carcinogens* (1981)



Carcinogenicity

N-Nitrosodimethylamine is *reasonably anticipated to be a human carcinogen* based on sufficient evidence of carcinogenicity from studies in experimental animals.

Cancer Studies in Experimental Animals

N-Nitrosodimethylamine caused tumors in numerous species of experimental animals, at several different tissue sites, and by several different routes of exposure. Tumors were observed in all species tested, including mice, rats, hamsters, guinea pigs, multimammate mice (genus *Mastomys*), rabbits, frogs, newts, and various fish. *N*-Nitrosodimethylamine caused tumors primarily of the liver, respiratory tract, kidney, and blood vessels (IARC 1972, 1978). Benign and malignant tumors of the liver (hepatocellular adenoma and carcinoma) or bile duct (cholangioma or cholangiocellular tumors) were observed following (1) oral administration in mice, rats, hamsters, rabbits, guinea pigs, and fish, (2) inhalation exposure in mice, (3) prenatal exposure in mice, (4) subcutaneous administration in hamsters, *Mastomys,* and newborn and suckling mice and rats, (5) intraperitoneal injection in adult and newborn mice and in newts, (6) intramuscular injection in rats, and (7) exposure via tank water in frogs and fish.

Exposure to *N*-nitrosodimethylamine by most of these routes also caused tumors of the respiratory tract: (1) oral exposure caused lung tumors in mice, (2) inhalation exposure caused lung tumors in mice and rats and nasal-cavity tumors in rats, (3) subcutaneous injection caused lung tumors in adult, newborn, and suckling mice and nasal-cavity tumors in adult hamsters, (4) intraperitoneal injection caused lung tumors in adult and newborn mice and nasal-cavity tumors in rats, and (5) prenatal exposure caused lung tumors in mice (IARC 1972, 1978).

N-Nitrosodimethylamine caused kidney tumors in rats and mice exposed orally or by inhalation or intraperitoneal injection and in rats exposed prenatally or by subcutaneous injection. Blood-vessel tumors (hemangioma or hemangiosarcoma) were observed in mice, rats, and hamsters after oral exposure; in hamsters and adult, newborn, and suckling mice after subcutaneous injection; and in mice after intraperitoneal injection. Addition of *N*-nitrosodimethylamine to the tank water of frogs caused tumors of the hematopoietic system (IARC 1972, 1978).

Since *N*-nitrosodimethylamine was listed in the *Second Annual Report on Carcinogens,* additional studies in experimental animals have been identified, which reported that *N*-nitrosodimethylamine caused tumors at additional tissue sites and in additional species. Tumors of the digestive gland and hematopoietic system were observed in mollusks exposed to *N*-nitrosodimethylamine in the tank water (Khudoley and Syrenko 1978), and ovarian tumors (granulosa-cell tumors) in female hamsters exposed by subcutaneous injection (Richter-Reichhelm *et al.* 1978). Liver tumors were observed in foxes after dietary exposure (Koppang *et al.* 1981) and in female toads after subcutaneous injection (Sakr *et al.* 1989), and lung and liver tumors were observed in rats after a single intraperitoneal injection (Noronha and Goodall 1983, Sykora *et al.* 1985, Driver and Swann 1987).

Cancer Studies in Humans

No epidemiological studies evaluating the relationship between human cancer and exposure specifically to *N*-nitrosodimethylamine were available when it was listed in the *Second Annual Report on Carcinogens.* Since then, a number of population-based case-control studies or ecological studies of cancer and dietary sources of *N*-nitrosodimethylamine (particularly, for example, cured, salted, or barbecued meat or fish) have been conducted in various countries. These studies focused mainly on cancer of the gastrointestinal tract, and the majority relied on estimated intake from self-reported dietary histories. Several case-control studies reported dose-related associations, some statistically significant, between estimated *N*-nitrosodimethylamine intake and oropharyngeal cancer (De Stefani *et al.* 1994), stomach cancer (Le Vecchia *et al.* 1995, Pobel *et al.* 1995, Larsson *et al.* 2006), esophageal cancer (Lu *et al.* 1987, Rogers *et al.* 1995), or colorectal cancer (Knekt *et al.* 1999). Ecological studies also suggested an association between high dietary *N*-nitrosodimethylamine intake and high rates of esophageal cancer in populations (Siddiqi *et al.* 1988, 1991, Lin *et al.* 2002a,b). A case-control study of lung cancer found a dose-related increase in risk associated with estimated dietary intake of *N*-nitrosodimethylamine among smokers and nonsmokers (De Stefani *et al.* 1996). Several studies adjusted for smoking or alcohol consumption, and interactive effects with these substances were noted in some analyses; however, the results may have been confounded by exposure to these substances or other factors, including other nitrosamines in the diet. No studies of occupational exposure to *N*-nitrosodimethylamine were identified.

Properties

N-Nitrosodimethylamine is a nitrosamine compound that exists at room temperature as a yellow liquid with a faint characteristic odor (Akron 2009). It is very soluble in water, alcohol, and ether, miscible with methylene chloride and vegetable oils, and soluble in lipids, chloroform, and most other organic solvents (HSDB 2009). It is stable in the dark in neutral or alkaline solution for at least 14 days, but is less stable in more acidic solutions or in light, especially ultraviolet light (IARC 1978). Physical and chemical properties of *N*-nitrosodimethylamine are listed in the following table.

Property	Information
Molecular weight	74.1[a]
Specific gravity	1.0048 at 20°C/4°C[a]
Melting point	< 25°C[b]
Boiling point	151°C to 153°C[a]
Log K_{ow}	–0.57[a]
Water solubility	1,000 g/L at 24°C[b]
Vapor pressure	2.7 mm Hg at 20°C[b]
Vapor density relative to air	2.56[a]

Sources: [a]HSDB 2009, [b]ChemIDplus 2009.

Use

N-Nitrosodimethylamine is used primarily as a research chemical (HSDB 2009). Before April 1, 1976, it was used as an intermediate in the electrolytic production of 1,1-dimethylhydrazine, a storable liquid rocket fuel containing approximately 0.1% *N*-nitrosodimethylamine as an impurity (IARC 1978). Other former uses of *N*-nitrosodimethylamine include use in control of nematodes, to inhibit nitrification in soil, in active metal anode-electrolyte systems (high-energy batteries), in the preparation of thiocarbonyl fluoride polymers, and as a plasticizer for rubber and acrylonitrile polymers, a solvent in the fiber and plastics industry, an antioxidant, a softener of copolymers, and an additive to lubricants (HSDB 2009).

Production

Commercial production of *N*-nitrosodimethylamine in the United States began in the mid 1950s for use in the manufacture of 1,1-dimethylhydrazine. The last commercial producer of *N*-nitrosodimethylamine closed its plant in 1976 (IARC 1978), and there is no evidence that *N*-nitrosodimethylamine is currently manufactured commercially in the United States (HSDB 2009). In 2009, *N*-nitrosodimethylamine was available from nine U.S. suppliers (ChemSources 2009). The United States International Trade Commission does not provide specific information on imports and exports of *N*-nitrosodimethylamine. Import and export data are reported for U.S. Census Bureau Schedule B category 29.29, which includes *N*-nitrosodimethylamine and related compounds (USCB 2018).

Exposure

The routes of potential human exposure to *N*-nitrosodimethylamine are ingestion, inhalation, and dermal contact (HSDB 2009). The general population may be exposed to unknown quantities of *N*-nitrosodimethylamine present in foods and beverages, tobacco smoke, herbicides, pesticides, drinking water, and industrial pollution (IARC 1978, ATSDR 1989). In addition, nitrosamines may be formed from amines reacting with nitrites in the human body as a result of ingestion of these precursors separately in food, water, or air. Intake of *N*-nitrosodimethylamine from exposure via air, diet, and smoking has been estimated at a few micrograms per day. *N*-Nitrosodimethylamine is present in a variety of foods, including cheese, soybean oil, various meat products, bacon, various cured meats, frankfurters, cooked ham, fish and fish products, spices used for meat curing, apple brandy, other alcoholic beverages, and beer. Concentrations in these foodstuffs have been measured at up to 850 µg/kg (in spices used in curing) (IARC 1978).

N-Nitrosodimethylamine has been detected in numerous drugs formulated with aminopyrine, including tablets, suppositories, injections, drops, and syrups, at concentrations ranging from less than 10 to 371 µg/kg. *N*-Nitrosodimethylamine was measured in mainstream cigarette smoke at 13 to 65 ng per cigarette for nonfiltered cigarettes and 5.7 to 43 ng for filtered cigarettes and in sidestream smoke at 680 to 823 ng for nonfiltered cigarettes and 1,040 to 1,770 ng for filtered cigarettes. It was found at concentrations of 90 to 240 ng/m^3 in smoke-filled rooms, such as bars, but at less than 5 ng/m^3 in residences (IARC 1978). Nitrosamines frequently are produced during rubber processing and may be present as contaminants in the final rubber product. Potential exposure depends on the ability of the nitrosamine to migrate from the product into the body. Dimethylamine-formulated pesticides and herbicides contained *N*-nitrosodimethylamine at 190 to 640 mg/L (190,000 to 640,000 µg/L) (ATSDR 1989).

N-Nitrosodimethylamine is widespread in the environment, but it is rapidly decomposed by sunlight and does not usually persist in ambient air or water exposed to sunlight (ATSDR 1989). *N*-Nitrosodimethylamine was found at concentrations of 0.25 µg/L in industrial wastewater from chemical factories, 0.02 to 0.82 µg/L in chlorinated drinking water, less than 0.01 µg/L in high-nitrate well water, and 0.012 to 0.34 µg/L in deionized water. *N*-Nitrosodimethylamine and other nitrosamines were found at very low concentrations in ion-exchange resins (Gough *et al.* 1977). Soil samples taken near industrial plants contained *N*-nitrosodimethylamine at concentrations of up to 15.1 ng/g (IARC 1978).

There is some potential for occupational exposure of laboratory, copolymer, lubricant, and pesticide workers to *N*-nitrosodimethylamine (IARC 1978, HSDB 2009). The National Occupational Exposure Survey (conducted from 1981 to 1983) estimated that 747 workers, including 299 women, potentially were exposed to *N*-nitrosodimethylamine (NIOSH 1990). Occupational Safety and Health Administration regulations concerning *N*-nitrosodimethylamine designate strict procedures to avoid worker contact (IARC 1978). Mixtures containing *N*-nitrosodimethylamine at 1.0% or more must be maintained in isolated or closed systems, workers must observe special hygiene rules, and certain procedures must be followed for movement of the material and in case of accidental spills and emergencies.

Regulations

Consumer Product Safety Commission (CPSC)

A voluntary standard provides that rubber pacifiers shall not contain more than 10 ppb of any single nitrosamine or more than 20 ppb of total nitrosamines.

Environmental Protection Agency (EPA)

Clean Air Act

National Emission Standards for Hazardous Air Pollutants: Listed as a hazardous air pollutant.

Clean Water Act

Effluent Guidelines: Nitrosamines are listed as a toxic pollutant.
Water Quality Criteria: Based on fish or shellfish and water consumption = 0.00069 µg/L; based on fish or shellfish consumption only = 3.0 µg/L.

Comprehensive Environmental Response, Compensation, and Liability Act

Reportable quantity (RQ) = 10 lb.

Emergency Planning and Community Right-To-Know Act

Toxics Release Inventory: Listed substance subject to reporting requirements.
Reportable quantity (RQ) = 10 lb.
Threshold planning quantity (TPQ) = 1,000 lb.

Resource Conservation and Recovery Act

Listed Hazardous Waste: Waste code for which the listing is based wholly or partly on the presence of *N*-nitrosodimethylamine = P082.

Listed as a hazardous constituent of waste.

Toxic Substances Control Act

Nitrosating agents distributed in commerce require warning labels and instructions on use.

Food and Drug Administration (FDA, an HHS agency)

Action levels for *N*-nitrosodimethylamine in barley malt and malt beverages range from 5 to 10 ppb.
The action level for *N*-nitrosamines in rubber baby-bottle nipples is 10 ppb.
In order to use nitrites and/or nitrates as food additives in curing premixes, a petition must be filed supported by data demonstrating that nitrosamines are not formed.

Mine Safety and Health Administration (MSHA, Dept. of Labor)

To control airborne exposure, *N*-nitrosodimethylamine shall not be used or stored except by competent persons under laboratory conditions approved by a nationally recognized agency acceptable to the Secretary of Labor.

Occupational Safety and Health Administration (OSHA, Dept. of Labor)

Potential occupational carcinogen: Engineering controls, work practices, and personal protective equipment are required.

Guidelines

American Conference of Governmental Industrial Hygienists (ACGIH)

Threshold limit value – time-weighted average (TLV-TWA) = exposure by all routes should be as low as possible.

National Institute for Occupational Safety and Health (NIOSH, CDC, HHS)

Listed as a potential occupational carcinogen.

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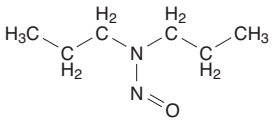
N-Nitrosodi-*n*-propylamine

CAS No. 621-64-7

Reasonably anticipated to be a human carcinogen

First listed in the *Second Annual Report on Carcinogens* (1981)



Carcinogenicity

N-Nitrosodi-*n*-propylamine is *reasonably anticipated to be a human carcinogen* based on sufficient evidence of carcinogenicity from studies in experimental animals.

Cancer Studies in Experimental Animals

N-Nitrosodi-*n*-propylamine caused tumors in two rodent species, at several different tissue sites, and by two different routes of exposure. In rats, it caused liver cancer (hepatocellular carcinoma) and benign and malignant tumors of the esophagus (papilloma and carcinoma) following administration in the drinking water or subcutaneous injection (IARC 1978). Subcutaneous injection of *N*-nitrosodi-*n*-propylamine also caused tumors of the lung and nasal and paranasal sinus cavities in hamsters and rats, tumors of the laryngobronchial tract in hamsters, and benign and malignant kidney tumors (adenoma and adenocarcinoma) in rats.

Since *N*-nitrosodi-*n*-propylamine was listed in the *Second Annual Report on Carcinogens,* additional studies in experimental animal have been identified, which reported that *N*-nitrosodi-*n*-propylamine caused tumors of the liver, esophagus, and respiratory tract by additional routes of exposure or in additional species. Liver tumors were observed in monkeys exposed by intraperitoneal injection (Adamson and Sieber 1979, 1982); cancer (carcinoma) of the liver, esophagus, and nasal cavity in rats exposed by stomach tube (Lijinsky and Reuber 1983); and tracheal tumors in male hamsters exposed by intratracheal instillation (Ishinishi *et al.* 1988). In addition, adminis-

tration of *N*-nitrosodi-*n*-propylamine in the drinking water caused forestomach tumors in male rats (Lijinsky *et al.* 1981).

Cancer Studies in Humans

No epidemiological studies were identified that evaluated the relationship between human cancer and exposure specifically to *N*-nitrosodi-*n*-propylamine.

Properties

N-Nitrosodi-*n*-propylamine is a nitrosamine compound that is a yellow liquid at room temperature (HSDB 2009). It is soluble in water, lipids, and organic solvents. It is stable in the dark in neutral or alkaline solution for at least 14 days, but is less stable in more acidic solutions or in light, especially ultraviolet light (IARC 1978). Physical and chemical properties of *N*-nitrosodi-*n*-propylamime are listed in the following table.

Property	Information
Molecular weight	130.2[a]
Specific gravity	0.9160 at 20°C/4°C[a]
Boiling point	206°C[b]
Log K_{ow}	1.36[a]
Water solubility	13 g/L at 24°C[b]
Vapor pressure	0.086 mm Hg at 20°C[b]

Sources: [a]HSDB 2009, [b]ChemIDplus 2009.

Use

N-Nitrosodi-*n*-propylamine is used in small quantities in laboratory research. It has no known commercial use (IARC 1978, ATSDR 1989, HSDB 2009).

Production

N-Nitrosodi-*n*-propylamine was first prepared in 1886, but it has never been produced in commercial quantities (IARC 1978, HSDB 2009). In 2009, it was available in small quantities for research purposes from eight U.S. suppliers (ChemSources 2009). The United States International Trade Commission does not provide specific information on imports and exports of *N*-nitrosodi-*n*-propylamine. Import and export data are reported for U.S. Census Bureau Schedule B category 29.29, which includes *N*-nitrosodi-*n*-propylamine and related compounds (USCB 2018).

Exposure

The primary routes of potential human exposure to *N*-nitrosodi-*n*-propylamine are inhalation, ingestion, and dermal contact (HSDB 2009). *N*-Nitrosodi-*n*-propylamine has been detected in extruded rubber products, cheese, and alcoholic beverages, and in the herbicides trifluralin, isopropalin, and oryzalin at low concentrations (17 to 190 ppm) (IARC 1978, ATSDR 1989, HSDB 2009). There is some evidence that *N*-nitrosodi-*n*-propylamine may be formed in the upper gastrointestinal tract following ingestion of foods containing nitrites and secondary amines (ATSDR 1989). It may also occur in cigarette smoke at low levels (about 1 ng per cigarette). *N*-Nitrosodi-*n*-propylamine is not commonly detected in the environment. According to the U.S. Environmental Protection Agency's Toxics Release Inventory, two facilities released a total of 2,379 lb of *N*-nitrosodi-*n*-propylamine to the environment in 1998, and one facility released 5 lb in 1999. Since 2001, releases have ranged from a low of 257 lb in 2002 to a high of 755 lb in 2005. In 2007, 250 lb was released to air and 500 lb to an off-site hazardous-waste landfill (TRI 2009). When released to the environment, *N*-nitrosodi-*n*-propylamine will undergo photochemical and biological degradation and will not persist. *N*-Nitrosodi-*n*-propylamine has been detected in some samples of wastewater from chemical plants (ATSDR 1989).

Occupational exposure to *N*-nitrosodi-*n*-propylamine may occur through inhalation and dermal contact during herbicide application (HSDB 2009) or production of extruded rubber parts (ATSDR 1989). *N*-Nitrosodi-*n*-propylamine was not detected in air samples collected at agricultural fields before, during, or after application of trifluralin. However, at an automobile plant where workers were involved in the production of extruded rubber parts, it was found in air samples at concentrations of 1.3 to 3.3 μg/m^3. In the vulcanization step of tire manufacturing, *N*-Nitrosodi-*n*-propylamine was measured at concentrations of up to 1.086 mg/m^3, resulting in an estimated daily intake of 0.0029 mg/kg of body weight for workers (Durmusoglu 2007). No data were available on the numbers of workers potentially exposed to *N*-nitrosodi-*n*-propylamine.

Regulations

Consumer Product Safety Commission (CPSC)

A voluntary standard provides that rubber pacifiers shall not contain more than 10 ppb of any single nitrosamine or more than 20 ppb of total nitrosamines.

Environmental Protection Agency (EPA)

Clean Water Act

Effluent Guidelines: Nitrosamines are listed as a toxic pollutant.
Water Quality Criteria: Based on fish or shellfish and water consumption = 0.0050 μg/L; based on fish or shellfish consumption only = 0.51 μg/L.

Comprehensive Environmental Response, Compensation, and Liability Act

Reportable quantity (RQ) = 10 lb.

Emergency Planning and Community Right-To-Know Act

Toxics Release Inventory: Listed substance subject to reporting requirements.

Resource Conservation and Recovery Act

Listed Hazardous Waste: Waste code for which the listing is based wholly or partly on the presence of *N*-nitrosodi-*n*-propylamine = U111.
Listed as a hazardous constituent of waste.

Toxic Substances Control Act

Nitrosating agents distributed in commerce require warning labels and instructions on use.

Food and Drug Administration (FDA, an HHS agency)

The action level for *N*-nitrosamines in rubber baby-bottle nipples is 10 ppb.
In order to use nitrites and/or nitrates as food additives in curing premixes, a petition must be filed supported by data demonstrating that nitrosamines are not formed.

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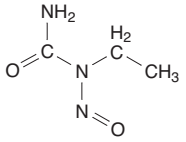
N-Nitroso-*N*-ethylurea

CAS No. 759-73-9

Reasonably anticipated to be a human carcinogen

First listed in the *Second Annual Report on Carcinogens* (1981)

Also known as *N*-ethyl-*N*-nitrosourea



Carcinogenicity

N-Nitroso-*N*-ethylurea is *reasonably anticipated to be a human carcinogen* based on sufficient evidence of carcinogenicity from studies in experimental animals.

Cancer Studies in Experimental Animals

N-Nitroso-*N*-ethylurea caused tumors in several species of experimental animals, at several different tissue sites, and by several different routes of exposure. It was carcinogenic in animals exposed perinatally and as adults. Perinatal exposure caused primarily nervous-system tumors, whereas tumors occurred at numerous tissue sites in adults, including the kidney and lymphoreticular system.

Tumors of the nervous system (brain, spinal cord, or peripheral nerves) were observed following oral exposure to *N*-nitroso-*N*-ethylurea in suckling rats, a single subcutaneous injection in newborn rats, and prenatal exposure in mice, rats, hamsters, and rabbits. In adult rodents, intraperitoneal injection of *N*-nitroso-*N*-ethylurea caused brain tumors in mice, and intravenous injection caused tumors of the brain and peripheral nerves in rats (IARC 1972, 1978).

Prenatal exposure to *N*-nitroso-*N*-ethylurea also caused benign lung tumors (adenoma), leukemia, and tumors of the liver, Harderian gland, and endocrine glands in mice; benign and malignant kidney tumors (adenoma, adenocarcinoma, and adenocarcinosarcoma) in rabbits; and benign tumors of the sweat glands (adenoma) and skin (papilloma) in pigs. Oral exposure caused leukemia in adult rats, kidney tumors (nephroblastoma) in suckling rats, and tumors of the kidney (nephroblastoma), eye, liver, muscle, and jaw in newborn opossums. Intravenous injection of *N*-nitroso-*N*-ethylurea caused tumors of the kidney, ovary, uterus, and vagina in rats and tumors of the ovary, uterus, bone, skin, and blood vessels in monkeys. Intraperitoneal injection caused thymic lymphoma and myeloid leukemia in rats and lymphoma and tumors of the liver, kidney, ovary, lung, Harderian gland, stomach in mice. In newborn mice, a single subcutaneous injection of *N*-nitroso-*N*-ethylurea caused lymphoma, liver cancer (hepatocellular carcinoma), and benign and malignant lung tumors (adenoma and adenocarcinoma) (IARC 1972, 1978).

Since *N*-nitroso-*N*-ethylurea was listed in the *Second Annual Report on Carcinogens,* additional studies in experimental animal have been identified, which confirmed the induction of several tumors types observed in earlier studies and reported that it caused tumors by additional routes of exposure, in additional species, and at additional tissue sites. Tumors were observed for the following additional routes of exposure or additional species:

- Intratracheal administration caused benign tracheal tumors (papilloma and polyps) in male hamsters (Grubbs *et al.* 1981).
- Dermal administration caused skin tumors in mice (Lijinsky 1982, Lijinsky and Reuber 1983).
- Implantation into the mammary gland caused mammary-gland cancer (adenocarcinoma) in female rats (Holtzman *et al.* 1985).
- Intracerebral injection caused brain tumors in adult rats (Druckrey 1973), and a single intracerebral injection caused spinal-cord tumors in newborn rats of both sexes (Pfaffenroth and Das 1979).
- Intravesicular administration caused urinary-bladder tumors in female rats (Lijinsky *et al.* 1992).
- Injection directly into the amniotic sac of pregnant mice caused benign lung tumors (alveologenic adenoma) in the offspring (Rossi *et al.* 1979).
- In fish, exposure in the tank water caused benign skin tumors (papilloma) (Beckwith *et al.* 2000).
- In gerbils, subcutaneous injection caused nervous-system tumors (oligodendroglioma) and skin cancer (melanoma) in newborns of both sexes and benign blood-vessel tumors (hemangioma) in adults of both sexes (Naito *et al.* 1985).

N-nitroso-*N*-ethylurea also caused tumors at the following additional tissue sites:

- Benign forestomach tumors (squamous-cell papilloma) were observed in male hamsters exposed by intraperitoneal injection, as well peripheral-nerve tumors in prenatally exposed hamsters (Likhachey *et al.* 1983, Diwan *et al.* 1996).
- Malignant placental tumors (choriocarcinoma) were observed in pregnant monkeys exposed by intravenous injection (Rice *et al.* 1981).
- Tumors of the lining of the peritoneal cavity (mesothelioma) were observed in orally exposed male rats, as well as mammary-gland tumors in orally exposed female rats (Lijinsky and Kovatch 1989).

Intraperitoneal injection of *N*-nitroso-*N*-ethylurea in rats caused mammary-gland tumors in males, ovarian tumors in females, nervous-system tumors in adult males and newborns, and possibly kidney tumors in newborns (Mandybur *et al.* 1978, Stoica and Koestner 1984, Stoica *et al.* 1985, Hasgekar *et al.* 1989). Prenatal exposure of mice to *N*-nitroso-*N*-ethylurea caused benign skin tumors (papilloma and sebaceous adenoma) in nude mice and cancer of the small intestine (adenocarcinoma) in mice of both sexes in other mouse strains (Anderson *et al.* 1982, Oomen *et al.* 1984, 1988, 1989). Prenatal exposure of rabbits caused tumors of the kidney and neural tissue (neurofibroma) (Fox *et al.* 1982).

Oral administration of *N*-nitroso-*N*-ethylurea to rats caused thyroid-gland tumors, forestomach cancer (squamous-cell carcinoma), and benign lung tumors (adenoma) in both sexes; tumors of the colon and skin in males; and ovarian tumors (Sertoli-cell tumors) in females (Maekawa *et al.* 1984, Lijinsky *et al.* 1985, Maekawa *et al.* 1986, Lijinsky and Kovatch 1989, 1996). Administration by stomach tube to hamsters caused benign and malignant forestomach tumors (papilloma and squamous-cell carcinoma) and blood-vessel cancer (hemangiosarcoma) in both sexes (Lijinsky *et al.* 1985).

Cancer Studies in Humans

No epidemiological studies were identified that evaluated the relationship between human cancer and exposure specifically to *N*-nitroso-*N*-ethylurea.

Properties

N-Nitroso-*N*-ethylurea is a nitrosamine compound that exists as yellow-pink or buff-yellow crystals at room temperature (HSDB 2009). It is soluble in water, chloroform, and other polar organic solvents, but insoluble in nonpolar organic solvents. It decomposes in alkaline solution at a rate that depends on pH (IARC 1978). The pure compound is sensitive to moisture and light and should be stored under refrigeration. Physical and chemical properties of *N*-nitroso-*N*-ethylurea are listed in the following table.

Property	Information
Molecular weight	117.1[a]
Melting point	103°C to 104°C (with decomposition)[a]
Log K_{ow}	0.23[a]
Water solubility	13 g/L at 25°C[b]
Vapor pressure	0.0183 mm Hg at 25°C[b]

Sources: [a]HSDB 2009, [b]ChemIDplus 2009.

Use

N-Nitroso-*N*-ethylurea has been used to synthesize diazoethane in the laboratory, and its mutagenic effects have been studied for promoting the growth of various plants (IARC 1978).

Production

N-Nitroso-*N*-ethylurea was first prepared in 1919 but has never been produced in commercial quantities in the United States (IARC 1978). In 2009, it was available in small quantities for research purposes from six U.S. suppliers (ChemSources 2009).

Exposure

The potential for human exposure is limited, because *N*-nitroso-*N*-ethylurea is not produced or used in large quantities in the United States (IARC 1978). Human exposure to *N*-nitroso compounds may occur through absorption from food, water, and air, and from formation in the human body from precursors ingested separately from food or water (IARC 1978). Exposure may also result from the consumption or smoking of tobacco. According to the U.S. Environmental Proection Agency's Toxics Release Inventory, environmental releases of *N*-nitroso-*N*-ethylurea totaled 169 lb in 1999 and 255 lb in 2001, but only 10 lb in 2005, 2006, and 2007 (TRI 2009). In air, *N*-nitroso-*N*-ethylurea exists solely as vapor and is degraded by reaction with photochemically produced hydroxyl radicals, with an estimated half-life of 3.2 days. It hydrolyzes in water, with a half-life of 1.5 hours at pH 7 at 20°C. Occupational exposure to *N*-nitroso-*N*-ethylurea may occur through inhalation or dermal contact during its use in research (HSDB 2009). No data were found on the numbers of workers potentially exposed to *N*-nitroso-*N*-ethylurea.

Regulations

Consumer Product Safety Commission (CPSC)

A voluntary standard provides that rubber pacifiers shall not contain more than 10 ppb of any single nitrosamine or more than 20 ppb of total nitrosamines.

Environmental Protection Agency (EPA)

Clean Water Act

Effluent Guidelines: Nitrosamines are listed as a toxic pollutant.

Water Quality Criteria: Based on fish or shellfish and water consumption = 0.0008 µg/L for nitrosamines; based on fish or shellfish consumption only = 1.24 µg/L for nitrosamines.

Comprehensive Environmental Response, Compensation, and Liability Act

Reportable quantity (RQ) = 1 lb.

Emergency Planning and Community Right-To-Know Act

Toxics Release Inventory: Listed substance subject to reporting requirements.

Resource Conservation and Recovery Act

Listed Hazardous Waste: Waste code for which the listing is based wholly or partly on the presence of *N*-nitroso-*N*-ethylurea = U176.

Listed as a hazardous constituent of waste.

Toxic Substances Control Act

Nitrosating agents distributed in commerce require warning labels and instructions on use.

Food and Drug Administration (FDA, an HHS agency)

The action level for *N*-nitrosamines in rubber baby-bottle nipples is 10 ppb.

In order to use nitrites and/or nitrates as food additives in curing premixes a petition must be filed supported by data demonstrating that nitrosamines are not formed.

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TRI. 2009. *TRI Explorer Chemical Report.* U.S. Environmental Protection Agency. Last updated: 3/19/09. http://www.epa.gov/triexplorer and select *N*-Nitroso-*N*-Ethylurea .

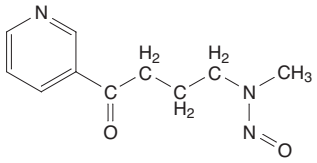
4-(*N*-Nitrosomethylamino)-1-(3-pyridyl)-1-butanone

CAS No. 64091-91-4

Reasonably anticipated to be a human carcinogen

First listed in the *Sixth Annual Report on Carcinogens* (1991)

Also known as NNK or 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone



Carcinogenicity

4-(*N*-Nitrosomethylamino)-1-(3-pyridyl)-1-butanone (NNK) is *reasonably anticipated to be a human carcinogen* based on sufficient evidence of carcinogenicity from studies in experimental animals.

Cancer Studies in Experimental Animals

NNK caused tumors in three rodent species and at several different tissue sites, primarily tumors of the lung and nasal cavity in rats and hamsters. In some studies, NNK caused tumors after a single injection, and dose-response relationships were observed for liver, lung, and nasal-cavity tumors in rats. Subcutaneous injection of NNK caused benign or malignant nasal-cavity tumors (squamous- or transitional-cell papilloma, neuroblastoma, rhabdomyosarcoma, esthesioneuroepithelioma, squamous-cell carcinoma, anaplastic carcinoma, or spindle-cell sarcoma) and lung cancer (squamous-cell carcinoma, adenocarcinoma, and adenosquamous-cell carcinoma) in rats and hamsters of both sexes (IARC 1985). Subcutaneous injection of NNK also caused cancer of the liver (hepatocellular carcinoma) and the blood vessels (hemangiosarcoma) in rats of both sexes and tracheal tumors in hamsters of both sexes after single or multiple injections. In female strain A mice (a strain with a high spontaneous incidence of lung tumors), intraperitoneal injection of NNK caused benign and malignant lung tumors (adenoma and carcinoma).

Since NNK was listed in the *Sixth Annual Report on Carcinogens,* additional studies have been identified in which NNK was carcinogenic by additional routes of exposure or in additional species. The majority of these studies confirmed that NNK primarily caused tumors of the lung, nasal cavity, and liver in rodents:

- Oral administration (by stomach tube or in the drinking water) caused lung tumors in strain A/J mice (Chung 1999, Sugimoto *et al.* 2003, Kim *et al.* 2004, Richie *et al.* 2006) and male rats (Rivenson *et al.* 1988, Lijinsky *et al.* 1991a, Hoffmann *et al.* 1993, Hecht *et al.* 1996), nasal-cavity tumors in rats of both sexes, and liver tumors in male rats (Lijinsky *et al.* 1991a).
- Swabbing of the oral cavity with NNK caused tumors of the lung, nasal cavity, and liver in rats (Prokopczyk *et al.* 1991).
- Administration onto the tongue caused lung tumors in mice (Padma *et al.* 1989).
- Intravesicular instillation caused lung and liver tumors in female rats (Lijinsky *et al.* 1991b).
- Intraperitoneal injection caused lung and liver tumors in newborn mice of both sexes (Anderson *et al.* 1991, Beebe *et al.* 1993).
- Prenatal exposure of hamsters caused nasal-cavity tumors (Correa *et al.* 1990, Schuller *et al.* 1994).
- In female mink, subcutaneous injection caused nasal-cavity tumors (Koppang *et al.* 1997).

NNK also was found to cause tumors at additional tissues sites. Prenatal exposure of hamsters caused adrenal-gland tumors in both sexes (Schuller *et al.* 1993, 1994) and caused tumors of the larynx and trachea, in addition to the nasal cavity (Correa *et al.* 1990). Administration of NNK in the drinking water of male rats also increased the combined incidences of leukemia and lymphoma (Hecht *et al.* 1996) and benign and malignant tumors of the pancreas (exocrine acinar adenoma and adenocarcinoma) (Rivenson *et al.* 1988, Hoffmann *et al.* 1993).

Cancer Studies in Humans

No epidemiological studies were identified that evaluated the relationship between human cancer and exposure specifically to NNK.

Properties

NNK is a tobacco-specific nitrosamine compound. In its pure form, NNK is a pale-yellow crystalline solid at room temperature (Akron 2009). It is soluble in water (SRC 2009). Physical and chemical properties of NNK are listed in the following table.

Property	Information
Molecular weight	207.2[a]
Melting point	71°C to 73°C[a]
Water solubility	40.5 g/L at 25°C[b]
Dissociation constant (pK_a)	10.57[a]

Sources: [a]Akron 2009, [b]SRC 2009.

Use

NNK has no known use other than as a laboratory chemical; it has been used as a positive-control substance in laboratory carcinogenicity studies (IARC 1985, 2007).

Production

Synthetic NNK is prepared by reacting sodium hydroxide and sodium nitrite with 4-(*N*-methyl)-1-(3-pyridyl)-1-butanone dihydrochloride or by reacting nicotine with sodium nitrite in aqueous solution. NNK is not produced commercially (IARC 1985). In 2009, it was available in small quantities for research purposes from six suppliers worldwide, including four U.S. suppliers (ChemSources 2009).

Exposure

Potential exposure to NNK is widespread among tobacco-product users and people exposed to sidestream smoke. NNK has been measured in tobacco at concentrations of 0.1 to 35 mg/kg. It was found in moist snuff at up to 18 mg/kg (dry weight), in dry snuff at up to 84.4 mg/kg, in leading U.S. brands of snuff at 0.2 to 8.3 mg/kg, in U.S. chewing tobacco products at up to 1.1 mg/kg (dry weight), in U.S. cigarettes at up to 1.27 mg/kg of dry tobacco (IARC 1985, 2007), and in Nigerian or American cigarettes at 55 to 317 ng per cigarette (Atawodi *et al.* 1995, Harris 2001). NNK was found in the tobacco of bidi cigarettes at concentrations ranging from 0.10 to 0.85 mg/kg, compared with 0.11 to 0.28 mg/kg for U.S. cigarettes (Wu *et al.* 2004).

NNK has been found in mainstream smoke from U.S. cigarettes at concentrations of up to 425 ng per cigarette (IARC 2007). NNK was measured in cigarette and cigar smoke at 0.9 to 7 mg/kg, in mainstream cigarette at 0.02 to 4.2 µg per cigarette, and in sidestream smoke at 0.2 to 15.7 µg per cigarette (IARC 1985). One study found NNK concentrations to be 45% to 73% lower in smoke from filtered than non-filtered cigarettes. NNK was measured in mainstream smoke from bidi cigarettes at 2.13 to 25.9 ng per cigarette and from U.S. cigarettes at 34.3 to 139 ng per cigarette when smoked at an equal rate; however, bidi cigarettes generally are smoked at about twice the rate of U.S. cigarettes, resulting in delivery of about the same total amount of NNK to the smoker (Wu *et al.* 2004).

NNK may also form in the mouth during tobacco chewing or oral snuff use. Concentrations of NNK in the saliva of women who used snuff ranged from 2.1 to 201 ng/g. NNK was detected in pancreatic juices from smokers at a mean concentration of 88.7 ng/mL (compared with 12.4 ng/mL in nonsmokers) and in cervical mucus from smokers at 46.9 ng/g of mucus (Prokopczyk *et al.* 1997, 2002). Metabolites of NNK were found in the urine of newborns whose mothers had smoked during pregnancy, but not in the urine of those born to nonsmoking mothers (Lackmann *et al.* 1999). Children in households with smokers also had significantly elevated urinary concentrations of NNK metabolites; the highest levels were found among African-American children (Sexton *et al.* 2004). The concentration of NNK metabolites was also significantly increased in the urine of nonsmoking hospitality workers on days when they worked in locations where smoking was allowed (Tulunay *et al.* 2005). Reduction of smoking reduced the urinary levels of NNK metabolites, but the reduction was less than expected based on the reduction in the number of cigarettes smoked per day (Hecht *et al.* 2004, Joseph *et al.* 2005).

Regulations

Consumer Product Safety Commission (CPSC)

A voluntary standard provides that rubber pacifiers shall not contain more than 10 ppb of any single nitrosamine or more than 20 ppb of total nitrosamines.

Environmental Protection Agency (EPA)

Clean Water Act

Effluent Guidelines: Nitrosamines are listed as a toxic pollutant.

Water Quality Criteria: Based on fish or shellfish and water consumption = 0.0008 µg/L for nitrosamines; based on fish or shellfish consumption only = 1.24 µg/L for nitrosamines.

Resource Conservation and Recovery Act

Listed as a hazardous constituent of waste.

Toxic Substances Control Act

Nitrosating agents distributed in commerce require warning labels and instructions on use.

Food and Drug Administration (FDA, an HHS agency)

The action level for *N*-nitrosamines in rubber baby-bottle nipples is 10 ppb.

In order to use nitrites and/or nitrates as food additives in curing premixes a petition must be filed supported by data demonstrating that nitrosamines are not formed.

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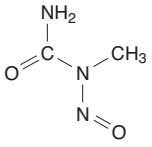
N-Nitroso-*N*-methylurea

CAS No. 684-93-5

Reasonably anticipated to be a human carcinogen

First listed in the *Second Annual Report on Carcinogens* (1981)

Also known as nitrosomethylurea or *N*-methyl-*N*-nitrosourea



Carcinogenicity

N-Nitroso-*N*-methylurea is *reasonably anticipated to be a human carcinogen* based on sufficient evidence of carcinogenicity from studies in experimental animals.

Cancer Studies in Experimental Animals

N-Nitroso-*N*-methylurea caused tumors in several species of experimental animals, at several different tissue sites, and by several different routes of exposure. It was carcinogenic in animals exposed perinatally or as adults. Perinatal exposure resulted mainly in nervous-system tumors, whereas tumors occurred at numerous tissue sites in adults, including the respiratory and digestive tracts.

Tumors of the nervous system were observed following prenatal exposure in rats, oral exposure in adult rats (peripheral nerves), and exposure by injection in newborn rats (peripheral nervous system) and adult rats, rabbits, and dogs (peripheral and central nervous system). Prenatal exposure also caused kidney tumors in rats and benign tumors of the lung (adenoma) and liver (hepatocellular adenoma) in mice, and exposed pregnant rats developed mammary-gland tumors (IARC 1972, 1978).

Administration of *N*-nitroso-*N*-methylurea by intratracheal instillation caused cancer of the nasopharyngeal tube, pharynx, larynx, bronchi, esophagus, forestomach, and trachea (epidermoid or large-cell anaplastic carcinoma) in hamsters. Intrarectal administration caused benign or malignant colon tumors (adenoma, adenocarcinoma, or squamous-cell carcinoma) in male rats and female mice and guinea pigs. In female mice, it also caused benign lung tumors (adenoma) and lymphoma. Lung tumors also resulted from exposure by injection in rats. In addition, injection exposure caused (1) digestive-tract tumors in rats, gerbils, and hamsters, (2) tumors of the pancreas, small intestine, and abdominal cavity in guinea pigs, (3) leukemia or lymphoma in newborn and adult mice and lymphoma in adult rats, (4) blood-vessel tumors in guinea pigs, rabbits, and dogs, (5) mammary-gland tumors in rats, and (6) tumors of the heart (sarcoma) and at the injection site in hamsters. Dermal exposure caused leukemia and benign and malignant skin tumors in mice and skin cancer (squamous or basal-cell carcinoma) in rats and hamsters. Intravesicular instillation caused benign and malignant urinary-bladder tumors (transitional-cell papilloma and carcinoma) in female rats (IARC 1972, 1978).

Since *N*-nitroso-*N*-methylurea was listed in the *Second Annual Report on Carcinogens,* additional studies in experimental animal have been identified that confirmed the induction of several tumor types previously observed and reported tumors at additional tissue sites, in additional species, and by additional routes of exposure:

- Tumors of the thymus were observed in rats following oral exposure (Lijinsky and Kovatch 1996).
- Tumors of the teeth (odontoma, odontoameloblastoma, and ameloblastoma) were observed in male rats exposed by injection (Smulow *et al.* 1983).
- Female shrews developed uterine and cervical tumors following intrarectal administration (Yang *et al.* 1996).
- In fish, addition of *N*-nitroso-*N*-methylurea to the tank water caused eye tumors (neuroblastoma) (Schwab *et al.* 1979).
- Placement of *N*-nitroso-*N*-methylurea–impregnated sutures in the ovaries of female rats caused benign or malignant ovarian tumors (adenoma or adenocarcinoma) (Tunca *et al.* 1985).
- Implantation of *N*-nitroso-*N*-methylurea, as well as injection, caused mammary-gland cancer (adenocarcinoma) in female rats (Thompson and Meeker 1983, Holtzman *et al.* 1985, Seaborn and Yang 1993, Takahashi *et al.* 1995).
- Administration of *N*-nitroso-*N*-methylurea directly into the stomach via a surgically formed external opening caused cancer of the forestomach (carcinoma) in rats of both sexes (Garcia-Gonzalez *et al.* 2000).

Oral administration, intrarectal administration, and injection of *N*-nitroso-*N*-methylurea in several species (adult monkeys, mice, rats, gerbils, or hamsters) caused tumors of the digestive tract (esophagus, glandular stomach, forestomach, small intestine, or colon) or respiratory tract (oropharynx or lung) (Adamson and Sieber 1979, Zimmerman *et al.* 1982, Eisenberg *et al.* 1983, Likhachey *et al.* 1983, Smulow *et al.* 1983, Yano *et al.* 1984, Wang *et al.* 1985, Verdeal *et al.* 1986, Beland *et al.* 1988, Bosland *et al.* 1992, Furukawa *et al.* 1992, Tatematsu *et al.* 1992, 1993, 1994, 1998, Lijinsky and Kovatch 1996, Tamano *et al.* 1996, Yang *et al.* 1996). Blood-vessel cancer (hemangioendothelial sarcoma) also was reported in mice (Tatematsu *et al.* 1993, 1998) and leukemia and tumors of the prostate, Zymbal gland, uterus, adrenal glands, and blood vessels in rats (Eisenberg *et al.* 1983, Verdeal *et al.* 1986, Bosland *et al.* 1992, Tamano *et al.* 1996).

Cancer Studies in Humans

No epidemiological studies were identified that evaluated the relationship between human cancer and exposure specifically to *N*-nitroso-*N*-methylurea.

Properties

N-Nitroso-*N*-methylurea is a nitrosamine compound that exists as a colorless to yellow crystal or plate at room temperature (HSDB 2009). It is soluble in water, alcohol, ether, acetone, benzene, chloroform, and other polar organic solvents and insoluble in nonpolar organic solvents. It decomposes in alkaline solution at a rate that depends on pH (IARC 1978). The pure compound is sensitive to moisture and

light and should be stored under refrigeration. Physical and chemical properties of *N*-nitroso-*N*-methylurea are listed in the following table.

Property	Information
Molecular weight	103.1[a]
Melting point	124°C (decomposes)[a]
Log K_{ow}	–0.03[a]
Water solubility	14.4 g/L at 24°C[a]
Vapor pressure	0.0293 mm Hg at 25°C[b]

Sources: [a]HSDB 2009, [b]ChemIDplus 2009.

Use

N-Nitroso-*N*-methylurea was once widely used to synthesize diazomethane in the laboratory; however, it has been replaced by other reagents for this use (IARC 1972, 1978, HSDB 2009). *N*-Nitroso-*N*-methylurea has been studied as a chemotherapeutic agent in cancer treatment, either alone or in combination with cyclophosphamide. Small quantities are used in research to study its mutagenic effects on plants.

Production

N-Nitroso-*N*-methylurea has never been produced commercially in the United States (IARC 1978, HSDB 2009). In 2009, it was available in small quantities for research purposes from eight U.S. suppliers (ChemSources 2009). The United States International Trade Commission does not provide specific information on imports and exports of *N*-nitroso-*N*-methylurea. Import and export data are reported for U.S. Census Bureau Schedule B category 29.29, which includes *N*-nitroso-*N*-methylurea and related compounds (USCB 2018).

Exposure

The potential for human exposure in the United States is limited, because *N*-nitroso-*N*-methylurea is not produced or used in large quantities (IARC 1978, HSDB 2009). Cancer patients potentially were directly exposed by injection when *N*-nitroso-*N*-methylurea was tested as a chemotherapeutic agent in conjunction with cyclophosphamide; however, no data were found on the frequency or extent of this testing (IARC 1978). According to the U.S. Environmental Protection Agency's Toxics Release Inventory, two facilities released a total of 170 lb of *N*-nitroso-*N*-methylurea to the environment in 1999, 96% of which was from one facility. Releases totaled 260 lb in 2001 and 10 lb in 2005, 2006, and 2007 (TRI 2009). In air, *N*-nitroso-*N*-methylurea exists solely as vapor and is degraded by reaction with photochemically produced hydroxyl radicals, with an estimated half-life of 10 days. In water, it hydrolyzes, with a half-life of 1.2 hours at pH 7 at 20°C.

Occupational exposure to *N*-nitroso-*N*-methylurea may occur through inhalation or dermal contact at facilities where it is used in research (HSDB 2009). During clinical testing for its use as a chemotherapeutic agent, health professionals such as pharmacists, physicians, and nurses could have been exposed during preparation and administration of the drug or during clean-up (IARC 1978).

Regulations

Consumer Product Safety Commission (CPSC)

A voluntary standard provides that rubber pacifiers shall not contain more than 10 ppb of any single nitrosamine or more than 20 ppb of total nitrosamines.

Environmental Protection Agency (EPA)

Clean Air Act

National Emission Standards for Hazardous Air Pollutants: Listed as a hazardous air pollutant.

Clean Water Act

Effluent Guidelines: Nitrosamines are listed as a toxic pollutant.

Water Quality Criteria: Based on fish or shellfish and water consumption = 0.0008 µg/L for nitrosamines; based on fish or shellfish consumption only = 1.24 µg/L for nitrosamines.

Comprehensive Environmental Response, Compensation, and Liability Act

Reportable quantity (RQ) = 1 lb.

Emergency Planning and Community Right-To-Know Act

Toxics Release Inventory: Listed substance subject to reporting requirements.

Resource Conservation and Recovery Act

Listed Hazardous Waste: Waste code for which the listing is based wholly or partly on the presence of *N*-nitroso-*N*-methylurea = U177.

Listed as a hazardous constituent of waste.

Toxic Substances Control Act

Nitrosating agents distributed in commerce require warning labels and instructions on use.

Food and Drug Administration (FDA, an HHS agency)

The action level for *N*-nitrosamines in rubber baby-bottle nipples is 10 ppb.

In order to use nitrites and/or nitrates as food additives in curing premixes, a petition must be filed supported by data demonstrating that nitrosamines are not formed.

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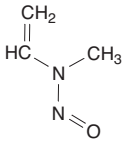
N-Nitrosomethylvinylamine

CAS No. 4549-40-0

Reasonably anticipated to be a human carcinogen

First listed in the *Second Annual Report on Carcinogens* (1981)

Also known as *N*-methylvinylnitrosoamine



Carcinogenicity

N-Nitrosomethylvinylamine *is reasonably anticipated to be a human carcinogen* based on sufficient evidence of carcinogenicity from studies in experimental animals.

Cancer Studies in Experimental Animals

N-Nitrosomethylvinylamine caused tumors in rats at several different tissue sites and by two different routes of exposure. Administration of *N*-nitrosomethylvinylamine in the drinking water caused cancer (carcinoma) of the tongue and pharynx and benign and malignant tumors of the esophagus (mainly squamous-cell carcinoma), and inhalation exposure caused cancer of the nasal cavity (squamous-cell carcinoma) (IARC 1978).

Cancer Studies in Humans

No epidemiological studies were identified that evaluated the relationship between human cancer and exposure specifically to *N*-nitrosomethylvinylamine.

Properties

N-Nitrosomethylvinylamine is a nitrosamine compound that is a yellow liquid at room temperature (HSDB 2009). It is soluble in water, lipids, and organic solvents. It is relatively unstable and decomposes in solution (up to 10% in 24 hours), and it is sensitive to light, especially ultraviolet light (IARC 1978). Physical and chemical properties of *N*-nitrosomethylvinylamine are listed in the following table.

Property	Information
Molecular weight	86.1[a]
Boiling point	47°C to 48°C at 30 mm Hg[b]
Log K_{ow}	–0.28[b]
Water solubility	30 g/L[b]
Vapor pressure	8.96 mm Hg at 25°C[b]

Sources: [a]HSDB 2009, [b]ChemIDplus 2009.

Use

N-Nitrosomethylvinylamine is used as a research chemical; no other uses were identified (IARC 1978, HSDB 2009).

Production

There is no evidence that *N*-nitrosomethylvinylamine has ever been produced commercially in the United States (IARC 1978, HSDB 2009). In 2009, it was available in small quantities for research purposes from one supplier worldwide, in the United States (ChemSources 2009).

Exposure

Exposure to *N*-nitrosomethylvinylamine is limited primarily to the individuals using it in research (HSDB 2009). According to the U.S. Environmental Protection Agency's Toxics Release Inventory, environmental releases of *N*-nitrosomethylvinylamine occurred only in 1999 (157 lb), 2002 (10 lb), and 2003 (26 lb) (TRI 2009).

Regulations

Consumer Product Safety Commission (CPSC)

A voluntary standard provides that rubber pacifiers shall not contain more than 10 ppb of any single nitrosamine or more than 20 ppb of total nitrosamines.

Environmental Protection Agency (EPA)

Clean Water Act

Effluent Guidelines: Nitrosamines are listed as a toxic pollutant.
Water Quality Criteria: Based on fish or shellfish and water consumption = 0.0008 µg/L for nitrosamines; based on fish or shellfish consumption only = 1.24 µg/L for nitrosamines.

Comprehensive Environmental Response, Compensation, and Liability Act

Reportable quantity (RQ) = 10 lb.

Emergency Planning and Community Right-To-Know Act

Toxics Release Inventory: Listed substance subject to reporting requirements.

Resource Conservation and Recovery Act

Listed Hazardous Waste: Waste code for which the listing is based wholly or partly on the presence of *N*-nitrosomethylvinylamine = P084.
Listed as a hazardous constituent of waste.

Toxic Substances Control Act

Nitrosating agents distributed in commerce require warning labels and instructions on use.

Food and Drug Administration (FDA, an HHS agency)

The action level for *N*-nitrosamines in rubber baby-bottle nipples is 10 ppb.
In order to use nitrites and/or nitrates as food additives in curing premixes a petition must be filed supported by data demonstrating that nitrosamines are not formed.

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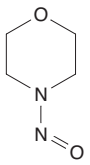
N-Nitrosomorpholine

CAS No. 59-89-2

Reasonably anticipated to be a human carcinogen

First listed in the *Second Annual Report on Carcinogens* (1981)



Carcinogenicity

N-Nitrosomorpholine is *reasonably anticipated to be a human carcinogen* based on sufficient evidence of carcinogenicity from studies in experimental animals.

Cancer Studies in Experimental Animals

N-Nitrosomorpholine caused tumors in several species of experimental animals, at several different tissue sites, and by several different routes of exposure. Tumors of the liver or bile duct were observed in several species and by several routes of exposure. Administration of *N*-nitrosomorpholine in the drinking water caused benign liver tumors (hepatocellular adenoma) in male mice and benign or malignant liver and bile-duct tumors (hepatocellular carcinoma, cholangiofibroma, or cholangiocarcinoma) in rats. Intravenous injection of *N*-nitrosomorpholine caused liver cancer (hepatocellular carcinoma) in rats, and addition of *N*-nitrosomorpholine to the tank water caused benign and malignant liver tumors (hepatocellular adenoma and carcinoma) in two species of fish (IARC 1978).

Administration of *N*-nitrosomorpholine in the drinking water also caused benign lung tumors (adenoma) in male mice and blood-vessel cancer (hemangiosarcoma and hemangioendothelioma) and kidney tumors (epithelial tumors) in rats. Tumors of the respiratory tract (primarily the nasal cavities and trachea) and upper digestive tract occurred in hamsters of both sexes exposed by subcutaneous injection, and cancer of the nasal cavity in rats exposed by intravenous injection (IARC 1978).

Since *N*-nitrosomorpholine was listed in the *Second Annual Report on Carcinogens,* additional studies in experimental animals have been identified, some of which reported induction of tumors by additional routes of exposure or at an additional tissue site:

- Administration of *N*-nitrosomorpholine in the drinking water or by stomach tube caused respiratory- or digestive-tract tumors in hamsters (Ketkar *et al.* 1983, Lijinsky *et al.* 1984, Cardesa *et al.* 1990).
- Inhalation exposure caused forestomach tumors in male rats, tracheal tumors in male hamsters, and liver tumors in female rats and male hamsters (Klein *et al.* 1990).
- Intratracheal instillation caused tracheal tumors in male hamsters (Ishinishi *et al.* 1988).
- Intravesicular instillation caused liver and nasal-cavity tumors in female rats (Lijinsky *et al.* 1991).
- Tumors of the esophagus occurred in female rats exposed to *N*-nitrosomorpholine in the drinking water (Lijinsky *et al.* 1988).

Cancer Studies in Humans

No epidemiological studies were identified that evaluated the relationship between human cancer and exposure specifically to *N*-nitrosomorpholine.

Properties

N-Nitrosomorpholine is a nitrosamine compound that exists as yellow crystals at room temperature (HSDB 2009). It is completely miscible with water and soluble in organic solvents. It is stable in the dark in neutral or alkaline solution for at least 14 days, but is less stable in more acidic solutions or in light, especially ultraviolet light (IARC 1978). Physical and chemical properties of *N*-nitrosomorpholine are listed in the following table.

Property	Information
Molecular weight	116.1[a]
Melting point	29°C[a]
Boiling point	224°C to 224.5°C at 747 mm Hg[a]
Log K_{ow}	–0.44[a]
Water solubility	1,000 g/L at 24°C[b]
Vapor pressure	0.036 mm Hg at 20°C[b]
Dissociation constant (pK_b)	12.41[c]

Sources: [a]HSDB 2009, [b]ChemIDplus 2009, [c]Akron 2009.

Use

N-Nitrosomorpholine is used as a research chemical. Although it was found to be effective as an antimicrobial agent, and patents were issued for its use as a solvent for polyacrylonitrile and as an intermediate in the production of *N*-aminomorpholine, there is no evidence that it is used commercially in the United States (IARC 1978).

Production

There is no evidence that *N*-nitrosomorpholine is produced commercially in the United States. In 2009, it was available in small quantities for research purposes from nine U.S. suppliers (ChemSources 2009).

Exposure

The routes of potential human exposure to *N*-nitrosomorpholine are dermal contact, ingestion, and inhalation (HSDB 2009). *N*-Nitrosamines are formed by reactions of precursors (nitrosating agents and primary or secondary amines) that are present in industrial processes, foods, or the human body (Schothorst and Somers 2005). *N*-Nitroso compounds have been identified in a variety of vegetables, fruits, cheeses, meats, and alcoholic beverages (Brunnemann *et al.* 1982b). *N*-Nitroso compounds may be formed from amines and quaternary ammonium salts by reaction with nitrosating agents, such as nitrite, in the stomach or during cooking processes. The degree of this potential exposure is unknown, but is assumed to be intermittent and at relatively low levels. *N*-Nitrosomorpholine was found in tobacco snuff at concentrations of 24 to 690 ppb (Brunnemann *et al.* 1982a) and in rubber nipples for baby bottles at 3.0 to 14.1 ppb (HSDB 2009). According to the U.S. Environmental Protection Agency's Toxics Release Inventory, 21 lb of *N*-nitrosomorpholine was released to the environment in 2005, of which 20 lb was released to a hazardous-waste landfill and 1 lb to an off-site hazardous-waste underground injection well (TRI 2009).

Workers in chemical research laboratories and in the rubber and tire manufacturing industry may be exposed to *N*-nitrosomorpholine. *N*-Nitrosomorpholine concentrations in air ranged from 0.07 to 5.1 μg/m^3 in a tire factory and from 0.6 to 27 μg/m^3 in an aircraft tire factory. *N*-Nitrosomorpholine was detected as a contaminant in analytical-grade dichloromethane at 10 to 32 μg/L and in chloroform at 2 to 376 μg/L (IARC 1978).

Regulations

Consumer Product Safety Commission (CPSC)

A voluntary standard provides that rubber pacifiers shall not contain more than 10 ppb of any single nitrosamine or more than 20 ppb of total nitrosamines.

Environmental Protection Agency (EPA)

Clean Air Act

National Emission Standards for Hazardous Air Pollutants: Listed as a hazardous air pollutant.

Clean Water Act

Effluent Guidelines: Nitrosamines are listed as a toxic pollutant.

Water Quality Criteria: Based on fish or shellfish and water consumption = 0.0008 µg/L for nitrosamines; based on fish or shellfish consumption only = 1.24 µg/L for nitrosamines.

Comprehensive Environmental Response, Compensation, and Liability Act

Reportable quantity (RQ) = 1 lb.

Emergency Planning and Community Right-To-Know Act

Toxics Release Inventory: Listed substance subject to reporting requirements.

Resource Conservation and Recovery Act

Listed as a hazardous constituent of waste.

Toxic Substances Control Act

Nitrosating agents distributed in commerce require warning labels and instructions on use.

Food and Drug Administration (FDA, an HHS agency)

The action level for *N*-nitrosamines in rubber baby-bottle nipples is 10 ppb.

In order to use nitrites and/or nitrates as food additives in curing premixes, a petition must be filed supported by data demonstrating that nitrosamines are not formed.

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Ketkar MB, Holste J, Preussmann R, Althoff J. 1983. Carcinogenic effect of nitrosomorpholine administered in the drinking water to Syrian golden hamsters. *Cancer Lett* 17(3): 333-338.

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Lijinsky W, Kovatch RM, Riggs CW, Walters PT. 1988. Dose response study with *N*-nitrosomorpholine in the drinking water of F-344 rats. *Cancer Res* 48(8): 2089-2095.

Lijinsky W, Thomas BJ, Kovatch RM. 1991. Local and systemic carcinogenic effects of alkylating carcinogens in rats treated by intravascular administration. *Jpn J Cancer Res* 82(9): 980-986.

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TRI. 2009. *TRI Explorer Chemical Report*. U.S. Environmental Protection Agency. Last updated: 3/19/09. http://www.epa.gov/triexplorer and select *N*-Nitrosomorpholine.

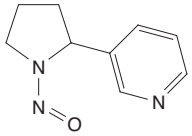
N-Nitrosonornicotine

CAS No. 16543-55-8

Reasonably anticipated to be a human carcinogen

First listed in the *Second Annual Report on Carcinogens* (1981)

Also known as NNN



Carcinogenicity

N-Nitrosonornicotine is *reasonably anticipated to be a human carcinogen* based on sufficient evidence of carcinogenicity from studies in experimental animals.

Cancer Studies in Experimental Animals

N-Nitrosonornicotine caused tumors in several species of experimental animals, at several different tissue sites, and by several different routes of exposure. Administration of *N*-nitrosonornicotine in the drinking water of rats of both sexes caused cancer of the nasal cavity (carcinoma in males and adenocarcinoma in females) and benign and malignant esophageal tumors (papilloma and carcinoma). Subcutaneous injection of *N*-nitrosonornicotine caused benign tracheal tumors (papilloma) in hamsters of both sexes, and intraperitoneal injection caused benign lung tumors (adenoma) in mice of both sexes (IARC 1978).

Since *N*-nitrosonornicotine was listed in the *Second Annual Report on Carcinogens,* additional experimental animal studies have been identified. *N*-Nitrosonornicotine was reported to cause nasal tumors in rodents by the following additional routes of exposure: (1) by stomach tube or dietary exposure in male rats (IARC 1985, Griciute *et al.* 1986), (2) by administration in the drinking water and by intraperitoneal injection in hamsters of both sexes (IARC 1985), and (3) by subcutaneous injection in male rats, male hamsters, and female mink (IARC 1985, Koppang *et al.* 1992, 1997, IARC 2007). The types of nasal tumors varied among the studies, but mainly consisted of the malignant tumor esthesioneuroepithelioma (also known as olfactory neuroblastoma), which arises from the olfactory nerves, and benign tumors (mainly adenoma). In addition, exposure to *N*-nitrosonornicotine by stomach tube or in the diet caused cancer of the esophagus (squamous-cell carcinoma) in male rats (IARC 1985, Griciute *et al.* 1986); subcutaneous injection caused benign lung tumors (adenoma) in rats of both sexes; and intraperitoneal injection caused benign tracheal tumors (papilloma) in male hamsters. *N*-Nitrosonornicotine administered by oral swabbing (of the tongue or cheek pouch) caused tumors of the lung, forestomach, and liver in male mice and in hamsters of both sexes (Padma *et al.* 1989).

Cancer Studies in Humans

No epidemiological studies were identified that evaluated the relationship between human cancer and exposure specifically to *N*-nitrosonornicotine.

Properties

N-Nitrosonornicotine is a nitrosamine compound that exists as a yellow oil at room temperature, but solidifies on standing in the cold (HSDB 2009). It is soluble in water (ChemIDplus 2009). Physical and chemical properties of *N*-nitrosonornicotine are listed in the following table.

Property	Information
Molecular weight	177.2[a]
Melting point	47°C[a]
Boiling point	154°C at 0.2 mm Hg[a]
Log K_{ow}	0.32[b]
Water solubility	1,000 g/L at 25°C[b]
Vapor pressure	0.2 mm Hg at 154°C[a]
Dissociation constant (pK_b)	8.84[c]

Sources: [a]HSDB 2009, [b]ChemIDplus 2009, [c]Akron 2009.

Use

The only known use of *N*-nitrosonornicotine is as a research chemical (IARC 1985).

Production

There is no evidence that *N*-nitrosonornicotine has been produced commercially in the United States (IARC 1985). In 2009, it was available in small quantities for research purposes from two U.S. suppliers (ChemSources 2009).

Exposure

N-Nitrosonornicotine has been found in a variety of tobacco products (chewing tobacco, snuff, cigarettes, and cigars) and in mainstream and sidestream smoke from cigars and cigarettes, in the saliva of chewers of betel quid with tobacco, and in the saliva of oral-snuff users (IARC 1978, 1985). Some of the *N*-nitrosonornicotine in saliva appears to be formed endogenously from nitrite in saliva and tobacco alkaloids. Thus, there is widespread exposure to *N*-nitrosonornicotine among users of tobacco products and those exposed to sidestream smoke.

N-Nitrosonornicotine is produced by nitrosation of nicotine during the curing, aging, processing, and smoking of tobacco. About half of the *N*-nitrosonornicotine originates in the unburnt tobacco, whereas the remainder is formed during burning. *N*-Nitrosonornicotine has been found in cigarettes at concentrations of up to 11.9 mg/kg, in snuff products at up to 77.1 mg/kg, and in chewing tobacco at up to 90.6 mg/kg. The differences in *N*-nitrosonornicotine concentrations in tobacco products are largely due to differences in the tobacco types used in a given product, agricultural practices, curing methods, and manufacturing processes. *N*-Nitrosonornicotine is formed primarily from its corresponding secondary amine (nornicotine) in the early stages of tobacco curing and processing. Some *N*-nitrosonornicotine is formed from the tertiary amine (nicotine) at the later stages of tobacco curing and fermentation. Levels of *N*-nitrosonornicotine are consistently higher in Burley than in Bright tobacco, regardless of the curing method. However, flue-curing of Bright tobacco produces nearly three times as much nitrosamine as air-curing of the same tobacco. *N*-Nitrosonornicotine has been found in cigarette smoke at up to 3.7 µg per cigarette (IARC 1978, 1985).

Regulations

Consumer Product Safety Commission (CPSC)

A voluntary standard provides that rubber pacifiers shall not contain more than 10 ppb of any single nitrosamine or more than 20 ppb of total nitrosamines.

Environmental Protection Agency (EPA)

Clean Water Act

Effluent Guidelines: Nitrosamines are listed as a toxic pollutant.
Water Quality Criteria: Based on fish or shellfish and water consumption = 0.0008 µg/L for nitrosamines; based on fish or shellfish consumption only = 1.24 µg/L for nitrosamines.

Emergency Planning and Community Right-To-Know Act

Toxics Release Inventory: Listed substance subject to reporting requirements.

Resource Conservation and Recovery Act

Listed as a hazardous constituent of waste.

Toxic Substances Control Act

Nitrosating agents distributed in commerce require warning labels and instructions on use.

Food and Drug Administration (FDA, an HHS agency)

The action level for *N*-nitrosamines in rubber baby-bottle nipples is 10 ppb.
In order to use nitrites and/or nitrates as food additives in curing premixes a petition must be filed supported by data demonstrating that nitrosamines are not formed.

References

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IARC. 2007. Some tobacco-specific *N*-nitrosamines. *In Smokeless Tobacco and Some Tobacco-specific* N-*Nitrosamines*. IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Humans, vol. 89. Lyon, France: International Agency for Research on Cancer. pp. 421-583.

Koppang N, Rivenson A, Reith A, Dahle HK, Evensen O, Hoffmann D. 1992. A study of tobacco carcinogenesis XLVIII. Carcinogenicity of *N'*-nitrosonornicotine in mink *(Mustela vison)*. *Carcinogenesis* 13(11): 1957-1960.

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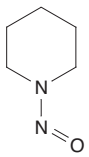
Padma PR, Lalitha VS, Amonkar AJ, Bhide SV. 1989. Carcinogenicity studies on the two tobacco-specific *N*-nitrosamines, *N'*-nitrosonornicotine and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone. *Carcinogenesis* 10(11): 1997-2002.

N-Nitrosopiperidine

CAS No. 100-75-4

Reasonably anticipated to be a human carcinogen

First listed in the *Second Annual Report on Carcinogens* (1981)



Carcinogenicity

N-Nitrosopiperidine is *reasonably anticipated to be a human carcinogen* based on sufficient evidence of carcinogenicity from studies in experimental animals.

Cancer Studies in Experimental Animals

N-Nitrosopiperidine caused tumors in several species of experimental animals, at several different tissue sites, and by several different routes of exposure. Tumors occurred mainly in the respiratory tract, upper digestive tract, and liver (IARC 1978). Benign lung tumors (adenoma) occurred in mice administered *N*-nitrosopiperidine in the diet or drinking water or by intraperitoneal injection. Benign and malignant nasal-cavity tumors (cholesteatoma, esthesioneuroepithelioma, and squamous-cell carcinoma) and cancer of the pharynx (carcinoma) were observed in rats exposed by subcutaneous or intravenous injection. When administered as a single dose to pregnant hamsters, *N*-nitrosopiperidine caused respiratory-tract tumors

at a much higher incidence in the mothers than in the offspring. Upper-digestive-tract tumors caused by *N*-nitrosopiperidine included cancer of the forestomach (squamous-cell carcinoma) and benign esophageal tumors (papilloma) following dietary administration to male mice and benign and malignant esophageal tumors (papilloma and squamous-cell carcinoma) in rats exposed via the drinking water or by subcutaneous or intravenous injection. Benign and/or malignant liver tumors (hepatocellular adenoma or carcinoma) occurred in male mice administered *N*-nitrosopiperidine in the diet and rats and monkeys administered *N*-nitrosopiperidine in the drinking water. Tumors of the respiratory tract, upper digestive tract, and liver also occurred in hamsters administered *N*-nitrosopiperidine by subcutaneous injection. One study reported blood-vessel cancer (hemangioendothelioma) in male mice exposed to *N*-nitrosopiperidine in the diet.

Since *N*-nitrosopiperidine was listed in the *Second Annual Report on Carcinogens,* additional studies in experimental animals have been identified. *N*-Nitrosopiperidine administered in the drinking water caused benign and malignant upper-respiratory-tract tumors in hamsters of both sexes (Cardesa *et al.* 1990) and liver cancer (hepatocellular carcinoma) in monkeys exposed by intraperitoneal injection or dietary administration (Adamson and Sieber 1979).

Cancer Studies in Humans

No epidemiological studies were identified that evaluated the relationship between human cancer and exposure specifically to *N*-nitrosopiperidine.

Properties

N-Nitrosopiperidine is a nitrosamine compound that exists as a yellow oil at room temperature (HSDB 2009). It is soluble in water, hydrochloric acid, organic liquids, and lipids. Physical and chemical properties of *N*-nitrosopiperidine are listed in the following table.

Property	Information
Molecular weight	114.2[a]
Specific gravity	1.0631 at 18.5°C/4°C[a]
Boiling point	219°C[a]
Log K_{ow}	0.36[a]
Water solubility	76.5 g/L at 24°C[b]
Vapor pressure	0.092 mm Hg at 20°C[b]

Sources: [a]HSDB 2009, [b]ChemIDplus 2009.

Use

N-Nitrosopiperidine is used as a research chemical (HSDB 2009); no other uses were identified.

Production

N-Nitrosopiperidine was first prepared in 1863 by the action of nitrogen dioxide on piperidine (IARC 1978). Although numerous patents have been issued for the production of *N*-nitrosopiperidine, there is no evidence that it has been manufactured commercially in the United States. In 2009, it was available in small quantities for research purposes from eight U.S. suppliers (ChemSources 2009). The United States International Trade Commission does not provide specific information on imports and exports of *N*-nitrosopiperidine. Import and export data are reported for U.S. Census Bureau Schedule B category 29.33, which includes *N*-nitrosopiperidine and related compounds (USCB 2018).

Exposure

Because only small quantities of *N*-nitrosopiperidine are produced for research, potential exposure appears to be limited. The general population may be exposed to low concentrations of *N*-nitrosopiperidine from cigarette smoke and certain foods (IARC 1978). Trace amounts of *N*-nitrosopiperidine were found in cigarettes, but it was not found in all brands of cigarettes tested. *N*-Nitrosopiperidine was found at concentrations of up to 64 µg/kg in meat and fish products such as bacon, bologna, wieners, and smoked cod. The presence of *N*-nitrosopiperidine in meat, cheese, and spices results from the preservative use of sodium nitrite, which reacts with the amines present in meats and cheese to form nitrosamines. According to the U.S. Environmental Protection Agency's Toxics Release Inventory, environmental releases of *N*-nitrosopiperidine were 14,756 lb in 1999 and 19,309 lb in 2001; most was released to on-site hazardous-waste landfills, and a small portion was released to off-site non-hazardous-waste landfills. In 2002 and thereafter, much smaller total quantities (≤ 500 lb) were released to off-site hazardous-waste landfills (TRI 2009).

Regulations

Consumer Product Safety Commission (CPSC)

A voluntary standard provides that rubber pacifiers shall not contain more than 10 ppb of any single nitrosamine or more than 20 ppb of total nitrosamines.

Environmental Protection Agency (EPA)

Clean Water Act

Effluent Guidelines: Nitrosamines are listed as a toxic pollutant.
Water Quality Criteria: Based on fish or shellfish and water consumption = 0.0008 µg/L for nitrosamines; based on fish or shellfish consumption only = 1.24 µg/L for nitrosamines.

Comprehensive Environmental Response, Compensation, and Liability Act

Reportable quantity (RQ) = 10 lb.

Emergency Planning and Community Right-To-Know Act

Toxics Release Inventory: Listed substance subject to reporting requirements.

Resource Conservation and Recovery Act

Listed Hazardous Waste: Waste code for which the listing is based wholly or partly on the presence of *N*-nitrosopiperidine = U179.
Listed as a hazardous constituent of waste.

Toxic Substances Control Act

Nitrosating agents distributed in commerce require warning labels and instructions on use.

Food and Drug Administration (FDA, an HHS agency)

The action level for *N*-nitrosamines in rubber baby-bottle nipples is 10 ppb.
In order to use nitrites and/or nitrates as food additives in curing premixes, a petition must be filed supported by data demonstrating that nitrosamines are not formed.

References

Adamson R, Sieber S. 1979. The use of nonhuman primates for chemical carcinogenisis studies. In *Regulatory Aspects of Carcinogenesis and Food Additives: the Delaney Clause*. San Francisco: Academic Press. pp. 275-302.

Cardesa A, Garcia-Bragado F, Ramirez J, Ernst H. 1990. Histological types of laryngotracheal tumors induced in Syrian golden hamsters by nitrosomorpholine and nitrosopiperidine. *Exp Pathol* 40(4): 267-281.

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ChemSources. 2009. *Chem Sources - Chemical Search.* Chemical Sources International. http://www.chemsources.com/chemonline.html and search on nitrosopiperidine. Last accessed: 10/7/09.

HSDB. 2009. *Hazardous Substances Data Bank.* National Library of Medicine. http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB and search on CAS number. Last accessed: 10/7/09.

IARC. 1978. *N*-Nitrosopiperidine. In *Some* N-*nitroso compounds*. IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans, vol. 17. Lyon, France: International Agency for Research on Cancer. pp. 287-301.

TRI. 2009. *TRI Explorer Chemical Report.* U.S. Environmental Protection Agency. Last updated: 3/19/09. http://www.epa.gov/triexplorer and select *N*-Nitrosopiperidine.

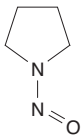
USCB. 2018. Chapter 29 - Organic Chemicals. In *U.S. Census Bureau - Foreign Trade Schedule B (2018)*. https://uscensus.prod.3ceonline.com and search on CAS number or substance name. Last accessed: 10/10/18.

N-Nitrosopyrrolidine

CAS No. 930-55-2

Reasonably anticipated to be a human carcinogen

First listed in the *Second Annual Report on Carcinogens* (1981)



Carcinogenicity

N-Nitrosopyrrolidine is *reasonably anticipated to be a human carcinogen* based on sufficient evidence of carcinogenicity from studies in experimental animals.

Cancer Studies in Experimental Animals

Oral exposure to *N*-nitrosopyrrolidine caused tumors in two rodent species and at two different tissue sites. Administered in the drinking water, it caused liver cancer (hepatocellular carcinoma) in several strains of rats (both sexes) and benign lung tumors (adenoma) in mice of both sexes (IARC 1978).

Since *N*-nitrosospyrrolidine was listed in the *Second Annual Report on Carcinogens,* additional studies in rodents have been identified. Liver tumors were observed in hamsters exposed to *N*-nitrosopyrrolidine in the drinking water; tumor incidence increased with increasing dose (Ketkar *et al.* 1982). *N*-Nitrosospyrrolidine administered by intraperitoneal injection to hamsters caused tumors of the larynx or trachea 25 weeks after a single injection and preneoplastic and neoplastic nasal-cavity lesions 25 weeks after two injections. In female strain A/J mice (a strain with a high spontaneous incidence of lung tumors), *N*-nitrosopyrrolidine administered by intraperitoneal injection increased the incidence of benign lung tumors and the number of tumors per animal (Hecht *et al.* 1988, Hoffmann *et al.* 1993).

Cancer Studies in Humans

No epidemiological studies were identified that evaluated the relationship between human cancer and exposure specifically to *N*-nitrosopyrrolidine.

Properties

N-Nitrosopyrrolidine is a nitrosamine compound that is a yellow liquid at room temperature (HSDB 2009). It is totally soluble in water, organic liquids, and lipids. It is stable at room temperature in the dark, but is sensitive to light, especially ultraviolet light (IARC 1978). Physical and chemical properties of *N*-nitrosopyrrolidine are listed in the following table.

Property	Information
Molecular weight	100.1[a]
Specific gravity	1.1[a]
Boiling point	214°C at 760 mm Hg[a]
Log K_{ow}	–0.19[a]
Water solubility	1,000 g/L at 24°C[b]
Vapor pressure	0.06 at 20°C[b]

Sources: [a]HSDB 2009, [b]ChemIDplus 2009.

Use

N-Nitrosopyrrolidine is used primarily as a research chemical and is not produced commercially in the United States (IARC 1978, HSDB 2009).

Production

N-Nitrosopyrrolidine was first prepared in 1888 by the reaction of pyrrolidine with potassium nitrate in a weak hydrochloric acid solution (IARC 1978). It is not produced commercially in the United States. In 2009, it was available in small quantities for research purposes from eight U.S. suppliers (ChemSources 2009). The United States International Trade Commission does not provide specific information on imports and exports of *N*-nitrosopyrrolidine. Import and export data are reported for U.S. Census Bureau Schedule B category 29.33, which includes *N*-nitrosopyrrolidine and related compounds (USCB 2018)

Exposure

N-Nitrosopyrrolidine is produced when foods preserved with or contaminated by nitrite, especially fatty foods, are prepared by heating. Exposure can occur through inhalation of vapors released during cooking or ingestion of food (IARC 1978). In recent years, lower concentrations of sodium nitrite in foods have resulted in lower concentrations of *N*-nitrosopyrrolidine in foods. For example, the *N*-nitrosopyrrolidine content of bacon decreased from approximately 67 µg/kg in 1971 through 1974 to 17 µg/kg in 1975 and 1976; when bacon is fried, an average of 50% of the *N*-nitrosopyrrolidine normally present in the meat is detected in the vapor. Dry premixed cures containing spices and sodium nitrite originally contained *N*-nitrosopyrrolidine at a concentration of 40 µg/kg, but the level increased to 520 µg/kg after six months of storage. *N*-Nitrosopyrrolidine was found in tobacco smoke at concentrations of up to 0.113 µg per cigarette and in pipe-bowl scrapings at up to 1.6 mg/kg of residue. Wastewater from chemical factories was reported to contain *N*-nitrosopyrrolidine at concentrations of 0.09 to 0.20 µg/L.

Regulations

Consumer Product Safety Commission (CPSC)

A voluntary standard provides that rubber pacifiers shall not contain more than 10 ppb of any single nitrosamine or more than 20 ppb of total nitrosamines.

Environmental Protection Agency (EPA)

Clean Water Act

Effluent Guidelines: Nitrosamines are listed as a toxic pollutant.

Water Quality Criteria: Based on fish or shellfish and water consumption = 0.016 µg/L; based on fish or shellfish consumption only = 34 µg/L.

Comprehensive Environmental Response, Compensation, and Liability Act

Reportable quantity (RQ) = 1 lb.

Resource Conservation and Recovery Act

Listed Hazardous Waste: Waste code for which the listing is based wholly or partly on the presence of *N*-nitrosopyrrolidine = U180.

Listed as a hazardous constituent of waste.

Toxic Substances Control Act

Nitrosating agents distributed in commerce require warning labels and instructions on use.

Food and Drug Administration (FDA, an HHS agency)

The action level for *N*-nitrosamines in rubber baby-bottle nipples is 10 ppb.

In order to use nitrites and/or nitrates as food additives in curing premixes a petition must be filed supported by data demonstrating that nitrosamines are not formed.

References

ChemIDplus. 2009. *ChemIDplus Advanced*. National Library of Medicine. http://chem.sis.nlm.nih.gov/chemidplus and select Registry Number and search on CAS number. Last accessed: 10/7/09.

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Hecht SS, Abbaspour A, Hoffman D. 1988. A study of tobacco carcinogenesis. XLII. Bioassay in A/J mice of some structural analogues of tobacco-specific nitrosamines. *Cancer Lett* 42(1-2): 141-145.

Hoffmann D, Djordjevic MV, Rivenson A, Zang E, Desai D, Amin S. 1993. A study of tobacco carcinogenesis. LI. Relative potencies of tobacco-specific *N*-nitrosamines as inducers of lung tumours in A/J mice. *Cancer Lett* 71(1-3): 25-30.

HSDB. 2009. *Hazardous Substances Data Bank*. National Library of Medicine. http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB and search on CAS number. Last accessed: 10/7/09.

IARC. 1978. *N*-Nitrosopyrrolidine. In *Some* N-*nitroso compounds*. IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans, vol. 17. Lyon, France: International Agency for Research on Cancer. pp. 313-326.

Ketkar MB, Schneider P, Preussmann R, Plass C, Mohr U. 1982. Carcinogenic effect of low doses of nitrosopyrrolidine administered in the drinking water to Syrian golden hamsters. *J Cancer Res Clin Oncol* 104(1-2): 75-79.

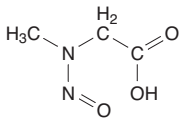
USCB. 2018. Chapter 29 - Organic Chemicals. In *U.S. Census Bureau - Foreign Trade Schedule B (2018)*. https://uscensus.prod.3ceonline.com and search on CAS number or substance name. Last accessed: 10/10/18.

N-Nitrososarcosine

CAS No. 13256-22-9

Reasonably anticipated to be a human carcinogen

First listed in the *Second Annual Report on Carcinogens* (1981)



Carcinogenicity

N-Nitrososarcosine is *reasonably anticipated to be a human carcinogen* based on sufficient evidence of carcinogenicity from studies in experimental animals.

Cancer Studies in Experimental Animals

N-Nitrososarcosine caused tumors in two rodent species, at several different tissue sites, and by two different routes of exposure. Dietary exposure to *N*-nitrososarcosine caused cancer of the nasal cavity (squamous-cell carcinoma) in mice of both sexes, and administration in the drinking water caused benign and malignant tumors of the esophagus (papilloma and squamous-cell carcinoma) in rats. Intraperitoneal injection of *N*-nitrososarcosine in newborn mice caused liver cancer (hepatocellular carcinoma) in males (IARC 1978).

Cancer Studies in Humans

No epidemiological studies were identified that evaluated the relationship between human cancer and exposure specifically to *N*-nitrososarcosine.

Properties

N-Nitrososarcosine is a nitrosamine compound that is a pale-yellow crystal at room temperature (HSDB 2009). It is soluble in water and polar organic solvents but is unstable in aqueous solution. It is sensitive to light, especially ultraviolet light (Akron 2009). Physical and chemical properties of *N*-nitrososarcosine are listed in the following table.

Property	Information
Molecular weight	118.1[a]
Melting point	66°C to 67°C[a]
Log K_{ow}	–0.78[b]
Water solubility	1,000 g/L at 25°C[b]
Vapor pressure	0.00261 mm Hg at 25°C[b]

Sources: [a]HSDB 2009, [b]ChemIDplus 2009.

Use

N-Nitrososarcosine is not used commercially in the United States, but has limited use in research (IARC 1978, HSDB 2009).

Production

There is no evidence that *N*-nitrososarcosine has been produced commercially in the United States (IARC 1978, HSDB 2009). In 2009, it was available in small quantities for research purposes from three U.S. suppliers (ChemSources 2009).

Exposure

The routes of potential human exposure to *N*-nitrososarcosine are inhalation, ingestion, and dermal contact (HSDB 2009). *N*-Nitrososarcosine is formed when nitrite-preserved foods containing primary or secondary amines are prepared by heating. Exposure could occur through inhalation during cooking or through ingestion of the prepared food. *N*-Nitrososarcosine has been detected in foods; in particular, it was found in smoked meat at concentrations of 2 to 56 µg/kg. It was also found in tobacco smoke at concentrations of 22 to 460 ng per cigarette. In air, *N*-nitrososarcosine exists predominantly in the gas phase and degrades by reaction with photochemically produced hydroxyl radicals, with a half-life of 1.9 days (IARC 1978, Tricker *et al.* 1991, HSDB 2009).

Regulations

Consumer Product Safety Commission (CPSC)

A voluntary standard provides that rubber pacifiers shall not contain more than 10 ppb of any single nitrosamine or more than 20 ppb of total nitrosamines.

Environmental Protection Agency (EPA)

Clean Water Act

Effluent Guidelines: Nitrosamines are listed as a toxic pollutant.

Water Quality Criteria: Based on fish or shellfish and water consumption = 0.0008 µg/L for nitrosamines; based on fish or shellfish consumption only = 1.24 µg/L for nitrosamines.

Resource Conservation and Recovery Act

Listed as a hazardous constituent of waste.

Toxic Substances Control Act

Nitrosating agents distributed in commerce require warning labels and instructions on use.

Food and Drug Administration (FDA, an HHS agency)

The action level for *N*-nitrosamines in rubber baby-bottle nipples is 10 ppb.

In order to use nitrites and/or nitrates as food additives in curing premixes a petition must be filed supported by data demonstrating that nitrosamines are not formed.

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Tricker AR, Ditrich C, Preussmann R. 1991. *N*-Nitroso compounds in cigarette tobacco and their occurrence in mainstream tobacco smoke. *Carcinogenesis* 12(2): 257-261.

N'-NITROSONORNICOTINE (NNN)

VOL.: 37 (1985) (p. 241)

CAS Nos: 80508-23-2; 16543-55-8; 84237-38-7
Chem. Abstr. Name: Pyridine, 3-(1-nitroso-2-pyrrolidinyl)-; pyridine, 3-(1-nitroso-2-pyrrolidinyl)-,(S)-; pyridine,3-(1-nitroso-2-pyrrolidinyl)-, (+,-)-

5. Summary of Data Reported and Evaluation

5.1 Exposures

N'-Nitrosonornicotine (NNN) has been found in a variety of tobacco products (chewing tobacco, snuff, cigarettes and cigars), in mainstream and sidestream smoke from cigars and cigarettes, in saliva of chewers of betel quid with tobacco and in saliva of oral-snuff users. Some of the NNN in saliva appears to be formed endogenously from nitrite in saliva and tobacco alkaloids. Thus, there is widespread exposure to NNN among users of tobacco products and those exposed to sidestream smoke.

5.2 Experimental data

NNN was tested for carcinogenicity in rats, mice and hamsters by different routes of administration in multiple experiments. Following its oral administration, NNN produced carcinomas of the upper digestive tract, mainly the oesophagus, and of the nasal cavity in rats and nasal-cavity tumours in hamsters. Following its subcutaneous administration, NNN produced primarily tumours of the nasal cavity in rats and tumours of the trachea in hamsters. Intraperitoneal injection produced lung tumours in mice and tumours of the nasal cavity and trachea in hamsters. There was evidence of a dose-response relationship after subcutaneous administration of NNN to rats.

Several metabolites of NNN were tested in mice by intraperitoneal injection, producing lung tumours. NNN-1-*N*-oxide was also tested in rats and hamsters by oral administration; it produced nasal-cavity and oesophageal tumours in rats.

NNN is mutagenic to *Salmonella typhimurium* in the presence of an exogenous metabolic system. It induces unscheduled DNA synthesis in primary cultures of rat hepatocytes.

5.3 Human data

No case report or epidemiological study of the carcinogenicity of NNN was available to the Working Group.

5.4 Evaluation

There is *sufficient evidence* for the carcinogenicity of *N'*-nitrosonornicotine to experimental animals.

No data on humans were available.

For definition of the italicized terms, see Preamble Evaluation.

Previous evaluation: Vol. 17 (1978)

Subsequent evaluation: Suppl. 7 (1987) (p. 68: **Group 2B**)

Synonyms

- 1'-Demethyl-1'-nitrosonicotine
- 1'-Desmethyl-1'-nitrosonicotine
- 1'-Nitroso-1'-demethylnicotine
- *N*-Nitrosonornicotine
- 1'-Nitrosonornicotine
- Nitrosonornicotine
- 1-Nitroso-2-(3-pyridyl)pyrrolidine
- 3-(1-Nitroso-2-pyrrolidinyl)pyridine

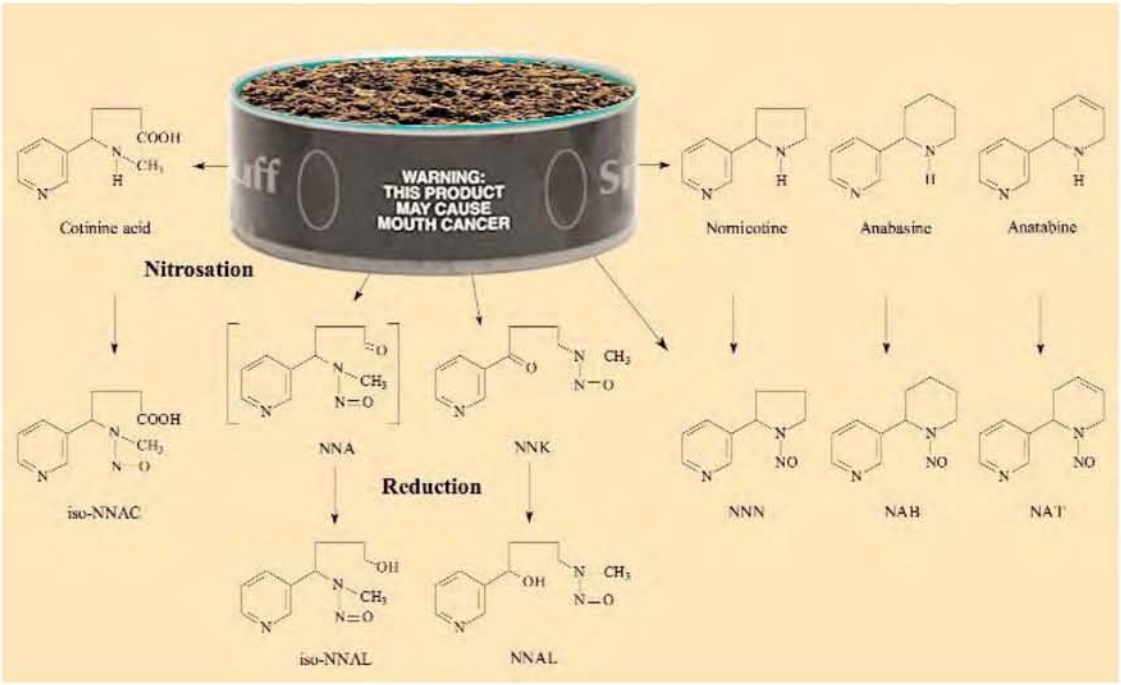
Last updated: 21 April 1998

WORLD HEALTH ORGANIZATION
INTERNATIONAL AGENCY FOR RESEARCH ON CANCER

IARC Monographs on the Evaluation of Carcinogenic Risks to Humans

VOLUME 89

Smokeless Tobacco and Some Tobacco-specific *N*-Nitrosamines



LYON, FRANCE
2007

WORLD HEALTH ORGANIZATION
INTERNATIONAL AGENCY FOR RESEARCH ON CANCER

IARC Monographs on the Evaluation of Carcinogenic Risks to Humans

VOLUME 89

Smokeless Tobacco and Some Tobacco-specific *N*-Nitrosamines

This publication represents the views and expert opinions
of an IARC Working Group on the
Evaluation of Carcinogenic Risks to Humans,
which met in Lyon,

5–12 October 2004

2007

IARC MONOGRAPHS

In 1969, the International Agency for Research on Cancer (IARC) initiated a programme on the evaluation of the carcinogenic risk of chemicals to humans involving the production of critically evaluated monographs on individual chemicals. The programme was subsequently expanded to include evaluations of carcinogenic risks associated with exposures to complex mixtures, life-style factors and biological and physical agents, as well as those in specific occupations.

The objective of the programme is to elaborate and publish in the form of monographs critical reviews of data on carcinogenicity for agents to which humans are known to be exposed and on specific exposure situations; to evaluate these data in terms of human risk with the help of international working groups of experts in chemical carcinogenesis and related fields; and to indicate where additional research efforts are needed.

The lists of IARC evaluations are regularly updated and are available on the Internet at http://monographs.iarc.fr/.

This programme has been supported by Cooperative Agreement 5 UO1 CA33193 awarded since 1982 by the United States National Cancer Institute, Department of Health and Human Services. Additional support has been provided since 1986 by the European Commission, Directorate-General EMPL (Employment, and Social Affairs), Health, Safety and Hygiene at Work Unit, and since 1992 by the United States National Institute of Environmental Health Sciences.

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The IARC Monographs Working Group alone is responsible for the views expressed in this publication.

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Cover legend:

Schematic diagramme of the metabolism of nicotine, an addictive chemical present in all tobacco products. The tobacco-specific *N*-nitrosamines NNK, NNN, NAB and NAT are reviewed in the second Monograph of this volume.

Cover design: Georges Mollon, IARC

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NOTE TO THE READER

The term ‘carcinogenic risk’ in the *IARC Monographs* series is taken to mean that an agent is capable of causing cancer under some circumstances. The *Monographs* evaluate cancer hazards, despite the historical presence of the word ‘risks’ in the title.

Inclusion of an agent in the *Monographs* does not imply that it is a carcinogen, only that the published data have been examined. Equally, the fact that an agent has not yet been evaluated in a monograph does not mean that it is not carcinogenic.

The evaluations of carcinogenic risk are made by international working groups of independent scientists and are qualitative in nature. No recommendation is given for regulation or legislation.

Anyone who is aware of published data that may alter the evaluation of the carcinogenic risk of an agent to humans is encouraged to make this information available to the Carcinogen Identification and Evaluation Group, International Agency for Research on Cancer, 150 cours Albert Thomas, 69372 Lyon Cedex 08, France, in order that the agent may be considered for re-evaluation by a future Working Group.

Although every effort is made to prepare the monographs as accurately as possible, mistakes may occur. Readers are requested to communicate any errors to the Carcinogen Identification and Evaluation Group, so that corrections can be reported in future volumes.

IARC WORKING GROUP ON THE EVALUATION OF CARCINOGENIC RISKS TO HUMANS: SMOKELESS TOBACCO AND SOME TOBACCO-SPECIFIC *N*-NITROSAMINES

Lyon, 5–12 October 2004

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PREAMBLE

IARC MONOGRAPHS PROGRAMME ON THE EVALUATION OF CARCINOGENIC RISKS TO HUMANS

PREAMBLE

1. BACKGROUND

In 1969, the International Agency for Research on Cancer (IARC) initiated a programme to evaluate the carcinogenic risk of chemicals to humans and to produce monographs on individual chemicals. The *Monographs* programme has since been expanded to include consideration of exposures to complex mixtures of chemicals (which occur, for example, in some occupations and as a result of human habits) and of exposures to other agents, such as radiation and viruses. With Supplement 6 (IARC, 1987a), the title of the series was modified from *IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans* to *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans*, in order to reflect the widened scope of the programme.

The criteria established in 1971 to evaluate carcinogenic risk to humans were adopted by the working groups whose deliberations resulted in the first 16 volumes of the *IARC Monographs series*. Those criteria were subsequently updated by further ad-hoc working groups (IARC, 1977, 1978, 1979, 1982, 1983, 1987b, 1988, 1991a; Vainio *et al.*, 1992).

2. OBJECTIVE AND SCOPE

The objective of the programme is to prepare, with the help of international working groups of experts, and to publish in the form of monographs, critical reviews and evaluations of evidence on the carcinogenicity of a wide range of human exposures. The *Monographs* may also indicate where additional research efforts are needed.

The *Monographs* represent the first step in carcinogenic risk assessment, which involves examination of all relevant information in order to assess the strength of the available evidence that certain exposures could alter the incidence of cancer in humans. The second step is quantitative risk estimation. Detailed, quantitative evaluations of epidemiological data may be made in the *Monographs*, but without extrapolation beyond the range of the data available. Quantitative extrapolation from experimental data to the human situation is not undertaken.

The term ‘carcinogen’ is used in these monographs to denote an exposure that is capable of increasing the incidence of malignant neoplasms; the induction of benign neo-

plasms may in some circumstances (see p. 19) contribute to the judgement that the exposure is carcinogenic. The terms 'neoplasm' and 'tumour' are used interchangeably.

Some epidemiological and experimental studies indicate that different agents may act at different stages in the carcinogenic process, and several mechanisms may be involved. The aim of the *Monographs* has been, from their inception, to evaluate evidence of carcinogenicity at any stage in the carcinogenesis process, independently of the underlying mechanisms. Information on mechanisms may, however, be used in making the overall evaluation (IARC, 1991a; Vainio *et al.*, 1992; see also pp. 25–27).

The *Monographs* may assist national and international authorities in making risk assessments and in formulating decisions concerning any necessary preventive measures. The evaluations of IARC working groups are scientific, qualitative judgements about the evidence for or against carcinogenicity provided by the available data. These evaluations represent only one part of the body of information on which regulatory measures may be based. Other components of regulatory decisions vary from one situation to another and from country to country, responding to different socioeconomic and national priorities. **Therefore, no recommendation is given with regard to regulation or legislation, which are the responsibility of individual governments and/or other international organizations.**

The *IARC Monographs* are recognized as an authoritative source of information on the carcinogenicity of a wide range of human exposures. A survey of users in 1988 indicated that the *Monographs* are consulted by various agencies in 57 countries. About 2500 copies of each volume are printed, for distribution to governments, regulatory bodies and interested scientists. The Monographs are also available from IARC*Press* in Lyon and via the Marketing and Dissemination (MDI) of the World Health Organization in Geneva.

3. SELECTION OF TOPICS FOR MONOGRAPHS

Topics are selected on the basis of two main criteria: (a) there is evidence of human exposure, and (b) there is some evidence or suspicion of carcinogenicity. The term 'agent' is used to include individual chemical compounds, groups of related chemical compounds, physical agents (such as radiation) and biological factors (such as viruses). Exposures to mixtures of agents may occur in occupational exposures and as a result of personal and cultural habits (like smoking and dietary practices). Chemical analogues and compounds with biological or physical characteristics similar to those of suspected carcinogens may also be considered, even in the absence of data on a possible carcinogenic effect in humans or experimental animals.

The scientific literature is surveyed for published data relevant to an assessment of carcinogenicity. The IARC information bulletins on agents being tested for carcinogenicity (IARC, 1973–1996) and directories of on-going research in cancer epidemiology (IARC, 1976–1996) often indicate exposures that may be scheduled for future meetings. Ad-hoc working groups convened by IARC in 1984, 1989, 1991, 1993 and

1998 gave recommendations as to which agents should be evaluated in the IARC Monographs series (IARC, 1984, 1989, 1991b, 1993, 1998a,b).

As significant new data on subjects on which monographs have already been prepared become available, re-evaluations are made at subsequent meetings, and revised monographs are published.

4. DATA FOR MONOGRAPHS

The *Monographs* do not necessarily cite all the literature concerning the subject of an evaluation. Only those data considered by the Working Group to be relevant to making the evaluation are included.

With regard to biological and epidemiological data, only reports that have been published or accepted for publication in the openly available scientific literature are reviewed by the working groups. In certain instances, government agency reports that have undergone peer review and are widely available are considered. Exceptions may be made on an ad-hoc basis to include unpublished reports that are in their final form and publicly available, if their inclusion is considered pertinent to making a final evaluation (see pp. 25–27). In the sections on chemical and physical properties, on analysis, on production and use and on occurrence, unpublished sources of information may be used.

5. THE WORKING GROUP

Reviews and evaluations are formulated by a working group of experts. The tasks of the group are: (i) to ascertain that all appropriate data have been collected; (ii) to select the data relevant for the evaluation on the basis of scientific merit; (iii) to prepare accurate summaries of the data to enable the reader to follow the reasoning of the Working Group; (iv) to evaluate the results of epidemiological and experimental studies on cancer; (v) to evaluate data relevant to the understanding of mechanism of action; and (vi) to make an overall evaluation of the carcinogenicity of the exposure to humans.

Working Group participants who contributed to the considerations and evaluations within a particular volume are listed, with their addresses, at the beginning of each publication. Each participant who is a member of a working group serves as an individual scientist and not as a representative of any organization, government or industry. In addition, nominees of national and international agencies and industrial associations may be invited as observers.

6. WORKING PROCEDURES

Approximately one year in advance of a meeting of a working group, the topics of the monographs are announced and participants are selected by IARC staff in consultation with other experts. Subsequently, relevant biological and epidemiological data are

collected by the Carcinogen Identification and Evaluation Unit of IARC from recognized sources of information on carcinogenesis, including data storage and retrieval systems such as MEDLINE and TOXLINE.

For chemicals and some complex mixtures, the major collection of data and the preparation of first drafts of the sections on chemical and physical properties, on analysis, on production and use and on occurrence are carried out under a separate contract funded by the United States National Cancer Institute. Representatives from industrial associations may assist in the preparation of sections on production and use. Information on production and trade is obtained from governmental and trade publications and, in some cases, by direct contact with industries. Separate production data on some agents may not be available because their publication could disclose confidential information. Information on uses may be obtained from published sources but is often complemented by direct contact with manufacturers. Efforts are made to supplement this information with data from other national and international sources.

Six months before the meeting, the material obtained is sent to meeting participants, or is used by IARC staff, to prepare sections for the first drafts of monographs. The first drafts are compiled by IARC staff and sent before the meeting to all participants of the Working Group for review.

The Working Group meets in Lyon for seven to eight days to discuss and finalize the texts of the monographs and to formulate the evaluations. After the meeting, the master copy of each monograph is verified by consulting the original literature, edited and prepared for publication. The aim is to publish monographs within six months of the Working Group meeting.

The available studies are summarized by the Working Group, with particular regard to the qualitative aspects discussed below. In general, numerical findings are indicated as they appear in the original report; units are converted when necessary for easier comparison. The Working Group may conduct additional analyses of the published data and use them in their assessment of the evidence; the results of such supplementary analyses are given in square brackets. When an important aspect of a study, directly impinging on its interpretation, should be brought to the attention of the reader, a comment is given in square brackets.

7. EXPOSURE DATA

Sections that indicate the extent of past and present human exposure, the sources of exposure, the people most likely to be exposed and the factors that contribute to the exposure are included at the beginning of each monograph.

Most monographs on individual chemicals, groups of chemicals or complex mixtures include sections on chemical and physical data, on analysis, on production and use and on occurrence. In monographs on, for example, physical agents, occupational exposures and cultural habits, other sections may be included, such as: historical perspectives, description of an industry or habit, chemistry of the complex mixture or taxonomy. Mono-

graphs on biological agents have sections on structure and biology, methods of detection, epidemiology of infection and clinical disease other than cancer.

For chemical exposures, the Chemical Abstracts Services Registry Number, the latest Chemical Abstracts primary name and the IUPAC systematic name are recorded; other synonyms are given, but the list is not necessarily comprehensive. For biological agents, taxonomy and structure are described, and the degree of variability is given, when applicable.

Information on chemical and physical properties and, in particular, data relevant to identification, occurrence and biological activity are included. For biological agents, mode of replication, life cycle, target cells, persistence and latency and host response are given. A description of technical products of chemicals includes trade names, relevant specifications and available information on composition and impurities. Some of the trade names given may be those of mixtures in which the agent being evaluated is only one of the ingredients.

The purpose of the section on analysis or detection is to give the reader an overview of current methods, with emphasis on those widely used for regulatory purposes. Methods for monitoring human exposure are also given, when available. No critical evaluation or recommendation of any of the methods is meant or implied. The IARC published a series of volumes, *Environmental Carcinogens: Methods of Analysis and Exposure Measurement* (IARC, 1978–93), that describe validated methods for analysing a wide variety of chemicals and mixtures. For biological agents, methods of detection and exposure assessment are described, including their sensitivity, specificity and reproducibility.

The dates of first synthesis and of first commercial production of a chemical or mixture are provided; for agents which do not occur naturally, this information may allow a reasonable estimate to be made of the date before which no human exposure to the agent could have occurred. The dates of first reported occurrence of an exposure are also provided. In addition, methods of synthesis used in past and present commercial production and different methods of production which may give rise to different impurities are described.

Data on production, international trade and uses are obtained for representative regions, which usually include Europe, Japan and the United States of America. It should not, however, be inferred that those areas or nations are necessarily the sole or major sources or users of the agent. Some identified uses may not be current or major applications, and the coverage is not necessarily comprehensive. In the case of drugs, mention of their therapeutic uses does not necessarily represent current practice, nor does it imply judgement as to their therapeutic efficacy.

Information on the occurrence of an agent or mixture in the environment is obtained from data derived from the monitoring and surveillance of levels in occupational environments, air, water, soil, foods and animal and human tissues. When available, data on the generation, persistence and bioaccumulation of the agent are also included. In the case of mixtures, industries, occupations or processes, information is given about all

agents present. For processes, industries and occupations, a historical description is also given, noting variations in chemical composition, physical properties and levels of occupational exposure with time and place. For biological agents, the epidemiology of infection is described.

Statements concerning regulations and guidelines (e.g., pesticide registrations, maximal levels permitted in foods, occupational exposure limits) are included for some countries as indications of potential exposures, but they may not reflect the most recent situation, since such limits are continuously reviewed and modified. The absence of information on regulatory status for a country should not be taken to imply that that country does not have regulations with regard to the exposure. For biological agents, legislation and control, including vaccines and therapy, are described.

8. STUDIES OF CANCER IN HUMANS

(*a*) *Types of studies considered*

Three types of epidemiological studies of cancer contribute to the assessment of carcinogenicity in humans — cohort studies, case–control studies and correlation (or ecological) studies. Rarely, results from randomized trials may be available. Case series and case reports of cancer in humans may also be reviewed.

Cohort and case–control studies relate the exposures under study to the occurrence of cancer in individuals and provide an estimate of relative risk (ratio of incidence or mortality in those exposed to incidence or mortality in those not exposed) as the main measure of association.

In correlation studies, the units of investigation are usually whole populations (e.g. in particular geographical areas or at particular times), and cancer frequency is related to a summary measure of the exposure of the population to the agent, mixture or exposure circumstance under study. Because individual exposure is not documented, however, a causal relationship is less easy to infer from correlation studies than from cohort and case–control studies. Case reports generally arise from a suspicion, based on clinical experience, that the concurrence of two events — that is, a particular exposure and occurrence of a cancer — has happened rather more frequently than would be expected by chance. Case reports usually lack complete ascertainment of cases in any population, definition or enumeration of the population at risk and estimation of the expected number of cases in the absence of exposure. The uncertainties surrounding interpretation of case reports and correlation studies make them inadequate, except in rare instances, to form the sole basis for inferring a causal relationship. When taken together with case–control and cohort studies, however, relevant case reports or correlation studies may add materially to the judgement that a causal relationship is present.

Epidemiological studies of benign neoplasms, presumed preneoplastic lesions and other end-points thought to be relevant to cancer are also reviewed by working groups. They may, in some instances, strengthen inferences drawn from studies of cancer itself.

(*b*) *Quality of studies considered*

The Monographs are not intended to summarize all published studies. Those that are judged to be inadequate or irrelevant to the evaluation are generally omitted. They may be mentioned briefly, particularly when the information is considered to be a useful supplement to that in other reports or when they provide the only data available. Their inclusion does not imply acceptance of the adequacy of the study design or of the analysis and interpretation of the results, and limitations are clearly outlined in square brackets at the end of the study description.

It is necessary to take into account the possible roles of bias, confounding and chance in the interpretation of epidemiological studies. By 'bias' is meant the operation of factors in study design or execution that lead erroneously to a stronger or weaker association than in fact exists between disease and an agent, mixture or exposure circumstance. By 'confounding' is meant a situation in which the relationship with disease is made to appear stronger or weaker than it truly is as a result of an association between the apparent causal factor and another factor that is associated with either an increase or decrease in the incidence of the disease. In evaluating the extent to which these factors have been minimized in an individual study, working groups consider a number of aspects of design and analysis as described in the report of the study. Most of these considerations apply equally to case–control, cohort and correlation studies. Lack of clarity of any of these aspects in the reporting of a study can decrease its credibility and the weight given to it in the final evaluation of the exposure.

Firstly, the study population, disease (or diseases) and exposure should have been well defined by the authors. Cases of disease in the study population should have been identified in a way that was independent of the exposure of interest, and exposure should have been assessed in a way that was not related to disease status.

Secondly, the authors should have taken account in the study design and analysis of other variables that can influence the risk of disease and may have been related to the exposure of interest. Potential confounding by such variables should have been dealt with either in the design of the study, such as by matching, or in the analysis, by statistical adjustment. In cohort studies, comparisons with local rates of disease may be more appropriate than those with national rates. Internal comparisons of disease frequency among individuals at different levels of exposure should also have been made in the study.

Thirdly, the authors should have reported the basic data on which the conclusions are founded, even if sophisticated statistical analyses were employed. At the very least, they should have given the numbers of exposed and unexposed cases and controls in a case–control study and the numbers of cases observed and expected in a cohort study. Further tabulations by time since exposure began and other temporal factors are also important. In a cohort study, data on all cancer sites and all causes of death should have been given, to reveal the possibility of reporting bias. In a case–control study, the effects of investigated factors other than the exposure of interest should have been reported.

Finally, the statistical methods used to obtain estimates of relative risk, absolute rates of cancer, confidence intervals and significance tests, and to adjust for confounding should have been clearly stated by the authors. The methods used should preferably have been the generally accepted techniques that have been refined since the mid-1970s. These methods have been reviewed for case–control studies (Breslow & Day, 1980) and for cohort studies (Breslow & Day, 1987).

(*c*) *Inferences about mechanism of action*

Detailed analyses of both relative and absolute risks in relation to temporal variables, such as age at first exposure, time since first exposure, duration of exposure, cumulative exposure and time since exposure ceased, are reviewed and summarized when available. The analysis of temporal relationships can be useful in formulating models of carcinogenesis. In particular, such analyses may suggest whether a carcinogen acts early or late in the process of carcinogenesis, although at best they allow only indirect inferences about the mechanism of action. Special attention is given to measurements of biological markers of carcinogen exposure or action, such as DNA or protein adducts, as well as markers of early steps in the carcinogenic process, such as proto-oncogene mutation, when these are incorporated into epidemiological studies focused on cancer incidence or mortality. Such measurements may allow inferences to be made about putative mechanisms of action (IARC, 1991a; Vainio *et al.*, 1992).

(*d*) *Criteria for causality*

After the individual epidemiological studies of cancer have been summarized and the quality assessed, a judgement is made concerning the strength of evidence that the agent, mixture or exposure circumstance in question is carcinogenic for humans. In making its judgement, the Working Group considers several criteria for causality. A strong association (a large relative risk) is more likely to indicate causality than a weak association, although it is recognized that relative risks of small magnitude do not imply lack of causality and may be important if the disease is common. Associations that are replicated in several studies of the same design or using different epidemiological approaches or under different circumstances of exposure are more likely to represent a causal relationship than isolated observations from single studies. If there are inconsistent results among investigations, possible reasons are sought (such as differences in amount of exposure), and results of studies judged to be of high quality are given more weight than those of studies judged to be methodologically less sound. When suspicion of carcinogenicity arises largely from a single study, these data are not combined with those from later studies in any subsequent reassessment of the strength of the evidence.

If the risk of the disease in question increases with the amount of exposure, this is considered to be a strong indication of causality, although absence of a graded response is not necessarily evidence against a causal relationship. Demonstration of a decline in

risk after cessation of or reduction in exposure in individuals or in whole populations also supports a causal interpretation of the findings.

Although a carcinogen may act upon more than one target, the specificity of an association (an increased occurrence of cancer at one anatomical site or of one morphological type) adds plausibility to a causal relationship, particularly when excess cancer occurrence is limited to one morphological type within the same organ.

Although rarely available, results from randomized trials showing different rates among exposed and unexposed individuals provide particularly strong evidence for causality.

When several epidemiological studies show little or no indication of an association between an exposure and cancer, the judgement may be made that, in the aggregate, they show evidence of lack of carcinogenicity. Such a judgement requires first of all that the studies giving rise to it meet, to a sufficient degree, the standards of design and analysis described above. Specifically, the possibility that bias, confounding or misclassification of exposure or outcome could explain the observed results should be considered and excluded with reasonable certainty. In addition, all studies that are judged to be methodologically sound should be consistent with a relative risk of unity for any observed level of exposure and, when considered together, should provide a pooled estimate of relative risk which is at or near unity and has a narrow confidence interval, due to sufficient population size. Moreover, no individual study nor the pooled results of all the studies should show any consistent tendency for the relative risk of cancer to increase with increasing level of exposure. It is important to note that evidence of lack of carcinogenicity obtained in this way from several epidemiological studies can apply only to the type(s) of cancer studied and to dose levels and intervals between first exposure and observation of disease that are the same as or less than those observed in all the studies. Experience with human cancer indicates that, in some cases, the period from first exposure to the development of clinical cancer is seldom less than 20 years; studies with latent periods substantially shorter than 30 years cannot provide evidence for lack of carcinogenicity.

9. STUDIES OF CANCER IN EXPERIMENTAL ANIMALS

All known human carcinogens that have been studied adequately in experimental animals have produced positive results in one or more animal species (Wilbourn *et al.*, 1986; Tomatis *et al.*, 1989). For several agents (aflatoxins, 4-aminobiphenyl, azathioprine, betel quid with tobacco, bischloromethyl ether and chloromethyl methyl ether (technical grade), chlorambucil, chlornaphazine, ciclosporin, coal-tar pitches, coal-tars, combined oral contraceptives, cyclophosphamide, diethylstilboestrol, melphalan, 8-methoxypsoralen plus ultraviolet A radiation, mustard gas, myleran, 2-naphthylamine, nonsteroidal estrogens, estrogen replacement therapy/steroidal estrogens, solar radiation, thiotepa and vinyl chloride), carcinogenicity in experimental animals was established or highly suspected before epidemiological studies confirmed their carcinogenicity in humans (Vainio *et al.*, 1995). Although this association cannot establish that all agents

and mixtures that cause cancer in experimental animals also cause cancer in humans, nevertheless, **in the absence of adequate data on humans, it is biologically plausible and prudent to regard agents and mixtures for which there is *sufficient evidence* (see p. 24) of carcinogenicity in experimental animals as if they presented a carcinogenic risk to humans**. The possibility that a given agent may cause cancer through a species-specific mechanism which does not operate in humans (see p. 27) should also be taken into consideration.

The nature and extent of impurities or contaminants present in the chemical or mixture being evaluated are given when available. Animal strain, sex, numbers per group, age at start of treatment and survival are reported.

Other types of studies summarized include: experiments in which the agent or mixture was administered in conjunction with known carcinogens or factors that modify carcinogenic effects; studies in which the end-point was not cancer but a defined precancerous lesion; and experiments on the carcinogenicity of known metabolites and derivatives.

For experimental studies of mixtures, consideration is given to the possibility of changes in the physicochemical properties of the test substance during collection, storage, extraction, concentration and delivery. Chemical and toxicological interactions of the components of mixtures may result in nonlinear dose–response relationships.

An assessment is made as to the relevance to human exposure of samples tested in experimental animals, which may involve consideration of: (i) physical and chemical characteristics, (ii) constituent substances that indicate the presence of a class of substances, (iii) the results of tests for genetic and related effects, including studies on DNA adduct formation, proto-oncogene mutation and expression and suppressor gene inactivation. The relevance of results obtained, for example, with animal viruses analogous to the virus being evaluated in the monograph must also be considered. They may provide biological and mechanistic information relevant to the understanding of the process of carcinogenesis in humans and may strengthen the plausibility of a conclusion that the biological agent under evaluation is carcinogenic in humans.

(*a*) *Qualitative aspects*

An assessment of carcinogenicity involves several considerations of qualitative importance, including (i) the experimental conditions under which the test was performed, including route and schedule of exposure, species, strain, sex, age, duration of follow-up; (ii) the consistency of the results, for example, across species and target organ(s); (iii) the spectrum of neoplastic response, from preneoplastic lesions and benign tumours to malignant neoplasms; and (iv) the possible role of modifying factors.

As mentioned earlier (p. 11), the *Monographs* are not intended to summarize all published studies. Those studies in experimental animals that are inadequate (e.g., too short a duration, too few animals, poor survival; see below) or are judged irrelevant to

the evaluation are generally omitted. Guidelines for conducting adequate long-term carcinogenicity experiments have been outlined (e.g. Montesano *et al.*, 1986).

Considerations of importance to the Working Group in the interpretation and evaluation of a particular study include: (i) how clearly the agent was defined and, in the case of mixtures, how adequately the sample characterization was reported; (ii) whether the dose was adequately monitored, particularly in inhalation experiments; (iii) whether the doses and duration of treatment were appropriate and whether the survival of treated animals was similar to that of controls; (iv) whether there were adequate numbers of animals per group; (v) whether animals of each sex were used; (vi) whether animals were allocated randomly to groups; (vii) whether the duration of observation was adequate; and (viii) whether the data were adequately reported. If available, recent data on the incidence of specific tumours in historical controls, as well as in concurrent controls, should be taken into account in the evaluation of tumour response.

When benign tumours occur together with and originate from the same cell type in an organ or tissue as malignant tumours in a particular study and appear to represent a stage in the progression to malignancy, it may be valid to combine them in assessing tumour incidence (Huff *et al.*, 1989). The occurrence of lesions presumed to be preneoplastic may in certain instances aid in assessing the biological plausibility of any neoplastic response observed. If an agent or mixture induces only benign neoplasms that appear to be end-points that do not readily progress to malignancy, it should nevertheless be suspected of being a carcinogen and requires further investigation.

(*b*) *Quantitative aspects*

The probability that tumours will occur may depend on the species, sex, strain and age of the animal, the dose of the carcinogen and the route and length of exposure. Evidence of an increased incidence of neoplasms with increased level of exposure strengthens the inference of a causal association between the exposure and the development of neoplasms.

The form of the dose–response relationship can vary widely, depending on the particular agent under study and the target organ. Both DNA damage and increased cell division are important aspects of carcinogenesis, and cell proliferation is a strong determinant of dose–response relationships for some carcinogens (Cohen & Ellwein, 1990). Since many chemicals require metabolic activation before being converted into their reactive intermediates, both metabolic and pharmacokinetic aspects are important in determining the dose–response pattern. Saturation of steps such as absorption, activation, inactivation and elimination may produce nonlinearity in the dose–response relationship, as could saturation of processes such as DNA repair (Hoel *et al.*, 1983; Gart *et al.*, 1986).

(*c*) *Statistical analysis of long-term experiments in animals*

Factors considered by the Working Group include the adequacy of the information given for each treatment group: (i) the number of animals studied and the number examined histologically, (ii) the number of animals with a given tumour type and (iii) length of survival. The statistical methods used should be clearly stated and should be the generally accepted techniques refined for this purpose (Peto *et al.*, 1980; Gart *et al.*, 1986). When there is no difference in survival between control and treatment groups, the Working Group usually compares the proportions of animals developing each tumour type in each of the groups. Otherwise, consideration is given as to whether or not appropriate adjustments have been made for differences in survival. These adjustments can include: comparisons of the proportions of tumour-bearing animals among the effective number of animals (alive at the time the first tumour is discovered), in the case where most differences in survival occur before tumours appear; life-table methods, when tumours are visible or when they may be considered 'fatal' because mortality rapidly follows tumour development; and the Mantel-Haenszel test or logistic regression, when occult tumours do not affect the animals' risk of dying but are 'incidental' findings at autopsy.

In practice, classifying tumours as fatal or incidental may be difficult. Several survival-adjusted methods have been developed that do not require this distinction (Gart *et al.*, 1986), although they have not been fully evaluated.

10. OTHER DATA RELEVANT TO AN EVALUATION OF CARCINOGENICITY AND ITS MECHANISMS

In coming to an overall evaluation of carcinogenicity in humans (see pp. 25–27), the Working Group also considers related data. The nature of the information selected for the summary depends on the agent being considered.

For chemicals and complex mixtures of chemicals such as those in some occupational situations or involving cultural habits (e.g. tobacco smoking), the other data considered to be relevant are divided into those on absorption, distribution, metabolism and excretion; toxic effects; reproductive and developmental effects; and genetic and related effects.

Concise information is given on absorption, distribution (including placental transfer) and excretion in both humans and experimental animals. Kinetic factors that may affect the dose–response relationship, such as saturation of uptake, protein binding, metabolic activation, detoxification and DNA repair processes, are mentioned. Studies that indicate the metabolic fate of the agent in humans and in experimental animals are summarized briefly, and comparisons of data on humans and on animals are made when possible. Comparative information on the relationship between exposure and the dose that reaches the target site may be of particular importance for extrapolation between species. Data are given on acute and chronic toxic effects (other than cancer), such as

organ toxicity, increased cell proliferation, immunotoxicity and endocrine effects. The presence and toxicological significance of cellular receptors is described. Effects on reproduction, teratogenicity, fetotoxicity and embryotoxicity are also summarized briefly.

Tests of genetic and related effects are described in view of the relevance of gene mutation and chromosomal damage to carcinogenesis (Vainio *et al.*, 1992; McGregor *et al.*, 1999). The adequacy of the reporting of sample characterization is considered and, where necessary, commented upon; with regard to complex mixtures, such comments are similar to those described for animal carcinogenicity tests on p. 18. The available data are interpreted critically by phylogenetic group according to the end-points detected, which may include DNA damage, gene mutation, sister chromatid exchange, micronucleus formation, chromosomal aberrations, aneuploidy and cell transformation. The concentrations employed are given, and mention is made of whether use of an exogenous metabolic system *in vitro* affected the test result. These data are given as listings of test systems, data and references. The data on genetic and related effects presented in the *Monographs* are also available in the form of genetic activity profiles (GAP) prepared in collaboration with the United States Environmental Protection Agency (EPA) (see also Waters *et al.*, 1987) using software for personal computers that are Microsoft Windows® compatible. The EPA/IARC GAP software and database may be downloaded free of charge from *www.epa.gov/gapdb*.

Positive results in tests using prokaryotes, lower eukaryotes, plants, insects and cultured mammalian cells suggest that genetic and related effects could occur in mammals. Results from such tests may also give information about the types of genetic effect produced and about the involvement of metabolic activation. Some end-points described are clearly genetic in nature (e.g., gene mutations and chromosomal aberrations), while others are to a greater or lesser degree associated with genetic effects (e.g. unscheduled DNA synthesis). In-vitro tests for tumour-promoting activity and for cell transformation may be sensitive to changes that are not necessarily the result of genetic alterations but that may have specific relevance to the process of carcinogenesis. A critical appraisal of these tests has been published (Montesano *et al.*, 1986).

Genetic or other activity detected in experimental mammals and humans is regarded as being of greater relevance than that in other organisms. The demonstration that an agent or mixture can induce gene and chromosomal mutations in whole mammals indicates that it may have carcinogenic activity, although this activity may not be detectably expressed in any or all species. Relative potency in tests for mutagenicity and related effects is not a reliable indicator of carcinogenic potency. Negative results in tests for mutagenicity in selected tissues from animals treated *in vivo* provide less weight, partly because they do not exclude the possibility of an effect in tissues other than those examined. Moreover, negative results in short-term tests with genetic end-points cannot be considered to provide evidence to rule out carcinogenicity of agents or mixtures that act through other mechanisms (e.g. receptor-mediated effects, cellular toxicity with regenerative proliferation, peroxisome proliferation) (Vainio *et al.*, 1992). Factors that

may lead to misleading results in short-term tests have been discussed in detail elsewhere (Montesano *et al.*, 1986).

When available, data relevant to mechanisms of carcinogenesis that do not involve structural changes at the level of the gene are also described.

The adequacy of epidemiological studies of reproductive outcome and genetic and related effects in humans is evaluated by the same criteria as are applied to epidemiological studies of cancer.

Structure–activity relationships that may be relevant to an evaluation of the carcinogenicity of an agent are also described.

For biological agents — viruses, bacteria and parasites — other data relevant to carcinogenicity include descriptions of the pathology of infection, molecular biology (integration and expression of viruses, and any genetic alterations seen in human tumours) and other observations, which might include cellular and tissue responses to infection, immune response and the presence of tumour markers.

11. SUMMARY OF DATA REPORTED

In this section, the relevant epidemiological and experimental data are summarized. Only reports, other than in abstract form, that meet the criteria outlined on p. 11 are considered for evaluating carcinogenicity. Inadequate studies are generally not summarized: such studies are usually identified by a square-bracketed comment in the preceding text.

(*a*) *Exposure*

Human exposure to chemicals and complex mixtures is summarized on the basis of elements such as production, use, occurrence in the environment and determinations in human tissues and body fluids. Quantitative data are given when available. Exposure to biological agents is described in terms of transmission and prevalence of infection.

(*b*) *Carcinogenicity in humans*

Results of epidemiological studies that are considered to be pertinent to an assessment of human carcinogenicity are summarized. When relevant, case reports and correlation studies are also summarized.

(*c*) *Carcinogenicity in experimental animals*

Data relevant to an evaluation of carcinogenicity in animals are summarized. For each animal species and route of administration, it is stated whether an increased incidence of neoplasms or preneoplastic lesions was observed, and the tumour sites are indicated. If the agent or mixture produced tumours after prenatal exposure or in single-dose experiments, this is also indicated. Negative findings are also summarized. Dose–response and other quantitative data may be given when available.

(*d*) *Other data relevant to an evaluation of carcinogenicity and its mechanisms*

Data on biological effects in humans that are of particular relevance are summarized. These may include toxicological, kinetic and metabolic considerations and evidence of DNA binding, persistence of DNA lesions or genetic damage in exposed humans. Toxicological information, such as that on cytotoxicity and regeneration, receptor binding and hormonal and immunological effects, and data on kinetics and metabolism in experimental animals are given when considered relevant to the possible mechanism of the carcinogenic action of the agent. The results of tests for genetic and related effects are summarized for whole mammals, cultured mammalian cells and nonmammalian systems.

When available, comparisons of such data for humans and for animals, and particularly animals that have developed cancer, are described.

Structure–activity relationships are mentioned when relevant.

For the agent, mixture or exposure circumstance being evaluated, the available data on end-points or other phenomena relevant to mechanisms of carcinogenesis from studies in humans, experimental animals and tissue and cell test systems are summarized within one or more of the following descriptive dimensions:

(i) Evidence of genotoxicity (structural changes at the level of the gene): for example, structure–activity considerations, adduct formation, mutagenicity (effect on specific genes), chromosomal mutation/aneuploidy

(ii) Evidence of effects on the expression of relevant genes (functional changes at the intracellular level): for example, alterations to the structure or quantity of the product of a proto-oncogene or tumour-suppressor gene, alterations to metabolic activation/inactivation/DNA repair

(iii) Evidence of relevant effects on cell behaviour (morphological or behavioural changes at the cellular or tissue level): for example, induction of mitogenesis, compensatory cell proliferation, preneoplasia and hyperplasia, survival of premalignant or malignant cells (immortalization, immunosuppression), effects on metastatic potential

(iv) Evidence from dose and time relationships of carcinogenic effects and interactions between agents: for example, early/late stage, as inferred from epidemiological studies; initiation/promotion/progression/malignant conversion, as defined in animal carcinogenicity experiments; toxicokinetics

These dimensions are not mutually exclusive, and an agent may fall within more than one of them. Thus, for example, the action of an agent on the expression of relevant genes could be summarized under both the first and second dimensions, even if it were known with reasonable certainty that those effects resulted from genotoxicity.

12. EVALUATION

Evaluations of the strength of the evidence for carcinogenicity arising from human and experimental animal data are made, using standard terms.

It is recognized that the criteria for these evaluations, described below, cannot encompass all of the factors that may be relevant to an evaluation of carcinogenicity. In considering all of the relevant scientific data, the Working Group may assign the agent, mixture or exposure circumstance to a higher or lower category than a strict interpretation of these criteria would indicate.

(*a*) *Degrees of evidence for carcinogenicity in humans and in experimental animals and supporting evidence*

These categories refer only to the strength of the evidence that an exposure is carcinogenic and not to the extent of its carcinogenic activity (potency) nor to the mechanisms involved. A classification may change as new information becomes available.

An evaluation of degree of evidence, whether for a single agent or a mixture, is limited to the materials tested, as defined physically, chemically or biologically. When the agents evaluated are considered by the Working Group to be sufficiently closely related, they may be grouped together for the purpose of a single evaluation of degree of evidence.

(i) *Carcinogenicity in humans*

The applicability of an evaluation of the carcinogenicity of a mixture, process, occupation or industry on the basis of evidence from epidemiological studies depends on the variability over time and place of the mixtures, processes, occupations and industries. The Working Group seeks to identify the specific exposure, process or activity which is considered most likely to be responsible for any excess risk. The evaluation is focused as narrowly as the available data on exposure and other aspects permit.

The evidence relevant to carcinogenicity from studies in humans is classified into one of the following categories:

Sufficient evidence of carcinogenicity: The Working Group considers that a causal relationship has been established between exposure to the agent, mixture or exposure circumstance and human cancer. That is, a positive relationship has been observed between the exposure and cancer in studies in which chance, bias and confounding could be ruled out with reasonable confidence.

Limited evidence of carcinogenicity: A positive association has been observed between exposure to the agent, mixture or exposure circumstance and cancer for which a causal interpretation is considered by the Working Group to be credible, but chance, bias or confounding could not be ruled out with reasonable confidence.

Inadequate evidence of carcinogenicity: The available studies are of insufficient quality, consistency or statistical power to permit a conclusion regarding the presence or absence of a causal association between exposure and cancer, or no data on cancer in humans are available.

Evidence suggesting lack of carcinogenicity: There are several adequate studies covering the full range of levels of exposure that human beings are known to encounter, which are mutually consistent in not showing a positive association between exposure to

the agent, mixture or exposure circumstance and any studied cancer at any observed level of exposure. A conclusion of 'evidence suggesting lack of carcinogenicity' is inevitably limited to the cancer sites, conditions and levels of exposure and length of observation covered by the available studies. In addition, the possibility of a very small risk at the levels of exposure studied can never be excluded.

In some instances, the above categories may be used to classify the degree of evidence related to carcinogenicity in specific organs or tissues.

(ii) *Carcinogenicity in experimental animals*

The evidence relevant to carcinogenicity in experimental animals is classified into one of the following categories:

Sufficient evidence of carcinogenicity: The Working Group considers that a causal relationship has been established between the agent or mixture and an increased incidence of malignant neoplasms or of an appropriate combination of benign and malignant neoplasms in (a) two or more species of animals or (b) in two or more independent studies in one species carried out at different times or in different laboratories or under different protocols.

Exceptionally, a single study in one species might be considered to provide sufficient evidence of carcinogenicity when malignant neoplasms occur to an unusual degree with regard to incidence, site, type of tumour or age at onset.

Limited evidence of carcinogenicity: The data suggest a carcinogenic effect but are limited for making a definitive evaluation because, e.g. (a) the evidence of carcinogenicity is restricted to a single experiment; or (b) there are unresolved questions regarding the adequacy of the design, conduct or interpretation of the study; or (c) the agent or mixture increases the incidence only of benign neoplasms or lesions of uncertain neoplastic potential, or of certain neoplasms which may occur spontaneously in high incidences in certain strains.

Inadequate evidence of carcinogenicity: The studies cannot be interpreted as showing either the presence or absence of a carcinogenic effect because of major qualitative or quantitative limitations, or no data on cancer in experimental animals are available.

Evidence suggesting lack of carcinogenicity: Adequate studies involving at least two species are available which show that, within the limits of the tests used, the agent or mixture is not carcinogenic. A conclusion of evidence suggesting lack of carcinogenicity is inevitably limited to the species, tumour sites and levels of exposure studied.

(*b*) *Other data relevant to the evaluation of carcinogenicity and its mechanisms*

Other evidence judged to be relevant to an evaluation of carcinogenicity and of sufficient importance to affect the overall evaluation is then described. This may include data on preneoplastic lesions, tumour pathology, genetic and related effects, structure–activity relationships, metabolism and pharmacokinetics, physicochemical parameters and analogous biological agents.

Data relevant to mechanisms of the carcinogenic action are also evaluated. The strength of the evidence that any carcinogenic effect observed is due to a particular mechanism is assessed, using terms such as weak, moderate or strong. Then, the Working Group assesses if that particular mechanism is likely to be operative in humans. The strongest indications that a particular mechanism operates in humans come from data on humans or biological specimens obtained from exposed humans. The data may be considered to be especially relevant if they show that the agent in question has caused changes in exposed humans that are on the causal pathway to carcinogenesis. Such data may, however, never become available, because it is at least conceivable that certain compounds may be kept from human use solely on the basis of evidence of their toxicity and/or carcinogenicity in experimental systems.

For complex exposures, including occupational and industrial exposures, the chemical composition and the potential contribution of carcinogens known to be present are considered by the Working Group in its overall evaluation of human carcinogenicity. The Working Group also determines the extent to which the materials tested in experimental systems are related to those to which humans are exposed.

(*c*) *Overall evaluation*

Finally, the body of evidence is considered as a whole, in order to reach an overall evaluation of the carcinogenicity to humans of an agent, mixture or circumstance of exposure.

An evaluation may be made for a group of chemical compounds that have been evaluated by the Working Group. In addition, when supporting data indicate that other, related compounds for which there is no direct evidence of capacity to induce cancer in humans or in animals may also be carcinogenic, a statement describing the rationale for this conclusion is added to the evaluation narrative; an additional evaluation may be made for this broader group of compounds if the strength of the evidence warrants it.

The agent, mixture or exposure circumstance is described according to the wording of one of the following categories, and the designated group is given. The categorization of an agent, mixture or exposure circumstance is a matter of scientific judgement, reflecting the strength of the evidence derived from studies in humans and in experimental animals and from other relevant data.

Group 1 — The agent (mixture) is carcinogenic to humans.
The exposure circumstance entails exposures that are carcinogenic to humans.

This category is used when there is *sufficient evidence* of carcinogenicity in humans. Exceptionally, an agent (mixture) may be placed in this category when evidence of carcinogenicity in humans is less than sufficient but there is *sufficient evidence* of carcinogenicity in experimental animals and strong evidence in exposed humans that the agent (mixture) acts through a relevant mechanism of carcinogenicity.

Group 2

This category includes agents, mixtures and exposure circumstances for which, at one extreme, the degree of evidence of carcinogenicity in humans is almost sufficient, as well as those for which, at the other extreme, there are no human data but for which there is evidence of carcinogenicity in experimental animals. Agents, mixtures and exposure circumstances are assigned to either group 2A (probably carcinogenic to humans) or group 2B (possibly carcinogenic to humans) on the basis of epidemiological and experimental evidence of carcinogenicity and other relevant data.

Group 2A — The agent (mixture) is probably carcinogenic to humans. The exposure circumstance entails exposures that are probably carcinogenic to humans.

This category is used when there is *limited evidence* of carcinogenicity in humans and *sufficient evidence* of carcinogenicity in experimental animals. In some cases, an agent (mixture) may be classified in this category when there is *inadequate evidence* of carcinogenicity in humans, *sufficient evidence* of carcinogenicity in experimental animals and strong evidence that the carcinogenesis is mediated by a mechanism that also operates in humans. Exceptionally, an agent, mixture or exposure circumstance may be classified in this category solely on the basis of *limited evidence* of carcinogenicity in humans.

Group 2B — The agent (mixture) is possibly carcinogenic to humans. The exposure circumstance entails exposures that are possibly carcinogenic to humans.

This category is used for agents, mixtures and exposure circumstances for which there is *limited evidence* of carcinogenicity in humans and less than *sufficient evidence* of carcinogenicity in experimental animals. It may also be used when there is *inadequate evidence* of carcinogenicity in humans but there is *sufficient evidence* of carcinogenicity in experimental animals. In some instances, an agent, mixture or exposure circumstance for which there is *inadequate evidence* of carcinogenicity in humans but *limited evidence* of carcinogenicity in experimental animals together with supporting evidence from other relevant data may be placed in this group.

Group 3 — The agent (mixture or exposure circumstance) is not classifiable as to its carcinogenicity to humans.

This category is used most commonly for agents, mixtures and exposure circumstances for which the *evidence of carcinogenicity* is *inadequate* in humans and *inadequate* or *limited* in experimental animals.

Exceptionally, agents (mixtures) for which the *evidence of carcinogenicity* is *inadequate* in humans but *sufficient* in experimental animals may be placed in this category

when there is strong evidence that the mechanism of carcinogenicity in experimental animals does not operate in humans.

Agents, mixtures and exposure circumstances that do not fall into any other group are also placed in this category.

Group 4 — The agent (mixture) is probably not carcinogenic to humans.

This category is used for agents or mixtures for which there is *evidence suggesting lack of carcinogenicity* in humans and in experimental animals. In some instances, agents or mixtures for which there is *inadequate evidence* of carcinogenicity in humans but *evidence suggesting lack of carcinogenicity* in experimental animals, consistently and strongly supported by a broad range of other relevant data, may be classified in this group.

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GENERAL REMARKS

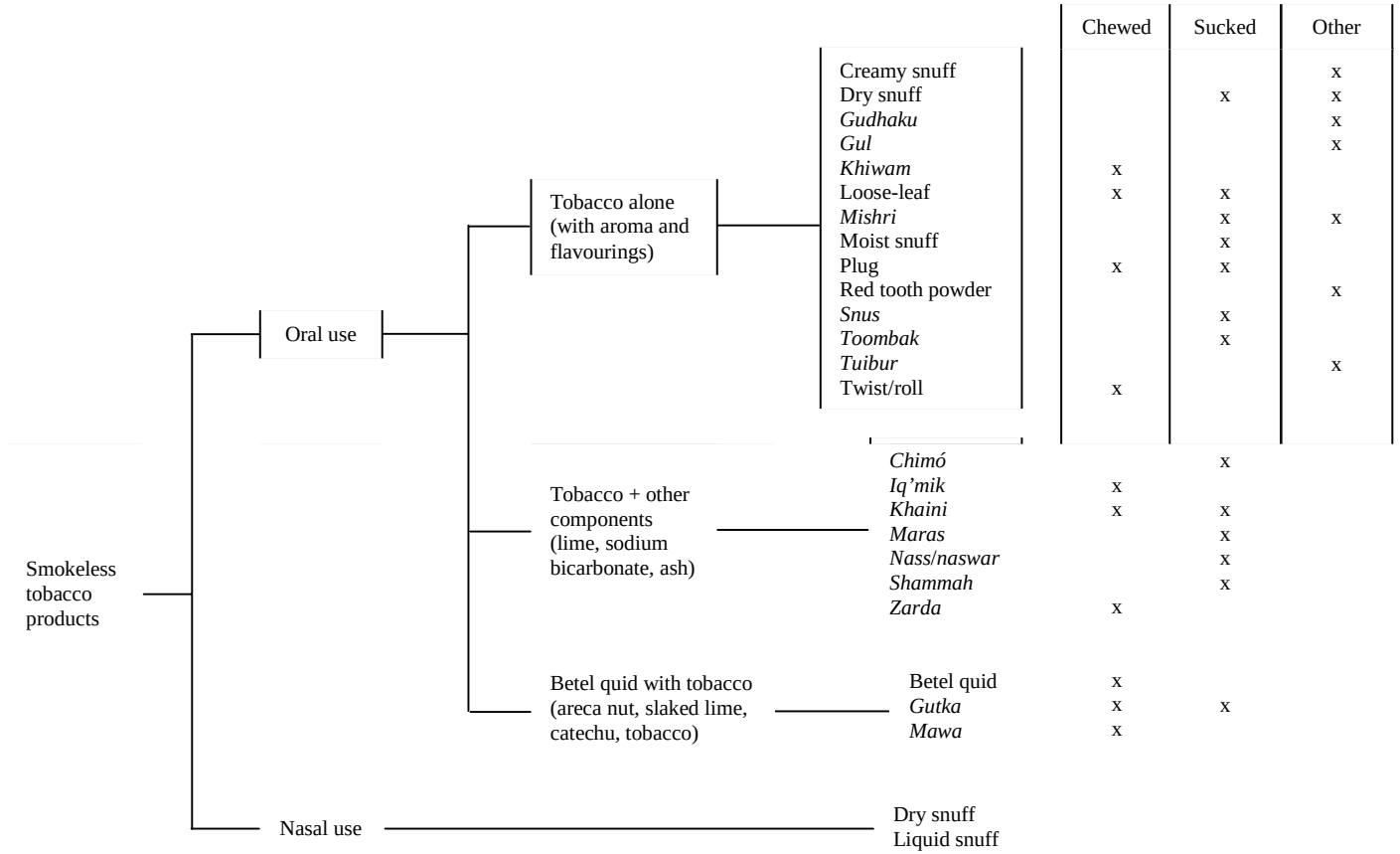
This eighty-ninth volume of the *IARC Monographs* is the third and last of a series on tobacco-related agents. Volume 83 reported on the carcinogenicity of tobacco smoke and involuntary smoking (second-hand smoke or environmental tobacco smoke) (IARC, 2004a). Volume 85 summarized the evidence on the carcinogenic risk of chewing betel quid with and without tobacco (IARC, 2004b). That volume explored the variety of products chewed in South Asia and other parts of the word that contain areca nut in combination with other ingredients, often including tobacco. In this eighty-ninth volume, the carcinogenic risks associated with the use of smokeless tobacco, including chewing tobacco and snuff, are considered in a first monograph. The second monograph reviews some tobacco-specific nitrosamines. These agents were evaluated earlier in Volume 37 of the *Monographs* (IARC, 1985), and information gathered since that time has been summarized and evaluated.

The agent termed 'smokeless tobacco' includes a large variety of commercially or non-commercially available products and mixtures that contain tobacco as the principal constituent and are used either orally or nasally without combustion. Figure 1 presents the forms of smokeless tobacco that are evaluated in this volume and their mode of use. For all products, except those that contain areca nut, the only known source of carcinogenic agents is the tobacco. (For tobacco chewed with betel quid or areca nut, see IARC, 2004b). The expression 'smokeless tobacco' was preferred to other terms such as 'non-smoking tobacco', 'non-smoked tobacco', 'unsmoked tobacco' and 'uncombusted tobacco', despite the imprecision of this term in the English language and the potential difficulty in its translation (see IARC, 1985).

The oral and nasal use of tobacco, either in leaf form for chewing or finely powdered as snuff, is as old as its use for smoking in pipes, cigars and cigarettes. In the first half of the twentieth century, the use of chewing tobacco and snuff in Europe and North America was overtaken by a huge increase in the smoking of cigarettes. In some parts of the world, particularly in South Asia, however, smokeless tobacco is still widely used. In addition, there has been a resurgence in the use of chewing tobacco and snuff in some European countries and in the USA during the last few decades.

In recent years, tobacco manufacturing companies have developed smokeless tobacco products with potential reduced exposure, also know as PREPs. These products are promoted and marketed by industry with claims that imply reduced risks and, subsequently,

Figure 1. Forms of smokeless tobacco evaluated in this volume



harm. The issue regarding the use of PREPs in the reduction of harm caused by tobacco has been reviewed (Henningfield *et al*., 2002; Tomar, 2002; Hatsukami *et al*., 2004).

Some health scientists have suggested that smokeless tobacco should be used for smoking cessation, and claim that its use would reduce the smoker's exposure to carcinogens and risk for cancer. They also attribute declines in smoking in Sweden to increased consumption of moist snuff in that country. However, as discussed in Section 1 of the monograph on Smokeless Tobacco, these claims are not supported by the available evidence.

Occupational exposure to unburnt tobacco may occur during tobacco manufacture, particularly in *bidi* factories in India, which are often very small-scale industries that have poor working conditions. The workers are mainly women and are exposed to tobacco by dermal contact, and also have airborne exposure to tobacco dust and volatile components. Studies of such industries have mainly reported on the concentration of tobacco dust and particulate matter in the ambient air in the factories, as well as biomonitoring of the workers, but no epidemiological studies on tobacco-related health risks in these workers have been carried out.

While in the Americas, Europe and Oceania, the leading cancers are those of the lung, breast, prostate and colorectum, cancer of the oral cavity is one of the leading malignancies in India and many other countries in South-East Asia, and ranks first in incidence among men and third among women, after cancer of the cervix and of the breast (IARC, 2003).

Oral leukoplakia is considered to be a precursor stage of oral cancer and is also prevalent in South Asia; this precancerous lesion is therefore also discussed in Section 2, Studies of Cancer in Humans. The term 'snuff-induced lesions', which is sometimes used in research articles, is avoided because of the ambiguity in the type of lesions to which it refers.

Tobacco-specific *N*-nitroso compounds have been detected at high concentrations in snuff and chewing tobacco and were evaluated in a previous monograph (IARC, 1985). New tobacco-specific nitrosamines have been identified and isolated since that time. However, only those for which there are sufficient mechanistic data to be able to draw a conclusion on their carcinogenicity were evaluated. Many other known carcinogens have been identified in various forms of tobacco (IARC, 2004a), including smokeless tobacco. The identification of nitrosamines as carcinogenic agents does not rule out the likelihood that other compounds present in tobacco may also contribute to their carcinogenicity.

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THE MONOGRAPHS

SMOKELESS TOBACCO

SMOKELESS TOBACCO

1. Description of Smokeless Tobacco Practices

1.1 Historical overview

The tobacco plant is thought to have originated on the mainland between North and South America. Its cultivation probably dates back at least 5000 years; tobacco seeds were discovered during archaeological excavations in both Mexico and Peru around 3500 BC, which shows that tobacco was an article of value to the inhabitants (Voges, 1984).

American Indians were probably the first people to smoke, chew and snuff tobacco, as early as the 1400s (Christen *et al.*, 1982). The Indians inhaled powdered tobacco through a hollow Y-shaped piece of cane or pipe by placing the forked ends into each nostril and the other end near the powdered tobacco. This instrument was called a '*tobago*' or '*tobaca*'. The word was later changed by the Spaniards to 'tobacco' (Christen *et al*., 1982).

1.1.1 *Tobacco chewing*

In 1499, Amerigo Vespucci found Indians on Margarita Island, off the coast of Venezuela, who chewed a green herb known as tobacco in order to quench their thirst, since it produced an increase in salivation; he also reported that the Indians chewed tobacco leaves to whiten their teeth and to alleviate hunger (Heimann, 1960; Stewart, 1967; Voges, 1984).

The practice of tobacco chewing was widespread in parts of Central and South America in the late 1500s (Voges, 1984). Columbus, in 1571, observed men in Veragua, later known as Costa Rica, who put a dry herb in their mouths and chewed it (Heimann, 1960). Use of plug tobacco was reported in Santo Domingo during the sixteenth century. Tobacco chewing seems to have been a common practice among the American Indians, especially when long distances had to be covered; it has been reported that tobacco was the support against hunger, thirst and fatigue when an American Indian would trek for 2 or 3 days with no other support. Several American tribes mixed either lime or finely-powdered and burned, fresh- or saltwater molluscs with their chewing tobacco (Curtis, 1935).

Among native Americans, chewing tobacco was thought to have several medicinal uses, such as to alleviate toothache, to disinfect cuts by spitting the tobacco juice and

saliva mixture onto the wound, and to relieve the effects of snake, spider and insect bites (Axton, 1975).

By 1531, the Spaniards were growing tobacco commercially in the West Indies and maintained a monopoly over the European markets until 1575, at which time the Portuguese began to grow large quantities of the commodity. Tobacco was soon grown in Europe as both a decorative and medicinal plant. In 1559, Jean Nicot, in whose honour the genus *Nicotiana* was named, was ambassador to Sebastian, King of Portugal. He grew tobacco and promoted the product in Europe for its magic 'cure-all' properties. By the early seventeenth century, tobacco had become one of the major exports of the American colonies (Christen *et al.*, 1982) and its use in various forms had spread throughout Europe, Turkey, Russia, Arabia, China, Alaska and the world (Axton, 1975). Portugese and Spanish sailing crews who were addicted to tobacco carried seeds, and planted them at ports.

When smoking was forbidden on British naval vessels because of the fire hazard, sailors turned to chewing tobacco and snuff. In Europe, tobacco was regarded as a prophylactic during the plague and, for those who did not like smoking, chewing was an alternative. Tobacco chewing was recommended for cleaning the teeth of women and children (Brooks, 1952). Chewing tobacco became popular in the USA only during the first half of the nineteenth century (Gottsegen, 1940). In spite of two centuries of pipe smoking and snuff use, by the mid-1850s, North Americans rejected the European practices in general, and British practices in particular, that entailed snuff boxes and formality; in addition, tobacco chewing was more convenient for Americans who trekked westward in their wagons. During the 1860s, tobacco was chewed in the form of either a plug or a twist. Of the 348 tobacco factories listed in the 1860 Census for Virginia and North Carolina, only seven manufactured smoking products (Heimann, 1960). American pioneers resorted to the use of a home-made sweet plug, so-named because the leaf was wadded into a hole in a log and laced with a sweetening agent (usually brandy or cane sugar), which, after removal of the fermented leaf, resulted in a tasty chew (Axton, 1975).

In 1797, Adam Clarke, a famous Methodist minister, appealed to all tobacco consumers and religious followers to avoid the use of tobacco for the sake of their health and their souls. This plea was also due to the fact that it had become unsafe to kneel when praying because chewers had made the floors unsanitary (Brooks, 1952).

During the latter part of the nineteenth century, the 'germ theory of infection' changed the course of chewing in America, and it was felt that expectorating on the floor and into a brass cuspidor could be a source of contamination and the spread of disease. By the 1890s, public outcry made tobacco chewing socially unacceptable behaviour and unlawful in most public places (Christen *et al.*, 1982). Anti-spitting laws were passed in New York and Philadelphia, USA, in 1896 and in Toronto, Canada, in 1904 (Kozlowski, 1981).

The market for chewing tobacco passed its peak in 1890, when some 3 lb (about 1.5 kg) of plug, twist or fine-cut chewing tobacco were chewed annually per capita in the USA (Heimann, 1960). Nevertheless, chewing remained the dominant form of tobacco use in America until the expansion of the cigarette industry in 1918 (Maxwell, 1980). In 1945, cuspidors were removed from all federal buildings by order of the US District Court

in Washington DC (Brooks, 1952). The apparent decline in tobacco chewing is exemplified by a memorandum of 14 September 1955 to the American Tobacco Company, stating, “It has become impossible to hire persons in the New York area to clean and maintain cuspidors ... it will be necessary to remove them promptly from the premises” (Heimann, 1960). During the second half of the 1960s through to the 1970s, however, a resurgence in tobacco chewing occurred in the USA (Christen & Glover, 1981).

1.1.2 *Snuff taking*

The native populations of Brazil were the first people known to use snuff. Using a cup and a pestle made from rosewood, the tobacco leaves were ground into a powder and acquired the delicate aroma of the wood. The resulting snuff was placed in ornately decorated bone tubes, one end of which was plugged to preserve the fragrance (Curtis, 1935). The American Indians inhaled powdered tobacco through a hollow Y-shaped piece of cane or pipe by placing the forked ends into each nostril and the other end near the powdered tobacco (Christen *et al.*, 1982).

Friar Ramón Pané, a Franciscan monk who travelled with Christopher Columbus on his second voyage to the New World in 1493, reported that the Caribbean Indians of the lesser Antilles used snuff (Christen *et al.*, 1982). In Haiti, snuff powder was used by medicine men for clearing nasal passages and as an analgesic (Stewart, 1967). Friar Pané’s return to Spain with snuff signalled the arrival in Europe of a practice that was to last for several centuries.

In 1519, Ocaranza found that Mexican Indians used tobacco powder to heal burns and wounds and, in 1525, Herrera observed that Mexican Indians held tobacco powder in their mouth to send them to sleep and reduce pain (Stewart, 1967).

The Dutch, who named the powdered tobacco ‘snuff’, were using the product by 1560 (Christen *et al.*, 1982). By the early 1600s, snuff had become an expensive commodity and its use had spread throughout South America, China, Japan and Africa. The origin of the process terms ‘carotte’ and ‘rappee’ goes back to the 1600s when tobacco for snuff was prepared in the form of a carrot to be rasped in the quantity desired for use (Curtis, 1935). In 1620, the Royal Snuff Factory was established in Seville, and this became the centre of the manufacture and development of this product (Voges, 1984). Snuff use expanded through Japan to China (Ching Dynasty) in the 1650s: palace artisans produced exquisitely carved, inlaid enamelled or painted snuff bottles with a tiny spoon attached to the bottle stopper; a small portion of snuff was placed on the left thumbnail and inhaled through the nose. The Chinese believed that snuff cured pains in the eyes and teeth, alleviated throat ailments, constipation and cold symptoms, and promoted sweating (Christen *et al.*, 1982).

By 1650, snuff use had also spread from France to England, Scotland and Ireland. The Irish called snuff ‘powder’ or ‘smutchin’; the Scots called it ‘sneeshin’ (Harrison, 1964). Jean Nicot is credited with introducing snuff to Catherine de Medici, Queen of France, to cure her headaches (Christen *et al.*, 1982).

Snuff use reached a peak in England during the reign of Queen Anne (1702–14), and was called the ‘final reason for the human nose’. It was at this time that ready-made snuff became available in England. It continued to be popular during the reign of George III, and his wife, Charlotte (1760–1820), referred to as ‘Snuffy Charlotte’, had an entire room in Windsor Castle devoted to her snuff stock. Lord Nelson, the Duke of Wellington, Marie Antoinette, Disraeli, Alexander Pope and Samuel Johnson all used snuff (Harrison, 1964). In diplomatic intrigue, poisons were sometimes placed in snuff. The aristocratic popularity of snuff led to a minor art form, in that snuff boxes became symbols that reflected the wealth and rank of their owner. The dandy, Lord Petersham, was said to own an annual set of 365 snuff boxes (Christen *et al.*, 1982).

The leading snuff supplier of the time provided King George IV with his own special blends, King’s Morning Mix, King’s Plain and King’s Carotte (Ryan, 1980). Home-made snuff was common. The tightly-rolled tobacco leaves (carotte) were often soaked in cinnamon, lavender or almond oils; tobacco was dried and ground by means of an iron hand-grater that resembled a modern cheese-grater. The proper manner of inhaling snuff was to place a small quantity on the back of the hand and sniff it up the nostrils to induce a sneeze (Christen *et al.*, 1982).

Although hundreds of varieties of snuff existed in Europe by the 1800s, these consisted of three basic types: Scotch snuff, which was a dry, strong, unflavoured and finely ground powder; Maccaboy, a moist and highly scented snuff; and Rapee, also known as Swedish snuff, a coarsely grated snuff (Heimann, 1960).

Snuff was introduced into Sweden in the middle of the seventeenth century, but its popularity among aristocrats reached a height during the eighteenth century, when use of nasal snuff became the highest fashion at the court of King Gustav III, among both men and women. The practice subsequently spread to the general Swedish population.

In many Swedish cities, snuff has been manufactured since the beginning of the eighteenth century. In Gothenburg, which is considered to be the centre of snuff production, manufacture started in about 1650 (Loewe, 1981). In 1795, Samuel Fiedler established a snuff mill in Gothenburg and began a small business, which later developed into three separate companies. At the end of the nineteenth century, the leading producer was Jacob Ljunglöf in Stockholm; his leading brand ‘Ettan’ became well known throughout Europe (Loewe, 1981). In 1914, the production of snuff in Sweden was taken over by the Swedish tobacco monopoly, which restored Gothenburg as its centre. A large factory was built around 1920, and expanded in 1979, for the production of snuff and chewing tobacco.

Since the beginning of the twentieth century, snuff has been used mainly orally in Sweden. In the 1950s and 1960s, use of moist snuff was prevalent predominantly among older men and was heading towards a ‘natural death’: the median age of consumers in 1969–70 was over 40 years (Nordgren & Ramström, 1990). However, the development of new products and intensive advertisement and promotion by Swedish Match, the country’s primary snuff manufacturer, starting in the late 1960s, led to a surge in the use of moist snuff among young men. By 1972–73, the median age of moist snuff users had dropped to 30 years (Nordgren & Ramström, 1990). More recently, Swedish Match has

been representing its moist snuff products as less harmful tobacco products than cigarettes (Henningfield & Fagerström, 2001). As discussed later in this section, the prevalence of smokeless tobacco use continues to increase in Sweden, particularly among young men.

Commercially manufactured snuff made its way to North America in 1611 by way of John Rolfe, husband of Pocahontas. Rolfe introduced the better Spanish variety of tobacco to ensure the survival of the Jamestown Colony in Virginia. Although most of the colonists in America never fully accepted the English style of snuff use, American aristocrats used snuff, and Dolly Madison was known to distribute samples of snuff to White House guests. During the 1800s until the mid 1930s, a communal snuff box was installed for members of the US Congress. The colonists also found it more to their taste to place snuff in their mouths rather than to sniff it (Christen *et al*., 1982).

The first snuff mills in America were constructed in Virginia in about 1730 (Heimann, 1960). The snuff was made from New England tobacco and its quality was said to equal that of the native Scottish varieties (Robert, 1949). Pierre Lorillard, a Huguenot, established a snuff mill in New York in 1760 and carefully guarded the secret of the ingredients and blends of his products (Christen *et al*., 1982).

Between 1880 and 1930, the production of snuff in the USA increased from 4 million lb (1.8 million kg) to more than 40 million lb (18 million kg) per year (Garner, 1951). By 1945, the American Snuff Company in Memphis, TN, claimed to be the largest snuff manufacturer in the world (Christen *et al*., 1982). Snuff was made predominantly from dark, air- and fire-cured leaves. Stems and leaves were aged in hogsheads and conditioned before being cut into strips of 1–2 in (2.5–5 cm) in width. The chopped leaves underwent further fermentation for about 2 months, during which time the tobacco lost its creosote-like odour and became more aromatic. It was next dried by passing it through steam-heated containers and then ground to a fine powder in a revolving steel drum. The powder was passed over silk cloth that contained as many as 96 threads per in (38 per cm). The coarse residue was returned to the mill for additional grinding before being packed into 100-lb (45-kg) bags for storage prior to repacking in smaller containers for retail sale. The dry and moist snuffs were used for dipping and placing in the mouth. Rappee or French snuff was used for inhaling, and Maccaboy snuff was both sucked and inhaled (Garner, 1951).

The use of smokeless tobacco products in the USA was widespread throughout the nineteenth century. Dental snuff was advertised to relieve toothache; to cure neuralgia, bleeding gums and scurvy; and to preserve and whiten teeth and prevent decay (Christen *et al*., 1982). With the advent of anti-spitting laws, loss of social acceptability and increased popularity of cigarette smoking, its use declined rapidly during the twentieth century.

Beginning in the mid-1970s, the US Tobacco Company (later renamed the US Smokeless Tobacco Co.), the leading manufacturer of smokeless tobacco products in the USA, developed new products, new images and an aggressive marketing campaign to expand its market (Connolly *et al*., 1986; Connolly, 1995). The marketing campaign included a ‘graduation’ marketing strategy that was designed to recruit new, young users with low-dose nicotine ‘starter’ moist snuff products and move them to higher-dosage products as they developed tolerance and addiction to nicotine (Connolly, 1995). The result was a nine-

fold increase in the prevalence of snuff use among young adult men (< 24 years old) between 1970 and 1987 (Giovino *et al*., 1994; Giovino, 1999). The United States Smokeless Tobacco Company continues to market its products for young men (Myers, 2003) and, in recent years, has also been marketing products for smokers as an alternative tobacco product, particularly for use when faced with smoking restrictions (Henningfield *et al*., 2002).

Tobacco was introduced into South Asia in the 1600s as a product to be smoked and was gradually used in many different forms (Bhonsle *et al*., 1992; Gupta & Ray, 2003). The chewing of betel quid (*pan*) was a popular practice that existed for over 2000 years and extended eastwards as far as the South Pacific Islands. After its introduction, tobacco soon became a new ingredient in betel quid, which has become the most commonly used form of smokeless tobacco in South Asia (Gupta & Ray, 2003; IARC, 2004a).

In Sudan, the introduction of *toombak* is historically attributed to a Koranic (Islamic) teacher, who came from Egypt, Timbuktu in Mali, Morocco, Turkey or Arabia, and dates back several centuries (Idris *et al*., 1998a). Another popular name for *toombak* is *sute*, which means 'sniffing of the product' in the local language, and indicates nasal usage when it was first introduced.

1.1.3 *Attitudes and beliefs regarding smokeless tobacco use*

The use of tobacco, including smokeless tobacco, has been controversial since its introduction. Therefore, a history of smokeless tobacco use is not complete without a discussion of the attacks on tobacco by various groups. In 1590 in Japan, tobacco was prohibited, and users lost their property or were jailed. James VI of Scotland, who became King James I of England and Ireland in 1603, was a strong anti-smoking advocate and increased taxes on tobacco by 4000% in an attempt to reduce the quantity imported into England. In 1633, the Sultan Murad IV of Turkey made any use of tobacco a capital offence, punishable by death from hanging, beheading or starvation, and maintained that tobacco caused infertility and reduced the fighting capabilities of his soldiers. The Russian Czar Michael Fedorovich, the first Romanov (1613–45), prohibited the sale of tobacco, and stated that users would be subject to physical punishment; persistent users would be killed. A Chinese law in 1638 threatened that anyone who possessed tobacco would be beheaded (Christen *et al*., 1982).

During the mid 1600s, Pope Urban VIII banned the use of snuff in churches, and Pope Innocent X attacked its use by priests in the Catholic Church. Other religious groups banned snuff use: John Wesley (1703–91), the founder of Methodism, attacked its use in Ireland; similarly, the Mormons, Seventh-Day Adventists, Parsees and Sikhs of India, Buddhist monks of Korea, members of the Tsai Li sect of China, and some Ethiopian Christian sects forbade the use of tobacco (Christen *et al*., 1982).

In Bavaria, Germany, in 1652, tobacco was available only on a doctor's prescription; Frederick the Great, King of Prussia, prevented his mother, the Dowager Queen of Prussia,

from using snuff at his coronation in 1790. Louis XV, ruler of France from 1723 to 1774, banned the use of snuff from the Court of France (Christen *et al.*, 1982).

In 1761, John Hill, a London physician and botanist, concluded that nasal cancer could develop as a consequence of snuff use. He reported five cases of 'polypusses, a swelling in the nostril that was hard, black and adherent with the symptoms of an open cancer' (Redmond, 1970).

1.2 Manufacture and use of smokeless tobacco products

Smokeless tobacco is consumed without burning the product, and can be used orally or nasally. Oral smokeless tobacco products are placed in the mouth, cheek or lip and sucked (dipped) or chewed. Tobacco pastes or powders are used in a similar manner and applied to the gums or teeth. Fine tobacco mixtures are usually inhaled and absorbed in the nasal passages. Table 1 lists smokeless tobacco products according to their mode of use.

Table 1. Classification of smokeless tobacco products by mode of use

Oral use			Nasal use (sniffing)
Sucking	Chewing	Other oral uses	
Chimó	Betel quid	Creamy stuff	Dry snuff
Dry snuff	*Gutka*	*Gudhaku*	Liquid snuff
Gutka	*Iq'mik*	*Gul*	
Khaini	*Khaini*	*Mishri*	
Loose-leaf	*Khiwam*	Red tooth powder	
Maras	Loose-leaf	*Tuibur*	
Mishri	*Mawa*		
Moist snuff	Plug		
Naswar	Tobacco chewing gum		
Plug	Twist or roll		
Shammah	*Zarda*		
Snus			
Tobacco tablets			
Toombak			

Smokeless tobacco products are used throughout the world (National Cancer Institute/Centers for Disease Control, 2002; Gupta & Ray, 2003). Table 2 presents an overview of their use by WHO region. It is worth noting that some products are known to be used by immigrants from certain regions where a product is used to other regions.

There are many different botanical classifications for tobacco plants. The genus *Nicotiana* is classified into three main subgenera, *N. rustica*, *N. tabacum* and *N. petuniodes*. Smokeless tobacco products use *N. tabacum*, and sometimes *N. rustica*. In the USA, tobacco is also classified by the curing method (e.g. flue-cured, air-cured, dry air-cured

tobacco) and by production areas (Virginia, North Carolina, Tennessee, Wisconsin) (Tso, 1990).

Table 2. Use of smokeless tobacco products by WHO region

Tobacco product	WHO Region[a]					
	AFRO	AMRO	EMRO	EURO	SEARO	WPRO
Oral use						
Betel quid with tobacco			X		X	X
Chimó		X				
Creamy snuff					X	
Dry snuff	X	X		X		
Gul					X	
Gudhaku					X	
Gutka					X	
Iq'mik		X				
Khaini					X	
Khiwam					X	
Loose leaf		X		X		
Maras				X		
Mawa					X	
Mishri					X	
Moist snuff		X		X		
Naswar	X		X	X		
Plug chewing tobacco		X				
Red tooth powder					X	
Shammah			X	X		
Tobacco chewing gum						X
Tobacco tablet		X				
Toombak	X					
Tuibur					X	
Twist/roll chewing tobacco		X				
Zarda			X		X	
Nasal use						
Dry snuff	X		X	X	X	
Liquid snuff	X					

[a] The countries included in each region are available at: http://www.who.int/about/regions/en/

Notes:
Some of these products are known to be used by immigrants from certain regions where a product is used to other regions of the world.
This table was compiled by the experts present at the meeting and is based on individual knowledge about use of these products and is not intended to be exhaustive or complete.

1.2.1 *Oral use*

Oral use of smokeless tobacco is practised in Africa, North America, South-East Asia, Europe and the Middle East, and consists of placing a piece of tobacco or tobacco product in the mandibular groove and either chewing or sucking it for a certain period of time: a 'chaw', which refers to a portion of tobacco the size of a golf ball, is generally chewed, whereas a 'quid' is usually a much smaller portion and is held in the mouth rather than chewed (Pindborg *et al*., 1992).

(*a*) *Betel quid with tobacco*

Betel quid with tobacco, commonly known as *paan* or *pan*, consists of four main ingredients: (i) betel leaf (*Piper betle*), (ii) areca nut (*Areca catechu*), (iii) slaked lime and (iv) tobacco. Of these, tobacco is the most important ingredient for regular users. Betel quid can be prepared by the vendor or at home. Various tobacco preparations are used in unprocessed, processed or manufactured forms. Tobacco may be used in raw, sun-dried or roasted form, then finely chopped or powdered and scented. Alternatively, tobacco may be boiled, made into a paste and scented with rosewater or perfume. The final product is placed in the mouth and chewed. Betel quid with tobacco is used in Central, East, South and South-East Asia, in the western Pacific and in migrant communities arising therefrom (Bhonsle *et al*., 1992; Gupta & Ray, 2003). Exposure to and the health effects of betel quid with or without tobacco are described in detail in a previous monograph (IARC, 2004a).

(*b*) Chimó

Chimó is specific to Venezuela. It contains tobacco leaf, sodium bicarbonate, brown sugar, ashes from the Mamón tree (*Melicocca bijuga*), and vanilla and anisette flavourings. The ingredients vary according to the region within Venezuela. Tobacco leaves are crushed and boiled for several hours, during which starch and fibre are discharged. The remaining portion becomes a concentrated product: 10 kg of tobacco yield 1 kg of 'pasta'. For maturation, *chimó* is then placed in natural containers or 'taparas' (the dried fruit from the Tapara tree) or is wrapped in banana leaves. The matured paste is 'seasoned' with the ingredients listed above. Finally, it is packaged in small tins or candy-like wrapped cylinders. A small amount of *chimó* is placed between the lip or cheek and the gum and left there for some time, usually 30 min. The mixture of *chimó* and saliva is spat out.

(*c*) *Creamy snuff*

Creamy snuff consists of finely ground tobacco mixed with aromatic substances, such as clove oil, glycerin, spearmint, menthol and camphor, salts, water and other hydrating agents. It is often used to clean teeth. The manufacturer recommends letting the paste linger in the mouth before rinsing. Creamy snuff is manufactured commercially and marketed as a dentifrice, and is commonly used as such by women in South Asia.

(*d*) *Dry snuff*

In Europe and the USA, tobacco (primarily Kentucky and Tennessee tobacco) is fire-cured, then fermented and processed into a dry, powdered form. The moisture content of the finished product is less than 10%. Dry snuff is packaged and sold in small metal or glass containers. Typically, in the USA, a pinch (called a 'dip') is held between the lip or cheek and gum. In Europe, it is commonly inhaled into the nostrils (see Section 1.2.2 Nasal use).

In India, dry snuff was once commonly used nasally, but is now used mainly orally. It is frequently prepared at home by roasting coarsely cut tobacco on a griddle and then powdering it. This pyrolysed snuff-like preparation, mainly used in Goa, Maharastra, Gujarat and eastern parts of India, is widely used by the poorer classes as a dentifrice (applied to the teeth and gums), especially by women, but tends to be used many times a day, due to its addictive properties. It is known as *bajjar* or *tapkir*/*tapkeer*.

In many regions of the world, dry snuff is used both orally and nasally.

In northern Africa, dry snuff is known as *naffa*, *tenfeha* or *nufha*.

(*e*) Gudhaku

Gudhaku is a paste made of powdered tobacco and molasses. It is available commercially and is stored in a metal container. *Gudhaku* is applied to the teeth and gums with the finger, predominantly by women in India in the States of Bihar, Orissa, Uttar Pradesh and Uttaranchal.

(*f*) Gul

Gul contains tobacco powder, molasses and other ingredients and is manufactured commercially. It is applied to the teeth for the purpose of cleaning and then to the gums many times during the day. *Gul* is used in South Asia, including the Indian Subcontinent.

(*g*) Gutka

Gutka is manufactured commercially and consists of sun-dried, roasted, finely chopped tobacco, areca nut, slaked lime and catechu mixed together with several other ingredients such as flavourings and sweeteners. The product is sold in small packets or sachets. It is held in the mouth, sucked and chewed. Saliva is generally spat out, but is sometimes swallowed. *Gutka* is used in South Asia, including the Indian Subcontinent, and by Asian expatriates in several parts of the world, especially Canada, the United Kingdom and the USA (IARC, 2004a).

(*h*) Iq'mik

Fire-cured tobacco leaves are mixed with punk ash, which is generated by burning a woody fungus that grows on the bark of birch trees. The separate ingredients are available at grocery stores and retail outlets, but are generally combined by the user before use. Users pinch off a small piece and chew the *iq'mik*. The user may pre-chew the *iq'mik* and

place it in a small box for later use by others, including children and sometimes teething babies. *Iq'mik* is used by native Americans in the northwestern parts of North America.

(*i*) Khaini

Khaini is made from sun-dried or fermented coarsely cut tobacco leaves. The tobacco used for *khaini* is from *N. rustica* and/or *N. tabacum*. The tobacco leaves are crushed into smaller pieces. A pinch of tobacco is taken in the palm of the hand, to which a small amount of slaked lime paste is added. The mixture is then rubbed thoroughly with the thumb. *Khaini* is usually prepared by the user at the time of use, but is also available commercially. It is held in the mouth and sucked or chewed. Areca nut may sometimes be added to *khaini* by the user. *Khaini* is used in South Asia, including the Indian Subcontinent.

(*j*) Khiwam

Khiwam (or *qimam*) consists of tobacco extract, spices and additives. The tobacco used for *khiwam* is from *N. rustica* and/or *N. tabacum*. Tobacco leaves are processed by removing their stalks and stems, then boiling and soaking them in water flavoured with spices (e.g. saffron, cardamom, aniseed) and additives such as musk. The resulting pulp is mashed, strained and dried into a paste. The paste is placed in the mouth and chewed. *Khiwam* may also be used in betel quid (IARC, 2004a). It is used in South Asia, including the Indian Subcontinent.

(*k*) *Loose-leaf*

Loose-leaf tobacco is manufactured commercially and consists of loose cigar tobacco leaves from Pennsylvania and Wisconsin that are air-cured, stemmed, cut or granulated, and loosely packed to form small strips of shredded tobacco. Most brands are sweetened and flavoured with liquorice, and are typically sold in pouches weighing about 3 oz. Loose-leaf tobacco is high in sugar content (approximately 35%). A piece of tobacco 0.75–1 in in diameter is placed between the cheek and lower lip, typically toward the back of the mouth. It is either chewed or held in place. Saliva is spat or swallowed. Loose-leaf is used in Europe and North America.

(*l*) Maras

In Turkey, a type of smokeless tobacco called *maras* is widely used in the south-eastern region, especially in the cities of Kahramanmaras and Gaziantep. First, sun-dried leaves of the tobacco plant species *N. rustica* L. — known locally as 'crazy tobacco' — are powdered and mixed with the ash of wood, in particular oak, walnut or grapevine, in 1:2 or 1:3 proportions (tobacco and oak, respectively). Then, water is sprinkled onto the mixture for humidification. A small amount of the mixture (approximately 1 g) is applied between the lower labial mucosa and gingiva for 4–5 min. This procedure is repeated many times during the day; some people even sleep with the powder in their mouth.

(*m*) Mawa

Mawa is a mixture of small pieces of sun-cured areca nut with crushed tobacco leaves and slaked lime. The resulting mixture is about 95% areca nut by weight. It is placed in the mouth and chewed for 10–20 min. *Mawa* is used in South Asia, including the Indian Subcontinent.

(*n*) Mishri

Mishri is made from tobacco that is baked on a hot metal plate until toasted or partially burnt, and then powdered. It is applied to the teeth and gums as a dentifrice, usually twice a day and more frequently in some cases. Users then tend to hold it in their mouths. *Mishri* is used in South Asia, including the Indian Subcontinent.

(*o*) *Moist snuff*

The tobacco is either air- or fire-cured, then processed into fine particles (‘fine-cut’) or strips (‘long-cut’). Tobacco stems and seeds are not removed. The final product may contain up to 50% moisture. Moist snuff is sold either loose or packaged in small, ready-to-use pouches called packets or sachets. A pinch (called a dip) or a pouch is placed and held between the lip or cheek and gum. Saliva may be swallowed or, more commonly, spat out. Moist snuff is used in Europe and North America, and is the most common form of smokeless tobacco in the USA (see Section 1.4.2).

Swedish-type moist snuff (*snus*) consists of finely ground dry tobacco (Kentucky and Virginia tobacco), mixed with aromatic substances, salts (sodium chloride), water, humidifying agents and chemical buffering agents (sodium carbonate). A pinch (called a dip) is placed between the gum and upper lip. The average user keeps snuff in the mouth for 11–14 h per day. In Sweden, the portions come in two doses, regular and ‘mini-portions’ (1.0 g and 0.5 g tobacco, respectively), or loose.

(*p*) Naswar

Naswar (or *nass*) is a mixture of sun-dried, sometimes only partially cured, powdered local tobacco (*N. rustica*), ash, oil, flavouring agents (e.g. cardamom, menthol), colouring agents (indigo) and, in some areas, slaked lime. It is made by pouring water into a cement-lined cavity to which lime is added, followed by tobacco. Colouring and flavouring agents are then added. The ingredients are then pounded and mixed with a heavy wooden mallet. The type of oil varies by region. Water is added and the mixture is rolled into balls. It is then usually placed under the tongue (in the floor of the mouth) and then sucked. *Naswar* is used widely in Afghanistan, Iran, Pakistan and the central Asian Republics, and in South Africa.

(*q*) *Plug chewing tobacco*

Plug is the oldest form of chewing tobacco. It is produced from the heavier grades of Burley and bright tobacco or cigar tobacco leaves harvested from the top of the plant.

Once the stems are removed, the leaves are immersed in a mixture of liquorice or sugar, pressed into a plug, covered by a wrapper leaf and re-shaped into bricks or flat blocks. Moist plug tobacco has at least 15% moisture content; plug or 'firm plug' tobacco has less than 15% moisture content. Sugar content is approximately 25%. Moist plug is chewed, or held between the cheek or lower lip and gum. Saliva is spat or swallowed. Moist plug is used primarily in North America.

(*r*) *Red tooth powder*

Red tooth powder is a fine tobacco powder that is red in colour and contains many additional ingredients including herbs and flavouring agents. It is manufactured commercially and marketed as a herbal product. Red tooth powder is used in South Asia as a dentifrice.

(*s*) Shammah

Shammah is a mixture of powdered tobacco, lime, ash, black pepper, oils and flavourings. The greenish-yellow powder is placed in the buccal or lower labial vestibule of the mouth. The user spits out insoluble debris. It is used in the Middle East, including some parts of southern Saudi Arabia and Yemen.

(*t*) *Snuff*

Two types of snuff are used orally: dry snuff and moist snuff; these are discussed under (*d*) and (*o*), respectively. Dry snuff may also be used nasally (see Section 1.2.2(*a*)).

(*u*) *Tobacco chewing gum*

Tobacco chewing gum was developed by the company Swedish Match in 2003 and marketed under the brand name 'Fire' as an alternative tobacco product and test marketed in Tokyo, Japan.

(*v*) *Tobacco tablets*

Tobacco tablets were introduced on the market in 2002 in the form of 10-piece blister card. They are made of compressed powdered tobacco, mint and eucalyptus and melt in the mouth. Each tablet contains approximately 1.3 mg nicotine (Nguyen *et al.*, 2002). Tobacco tablets are also known by the brand names Ariva® and Cigalett®.

(*w*) Toombak

Toombak is a moist tobacco product used primarily in Sudan. It consists of tobacco (*N. rustica* and/or *N. glauca*) and sodium bicarbonate. Tobacco leaves are harvested and left in a field to dry uniformly. The leaves are then tied into bundles, sprinkled with water and stored for a couple of weeks at 30–45 °C to allow fermentation. They are then ground and matured for up to 1 year. After maturation, *toombak* vendors (in *toombak* shops) place the product in bowls and gradually add sodium bicarbonate until the mixture is approxi-

mately four parts of tobacco to one part of sodium bicarbonate. The mixture is blended by hand and constantly tested with the tips of the fingers until it becomes moist and hardened. The *toombak* is then placed in an air-tight container shortly before sale.

Toombak is rolled into a ball that weighs about 10 g and is called a *saffa*. The *saffa* is held between the gum and the lip or cheek, or under the tongue on the floor of the mouth. It is sucked slowly for 10–15 min. Male users periodically spit, while female users typically swallow the saliva generated. The user usually rinses his/her mouth with water after the *saffa* is removed. Commercial names for *toombak* include El-Sanf (of high quality), Wad Amari (accrediting the person who was believed to have introduced it) and Sultan El-Khaif (the power to improve one's state of mind) (Idris *et al*., 1998a).

(*x*) Tuibur

Tuibur (or *hidakphu*) is tobacco water for oral use. Tobacco smoke is passed through water and the water is used for gargling or sipping. *Tuibur* is commonly used in the north-eastern states of India (Manipur, Mizoram, Sikkim, Tripura) (Mahanta *et al*., 1998).

(*y*) *Twist/roll chewing tobacco*

Twist/roll chewing tobacco is hand-made by commercial manufacturers. Dark, air- or fire-cured leaf Burley tobacco is treated with a tar-like tobacco leaf extract and flavours, and twisted into rope-like strands that are dried. The product is sold by the piece in small (about 50 g) or larger sizes based on the number of leaves in the twist. Twist/roll is used in North America.

(*z*) Zarda

Zarda consists of tobacco, lime, spices and vegetable dyes. Tobacco leaves are broken up and boiled with lime and spices until dry. The mixture is dried and coloured with vegetable dyes. *Zarda* is generally chewed mixed with finely chopped areca nuts and spices. It is often used as an ingredient in betel quid. *Zarda* is commonly used in India and the Middle East, and is known as *dokta* in West Bengal.

1.2.2 *Nasal use*

(*a*) *Dry snuff*

Tobacco (primarily Kentucky and Tennessee tobacco) is fire-cured, then fermented and processed into a dry, powdered form. The moisture content of the finished product is less than 10%. It is packaged and sold in small metal or glass containers. In Europe, dry snuff is commonly inhaled into the nostrils. In many regions of the world, it is used both orally and nasally.

(*b*) *Liquid snuff*

Liquid snuff was reported to be used by the Nandi tribe in East Africa. It is used nasally (Hou-Jensen, 1964).

1.3 Chemical composition of smokeless tobacco

1.3.1 *General overview*

The type of tobacco used in a particular product has a decisive influence on its chemical composition. That of leaf tobacco varies with genetic make-up, environmental conditions and every step of production and handling (Tso, 1990). The classification of leaf tobacco commonly used in smokeless tobacco products is primarily based on curing methods (e.g. air-, flue- and fire-cured tobacco) and tobacco types (e.g. Burley, Wisconsin, Pennsylvania air-cured tobacco, dark fire-cured tobacco, Virginia flue-cured tobacco).

The first summary of chemical components found in tobacco and tobacco smoke was prepared by Stedman in 1968. Since then, frequent additions have been made to the list and, in 1988, the number of compounds identified in tobacco totaled 3044 (Roberts, 1988). The latter count has not been confirmed by independent research. Moreover, Roberts (1998) does not list many of the constituents that are currently known to be present in tobacco (e.g. volatile *N*-nitrosamines, tobacco-specific *N*-nitrosamines, *N*-nitrosamino acids). Hoffman *et al.* (2001) expanded the list to include 23 *N*-nitrosamines and 28 pesticides, which brought the number to 3095 constituents in tobacco. The identification of each single compound is an arduous task and requires a vigorous confirmation protocol that uses state-of-the-art instrumentation as well as synthesis.

During preparation for product manufacture, tobacco leaves, stems and other ingredients are blended to achieve a specific nicotine content, pH, taste, flavour and aroma. These features are critical for acceptance of the product by the user. For cigarettes, it has been demonstrated that the type of tobacco blend significantly affects these features as well as the toxicity of the product (Abdallah, 2003; Baker & Smith, 2003). The pH strongly influences the concentration of unprotonated nicotine, the bioavailable form of nicotine (Djordjevic *et al.*, 1995; Henningfield *et al*., 1995; Richter & Spierto, 2003), while the nitrite content influences the levels of nitrosamines in the product (Fischer *et al*., 1989; Burton *et al*., 1994; Hoffmann *et al*., 1995).

A choice of 60 *N. tabacum* species and 100 varieties of tobacco can be blended. However, the majority of commercial tobacco products use *N. tabacum* species, which are grown in North America and throughout the world. The alkaloid content in *N. tabacum* species varies greatly. From a random examination of 152 cultivated varieties, a range of alkaloid content between 0.17 and 4.93% was found. Tobacco types, plant parts, cultural practices, degree of ripening and fertilizer treatment are among some prominent factors that determine the level of alkaloids in *Nicotiana* plants. Every step in tobacco production that affects plant metabolism influences the level of alkaloid content to a certain degree. Cured tobacco lines can contain between 0.2 and 4.75% nicotine by weight, depending on

plant genetics, growing conditions, degree of ripening, fertilizer treatment and leaf position on the stalk (Tso, 1990; Stratton *et al*., 2001).

N. rustica species is cultivated in some parts of eastern Europe, Asia Minor and Africa, and the cured leaves may contain up to 12% nicotine. In greenhouse-grown plants, *N. rustica* can accumulate up to 5.3 mg nicotine/g tobacco (98.2% of total alkaloids) and in field-grown plants up to 24.8 mg nicotine (97.1% of total alkaloids) (Sisson & Severson, 1990). *Toombak*, which contains *N. rustica* tobacco, was reported to contain the highest levels of nicotine (up to 102.4 mg/g dry wt) and nicotine-derived tobacco-specific nitrosamines ever measured in consumer products (Idris *et al*., 1991; Prokopczyk *et al.*, 1995).

The chemical composition of tobacco undergoes substantial changes during growing, curing, processing and storing (Burton *et al*., 1983, 1989a,b; Peele *et al*., 1995; Walton *et al*., 1995; Wiernik *et al*., 1995; Peele *et al*., 2001; Bush *et al*., 2001). The purpose of curing is to produce a dried leaf of suitable physical properties and chemical composition. At the beginning of curing, a tobacco leaf is metabolically active and continues to live until biochemical processes are arrested by thermal effects or desiccation. In curing, the starch content of the leaves declines drastically, while the amount of reducing sugars increases by 100%. Protein and nicotine contents decrease slightly. The bulk of the processed tobacco leaf before fermentation consists of carbohydrates (about 50%) and proteins. Fermentation of cured tobacco causes the contents of carbohydrates and polyphenols in the leaves to diminish. Other major components are alkaloids (0.5–5.0%), which include nicotine as the predominant compound (85–95% of total alkaloids), terpenes (0.1–3.0%), polyphenols (0.5–4.5%), phytosterols (0.1–2.5%), carboxylic acids (0.1–0.7%), alkanes (0.1–0.4%), aromatic hydrocarbons, aldehydes, ketones, amines, nitriles, *N*- and *O*-heterocyclic hydrocarbons, pesticides, alkali nitrates (0.01–5%) and at least 30 metallic compounds (Brunnemann & Hoffmann, 1992; IARC, 2004b).

Because of the disappearance of carbohydrates and polyphenols during fermentation, heavy casings [additives applied during processing] such as molasses, liquorice and fruit extracts are added to tobacco to meet the consumer's requirements (e.g. they improve taste, flavour and aroma, and prolong shelf-life). Many smokeless tobacco formulations use plant extracts or chemicals as flavouring agents (Mookherjee & Wilson, 1988; Roberts, 1988; Sharma *et al*., 1991). Tobacco additives may include methyl or ethyl salicylate, β-citronellol, 1,8-cineole, menthol, benzyl benzoate, eugenol and possibly coumarin, among others (LaVoie *et al.*, 1989; Stanfill *et al.*, 2006). Eugenol (ranging from < 0.00005 to 25 706 μg/g in Dentobac Creamy Snuff sold in India; Gupta, 2004) and menthol are used to numb the throat and facilitate tobacco use (Ahijevych & Garrett, 2004; Wayne & Connolly, 2004). Ascorbic acid is added to tobacco as an antimicrobial agent whereas the addition of sodium propionate serves as a fungicide. Other additives, such as ammonia, ammonium carbonate and sodium carbonate, are applied to control nicotine delivery by raising pH and subsequently the level of unprotonated nicotine which is the form of nicotine that is most readily absorbed through the mouth into the bloodstream (Djordjevic *et al.*, 1995; Henningfield *et al.*, 1995). However, the formulation of most of the additives, including flavours, remains a trade secret.

1.3.2 *Carcinogenic compounds in smokeless tobacco*

To date, 28 carcinogens have been identified in smokeless tobacco (Table 3; adapted from Brunnemann & Hoffmann, 1992). The major and most abundant group of carcinogens are the non-volatile alkaloid-derived tobacco-specific *N*-nitrosamines (TSNA) and *N*-nitrosoamino acids. Other carcinogens reportedly present in smokeless tobacco include volatile *N*-nitrosamines, certain volatile aldehydes, traces of some polynuclear aromatic hydrocarbons such as benzo[*a*]pyrene, certain lactones, urethane, metals, polonium-210 and uranium-235 and -238 (see Brunnemann & Hoffmann, 1992 for review).

There are three major types of nitroso compounds in smokeless tobacco: (*a*) non-volatile TSNA, including 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and *N*′-nitrosonornicotine (NNN); (*b*) *N*-nitrosamino acids, including *N*-nitrososarcosine (NSAR), 3-(methylnitrosamino)propionic acids (MNPA) and 4-(methylnitrosamino)butyric acids (MNBA); and (*c*) volatile *N*-nitrosamines, including *N*-nitrosodimethylamine (NDMA), *N*-nitrosopyrrolidine (NPYR), *N*-nitrosopiperidine (NPIP) and *N*-nitrosomorpholine (NMOR).

TSNA are present in fresh green tobacco leaves in *N. tabacum* species, at levels of up to 0.39 μg/g NNN and 0.42 μg/g NNK in the top leaves of tobacco (flue-cured type) grown in the USA (Djordjevic *et al*., 1989a), up to 0.035 μg/g NNN and 0.0115 μg/g NNK in *N. tabacum* grown in India (Bhide *et al*., 1987a) and up to 46.1 μg/g NNN and 2.34 μg/g NNK in *N. rustica* species grown in India (Bhide *et al*., 1987a). However, TSNA are formed primarily during tobacco curing, fermentation and ageing, from their alkaloid precursors (namely, nicotine, nornicotine, anatabine and anabasine) and from nitrite/nitrate. The nitrate or nitrite content, the mode of curing and the various steps of processing are therefore the determining factors for the yields of TSNA in tobacco (Burton *et al*., 1989a,b; Fischer *et al*., 1989; Chamberlain & Chortyk, 1992; Djordjevic *et al*., 1993a; Burton *et al*., 1994; Peele *et al*., 2001; Li & Bush, 2004). NNN, *N*′-nitrosoanatabine (NAT) and *N*′-nitrosoanabasine (NAB) are formed primarily from the corresponding secondary amines in the early stages of tobacco processing; some NNN and the majority of NNK are formed from the tertiary amine nicotine at the later stage of tobacco curing and fermentation (Spiegelhalder & Fischer, 1991).

In addition to these three groups of compounds, smokeless tobacco contains *N*-nitrosodiethanolamine (NDELA), which is formed from diethanolamine, a residual contaminant in tobacco. In 1981, the levels of NDELA were up to 224 ng/g in chewing tobacco and up to 6840 ng/g in fine-cut moist snuff. Treatment of Burley leaves with the sucker growth inhibitor maleic hydrazide significantly increased the hydrazine content. Although a tolerance of 80 ppm for maleic hydrazide was established in at least three European countries and the USA, concentrations up to 269 ppm were reported for the flue-cured tobacco harvested in Georgia, USA, in 1990 (Sheets, 1990). As the use of maleic hydrazide–diethanolamine as a sucker growth-controlling agent was gradually reduced, the concentration of NDELA decreased to less than 100 ng/g in 1990 (Brunnemann & Hoffmann, 1991).

Table 3. Chemical agents identified in smokeless tobacco products

Agent	Type of tobacco where it has been detected	Concentration (ng/g)	*IARC Monographs* evaluation of carcinogenicity			*Monographs* volume, year
			In animals	In humans	IARC Group	
Benzo[*a*]pyrene	NT, MS, DS, MI[a]	> 0.1–90	S	I	1	Vol. 92 (in prep.)
α-Angelica lactone	NT	Present	–	–	–	–
β-Angelica lactone	NT	Present	–	–	–	–
Coumarin	NT	600	L	I	3	Vol. 77 (2000)
Ethyl carbamate (urethane)	CT	310–375	S	I	2A	Vol. 96 (in prep.)
Volatile aldehydes						
Formaldehyde	NT, MS, DS	1600–7400	S	S	1	Vol. 88 (2006)
Acetaldehyde	NT, MS, DS	1400–27 400	S	I	2B	Vol. 71 (1999)
Crotonaldehyde	MS, DS	200–2400	I	I	3	Vol. 63 (1995)
Volatile N-*nitrosamines*						
N-Nitrosodimethylamine (NDMA)	CT, MS	ND–270	S	I	2A	Suppl. 7 (1987)
N-Nitrosopyrrolidine (NPYR)	CT, MS	ND–860	S	I	2B	Suppl. 7 (1987)
N-Nitrosopiperidine (NPIP)	CT, MS	ND–110	S	I	2B	Suppl. 7 (1987)
N-Nitrosomorpholine (NMOR)	CT, MS	ND–690	S	I	2B	Suppl. 7 (1987)
N-Nitrosodiethanolamine (NDELA)	CT, MS	40–6800	S	I	2B	Vol. 77 (2000)
N-*Nitrosamino acids*						
N-Nitrososarcosine (NSAR)	MS	ND–6300	S	I	2B	Suppl. 7 (1987)
3-(*N*-methylnitrosamino) propionic acid (MNPA)	CT, MS	200–70 000	–	–	–	–
4-(*N*-methylnitrosamino) butyric acid (MNBA)	CT, MS	ND–17 500	–	–	–	–
Nitrosoazetidine-4-carboxylic acid (NAzCA)	CT, MS	4–140	–	–	–	–

Table 3 (contd)

Agent	Type of tobacco where it has been detected	Concentration (ng/g)	*IARC Monographs* evaluation of carcinogenicity			*Monographs* volume, year
			In animals	In humans	IARC Group	
Tobacco-specific N-*nitrosamines (TSNA)*						
N′-Nitrosonornicotine (NNN)	CT, MS	400–3 085 000	S	–	1	Vol. 89
4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)	CT, MS	ND–7 870 000	S	–	1	Vol. 89
4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL)	MS	0.07–22 900	–	–	–	Vol. 89
N′-Nitrosoanabasine (NAB)	ST, MS	Present–2 370 000	L	I	3	Vol. 89
Inorganic compounds						
Arsenic	NT	500–900	L	S	1	Suppl. 7 (1987)
Nickel compounds	ST, MS	180–2700	S	S	1	Vol. 49 (1990)
Radioelements		*(pCi/g)*				
Polonium-210	NT, MS, DS	0.16–1.22	S	I	1[b]	Vol. 78 (2001)
Uranium-235	MS	2.4	L	I	1[b]	Vol. 78 (2001)
Uranium-238	MS	1.91	L	I	1[b]	Vol. 78 (2001)
Beryllium	NA	NA	S	S	1	Vol. 58 (1993)

Updated from Bhide *et al.* (1984a); Nair, U.J. *et al.* (1987); Idris *et al.* (1991); Brunnemann & Hoffmann (1992)

CT, chewing tobacco; DS, dry snuff; I, inadequate; L, limited; MI, *mishri*; MS, moist snuff; NA, not available; ND, not detected; NT, natural tobacco; S, sufficient; ST, smoking tobacco

[a] Concentrations up to 119 000 ng/g in *mishri* (Nair, U.J. *et al.*, 1987)

[b] Evaluation of internally deposited α-particle-emitting radionuclides

Polycyclic aromatic hydrocarbons (PAHs) originate primarily from polluted air and perhaps from fire-curing of some tobaccos.

Formaldehyde, acetaldehyde and crotonaldehyde, which are themselves probable or known human carcinogens, probably contribute to the carcinogenic potential of smokeless tobacco. It is known that tobacco contains a large spectrum of alkyl aldehydes that contribute to its aroma and are formed from amino acids and sugars by heating during tobacco processing (Coleman & Perfetti, 1997).

The α- and β-angelica lactones have been reported in natural tobacco (Weeks *et al*., 1989). A minor group of polyphenols in tobacco are coumarins, of which scopoletin is the major representative. The presence of urethane in fermented Burley tobacco (up to 400 ng/g) is not unexpected since the fermentation of food and beverages leads to the formation of this compound. Both air- and flue-cured tobaccos contain hydrazines.

Radioactive polonium-210, which decays to radon, originates from soil that is fertilized with phosphates rich in radium-226 (Tso *et al*., 1966).

1.3.3 *Smokeless tobacco products*

(*a*) *Nicotine, pH and unprotonated nicotine*

All smokeless tobacco products contain nicotine as a major constituent, which is addictive (Henningfield *et al*., 1997; Hatsukami & Severson, 1999). The level of unprotonated nicotine affects the rate and degree of nicotine absorption (see Section 4.1).

Djordjevic *et al*. (1995) analysed 17 brands of moist snuff purchased in Westchester County, New York (USA) in 1994. In addition, samples of the five leading brands were purchased in six areas of the USA (Alameda, CA; Boston, MA; Denver, CO; Lansing, MI; Lexington, KY; Westchester, NY) and analysed separately to determine geographic variations. The nicotine content in 17 brands ranged from 0.47% dry wt (in Hawken Wintergreen) to 3.43% (in Skoal Long Cut Mint), which corresponds to 3.4 mg/g and 14.5 mg/g, respectively; the pH ranged from 5.39 (in Skoal Bandits Classic) to 7.99 (in Kodiak Wintergreen); unprotonated nicotine ranged from 0.23% of total nicotine (in Skoal Bandits Classic) to 48.3% (in Kodiak Wintergreen). The average values for the five best-selling brands of moist snuff in the USA in 1994 are summarized in Table 4.

Similar findings were reported by Henningfield *et al*. (1995) for products purchased at three locations (Baltimore, MD; Boston, MA; Lansing MI; Table 4). Both studies show that nicotine-dosing capability varies remarkably between products and that it is governed predominantly by nicotine content and pH level.

The Centers for Disease Control and Prevention (CDC) carried out an analysis of 18 smokeless tobacco products (eight brands of moist snuff and 10 of loose-leaf chewing tobacco) (Richter & Spierto, 2003). Among moist snuff brands, Timber Wolf Long Cut Straight contained the highest amount of nicotine (13.54 mg/g) followed by Copenhagen snuff and Skoal (12.71 mg/g and 12.94 mg/g, respectively). Consistent with the findings by Djordjevic *et al*. (1995), the highest pH was measured for Kodiak Wintergreen (pH, 8.28), which also had the highest quantity of unprotonated nicotine (64.5%; 5.81 mg/g).

Table 4. Nicotine content and pH of the five leading brands purchased at different locations in the USA

Constituents	Skoal Bandits Straight	Hawken Wintergreen	Skoal Original Fine Cut Wintergreen	Copenhagen Snuff	Kodiak Wintergreen
Djordjevic et al. *(1995)*[a]					
pH	5.37 ± 0.12	5.71 ± 0.1	7.46 ± 0.14	8.00 ± 0.31	8.19 ± 0.11
Nicotine (% dry wt)	2.29 ± 0.46	0.46 ± 0.02	2.81 ± 0.34	2.91 ± 0.18	2.5 ± 0.22
Nicotine (mg/g)	10.1 ± 0.8	3.2 ± 0.2	11.9 ± 1.3	12.0 ± 0.7	10.9 ± 0.8
Unprotonated nicotine (%)[b]	0.23 ± 0.05	0.5 ± 0.11	22.0 ± 5.73	49.0 ± 16.7	59.7 ± 6.01
Henningfield et al. *(1995)*	Skoal Bandits Wintergreen				
pH	6.9		7.6	8.6	
Nicotine (mg/g)	7.5		10.4	11.4	
Unprotonated nicotine (%)[b]	7.05		27.55	79.17	
Unprotonated nicotine (mg/g)	0.53		2.87	9.03	

[a] All values are mean ± standard deviation.
[b] The percentage of unprotonated nicotine was calculated according to the Henderson-Hasselbach equation and by using a pKa value of 8.02 for nicotine (Henningfield *et al*., 1995).

The lowest pH and amount of free nicotine were reported for Hawken Wintergreen (pH, 5.35; 0.20% free nicotine or 0.01 mg/g).

Another CDC study (CDC, 1999a) also reported that Copenhagen snuff and Kodiak Wintergreen had the highest pH (8.18 and 8.35, respectively) and the highest concentration of unprotonated nicotine (6.23 and 5.83 mg/g tobacco, respectively); Skoal Bandits Straight and Hawken Wintergreen had the lowest pH (5.52 and 5.24, respectively) as well as the lowest concentration of unprotonated nicotine (0.025 and 0.007 mg/g tobacco, respectively).

In 1996, Massachusetts enacted a tobacco product disclosure law which required manufacturers of cigarettes and smokeless tobacco products to disclose the ingredients and nicotine content by brand for average consumers. The Massachusetts Department of Public Health (MDPH) promulgated regulations in 1996 that required cigarette and smokeless tobacco manufacturers to file annual reports on nicotine yield by brand (MDPH, 2004). The requirements for reporting on smokeless tobacco were based on federal rules published by the CDC, adopted in 1996 and revised in 1999 (CDC, 1999b). Unlike Massachusetts, where disclosure of nicotine is a public record, data reported to the CDC remain private. Annual reports submitted by all smokeless tobacco manufacturers who sold products in Massachusetts from 1997–2003 contributed the most comprehensive data base on the levels of total nicotine (expressed as % and mg/g adjusted for moisture), tobacco pH and the levels of unprotonated nicotine (expressed as % of total nicotine and mg/g dry wt) in smokeless tobacco. Tables 5–7 list the pH, and total and unprotonated nicotine content of individual brands of, respectively, chewing tobacco, dry snuff and moist snuff sold in the Common-

Table 5. Chemical composition of chewing tobacco sold in Massachusetts (USA) in 2003

Manufacturer	Brand name	Sub-brand	Moisture content (%)	Nicotine (% dry wt)	Nicotine (mg/g)	pH	% unprotonated nicotine	Total unprotonated nicotine (mg/g)
Conwood Company	Bloodhound	Plug	21.10	1.72	13.54	5.37	0.22	0.03
	Bull of the Woods	Plug	20.73	2.12	16.78	5.16	0.14	0.02
	Cannon Ball	Plug	20.62	1.68	13.37	5.23	0.16	0.02
	Cotton Bowl Twist	Chewing Tobacco	14.57	4.65	39.74	5.21	0.15	0.06
	Cumberland Twist	Chewing Tobacco	22.35	1.56	12.12	5.70	0.48	0.06
	Hawken	Wintergreen Smokeless Tobacco	28.57	0.60	4.31	5.77	0.56	0.02
	HB Scott	Loose Leaf	23.95	0.60	4.53	6.09	1.16	0.05
	Levi Extra	Loose Leaf	23.85	0.67	5.13	6.13	1.27	0.07
	Levi Garrett	Plug	22.48	0.84	6.51	5.93	0.81	0.05
		Loose Leaf	24.13	0.71	5.40	6.02	0.99	0.05
	Lieberman's	Loose Leaf	19.58	1.12	8.99	6.76	5.21	0.47
	Mammoth Cave Twist	Chewing Tobacco	16.77	3.88	32.28	5.10	0.12	0.04
	Morgan's	Loose Leaf	23.97	0.45	3.41	6.00	0.95	0.03
	Peachey	Loose Leaf	24.02	0.62	4.68	5.73	0.51	0.02
	Taylor's Pride	Plug	22.15	0.79	6.18	5.94	0.82	0.05
		Loose Leaf	23.82	0.62	4.76	5.79	0.59	0.03
	Union Workman	Loose Leaf	23.53	0.52	3.97	5.89	0.74	0.03
National Tobacco	Beech-Nut	Regular	24.36	0.77	7.71	5.83	0.64	0.05
		Wintergreen	25.25	0.55	5.54	5.97	0.88	0.05
	Durango	Regular	24.61	0.59	5.93	5.96	0.86	0.05
	Havana Blossom	NR	22.43	1.64	16.37	5.95	0.84	0.14
	Trophy	NR	24.04	0.56	5.58	6.02	0.99	0.06
RBJ Sales Inc.	24-C	Course Cut	23.34	0.49	4.93	5.70	0.51	0.02
	757	Sweet Chew	22.71	0.56	5.59	5.94	0.82	0.05
	Black Wild Cherry	Loose Leaf	24.32	0.49	4.85	5.64	0.42	0.02
	Butternut	Loose Leaf	22.75	0.53	5.25	5.99	0.53	0.05

Table 5 (contd)

Manufacturer	Brand name	Sub-brand	Moisture content (%)	Nicotine (% dry wt)	Nicotine (mg/g)	pH	% unprotonated nicotine	Total unprotonated nicotine (mg/g)
RBJ Sales Inc. (contd)	Fred's Choice	Chewing Tobacco	27.79	0.50	5.05	5.78	0.59	0.03
	L-50	Mellow Chew	22.11	0.50	4.98	5.62	0.41	0.02
	Stoker's	Apple Loose Leaf	24.10	0.51	5.14	5.67	0.74	0.35
		Peach Loose Leaf	22.47	0.54	5.43	5.96	0.88	0.05
		Red Course Cut	25.31	0.49	4.89	5.72	0.55	0.03
		Tequila Sunrise Chew	26.96	0.49	4.87	5.76	0.57	0.03
	Tennessee	Chew	25.31	0.49	4.89	NR	0.55	0.03
	Tropical Chew	Chewing Tobacco	25.13	0.46	4.63	5.87	0.73	0.03
Swedish Match North America	Apple	Thick Plug	16.98	1.45	12.00	5.33	0.21	0.03
		Thin Plug	16.81	1.36	11.28	5.28	0.18	0.02
	Browns	Mule Plug	20.89	1.23	9.73	5.34	0.21	0.02
	Cup	Plug	23.83	2.68	20.43	5.07	0.11	0.02
	Day's Work	Plug	21.40	1.53	12.00	5.24	0.17	0.02
	Exalt Original Snuff	NR	23.87	3.32	25.30	6.80	6.13	1.54
	Exalt Peppermint Snuff	NR	20.46	2.22	17.65	6.91	9.52	1.77
	Granger	Select Loose Leaf	24.35	0.74	5.60	6.07	1.13	0.06
	J.D.'s Blend	Loose Leaf	27.16	0.61	4.48	6.41	2.47	0.11
	Original Natural Leaf	Plug	17.99	1.51	12.35	5.70	0.49	0.06
	Pay Car	Loose Leaf	25.41	1.13	8.45	5.90	0.76	0.06
	Red Horse	Loose Leaf	25.68	1.06	7.85	5.94	0.82	0.07
	Red Man	Plug	21.45	1.00	7.87	5.89	0.74	0.06
		Loose Leaf	25.83	1.17	8.70	6.01	1.01	0.09
		Select Loose Leaf	26.27	0.52	3.83	6.35	2.11	0.08
		Golden Blend Loose Leaf	25.84	1.05	7.75	6.22	2.12	0.17
		Golden Blend Totems	19.45	0.51	4.10	6.33	2.25	0.10
	Southern Pride	Loose Leaf	25.76	0.75	5.55	6.23	2.20	0.13
	Spark	Plug	20.07	1.18	9.45	5.95	0.85	0.08
	Tinsley	Plug	18.82	1.48	12.05	5.52	0.31	0.04
	Union Standard	Plug	16.88	0.98	8.18	5.80	0.67	0.06
		Loose Leaf	25.22	1.11	8.30	5.94	0.84	0.07
	WNT	Thick Plug	18.10	1.50	12.30	5.56	0.35	0.04
	Work Horse	Loose Leaf	25.24	1.10	8.23	5.86	0.71	0.06

Table 5 (contd)

Manufacturer	Brand name	Sub-brand	Moisture content (%)	Nicotine (% dry wt)	Nicotine (mg/g)	pH	% unprotonated nicotine	Total unprotonated nicotine (mg/g)
Swisher International	Best Buy	Chewing Tobacco	25.04	0.94	7.06	5.83	0.67	0.05
	Bowie	Chewing Tobacco	24.60	0.91	6.88	5.86	0.71	0.05
	Chattanooga	Chewing Tobacco	24.88	1.06	7.97	5.77	0.69	0.05
	Earl Caulfield's Country Flavors	Classic Bourbon	24.44	0.97	7.30	5.57	0.41	0.03
		Orchard Blend	24.60	0.91	6.88	5.86	0.71	0.05
	Jackson's Apple Jack	NR	25.29	0.91	6.81	5.74	0.54	0.04
	Lancaster Premium	NR	25.04	0.94	7.06	5.83	0.67	0.05
	Old Reliable Elephant Butts	NR	19.22	2.96	23.97	5.79	0.59	0.14
	Penn Cigar Clippings	NR	18.65	3.23	26.23	5.71	0.50	0.13
	Silver Cup	NR	21.88	1.69	13.17	5.62	0.41	0.05
	Standard Clippings	NR	20.07	2.08	16.65	5.81	0.62	0.10
	Starr Value	Chewing Tobacco	25.04	0.94	7.06	5.83	0.67	0.05
	Superior	Quality Chew	25.04	0.94	7.06	5.83	0.67	0.05
	Swisher Sweets	Chewing Tobacco	25.04	0.94	7.06	5.83	0.67	0.05
	Whalen Plain Scrap	NR	18.65	3.23	26.23	5.71	0.50	0.13
	XX Black	NR	18.65	3.23	26.23	5.71	0.50	0.13

NR, not reported
From MDPH (2004)

Table 6. Chemical composition of dry snuff sold in Massachusetts (USA) in 2003

Manufacturer	Brand name	Sub-brand	Moisture content (%)	Nicotine (% dry wt)	Nicotine (mg/g)	pH	% unprotonated nicotine	Total unprotonated nicotine (mg/g)
Conwood Company	Dental	Scotch Dry Snuff	6.00	1.82	17.12	6.27	1.75	0.30
		Sweet Dry Snuff	6.11	1.70	15.99	5.82	0.63	0.10
	Honest	Scotch Dry Snuff	5.93	1.94	18.27	6.25	1.67	0.31
	Peach	Sweet	6.10	1.31	12.29	6.05	1.06	0.13
	Tube Rose	Sweet Dry Snuff	6.61	1.59	14.82	5.89	0.74	0.11
	W.E. Garrett	Sweet	6.13	1.66	15.58	5.79	0.59	0.09
		Dry Scotch Snuff	5.38	2.25	21.33	5.92	0.79	0.17
Swisher International	Buttercup[a]	Sweet Scotch Snuff	7.30	1.57	14.54	5.96	0.89	0.13
	Dixie[a]	Sweet Snuff	8.20	1.36	12.45	5.41	0.24	0.03
	Ladies Choice	Extra Strong Scotch Snuff	7.84	2.51	23.17	6.22	1.58	0.36
	Lorillard	High Toast Scotch Snuff	7.00	1.88	17.53	6.39	2.31	0.41
		Sweet Scotch Snuff	7.17	1.61	14.88	6.09	1.20	0.18
	Navy	Sweet Scotch Snuff	7.59	1.83	16.91	6.28	1.80	0.31
		Plain Scotch Snuff	7.61	2.69	24.84	6.60	3.87	0.97
	Railroad Mills	Sweet Scotch Snuff	7.12	1.79	16.61	6.28	1.81	0.30
		Plain Scotch Snuff	7.61	2.69	24.84	6.60	3.87	0.97
	Ralph's	Scotch Snuff	8.56	2.34	21.42	6.16	1.41	0.30
	Society	Sweet Scotch Snuff	7.30	1.57	14.54	5.96	0.89	0.13
	Square	Snuff	8.56	2.34	21.42	6.16	1.41	0.30
	Starr	Scotch Snuff	7.70	1.14	10.48	7.51	29.56	3.08
	Strawberry	Sweet Snuff	7.17	1.61	14.88	6.09	1.20	0.18
	Superior	Scotch Snuff	7.84	2.51	23.17	6.22	1.58	0.36
	Three Thistles	Sweet Scotch Snuff	7.12	1.79	16.61	6.28	1.81	0.30
		Strong Scotch Snuff	8.56	2.34	21.42	6.16	1.41	0.30
	Tops	Sweet Snuff	7.35	1.55	14.31	5.99	1.08	0.15
		Mild Scotch Snuff	8.38	1.78	16.30	5.50	0.31	0.05
	Wild Cherry	Sweet Scotch Snuff	8.15	2.55	23.38	6.20	1.48	0.35

Table 6 (contd)

Manufacturer	Brand name	Sub-brand	Moisture content (%)	Nicotine (% dry wt)	Nicotine (mg/g)	pH	% unprotonated nicotine	Total unprotonated nicotine (mg/g)
US Tobacco	Bruton	White Label	7.55	1.20	11.14	7.61	28.48	3.12
	Carhart's Choice	NR	7.20	1.33	12.38	7.23	14.07	1.74
	Devoe	Sweet	7.20	1.33	12.38	7.23	14.07	1.74
		Eagle	7.55	1.20	11.14	7.61	28.48	3.12
	Red Seal	Scotch	7.44	1.47	13.70	7.29	19.76	2.51
	Revel[a]	Mild	5.89	0.49	4.70	7.96	46.46	2.18
		Regular	5.71	1.06	10.11	7.67	30.91	3.13
	Rooster	Scotch	7.23	1.33	12.51	6.82	5.92	0.74

From MDPH (2004)

[a] Reported as moist snuff in original article

Table 7. Chemical composition of moist snuff sold in Massachusetts (USA) in 2003

Manufacturer	Brand name	Sub-brand	Moisture content (%)	Nicotine (% dry wt)	Nicotine (mg/g)	pH	% unprotonated nicotine	Total unprotonated nicotine (mg/g)
Conwood Company	Cougar	Fine Cut Natural	55.12	2.35	10.53	8.14	56.86	5.99
		Long Cut Natural	55.17	2.30	10.32	8.13	56.30	5.81
		Long Cut Wintergreen	55.48	2.64	11.75	8.03	50.58	5.94
		Wintergreen	55.72	3.04	13.46	7.98	47.70	6.42
	Grizzly	Fine Cut Natural	54.73	3.39	15.35	7.83	39.23	6.02
		Long Cut Wintergreen	53.85	2.56	11.81	8.23	61.86	7.31
	Kodiak	Wintergreen	53.90	2.30	10.60	8.33	67.12	7.12
		Ice	54.30	2.49	11.36	8.09	54.02	6.14
		Straight	54.30	2.49	11.36	[8.19][a]	59.66	6.78
	Xtreme	Wintergreen	54.47	2.52	11.49	8.38	69.61	8.00
RBJ Sales Inc.	Stoker's	Smokeless Regular Moist	45.67	2.29	22.93	5.49	0.44	0.10
	Yukon	Fine Cut	49.52	2.06	20.55	7.06	10.27	2.13
		Long Cut	51.30	1.86	18.55	6.85	6.57	1.22
Swedish Match North America	Longhorn Fine Cut	Natural	51.18	3.35	16.35	8.08	53.39	8.57
	Longhorn Long Cut	Wintergreen	53.45	3.14	14.60	7.94	45.26	6.71
	Renegades	Moist Snuff	54.22	3.31	15.18	7.35	17.60	2.73
	Sequoia	Cinnamon Ice Snuff	51.62	3.13	15.15	7.44	20.84	3.15
		Mountain Cider Snuff	51.90	3.04	14.60	7.08	11.00	1.58
	Sequoia Artic Wintergreen	Wintergreen Snuff	54.63	2.82	12.80	8.09	54.02	6.92
	Timberwolf	Fine Cut Natural	52.41	3.66	17.43	7.84	39.90	6.98
		Long Cut Natural	52.03	3.46	16.60	7.91	43.94	7.36
		Long Cut Wintergreen	54.72	3.64	16.50	7.94	45.60	7.59
		Cool Wintergreen	53.91	3.46	15.95	7.89	42.49	6.79
		Fine Cut Wintergreen	54.83	3.65	16.47	7.96	46.42	7.88
		Long Cut Mint	54.09	3.50	16.08	7.77	36.15	5.83
		Long Cut Straight	54.58	3.70	16.83	7.87	41.40	7.02

Table 7 (contd)

Manufacturer	Brand name	Sub-brand	Moisture content (%)	Nicotine (% dry wt)	Nicotine (mg/g)	pH	% unprotonated nicotine	Total unprotonated nicotine (mg/g)
Swisher International	Best Buy	Wintergreen	54.80	2.16	9.75	7.35	19.88	1.94
		Natural	54.48	2.83	12.96	7.91	44.46	5.75
		Long Cut Straight	54.86	2.29	10.30	7.33	18.86	1.98
		Long Cut Cherry	54.20	2.17	9.94	7.64	30.48	2.93
	Bowie	Long Cut Wintergreen	54.80	2.16	9.75	7.35	19.88	1.94
		Natural	54.48	2.83	12.96	7.91	44.46	5.75
	Cheyenne	Long Cut Wintergreen	54.80	2.16	9.75	7.35	19.88	1.94
		Natural	54.48	2.83	12.96	7.91	44.46	5.75
	Cooper	Long Cut Wintergreen	54.93	2.21	9.93	7.15	13.04	1.33
	Finest Quality	Long Cut Wintergreen	54.93	2.21	9.93	7.15	13.04	1.33
		Natural	54.50	2.80	12.74	8.01	49.78	6.38
		Long Cut Mint	53.40	2.09	9.71	7.23	17.10	1.65
		Long Cut Cherry	54.10	2.27	10.42	7.52	24.74	2.56
	Gold River[b]	NR	23.92	1.50	11.41	5.71	0.58	0.06
	Hunter	Long Cut Wintergreen	55.40	2.43	10.84	7.59	27.51	2.98
		Natural	54.50	2.80	12.74	8.01	49.78	6.38
	Kayak	Fine Cut Natural	54.50	2.80	12.74	8.01	49.78	6.38
		Long Cut Wintergreen	55.31	2.40	10.71	7.62	28.87	3.08
	Lorillard	Maccoboy Snuff	41.70	2.13	12.42	6.80	5.76	0.73
	Mail Pouch	NR	21.58	1.76	13.80	5.73	0.56	0.08
		Country Blend	25.04	0.94	7.06	5.83	0.67	0.05
		Select	25.04	0.94	7.06	5.83	0.67	0.05
	Our Best	Long Cut Wintergreen	54.80	2.16	9.75	7.35	19.88	1.94
		Natural	54.48	2.83	12.96	7.91	44.46	5.75
		Fine Cut Wintergreen	54.04	2.58	11.86	7.49	24.60	2.88
	Our Pride	Natural	54.48	2.83	12.96	7.91	44.46	5.75
		Fine Cut Wintergreen	54.04	2.58	11.86	7.49	24.60	2.88

Table 7 (contd)

Manufacturer	Brand name	Sub-brand	Moisture content (%)	Nicotine (% dry wt)	Nicotine (mg/g)	pH	% unprotonated nicotine	Total unprotonated nicotine (mg/g)
Swisher International (contd)		Long Cut Straight	54.86	2.29	10.30	7.33	18.86	1.98
	Railroad Mills	Maccoboy Snuff	41.82	2.08	12.14	6.73	4.94	0.59
		Checkerberry Snuff	40.80	1.21	7.18	7.21	18.57	1.33
	Redwood	Fine Cut	54.48	2.83	12.96	7.91	44.46	5.75
		Long Cut	54.63	2.64	11.96	7.75	38.17	4.53
	Silver Creek	Long Cut Wintergreen	54.80	2.16	9.75	7.35	19.88	1.94
		Fine Cut Wintergreen	54.04	2.58	11.86	7.49	24.60	2.88
		Long Cut Straight	54.86	2.29	10.30	7.33	18.86	1.98
		Long Cut Cherry	54.20	2.17	9.94	7.64	30.48	2.93
	Silverado	Natural Pouches	48.90	2.47	12.58	6.95	8.23	1.03
		Wintergreen Pouches	50.87	2.42	11.87	7.08	10.91	1.29
	Starr Value	Long Cut Wintergreen	54.80	2.16	9.75	7.35	19.88	1.94
		Natural	54.48	2.83	12.96	7.91	44.46	5.75
	Superior Value	Long Cut Wintergreen	54.80	2.16	9.75	7.35	19.88	1.94
		Natural	54.48	2.83	12.96	7.91	44.46	5.75
		Long Cut Cherry	54.20	2.17	9.94	7.64	30.48	2.93
	Swisher Sweets	Long Cut Wintergreen	54.80	2.16	9.75	7.35	19.88	1.94
		Long Cut Straight	54.86	2.29	10.30	7.33	18.86	1.98
	Tub[b]	NR	20.07	2.08	16.65	5.81	0.62	0.10
US Tobacco	Copenhagen	Fine Cut	54.35	2.87	13.12	7.87	43.03	5.56
		Long Cut	54.60	2.95	13.42	7.54	26.12	3.51
		Long Cut Black	54.17	2.91	13.42	7.18	13.35	1.79
		Pouch	53.78	2.16	9.99	7.63	31.04	3.01
	Husky	Long Cut Wintergreen	54.75	3.14	13.96	7.33	16.80	2.35
		Natural	54.37	3.04	13.70	7.71	32.88	4.50
	Red Seal	Long Cut Natural	55.76	3.01	13.50	7.51	24.71	3.33
		Long Cut Wintergreen	54.43	2.95	13.62	7.42	20.61	2.82

Table 7 (contd)

Manufacturer	Brand name	Sub-brand	Moisture content (%)	Nicotine (% dry wt)	Nicotine (mg/g)	pH	% unprotonated nicotine	Total unprotonated nicotine (mg/g)
US Tobacco (contd)		Natural	55.01	2.93	13.35	7.52	25.61	3.40
		Fine Cut Wintergreen	54.59	2.90	13.37	7.44	21.35	2.86
		Long Cut Mint	55.10	3.05	13.89	7.51	24.85	3.42
		Long Cut Straight	54.81	3.02	13.84	7.35	17.83	2.48
	Rooster	Wintergreen	55.77	2.81	12.62	7.43	20.79	2.62
		Berry	55.24	2.75	12.47	7.44	21.34	2.67
		Mint	55.73	2.80	12.51	7.54	25.19	3.16
	Skoal	Long Cut Wintergreen	54.52	3.00	13.82	7.48	22.75	3.15
		Fine Cut Wintergreen	54.45	2.81	12.98	7.38	19.17	2.47
		Long Cut Mint	54.62	2.97	13.69	7.44	21.06	2.88
		Long Cut Straight	54.84	2.99	13.69	7.54	25.64	3.52
		Long Cut Cherry	54.03	2.90	13.51	7.44	21.00	2.85
		Pouch	55.53	2.62	11.68	7.60	27.96	3.25
		Bandits Mint	49.00	1.75	8.96	7.00	8.79	0.79
		Bandits Straight	48.60	1.94	9.99	5.50	0.31	0.03
		Bandits Wintergreen	48.56	1.77	9.11	6.80	5.72	0.52
		Fine Cut Key	54.88	3.00	13.68	7.64	32.42	4.40
		Fine Cut Straight	54.56	2.88	13.29	7.41	20.42	2.71
		Long Cut Berry	54.27	2.94	13.59	7.16	12.29	1.67
		Long Cut Classic	55.18	3.18	14.45	8.03	49.96	7.18
		Long Cut Spearmint	54.68	3.08	13.79	7.33	18.03	2.50
		Long Cut Vanilla	55.16	2.92	12.93	7.50	23.61	3.05
		Pouch Berry	54.55	2.96	13.29	7.40	19.81	2.63
	WB Cut	Long Cut Cherry	34.61	3.68	24.29	5.50	0.30	0.07

From MDPH (2004)

[a] Reported as 0.0819 in original document

[b] Reported as dry snuff in original document

wealth of Massachusetts in 2003; Table 8 presents the mean values for each type of tobacco product.

Table 8. Ranges of pH and nicotine concentration in smokeless tobacco products sold in Massachusetts (USA) in 2003

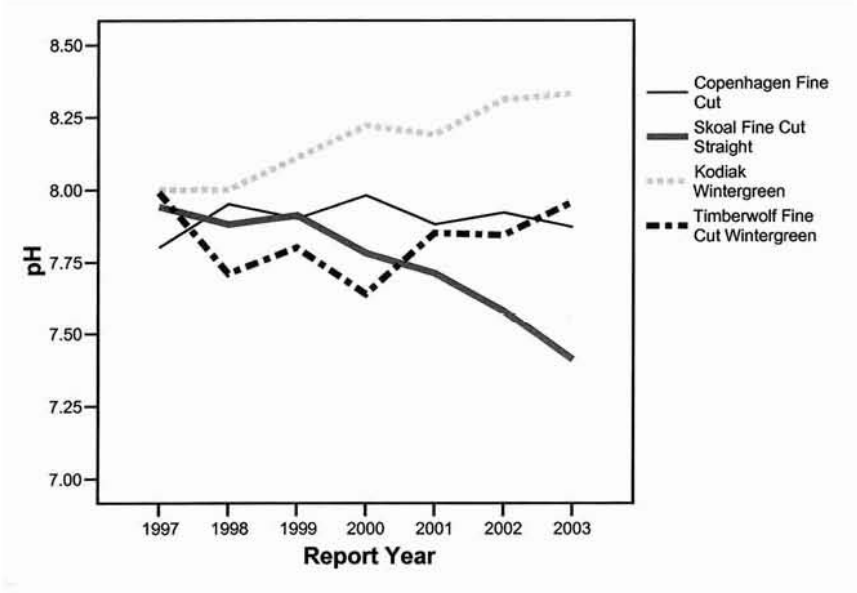
Constituent	Chewing tobacco (*n* = 74) Mean (range)	Dry snuff (*n* = 33) Mean (range)	Moist snuff (*n* = 106) Mean (range)
Moisture (%)	22.8 (14.57–28.57)	8.2 (5.38–23.9)[a]	52.6 (21.58–55.77)[b]
Nicotine (%, dry wt)	1.22 (0.45–4.65)	1.82 (1.14–2.69)	2.58 (0.49–3.70)
Nicotine (mg/g product)	9.9 (3.41–39.74)	16.8 (10.48–24.84)	12.6 (4.70–24.29)
pH	5.82 (5.07–6.91)	6.36 (5.50–7.61)	7.43 (5.41–8.38)
Unprotonated nicotine (mg/g product)	0.11 (0.02–1.77)	0.71 (0.05–3.12)	3.52 (0.03–8.57)

From MDPH (2004)
[a] Two Swisher International products contained over 20% moisture.
[b] Four moist snuff brands contained 5.71–8.2% moisture and were therefore excluded from the statistical analysis.

On average, moist snuff contained the highest percentage of moisture (mean, 52.6%; range, 21.58–55.77%) and nicotine (mean, 2.58% dry wt; range, 0.49–3.7%) (Table 8). Dry snuff had the lowest moisture content (mean, 8.2%; range, 5.38–23.9%) but middle range of nicotine (mean, 1.82%; range, 1.14–2.69%). Chewing tobacco had the lowest nicotine content (mean, 1.22%; range 0.45–4.65%). Moist snuff had, on average, the highest pH (7.43 versus 6.36 and 5.82 in dry snuff and chewing tobacco, respectively). Because of the high pH, the levels of unprotonated nicotine in moist snuff averaged 3.52 mg/g product (range, 0.03–8.57 mg/g); this is fivefold higher than that in dry snuff and 32-fold higher than that in chewing tobacco. The highest concentration of unprotonated nicotine was reported for Longhorn Fine Cut Natural, which is marketed by Swedish Match North America (Table 7).

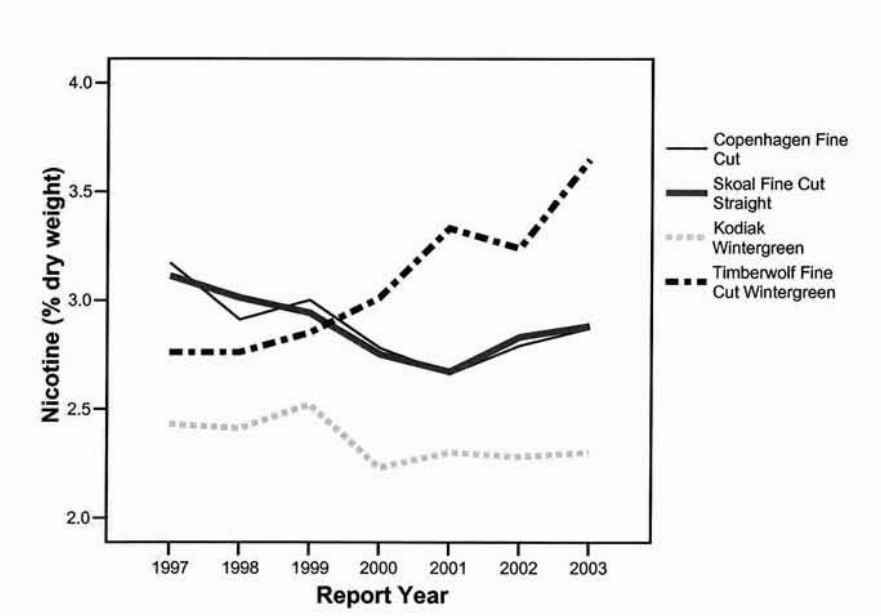
Regular and comprehensive reporting on the chemical composition of smokeless tobacco products to the MDPH enables analysis of trends in chemical composition over time and comparison of the levels of specific constituents between different brands or types of products. The trends for pH and nicotine content (both total and unprotonated) in the four leading brands of moist snuff in the USA (Copenhagen Fine Cut, Skoal Straight Fine Cut, Kodiak Wintergreen and Timberwolf Fine Cut Wintergreen) (Maxwell Tobacco Facts Book, 2002) from 1997 to 2003 are presented in Figures 1–3 (MDPH, 2004). Nicotine content (% dry wt) in three of the brands did not change notably between 1997 and 2003, while it increased steadily in Timber Wolf from 2.8 to 3.6% during the same period.

Figure 1. The pH of leading US moist snuff products (1997–2003)



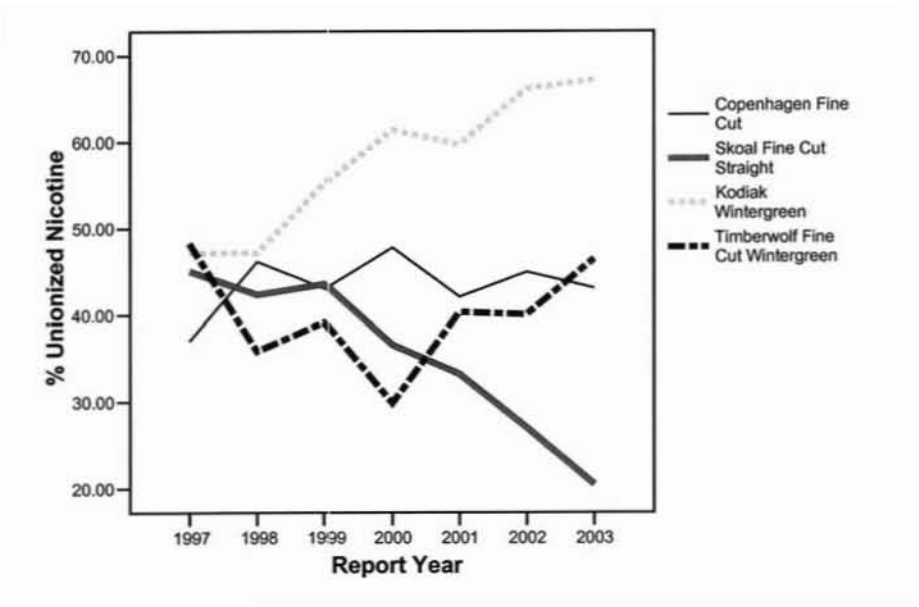
From MDPH (2004)

Figure 2. The nicotine content (% dry weight) in leading US moist snuff products (1997–2003)



From MDPH (2004)

Figure 3. The unprotonated nicotine content (% of the total nicotine) in leading US moist snuff brands (1997–2003)



From MDPH (2004)

The pH values for Copenhagen fine cut were constant between 1997 and 2003 (Figure 2), while the pH of Skoal Fine Cut Straight dropped significantly during the same period. Of the four brands, Kodiak has had the highest pH since 1999, and the pH of the Timber Wolf fluctuated between 7.6 and 8.0. The latter observation underlines the importance of monitoring the composition of all products rather than using one brand as a proxy for different types of smokeless tobacco product, or sub-brands of a brand family.

As shown in Figure 3, the levels of unprotonated nicotine were the highest in Kodiak Wintergreen, and increased from 35.19% total nicotine in 1997 to 60.27% total nicotine in 2003. This pattern parallels the trend in pH. On average, the levels of unprotonated nicotine in Copenhagen and Skoal brand families decreased steadily overtime. However, for the individual brands, this trend was only true for Skoal Fine Cut Straight, and not for Copenhagen Fine Cut, similar to the observation regarding pH. The percentage of unprotonated nicotine for Timberwolf also parallels the pH (Figures 2 and 3). As a result of the constant interplay of pH, nicotine content and moisture in tobacco products, the levels of unprotonated nicotine vary from product to product and from year to year.

In summary, the data from the MDPH show that pH and unprotonated nicotine content are brand- and company-specific. pH appears to be the primary determinant of nicotine absorption (Tomar & Henningfield, 1997). Among the 562 components reported on the list of additives for smokeless tobacco products (House of Representatives, 1994), several salts (e.g. ammonium, sodium and potassium salts) may alter the pH of smokeless

tobacco. Moreover, smokeless tobacco contains components that are intended to control delivery of nicotine to the body (Food and Drug Administration, 1996). However, exposure of users to tobacco toxins does not depend only on their concentration in a particular product but also how the product has been used. Smokeless tobacco users who dip or chew eight to 10 times a day may be exposed to the same amount of nicotine as individuals who smoke 30–40 cigarettes a day (DHHS, 1986). Lemmonds *et al.* (2005) examined the relationship between topographical measures of oral smokeless tobacco and biomarkers of exposure to tobacco and carcinogens. The major finding of the study was that frequency and duration measures of smokeless tobacco use are significantly correlated with total cotinine, a major metabolite of nicotine. Fifty-four male snuff users of 2.8 tins/week (6.1 dips/day) excreted on average 20.1 nmol cotinine/mg creatinine (or 3.3 nmol cotinine per dip) in urine compared with 27 nmol cotinine/mg creatinine excreted by smokers who consumed on average 27.9 cigarettes/day (or 1.07 nmol creatinine per cigarette) (Hecht *et al.*, 2005). Thus, snuff dippers are exposed to 3.08-fold higher amounts of nicotine than cigarette smokers. This high exposure to nicotine needs to be taken into consideration when recommending nicotine replacement therapy to those who contemplate quitting snuff use. Moreover, it has been shown that increasing the nicotine concentration in the presence of alcohol significantly increases the penetration of NNN across the oral mucosa (Du *et al.*, 2000).

The latest information on the chemical composition of 14 varieties of smokeless tobacco products used in India, including pH and nicotine content, was made available in a report to the WHO South-East Asian Regional Office (Gupta, 2004; Table 9). Some products had a pH of up to 10.1 and a nicotine content of up to 10.2 mg/g.

Ayo-Yusuf *et al.* (2004) reported on the pH and nicotine content of moist snuff products consumed in South Africa. The pH ranged from 7.1 to 10.1, the nicotine content from 0.8 to 1.6% wet wt [11.6–29.3 mg/g dry wt, as adjusted for moisture content] and from 10.1 to 99.1% in the unprotonated form.

A new product that is on the market, tobacco tablets, also referred to as Ariva® or Cigalett®, contain 1.3 mg nicotine per tablet and have a pH of 8.4. The 'buffering capacity' of Ariva® is sufficient to control the acidic pH of human saliva (Nguyen *et al.*, 2002).

(*b*) *Tobacco-specific* N-*nitrosamines (TSNA)*

Hoffmann *et al.* (1995) provided the most comprehensive insight into the levels of major tobacco carcinogens in the leading brands of moist snuff sold in the USA. The purpose of the study was threefold: (*a*) to determine the concentrations of major carcinogenic TSNA and *N*-nitrosamino acids in each of the five most popular brands of moist snuff (Table 10); (*b*) to analyse quantitative differences in selected snuff components (e.g. NNK and NNN) between two major categories of moist snuff: a category that comprised those brands known to have high levels of unprotonated nicotine (Copenhagen, Skoal Fine Cut and Kodiak) versus a category that comprised those brands known to have low levels (Hawken and Skoal Bandits); and (*c*) to compare the concentration of nicotine, NNN, NNK and total TSNA between these two categories. Concentrations (mean ± standard deviation

Table 9. Chemical composition of smokeless tobacco products used in India

Constituent	Minimum value	Brand	Maximum value	Brand
pH	5.21	Baba Zarda 120	10.1	Lime Mix – Miraj Tobacco
Ammonia (μg/g)	4.04	Baidhyanath Red Tooth Powder	5280	Gai Chhap Zarda
Total carbonate (μg/g)	140	Dabur Red Tooth Powder	2040	Baba Zarda 120
Nicotine (mg/g)	1.24	Raja Khaini[a]	10.16	Dentobac Creamy Snuff
NNN (μg/g)	ND	Click Eucalyptus[b]	7.36	Baba Zarda 120
NNK (μg/g)	ND	Click Eucalyptus[b]	4.88	IPCO Creamy Snuff
Benzo[*a*]pyrene (μg/g)	< 0.0001	Click Eucalyptus	0.94	IPCO Creamy Snuff
Cadmium (μg/g)	0.03	Click Eucalyptus	0.5	Baba Zarda 120
Arsenic (μg/g)	0.07	Click Eucalyptus	1.53	Shahin Mishri
Nitrate (μg/g)	< 0.1	Dabur Red Tooth Powder	13.85	Lime Mix – Miraj Tobacco

From Gupta (2004)
ND, not detected; NNK, 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone; NNN, *N*′-nitrosonornicotine
[a] *Tuibur* contained no detectable amounts of nicotine.
[b] Click Eucalyptus and six other compounds in this report contained nitrosamines other than tobacco-specific *N*-nitrosamines.

[SD]) of nicotine, NNN, NNK and total TSNA in the two categories were as follows: nicotine, 11.6 ± 1.06 mg/g versus 6.96 ± 3.62 mg/g ($p = 0.0017$); NNN, 7.74 ± 1.70 μg/g versus 4.17 ± 1.35 μg/g ($p < 0.0001$); NNK, 1.23 ± 0.68 μg/g versus 0.61 ± 0.41 μg/g ($p = 0.012$); and total TSNA (including NNN, NNK, NAB and NAT), 14.3 ± 3.82 μg/g versus 6.3 ± 2.56 μg/g ($p < 0.001$). In another study, moist snuff with a high pH and high unprotonated nicotine content, purchased in 2000, contained 15.4 μg/g dry wt NNN and 2.5 μg/g dry wt NNK (Brunnemann *et al.*, 2002). The brand Conwood's Grizzly contained 70.8 μg/g NNN and 10.1 μg/g NNK (Brunnemann *et al.*, 2004).

Table 11 shows an international comparison of the concentrations of two carcinogenic TSNA, NNN and NNK, as well as of tobacco pH (as determined in an aqueous tobacco suspension). The ranges for all three measures are wide and are product-specific and country-specific. The highest values of pH were measured in *naswar* from Uzbekistan (Brunnemann *et al.*, 1985), *toombak* from Sudan (Idris *et al.*, 1998a) and new moist snuff brands recently introduced in South Africa (Ayo-Yusuf *et al.*, 2004). The highest concentrations of NNN and NNK were measured in some moist snuff brands in the USA (135 and 17.8 μg/g tobacco, respectively). However, values as high as 3085 and 7870 μg/g dry wt tobacco, respectively, have been measured in home-made *toombak*.

Although there has been a decline in the concentrations of nitrosamines in some smokeless tobacco products in Sweden and the USA since the 1980s (Djordjevic *et al.*, 1993b; Brunnemann *et al.*, 2004; Österdahl *et al.*, 2004), the trend may not apply to other

Table 10. Levels of tobacco-specific *N*-nitrosamines and *N*-nitrosamino acids in the five leading brands sold in the USA, 1994

Constituents (μg/g dry wt)	Skoal Bandits Straight	Hawken Wintergreen	Skoal Original Fine Cut Wintergreen	Copenhagen Snuff	Kodiak
Tobacco-specific N-*nitrosamines (TSNA)*					
NNN	5.09 ± 1.03	3.07 ± 0.3	8.18 ± 1.33	8.73 ± 1.44	6.3 ± 1.06
NNK	0.92 ± 0.26	0.23 ± 0.04	1.25 ± 0.13	1.89 ± 0.62	0.55 ± 0.15
Total TSNA	8.19 ± 1.72	4.08 ± 0.44	14.9 ± 2.5	17.24 ± 2.97	10.96 ± 2.44
Nitrite nitrogen	1.3 ± 0.4	1.4 ± 0.8	64.5 ± 41.9	672.0 ± 296.8	2.77 ± 1.13
N-*Nitrosamino acids (NNAC)*					
NSAR	0.02 ± 0.01	0.07 ± 0.01	0.04 ± 0.0	0.06 ± 0.01	0.04 ± 0.01
MNPA	10.96 ± 1.80	5.62 ± 0.71	2.39 ± 0.34	2.62 ± 0.62	2.23 ± 0.32
MNBA	0.1 ± 0.08	0.33 ± 0.06	0.23 ± 0.06	0.34 ± 0.1	0.19 ± 0.04
NPRO	1.9 ± 0.42	4.89 ± 0.52	4.6 ± 0.8	5.67 ± 1.29	2.39 ± 0.63
Iso-NNAC	0.07 ± 0.02	0.14 ± 0.03	0.13 ± 0.07	0.31 ± 0.12	0.14 ± 0.02
Total NNAC	13.45 ± 2.07	11.56 ± 1.28	8.15 ± 1.3	10.47 ± 2.7	5.7 ± 1.07

From Hoffmann *et al*. (1995)
MNBA, 4-(*N*-methylnitrosamino)butyric acids; MNPA, 3-(*N*-methylnitrosamino)propionic acids; NNK, 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone; NNN, *N*′-nitrosonornicotine; NPRO, *N*-nitrosoproline; NSAR, *N*-nitrososarcosine

products and countries. For example, the concentrations of NNN and NNK in the two leading snuff brands in the USA were reduced significantly by 70–90% from 1980 to 1992, based on dry weight (Djordjevic *et al*., 1993b). However, samples of a new brand of moist snuff introduced on the US market in the 1990s contained very high amounts of NNN and NNK (up to 57.1 and 16.4 μg/g dry wt, respectively) (Hoffmann *et al*., 1991). Moreover, the commercial brand Conwood's Grizzly, purchased in the USA in 2003, contained 70.8 μg/g dry wt NNN and 10.1 μg/g dry wt NNK (Brunnemann *et al*., 2004). In Sweden, the concentrations of NNN and NNK in moist snuff decreased, respectively, from 3.8 and 0.8 μg/g in 1983 to 0.49 μg/g and 0.19 μg/g wet wt in 2002 (87% and 76% decrease, respectively; Österdahl *et al*., 2004). Values for NNN and NNK of up to 3085 and 7870 μg/g, respectively, were reported in *toombak* (Idris *et al*., 1991, 1998a). The latest report by Stepanov *et al.* (2006) shows the wide range of TSNA concentrations in 19 brands of new and conventional smokeless tobacco products purchased in retail stores in the USA or online from Snus Worldwide, Sweden. Levels of NNN ranged from 0.019 μg/g wet wt in Ariva® hard snuff to 4.5 μg/g in Skoal Long Cut; those of NNK ranged from 0.032 μg/g in Revel to 1.6 μg/g in Copenhagen Long Cut; and those of NAT ranged from 0.12 μg/g in Ariva® to 4.1 μg in Skoal Long Cut Straight. Stepanov *et al.* (2005) also reported a wide range of TSNA concentrations in smokeless tobacco products from India: NNN concentrations ranged from not detected in *supari* and a sample of a

Table 11. International comparison of the pH and concentration ranges of *N*-nitrosonornicotine (NNN) and 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in smokeless tobacco products (μg/g tobacco)

Country	Type of product	pH	Concentration (μg/g tobacco)			References
			Reported as[a]	NNN	NNK	
Belgium	Chewing tobacco		Dry	7.38	0.13	Ohshima *et al*. (1985)
Canada	Moist snuff	7.5–8.23	Dry	50.4–79.1	3.2–5.8	Brunnemann *et al*. (1985)
	Chewing tobacco	5.28	Dry	2.09	0.24	
Denmark	Chewing tobacco		Wet	0.08–1.6	0.02–1.9	Österdahl *et al*. (2004)
Germany	Chewing tobacco	5.01–5.05	Dry	1.4–2.3	0.03–0.30	Brunnemann *et al*. (1985); Tricker *et al*. (1988)
	Dry snuff		Dry	2.4–18.8	0.58–6.4	
			Wet	0.68	0.10	Tricker & Preussmann (1991); Österdahl *et al*. (2004)
India	Moist snuff		Wet	0.56	0.24	Stepanov *et al*. (2005)
	Chewing tobacco	4.36–6.42	Dry	0.47–0.85	0.13–0.60	Brunnemann *et al*. (1985); Tricker *et al*. (1988)
			Wet	15.3–24.4	2.7–6.5	Nair *et al*. (1989)
	Dry snuff		Wet	137–1 356	110–245	Nair *et al*. (1989)
	Khaini		Dry	25.8–40.0	0.11–5.3	Stich *et al*. (1992)
			Wet	39.4–76.9	2.3–28.4	Stepanov *et al*. (2005)
	Khiwam		Dry	2.5–8.95	0.1–1.03	Tricker & Preussmann (1989)
	Gutka		Wet	0.09–1.1	0.04–0.43	Stepanov *et al*. (2005)
			NR	1.9–5.7	10.7–11.5	Gupta (2004)
	Mishri		Dry	0.3–7.0	0.29–1.1	Nair, U.J. *et al*. (1987); Tricker *et al*. (1988)
			Wet	4.21	0.87	Stepanov *et al*. (2005)
			NR	4.02–4.47		Gupta (2004)

Table 11 (contd)

Country	Type of product	pH	Concentration (μg/g tobacco)			References
			Reported as[a]	NNN	NNK	
India (contd)	*Supari*		Wet	ND	ND	Stepanov *et al*. (2005)
			NR	1.9–2.5	4.9–11.6	Gupta (2004)
	Creamy snuff/ toothpaste		Wet	2.5–48.7	1.3–12.5	Nair *et al*. (1989); Stepanov *et al*. (2005)
					4.4–4.9	Gupta (2004)
	Tooth powder		Wet	ND–0.04	ND	Stepanov *et al*. (2005)
	Tuibur		NR	19.7–20.1		Gupta (2004)
	Zarda		Dry	0.4–79	0.22–24.1	Tricker & Preussmann (1988); Tricker *et al*. (1988)
			Wet	4.8–19.9	1.1–3.1	Stepanov *et al*. (2005)
			NR	6.6–7.4		Gupta (2004)
	Other		Wet	1.74–19.2	0.08–2.6	Stepanov *et al*. (2005)
Norway	Moist snuff		Wet	21	3.3	Österdahl *et al.* (2004)
Uzbekistan	*Nass*	11.0–11.8	Dry	0.12–0.52	0.02–0.13	Brunnemann *et al*. (1985)
South Africa	Low-TSNA moist snuff	7.1–10.1	Dry	1.05–2.07	0.27–0.29	Ayo-Yusuf *et al*. (2004); Brunnemann *et al.* (2004)
Sudan	*Toombak*	8.0–11	Dry	141–3 085	188–7 870	Idris *et al*. (1991); Prokopczyk *et al*. (1995)
Sweden	Moist snuff	7.3–8.68	Dry	1.12–154[b]	0.19–2.95	Brunnemann *et al*. (1985); Ohshima *et al*. (1985); Tricker *et al*. (1988); Hoffmann *et al.* (1991); Tricker & Preussmann (1991); Brunnemann & Hoffmann (1992); Djordjevic *et al*. (1993b); Connolly (2001)
			Wet	0.49–4.4	0.19–1.3	Österdahl & Slorach (1988); Österdahl *et al*. (2004)

Table 11 (contd)

Country	Type of product	pH	Concentration (µg/g tobacco)			References
			Reported as[a]	NNN	NNK	
Sweden (contd)	Low-TSNA moist snuff		Wet	0.15–2.3	0.03–0.36	Österdahl *et al.* (2004); Stepanov *et al.* (2006)
	Chewing tobacco		Wet	0.7–1.7	0.01–0.46	Österdahl *et al.* (2004)
Thailand	Chewing tobacco		Dry	0.5	0.1	Tricker *et al.* (1988)
United Kingdom	Moist snuff		Dry	1.1–52.0	0.4–13.0	Hoffmann *et al.* (1988); Brunnemann & Hoffmann (1992)
	Chewing tobacco		Dry	0.9	0.3	Tricker *et al.* (1988)
	Dry snuff		Dry	2.4–16.0	0.58–4.3	Tricker & Preussmann (1991); Brunnemann & Hoffmann (1992)
			Wet	1.8	0.26	Österdahl *et al.* (2004)
USA	Moist snuff	5.2–8.88	Dry Wet	ND–147 0.71–63	ND–17.8 0.06–13	Brunneman *et al.* (1985); Ohshima *et al.* (1985); Hoffmann *et al.* (1986); Adams *et al.* (1987); Brunnemann *et al.* (1987a,b); Chamberlain *et al.* (1988); Hoffmann *et al.* (1988); Tricker *et al.* (1988); Andersen *et al.* (1989); Djordejvic *et al.* (1989a); Hoffmann *et al.* (1991); Brunnemann & Hoffmann (1992); Prokopczyk *et al.* (1992a); Djordjevic *et al.* (1993b); Hoffmann *et al.* (1995); Prokopczyk *et al.* (1995); Connolly (2001); Brunnemann *et al.* (2002, 2004); Österdahl *et al.* (2004); Stepanov *et al.* (2006)
	Low-TSNA moist snuff		Wet	0.62–0.64	0.032–0.033	Stepanov *et al.* (2006)

Table 11 (contd)

Country	Type of product	pH	Concentration (μg/g tobacco)			References
			Reported as[a]	NNN	NNK	
USA (contd)	Chewing tobacco	0.6–6.37	Dry	0.67–6.5	ND–1.05	Brunnemann *et al*. (1985); Chamberlain *et al*. (1988); Andersen *et al*. (1989); Djordjevic *et al*. (1989a); Brunnemann & Hoffmann (1992)
			Wet	0.25–1.1	0.08–0.11	Österdahl *et al*. (2004)
	Dry snuff	5.8–6.3	Dry	9.4–116.1	0.88–84.4	Adams *et al*. (1987); Brunnemann *et al*. (1987a); Andersen *et al*. (1989); Djordjevic *et al*. (1989a)
	Hard snuff/ lozenges		NR	0.02–0.06	0.037–0.043	Stepanov *et al*. (2006)

ND, not detected; NR, not reported; TSNA, tobacco-specific *N*-nitrosamines

[a] Reported as ng/g of dry wt (Dry) or wet wt (Wet) of tobacco

[b] The Working Group was doubtful about the validity of this value; the next highest value was 20 900 ng/g (Ohshima *et al*., 1985).

tooth powder to 76.9 μg/g wet wt in *khaini*; those of NNK ranged from not detected to 28.4 μg/g in *khaini*.

In recent years, the Swedish Match Company has developed a new method for manufacturing oral snuff that uses select blends of tobacco as well as a new processing method. Instead of the dark fire-cured tobacco commonly used in US snuff, Swedish Match uses tobacco with a low nitrate content, which itself reduces TSNA levels. In addition, the tobacco is processed in a heated closed system that resembles pasteurization of milk, which eliminates bacteria that may be indirectly responsible for the formation of the nitrosamines (Parsons *et al*., 1986; Gothia, 2004). The company also encourages retailers to refrigerate packages to prevent the formation of TSNA during storage (see below).

In 2001, the MDPH initiated a study aimed at comparing traditional snuff brands with PREPs (Stratton *et al*., 2001). The study found that the levels of NNN, NNK, NAT and NAB in moist snuff produced by the new manufacturing process (Swedish Match brand Ettan) were up to 45 times lower than those in leading products manufactured under standard processes in the USA (Table 12).

Table 12. Levels of tobacco-specific *N*-nitrosamines (TSNA) in the five leading brands in the USA versus PREP

Company	Brand[a]	NNN (μg/g)	NNK (μg/g)	Total TSNA[b] (μg/g)
Conwood Company	Kodiak	7.4	0.97	16.6
Swedish Match North America	Timber Wolf	3.0	0.95	7.5
Swisher International	Silver Creek	41.4	17.8	127.9
US Tobacco	Copenhagen	14.3	3.4	41.1
	Skoal	20.8	14.3	64.0
Swedish Match	Ettan (PREP)	1.12	0.53	2.8

From Connolly (2001)
NNK, 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone; NNN, N′-nitrosonornicotine; PREP, potential reduced exposure product
[a] Snuff manufactured in the USA was purchased in the Commonwealth of Massachusetts; Ettan was purchased in Sweden.
[b] Total TSNA includes NNN, NNK, *N*-nitrosoanatabine and *N*-nitrosoanabasine.

In Sweden, all moist snuff brands on the market in 2002 contained low amounts of TNSA: NNN, 0.15–0.61 μg/g wet wt; and NNK, 0.03–0.36 μg/g wet wt. NNN concentrations in moist snuff decreased consistently from 1983 to 2002 from 3.8 to 0.49 μg/g wet wt and those of NNK from 0.80 to 0.19 μg/g wet wt (Österdahl *et al*., 2004).

Levels of TSNA in new oral snuff brands do not always parallel nicotine content (see Table 7 for the nicotine content and Table 12). For example, Taxi, a very high nicotine-delivery product manufactured by Swedish Match for the South African market, contains low

levels of TNSA: NNN, 2.07 μg/g dry wt; and NNK, 0.29 μg/g dry wt (Brunnemann *et al*., 2004).

(*c*) N-*Nitrosamino acids*

The amino acids present in tobacco, and probably also the proteins with secondary amino groups, are amenable to *N*-nitrosation. Since 1985, numerous studies have reported the presence of nitrosamino acids in smokeless tobacco products. Levels of *N*-nitrosoamino acids in smokeless tobacco products worldwide are presented in Table 13. To date, 11 *N*-nitrosamino acids have been identified in smokeless tobacco: NSAR, *N*-nitrosoazetidine-4-carboxylic acid (NAzCA), MNPA, MNBA, *N*-nitrosoproline (NPRO), *N*-nitrosohydroxyproline (NHPRO), *N*-nitrosopipecolic acid (NPIC), *N*-nitrosothiazolidine- 4-carboxylic acid (NTCA), *N*-nitroso-2-methylthiazolidine-4-carboxylic acid (MNTCA), 4-(methylnitrosamino)-4-(3-pyridyl)butyric acid (*iso*-NNAC) and 2-(methylnitrosamino)-3-phenylpropionic acid (MNPhPA) (Ohshima *et al*., 1985; Tricker & Preussmann, 1988; Djordjevic *et al*., 1989b; Tricker & Preussmann, 1989, 1991; Hoffmann *et al*., 1995). Of these, the following have been established as carcinogens in experimental animals: NSAR, MNPA, MNBA and NAzCA. The concentration of the nitrosamino acids depends on the nitrate or nitrite content of the tobacco; in addition, they are formed during prolonged storage, particularly under adverse conditions of temperature and relative humidity (Djordjevic *et al*., 1993a).

The highest concentrations of *N*-nitrosamino acids in moist snuff purchased in the USA were found in Skoal Bandits Straight and Hawken Wintergren (13.45 and 11.56 μg/g, respectively) and the lowest in Kodiak (5.7 μg/g), which is opposite to the trend observed for TNSA (Hoffmann *et al*., 1995).

(*d*) *Volatile* N-*nitrosamines*

Volatile *N*-nitrosamines are formed from volatile amines and nitrosating agents. The levels of volatile *N*-nitrosamines in smokeless tobacco products worldwide are presented in Table 14. The highest amounts were found in moist snuff (NDMA up to 265 ng/g dry wt and NPYR up to 860 ng/g dry wt; see also Table 3). The presence of NMOR (see IARC, 1987) indicates contamination with morpholine either from additives or from diffusion of containers coated with morpholine-containing wax (Brunnemann *et al*., 1985; Brunnemann & Hoffmann, 1991).

(*e*) *Other carcinogenic compounds*

In smokeless tobacco products from the USA, the levels of benzo[*a*]pyrene ranged from < 0.1 to 63 ng/g in moist snuff (Hoffmann *et al*., 1986) and up to 90.5 ng/g in dry snuff (Brunemann & Hoffmann, 1992; Table 3). Bhide *et al*. (1984a) reported on the whole range of PAHs in Indian smokeless tobacco products such as *mishri* and snuff: benzo[*a*]pyrene, 7.6–66 ng/g; benzofluoranthenes (*b* + *j* + *k*), 35–231 ng/g; indeno[1,2,3-*cd*]pyrene, 4.3–24 ng/g; benz[*a*]anthracene, 19–79 ng/g; chrysene and triphenylene, 37–

Table 13. Comparison of the major carcinogenic *N*-nitrosamino acids in smokeless tobacco (µg/g dry wt) across countries

Country	Type of product	NSAR	MNPA	MNBA	Reference
Belgium	Chewing tobacco	NE	1.63	0.09	Ohshima *et al.* (1985)
Germany	Nasal snuff	ND–0.085	0.49–4.26	0.08–0.41	Tricker & Preussmann (1991); Brunnemann & Hoffmann (1992)
India	*Zarda* *Khiwam*	ND–0.35 0.01–0.04	0.02–18.0 0.26–1.38	ND–2.04 0.01–0.19	Tricker & Preussmann (1988, 1989, 1991)
Sweden	Moist snuff	0.01–0.68	0.38–4.40	0.03–0.26	Ohshima *et al.* (1985); Hoffmann *et al.* (1991); Tricker & Preussmann (1991); Brunnemann & Hoffmann (1992)
United Kingdom	Moist snuff Nasal snuff	0.03–1.1 ND–0.04	1.36–19.0 1.0–2.8	0.06–8.0 0.1–0.28	Tricker & Preussmann (1991); Brunnemann & Hoffmann (1992)
USA	Moist snuff Chewing tobacco Dry snuff	ND–6.3 NE NE	0.15–70.0 0.6 1.2–4.5	ND–17.5 0.03 0.14–0.46	Ohshima *et al.* (1985); Djordjevic *et al.* (1989b); Hoffmann *et al.* (1991); Brunnemann & Hoffmann (1992); Djordjevic *et al.* (1993a,b, 1994); Hoffmann *et al.* (1995)

MNBA, 4-(*N*-methylnitrosamino)butyric acids; MNPA, 3-(*N*-methylnitrosamino)propionic acids; ND, not detected; NE, not evaluated; NSAR, *N*-nitrososarcosine

Table 14. Comparison of the major carcinogenic volatile *N*-nitrosamines in smokeless tobacco (ng/g dry wt) across countries

Country	Type of product	NDMA	NPYR	NMOR	Reference
Canada	Moist snuff	23–72.8	321–337	21.9–32.8	Brunnemann *et al.* (1985)
	Chewing tobacco	ND	ND	ND	
Denmark	Chewing tobacco	5.5	16	ND	
Germany	Nasal snuff	2.0–82	1.5–75	ND	Brunnemann *et al.* (1985); Tricker & Preussmann (1991); Brunnemann & Hoffmann (1992)
	Chewing tobacco	ND	ND	ND	
India	*Zarda*	2.0–31	6.0–69	ND	Brunnemann *et al.* (1985); Nair, U.J. *et al.* (1987); Tricker & Preussmann (1988, 1989, 1991)
	Khiwam	1.5–28	11–250	NE	
	Chewing tobacco	ND–0.56	1.55–4.48	ND	
	Mishri	12–80	21–99	NE	
Norway	Moist snuff	130	8.9	32.0	Brunnemann & Hoffmann (1992)
Sweden	Moist snuff	ND–63	ND–155	ND–44	Brunnemann *et al.* (1985); Hoffmann *et al.* (1991); Tricker & Preussmann (1991); Brunnemann & Hoffmann (1992); Djordjevic *et al.* (1993a)
	Chewing tobacco	0.2	0.8	0.4	
United Kingdom	Moist snuff	6.0–212	64–860	ND–1.5	Hoffmann & Brunnemann (1988); Tricker & Preussmann (1991); Brunnemann & Hoffmann (1992)
	Nasal snuff	4.5–82	1.5–130	ND	
USA	Moist snuff	ND–265	ND–575	ND–690	Brunnemann *et al.* (1985); Hoffmann *et al.* (1986, 1987); Hoffmann & Brunnemann (1988); Brunnemann & Hoffmann (1991); Hoffmann *et al.* (1991); Brunnemann & Hoffmann (1992)
	Chewing tobacco	4.12–64	ND–0.8	ND–0.6	
	Dry snuff	ND–19	72–148	ND–39	
Former USSR (Central Asian Republics)	*Nass*	ND	1.74–8.82	ND	Brunnemann *et al.* (1985)

ND, not detected; NDMA, *N*-nitrosodimethylamine; NE, not evaluated; NMOR, *N*-nitrosomorpholine; NPYR, *N*-nitrosopyrrolidine

192 ng/g; benzo[*e*]pyrene, 10–110 ng/g; pyrene, 60–169 ng/g; fluoranthene, 55–218 ng/g; and benzo[*ghi*]perylene, 5.6–17 ng/g.

Hoffmann *et al*. (1987) reported the levels of select volatile aldehydes in smokeless tobacco products: formaldehyde, 3.9–6.8 μg/g in moist snuff and 1.6–7.4 μg/g in dry snuff; acetaldehyde, 2.4–7.4 μg/g in moist snuff and 1.4–3.9 μg/g in dry snuff; and crotonaldehyde, 1.0–2.4 μg/g in moist snuff and 0.2–0.6 μg/g in dry snuff.

Uranium was reported in five samples of Indian snuff at a concentration of about 3 pCi/g tobacco (Sharma *et al*., 1985). Hoffmann *et al*. (1987) reported 0.16–1.22 pCi/g polonium-210 in commercial moist snuff and 0.23–0.39 pCi/g in commercial dry snuff in the USA.

(*f*) *Effect of storage conditions on the levels of* N-*nitrosamines*

The effect of storage conditions on the formation of TSNA in smokeless tobacco was studied in moist and dry snuff and in chewing tobacco.

In a study of the effects of ageing and storage on the levels of TSNA, *N*-nitrosamino acids and volatile *N*-nitrosamines in commercial moist snuff from the USA, it was found that during storage at 4 °C none of these compounds increased significantly (Djordjevic *et al*., 1993a). However, at higher temperatures, the levels of *N*-nitrosamines and nitrite in the moist snuff increased significantly over time. After 8 weeks of storage at 37 °C, the levels of NNN and NNK had risen threefold (from 6.24 to 18.7 μg/g), those of the *N*-nitrosamino acids MNPA and MNBA had risen 5.2-fold (from 3.13 to 16.3 ppm) and those of volatile *N*-nitrosamines had risen 10-fold (from 0.02 to 0.2 μg/g); moist snuff stored for 8 weeks at 37 °C contained 0.0386 μg/g NDMA, 0.0714 μg/g NPYR and 0.0176 μg/g NMOR. The concentration of 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanol (NNAL), a metabolite of NNK, doubled during storage at 37 °C from 0.29 to 0.65 μg/g. In a study conducted by the MDPH (Connolly, 2001), the effect of ageing of snuff was examined over 2, 4 and 6 months. Levels of total TSNA, including NNN, NNK, NAT and NAB, in the leading US brand Copenhagen increased 137%. No significant changes were observed in TSNA levels in Ettan, the Swedish Match moist snuff brand, when subjected to storage under adverse conditions. An earlier study revealed that levels of both NNN and NNK in moist snuff increased 21 and 12-fold, respectively, within the first 24 weeks of storage; in contrast, levels of nicotine decreased 1.3-fold during the same period. Concentrations of NNN and NNK in chewing tobacco and dry snuff during 24 weeks of storage increased 1.5- and 1.8-fold, respectively (Andersen *et al*., 1989).

1.3.4 *Kentucky (KY) reference smokeless tobacco products*

For research purposes, a series of reference smokeless tobacco products was developed and manufactured by the Tobacco and Health Research Institute (1987) at the University of Kentucky, Lexington, KY (USA) in the late 1980s. Each reference product, i.e. moist snuff, dry snuff and loose-leaf chewing tobacco, was custom made to mimic the chemical composition of commercial products in the respective category. However, speci-

fic flavourings and additives, including those used by manufacturers to influence levels of unprotonated nicotine, were not included in KY reference products. KY reference smokeless tobacco products contain the following ingredients:

Loose-leaf chewing tobacco (**1S1)**: Wisconsin air-cured tobacco, 17.4%; Pennsylvania air-cured tobacco, 15.47%; crushed Burley tobacco stems, 5.8%; glycerin, 3.75%; sucrose, 23.01%; dextrose, 1.7%; maltose, 1.3%; other corn syrup solids, 6.21%; salt, 1.6%; sodium propionate, 0.28%; water, 23.48%.

Dry snuff (**1S2)**: dark-fired tobacco, 22.75%; fire-cured Virginia tobacco, 19.66%; air-cured stems, 33.03%; fire-cured stems, 15.2%; salt, 0.36%; water, 9.0%.

Moist snuff (1S3): dark-fired tobacco, 25.73%; air-cured tobacco, 7.83%; Burley stems, 3.73%; sodium carbonate, 0.51%; sodium chloride, 7.4%; water, 54.80%.

As the blending recipe for KY reference products shows, loose-leaf chewing tobacco and moist snuff contain about 30% of tobacco by weight whereas dry snuff contains 75% of tobacco. The chemical composition of these reference products is shown in Table 15. In addition to data on nicotine, total nitrogen, nitrate nitrogen, total sugars, reducing sugars, moisture, pH, ash, potassium, sodium and calcium (Tobacco and Health Research Institute, 1987), the levels of selected TSNA and *N*-nitrosamino acids are also presented (Djordjevic *et al*., 1989b; Brunnemann *et al*., 2002).

1.3.5 *Pesticide residues*

Maximum allowable limits for pesticides on tobacco (e.g. maleic hydrazide, chlordane, dichlorodiphenyltrichloroethane, dichlorodiphenyldichloroethylene, dieldrin, endrine, heptachlor) in Germany, Italy, Spain and the USA are summarized by Sheets (1990).

1.4 Production, consumption and prevalence of use of smokeless tobacco products

This section presents data on sales, consumption and prevalence of use of smokeless tobacco products. Where possible, data are presented separately for each product type. In some countries and surveys, consumption was not measured or reported separately and thus overall consumption or prevalence of use of smokeless tobacco is reported. In most countries, surveys do not specify which type of snuff is used, but the overwhelming majority of snuff is of the moist variety and is taken orally.

Data on prevalence of smokeless tobacco use among youths in South America (Section 1.4.2(*c*)), South Asia (Section 1.4.3) and Africa (Section 1.4.4) rely primarily on the Global Youth Tobacco Survey (GYTS). The GYTS project was developed by WHO and the CDC in the USA. It is an international surveillance project designed to enhance the capacity of countries to monitor tobacco use among youths, and to guide the implementation and evaluation of tobacco prevention and control programmes. The GYTS has been completed in 120 countries. It uses a two-stage cluster sample survey design that produces representative samples of students in grades associated with the ages of 13–15 years.

Table 15. Chemical composition of Kentucky reference smokeless tobacco products

Constituent (%)	Chewing tobacco (loose-leaf) (1S1)	Dry snuff (1S2)	Moist snuff (1S3)
Nicotine	0.76, 0.95[a]	1.49, 1.6[a]	1.25, 2.51[a], 2.52[b]
Total nitrogen	1.20	2.59	1.33
Nitrate nitrogen	0.20	0.74	0.28
Total sugars	26.5	0.67	0.2
Reducing sugars	4.18	0.52	0.04
Moisture	23.2, 18.8[a]	8.79, 10.0[a]	55.0, 58.7[a], 52.0[b]
pH	6.42, 6.3[a]	6.37, 6.5[a]	8.01, 7.7[a], 6.93[b]
Ash	11.0	22.1	17.3
Potassium	2.09	5.91	1.78
Sodium	0.78	0.28	2.8
Calcium	1.37	2.93	1.44
Tobacco-specific N-*nitrosamines (TSNA)*			
NNN (μg/g)[c]	2.4[a]	81.3[a]	10.9[a], 12.6[b], 8.8[d]
NNK (μg/g)[c]	0.17[a]	20.3[a]	0.82[a], 2.2[b], 2.1[d]
Total TSNA (μg/g)[c]	3.6[a]	137.5[a]	19.6[a], 20.8[b]15.8[d]
N-*Nitrosamino acids (NNAC)*			
MNPA (μg/g)[c]	1.0[a]	13.1[a]	4.6[a]
MNBA (μg/g)[c]	0.05[a]	1.54[a]	0.4[a]
NPRO (μg/g)[c]	0.7[a]	15.4[a]	6.6[a]
Iso-NNAC (μg/g)[c]	0.03[a]	0.95[a]	0.13[a]
Total NNAC (μg/g)[c]	1.8[a]	31.9[a]	11.9[a]

From Tobacco and Health Research Institute (1987), unless otherwise stated
MNBA, 4-(*N*-methylnitrosamino)butyric acids; MNPA, 3-(*N*-methylnitrosamino)-propionic acids; NNK, 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone; NNN, *N*′-nitrosonornicotine; NPRO, *N*-nitrosoproline
[a] From Djordjevic *et al*. (1989b)
[b] From Brunneman & Hoffmann (2002)
[c] Per dry weight
[d] From Connolly (2001)

The prevalence measures used in this study included: current cigarette smoking — defined as ‘The percentage of students who smoked cigarettes on 1 or more days during the past 30 days’ and current other tobacco use — defined as ‘The percentage of students who had used any form of tobacco products other than cigarettes during the past 30 days’. Thus, other tobacco products include smokeless tobacco products as well as other smoking products.

1.4.1 *Europe*

Trends on sales of chewing tobacco in six European countries and of snuff in 13 countries are given in Tables 16 and 17, respectively. For three of these countries (Austria, Finland and France), the reports combined sales of chewing tobacco and snuff.

For many countries, no data were available on the consumption of smokeless tobacco products. For most countries included in Tables 16 and 17, no additional information was available on the use of smokeless tobacco other than annual sales, and those countries are not listed separately in this section, which includes a discussion of available data for those countries for which data on the prevalence of smokeless tobacco use were available. Estimates of annual per-capita consumption and prevalence of use of smokeless tobacco in these countries are given in Table 18 and 19, respectively.

(*a*) *Denmark*

Sales of chewing tobacco in Denmark have been declining since the early 1900s (Table 16). In 1995, snuff and chewing tobacco comprised 0.5% of all tobacco sales by weight in Denmark (Forey *et al*., 2002). There are few recent reports on prevalence of use. Among employed men who participated in the Copenhagen Male Study in 1985–86 (mean age, 63 years; range, 53–74 years), an estimated 3.5% reported chewing tobacco or using snuff without smoking (Suadicani *et al*., 1997).

(*b*) *Finland*

Per-capita consumption of moist snuff was relatively constant in Finland from 1970 to 1987 at 6–8 g per person aged 15 years and older, after which it increased to 22–29 g per person for the period 1988–94 (Wicklin, 2005) (Table 18). A 1987 survey of 14–18-year-olds found that use of snuff varied widely among regions in Finland: the proportion of boys who had tried snuff ranged from 17% in eastern and central Finland to 41% in Lapland, and regular use of snuff was reported by 2% of boys in eastern and central Finland, 4% in western Finland, 7% in Uusimaa and 10% in Lapland (Karvonen *et al*., 1993). Although the proportion of girls who had tried snuff ranged from 5 to 15% among the regions, regular use was reported by no more than 1% in any region.

A survey of 793 first- and second-year students in four senior high schools in the Turku region (mean age, 16.6 years) was conducted in December 1994, before the ban on snuff sales was enacted in Finland on 1 March 1995 (Merne *et al*., 1998). A cross-sectional survey was conducted in the same schools 1 year later, in December 1995. The study showed a prevalence of snuff use of 9% in 1994 (19% of the boys and 1% of the girls) and of 8% in 1995 (sex-specific prevalence not reported). Of students who reported the use of snuff before the ban, 10% reported to have quit because of the ban, 20% reported reducing their use, 12% reported switching to cigarettes and 5% reported switching to other drugs.

In 2002, 1.2% of adult men in Finland used snuff daily, and prevalence of daily use was highest among men aged 25–34 years (2.3%) (Patja & Vartianen, 2003). Occasional use of snuff was reported by 6% of men and boys aged 15–24 years. Among women, 1.1%

Table 16. Sales of chewing tobacco in selected European countries (tonnes)

Year	Austria	Denmark[a]	Finland	France	Norway[a]	Sweden[a]
	Chewing tobacco (includes snuff after 1985)	Chewing tobacco	Chewing tobacco and snuff	Chewing tobacco (includes snuff after 1970)	Chewing tobacco	Chewing tobacco
1920		1410				360
1925	320	1130				230
1930	360	950			910	140
1935	270	730		860	640	90
1940	320	590		540	540	90
1945	50	360		450	180	50
1950	140	410		590	320	50
1955	90	320		540	230	50
1960	50	270		590	180	50
1965	50	180		500	140	0
1970	0	140		500	90	0
1975	9	93	27	685	63	14
1976	9	82	28	691	69	14
1977	6	76	28	671	69	15
1978	2	47	31	655	59	15
1979	2	65	31	581	60	18
1980	2	61	23	516	57	18
1981	1	58	27	427	55	22
1982	1	52	27	416	48	23
1983	1	49	25	416	43	24
1984	1	44	25	391	42	22
1985	1	43	27	404	40	20
1986	8	39	28	372	36	19
1987	7	37	23	374	31	18
1988	8	32	116	382	30	16
1989	8	29	104	380	26	15
1990	8	26	87	397	24	14
1991	9	25	92	394	20	13
1992	9	22	109	404	19	13
1993	10	19	94	391	20	13
1994	10	16	91	370	18	13
1995	10	14	91	381	17	12
1996		13			16	12
1997		10			15	12
1998		9			14	14
1999		8			14	14

Table 16 (contd)

Year	Austria	Denmark[a]	Finland	France	Norway[a]	Sweden[a]
	Chewing tobacco (includes snuff after 1985)	Chewing tobacco	Chewing tobacco and snuff	Chewing tobacco (includes snuff after 1970)	Chewing tobacco	Chewing tobacco
2000		7			12	14
2001		7			12	13
2002		6			12	13
2003		6			12	13
2004		6			13	13

From Forey *et al*. (2002), unless otherwise specified
[a] Data after 1995 from Wicklin (2005)

used snuff occasionally and 0.6% used it daily. Among 16-year-olds, 3.3% of boys used snuff daily and 9% reported occasional use; 1% of girls used snuff occasionally but none reported daily use.

(*c*) *Norway*

Data on use of snuff in Norway has been collected by Statistics Norway since 1985 (Kraft & Svendsen, 1997). Most recent data from national surveys indicate that, in 2004–05, 10% of boys and men aged 16–74 years used snuff: 5% used it daily and 5% occasionally (Directorate of Health and Social Affairs, 2006a).

Among boys and men aged 16–24 years, the prevalence of daily or occasional snuff use increased from 9% in 1985 to 15% in 1994 (Kraft & Svendsen, 1997), and to 33% in 2004–05, the highest of any age group (Table 20) (Directorate of Health and Social Affairs, 2006a). Between 1983 and 2001, the prevalence of daily smoking by boys and men aged 16–24 years remained relatively constant at 28–32% (Directorate of Health and Social Affairs, 2006b), which suggests that the rise in snuff use among young men in Norway was not accompanied by a decline in smoking. The prevalence of occasional or daily use of snuff in men aged 16–44 years has increased steadily since 1988 and has more than doubled between 1985 and 2003 (Directorate of Health and Social Affairs, 2006a). Among men aged 65–74 years, the prevalence of daily or occasional snuff use declined from 12 to 6% between 1985 and 1994 (Kraft & Svendsen, 1997) and was 1% in 2004–05 (Directorate of Health and Social Affairs, 2006a).

The National Council on Tobacco and Health of Norway conducts surveys of tobacco use among Norwegian lower secondary school youths corresponding to the ages of 13–15 years (Braverman *et al*., 2001; Directorate of Health and Social Affairs, 2003, 2006c). The surveys are administered at 5-year intervals, and items on snuff were added in 1985.

Table 17. Sales of snuff in selected European countries (tonnes)

Year	Austria	Bulgaria	Denmark[a]	Finland	France	Iceland[a]	Ireland	Italy	Norway[a,b]	Poland	Portugal	Sweden[a,b]	United Kingdom
	Snuff (includes chewing tobacco and snuff after 1986)	Snuff	Snuff	Snuff (includes chewing tobacco and snuff after 1970)	Snuff (includes chewing tobacco and snuff after 1970)	Snuff (dry snuff used nasally)	Snuff	Snuff	Snuff	Snuff	Snuff	Snuff	Snuff
1920		3	180	50			140					6530	
1925	140	1	270	90			140	2090		390		5310	
1930	140	0	360	90			140	1720	450			4850	410
1935	90		450	50	2180	32	90	1320	410			4490	450
1940	90		500	50	1410	18	90	1090	540		50	3900	450
1945	0		540	0	860	32	90	730	270		50	3490	500
1950	50		500	50	770	36	50	540	540		50	3130	320
1955	0		450	50	590	36	50	540	540		0	2860	320
1960	0		450	0	410	32	50	450	450		0	2680	270
1965	0		360	50	320	32	0	320	410		0	2490	230
1970	0		270	50	180	27	0	180	320		0	2490	180
1975	5		223	27	685	17		130	263			2943	180
1976	6		207	28	691	17		120	267			3189	140
1977	7		198	28	671	16		110	283			3361	140
1978	6		185	31	655	14		100	268			3442	180
1979	6		170	31	581	14		100	260			3550	90
1980	7		159	23	516	15		90	263			3665	90
1981	8		149	27	427	15		80	270			3759	90
1982	8		142	27	416	15		80	248			3929	100
1983	8		131	25	416	15		70	247			4029	100
1984	8		117	25	391	15			274			4332	100
1985	7		107	27	404	13			292			4560	100
1986	8		98	28	372	12			279			4673	0
1987	7		84	23	374	13			270			4695	0
1988	8		76	116	382	12			279			4594	0

Table 17 (contd)

Year	Austria	Bulgaria	Denmark[a]	Finland	France	Iceland[a]	Ireland	Italy	Norway[a,b]	Poland	Portugal	Sweden[a]	United Kingdom
	Snuff (includes chewing tobacco and snuff after 1986)	Snuff	Snuff	Snuff (includes chewing tobacco and snuff after 1970)	Snuff (includes chewing tobacco and snuff after 1970)	Snuff (dry snuff used nasally)	Snuff	Snuff	Snuff	Snuff	Snuff	Snuff	Snuff
1989	8		75	104	380	12			285			4606	0
1990	8		70	87	397	12			286			4632	0
1991	9		62	92	394	13			283			4836	0
1992	9		52	109	404	13			263			5007	0
1993	10		48	94	391	12		36	295			5034	0
1994	10		40	91	370	13			301			5238	0
1995	10		37	91	381	12			314			5407	0
1996			36			13			346			5637	
1997			35			11			354			5328	
1998			33			12			343			5349	
1999			31			10			361			5691	
2000			31			10			358			6229	
2001			30			10			386			6462	
2002			30			11			419			6752	
2003			29			12			468			6813	
2004			28			13			550			6850	

From Forey *et al.* (2002), unless otherwise specified

[a] Data after 1995 from Wicklin (2005). Figures for 2003 and 2004 have not been validated (returns not available).

[b] Data after 1995 also available from Directorate of Health and Social Affairs (2005)

Table 18. Estimated per-capita consumption of smokeless tobacco in selected European countries (g per person aged ≥ 15 years)

Year	Denmark	Finland	Norway	Sweden
	Smokeless tobacco[a]	Moist snuff	Moist snuff	Moist snuff
1970	76	7	107	393
1971	70	8	108	409
1972	66	6	99	414
1973	61	6	94	420
1974	58	7	94	434
1975	57	7	86	452
1976	53	8	87	488
1977	50	8	92	511
1978	47	8	86	520
1979	42	8	83	533
1980	39	6	83	547
1981	37	7	85	558
1982	35	7	77	580
1983	32	6	76	593
1984	28	6	84	635
1985	26	7	88	666
1986	23	7	84	679
1987	20	6	80	679
1988	18	29	82	661
1989	18	26	83	658
1990	16	22	83	658
1991	15	23	82	684
1992	12	27	76	707
1993	11	23	85	708
1994	9	22	86	732
1995	9		90	754
1996	8		98	785
1997	8		100	741
1998	8		96	742
1999	7		101	788
2000	7		99	859
2001	7		107	887
2002	7		115	921
2003	7		128	924
2004	6		149	922

From Wicklin (2005)

[a] See Tables 16 and 17 for relative contributions of chewing tobacco and moist snuff.

Table 19. Prevalence (%) of daily use of moist snuff in three Nordic countries[a]

Age (years)	Sweden		Norway 2001–2003	Finland
	Men	Women	Men	Men
16–24	26.5	4.7	10.9	4
25–34	32.6	3.2	11.8	7.4
35–44	31.4	4.4	10.9	2.3
45–54	24.4	4	3.5	0
55–64	18.2	0.8	2.2	0.3
65–74	9	0.6	0.5	NR
75–84	4.5	0.4	NR	NR
Total	23	3	7.3	2.5

From Wicklin (2005)
NR, not reported
[a] Data for Denmark not available

Table 20. Prevalence (%) by age of men who use snuff in Norway, 2004–2005

Age (years)	Daily	Occasionally	Any current use
16–24	16	17	33
25–34	17	11	28
35–44	10	5	15
45–54	4	7	11
55–64	2	5	7
65–74	0	1	1

From Directorate of Health and Social Affairs (2006a)

Experimental use and current use of snuff declined between 1985 and 1990 for boys and girls and then increased slightly between 1990 and 2000 (Table 21). In 2000, 15.7% of boys and 1.9% of girls aged 13–15 years reported current snuff use. The prevalence of current use increased with increasing grade in school in both sexes.

Cross-sectional surveys of military personnel in the late 1980s found a very high prevalence of snuff use relative to the general male population. A 1986 survey of Norwegian Army conscripts found that 33% used snuff (10% daily and 23% occasionally); 82% of snuff users also smoked (Schei *et al*., 1990). Similarly, prevalence was relatively high among military officers in a 1989 survey, with 23% reporting current use (15% daily and 8% occasionally) (Schei, 1992).

Table 21. Prevalence (%) by sex, grade and year of survey of youths in Norway who have tried snuff or use it currently

Parameter[a]	1985	1990	1995	2000[b]	2005[c]
Have tried snuff					
Boys, total	39.5	24.9	27.3	30.8	31.1
Grade 8	27.1	15.0	15.8	18.8	15.4
Grade 9	39.7	24.9	26.0	32.9	31.6
Grade 10	51.7	34.9	40.1	40.8	47.0
Girls, total	15.6	9.4	9.3	10.6	16.5
Grade 8	9.0	3.4	4.0	6.1	7.0
Grade 9	14.1	8.4	8.3	10.3	16.6
Grade 10	23.8	16.5	15.5	16.0	25.8
Total	27.6	17.2	18.3	20.8	24
Currently use snuff[d]					
Boys, total	17.3	9.8	11.9	15.7	16.2
Grade 8	9.9	4.5	4.5	7.1	4.9
Grade 9	17.0	9.6	11.5	17.3	15.1
Grade 10	25.1	15.2	19.7	22.8	29.2
Girls, total	3.2	2.2	2.5	1.9	5.2
Grade 8	1.5	0.7	1.3	1.4	1.4
Grade 9	3.1	2.0	2.0	1.8	4.4
Grade 10	4.9	3.8	4.2	2.6	9.8
Total	10.3	6.0	7.2	8.9	10.9

From Braverman *et al*. (2001), unless otherwise specified
[a] Based on school start at age 6 years
[b] From Directorate of Health and Social Affairs (2001, 2003)
[c] From Directorate of Health and Social Affairs (2006c) [data added after meeting as it became available]
[d] Includes daily or occasional use

(*d*) *Sweden*

After declining from 6500 tonnes to 2500 tonnes between 1920 and 1967, annual sales of moist snuff (*snus*) in Sweden increased back to 6800 tonnes in 2002 (Table 17). Accordingly, per-capita consumption of moist snuff between 1970 and 2004 increased steadily from 393 to 922 g per person (Table 18) (Wicklin, 2005).

The most recent official Swedish national survey on the prevalence of moist snuff use among adults was conducted in 2004–05. In 1996–97, 20.0% of men and 0.9% of women aged 16–84 years used moist snuff daily and 5.4% of men and 1.1% of women used it occasionally (Wicklin, 2006) (Table 22). The prevalence of daily moist snuff use among men increased from 16.7% in 1988–89 to 20.0% in 1996–97 and 22% in 2004.

The most recent age-specific official data on moist snuff use among men in Sweden (Wicklin, 2006) show that, in 2004, the prevalence of daily moist snuff use was highest among men aged 35–44 years (29%) and lowest among men aged 75–84 years (6%). This

Table 22. Prevalence (%) of use of *snus* among persons aged 16–84 years in Sweden, 1980–1997 (SCB/ULF surveys[a])

Year	Daily	Occasionally	Any current use
Men			
1980–81	[b]	[b]	16.6
1988–89	16.7	4.5	21.2
1996–97	20.0	5.4	25.4
Women			
1988–89	0.7	0.7	1.4
1996–97	0.9	1.1	2.0

From Wicklin (2006)

[a] SCB/ULF (living condition) surveys are conducted annually. Questions concerning *snus* use are included every seventh and eighth year. Questions concerning *snus* use are scheduled for inclusion in 2004–05. ULF surveys are conducted using personal and telephone interviews. Every year, approximately 6000 interviews are conducted. The results are reported as an average of two years' survey results.

[b] In 1980–1981, respondents were asked "Do you use *snus*?" but were not asked about daily or occasional use.

pattern is slightly different from that seen in 1988–89 and 1996–97 (Table 23), when the prevalence was highest among men aged 25–34 years and was lowest among men aged 55–64 years and 65–74 years, respectively. Between 1989 and 2004, the prevalence among men aged 16–24 years remained at 21–23% and increased among men aged 35–64 years. Moist snuff use was most prevalent among skilled and unskilled workers of all occupational groups; the survey showed some regional variations of moist snuff use.

Although unofficial trends of the prevalence of moist snuff use in Sweden are also available from the mail-based TEMO surveys conducted by the Statistical Bureau VECA[HB] and sponsored by the Swedish Match Company (Wicklin, 2006). The sample size in 2004 was about 12 000, but response rates were not reported; 20.4% of men aged 16–75 years reported daily use of snuff and 4.0% reported occasional use (Table 24). Daily moist snuff use was reported by 3.4% of women in that age range and 2.9% of women reported using it occasionally.

Data on daily use of moist snuff among young people in Sweden have been collected since 1981 during the School Children's Drug Habits surveys conducted by the Swedish Council for Information on Alcohol and Other Drugs (Wicklin, 2006). The prevalence of daily snuff use in 2003 was 2% among 12–13-year-old boys and was not reported for girls at that age. Among students aged 15–16 years, daily use of moist snuff remained relatively constant among boys aged 15–16 years, in the range of 11–14% until 1998, with a possible trend toward increasing moist snuff use in more recent years. Moist snuff use among 15–16-year-old girls remained relatively constant over time and was in the range of 0–2%.

Table 23. Prevalence (%) by selected demographic characteristics of daily use of *snus* among men aged 16–84 years in Sweden, 1988–89 and 1996–97 (SCB/ ULF surveys[a])

	1988–89	1996–97
Age (years)		
16–24	23.0	21.2
25–34	25.0	29.4
35–44	18.6	24.8
45–54	10.9	19.1
55–64	8.9	10.0
65–74	10.5	7.8
75–84	12.6	10.2
Profession		
Unskilled workers	21.7	22.4
Skilled workers	23.6	25.6
Total workers	22.6	24.1
Office staff, low	11.7	19.0
Office staff, all others	10.9	14.9
Total staff	11.0	15.9
Entrepreneurs	15.3	20.2
Farmers	14.6	12.4
Students	13.3	16.1
Others	11.6	12.9
Geographical region		
Stockholm	13.0	15.3
Göteborg/Malmö	12.7	16.8
Större kommuner	16.7	20.2
Södra mellanbygden	19.1	21.4
Norra tätbygden	21.7	17.5
Norra glesbygden	24.0	27.4
Overall	16.7	20.0

From Wicklin (2006)

[a] SCB/ULF (living condition) surveys are conducted annually. Questions concerning *snus* use are included every seventh and eighth year. Questions concerning *snus* use are scheduled for inclusion in 2004–2005. ULF surveys are conducted using personal and telephone interviews. Every year, approximately 6000 interviews are conducted. The results are reported as an average of survey results of 2 years.

(*e*) *United Kingdom*

The use of chewing tobacco is relatively rare in the general population of the United Kingdom, although use of various forms of oral tobacco is common in some immigrant communities in the form of chewing betel quid with tobacco.

Table 24. Prevalence (%) of use of *snus* by sex in Sweden, 1970–2004 (TEMO surveys[a])

Year[b]	Age groups[c]	Use *snus* daily[d]	Use *snus* occasionally[d]	Total
Men				
1970	16–67	–	–	12.1
1972	15–67	–	–	15.4
1980	15–69	–	–	16.6
1990	15–70	–	–	20.9
2000	16–75	16.5	7.4	23.9
2004	16–75	20.4	4.0	24.4
Women				
1972	15–67	–	–	0.2
1980	15–69	–	–	0.5
1990	15–70	–	–	2.6
2000	16–75	2.2	1.9	4.1
2004	16–75	3.4	2.9	6.3

From Wicklin (2006)

[a] The surveys were conducted by mail. The number of replies (from both men and women) has been around 12 000. Response rates are not reported.

[b] Data for 1970 are based on surveys in the autumn of 1969 and the spring of 1970. Data for 1972 are based on surveys in the autumn 1971 and the spring of 1972.

[c] The age groupings have been changed several times.

[d] Questions concerning daily and occasional *snus* use were first introduced in the TEMO surveys in 1997.

1.4.2 *North and South America*

Data on sales of snuff and chewing tobacco are available for Canada and the USA (Table 25). The USA are the leading producer of snuff worldwide, and have experienced substantial increases in sales of snuff in recent decades, from 10 840 tonnes in 1980 to 33 520 tonnes in 2003 (209% increase) (Forey *et al.*, 2002; Department of Agriculture, 2003).

Estimates of annual per-capita consumption of smokeless tobacco are available for the USA only (Table 26).

(*a*) *Canada*

Recent Canadian national data on consumption of smokeless tobacco and prevalence of use are reported only in aggregate and not by product type (Table 25). Sales of smokeless tobacco products in Canada have remained relatively constant from 1989 to 2003 other than some fluctuation in 2000 and 2001 (Tobacco Control Programme, 2004). In 1992–97, chewing tobacco generally accounted for about 20–30% of the smokeless tobacco market by weight; the majority of the market was snuff (Wyckham, 1999).

Table 25. Sales of chewing tobacco and snuff in North America (tonnes)

Year	Canada[a]		USA[b]	
	Plug tobacco (includes all smokeless tobacco after 1975)	Snuff (includes all smokeless tobacco after 1975)	Chewing tobacco	Snuff
1920	2990	320		16 370
1925	3860	360		17 150
1930	2680	450		18 190
1935	1770	360		17 280
1940	1410	360		17 190
1945	1450	450		19 780
1950	1040	410	38 960	18 140
1955	680	360	35 150	17 690
1960	500	410	28 940	15 740
1965	410	410	28 980	13 380
1970	270	360	30 930	12 110
1975		NA	36 560	11 430
1976		938	38 100	11 700
1977		1207	40 230	11 070
1978		699	41 910	11 020
1979		741	45 770	10 840
1980		770	48 040	10 840
1981		750	48 310	11 570
1982		726	39 920	19 910
1983		713	39 280	20 730
1984		745	39 600	21 640
1985		695	38 560	22 040
1986		629	35 700	21 180
1987			34 610	20 460
1988			33 880	21 680
1989		284	33 070	22 320
1990		261	32 070	23 270
1991		228	32 340	24 220
1992		215	30 710	25 170
1993		224	28 940	25 760
1994		239	28 030	26 580
1995		250	28 210	26 940
1996		257	27 200	27 900
1997		252	25 800	28 200
1998		255	23 800	29 100
1999		259	22 800	29 700
2000		154	22 000	31 100
2001		312	21 100	31 600
2002		272	19 600	32 500
2003		236	18 300	33 500

Data from Forey *et al*. (2002), unless otherwise specified

[a] Data for 1976–86 from Millar (1989); data for 1989–2003 from Tobacco Control Programme (2004)

[b] Data after 1995 from Department of Agriculture (2006)

Table 26. Estimated[a] per-capita consumption of smokeless tobacco in the USA (g per person aged ≥ 15 years)

Year	USA	
	Chewing tobacco	Snuff
1970	212	83
1971	219	81
1972	216	76
1973	217	74
1974	224	70
1975	227	71
1976	232	71
1977	241	66
1978	246	65
1979	264	63
1980	273	62
1981	271	65
1982	221	110
1983	215	114
1984	215	117
1985	207	118
1986	189	112
1987	182	107
1988	177	113
1989	171	115
1990	164	119
1991	164	123
1992	154	127
1993	144	128
1994	138	131
1995	138	131
1996	131	134
1997	123	134
1998	112	137
1999	106	139
2000	99	141
2001	94	141
2002	86	143

From Department of Agriculture (2003); Department of Commerce (2004); DHHS (2004a)

[a] Data calculated by the Working Group

A 1994 survey on smoking in Canada found that about 1% of the male population aged 15 years and older used smokeless tobacco products currently, which was unchanged from the 1986 prevalence (Wyckham, 1999). In 1986, use of chewing tobacco was slightly more prevalent (0.7%) than that of snuff (0.4%) among men aged 15 years and older, with a prevalence of 1.8% for those over 65 years of age (Millar, 1989). Use of chewing tobacco was slightly more prevalent among men in the Atlantic region (2.0%) and Prairies (1.1%) than in other regions. More recently, the Canadian Tobacco Use Monitoring Surveys enquired whether respondents had ever tried chewing tobacco, pinch or snuff; in 1999–2003, 13–15% of men and 2–3% of women aged 15 years and older reported ever trying these products (Tobacco Control Programme, 2004).

Relatively high use of chewing tobacco and other smokeless tobacco products has been reported among some native populations in some localities, among college athletes and among some young people who use other forms of tobacco. A survey of native Canadians in northern Saskatchewan found that 15% of boys and men and 7% of girls and women aged 7–21 years used chewing tobacco and 23% and 14%, respectively, used snuff (Hoover *et al*., 1990). Similar findings were reported from a 1987 survey of 5–19-year-olds in the Canadian Arctic, in which 11% of boys and 2% of girls currently used chewing tobacco and 13% and 5%, respectively, used snuff; the prevalence was more than 10 times higher among Dene or Métis and Inuit children than among non-native children (Millar, 1990). In a 1989 random telephone survey of boys and girls aged 11–19 years in northeastern Ontario, 4.5% of respondents reported current use of chewing tobacco and 1.1% used snuff (Blackford *et al*., 1994). In a longitudinal panel survey in Calgary, the prevalence of smokeless tobacco use was 1.1% in grade 6 (boys, 1.6%; girls, 0.7%), 2.2% in grade 7 (boys, 3.1%; girls, 1.1%) and 4.2% in grade 8 (boys, 6.9%; girls, 1.6%) (Abernathy & Bertrand, 1992).

A survey of 754 athletes at 10 English Canadian universities found that, among men, smokeless tobacco was used by 47.2% of hockey players, 36.2% of football players, 22.0% of soccer players, 12.0% of volleyball players and 6.8% of track or cross-country athletes (Spence & Gauvin, 1996). Use among female university athletes in some sports was relatively high compared with the general Canadian population, including track or cross-country (6.2%), basketball (4.0%), soccer (3.3%) and volleyball (2.4%). A 1987 survey of Ontario students aged 13–19 years found that smokeless tobacco use was uncommon in the general student population (2.6% of boys, 0.6% of girls), but the prevalence was relatively high among students who currently smoked (9.6% of boys, 1.9% of girls) (Adlaf & Smart, 1988).

(*b*) *USA*

Information on sales and per-capita consumption of chewing tobacco and snuff in the USA between 1920 and 2003 are presented in Tables 25 and 26, respectively.

In 2000, 4.4% of men and 0.3% of women in the USA were current users of smokeless tobacco products (Table 27). Current use was more common among men aged 18–44 years (5.0–5.8%) than among men aged 45 years and older (2.8–3.1%). Non-Hispanic white men were more likely to be current users (5.5%) than were men in other racial or

Table 27. Prevalence by selected demographic characteristics of current use of smokeless tobacco in the USA, 2000 (National Health Interview Survey)

Characteristic	Men		Women		Total	
	%	(95% CI)	%	(95% CI)	%	(95% CI)
Age (years)						
18–24	5.0	(± 1.5)	0.0	(± 0.0)	2.5	(± 0.8)
25–44	5.8	(± 0.7)	0.2	(± 0.1)	2.9	(± 0.4)
45–64	3.1	(± 0.6)	0.4	(± 0.2)	1.7	(± 0.3)
≥ 65	2.8	(± 0.8)	0.7	(± 0.3)	1.6	(± 0.4)
Region[a]						
Northeast	2.2	(± 0.5)	0.1	(± 0.1)	1.1	(± 0.3)
Midwest	4.4	(± 0.7)	0.1	(± 0.1)	2.1	(± 0.3)
South	6.7	(± 0.8)	0.7	(± 0.2)	3.6	(± 0.4)
West	2.6	(± 0.9)	0.1	(± 0.1)	1.3	(± 0.5)
Education						
Less than high school diploma	5.7	(± 1.2)	1.1	(± 0.4)	3.4	(± 0.6)
High school or GED diploma	5.6	(± 0.8)	0.2	(± 0.1)	2.7	(± 0.4)
More than high school diploma	3.4	(± 0.5)	0.1	(± 0.1)	1.7	(± 0.2)
Race or ethnicity						
Hispanic	0.8	(± 0.5)	0.0	(± 0.0)	0.4	(± 0.2)
White, non-Hispanic	5.5	(± 0.5)	0.2	(± 0.1)	2.7	(± 0.3)
Black, non-Hispanic	1.3	(± 0.6)	1.3	(± 0.5)	1.3	(± 0.4)
Other	2.2	(± 1.5)	0.5	(± 0.6)	1.4	(± 0.8)
Location of residence[b]						
MSA	3.3	(± 0.4)	0.2	(± 0.1)	1.7	(± 0.2)
Non-MSA	9.0	(± 1.3)	0.6	(± 0.2)	4.5	(± 0.6)
Total	4.4	(± 0.4)	0.3	(± 0.1)	2.3	(± 0.2)

From Tomar (2003a)

CI, confidence interval; GED, General Educational Development

[a] Northeast: Maine, New Hampshire, Vermont, Massachusetts, Rhode Island, Connecticut, New York, New Jersey, Pennsylvania; Midwest: Ohio, Indiana, Illinois, Michigan, Wisconsin, Minnesota, Iowa, Missouri, North Dakota, South Dakota, Nebraska, Kansas; South: Delaware, Maryland, District of Columbia, Virginia, West Virginia, North Carolina, South Carolina, Georgia, Florida, Kentucky, Tennessee, Alabama, Mississippi, Arkansas, Louisiana, Oklahoma, Texas; West: Montana, Idaho, Wyoming, Colorado, New Mexico, Arizona, Utah, Nevada, Washington, Oregon, California, Alaska, Hawaii.

[b] MSA, metropolitan statistical area; an MSA is a county or group of contiguous counties that contain at least one city with a population of 50 000 or more or includes a Census Bureau-defined urbanized area of at least 50 000 with a metropolitan population of at least 100 000.

ethnic groups (0.8–2.2%), although the sample size was insufficient to permit meaningful national estimates for some racial and ethnic groups that may have high levels of use, such as American Indians. The prevalence of smokeless tobacco use was higher among men with a high school education or less (5.6–5.7%) than among those with at least some post-high school education (3.4%). Prevalence of smokeless tobacco use was higher among men in the South (6.7%) than in all other geographic regions (2.2–4.4%), and was much higher among men who lived outside of metropolitan statistical areas (9.0%) than among urban men (3.3%) (Tomar, 2003a).

Based on combined unpublished data from the January and May 2000 Current Population Survey Tobacco Use Supplements, the prevalence of smokeless tobacco use among adult men in 2000 was highest in West Virginia (13.9%), Montana (13.1%), Wyoming (13.3%), Mississippi (9.4%) and Tennessee (9.2%) and lowest in Massachusetts (0.2%), Rhode Island (0.5%), New Jersey (0.6%), Connecticut (1.0%) and Hawaii (0.8%).

Similar to the pattern observed among adults, adolescent smokeless tobacco users in the USA are predominantly boys. In 1986–87, 6.1% of boys and 0.1% of girls aged 12–17 years reported current use of smokeless tobacco (Tomar *et al*., 1997). All recent national surveys of young people report the prevalence of 'smokeless tobacco' use, which includes snuff or chewing tobacco, and generally do not enquire about the products separately. The prevalence of current smokeless tobacco use among male high-school students has declined from 20.4% in 1993 (Kann *et al*., 1995) to 11.0% in 2003 (Grunbaum *et al*., 2004) (Table 28). Use of smokeless tobacco ranged from 9.1 to 13.3% for boys in grades

Table 28. Prevalence by sex, race or ethnicity and grade of use[a] of smokeless tobacco among high school students in the USA, 2003 (Youth Risk Behavior Survey)

Characteristics	Girls	Boys	Total
	% (95% CI)	% (95% CI)	% (95% CI)
Race or ethnicity			
White, non-Hispanic	1.6 (± 1.2)	13.2 (± 3.3)	7.6 (± 1.9)
Black, non-Hispanic	2.0 (± 1.1)	4.1 (± 1.8)	3.0 (± 1.1)
Hispanic	3.3 (± 2.1)	6.1 (± 3.5)	4.7 (± 2.7)
Grade			
9	3.8 (± 2.4)	9.1 (± 3.7)	6.6 (± 2.5)
10	1.0 (± 0.7)	9.6 (± 3.1)	5.4 (± 1.6)
11	2.0 (± 1.6)	13.3 (± 3.1)	7.8 (± 2.2)
12	1.3 (± 0.8)	12.7 (± 3.5)	7.1 (± 1.8)
Total	2.2 (± 1.2)	11.0 (± 2.3)	6.7 (± 1.5)

From Grunbaum *et al*. (2004)
CI, confidence interval
[a] Used chewing tobacco, snuff or dip on at least 1 of the 30 days preceding the survey.

9 to 12, while among high-school girls it ranged from 1.0 to 3.8% across grades. The prevalence was substantially higher among male non-Hispanic white students (13.2%) than among male Hispanic (6.1%) or non-Hispanic black students (4.1%) (Grunbaum *et al*., 2004). A nationally representative cohort study conducted in the early 1990s estimated that, each day, 2200 young people in the USA first try smokeless tobacco and about 830 become regular users (Tomar & Giovino, 1998).

(i) *Chewing tobacco*

Detailed data for production of the three major forms of chewing tobacco in the USA during 1981–2003 are presented in Table 29. Loose-leaf chewing tobacco remained the predominant form throughout that period, and comprised 94% of the chewing tobacco market by weight. However, production declined for all chewing tobacco products during

Table 29. Production of chewing tobacco in the USA, by major category

Year	Output (millions of kg)			
	Plug	Twist	Loose-leaf	Total
1981	8.1	0.8	31.9	40.8
1982	7.1	0.8	33.1	41.0
1983	6.4	0.8	32.2	39.4
1984	5.7	0.8	33.7	40.3
1985	5.1	0.7	33.6	39.4
1986	4.7	0.6	31.6	36.9
1987	4.5	0.6	30.5	35.7
1988	4.0	0.6	29.8	34.4
1989	3.7	0.6	29.4	33.8
1990	3.3	0.6	29.2	33.1
1991	3.0	0.5	29.2	32.7
1992	2.7	0.5	27.9	31.2
1993	2.4	0.5	26.3	29.2
1994	2.1	0.5	25.8	28.4
1995	1.8	0.5	26.0	28.4
1996	1.8	0.5	25.4	27.7
1997	1.6	0.5	24.4	26.4
1998	1.4	0.5	22.3	24.2
1999	1.3	0.4	21.4	23.1
2000	1.2	0.4	20.9	22.4
2001	1.1	0.4	19.9	21.3
2002	1.0	0.4	18.7	20.0
2003	0.8	0.3	18.4	19.5

From Department of Agriculture (2006)

that time. Per-capita consumption followed the same trend, declining by 68% from 273 g per person aged 15 years or older in 1980 to 86 g per person in 2002 (Table 26).

Use of chewing tobacco in the USA is primarily practised by men although there are examples of subpopulations of women in which use is relatively prevalent, particularly some American Indian and Alaskan Native communities (Schinke *et al*., 1987; Lanier *et al*., 1990). Prevalence of tobacco chewing appears to be declining in the USA after having reached a peak of 4.1% in 1987 (Table 30). In 2000, current use of chewing tobacco was reported by 2.5% of men and 0.1% of women; it tended to be slightly higher for men aged 25–34 years than in other age groups. Table 31 presents more detailed characteristics of the prevalence of use of different types of smokeless tobacco among men in 2000 in the USA (unpublished data from the 2000 National Health Interview Survey). Use of chewing tobacco was more prevalent among non-Hispanic white men than among other racial or ethnic groups, among men with less than a high school education than among more educated men, and among rural men than among urban men. Approximately one-half of the men who reported current use of chewing tobacco used those products on a daily basis.

(ii) *Snuff*

Moist snuff is the predominant form of snuff sold in the USA. It comprised 95% of the snuff market by weight in 2001 (Federal Trade Commission, 2003). Sales of dry snuff declined steadily from 3678.7 tonnes in 1986 to 1526.2 tonnes in 2001, while moist snuff sales increased gradually from 16 391.0 to 28 980.0 tonnes during that period (Tables 25 and 32). Except for a slight decline in the mid-1980s, per-capita consumption of snuff (moist and dry) in the USA has increased every year since 1981 except for some decline in 1986–89 (Table 26).

Trends in the prevalences of use of smokeless tobacco between 1970 and 2000 by sex and age are given in Table 30. This includes only individuals who do not smoke cigarettes, but who may smoke cigar or pipes.

In 2000, current snuff use was highest among men aged 18–24 years (3.6%) and was more prevalent than chewing tobacco use among men aged 18–44 years (Table 31). Snuff use was more prevalent among men in southern regions of the USA than in other regions, among men with a high school diploma or equivalent than among those with a higher education, among non-Hispanic whites than among other racial or ethnic groups and among men who resided outside of metropolitan statistical areas (i.e. primarily rural areas) than those who lived in metropolitan areas. About 60–65% of men who were current snuff users used those products on a daily basis, except for the youngest age group (18–24 years) (unpublished data from the 2000 National Health Interview Survey).

(iii) *Population groups with a high prevalence of use*

There are groups within the USA with exceptionally high prevalences of use of smokeless tobacco. A review of studies of professional baseball players conducted between 1987 and 1998 reported a prevalence of smokeless tobacco use of 35–46%, including both chewing tobacco and snuff (Greene *et al*., 1998), although snuff is used much more com-

Table 30. Prevalence (%) by sex and age of current[a] use of chewing tobacco or snuff[b] among adults in the USA

	1970	1987	1991	2000
Chewing tobacco				
Men (age in years)				
18–24	1.8	5.5	4.1	2.9
25–34	2.2	3.3	3.1	3.5
35–44	3.3	3.1	2.5	2.6
45–64	4.2	3.9	2.4	1.8
≥ 65	9.4	5.4	3.9	2.0
Total	3.9	4.1	3.1	2.5
Women (age in years)				
18–24	0.3	0.1	0.1	0.0
25–34	0.3	0.1	0.0	0.2
35–44	0.5	0.3	0.1	0.0
45–64	0.6	0.2	0.4	0.1
≥ 65	1.0	0.7	0.6	0.2
Total	0.5	0.3	0.3	0.1
Snuff				
Men (age in years)				
18–24	0.7	6.4	6.2	3.4
25–34	0.5	3.6	4.8	3.7
35–44	0.8	2.5	2.9	3.0
45–64	1.8	1.6	1.4	1.7
≥ 65	4.0	2.2	2.2	0.9
Total	1.5	3.1	3.3	2.5
Women (age in years)				
18–24	0.2	0.3	0.2	0.0
25–34	0.3	0.1	0.1	0.1
35–44	0.6	0.2	0.1	0.2
45–64	1.8	0.4	0.3	0.3
≥ 65	4.0	1.5	1.3	0.5
Total	1.4	0.5	0.4	0.2
Chewing tobacco or snuff				
Men (age in years)				
18–24	2.2	8.9	8.4	5.0
25–34	2.5	6.0	6.9	6.6
35–44	3.9	4.8	4.9	5.1
45–64	5.8	5.0	3.7	3.1
≥ 65	12.7	6.9	5.6	2.8
Total	5.2	6.1	5.6	4.4

Table 30 (contd)

	1970	1987	1991	2000
Women (age in years)				
18–24	0.4	0.3	0.2	0.0
25–34	0.5	0.2	0.1	0.2
35–44	1.0	0.3	0.2	0.2
45–64	2.3	0.6	0.6	0.4
≥ 65	4.8	1.9	1.7	0.7
Total	1.8	0.6	0.6	0.3

From Giovino *et al.* (1994); unpublished data from 2000 National Health Interview Survey

[a] Current is defined as used at least 20 times and now used every day or on some days.

[b] The figures represent users of smokeless tobacco products who did not smoke cigarettes; they may have smoked cigars or pipes.

monly than chewing tobacco among this group (75%–90% of current users). In another study of professional baseball players conducted in 1988 (Ernster *et al.*, 1990), 42% of players reported current use of any type of smokeless tobacco and, among users, 43% reported using both. In a study conducted in 1999, 31% of professional baseball players reported current use of smokeless tobacco, 82% of whom were using snuff (Cooper *et al.*, 2003).

High rates of smokeless tobacco use have also been reported among college athletes (Levenson-Gingiss *et al.*, 1989; Walsh *et al.*, 1994; Hannam, 1997; Green *et al.*, 2001). More than 20% of National Collegiate Athletic Association student athletes reported current use of smokeless tobacco in 1996, with a range of 6–55% among male teams and 1–22% among female teams (Green *et al.*, 2001). Those studies of college athletes that examined product type reported that exclusive snuff use was more common than exclusive chewing tobacco use, but dual use of products was common (Levenson Gingiss & Gottlieb, 1991; Walsh *et al.*, 1994; Chakravorty *et al.*, 2000).

Elevated use of smokeless tobacco has also been reported among high-school athletes compared with non-athletes (Davis *et al.*, 1997; Melnick *et al.*, 2001; Castrucci *et al.*, 2004). Similar to patterns among college athletes, high-school baseball players who used smokeless tobacco were much more likely to use exclusively snuff (40%) or to use both snuff and chewing tobacco (52%) than to use exclusively chewing tobacco (8%) (Walsh *et al.*, 2000).

There are indications that the prevalence of smokeless tobacco use is also relatively high among military personnel in the USA and ranges from 15 to 46% (Ballweg & Bray, 1989; Forgas *et al.*, 1996; Kenny *et al.*, 1996; Grasser & Childers, 1997; Chisick *et al.*, 1998; Kao *et al.*, 2000). The few studies that examined product type reported at least a 3:1

ratio of use of snuff to use of chewing tobacco (Kenny *et al*., 1996; Grasser & Childers, 1997).

Table 31. Prevalence (%) by selected demographic characteristics of current use of smokeless tobacco among men aged 18 years and older in the USA, 2000 (National Health Interview Survey)

Characteristic	Smokeless tobacco		Chewing tobacco		Snuff	
	Any current use	Daily use	Any current use	Daily use	Any current use	Daily use
Age (years)						
18–24	5.3	2.3	3.0	0.9	3.6	1.5
25–44	5.9	3.5	3.2	1.4	3.4	2.2
45–64	3.3	2.0	1.9	1.0	1.7	1.1
≥ 65	3.0	1.9	2.2	1.4	1.0	0.6
Region[a]						
Northeast	2.4	1.4	1.5	0.6	1.2	0.8
Midwest	4.5	2.6	2.6	1.2	2.6	1.6
South	6.8	4.0	3.6	1.7	4.1	2.5
West	2.8	1.3	2.1	0.8	1.2	0.6
Education						
Less than high school diploma	5.8	3.6	3.9	2.0	2.8	1.7
High school or GED diploma	5.7	3.5	2.7	1.1	3.8	2.4
More than high school diploma	3.6	1.8	2.2	1.0	1.9	1.0
Race or ethnicity						
Hispanic	0.9	0.2	0.5	0.0	0.5	0.2
White, non-Hispanic	5.7	3.4	3.2	1.6	3.3	2.0
Black, non-Hispanic	1.4	0.6	1.0	0.4	0.4	0.3
Other	2.2	0.9	1.5	0.1	1.4	0.9
Location of residence[b]						
MSA	3.4	1.9	2.0	0.9	1.9	1.1
Non-MSA	9.3	5.3	5.2	2.4	5.4	3.3
Total	4.6	2.6	2.6	1.2	2.6	1.6

Unpublished data from the 2000 National Health Interview Survey
GED, General Educational Development
[a] Northeast: Maine, New Hampshire, Vermont, Massachusetts, Rhode Island, Connecticut, New York, New Jersey, Pennsylvania; Midwest: Ohio, Indiana, Illinois, Michigan, Wisconsin, Minnesota, Iowa, Missouri, North Dakota, South Dakota, Nebraska, Kansas; South: Delaware, Maryland, District of Columbia, Virginia, West Virginia, North Carolina, South Carolina, Georgia, Florida, Kentucky, Tennessee, Alabama, Mississippi, Arkansas, Louisiana, Oklahoma, Texas; West: Montana, Idaho, Wyoming, Colorado, New Mexico, Arizona, Utah, Nevada, Washington, Oregon, California, Alaska, Hawaii.
[b] MSA, metropolitan statistical area; an MSA is a county or group of contiguous counties that contain at least one city with a population of 50 000 or more or includes a Census Bureau-defined urbanized area of at least 50 000 with a metropolitan population of at least 100 000.

Table 32. Sales of snuff in the USA, by category[a] (tonnes)

Year	Dry snuff	Moist snuff
1986	3678.7	16 391.0
1987	3290.9	16 465.1
1988	3206.8	17 887.1
1989	3286.2	18 605.4
1990	2805.7	19 856.6
1991	2645.9	20 950.5
1992	2550.7	22 003.6
1993	2266.6	22 771.2
1994	2183.7	23 600.1
1995	2036.7	24 102.5
1996	1913.6	24 895.4
1997	1843.2	25 074.4
1998	1715.4	25 486.1
1999	1620.4	26 523.3
2000	1571.1	27 888.2
2001	1526.2	28 980.0

From Federal Trade Commission (2003)
[a] Includes sales by the five major US manufacturers.

(*c*) *South America*

Limited information is available from the GYTS on the prevalence of the use of smokeless tobacco and non-cigarette tobacco for selected countries in Latin America (Table 33).

In a cross-sectional survey in schools in Venezuela, *chimó* was used by 13.5% of boys in grades 6–9 (~13–16 years old), including 10% of boys in grade 6 (Granero *et al.*, 2003).

1.4.3 *South Asia*

The prevalence of smokeless tobacco use is high in South Asia. Also, new forms of smokeless tobacco have been emerging over the last few decades to entice new consumers. Increasing use has been reported not only among men, but also among children, teenagers, women of reproductive age and immigrants of South Asian origin wherever they have settled (Gupta, 1992). In the WHO South-East Asia Region, over 250 million people use smokeless tobacco products, which represents 17% of the total population; of those, 95% live in India (82%) or Bangladesh (13%) (Sinha, 2004).

Data from national or sub-national surveys or data from studies with large sample sizes are presented in this section.

Table 33. Prevalence (%) of use of smokeless and non-cigarette tobacco products among 13–15-year-old schoolchildren in Latin America (Global Youth Tobacco Survey)

Country	Year of survey	Current use[a] of non-cigarette tobacco products (± 95% CI)
Antigua and Barbuda	2000	9.6 (± 2.2)
Argentina		
Buenos Aires	2003	8.2 (± 1.9)
Capital Federal	2003	6.2 (± 2.0)
Barbados	2002	10.3 (± 2.5)
Bolivia		
Cochabamba	2003	11.3 (± 2.5)
El Alto	2003	11.3 (± 1.4)
La Paz	2003	8.2 (± 1.3)
Oruro	2003	8.6 (± 1.9)
Santa Cruz	2003	9.7 (± 2.1)
Brazil		
Aracaju	2002	3.4 (± 1.6)
Curitiba	2002	3.4 (± 0.7)
Fortaleza	2002	3.4 (± 1.4)
Goiania	2002	5.7 (± 2.4)
Matto Grosso do Sul	2002	4.9 (± 1.5)
Paraiba	2002	3.5 (± 1.8)
Rio Grande do Norte	2002	4.9 (± 1.7)
Rio Grande do Sul	2002	6.0 (± 1.9)
Tocantins	2002	4.0 (± 2.3)
Chile		
Concepcion	2003	6.4 (± 2.4)
Coquimbo	2003	2.6 (± 0.9)
Santiago	2003	4.9 (± 1.3)
Valparaiso - Viña del Mar	2003	3.7 (± 1.2)
Columbia	2001	5.1 (± 1.0)
Costa Rica	2002	5.5 (± 0.8)
Cuba	2001	6.1 (± 1.2)
Dominica	2000	10.7 (± 2.3)
Ecuador		
Guayaquil	2001	8.2 (± 2.0)
Quito	2001	10.0 (± 1.8)
Zamora	2001	17.6 (± 3.1)
El Salvador	2003	8.4 (± 2.0)
Grenada	2000	8.7 (± 1.8)
Guatamala		
Chimal Tenago	2002	5.3 (± 2.0)
Guatamala City	2002	5.6 (± 1.4)
Guyana	2000	8.4 (± 2.2)
Haiti	2001	10.7 (± 4.6)

Table 33 (contd)

Country	Year of survey	Current use[a] of non-cigarette tobacco products (± 95% CI)
Honduras		
San Pedro Sula La Ceiba	2003	10.5 (± 3.1)
Tegucigalpa	2003	9.9 (± 1.7)
Jamaica	2001	7.8 (± 1.8)
Mexico		
Chetmul	2003	6.5 (± 1.7)
Cuernavaca	2003	6.9 (± 1.9)
Guadalajara	2003	5.5 (± 1.4)
Juarez	2003	10.8 (± 2.1)
Mexico City	2003	7.6 (± 1.5)
Nuevo Laredo	2003	5.0 (± 1.4)
Oaxaca	2003	7.6 (± 1.7)
Puebla	2003	8.6 (± 2.9)
Tapachula	2003	5.9 (± 1.5)
Tijuana	2003	7.4 (± 1.4)
Montserrat	2000	9.4
Nicaragua	2003	8.4 (± 1.4)
Panama	2002	9.8 (± 1.5)
Paraguay		
Alto Parana Ituapua	2003	12.4 (± 1.5)
Amambay Caaguazu	2003	13.2 (± 2.5)
Asuncion	2003	10.0 (± 2.0)
Central	2003	10.3 (± 1.4)
Peru	2003	7.2 (± 1.2)
St Kitts and Nevis	2002	13.7 (± 2.6)
St Lucia	2001	7.1 (± 2.1)
St Vincent and the Grenadines	2001	12.7 (± 2.8)
Suriname	2000	6.0 (± 1.7)
Trinidad and Tobago	2000	4.8 (± 1.0)
Uruguay		
Colonia	2001	6.5 (± 3.0)
Maldonado	2001	8.4 (± 2.3)
Montevideo	2001	10.2 (± 2.1)
Rivera	2001	7.3 (± 2.0)
Venezuela	1999	8.7 (± 1.5)
Virgin Islands (United Kingdom)	2001	8.2 (± 3.1)
Virgin Islands (USA)	2004	6.2 (± 1.4)

Updated from Global Youth Tobacco Survey Collaborative Group (2002). The values shown in this table may differ slightly from those available for individual countries. This results from the fact that data included in cross-country reports are limited to respondents 13–15 years of age. Materials that relate to a single country, such as the factsheets and single country reports available on the CDC website, include the full sample of students who completed the survey, and may include students aged 12 or 16 years.

[a] Current use is defined as used at least once in the 30 days preceding the survey.

(*a*) *Bangladesh*

Zarda, *khiwam* and *gul* are manufactured in Bangladesh and are also imported from India (Sinha, 2004).

In Bangladesh, 20–30% of women in rural areas are estimated to use smokeless tobacco, predominantly as part of a betel quid (Islam & Al-Khateeb, 1995). Among 638 respondents in a community-based intervention study on non-communicable diseases, 26% reported chewing tobacco products. Among users, 85% chewed daily and 15% occasionally (Table 34) (Rahman *et al*., 2001).

Table 34. Prevalence of chewing in Dhaka Metropolitan City, Bangladesh

Chewing frequency	Total	%
Total	638	100.0
No chewing	472	74.0
Chewing	166	26.0
Daily	141	85.0
Once a week at least	17	10.2
Less than once a week	8	4.8

From Rahman *et al.* (2001)

Among 11 409 respondents in a baseline community-based health behaviour surveillance study conducted in rural and urban areas, 169 (1.5%) reported current use of *gul* (urban 2%, rural 0.5%); application of *gul* was reported most frequently (5.2%) by urban women of lower socioeconomic classes. In addition, four people reported use of snuff (Rahman *et al.*, 2004).

A cross-sectional survey conducted among tobacco users in selected population groups in Bangladesh in 2003 showed use of treated tobacco leaf by 41.9%, raw dried tobacco leaf by 17.4% and powdered tobacco leaf by 3.9% (Table 35).

Among rickshaw pullers, 42.7% reported applying *gul* and 45% used betel quid with tobacco (Sinha, 2004).

(*b*) *Bhutan*

Tobacco consumption in Bhutan has changed from smoking to other forms such as oral use. Despite a total ban of sales of tobacco in Bhutan, packages of *zarda* used to be on sale in the Thimphu vegetable market. Many people, including young boys and monks, chew *zarda* and scented *khaini* (Sinha, 2004). A recent study showed that 8% of people in Bhutan chew or sniff tobacco (7% women, 10% men). Smoking prevalence is estimated to be 1% (Ugen, 2003).

Table 35. Type of tobacco product used among tobacco users in Bangladesh, 2003

Type of tobacco product	Individual[a]	Family[a]
Cigarette	382 (49.2%)	391 (39.5%)
Bidi	207 (26.4%)	225 (22.7%)
Hookah	6 (0.7%)	6 (0.6%)
Treated tobacco leaf [b]	326 (41.9%)	363 (36.7%)
Raw dried tobacco leaf [b]	135 (17.4%)	186 (18.8%)
Powdered tobacco leaf	30 (3.9%)	46 (4.6%)
Total	777 (100%)	990 (100%)

From WHO SEARO (2003)
[a] The modalities of tobacco use were documented by the subjects about themselves (individual) or by them about their family members (family).
[b] As constituents of betel quid

(*c*) *India*

India is one of the major producers of chewing tobacco in Asia. A specific feature of tobacco production in India is the variety in the types of tobacco produced. The presence of a strong domestic demand for tobacco product for chewing and application to a relatively large extent influences the cultivation of tobacco for these uses. Tobacco used for chewing and application is grown in Tamil Nadu, Uttar Pradesh, Bihar, West Bengal and Orissa (Reddy & Gupta, 2004).

In 2002, 40.6% of the tobacco production was used in cigarettes, 33.3% in *bidi* production and 12.4% was used for smokeless forms of chewing, snuffing and applied tobacco (Table 36; Reddy & Gupta, 2004). Between 1976 and 1994, chewing tobacco

Table 36. Tobacco production by type of tobacco in India, 2002

Type	Quantity (million kg)	%
Cigarettes	244	40.6
Bidi	200	33.3
Cigar	22	3.7
Hookah	60	10.0
Chewing tobacco	65	10.8
Snuff	10	1.6
Total	601	100

From Reddy & Gupta (2004)

production represented between 11% and 19% of total tobacco production, but production has increased substantially since 1995 (Table 37). In 2002, 65 million kg of chewing tobacco and 10 million kg of snuff tobacco were produced in India (Table 36). This increase was accompanied by a huge growth in the export of both chewing tobacco (9-fold increase between 1995 and 2005) and snuff tobacco (18-fold increase during the same period) (Table 38; Reddy & Gupta, 2004; Tobacco Board, 2006).

Table 37. Production of smokeless tobacco (in millions of kg) in India (derived estimates[a]) and percentage of total tobacco production

Years	Chewing		Snuff	
	Production	%	Production	%
1976–77	80.1	19.1	5	1.2
1977–78	70.8	14.3	6	1.6
1978–79	70	15.4	6	1.3
1979–80	72	16.4	7.6	1.7
1980–81	85.3	17.7	7.5	1.6
1981–82	77	14.8	7.6	1.5
1982–83	76.2	13.1	8.9	1.5
1983–84	78.7	16.0	9.2	1.9
1984–85	89	18.3	6.5	1.3
1985–86	75	17.0	7.9	1.8
1986–87	78	16.9	7.5	1.6
1990–91	78.8	14.2	11.8	2.1
1991–92	79.0	13.5	14.4	2.5
1992–93	71.2	11.9	13.3	2.2
1993–94	65.7	11.7	11.8	2.1
1994–95	138.3	24.4	11.7	2.1
1995–96	118.8	22.2	11.0	2.0
1996–97	156.6	26.1	10.0	1.7
1997–98[b]	108.5	18.9	11.0	1.9

[a] Calculated by the Working Group based on data from Directorate of Tobacco Development (1976–98)
[b] Provisional

Large variations in the prevalences and patterns of smokeless tobacco use occur across the country. Apart from regional preferences due to different socio-cultural norms, the preference for smokeless rather than smoked tobacco is inversely related to education and income (Gupta, 1996). Per-capita consumption of smokeless tobacco has increased among the lower socioeconomic classes between 1961 and 2000 in both rural and urban areas (data from the National Sample Survey Organization, cited in Gupta & Ray, 2003).

Table 38. Exports of tobacco from India by product (in tonnes)

Tobacco product	1995–96	1998–99	2001–2002	2004–2005
Cigarettes	884	1432	2883	7 190
Bidi	676	998	961	1062
Hookah/tobacco paste	9376	12 811	8910	10 600
Chewing tobacco/*zarda*	424	1191	2640	3778
Cut tobacco	512	2506	683	2034
Snuff	6	19	19	110
Total	11 883	18 957	16 076	24 774

From Tobacco Board (2006)

Six sets of data may allow an estimation of the prevalence of smokeless tobacco use in India: (*a*) large sub-national cross-sectional and cohort studies, (*b*) the National Family Health Survey, (*c*) the WHO sub-national study, (*d*) the National Sample survey on household consumer expenditure, (*e*) the Global Youth Tobacco Survey and (*f*) the Sample Registration system (unpublished). The last set of data is not discussed here.

(i) *Sub-national cross-sectional and cohort studies*

It has been estimated that approximately one-third of women and two-thirds of men in India use tobacco in one form or another (WHO, 1997). In prevalence surveys in 10 rural areas in eight states of India, smokeless tobacco was used by 3–53% of men and 3–49% of women (Table 39). In these areas, 2–26% of men and 0–4% of women also reported both smoking and smokeless tobacco use (Gupta & Ray, 2003). In a cross-sectional and cohort study in Mumbai, the prevalence of smokeless tobacco use in 1992–94 was 57.1% among women and 45.7% among men (Gupta, 1996). In another cross-sectional survey in a suburb of Trivandrum, Kerala, where residents were mostly of lower socioeconomic status, chewing practices were reported by 26.8% of men (n = 25 453) and 26.4% of women (n = 34 441), mainly in the form of betel quid with tobacco (Sankaranarayanan *et al.*, 2000).

(ii) *National Family Health Survey*

In the National Family Health Survey-2 conducted in 1998–99, 315 597 individuals aged 15 years or older from 91 196 households were sampled (Rani *et al.*, 2003). Among the study population, 20% (28.1% of men and 12.0% of women) reported chewing tobacco/*pan masala*; however, the prevalence may be underestimated by almost 11% for men and 1.5% for women because of the use of household informants. The prevalence of chewing tobacco/*pan masala* varied significantly (7–60%) between states (Table 40). Chewing of tobacco/*pan masala* was relatively more common (> 16%) in the central, eastern, western (except Goa) and northeastern states (except Tripura) compared with the northern and southern states. The prevalence of chewing tobacco/*pan masala* was significantly higher in rural, poorer and less educated populations compared with urban, wealthier

Table 39. Prevalence (%) of use of various types of tobacco in 10 areas in eight states of India

Area	Chewed[a] or applied	Smoked	Mixed	Total users	Reference
Men					
Bhavnagar, Gujarat	9	56	6	71	Mehta *et al.* (1969)
Darbhanga, Bihar	28	24	26	78	Mehta *et al.* (1969)
Ernakulam, Kerala	14	45	22	81	Mehta *et al.* (1969)
Goa	3	61	5	69	Bhonsle *et al.* (1976)
Mainpuri, Uttar Pradesh	21	41	20	82	Wahi (1968)
Mumbai (urban), Maharashtra	46	14	10	69	Gupta (1996)
Pune, Maharashtra	53	6	2	62	Mehta *et al.* (1972)
Singbhum, Jharkhand	17	50	14	81	Mehta *et al.* (1969)
Srikakulam, Andhra Pradesh	4	70	7	81	Mehta *et al.* (1969)
Trivandrum (urban), Kerala	27	56	NR	83	Sankaranarayanan *et al.* (2000)
Women					
Bhavnagar, Gujarat	15	–[b]	–	15	Mehta *et al.* (1969)
Darbhanga, Bihar	7	41	4	51	Mehta *et al.* (1969)
Ernakulam, Kerala	38	1	1	39	Mehta *et al.* (1969)
Goa	23	24	2	49	Bhonsle *et al.* (1976)
Mainpuri, Uttar Pradesh	9	11	1	21	Wahi (1968)
Mumbai (urban), Maharashtra	57	–	–	57.5	Gupta (1996)
Pune, Maharashtra	49	–	–	49	Mehta *et al.* (1972)
Singbhum, Jharkhand	26	5	2	33	Mehta *et al.* (1969)
Srikakulam, Andhra Pradesh	3	64	–	67	Mehta *et al.* (1969)
Trivandrum (urban), Kerala	26	2	NR	28	Sankaranarayanan *et al.* (2000)

Adapted from Gupta & Ray (2003)
NR, not reported
[a] Including betel quid with tobacco
[b] –, prevalence < 0.5%

and more educated populations in both men and women. The socioeconomic gradients (household wealth, education) had more impact for women than for men. The prevalence of chewing tobacco/*pan masala* was higher among tribal populations than among other communities (Table 41). In a multivariate analysis, the older population (≥ 25 years) had a greater likelihood of chewing tobacco compared with the younger population (15–24 years). Muslim women were more likely to chew tobacco than Hindu women, and the Sikh religion emerged as one of the strongest predictors among women for not chewing tobacco. The differentials by state of residence also persisted in the multivariate analysis. No significant association was observed between urban or rural residence and chewing of tobacco/*pan masala* among men after controlling for other characteristics. However, rural women were less likely to chew tobacco than urban women (Rani *et al*., 2003).

Table 40. Prevalence of chewing[a] in India by state and by sex (National Family Health Survey, 1998–99)

Region/State	Men	Women
	% (95% CI)	% (95% CI)
North		
Haryana	8.1 (6.7–9.8)	0.9 (0.6–1.3)
Himachal Pradesh	7.8 (6.7–9.1)	0.5 (0.3–0.8)
Jammu & Kashmir	7.3 (5.8–9.1)	0.9 (0.6–1.3)
New Delhi	13.1 (11.5–14.9)	2.5 (1.9–3.2)
Punjab	9.3 (8.0–10.8)	0.2 (0.1–0.4)
Rajasthan	19.0 (17.7–20.4)	3.8 (2.9–4.9)
Central		
Madhya Pradesh	40.3 (38.7–42.0)	14.4 (12.7–16.2)
Uttar Pradesh	36.3 (34.6–38.0)	10.9 (10.1–11.8)
East		
Bihar	51.8 (50.1–53.5)	6.7 (6.0–7.6)
Orissa	49.0 (46.7–51.4)	34.3 (31.9–36.9)
West Bengal	23.2 (20.9–25.6)	15.1 (13.5–17.0)
Northeast		
Arunachal Pradesh	51.6 (47.9–55.3)	33.1 (29.6–36.7)
Assam	47.8 (44.7–51.0)	24.3 (22.1–26.6)
Manipur	34.1 (31.1–37.3)	19.2 (15.5–23.5)
Meghalaya	16.9 (13.8–20.5)	27.6 (23.8–31.7)
Mizoram	60.2 (56.5–63.8)	60.7 (57.2–64.0)
Nagaland	45.0 (41.3–48.8)	16.5 (13.7–19.7)
Sikkim	39.5 (36.5–42.7)	18.6 (16.2–21.2)
Tripura	10.8 (8.9–13.1)	5.2 (3.3–8.1)
West		
Goa	7.7 (6.0–9.9)	8.0 (6.3–10.2)
Gujarat	24.6 (22.8–26.4)	8.0 (7.0–9.2)
Maharashtra	34.1 (32.3–36.0)	18.0 (16.1–20.0)
South		
Andhra Pradesh	10.7 (9.4–12.0)	9.9 (8.4–11.7)
Karnataka	13.8 (12.1–15.6)	14.1 (12.7–15.7)
Kerala	9.4 (8.3–10.7)	10.1 (9.1–11.2)
Tamil Nadu	12.9 (11.5–14.5)	10.7 (9.3–12.2)

From Rani *et al.* (2003)
CI, confidence interval
[a] Tobacco or *pan masala*

Table 41. Prevalence (%) of chewing of tobacco/*pan masala* in India (National Family Health Survey, 1998–99)

Variable	Prevalence in % (95% CI)	
	Men	Women
Age (years)		
15–24	4.4 (4.2–4.6)	14.3 (13.8–14.9)
25–39	17.2 (16.8–17.6)	31.6 (30.9–32.3)
40–59	25.7 (25.2–26.2)	35.3 (34.5–36.1)
≥ 60	22.4 (21.7–23.0)	37.4 (36.3–38.5)
Residence		
Urban	20.7 (19.7–21.7)	8.6 (7.9–9.3)
Rural	31.1 (30.4–31.8)	13.3 (12.8–13.8)
Economic status		
Richest 20%	16.4 (15.6–17.2)	4.8 (4.5–5.2)
Second richest	22.8 (22.0–23.7)	9.3 (8.7–9.8)
Middle	28.1 (27.3–28.9)	12.6 (12.0–13.2)
Second poorest	34.4 (33.4–35.4)	15.6 (14.4–15.9)
Poorest 20%	41.9 (40.7–43.2)	19.6 (18.7–20.6)
Years of schooling		
≥ 11	16.9 (16.2–17.7)	1.6 (1.3–1.9)
6–10	23.7 (23.1–24.3)	4.3 (4.0–4.6)
1–5	33.0 (32.1–33.9)	11.5 (10.8–12.1)
No education	38.6 (37.6–39.6)	17.2 (16.6–17.8)
Caste		
Forward caste	24.2 (23.4–25.1)	9.5 (8.9–10.1)
Scheduled caste	30.4 (29.1–31.8)	14.6 (13.7–15.5)
Scheduled tribe	41.1 (39.1–43.1)	20.8 (19.2–22.4)
Other backward castes	28.3 (27.2 –29.4)	10.8 (10.1–11.4)
Religion		
Hindu	29.1 (28.6–29.7)	12.0 (11.5–12.5)
Muslim	25.5 (24.0–27.1)	13.0 (12.1–14.0)
Christian	9.3 (8.0–10.8)	0.1 (0.04–0.3)
Sikh	19.1 (17.0–21.4)	11.5 (10.1–13.2)
Other	31.5 (27.6–35.7)	18.4 (15.3–21.9)

From Rani *et al.* (2003)
CI, confidence interval

(iii) *WHO Sub-national Study*

In a WHO study (Chaudhry, 2001), 35 288 respondents in Karnataka and 29 931 respondents in Uttar Pradesh (aged ≥ 10 years) were surveyed. Tobacco was predominantly used in smokeless form among women of all ages and among men under 30 years of age, both in urban and rural areas. The overall prevalence of current use of smokeless tobacco was 13.9% in Karnataka (13.4% among men, 14.4% among women) (Table 42) and 17.5% in Uttar Pradesh (24.3% among men, 6.6% among women) (Table 43). In Karnataka, the

Table 42. Prevalence by rural/urban area, age and sex of current use of smokeless tobacco in Karnataka, India (WHO Sub-national Study, 2001)

Age group (years)	Urban						Rural					
	Men		Women		Total		Men		Women		Total	
	Sample (no.)	Prevalence (%)	Sample (no.)	Prevalence (%)	Sample (no.)	Prevalence (%)	Sample (no.)	Prevalence (%)	Sample (no.)	Prevalence (%)	Sample (no.)	Prevalence (%)
10–14	281	1.8	224	0.4	505	1.2	925	1.0	818	0.0	1743	0.5
15–19	419	10.3	397	1.0	816	5.8	1700	8.6	1488	0.4	3188	4.8
20–24	432	15.7	403	1.7	835	9.0	1653	16.3	1627	2.8	3280	9.6
25–29	366	18.9	414	2.4	780	10.1	1652	16.9	1634	5.8	3286	11.4
30–34	278	18.0	333	4.8	611	10.8	1394	12.3	1296	11.7	2690	12.0
35–39	313	11.8	381	7.9	694	9.7	1396	13.8	1470	14.1	2866	14.0
40–44	325	16.9	285	12.6	610	14.9	1262	12.3	1208	23.9	2470	18.0
45–49	305	13.4	216	17.1	521	15.0	1187	14.2	964	26.7	2151	19.8
50–54	233	8.2	163	19.0	396	12.6	985	16.1	927	32.8	1912	24.2
55–59	113	8.0	79	12.7	192	9.9	582	17.7	452	39.8	1034	27.4
60–64	108	9.3	146	22.6	254	16.9	792	13.9	660	39.8	1452	25.7
65–69	57	10.5	45	37.8	102	22.5	341	19.6	266	40.6	607	28.8
≥ 70	98	8.2	64	29.7	162	16.7	576	21.7	478	43.5	1054	31.6
All ages	3328	12.6	3150	8.0	6478	10.4	14 445	13.5	13 288	15.9	27 733	14.7

From Chaudhry (2001)

Table 43. Prevalence by rural/urban area, age and sex of current use of smokeless tobacco in Uttar Pradesh, India (WHO Sub-national Study, 2001)

Age group (years)	Urban						Rural					
	Men		Women		Total		Men		Women		Total	
	Sample (no.)	Prevalence (%)	Sample (no.)	Prevalence (%)	Sample (no.)	Prevalence (%)	Sample (no.)	Prevalence (%)	Sample (no.)	Prevalence (%)	Sample (no.)	Prevalence (%)
10–14	279	3.2	118	0.8	397	2.5	1641	2.3	681	0.3	2322	1.7
15–19	381	13.9	181	0.6	562	9.6	1937	17.9	792	1.0	2729	13.0
20–24	411	23.6	247	2.4	658	15.7	1905	27.6	1332	2.3	3237	17.1
25–29	351	30.5	256	3.5	607	19.1	1812	30.0	1299	3.5	3111	18.9
30–34	282	25.2	220	6.8	502	17.1	1473	30.8	1203	4.2	2676	8.9
35–39	265	26.8	233	9.9	498	18.9	1406	27.5	1006	6.4	2412	18.7
40–44	269	28.3	187	7.5	456	19.7	1235	27.0	812	9.0	2047	19.8
45–49	231	21.6	141	11.3	372	17.7	1014	27.1	700	12.4	1714	21.1
50–54	173	25.4	89	11.2	262	20.6	885	28.0	466	13.5	1351	23.0
55–59	104	23.1	73	13.7	177	19.2	541	26.6	395	14.7	936	21.6
60–64	109	22.9	57	26.3	166	24.1	647	31.4	328	18.6	975	27.1
65–69	70	27.1	38	23.7	108	25.9	348	30.2	245	12.7	593	22.9
≥ 70	89	36.0	31	19.4	120	31.7	652	32.5	230	16.1	882	28.2
All ages	3014	22.5	1871	7.2	4885	16.6	15 496	24.6	9489	6.4	24 985	17.7

From Chaudhry (2001)

prevalence of use of smokeless tobacco was higher among women compared with men in the age groups above 40 years. In Uttar Pradesh, the proportion of men who used smokeless tobacco was higher than that of women in all age groups. In both regions, prevalence of smokeless tobacco use by women increased with age; for men, prevalence was highest in the age groups 25–29 years and above 70 years. Trends were similar in urban and rural areas. The prevalence of smokeless tobacco use was generally lower among educated women, especially in Karnataka. Clear-cut trends in reduced prevalence with increasing education were not observed in all age groups among men. Muslim men in Karnataka showed a higher overall prevalence compared with Hindus, while in Uttar Pradesh, a higher proportion of Hindu men compared with Muslims used smokeless tobacco. The reverse trend was observed among women in the two states [data for other religions were based on too few numbers to be reliable]. Variations in prevalence according to family income did not follow any specific trend, but the prevalence was comparatively lower in both states among women with higher family income (Chaudhry, 2001).

(iv) *National Sample Survey Organisation*

The National Sample Survey Organisation conducted its fifth quinquennial nationwide survey of household consumer expenditure in India during 1993–94. Interviews were conducted in 115 354 households in 6951 villages and in 4650 urban blocks. Prevalence of use of chewing tobacco was 11.2% and 6.3% among men in rural and urban areas, respectively, and 3.9% and 2.0%, respectively, for women (Table 44) (National Sample Survey Organisation, 1998). The prevalence of tobacco use was underestimated because only one household respondant answered for all inhabitants of the household.

Table 44. Prevalence (%) by rural/urban area and sex of use of tobacco in various forms in India (National Sample Survey Organisation)

Form of tobacco consumption	Use	1987–88				1993–94			
		Rural		Urban		Rural		Urban	
		Men	Women	Men	Women	Men	Women	Men	Women
Chewing tobacco[a]	Regular	11.7	5.0	6.3	2.9	11.2	3.9	6.3	2.0
	Casual	1.3	0.6	1.0	0.4	1.4	0.5	0.9	0.3
Snuff	Regular	0.7	0.8	0.4	0.5	0.5	0.6	0.3	0.3
	Casual	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0
Burnt tobacco powder/paste	Regular	2.7	3.1	1.4	1.6	2.7	2.5	1.2	1.0
	Casual	0.3	0.2	0.2	0.1	0.3	0.1	0.1	0.1
Tobacco in any form	Regular	38.7	10.3	23.9	5.3	31.9	8.1	21.6	3.7
	Casual	1.6	0.8	1.8	0.6	1.7	0.7	1.6	0.3

From National Sample Survey Organisation (1998)
[a] Including betel quid with tobacco

Comparison of the data from 1987–88 and 1993–94 (National Sample Survey Organisation, 1998; Gupta & Sankar, 2004) revealed no significant change in overall use of smokeless tobacco during this period (Table 44). Other reports suggest that there has been a shift towards use by younger people and at a very early age. For example, the prevalence of *mawa* use rose from 4.7% in 1969, mainly among older women, to 19% in 1993–94, mainly among younger generations (Gupta, 2000); in a survey conducted among rural children in three states, snuff was ever used by 38% of boys and 12% of girls aged 10–20 years (Krishnamurthy *et al.*, 1997); a comparative study of the prevalence of tobacco use in a rural area in Bihar showed that the prevalence of total tobacco use remained the same between 1967 and 2000, but that there had been a remarkable shift towards the use of smokeless tobacco (Sinha *et al.*, 2003a).

(v) *Global Youth Tobacco Survey (GYTS) and Global School Personnel Survey (GSPS)*

The Global School Personnel Survey (developed by WHO/CDC) is a cross-sectional survey that employs a cluster sample design to produce a representative sample of school personnel drawn from the same schools that were selected for GYTS. All school personnel (including non-teaching staff) in the selected schools were eligible to participate (Sinha *et al*., 2002).

In the eight northeastern states of India, daily use of smokeless tobacco among school personnel varied from 8.9 (Sikkim) to 49.4% (Mizoram) among men and from 1.6 (Manipur) to 80.3% (Mizoram) among women (Table 45) (Sinha *et al*., 2003b). In five of the eight states, the prevalence of daily use of smokeless tobacco among men and women was similar. In the eastern region, daily use of smokeless tobacco among school personnel varied from 7.8 (West Bengal) to 58.7% (Bihar) in men and from 1.0 (West Bengal) to 53.4% (Bihar) in women (Sinha *et al*., 2002, 2003b; Sinha & Gupta, 2004a; Sinha & Roychoudhury, 2004). The prevalence of use of each type of products is detailed in Table 46.

Smokeless tobacco use among students varied between states from 2.8 (Goa) to 55.6% (Bihar) (Table 47). Among boys, it varied from 2.7 (Delhi) to 57.6% (Bihar) and, among girls, from 2.1 (Goa) to 49.2% (Bihar). In 11 of 13 states, prevalences of smokeless tobacco use among boys and girls were similar; boys in Meghalaya and Uttaranchal reported significantly more smokeless tobacco use than girls (Arora *et al*., 2001; Sinha & Gupta, 2002a,b; Sinha *et al*., 2003c; Pednekar & Gupta, 2004; Sinha & Gupta, 2004b; Sinha *et al*., 2004a).

The use of tobacco products as dentrifice among students aged 13–15 years varied widely between states (Table 48). The prevalence among boys compared with that among girls was notably higher in Orissa and Uttaranchal, marginally higher in nine states and marginally lower in three states. Of the specific products, tobacco toothpaste (creamy snuff) and tooth powder (*lal dant manjan*) were common in all 14 states; the prevalence of use ranged from 2 to 32% and from 2 to 29%, respectively. *Gul* was used in eight states and the prevalence of its use ranged from 2 to 6%. Other dentifrice products containing tobacco were: *mishri* and *bajjar* in Goa and Maharashtra; *gudhaku* in Bihar, Orissa, Uttar

Table 45. Prevalence (%) by state of current use of smokeless tobacco[a] among school personnel in the northeastern and eastern regions of India (Global School Personnel Survey, 2001)

Region	Sample size	Prevalence (± 95% CI)					
		Men		Women		Total	
		Daily	Occasional	Daily	Occasional	Daily	Occasional
Northeastern region							
Arunachal Pradesh	533	28.9 (± 8.8)	19.0 (± 11.9)	25.1 (± 20.0)	23.9 (± 13.0)	28.2 (± 5.3)	19.9 (± 8.7)
Assam	782	10.1 (± 3.2)	34.3 (± 6.6)	13.5 (± 9.4)	37.0 (± 14.2)	10.7 (± 3.7)	34.7 (± 5.9)
Manipur	395	21.8 (± 9.5)	53.2 (± 14.1)	1.6 (± 2.7)	74.2 (± 9.9)	14.2 (± 5.9)	61.1 (± 9.2)
Meghalaya	447	30.8 (± 10.5)	20.5 (± 6.4)	17.4 (± 14.0)	39.2 (± 13.4)	24.9 (± 8.7)	28.8 (± 4.7)
Mizoram	307	49.4 (± 10.5)	29.8 (± 12.0)	80.3 (± 13.8)	6.9 (± 6.2)	57.8 (± 8.8)	23.7 (± 8.0)
Nagaland	426	18.5 (± 6.5)	31.3 (± 10.2)	18.1 (± 20.2)	14.4 (± 6.6)	18.3 (± 10.2)	25.4 (± 8.5)
Sikkim	342	8.9 (± 4.3)	45.3 (± 14.3)	51.9 (± 12.0)	21.7 (± 11.3)	17.6 (± 6.7)	40.6 (± 10.6)
Tripura	562	38.3 (± 11.5)	17.2 (± 6.8)	6.8 (± 2.0)	17.7 (± 10.4)	31.2 (± 9.2)	17.5 (± 5.7)
Eastern region							
Bihar	637	58.7 (± 6.3)		53.4 (± 16.1)		57.3 (± 7.5)	
Orissa	517	28.1 (± 13.3)	16.9 (± 9.0)	5.0 (± 5.2)	3.4 (± 3.6)	24.2 (± 11.3)	14.6 (± 8.1)
West Bengal	663	7.8		1.0		5.8	

From Sinha *et al.* (2002; 2003b); Sinha & Gupta (2004a); Sinha & Roychoudhury (2004)
CI, confidence interval
[a] Including betel quid with tobacco

Table 46. Prevalence (%) by type of tobacco product of smokeless tobacco use by school personnel in the north-eastern states of India (Global School Personnel Survey, 2001)

	Prevalence (± 95% CI)							
	Arunachal Pradesh	Assam	Manipur	Meghalaya	Mizoram	Nagaland	Sikkim	Tripur
Betel quid	70.8 (± 9.6)	69.5 (± 6.4)	54.7 (± 9.6)	55.4 (± 4.4)	20.2 (± 3.7)	69.3 (± 5.5)	15.7 (± 7.6)	54.9 (± 8.5)
Gutka	4.1 (± 1.7)	8.6 (± 2.9)	17.9 (± 12.0)	5.1 (± 1.7)	24.8 (± 3.2)	8.3 (± 3.5)	34.4 (± 4.5)	21.0 (± 6.8)
Smokeless tobacco without areca nut	24.3	15.6	27.3	39.4	54.3	20.3	49.9	22.5
Khaini	17.3 (± 8.8)	7.0 (± 1.8)	14.7 (± 2.6)	9.1 (± 2.4)	22.3 (± 2.2)	15.9 (± 5.2)	18.9 (± 5.9)	10.7 (± 2.8)
Gul	0.4 (± 0.4)	2.2 (± 2.0)	3.3 (± 2.9)	12.0 (± 6.5)	16.4 (± 3.2)	2.6 (± 1.5)	15.0 (± 3.8)	1.1 (± 1.0)
Snuff	1.9 (± 1.8)	–	–	–	9.3 (± 1.7)	–	3.0 (± 2.2)	1.2 (± 1.0)
Tuibur	4.1 (± 1.5)	6.1 (± 2.3)	8.4 (± 2.9)	13.1 (± 4.4)	5.6 (± 1.9)	0.6 (± 0.5)	12.4 (± 1.8)	1.1 (± 0.6)
Others	0.6 (± 0.6)	0.3 (± 0.3)	0.9 (± 0.7)	5.2 (± 1.9)	0.7 (± 0.7)	1.2 (± 0.9)	0.6 (± 0.5)	8.4 (± 4.0)
Multiple use	0.8 (± 0.8)	6.3 (± 6.0)	–	–	0.6 (± 0.6)	2.0 (± 2.0)	–	1.7 (± 0.9)
Total (no.)	253	327	243	219	227	180	222	211

From Sinha *et al.* (2003b)
CI, confidence interval

Table 47. Prevalence (%) by state of current use of smokeless tobacco among students in India (Global Youth Tobacco Survey)

Category	Sample size	Prevalence (± 95% CI)		
		Boys	Girls	Total
Arunachal Pradesh	2314	35.0 (± 10.4)	40.2 (± 8.0)	37.2 (± 5.9)
Assam	2177	29.3 (± 5.7)	20.4 (± 5.5)	25.3 (± 5.2)
Bihar	2636	57.6 (± 8.6)	49.2 (± 11.5)	55.6 (± 7.5)
Delhi	1731	2.7 (± 1.2)	2.5 (± 1.7)	2.8 (± 1.2)
Goa	2256	3.3 (± 1.6)	2.1 (± 1.4)	2.8 (± 1.2)
Manipur	1743	51.5 (± 11.4)	40.1 (± 14.5)	46.1 (± 10.2)
Meghalaya	2080	43.0 (± 7.0)	26.8 (± 7.2)	35.3 (± 7.4)
Mizoram	2295	45.7 (± 5.1)	40.1 (± 6.0)	42.9 (± 4.4)
Nagaland	2221	52.5 (± 7.5)	47.2 (± 6.3)	49.9 (± 4.9)
Sikkim	2236	42.5 (± 7.0)	31.8 (± 4.6)	37.7 (± 3.7)
Tripura	1866	39.7 (± 10.4)	29.4 (± 11.2)	35.1 (± 8.7)
Uttaranchal	2641	20.8 (± 11.4)	11.5 (± 6.7)	17.6 (± 9.2)
Uttar Pradesh	4542	21.6 (± 7.1)	14.5 (± 8.3)	19.7 (± 6.3)

From Arora *et al.* (2001); Pednekar & Gupta (2004); Sinha *et al.* (2003c); Sinha & Gupta (2002a,b); Sinha & Gupta (2004b); Sinha *et al.* (2004a)
CI, confidence interval

Table 48. Prevalence (%) of application of tobacco products as dentifrice in 14 states in India (Global Youth Tobacco Survey, 2000–2002)

State	Prevalence (95% CI)			
	Toothpaste	*Gul*	Tooth powder	Others
Arunachal Pradesh	23 (18–27)	2 (1–3)	4 (2–5)	–
Assam	11 (9–14)	3 (1–5)	4 (3–6)	–
Bihar	10 (7–12)	6 (4–7)	49 (43–54)	4 (3–6)
Goa	2 (1–2)	–	2 (1–2)	3 (2–4)
Maharashtra	2 (1–3)	–	2 (1–3)	9 (7–12)
Manipur	25 (22–28)	–	2 (0–3)	5 (1–9)
Meghalaya	18 (12–25)	1 (0–1)	4 (2–5)	–
Mizoram	12 (9–15)	–	9 (6–12)	4 (2–7)
Nagaland	32 (23–40)	3 (2–4)	5 (4–7)	–
Orissa	10 (8–12)	1 (1–2)	25 (23–28)	4 (2–6)
Sikkim	8 (5–11)	–	2 (1–3)	1 (1–1)
Tripura	25 (19–31)	–	3 (1–4)	1 (1–2)
Uttar Pradesh	10 (8–12)	2 (1–3)	29 (24–33)	16 (9–22)
Uttaranchal	18 (14–21)	2 (1–3)	29 (26–32)	11 (5–16)

From Sinha *et al.* (2004b)
CI, confidence interval

Pradesh and Uttaranchal; and tobacco water (*tuibur*) in Manipur, Mizoram, Sikkim and Tripura (Sinha *et al.*, 2004b).

The current use of smokeless tobacco among the participants of the GSPS (Sinha *et al.*, 2003b) and GYTS (Sinha *et al.*, 2003c) surveys in eight of the states is detailed below.

Arunachal Pradesh

In Arunachal Pradesh, betel quid was the most popular form of smokeless tobacco among men (73.6%) and women (51.4%). *Khaini* was used exclusively by men (19.8%), while *tuibur* (32.6%) and snuff (15.2%) were used exclusively by women (Sinha *et al.*, 2003b).

Current use of smokeless tobacco was reported by 37.2% of students (35.0% of boys, 40.2% of girls), whereas smoking was reported by 22.8% (31.8% of boys, 8.3% of girls). Smokeless tobacco use exclusively in the form of chewing was reported by 55.2% and use exclusively in the form of application was reported by 28.8%. The remainder used several forms of smokeless tobacco. Among chewers, *gutka* was the most popular product (49.8%), followed by *tamol* and a tobacco mixture (31%). Among appliers, 79.7% applied tobacco toothpaste, 12.3% applied red tooth powder and 8% applied *gul* (Sinha *et al.*, 2003c).

Assam

In Assam, the most popular form of smokeless tobacco use among men was betel quid (75.5%), followed by *khaini* (7.9%) and *gutka* (7.8%). Among women, betel quid (36.3%) was commonest, followed by *tuibur* (35.7%), *gul* (13.5%) and *gutka* (13.4%). *Gul* and *tuibur* were used primarily by women (Sinha *et al.*, 2003b).

Current use of smokeless tobacco was reported by 25.3% of students (29.3% of boys, 20.4% of girls). Current smoking was reported by 19.7% of students (28.6% of boys, 8.9% of girls). Smokeless tobacco use exclusively in the form of chewing was reported by 48.5% and that exclusively in the form of application by 18.8%. The remainder used several forms of smokeless tobacco. Among chewers, *gutka* was the most popular product (54.4%), followed by *tamol* and a tobacco mixture (28.9%). Among appliers, 58.5% applied tobacco toothpaste, 25% applied red tooth powder and 16.3% applied *gul* (Sinha *et al.*, 2003c).

Manipur

In Manipur, betel quid (54.7%) was the most popular form of smokeless tobacco among both men and women. The prevalence of *gutka* use among men was higher (24.1%) than that among women (2.6%), while the prevalence of *khaini* use among women was higher (29.1%) than that in men (8.9%). *Tuibur* was used predominantly by women (27.5%) (Sinha *et al.*, 2003b).

Current smokeless tobacco use was reported by 46.1% (51.5% of boys, 40.1% of girls), whereas smoking was reported by 26.8% (40.8% of boys, 10.7% of girls). Smokeless tobacco use exclusively in the form of chewing was reported by 53.2% and that exclu-

sively in the form of application by 31.9%. The remainder used several forms of smokeless tobacco. Among chewers, *gutka* (23.7%) was the most popular product (17.9% of boys, 30.2% of girls), followed by *tamol* and a tobacco mixture (18.1% overall, 28.0% of boys, 6.8% of girls). Among appliers, 18.3% of boys and 32.6% of girls applied tobacco toothpaste (Sinha *et al.*, 2003c).

Meghalaya

In Meghalaya, betel quid (55.4%) was the most popular form of smokeless tobacco, followed by *tuibur* (13.1%), *gul* (12.0%) and *khaini* (9.1%). *Gul* and *tuibur* were used primarily by women (Sinha *et al.*, 2003b).

Current smokeless tobacco use was reported by 35.3% (43.0% of boys, 26.8% of girls), whereas smoking was reported by 21.4% (32.1% of boys, 9.9% of girls). Smokeless tobacco use exclusively in the form of chewing was reported by 55.2% (62.1% of boys, 47.7% of girls) and that exclusively in the form of application by 22.9% (28% of boys, 17.6% of girls). The remainder used several forms of smokeless tobacco. Chewing was mainly in the form of *gutka* (19.4%), *tamol* with tobacco (9.2%, > 80% of boys) and *tamol* without tobacco (21%). Tobacco was applied by 18.2% as tobacco toothpaste and by 3.9% as red tooth powder (Sinha *et al.*, 2003c).

Mizoram

In Mizoram, among smokeless tobacco users, 24.8% used *gutka*, 22.3% used *khaini*, 20.2% used betel quid, 16.4% used *gul* and 9.3% used snuff. The use of *gutka* and snuff was reported slightly more frequently among women while that of betel quid and *gul* was more frequent among men (Sinha *et al.*, 2003b).

Current smokeless tobacco use was reported by 42.9% (45.7% of boys, 40.1% of girls), whereas current smoking was reported by 34.5% (40.7% of boys, 28.2% of girls). Smokeless tobacco use exclusively in the form of chewing was reported by 60.7% and that exclusively in the form of application by 25.0%. The remainder used several forms of smokeless tobacco. Among chewers, use of *gutka* (20%) was reported to be the most popular, followed by *pan* with tobacco (12.9%). Among appliers, the majority preferred tobacco toothpaste (11.8%) (Sinha *et al.*, 2003c).

Nagaland

In Nagaland, betel quid (69.3%), *khaini* (15.9%) and *gutka* (8.3%) were the most prevalent forms of smokeless tobacco used. Betel quid was more common among women, whereas *khaini* was used almost exclusively by men (Sinha *et al.*, 2003b).

Current smokeless tobacco use was reported by 49.9% (52.5% of boys, 47.2% of girls), whereas smoking was reported by 29.6% (38.7% of boys, 19.7% of girls). Smokeless tobacco use exclusively in the form of chewing was reported by 62.4% and that exclusively in the form of application by 40%. The remainder used several forms of smokeless tobacco. Among chewers, 28.1% reported chewing *gutka* and 8% reported use

of *pan* with tobacco. Among appliers, the predominant form was tobacco toothpaste (31.7%) (Sinha *et al.*, 2003c).

Sikkim

In Sikkim, *gutka* was the preferred (34.4%) form of smokeless tobacco, followed by *khaini* (18.9%), betel quid (15.7%), *gul* (15.0%) and *tuibur* (12.4%). *Gutka*, *khaini* and *tuibur* were used mainly by men while betel quid and *gul* were used primarily by women (Sinha *et al.*, 2003b).

Current smokeless tobacco use was reported by 37.7% (42.5% of boys, 31.8% of girls), whereas smoking was reported by 23.6% (32.9% of boys, 12.1% of girls). Smokeless tobacco use exclusively in the form of chewing was reported by 48.3% and that exclusively in the form of application by 11.3%. Among chewers, *tamol* and tobacco mixture were reported to be the most popular (52.3%), followed by *gutka* (33.5%). Among appliers, 69.2% applied tobacco toothpaste, 21.4% applied red tooth powder and 9.4% applied *tuibur* (Sinha *et al.*, 2003c).

Tripura

In Tripura, betel quid was the most popular (54.9%) form of smokeless tobacco, followed by *gutka* (21.0%) and *khaini* (10.7%). Betel quid was more popular among men while *khaini* was more popular among women (Sinha *et al.*, 2003b).

Current smokeless tobacco use was reported by 35.1% (39.7% of boys, 29.4% of girls), whereas smoking was reported by 21.2% (28.6% of boys, 12.4% of girls). Smokeless tobacco use exclusively in the form of chewing was reported by 57.5% and that exclusively in the form of application by 28.8%. Among chewers, *gutka* was the most popular (21.3%), followed by *tamol* with tobacco (10.5%; 17.0% of boys, 2.6% of girls) and *tamol* without tobacco (23.0%; 23.7% of boys, 22.1% of girls). Thus, boys equally used *tamol* with tobacco or without tobacco, whereas girls preferred *tamol* without tobacco. Among appliers, the majority preferred tobacco toothpaste (25%) (Sinha *et al.*, 2003c).

(vi) *Type of tobacco used by sex and region*

Bhonsle *et al.* (1992) reviewed available data from the 1970s on the prevalence of smokeless tobacco use by type of tobacco. *Khaini* use among men ranged from < 0.5% (Andhra Pradesh) to 44% (Bihar); that among women ranged from < 0.5 (Gujarat, Kerala) to 10% (Jharkhand). Chewing tobacco leaf varied among men from < 0.5 (Bihar, Goa, Gujarat, Jharkhand) to 9% (Andhra Pradesh) and among women from < 0.5 (Gujarat, Jharkhand) to 2% (Andhra Pradesh, Kerala). Applied tobacco (*bajjar* and *gudhaku*) was used by 1% of men and by 14–16% of women in Gujarat and Jharkhand (Tables 49 and 50; Bhonsle *et al.*, 1992).

Among 6271 school children in Goa (western India), 731 were tobacco users. Of these, 56% of boys and 66% of girls used *mishri* and almost half in both groups used creamy snuff (Table 51) (Vaidya *et al.*, 1992). Among 9097 adults (≥ 15 years) in a rural site in Bihar (eastern India), one third (32.7%) used smokeless tobacco, of whom 11.4%

Table 49. Prevalence of use of smokeless tobacco and other chewing products among men in selected states in India

	Gujarat		Kerala		Andhra Pradesh		Jharkhand		Bihar		Goa	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Pan with tobacco	101	2	1640	33	245	4	84	2	301	6	144	6
Pan without tobacco	242	5	15	< 0.5	134	3	–	–	–	–	48	2
Khaini	300	6	–	–	2	< 0.5	1308	27	2149	44	–	–
Tobacco leaf	30	< 0.5	104	2	484	9	9	< 0.5	6	< 0.5	12	< 0.5
Bajjar	52	1	–	–	–	–	–	–	–	–	–	–
Gudhaku	–	–	–	–	–	–	54	1	–	–	–	–
Areca nut	68	1	–	–	–	–	3	< 0.5	184	4	–	–
Multiple products	7	< 0.5	–	–	3	< 0.5	35	< 0.5	24	< 0.5	–	–
No chewing practice	4427	85	3152	64	4481	84	3307	69	2192	45	2311	92
Total	5227		4911		5349		4800		4856		2515	

From Bhonsle *et al.* (1992)

Table 50. Prevalence of use of smokeless tobacco and other chewing products among women in selected states in India

	Gujarat		Kerala		Andhra Pradesh		Jharkand		Bihar		Goa	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Pan with tobacco	1	< 0.5	1881	35	135	3	71	1	96	2	780	27
Pan without tobacco	6	< 0.5	41	< 0.5	25	< 0.5	–	–	–		131	4
Khaini	5	< 0.5	3	< 0.5	–	–	512	10	371	7	–	–
Tobacco leaf	5	< 0.5	118	2	116	2	7	< 0.5	–		18	1
Bajjar	666	14	–	–	–	–	–	–	–		–	–
Gudhaku	–	–	–	–	–	–	833	16	–		–	–
Areca nut	12	< 0.5	2	< 0.5	–	–	1	< 0.5	68	1	–	–
Multiple products	–	–	–	–	2	< 0.5	23	< 0.5	–		–	–
No chewing practice	4149	86	3331	62	4542	94	3801	72	4946	90	2005	68
Total	4844		5376		4820		5248		5481		2934	

From Bhonsle *et al.* (1992)

used *khaini* and 18.9% used tooth powder that contained tobacco (Table 52) (Sinha *et al.*, 2003a).

Table 51. Prevalence of use of different types of tobacco among schoolchildren in Goa, India

Tobacco product	Boys		Girls	
	No.	%	No.	%
Smoking	13	3	5	2
Mishri	256	56	177	66
Creamy snuff	212	46	128	47
Chewing	66	14	36	13
Single	388	84	219	81
Multiple	73	16	51	19
Total	461	100	270	100

From Vaidya *et al.* (1992)

Table 52. Prevalence of use (%) of different types of tobacco among adults (≥ 15 years) in Bihar, India

Tobacco type	Women	Men	Total
Non-user	55.0	25.9	39.6
Smoked tobacco	23.4	31.6	27.7
Bidi	84.1	82.0	82.9
Others	15.9	18.0	17.1
Smokeless tobacco	21.7	42.6	32.7
Tobacco tooth powder	41.3	8.8	18.9
Khaini	20.0	7.5	11.4
Pan masala	12.1	57.1	43.1
Others	26.6	26.6	26.6
Total (no.)	2586	2910	5496

From Sinha *et al.* (2003a)

A population-based cross-sectional survey was conducted in the city of Mumbai among 99 598 individuals aged 35 years and older during 1992–94 (Gupta, 1996). A high percentage of women used tobacco (57.5%), almost solely in the smokeless form. About one fifth (20%) of the population (26.5% of women; 10.3% of men) used *mishri* alone and 3.7% (1.1% of women; 7.5% of men) used tobacco leaf and lime (Table 53).

Table 53. Prevalence of use of smokeless tobacco and other chewing products in Mumbai, India

	Women		Men		Total	
	No.	%	No.	%	No.	%
Multiple tobacco practices	2013	3.3	2993	7.4	5006	5.0
Mishri	15 740	26.5	140	10.3	19 880	20.0
Mishri + betel quid with tobacco	10 687	18.0	4976	12.4	15 663	15.7
Betel quid with tobacco	3527	5.9	5871	14.7	9398	9.4
Khaini	640	1.1	2997	7.5	3637	3.7
Others with tobacco	1200	2.0	1144	2.9	2344	2.4
Areca nut without tobacco[a]	306	0.5	176	0.4	482	0.5
No chewing practice	25 414	42.7	17 774	44.4	43 188	43.4
Total	59 527	100	40 071	100	99 598	100

From Gupta (1996)
[a] Most frequently as betel quid without tobacco

Among 539 patients who entered hospital in Kerala and were recruited as controls for a case–control study, seven reported use of nasal snuff (Sankaranarayanan *et al.*, 1989a).

(*d*) *Indonesia*

In Indonesia, smokeless tobacco is used mainly as part of a betel quid and mostly in rural areas. Betel quid with tobacco and chewing tobacco were identified as smokeless tobacco products used by a small number of respondents both in Jakarta and Sukabumi. Of 5899 tobacco users, less than 0.5% (22 persons) had used chewing tobacco (Sinha, 2004).

(*e*) *Malaysia*

A cross-sectional survey was conducted to document the use of smokeless tobacco among Kadazan women in a rural area in the state of Sabah, East Malaysia (Gan, 1995). Of the 472 women interviewed, 328 chewed; 60% of all women included tobacco as an ingredient in their chew, while 10% did not. Tobacco with lime was used by 2.3% of women and tobacco only by 1.1%. Women with a low education were more likely to be chewers. The chewing practice was usually acquired during the teenage years and was perceived mainly as a cultural norm. The majority of tobacco chewers (46.3%) used three or four fresh preparations per day. Tobacco use increased with increase in age (Table 54).

In a similar survey among the indigenous people of Sabah State, 845 Bajaus (414 men, 431 women) were interviewed (Gan, 1998). Of these, 74.4% of men smoked compared with 3.3% of women and 77% of women used smokeless tobacco compared with 4.3% of men. Tobacco was commonly used in the form of a betel quid. Among chewers,

Table 54. Prevalence of tobacco chewing by Kadazan and Bajaus women in Sabah, Malaysia

Ingredients used in chew	Kadazan (*n* = 472)		Bajaus (*n* = 431)	
	No. with chewing habit	%	No. with chewing habit	%
Smokeless tobacco	281	59.5	332	77
Tobacco, betel leaf, areca nut, lime, *gambir*	148	31.3	183	42.4
Tobacco, betel leaf, areca nut, lime	108	22.9	137	31.8
Tobacco, betel leaf, areca nut	1	0.2	1	0.2
Tobacco and lime	11	2.3	3	0.7
Tobacco and areca nut	8	1.7	2	0.5
Tobacco only	5	1.1	6	1.4
Various combinations of above ingredients without tobacco	47	10.0	14	3.2

From Gan (1995, 1998)

half (51.2%) used fewer than five quids per day. Only nine women used tobacco without areca nut (tobacco only, 1.4%; tobacco with lime, 0.7%). The prevalence of smokeless tobacco use was significantly lower among the better educated and increased with increasing age (Table 54).

(*f*) *Myanmar*

Zarda is manufactured in Myanmar and is also imported from India (Sinha, 2004).

The WHO Sentinel Prevalence Survey of Tobacco Use in Myanmar (WHO SEARO, 2001) covered a sample of 6600 individuals (2903 men, 3697 women) in the Hinthada district from the Delta region and the Pakkuku township from the Dry zone region. Among current tobacco users, two-thirds reported smoking and one-third reported chewing. Among chewers, most chewed tobacco with areca nut (31%) and only 2% chewed raw tobacco. Among the respondents, 21.2% (33.8% of men, 11.2% of women) reported ever use of smokeless tobacco and 14.9% reported current use. Current smokeless tobacco use was nearly three times more prevalent among men than among women both in rural and urban areas. Use of smokeless tobacco was not reported by any respondent aged 10–14 years.

In the GYTS conducted in 2004, smokeless tobacco use was reported by 10.8% of students aged 13–15 years. Boys reported significantly more smokeless tobacco use than girls (18.1% versus 3.6%) (Kyaing, 2005).

(*g*) *Nepal*

Several smokeless tobacco products — *khaini*, *gutka* and *zarda* — are consumed in Nepal. Although they are fairly new to the hill population, they are becoming increasingly

popular in all parts of the country. Between 1996 and 1999, imports of *khaini* and *zarda* into Nepal, mostly from India, increased 72-fold (Karki *et al.*, 2003).

Studies on the economics of tobacco use in Nepal revealed that there are no national or sub-national data from Nepal. A prevalence of 9.4% for *khaini* use and of 31.6% for smoking has been reported from a survey of 6000 people aged 10 years or over (Karki *et al.*, 2003).

A cross-sectional survey was conducted in Dharan municipality (eastern Nepal) in 2001–2002 (Niraula, 2004). A representative sample of 2340 women aged 15 years and above was selected. Of these, 12.9% were cigarette smokers and 14.1% were smokeless tobacco users. The prevalence of tobacco chewing increased from 6.0% in the 15–24-year age group to peak at 25.3% in the 35–44-year age group, after which it decreased gradually. Tobacco chewing was more common among women who were involved in business (30.5%) than among others. Muslims were more likely and Christians were less likely to use tobacco than Buddhists (Table 55).

Nearly one student in 10 (9.3%) aged 13–15 years from the GYTS survey in Nepal reported current smokeless tobacco use. The prevalence of use among boys was significantly higher than that among girls (11.8% versus 5.6%) (Pandey & Pathak, 2001).

Among secondary school students of the sub-metropolitan city of Pokhara, ever use of *gutka* and *khaini* was reported by 41.2% and 3.0%, respectively. Smokeless tobacco use was more frequent among boys than girls (56.4% versus 31.2%). Non-governmental school students were more likely to use smokeless tobacco than governmental school students (Table 56; Paudel, 2003).

(*h*) *Pakistan*

Tobacco chewing alone, tobacco chewing with *pan* and tobacco chewing with smoking was reported by 2.2, 14.8 and 0.5% respondents, respectively, in a population sample of 10 749 people in Karachi (Mahmood *et al*., 1974) (Table 57). In a survey conducted in 1980 among 990 residents in Karachi, about 60% of men and 38% of women used tobacco; of these, about 11% of men and 31% of women chewed tobacco either on its own (1–2%), with *pan* (6.4–27%) or in association with smoking (2.2–2.5%) (Mahmood, 1982).

(*i*) *People's Republic of China*

China is the largest producer of tobacco in Asia (Shafey *et al.*, 2003). No additional information on China was available to the Working Group.

(*j*) *Sri Lanka*

In the WHO Sentinel Prevalence Survey of Tobacco Use in Sri Lanka (cited in Sinha, 2004), a total sample population of 5886 people (49.3% men, 50.7% women) was investigated. Current use of smokeless tobacco products was mainly a rural phenomenon (seven times more prevalent among men and six times more prevalent among women; Table 58), and prevalence among men was almost twice that among women. The trend

indicated a decrease in the current use of smokeless tobacco with education and economic level and an increase with increasing age.

Table 55. Prevalence by sociodemographic characteristics of tobacco chewing among women (≥ 15 years) in Dharan, Nepal, 2002

Characteristic	No. of women	Prevalence (%)
All	2340	14.1
Age group (years)		
15–24	933	6.0
25–34	582	21.1
35–44	250	25.2
45–54	279	23.3
55–64	190	9.5
≥ 65	106	7.9
Marital status		
Unmarried	725	1.4
Married	1376	21.1
Separated	35	22.9
Divorced	8	12.5
Widowed	196	10.7
Occupation		
Housewife	1312	18.3
Business	187	30.5
Service	137	10.2
Labourer	78	21.8
Student	605	0.5
Unemployed	21	0.0
Ethnicity		
Hill native castes	1219	15.1
Major Hill castes	759	12.9
Hill occupational castes	156	14.1
Terai castes	206	13.1
Religion		
Hindu	1659	14.4
Christian	157	3.8
Kirat	300	16.0
Buddhist	216	16.7
Muslim	8	25.0

From Niraula (2004)

Table 56. Prevalence (%) of ever use of tobacco by type of product among 2032 secondary school students in Nepal

Category		Prevalence (± 95% CI)	
		Surti, khaini	*Pan masala, gutka*
Sex	Boys	5.0 (± 1.4)	51.4 (± 3.1)
	Girls	0.9 (± 0.7)	30.3 (± 3.0)
School	Government	2.4 (± 0.9)	31.3 (± 2.7)
	Non-government	4.0 (± 1.4)	56.3 (± 3.5)
Caste	Brahmin/Chhetri	2.6 (± 1.1)	38.6 (± 3.2)
	Gurung/Magar	3.5 (± 1.4)	45.6 (± 3.7)
	Newar	2.4 (± 3.2)	40.5 (± 8.9)
	Others	3.3 (± 2.6)	37.9 (± 6.3)
Total		3.0 (± 0.8)	41.2 (± 2.2)

From Paudel (2003)

Table 57. Prevalence (%) of use of different tobacco products in Karachi, Pakistan, 1967–72

Habit	Men	Women	Total
Sample size (n)	5802	4947	10 749
No tobacco use	36.9	56.8	46.0
Pan	4.2	11.5	7.6
Tobacco chewing	2.6	1.9	2.2
Smoking	30.3	2.2	17.4
Pan + tobacco chewing	6.1	25.0	14.8
Pan + smoking	8.9	0.4	5.0
Tobacco chewing + smoking	0.7	0.1	0.5
Pan + tobacco chewing + smoking	8.7	0.9	5.1
Unknown	1.6	1.2	1.4

From Mahmood *et al.* (1974)

(*k*) *Uzbekistan*

In a survey conducted in Samarkand Oblast, all men aged 55–69 years who were residents in one local authority district were invited to participate. Of 1569 men, 636 (41%) reported *naswar* use and 259 (17%) were cigarette smokers (Zaridze *et al*., 1985).

Table 58. Prevalence by selected sociodemographic characteristics of current use of smokeless tobacco in Sri Lanka, 2001

Characteristic	Prevalence (%)
Sample size (n)	5886
Urban	
Men	3.7
Women	1.7
Rural	
Men	26.4
Women	12.0
Education	
No schooling	41.6
Primary	33.9
Secondary	8.9
Higher secondary	1.9
University	2.3
Monthly income (Rs)	
< 3000	17.0
3001–6000	12.7
6001–9000	10.4
9001–12 000	7.7
≥ 12 001	2.4

Adapted from Sinha (2004)

1.4.4 *Africa*

The two major tobacco producing countries in Africa are Malawi and Zimbabwe (Shafey *et al.*, 2003). The most widely grown type of tobacco in Zimbabwe is flue-cured Virginia, while Malawi is the largest producer of Burley tobacco in Africa. (Burley tobacco accounts for just under 15% of global tobacco production.) Worldwide, approximately 11–12 million farmers cultivate tobacco, about 18 000 of whom are in Zimbabwe and 375 000 in Malawi (Jaffee, 2003). Exports of tobacco leaf increased continuously in both countries between 1980–82 and 1997–99, by 130% in Malawi and by 69% in Zimbabwe (Jaffee, 2003). According to different sources, Zimbabwe produced 210 000–230 000 tonnes of tobacco leaves and Malawi 99 000–125 000 tonnes in 2000 (Jaffee, 2003; Shafey *et al.*, 2003). Tobacco accounts for over 30% of the export revenue of Zimbabwe and 75% of that of Malawi (Shafey *et al*., 2003).

Data from countries that participated in the GYTS are presented in Table 59 (Global Youth Tobacco Survey Collaborating Group, 2003).

Table 59. Prevalence (%) of use[a] of non-cigarette tobacco among students aged 13–15 years in African countries, 1999–2002 (Global Youth Tobacco Survey)

Country	Year of survey	Prevalence (± 95% CI)	
		Boys	Girls
Africa		11.0	9.2
Botswana	2002	10.1 (± 3.1)	9.2 (± 2.4)
Burkina Faso			
Ouagadougou	2001	7.9 (± 3.3)	6.3 (± 2.5)
Bobo Dioulasso	2001	5.9 (± 2.6)	5.5 (± 2.7)
Egypt	2001	18.3 (± 4.4)	12.0 (± 3.9)
Ghana	2000	13.6 (± 4.0)	15.5 (± 4.3)
Kenya	2001	9.0 (± 3.1)	8.9 (± 2.8)
Lesotho	2002	12.3 (± 2.9)	14.8 (± 2.3)
Malawi			
Blantyre	2001	14.4 (± 4.4)	15.2 (± 3.8)
Lilongwe	2001	12.8 (± 3.4)	12.7 (± 2.7)
Mali			
Bamako	2001	13.1 (± 3.8)	4.8 (± 2.5)
Mauritania	2001	15.8 (± 2.7)	13.4 (± 3.1)
Morocco	2001	10.4 (± 1.4)	7.6 (± 1.8)
Mozambique			
Maputo	2002	5.4 (± 1.7)	6.0 (± 1.7)
Gaza Inhambe	2002	5.7 (± 2.4)	7.8 (± 2.0)
Niger	2001	6.7 (± 2.4)	7.5 (± 3.4)
Nigeria			
Cross River State	2001	18.6 (± 4.6)	9.4 (± 3.3)
Senegal	2002	7.3 (± 2.1)	2.9 (± 1.0)
Seychelles	2002	13.0 (± 3.8)	5.5 (± 2.3)
South Africa	2002	14.8 (± 2.4)	11.9 (± 1.9)
Sudan	2001	17.2 (± 3.4)	10.4 (± 2.6)
Swaziland	2001	8.9 (± 2.2)	5.2 (± 0.2)
Togo	2002	9.5 (± 2.5)	7.1 (± 2.1)
Tunisia	2001	11.3 (± 2.2)	3.1 (± 0.8)
Uganda			
Arua	2002	23.8 (± 7.3)	20.0 (± 6.5)
Kampala	2002	9.7 (± 1.2)	9.8 (± 3.1)
Mpigi	2002	10.9 (± 3.4)	9.3 (± 1.8)
Zimbabwe			
Harare	1999	11.0 (± 4.1)	8.4 (± 4.4)
Manicaland	1999	11.6 (± 5.7)	13.9 (± 4.4)
Zambia	2002	17.0 (± 3.4)	17.4 (± 4.0)

From Global Youth Tobacco Survey Collaborative Group (2003)
CI, confidence interval
[a] Use is defined as used at least once in the 30 days preceding the survey.

(*a*) *Kenya*

A small study conducted among five ethnic groups in Kenya assessed differences in smokeless tobacco use related to gender and generation. In four of the five groups, little or no difference was observed in the prevalence of smokeless tobacco use between generations (except for the Gikuyu) or between sexes (except for the Luo) (Kaplan *et al.*, 1990).

(*b*) *South Africa*

In South Africa, smokeless tobacco is more commonly used through the nose and less commonly orally (Ayo-Yusuf *et al.*, 2004). Between 1992 and 1995, the consumption of snuff in South Africa increased by about 30% from 1.1 million kg to 1.5 million kg (Tobacco Board, 1992, 1994/95, cited in Ayo-Yusuf *et al.*, 2004).

A national household survey provided cross-sectional data on a representative sample of the population of South Africa (Table 60). Of 13 826 participants (5753 men, 8073 women), 6.7% (0.9% of men, 10.2% of women) used snuff or chewed tobacco. Smokeless tobacco was used predominantly by African and coloured women and the prevalence increased with age to peak at 28.9% and 9.7%, respectively, in women older than 64 years. The age-standardized prevalence of use of smokeless tobacco in South Africa was higher for Africans (8.4%) than for other ethnic groups (coloureds, 1.9%; whites, 0.8%; Asians, 0.2%) (Steyn *et al.*, 2002).

A telephone survey of 300 tobacco users each in Seshego (black area) and Pietersburg (white area) revealed that 3% of the white tobacco users used snuff while almost half (46.7%) of the blacks used snuff (Peltzer, 1999). The typical form of using snuff in this survey was sniffing (86.7%); placing snuff in the mouth was practiced by men only (13.3%).

In a sample of 330 grade-10 and 382 grade-12 students from a rural population (age range, 13–23 years), 4.0% of boys and 8.4% of girls were current snuff users (Peltzer, 2003). Twenty-four (3.4%) of the participants were current snuff users only, 31 (4.3%) were current smokers only and 17 (2.4%) currently used both snuff and cigarettes (Table 61). The preferable mode of taking snuff was by sniffing, followed by mouth only and both sniffing and by mouth. The prevalence of smokeless tobacco use was not significantly different between grade-10 and grade-12 students (Peltzer, 2003).

A structured questionnaire was administered by means of face-to-face interviews to 30 randomly selected households in a rural population (125 adults over the age of 30 years). Of the respondents, 20.8% were active oral snuff dippers (Table 62). No significant difference was observed in the prevalence between sexes ($p > 0.05$). None of the snuff dippers chewed or smoked tobacco. Among the snuff dippers, the vast majority (85%) placed the snuff in the lower labial sulcus; the remainder placed it in the lower buccal sulcus. About half of them prepared the snuff themselves and the other half acquired it commercially. The mean age of snuff dippers was 62.7 years (range, 36–95 years) and mean duration of use was 21.5 years; dipping lasted for about 2 h per day with an average of 35 min per dip (Ayo-Yusuf *et al.*, 2000).

Table 60. Prevalence of use of smokeless tobacco[a] in South Africa, 1998

Age group (years)	Men										Women									
	African		Coloured		White		Asian		Total		African		Coloured		White		Asian		Total	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
15–24	1493	0.2	207	0.8	93	2.3	46	0	1844	0.4	1709	3.2	224	2.4	97	0	70	0	2102	2.8
25–34	809	0	158	0	87	1.2	32	0	1091	0.1	1256	8.6	227	0.6	97	2.4	56	0	1634	7.0
35–44	737	0.5	154	0	85	0	39	0	1016	0.4	1022	12.7	197	2.3	94	0.2	61	0	1396	9.8
45–54	480	2.5	103	0	99	2.5	31	0	715	2.1	773	18.8	161	1.3	110	0	41	0	1088	13.5
55–64	359	2.1	80	0	70	0	19	–[b]	529	1.6	712	21.6	102	6.5	113	0	35	0	938	16.3
≥ 65	405	4.3	70	1.9	66	0	16	–	558	3.2	701	28.9	97	9.7	89	0	16	–	915	22.9
All ages	4283	1.0	772	0.4	500	1.2	183	0	5753	0.9	6173	12.6	1008	2.9	603	0.4	279	0	8073	10.2

From Steyn *et al.* (2002)

[a] Smokeless tobacco is defined as snuff or chewing tobacco.

[b] Subgroups with fewer than 30 people

Table 61. Status of snuff use and age at start by sex among students in South Africa

Tobacco use status	Boys No. (%)	Girls No. (%)
Sample size	328	384
Ever use	42 (12.8)	77 (20.1)
Current use	13 (4.0)	32 (8.4)
Mean age at start (years)	13.3 (4.9)	11.9 (5.7)

From Peltzer (2003)
[The Working Group noted some discrepancies between the text and table in the prevalence of current snuff users and current smokers.]

Table 62. Prevalence and pattern of snuff dipping in a rural population in South Africa

	Men	Women	Total
Respondents	62	63	125
Snuff dipper			
No.	11	15	26
Prevalence (%)	[17.7	23.8	20.8]

From Ayo-Yusuf *et al.* (2000)

(*c*) *Sudan*

Idris *et al*. (1994) conducted a cross-sectional survey on the use of *toombak* in a random population sample in the Nile State in northern Sudan. In a preliminary report on 2000 households with 5500 adults, about 40% of the men dipped *toombak*, and 9% were both cigarette smokers and *toombak* dippers. *Toombak* was particularly prevalent (> 45%) among men aged 40 years or older. Among women, *toombak* use was popular only in the older age groups; up to 10% of women aged 60 years and over used *toombak* (Idris *et al*., 1994).

A later report included results from 4535 households with 21 594 individuals aged 4 years and over (Table 63). In 60% of all households at least one member used *toombak*. The prevalence of *toombak* use in the entire population aged 4 years or older was 12.6%. The prevalence of *toombak* use was low (1.7%) among children and adolescents (4–17 years) and was highest in the oldest age group (70 years and older). Among the adult population aged 18 years and older, the prevalence of *toombak* use was significantly higher among men (34.1%) than among women (2.5 %), and was significantly higher in rural than in urban areas (35.4% versus 23.5% in men). The highest rates of *toombak* use

were found in the rural areas among men aged 30 years and older (mean, 46.6%; range, 45.3–47.1%) (Idris *et al*., 1998b).

Table 63. Prevalence (%) by age and area of residence of current use of *toombak* among men and women in Sudan, 1992

Age	Men		Women	
	Sample size	Prevalence	Sample size	Prevalence
Rural				
4–17	2728	1.9		
18–19	349	16.0		
20–21	391	26.1		
22–29	1236	32.5		
30–39	981	45.9		
40–49	679	47.0		
50–59	495	47.1		
60–69	386	45.3		
70–79	269	47.0		
≥ 18	4786	35.4		
All ages	7514	23.0	7232	1.0
Urban				
4–17	1067	1.0		
18–19	207	3.4		
20–21	196	9.7		
22–29	600	19.0		
30–39	552	27.5		
40–49	386	28.2		
50–59	278	31.3		
60–69	201	32.3		
70–79	67	46.3		
≥ 18	2487	23.5		
All ages	3554	16.7	3294	2.3
Combined				
4–17	3795	1.7		
18–19	556	11.3		
20–21	587	20.6		
22–29	1836	30.0		
30–39	1533	39.3		
40–49	1065	40.2		
50–59	773	41.4		
60–69	587	40.9		
70–79	336	47.0		
≥ 18	7273	34.1	[6731]	[2.5]
All ages	11 068	23.0	10 526	1.7

From Idris *et al.* (1998b)

(*d*) *Tunisia*

A cross-sectional study of a representative national sample of 5696 subjects aged 25 years and over was conducted in 1996, in which data were collected by means of a questionnaire. Tobacco use was reported by 30.4% of the respondents; 5.8% consumed 'traditional' tobacco, which was defined as tobacco in the form of snuff (*neffa*), chewing tobacco and/or a waterpipe. In this geographical area, *neffa* is the predominant form of snuff used. Use of 'traditional' tobacco was influenced by age, sex, level of education and rural or urban environment (Table 64). The proportion of men who only consumed 'traditional' tobacco increased from 2.4% in the 25–34-year age group to 20.4% in the ≥ 55-year age group; the corresponding values for women were 0.1% and 14.3%, respectively. The consumption of 'traditional' tobacco was more widespread in rural than in urban areas and was relatively high among poorly educated men from economically deprived backgrounds (Fakhfakh *et al*., 2002).

Table 64. Prevalence (%) by socioeconomic characteristics and age of use of 'traditional' tobacco[a] in 5696 subjects in Tunisia, 1996

	Men	Women
Overall prevalence	7.9	3.7
Location		
Rural	14.3	6.0
Urban	3.4	[2.1][b]
Education		
Illiterate	18.0	6.5
Primary	4.8	0.7
Secondary	2.6	
Tertiary	3.6	
Occupation		
Unemployed	13.9	3.8
Manual worker, service personnel	7.0	3.9
Employee, middle management	2.5	1.0
Employee, senior management	6.9	2.6
Age (years)		
25–34	2.4	0.1
35–44	4.7	0.4
45–54	5.7	2.5
≥ 55	20.4	14.3

From Fakhfakh *et al*. (2002)

[a] 'Traditional' tobacco is defined as snuff, chewing tobacco and waterpipe.

[b] Value given as 22.1% in the original source. The Working Group believed that the correct value is one order of magnitude lower.

(*e*) *Other countries*

In Algeria, an estimated 90% of tobacco production is used for the manufacture of snuff, and 24% of all tobacco consumed is in the form of snuff (WHO, 1997).

In Libya, apporoximately 140 tonnes of chewing tobacco are consumed every year (WHO, 1997).

Shammah is a traditional form of chewing tobacco that is used very commonly in southern Saudi Arabia and in Yemen (Hannan *et al*., 1986; Ibrahim *et al*., 1986).

In Lesotho, according to a 1992 survey in rural areas, prevalence of smokeless tobacco use was 2.7% for nasal snuff and 0.3% for oral snuff in those aged 15–29 years, 19.6% for nasal snuff and 2.1% for oral snuff in those aged 30–44 years and 28.5% for nasal snuff and 8.7% for oral snuff in those aged 45 years and over (WHO, 1997).

In Swaziland, Zambia and Zimbabwe, snuff taking is common in rural areas, particularly among older persons (WHO, 1997).

1.4.5 *Association between smokeless tobacco use and cigarette smoking*

Because the use of smokeless tobacco or cigarettes are both associated with nicotine delivery and addiction, interrelationships between smokeless tobacco use and smoking may help to explain long-term historical patterns and trends in the use of these products by populations of various cultures. Some observations suggest that certain smokeless tobacco products may serve as an effective method to quit smoking (Kozlowski *et al.*, 2003). Others have attributed the decline in smoking that has occurred in Sweden since the early 1980s to the expansion of moist snuff use in that country (Bates *et al*., 2003). However, some researchers have suggested that smokeless tobacco may actually serve as starter product for nicotine addiction among young people in the USA, which could lead to subsequent smoking (Tomar, 2003b), and is rarely used as a smoking cessation strategy (Tomar & Loree, 2004); others have questioned whether snuff played any significant role in reducing smoking in Sweden (Tomar *et al*., 2003; Lambe, 2004). The interrelationship between smokeless tobacco use and smoking, together with recommendations by tobacco manufacturers or those who advocate that tobacco users switch from one product type to another, may have significant implications for exposure to carcinogens among individuals and populations. Sweden and the USA provide the only two examples of nations in which commercial moist snuff products are widely promoted, available and used, and from which there are available epidemiological data to examine the interrelationship between the use of moist snuff and cigarette smoking.

(*a*) *Data from Sweden*

A number of reports indicate that dual use of moist snuff and cigarettes is fairly prevalent in Sweden. In 1985–87, 47% of all male snuff dippers were also smokers compared with 36% of non-snuff users who were smokers (Nordgren & Ramström, 1990). More recent, official national data on the prevalence of dual use could not be located, although a Swedish survey of current and former smokers commissioned by the Swedish Cancer

Society and Pharmacia AB in 2000 found that 19.8% of male current smokers also used moist snuff (Gilljam & Galanti, 2003). A census of ninth grade students (aged 15–16 years) in the County of Stockholm found that 14.3% of boys were exclusively smokers, 5.7% were exclusively snuff dippers and 13.8% used both cigarettes and snuff (Galanti *et al*., 2001a), that is, 71% of boys who used snuff also smoked and 49% of boys who smoked also used snuff.

Some data indicate that snuff use may be a precursor to smoking among young men in Sweden. In a cohort study conducted in the County of Stockholm that began in 1997, 2883 students in the fifth grade were recruited and followed-up 1 year later (Galanti *et al*., 2001b). At baseline, 22% of boys and 15% of girls had ever smoked and 8 and 3%, respectively, had ever used oral moist snuff. One year later, the overall prevalence of smoking had increased markedly, as had the transition to more advanced stages of smoking, especially among girls. The authors concluded that, in most cases, experimentation with oral snuff among boys marked the transition to cigarette smoking.

The extent to which snuff use may account for the decline in smoking in Sweden during the past few decades is unclear. Ramström (2000) reported that, in national surveys of the Swedish population in 1987 and 1988, respondents who had ever used tobacco were asked whether their primary tobacco use was smoking or snuff dipping. Among men aged 18–34 years, 43% were ever daily smokers; of these, 21.5% were former smokers and 21.5% were current daily smokers. Fifty-one per cent of women of the same age were ever daily smokers: 18.5% were former smokers and 32.5% were current daily smokers. From this observation, the author concluded that "Since the one major difference between men and women in Sweden is the widespread use of snuff among men and virtually no snuff use among women, it seems probable that male snuff use has kept down onset of smoking and increased smoking cessation" (Ramström, 2000). Similarly, the review by Foulds *et al*. (2003) cited ecological data on trends of sales of snuff and cigarettes, unadjusted data on prevalence of smoking and male snuff use and sequential cross-sectional surveys from a study in northern Sweden [the Working Group noted that this study was funded by the smokeless tobacco industry] (Rodu *et al*., 2002) as being "strongly suggestive of *snus* having a direct effect on the changes in male smoking and health". [Most conclusions that suggest that snuff played a significant role in reducing cigarette smoking are based largely on ecological or cross-sectional studies.]

Several studies in Sweden examined the possible contribution of snuff to quitting smoking. In a 1-year cohort study of 12 507 persons aged 47–68 years at baseline in 1992–94, Lindström *et al.* (2002a) examined predictors of smoking cessation or change to intermittent (non-daily) smoking among 3550 daily smokers. At baseline, 7.0% of all men and 0.4% of all women were snuff users. At the 1-year follow-up, 7.2% of daily smokers had quit and 6.5% had become intermittent smokers. In a multiple logistic regression analysis that controlled for sex and other demographic characteristics, daily smokers who remained so were less likely than the total population to be snuff users at baseline (odds ratio, 0.67; 95% confidence interval [CI], 0.51–0.87); daily smokers who became intermittent smokers were more likely than the general population to be snuff dippers at baseline

(odds ratio, 1.94; 95% CI, 1.07–3.51); and daily smokers who quit smoking did not differ from the total population in their use of snuff at baseline (odds ratio, 1.1; 95% CI, 0.54–2.26). The study did not report changes in snuff use during the period of follow-up. The authors concluded that sex differences in snuff consumption could provide "… a substantial, although not major, fraction of the explanation for why there has been an increase in smoking cessation in recent years among men but not among women, although we believe that other social and work-related factors may be even more important". Another analysis of the same cohort focused on intermittent smokers at baseline (Lindström *et al.*, 2002b), who accounted for 4.8% of the cohort of 699 people. At the 1-year follow-up, 59.9% of intermittent smokers were still intermittent smokers (intermittent/intermittent), 15.9% had become daily smokers (intermittent/daily) and 19.2% had stopped smoking (intermittent/stopped). Among intermittent/intermittent, 11.5% were snuff users at baseline, as were 9.5% of intermittent/daily, 9.0% of intermittent/stopped and 3.0% of the total cohort which included daily smokers, former smokers and never smokers. In multivariate logistic regression modelling, snuff use was a moderately strong correlate of intermittent smoking compared with the reference group regardless of smoking status at follow-up: odds ratios were 3.40 (95% CI, 1.70–6.81) for intermittent/daily, 4.22 (95% CI, 3.00–5.94) for intermittent/intermittent and 3.20 (95% CI, 1.79–5.71) for intermittent/stopped. The investigators did not report changes in snuff use during the follow-up and did not explicitly compare changes in smoking status as a function of snuff use at baseline. From these two studies, it may be concluded that: (*a*) snuff use may have been more common among intermittent smokers aged 45–69 years than among the rest of the adult population of that age group, but did not seem to be associated with subsequent cessation or prevent transition to daily smoking; and (*b*) snuff use was less common among daily smokers who remained daily smokers than among the general population, but was associated only with their transition to intermittent smoking and not with smoking cessation at the 1-year follow-up.

In a similar study, 5104 persons aged 16–84 years were interviewed in 1980–81 and then followed up in 1988–89 (Tillgren *et al.*, 1996). The cohort included 1546 daily smokers, 418 men who were daily snuff users and 129 men who used both snuff and cigarettes. At follow-up, 5% of male smokers had switched to snuff and 2% had started using snuff in addition to cigarettes, and 5% of non-tobacco users had started using snuff. Among male exclusive snuff users, 26% had quit all tobacco use and 10% had taken up cigarettes in addition to (5%) or instead of (5%) snuff. Among male dual product users, 56% either had continued dual product use or exclusively smoked, 31% exclusively used snuff and 13% had quit all tobacco use.

Rodu *et al.* (2003) reported findings from the MONICA cohort study: persons aged 25–64 years at study entry and who joined the cohort in 1986, 1990 and 1994 were followed up until 1999. Among all 308 men who smoked at entry to the study, 19% exclusively used snuff and 24% used no tobacco product at follow-up. Among 195 male smokers who had never used snuff at entry [63% of all male smokers], 57% were still exclusively smokers at follow-up, 8% had switched to snuff, 6% used both cigarettes and

snuff and 29% used no tobacco product. Among 423 women who smoked at entry to the study, 3% exclusively used snuff and 27% used no tobacco products at follow-up.

The most recent evidence that snuff may be a factor in the decline in smoking in Sweden over the past 20 years derives from cross-sectional studies. Gilljam and Galanti (2003) reported results from a survey in 2000 of 1000 former and 985 current daily smokers aged 25–55 years. Among men, more former smokers than current smokers had ever used snuff (54.7% versus 44.8%; $p = 0.003$) or currently used snuff (28.9% versus 19.8%; $p = 0.002$). Among men, snuff had been used at the most recent attempt to quit smoking by 28.7% of former smokers and 23.0% of current smokers ($p = 0.072$). The study found that having used snuff at the most recent attempt to quit was associated with an increased likelihood of abstinence among men (odds ratio, 1.54; 95% CI, 1.09–2.20). [The authors did not report an association between snuff use and cigarette smoking separately for women, but it could be calculated from the data reported in the tables. Snuff use was much less common among women than among men and did not differ between current and former smokers: 13.1% of women reported ever using snuff, including 14.1% of current smokers and 12.1% of former smokers, and 2.9% of women were current snuff users, including 2.5% of current smokers and 3.3% of former smokers. Use of snuff at the most recent attempt to quit smoking was reported by 4.8% of female current smokers who had attempted to quit and 4.5% of female former smokers.] These findings suggest that snuff use may be associated with smoking cessation among Swedish men but not women.

(*b*) *Data from the USA*

Many cross-sectional studies in the USA have reported moderate-to-strong degrees of association between concurrent smoking and use of smokeless tobacco in the adolescent population (Lichtenstein *et al*., 1984; Ary *et al*., 1987; Jones & Moberg, 1988; Murray *et al*., 1988; Olds, 1988; Ary *et al*., 1989; Colborn *et al*., 1989; Glover *et al*., 1989; Peterson *et al*., 1989; Riley *et al*., 1989; Sussman *et al*., 1989; Severson, 1990; Lee *et al*., 1994; Hatsukami *et al*., 1999; Coogan *et al*., 2000; Ringel *et al*., 2000). These studies, however, used a wide range of definitions of tobacco use and were often unable to establish a temporal relationship with the initiation of use of each tobacco product. Relatively few reports of longitudinal investigations into the relationship between smoking and smokeless tobacco have been published.

Some longitudinal studies found that the use of smokeless tobacco was predictive of the onset of or increase in cigarette smoking (Ary *et al*., 1987; Dent *et al*., 1987; Ary *et al*., 1989; Haddock *et al*., 2001), while others reported that smoking was predictive of initiation of experimentation with or regular use of smokeless tobacco (Ary *et al*., 1987; Dent *et al*., 1987; Ary, 1989; Sussman *et al*., 1989; Tomar & Giovino, 1998).

Two recent cohort studies suggest that use of smokeless tobacco may be a predictor of subsequent smoking among young men in the USA. In a cohort study of 7865 Air Force recruits with a mean age of 19 years at baseline, Haddock *et al*. (2001) considered regular smokeless tobacco use to be use of these products at least once per day; the 1-year measure of smoking outcome was defined as any smoking within the preceding 7 days.

Among current smokeless tobacco users, 27% initiated smoking, compared with 26.3% of former smokeless tobacco users and 12.9% of never users. After adjustment for demographic characteristics among recruits who had never been daily smokers, current users (odds ratio, 2.33; 95% CI, 1.84–2.94) and former users (odds ratio, 2.27; 95% CI, 1.64–3.15) of smokeless tobacco products were significantly more likely than never users to initiate smoking. Current or former smokeless tobacco use was a much stronger predictor of initiation of smoking than a range of other behaviours, including rebelliousness, use of a seat belt, alcoholic beverage consumption, binge drinking, level of physical activity and fruit and vegetable intake.

A recent nationally representative cohort study of adolescent boys and young adult men in the USA examined the longitudinal relationship between use of smokeless tobacco and initiation of smoking (Tomar, 2003b). Data were from the 1989 Teenage Attitudes and Practices Survey and its 1993 follow-up that comprised 7960 people aged 11–19 years at baseline. Analyses were limited to 3996 boys and men with complete data on smoking and smokeless tobacco use at both interviews. Young men who were not smokers in 1989 but regularly used smokeless tobacco were more than three times more likely than never users to be current smokers 4 years later (23.9% versus 7.6%; adjusted odds ratio, 3.45; 95% CI, 1.84–6.47). In contrast, 2.4% of current smokers and 1.5% of never smokers at baseline had become current regular smokeless tobacco users by follow-up. More than 80% of baseline current smokers were still smokers 4 years later and less than 1% had switched to smokeless tobacco; in contrast, 40% of baseline current regular smokeless tobacco users became smokers either in addition to or in place of smokeless tobacco use. The results suggest that smokeless tobacco may be a starter product for subsequent smoking among young men and boys in the USA, but may have little effect on quitting smoking in that age group.

Another analysis (O'Connor *et al*., 2003) of the same cohort as that analysed by Tomar (2003b) suggested that smokeless tobacco was no longer a statistically significant predictor of initiation of smoking when psychosocial risk factors were included in multiple logistic regression modelling. In modelling of predictors of current smoking among men and boys who had never experimented with cigarettes at baseline, an adjusted odds ratio of 1.97 (95% CI, 0.69–5.65) was found for those who reported regular use of smokeless tobacco. The study also suggested an association between smokeless tobacco use and established risk factors for initiation of smoking, such as having a smoker in the household (odds ratio, 1.52; 95% CI, 1.10–2.11). Another analysis also suggested a positive association between regular smokeless tobacco use and initiation of smoking in a model that included experimentation with cigarettes, school performance, depressive symptoms, having a smoker in the household and several markers of risk-taking behaviour (odds ratio, 1.68; 95% CI, 0.83–3.41).

A repeat of the analytic approach of O'Connor *et al*. (2003) that limited the analysis to boys under 16 years of age at baseline found that boys who had used smokeless tobacco were significantly more likely than non-users to be current smokers at follow-up (odds ratio, 1.67; 95% CI, 1.03–2.70) in multivariable modelling that included race or ethnicity,

geographical region of residence, experimentation with cigarettes, school performance, having a smoker in the house, depressive symptoms and two markers for risk-taking behaviour (Tomar, 2003c).

This series of analyses showed that smokeless tobacco use was an independent predictor of cigarette smoking among adolescent boys with a strength of association that was comparable with that of other established risk factors. However, regular use of smokeless tobacco was relatively uncommon at baseline in this cohort study and therefore the parameter estimates were fairly imprecise.

In an analysis of cross-sectional data from the 1987 National Health Interview Survey (NHIS), Kozlowski *et al*. (2003) found a significant association between ever use of smokeless tobacco and current smoking (odds ratio, 1.35; 95% CI, 1.05–1.74), but no association when men who had used cigarettes before smokeless tobacco were excluded from the analysis (odds ratio, 0.79; 95% CI, 0.56–1.11). On this basis, the authors concluded that the order of product use must be considered and that use of smokeless tobacco was unlikely to predict smoking. [The Working Group noted that the analysis did not exclude the many persons at any given age who had already become smokers; the large majority of men in the USA who initiate smoking do so without ever using smokeless tobacco, but that does not rule out the use of smokeless tobacco as a risk factor for nicotine addiction and initiation of smoking. The analytic approach of Kozlowski *et al*. (2003) was analogous to conducting a case–control study in which a very large proportion of the control group actually had the disease; such misclassification generally biases results toward the null (Rothman & Greenland, 1998).]

Tomar and Loree (2004) subsequently modelled smokeless tobacco use as a possible predictor of smoking by excluding from the analysis those who were already smokers at a particular age, and then examined whether smokeless tobacco use predicted subsequent smoking. In contrast to the conclusion of Kozlowski *et al*. (2003), Tomar and Loree (2004) found that white boys who used smokeless tobacco at age 15 years but had never smoked were significantly more likely than non-users of smokeless tobacco to become smokers subsequently, after controlling for age, geographical region and educational attainment (odds ratio, 1.80; 95% CI, 1.15–2.82). Similar results were found when the analysis was repeated for age 16 years (odds ratio, 1.53; 95% CI, 1.03–2.30) or age 17 years (odds ratio, 1.87; 95% CI, 1.17–2.98).

Only one study in the USA has explicitly examined the effectiveness of snuff use as a method for smoking cessation (Tilashalski *et al*., 1998). This pilot study found that 16 of the 63 subjects (25%) in the study had quit smoking at the 1-year follow-up by using snuff and six subjects (10%) had quit smoking by using some other method. [The study did not include a control group.] In a 7-year follow-up of 62 of the original 63 subjects, 28 (45%) had quit smoking, although fewer than half of subjects (n = 12) had reportedly done so by using snuff (Tilashalski *et al*., 2003).

Fiore *et al*. (1990) reported findings from the 1986 Adult Use of Tobacco Survey on the methods that smokers used to quit. In the mid-1980s, 6.8% of former smokers who had successfully quit smoking for at least 1 year had substituted cigarettes with other

tobacco products (including snuff, chewing tobacco, pipes or cigars) during any attempt to quit and 4.0% during their last attempt to quit. However, the proportions were very similar among those who relapsed: 6.8% of smokers who had made a serious attempt to quit in the past year but were not successful had tried substituting other tobacco products at any attempt and 2.1% had tried that strategy at their last attempt to quit.

The most recent direct measurement of the extent of smokeless tobacco use in the USA as a method for quitting smoking derives from the 2000 NHIS. Tomar and Loree (2004) examined changes in tobacco use within the male birth cohorts that were included in the cross-sectional analysis of Kozlowski *et al*. (2003) of data from the 1987 NHIS. A comparison of the prevalence of tobacco use among men aged 23–34 years in 1987 with that of 36–47-year-olds in 2000 revealed a very small decline in the prevalence of current smoking among this birth cohort, from 34.1% (95% CI, 31.9–36.3%) in 1987 to 31.0% (95% CI, 29.1–32.9%) in 2000; the prevalence of current snuff use declined during the same period from 5.8% (95% CI, 4.6–7.0%) to 2.5% (95% CI, 1.9–3.1%). Former smokers in the 2000 NHIS were asked what method they had used to quit smoking completely. Only 1.2% (95% CI, 0.1–2.3%) of male former smokers aged 36–47 years in 2000 reported switching to snuff or chewing tobacco to quit smoking. Of male current smokers in that age group who had unsuccessfully tried to quit, 0.3% (95% CI, 0.0–0.7%) reported switching to smokeless tobacco on their last attempt to quit. In a birth cohort in which 15.5% of men, who included 19.0% of ever smokers, had used smokeless tobacco by the age of 34 years, this practice accounted for a very small proportion of smoking cessation. The authors calculated that the number of men in this birth cohort who used smokeless tobacco, apparently for reasons other than smoking cessation, was up to 68 times greater than the number who used it to quit smoking. The number of men in this birth cohort for whom smokeless tobacco was a probable starter product for smoking was estimated to be about 17 times that of men who reported quitting smoking by using smokeless tobacco.

A recent cross-sectional study examined the associations between snuff use and smoking in a representative sample of men in the USA (Tomar, 2002). The 13 865 subjects were men aged 18 years and older in the 1998 NHIS. Multiple logistic regression modelling was used to examine the association between the use of snuff and cessation of smoking. The study reported that, in 1998, 26.4% of men in the USA smoked, 3.6% used snuff and 1.1% used both products. After adjusting for age and race or ethnicity, the prevalence of current smoking was higher among former snuff users (39.4%) and occasional users (38.9%) than among daily users (19.2%) or never users (25.4%). Daily snuff users were significantly more likely than never users to have quit smoking in the preceding 12 months (odds ratio, 4.2; 95% CI, 2.2–8.3). Occasional snuff users were more likely than never users to have tried to quit smoking in the preceding year (odds ratio, 1.7; 95% CI, 1.0–2.8) but tended to be less likely to succeed (odds ratio, 0.5; 95% CI, 0.2–1.3). After adjustment for age and race or ethnicity, smokers who used snuff daily smoked significantly fewer cigarettes per day on average than those who never used snuff (11.4 versus 18.4 cigarettes; $p = 0.0001$). Men were nearly three times more likely to be former snuff users who currently smoked (2.5%) than to be former smokers who currently used snuff

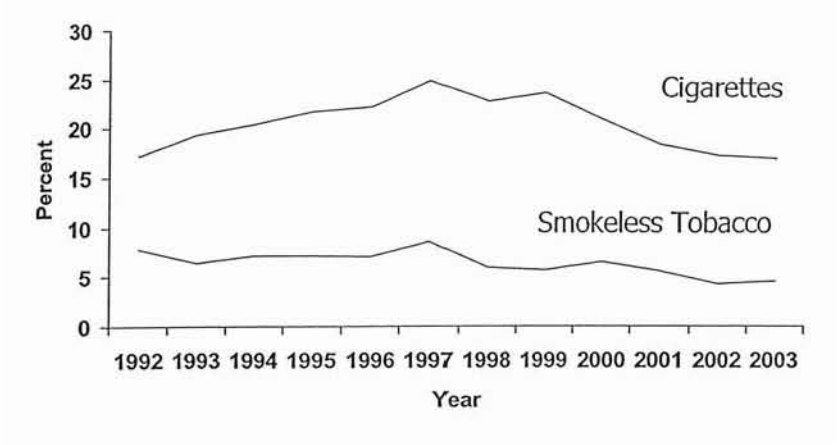
(0.9%). The author concluded that although some men may use snuff to quit smoking, men in the USA more commonly switched from snuff use to smoking.

Wetter *et al*. (2002) examined the characteristics, tobacco use patterns over time and predictors of tobacco cessation among 220 male concomitant users of cigarettes and smokeless tobacco in a large, randomized, worksite-based, matched-pair cancer prevention trial (n = 4886). High levels of dual use were found: 20% of smokeless tobacco users were also smokers (4% of the total study population). Compared with exclusively smokeless tobacco users, dual users were significantly more likely to be unmarried, to drink more alcoholic beverages, to live with a smoker and to use less smokeless tobacco per day, but had higher estimated exposure to nicotine. Dual users appeared to be less ready to change their use of smokeless tobacco than exclusively smokeless tobacco users. At the 4-year follow-up, exclusively smokeless tobacco users were the most likely (20.1%) and dual users were the least likely (11.3%) to have quit all tobacco use; 15.7% of exclusively smokers had quit. Among men who were dual users at baseline, 44.3% were still dual users at the 4-year follow-up, 27.0% were exclusively smokers and 17.4% were exclusively smokeless tobacco users. Men who were exclusively smokers or smokeless tobacco users at baseline showed little inclination to switch products completely, and comparable proportions added use of the other product: 4.6% of baseline smokers began using smokeless tobacco exclusively or in combination with cigarettes and 3.4% of baseline smokeless tobacco users began to smoke either exclusively or in combination with smokeless tobacco. Traditional measures of nicotine dependence (e.g. number of cigarettes or smokeless tobacco uses per day) that predicted cessation among exclusive smokers or smokeless tobacco users were not related to smoking cessation among dual users. Whether due to subject characteristics or the nature of dual product use, dual users in this study had the lowest tobacco cessation rates and tended to shift product use in both directions.

Dual tobacco use has been found to be fairly prevalent in specific subpopulations in the USA, such as in certain Native American populations, among whom 18% of current smokers also used smokeless tobacco and 26% of smokeless tobacco users also smoked (Spangler *et al*., 2001a).

At the population level, a possible effect of the substitution of smokeless tobacco for cigarettes could be manifested by a trend of increasing prevalence of smokeless tobacco use and declining prevalence of smoking. The possibility of such a pattern was explored by examining survey data collected among senior high school pupils as part of the Monitoring the Future Project, which has been conducted since 1975 by the University of Michigan under contract with the National Institute on Drug Abuse (Johnston *et al*., 2003). Data on cigarette smoking have been collected since the inception of the study and those on smokeless tobacco use since 1986. Trends in daily tobacco use among male senior high school pupils in the USA do not support a substitution effect of one product for another (Figure 4). The prevalence of daily smokeless tobacco use remained relatively constant from 1992 to 1996, and was 6–7% for young men. Following a slight increase in 1997 to 8.6%, the prevalence has declined gradually and was 4.3% in 2002. The prevalence of daily cigarette smoking increased from 17.2% in 1992 to 24.8% in 1997, after

Figure 4. Trends in prevalence of daily use of cigarettes or smokeless tobacco among male high school seniors. Monitoring the Future Project, 1992–2003

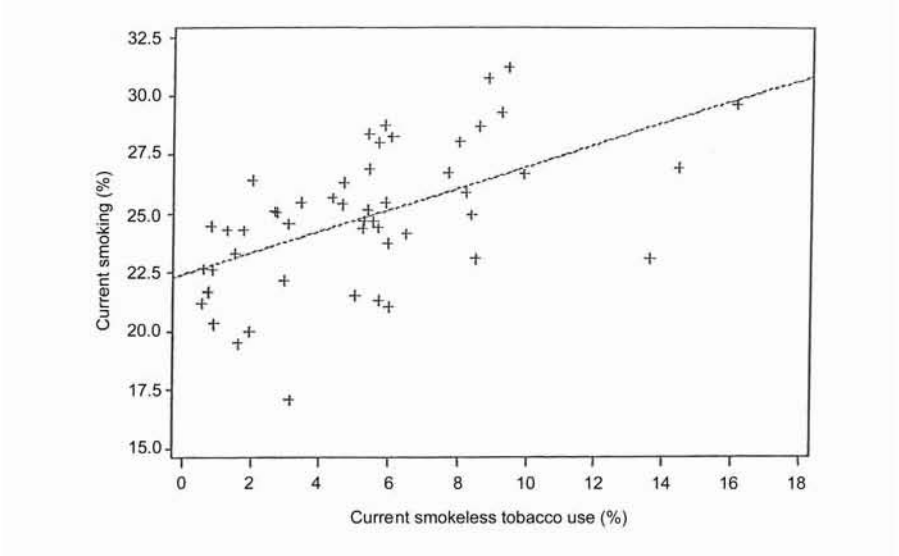


From Johnston *et al.* (2003)

which it began to decline and returned to 17.2% in 2002. At the population level, therefore, it appears that daily use of either cigarettes or snuff has been declining since 1997.

Another approach to the association between smokeless tobacco use and cigarette smoking in populations is to examine their prevalence by state. This was investigated by using data from the September 1998 and January and May 2000 Tobacco Use Supplements to the Current Population Survey. The Current Population Survey was conducted for the Bureau of Labor Statistics by the US Census Bureau and the Tobacco Use Supplements were developed and sponsored by the National Cancer Institute. Linear regression analysis revealed a statistically significantly positive association ($\beta = 0.456$; $p < 0.0001$; $R^2 = 0.2984$) between state-level prevalence of smokeless tobacco use and cigarette smoking among men aged 18 years and older (Figure 5). Similarly, there was a significantly positive association ($\beta = 1.291$; $p < 0.0001$; $R^2 = 0.2841$) between the prevalence of daily use of snuff and the prevalence of daily cigarette smoking among men in the states. The association between state prevalence of smokeless tobacco use and cigarette smoking was nearly identical when analyses were limited to white men. Although cultural and economic factors may affect the use of either tobacco product within states, the ecological patterns of use do not support the existence of widespread product substitution or a 'preventive' effect, in which higher prevalence of smokeless tobacco use is associated with lower prevalence of cigarette smoking (Tomar & Loree, 2004; Tomar, 2007).

Figure 5. Linear regression model of prevalence[a] of current[b] smokeless tobacco use on prevalence of current cigarette smoking among men aged 18 years and older in 50 states, USA, 1998 and 2000



From unpublished data from the September 1998 and January and May 2000 Current Population Survey Tobacco Use Supplements (combined), US Census Bureau and National Cancer Institute

[a] Age-adjusted (five categories) to 2000 US standard population

[b] Used smokeless tobacco (chewing tobacco or snuff) or cigarettes every day or on some days at the time of the interview

Model current smoking = 22.424 + 0.4567 * Smokeless tobacco use

Model $R^2 = 0.2984$

(*c*) *Summary*

In many ways, the recent histories of snuff use in Sweden and the USA are very similar. In both countries, the products were heading towards extinction in the late 1960s, when the development of new products, new images and aggressive marketing led to a new surge in sales. In both countries, these products were adopted largely by young men.

The primary difference between the countries is that the prevalence of daily use of snuff grew to a much larger extent in Sweden, perhaps due to a long history of snuff use and greater cultural acceptance of snuff dipping. The difference may also be attributable to the dominance of a single domestic tobacco company, Swedish Match, in both the cigarette and snuff markets. Swedish Match may have been willing to expand one market (moist snuff) by fostering a transfer of customers from the cigarette market; the company even sold its cigarette business to an Austrian tobacco company in 2000 (Henningfield & Fagerström, 2001). The exact role that snuff has played in reducing the prevalence of smoking in Sweden is unclear, but it has probably been overstated (Tomar *et al*., 2003).

The decline in smoking in Sweden during the past two decades occurred in an environment of increased taxation on cigarettes, increased availability of treatment, expansion of clean indoor air policies and increased communication about the dangers of smoking in Sweden (Henningfield & Fagerström, 2001). Evidence from ecological studies that the increasing prevalence of moist snuff use in Sweden has led to a decline in smoking is inconclusive because of the methodological limitations of ecological studies, which do not directly measure changes in behaviours by individuals. Data from the few available Swedish cohort studies do not support a conclusion that moist snuff was a major factor in the decline in smoking, and in even in areas of Sweden that have a relatively high use of moist snuff, adult smokers who have no previous history of snuff use rarely adopted these products. In the USA, cohort studies of young men suggest that a high proportion of young smokeless tobacco users subsequently initiate smoking, but very few smokers switch to using smokeless tobacco. Consistent with cohort studies, cross-sectional studies in the USA suggest that smokeless tobacco use is rarely used to quit smoking, even among birth cohorts with a substantial history of using those products. It is less clear what the effects might be if moist snuff is aggressively marketed in societies that have little previous experience with these products. Recent history suggests that snuff use will probably gain much more popularity among young men who have never used tobacco or are in the early stages of initiation of tobacco use than among middle-aged smokers who are looking for a cessation strategy.

1.4.6 *Occupational exposure to unburnt tobacco*

The manufacture of *bidis* is one of the largest cottage industries in India and provides employment to more than 3 million people (Govekar & Bhisey, 1992). On average, a *bidi* roller makes 500–1000 *bidis* per day and handles 225–450 g of tobacco flakes, and is thus exposed by dermal contact. In addition, the workers also receive airborne exposure to tobacco dust and volatile components.

(*a*) *Exposure to tobacco dust*

Several studies conducted in various countries suggest that tobacco workers are exposed to tobacco dust and particulate matter (Mengesha & Bekele, 1998; Uitti *et al*., 1998; Mustajbegovic *et al*., 2003; Yanev & Kostianev, 2004; Zuskin *et al*., 2004) (Table 65). In a study that assessed the extent of exposure to tobacco dust among workers in *bidi* tobacco processing plants (Bhisey *et al*., 1999a), the mean concentration of inspirable dust particles was 150 times higher than that in the control environment.

Yanev and Kostianev (2004) determined that the majority of tobacco dust particles had a size of 0.3 μm (range, 0.05–16 μm), and some anisometric forms ranged in size from 0.1 to 2.0 μm.

Table 65. Dust levels in tobacco factories

Country	Job task	No. of samples	Measurement	Mean concentration ($mg/m^3 \pm SD$)	Reference
Ethiopia	Blending	5	Respirable dust area samples	1.83 ± 1.69	Mengesha & Bekele (1998)
	Making	4		0.48 ± 0.31	
	Packing	3		0.29 ± 0.11	
				Total	
Croatia	Sorting	4	Total and respirable dust area samples	14.4	Mustajbegovic *et al.* (2003); Zuskin *et al.* (2004)
	Placing on belts	4		8.5	
	Grinding/shredding	4		4.4	
	Overall	12		9.1	
				Respirable	
				2.4	
				2.1	
				1.1	
				1.9	

SD, standard deviation

(*b*) *Biomonitoring of* bidi *industry workers*

Exposure to tobacco-specific compounds and to electrophilic moities through the occupational use of tobacco can be determined among *bidi* rollers by measuring urinary cotinine and thioethers, respectively. A series of studies have measured occupational exposure of *bidi* workers to nicotine and carcinogens through biomonitoring.

Ghosh *et al*. (1985) conducted a study of tobacco processing workers in India. Among non-tobacco users, none of the control subjects had detectable levels of urinary nicotine or cotinine; levels in exposed workers were 3.13 μg/mL and 3.4 μg/mL, respectively. The mean urinary nicotine and cotinine levels were higher among workers than among controls.

Urinary cotinine and thioethers were determined in samples from two groups of *bidi* rollers and controls from the same community (Bhisey & Govekar, 1991). None of the subjects used tobacco in any form. One group of *bidi* rollers lived in the most densely populated part of Mumbai and worked in a poorly ventilated room, while the other lived in an area with open spaces and worked singly in open courtyards. Urinary cotinine was not detected in control samples while it was present in most samples from *bidi* rollers. In both groups of *bidi* rollers, workers who rolled up to 1000 *bidis* per day showed higher urinary thioether excretion than those who made up to 500 *bidis* per day.

The same authors conducted a larger study that included a greater number of subjects (Govekar & Bhisey, 1992). Among those who had no personal use of tobacco, cotinine was not detected in the urine samples of workers who did not roll *bidis* but was present in samples of workers who did. Among tobacco users, the levels of urinary cotinine were similar in *bidi* rollers and non-*bidi* rollers. Mean urinary thioether levels were signifi-

cantly elevated among *bidi* rollers with or without personal use of tobacco compared with samples from the respective workers who did not roll *bidis*.

In another study (Bagwe & Bhisey, 1993), occupational exposure to tobacco was evident from the higher mean salivary cotinine levels that were observed in samples from *bidi* rollers and tobacco processing plant workers who did not report any personal tobacco use compared with their respective non-occupationally exposed counterparts.

A more recent study confirmed the findings for cotinine in saliva and urine and for thioethers (Bhisey *et al*., 1999a).

Nicotine and cotinine levels were measured in blood and urine samples from 10 healthy nonsmoking tobacco harvesters and five healthy nonsmoking controls at six time-points during a regular working shift (D'Alessandro *et al*., 2001). Maximum values of plasma and urinary nicotine were 3.45 ± 0.84 and 158.3 ± 42.5 ng/mL, respectively. The maximum values for cotinine were 20.54 ± 9.55 and 108.84 ± 47.02 ng/mL, respectively. The levels of plasma and urinary nicotine and those of urinary cotinine were significantly higher in samples from tobacco harvesters than in those from unexposed controls.

1.5 Regulations

1.5.1 *Framework Convention on Tobacco Control*

The first international tobacco control treaty, the Framework Convention on Tobacco Control (FCTC), was adopted unanimously by the 192 Member States of the World Health Organization in May 2003 and was opened for signature for a 1-year period. At closure, on 29 June 2004, 168 countries had signed the treaty. The Convention entered into force on 27 February 2005, 90 days after it had been acceded to, ratified, accepted and approved by 40 States. The FCTC provides a comprehensive regulatory structure for all forms of tobacco use, including smokeless tobacco (Part 1, Article 1F). Throughout the FCTC, the term 'tobacco products' is used to include specifically smokeless tobacco together with combusted tobacco products. The treaty will lay the legal framework in each country that ratifies the Convention for regulation to restrict or eliminate the use of any form of tobacco and to promote healthy tobacco-free lifestyles (WHO, 2003a).

1.5.2 *Australia and New Zealand*

In 1986, the South Australian Government became the first government in the world to ban smokeless tobacco. The ban subsequently became national in 1991 (Chapman & Wakefield, 2001).

New Zealand has also banned smokeless tobacco (WHO, 1997).

1.5.3 *Europe*

(*a*) *European Union*

Since 2001, smokeless tobacco has been regulated in the European Union under Directive 2001/37/EC, which supercedes Council Directive 89/622/EEC of 13 November 1989 and Directive 92/41/EEC of 15 May 1992 (European Parliament and Council, 2001). Article 2.4 of the 2001 directive defines 'tobacco for oral use' as "… all products for oral use, except those intended to be smoked or chewed, made wholly or partly of tobacco, in powder or in particulate form or in any combination of those forms, particularly those presented in sachet portions or porous sachets, or in a form resembling a food product." Article 8 of Directive 2001/37/EC requires that Member States prohibit the marketing of tobacco for oral use (as defined above), but explicitly exempts Sweden and the EFTA (European Free Trade Association) country Norway. Previously, all snuff packages had to carry the health warning "causes cancer" (Directive 92/41/EEC). This was changed in the 2001 Directive, which requires that smokeless tobacco products carry the following warning: "This tobacco product can damage your health and is addictive". The warning must cover at least 30% of the package.

Manufacturers and importers of tobacco products are required to submit to the Member States, on a yearly basis, a list of all ingredients and quantities thereof used in the manufacture of tobacco products, together with toxicological data on their effects on health and any addictive effects. This list must be accompanied by a statement that sets out the reasons for their inclusion. It must also be made public and be submitted to the Commission on a yearly basis (Article 6).

Texts, names, trade marks and figurative or other signs that suggest that a particular tobacco product is less harmful than others is prohibited on the packaging of tobacco products (Article 7).

(*b*) *Sweden*

Most regulations that govern the marketing and contents of smokeless tobacco in Sweden stem from provisions of the Swedish Tobacco Act. English language text of the provisions of the Swedish Tobacco Act is available through the website of the WHO Regional Office for Europe (WHO EURO, 2004). The Swedish Tobacco Act bans the advertisement of all tobacco products on national television, cable and radio, in local magazines and newspapers and in cinemas. Advertising on billboards, outdoor walls and at the point of sale are not permitted to "be invasive, enticing or encourage use of tobacco". Businesses may not market such products as shoes and clothing if they include a tobacco trademark (brand stretching).

The Swedish Tobacco Act also regulates the contents and packaging of all tobacco products: it requires the manufacturers to list the general ingredients on each package and, in accordance with EU Directive 2001/37/EC, requires a health warning label stating "This tobacco product can damage your health and is addictive". Sales of all tobacco products are restricted to persons aged 18 years and older and merchants are required to request

purchasers to provide proof of age. Guideline No. 7 of the National Board for Consumer Policies prohibits sponsorship of events by tobacco brands.

(*c*) *Norway*

The Norwegian Tobacco Act and regulation on the prohibition of tobacco advertising contains provisions on the marketing of smokeless tobacco. A translation of this legislation is available through the website of the WHO Regional Office for Europe (WHO EURO, 2004). The Norwegian Tobacco Act and above-mentioned regulation bans all forms of advertisement of tobacco products. Tobacco products must not be included in the advertising of other goods and services, and all free distribution of tobacco products is prohibited. Indirect advertising of tobacco products was also forbidden as of 1 January 1996. It is prohibited to produce in or import into Norway new types of product that contain tobacco or nicotine.

The Norwegian Tobacco Act also regulates the contents and packaging of all tobacco products. The provisions require the manufacturer to provide information of the general ingredients on each package. A health warning is also required on smokeless tobacco: "This tobacco product can damage your health and is addictive".

Tobacco products cannot to be sold to persons under 18 years of age.

1.5.4 *North America*

(*a*) *Canada*

The most recent regulations in Canada on information on tobacco products were enacted in June 2000 (Health Canada, 2000, 2001).

These regulations require that every manufacturer of chewing tobacco or oral snuff include one of the following bilingual warnings on every package: (*a*) "THIS PRODUCT IS HIGHLY ADDICTIVE" and "CE PRODUIT CRÉE UNE FORTE DÉPENDANCE"; (*b*) "THIS PRODUCT CAUSES MOUTH DISEASE" and "CE PRODUIT CAUSE DES MALADIES DE LA BOUCHE"; (*c*) "THIS PRODUCT IS NOT A SAFE ALTERNATIVE TO CIGARETTES" and "CE PRODUIT N'EST PAS UN SUBSTITUT SÉCURITAIRE À LA CIGARETTE"; or (*d*) "USE OF THIS PRODUCT CAN CAUSE CANCER" and "L'USAGE DE CE PRODUIT PEUT CAUSER LE CANCER".

Every manufacturer of nasal snuff is required to display one of the following bilingual health warnings on every package: (*a*) "THIS PRODUCT IS NOT A SAFE ALTERNATIVE TO CIGARETTES" and "CE PRODUIT N'EST PAS UN SUBSTITUT SÉCURITAIRE À LA CIGARETTE"; (*b*) "THIS PRODUCT CONTAINS CANCER CAUSING AGENTS" and "CE PRODUIT CONTIENT DES AGENTS CANCÉRIGÈNES"; (*c*) "THIS PRODUCT MAY BE ADDICTIVE" and "CE PRODUIT PEUT CRÉER UNE DÉPENDANCE"; or (*d*) "THIS PRODUCT MAY BE HARMFUL" and "CE PRODUIT PEUT ÊTRE NOCIF".

Every manufacturer of chewing tobacco or snuff is also required to display on every package of chewing tobacco or snuff that they manufacture the mean amount of toxic

constituents (nitrosamines, lead and nicotine) contained in the product, expressed in milligrams, micrograms or nanograms per gram of chewing tobacco or snuff and determined in accordance with the official method set out for that toxic constituent.

(*b*) *USA*

Most of the current federal regulations on the marketing of smokeless tobacco products were adopted as part of the Federal Comprehensive Smokeless Tobacco Health Education Act of 1986 (Public Law 99-252), which was signed into law in February 1986 (DHHS, 1989). The Act requires that one of three warnings be displayed on all packages and advertisements (except billboards) of smokeless tobacco. The three package warnings are: "WARNING: This product may cause mouth cancer; WARNING: This product may cause gum disease and tooth loss; and WARNING: This product is not a safe alternative to cigarettes." It requires that the three package warnings "be randomly displayed…in each 12-month period in as equal a number of times as is possible on each brand of the product and be randomly distributed in all parts of the USA in which such product is marketed." On advertisements, the law requires rotation of each warning every 4 months for each brand. The warnings on advertisements are required to appear in a circle-and-arrow format recommended earlier by the Federal Trade Commission for cigarette warnings. The Act prohibits Federal agencies or State or local jurisdictions from requiring any other health warnings on packages and advertisements (except billboards) of smokeless tobacco. No other Federal, State or local actions were pre-empted by the Act. The Federal Trade Commission issued regulations implementing the law on 4 November 1986.

The Comprehensive Smokeless Tobacco Health Education Act of 1986 also required that the manufacturers, packagers and importers of smokeless tobacco products provide annually a list of additives used in the manufacture of these products to the Secretary of Health and Human Services. The Secretary is required to treat the lists as "trade secret or confidential information", but may report to Congress on research activities concerning the health risks of these additives. However, the Secretary is granted no specific authority to regulate any of the additives. It also required that manufacturers provide to the Secretary of Health and Human Services a specification of the nicotine content of smokeless tobacco products, but it does not require that nicotine content be listed on packages or in advertisements. The list is an amalgamation of all additives used by any manufacturer in any type of smokeless tobacco product and is not brand-specific. It also contains no information on quantity or concentration of these 500 'ingredients' in any product. More recently, manufacturers of smokeless tobacco were required to use a standardized protocol to determine the nicotine concentration, pH and moisture content in all of their smokeless tobacco products and to provide that information annually to the CDC (1999b). Similarly to the information on product additives, however, CDC is prohibited from releasing that information to the public.

The Comprehensive Smokeless Tobacco Health Education Act of 1986 also prohibited the advertisement of smokeless tobacco products on television or radio.

The legal age at which persons can purchase smokeless tobacco in the USA is currently set at the state level. As of 1998, all states and the District of Columbia prohibit the sale of smokeless tobacco products to persons under the age of 18 years (Fishman *et al.*, 1999). In 1992, Congress passed a provision of the 1992 Alcohol, Drug Abuse, and Mental Health Administration Reorganization Act (the 'Synar Amendment') that addressed the access of minors to tobacco products. The final Synar regulation, issued in 1996, requires states to conduct annually random, unannounced inspections on a representative sample of retail tobacco outlets to assess the extent of sales to minors, and to show they have significantly reduced them to specified target levels (Fishman *et al*., 1999). On 23 August 1996, the US Food and Drug Administration issued a regulation to restrict the sale and promotion of cigarettes and smokeless tobacco products to children and adolescents (Kessler *et al*., 1996). The first two provisions of the regulation made it illegal for retailers to sell cigarettes or smokeless tobacco to anyone under the age of 18 years and required that they check the photographic identification of anyone under the age of 27 years. These two provisions went into effect on 28 February 1997 and remained in effect until 21 March 2000, when the US Supreme Court ruled that the Food and Drug Administration lacked the statutory authority to regulate cigarettes and smokeless tobacco (Natanblut *et al*., 2001). While the provision was in effect, compliance checks conducted in 110 000 establishments in 36 states and the District of Columbia found that the rate of sales to minors was higher for smokeless tobacco (38%) than for cigarettes (24%) (Clark *et al*., 2000).

In November 1998, the US Smokeless Tobacco Company, the largest manufacturer of smokeless tobacco products in the USA, reached a legal settlement with attorneys general for 46 states, the District of Columbia and several US territories (National Association of Attorneys General, 1998). This settlement, known as the Smokeless Tobacco Master Settlement Agreement, included a number of provisions that were intended to reduce the promotion and accessibility of smokeless tobacco products to minors. These provisions include: (*a*) the prohibition of the targeting of youths by advertising and promotion; (*b*) a ban on the use of cartoon characters in tobacco advertisements or packaging; (*c*) limitations on tobacco brand name sponsorships, including prohibition of the sponsorship of certain athletic events and concerts; (*d*) the elimination of outdoor advertising and transit advertisements; (*e*) the prohibition of payments related to tobacco products and media, including product placement in motion pictures and television; (*f*) a ban on tobacco brand name merchandise, including clothing; (*g*) a ban on the access of youths to free samples of smokeless tobacco; (*h*) a ban on gifts to under age persons based on proofs of purchase, including coupons; (*i*) limitations of third-party use of smokeless tobacco brand names; (*j*) a ban on the use of nationally recognized or established non-tobacco brand names as the brand name for a tobacco product; (*k*) the prohibition of the provision of tobacco products to sports teams; (*l*) the promulgation or reaffirmation of corporate cultural commitments related to access and consumption of youths, including the identification of an executive level manager to be responsible for identifying methods to reduce the use of tobacco by youths; (*m*) limitations on lobbying, including a prohibition of opposition by the US Smokeless Tobacco company to the passage of state or local legislative proposals

or administrative rules that are intended to reduce access to and use of tobacco products by youths; (*n*) the regulation and oversight of new tobacco-related trade associations; (*o*) the prohibition of agreements to suppress research; and (*p*) the prohibition of material misrepresentations of fact regarding the health consequences of using any tobacco product.

1.5.5 *Asia*

(*a*) *Overview of regulations on tobacco in Asia*

The status of regulations on tobacco products in Asia in 2003 is given in Table 66 (Shafey *et al.*, 2003). Some countries have regulations that are related to tobacco advertisement. In 11 countries, the contents or designs of tobacco advertisements are restricted. While six countries have banned the sponsorship of events by tobacco trans-nationals, no restrictions exist in eight. Sales of tobacco to minors are not regulated in nearly one-third of the countries, and verification of age at the point of sale is not enforced in any Asian country. Other provisions that are not regulated in some countries in the region include sale by minors in 11 countries, free products in 14 countries, misleading information on packaging in 15 countries and brand-stretching in 16 countries. [Brand-stretching is defined as the use of tobacco brand names on non-tobacco merchandise or services.]

Table 66. Status of regulations on tobacco products in Asia, 2003

	Banned	Restricted	Not Regulated	Unknown
Advertisements				
in certain media	18	21	6	5
to certain audiences	16	5	8	21
in certain locations	10	15	13	12
content or design	–	11	7	32
Sponsorship for certain audiences	11	2	16	21
Sponsorship advertising of events	6	2	8	34
Brand-stretching	7	2	16	25
Sales to minors	23	–	16	11
Sales by minors	1	–	11	38
Place of sales	–	10	12	28
Free products	14	3	14	19
Misleading information on packaging	1	–	15	31

Adapted from Shafey *et al.* (2003)
Countries include: Afghanistan, Armenia, Azerbaijan, Bahrain, Bangladesh, Bhutan, Brunei, Cambodia, China, Hong Kong, India, Indonesia, Iran, Iraq, Israel, Japan, Jordan, Kazakhstan, Korea (Democratic People's Republic of and Republic of), Kuwait, Kyrgyzstan, Laos, Lebanon, Malaysia, Maldives, Mongolia, Myanmar, Nepal, Oman, Pakistan, the Philippines, Qatar, Saudi Arabia, Singapore, Sri Lanka, Syria, Taiwan (China), Tajikistan, Thailand, Turkey, Turkmenistan, United Arab Emirates, Uzbekistan, Viet Nam, West Bank and Yemen

Few countries in Asia have comprehensive anti-tobacco laws that are strengthened by key principles such as taxation, advertising bans, smoking restrictions and effective cessation and education programmes. Egypt, Pakistan and Qatar in the WHO Eastern Mediterranean Region (EMRO) adopted tobacco control laws in 2002. In the WHO South-East Asian Region (SEARO) in 2003, only Thailand had a comprehensive tobacco control policy that included smokeless tobacco products (Shafey *et al.*, 2003); India, Myanmar and Sri Lanka have since followed (WHO Tobacco Free Initiative SEARO website). The Bangladesh Act does not cover smokeless tobacco products.

A number of countries in Asia have taken initiatives specifically to control the use of smokeless tobacco (Table 67). The manufacture of all types of smokeless tobacco product is prohibited in Israel, Taiwan (China) and Thailand, while the manufacture of nasal snuff is allowed in Hong Kong (Special Administrative Region) and Singapore. The promotion of smokeless tobacco products is not permitted in Hong Kong, Singapore, Taiwan (China) or Thailand. In addition to these four states, the sale of smokeless tobacco is not allowed in Bahrain, Bhutan, Israel or Turkey. The import of smokeless tobacco products is prohibited in Hong Kong, Iran, Israel, Japan, Kuwait, Saudi Arabia, Singapore, Taiwan (China), Thailand and the United Arab Emirates. Regulations in India, Thailand and Turkey are detailed below.

Table 67. Available information on legislative action to control the use of smokeless tobacco in Asian countries

Country	Year	Manufacture	Promotion	Sale	Import
Bahrain				+	
Bhutan				+	
Hong Kong, SAR	1987	+*	+	+	+
India[a]		+	+		
Iran					+
Israel	1986	+	+	+	+
Japan					+
Kuwait					+
Saudi Arabia	1990				+
Singapore	1987	+*	+	+	+
Taiwan (China)	1990	+	+	+	+
Thailand	1992	+	+	+	+
Turkey				+	
UAE					+

From WHO (1988); Masironi (1992); WHO (1997); World Bank (2000); Ugen (2003)
+, prohibited; *, except for nasal snuff
SAR, Special Administrative Region; UAE, United Arab Emirates
[a] See also Table 68

Bans on spitting are one of the measures that may influence the prevalence of smokeless tobacco use. In Singapore and in Goa, Tamil Nadu and West Bengal in India, spitting is prohibited in public places and in Maharashtra, India, in police stations only (Table 68). However, implementation is poor in India.

(*b*) *India*

Legislation in India began with the promulgation of the Cigarette Act, 1975 (Regulation of Production, Supply and Distribution Act). Following the example of the state of Maharashtra in 1987, some other states (Goa, Delhi) took initiatives to prevent smoking and spitting on government premises and have conducted educational campaigns against tobacco use. In June 1999, Indian railways, which operated under the Government of India, banned the sale of tobacco on railway platforms. In September 2000, the Government amended the Cable Network Rules and banned television advertisements for tobacco. Tobacco chewing is prohibited in schools that are run by the Union Government of India.

The Cigarettes and Other Tobacco Products Act, 2003 (Government of India, 2003) prohibits direct advertising in all media and sports sponsorship by tobacco companies. It also prohibits smoking in public places. It disallows the sale of tobacco in any form to persons under 18 years of age and within 100 yards of educational institutions. It also disallows the sale of tobacco in any form by persons under 18 years of age. Clear health warnings in local languages and in English are mandatory on all packages.

Recently, beginning with Tamil Nadu in 2001, banning orders have been issued in several states against the sale, manufacture and storage of *gutka* and, in some states, other forms of chewing tobacco and *pan masala* for a certain period of time (Gupta, 2001; Gupta & Ray, 2002). The production, sale, storage, distribution and use of smokeless tobacco products have been banned in Bihar, Andhra Pradesh, Goa, West Bengal, Tamil Nadu, Kerala, Maharashtra and Rajasthan (Table 68), but opposition by industry through the courts has forced these states to modify the ban or postpone its implementation until the Supreme Court reaches a decision.

Unmanufactured tobacco that does not bear any brand name and is used mainly for chewing is exempt from excise duty. Chewing tobacco and snuff that have a brand name are subject to 50% ad-valorem excise duty. Until 1994–95, chewing tobacco with a brand name was taxed (basic and additional excise duty tax) at 40% (Government of India, 2001; Reddy & Gupta, 2004).

(*c*) *Thailand*

Thailand has been a leader in formulating comprehensive control of tobacco, including smokeless tobacco. In 1992, the Tobacco Products Control Act B.E. 2535 was enacted with provisions to: prohibit the sale of tobacco products to persons under 18 years of age; prohibit sale promotions, e.g. exchanges, additions, offers to attend games or shows free of charge, or services to buyers or persons returning tobacco products for exchange or redemption; prohibit free samples; prohibit advertisement in all media except live broadcasts from abroad and foreign publications; prohibit the manufacture, import and advertise-

Table 68. Regulation of smokeless tobacco products in selected states in India

State	Year	Period in years	Products[a]	Production	Sale	Storage	Distribution	Use	Spitting	Advertising	Reference
India	2003		3	+	+[b]		+			+	Government of India (2003)
Andhra Pradesh	2002		3	+	+	+	+	+			Government of Andhra Pradesh (2002)
Bihar	2003	5	1	+	+	+	+				Government of Bihar (2003)
Goa	2003			+	+	+	+	–	+		Government of Goa (2003)
Kerala	1999			+	+	+					Government of Kerala (1999)
Maharashtra	2002	5	1	+	+	+			+[c]		Goverment of Maharashtra (2002)
Rajasthan	1950		2	–	+[d]	–		–	–	–	Government of Rajasthan (1950)
Tamil Nadu	2001	5	3	+	+[b]	+			+		Government of Tamil Nadu (2003)
West Bengal	2001		3	–	+[b]	+	+		+	+	Government of West Bengal (2001)

+, banned; –, unrestricted

[a] 1, *gutka*, *pan masala* with and without tobacco; 2, any smokeless tobacco product; 3, chewing tobacco

[b] Minors < 18 years of age

[c] In police stations only

[d] Minors < 16 years of age

ment of goods that imitate tobacco products and their packages. In Section 11, the composition of tobacco products must be in accordance with Ministerial Rules; and in Section 12, the packages of tobacco products must exhibit labels in accordance with the Ministerial Announcement. The Ministerial Rule pursuant to Section 11 was passed and became effective on 1 February 1997. This rule mandates manufacturers to disclose the ingredients of every brand of their products to the Ministry of Public Health. The Ministerial Announcements persuant to Section 12 were passed and became effective on 25 September 1993, and another announcement became effective on 16 October 1997 (WHO SEARO, 2004).

(*d*) *Turkey*

A strong anti-tobacco law (No. 4207) was enacted in Turkey in 1996. Sales of smokeless tobacco are banned, as is the advertisement of tobacco on radio and television and in government buildings. However, advertising is permitted in print media. Indirect advertising (using tobacco or tobacco products and their brand names) and any tobacco campaign that will promote and motivate the use of tobacco or tobacco products are banned. Restrictions on the access of minors to tobacco products were strengthened by increasing the minimum age at which tobacco or tobacco products may be bought to 18 years. Turkish radio and television and private television channels have to broadcast on the harmful effects of the use of tobacco and its products for at least 90 min per month (World Bank, 2000).

1.5.6 *Africa*

(*a*) *Comprehensive anti-tobacco laws*

Botswana, Mali, Mauritius and South Africa have comprehensive anti-tobacco laws that are based on key principles such as taxation, advertising bans, smoking restrictions, and effective cessation and education programmes (Shafey *et al*., 2003).

(*b*) *Tobacco advertisements in certain media*

Only a few countries, namely Algeria, Cape Verde, Libya, Morocco, Mozambique, Niger, South Africa, Sudan and Tunisia, have banned tobacco advertising in certain media. This represents 16.6% of the 54 African countries (Shafey *et al.*, 2003). In Algeria, advertising of tobacco has been banned since 1985. In Egypt, a complete ban on television and radio advertisements for tobacco has been in force since 1977 (WHO, 1997).

In 27 (58.7%) of the 46 countries in the WHO African Region, the contents or designs of tobacco advertisements are not regulated. While three countries (6.5%) have banned the sponsorship of events by tobacco trans-nationals, no restrictions have been imposed in 29 (63%) (Shafey *et al.*, 2003).

(*c*) *Other provisions*

Other provisions that are not regulated in a majority of countries in Africa include sale by minors in 32 countries (70%), sales of tobacco to minors in 29 countries (63%) (verification of age at the point of sale is not enforced in any African country), free products in 31 countries (67.3%), brand-stretching in 27 countries (58.7%), misleading information on packaging in 32 countries (70%), place of sale in 31 countries (67%), health warnings and messages in 25 countries (54.3%) and the indication of the amount of contents or constituents other than tar and nicotine on packaging in 32 countries (69%) (Shafey *et al.*, 2003).

In Uganda, excise tax on tobacco use was increased by 45% in 1993 (WHO, 1997).

None of the African countries is known to have constituted a National Tobacco Control Committee, none requires constituent disclosures for public or confidential use and none has provisions to enable litigation or measures to reduce the smuggling of tobacco.

2. Studies of Cancer in Humans

2.1 Introduction

Studies that have investigated the association between the use of smokeless tobacco and cancer have often faced a problem of small numbers of cases, which has often precluded an analysis of specific and relevant subgroups; alternatively, when such analyses were carried out, they resulted in imprecise relative risk estimates. This is of particular concern in relation to specific cancer sites, and also to an analysis of categories of smokeless tobacco use as well as to stratification for cigarette smoking and alcoholic beverage consumption. As an example, associations with smokeless tobacco use should preferably have been analysed in never smokers. In making its evaluation, the Working Group gave greatest weight to studies that adequately addressed potential confounding by smoking. In addition, of the studies that were reviewed previously (IARC, 1985), only those that addressed such potential confounding have been included and re-evaluated in this monograph.

The Working Group also considered the possibility of confounding by human papillomavirus (HPV), since there is sufficient evidence in humans that HPV 16 causes cancer of the oral cavity and oropharynx (IARC, 2007). In a systematic review of the detection of HPV DNA in squamous-cell carcinoma of the head and neck, the prevalence of HPV was only 24% in oral and 36% in oropharyngeal cancer (Kreimer *et al.*, 2005), which limits the proportion of cases that can be attributed to this virus. Moreover, negative associations between HPV DNA, tobacco smoking and alcoholic beverage consumption (Gillison *et al.*, 2000) and between HPV, tobacco smoking and *pan* chewing (Herrero *et al.*, 2003) have been observed. Therefore, the Working Group concluded that positive confounding by HPV is unlikely to account for a strong association of these cancers with tobacco chewing.

In this section, case series studies are generally included only if no analytical studies were available from that region or when cancer at the site where the smokeless tobacco was placed was considered.

2.2 Oral use

2.2.1 *Cancer of the oral cavity and pharynx*

The characteristics of cohort studies are summarized in Table 69 and results from these studies on oral and pharyngeal cancer are presented in Table 70.

The design and results of case–control studies on use of smokeless tobacco and cancer of the oral cavity and pharynx are summarized in Table 71.

(*a*) *North and South America*

(i) *Cohort studies*

The US Veterans cohort comprised 293 958 veterans who served in the US Armed Forces during 1917–40, were aged 31–84 years in 1953 and held US government life insurance policies in 1953 (Zahm *et al.*, 1992). Most policy holders were men (99.5%) and nearly all were white. The results on smokeless tobacco were based on 248 046 (84%) veterans who responded to the questionnaire mailed in 1954 or the questionnaire mailed in 1957 to 1954 non-respondents. The cohort was followed up for vital status from 1954 (or 1957) through to 1980, and follow-up was 96% complete; death certificates were available for 97% of the deceased cohort members and identified 129 deaths from oral cancer. The relative risk for oral cancer (ICD-7 140-144) was 3.0 (95% CI, 2.0–4.5) for users of chewing tobacco or snuff and those for infrequent use and frequent use were 1.9 (95% CI, 1.0–3.5) and 3.4 (95% CI, 2.1–5.6), respectively. The corresponding relative risks for the pharynx were 8.7 (95% CI, 4.1–8.3), 4.5 (95% CI, 1.7–11.7) and 11.2 (95% CI, 5.0–25.0), respectively. For early age at first use (≤ 14 years of age), the relative risk was 20.7 (95% CI, 8.0–53.7). [The Working Group noted that the results were not adjusted for tobacco smoking or alcoholic beverage consumption.]

NHANES I was a national probability sample survey of the non-institutionalized US population that oversampled the elderly, poor and women of childbearing age (Accortt *et al.*, 2002). A total of 14 407 adults, aged 25–74 years, underwent health examinations between 1971 and 1975. Of the participants, 13 861 persons (96%) were successfully traced in at least one of the NHANES I Epidemiological Follow-up Studies (NHEFS) in 1982–84, 1986, 1987 or 1992. Death certificates were available for 98% of the decedents. A random sample of 3847 of the cohort was asked about smokeless tobacco use at baseline. In the 1982–84 follow-up, information on smokeless tobacco use was obtained to infer baseline behaviour for study participants who were not part of the original random sample. Participants were considered to be users of smokeless tobacco if they currently used smokeless tobacco at baseline or had ever used it according to the 1982–84 questionnaire. The analysis was restricted to the 6805 black and white subjects, aged 45 years and

Table 69. Descriptions of cohort studies of smokeless tobacco use

Location Reference, name of cohort	Cohort description	Assessment of smokeless tobacco use	Follow-up and outcome	Neoplasms reported (no.)
North America				
Hsing *et al.* (1990); Kneller *et al.* (1991); Zheng *et al.* (1993), Lutheran Brotherhood cohort	26 030 white men aged ≥ 35 years who purchased life insurance from Lutheran Brotherhood Insurance Society, mostly from California, upper Midwest and northeastern USA.	17 818 (68.5%) responded to mailed questionnaire; few differences among responders and non-responders in demographic characteristics.	Vital status follow-up, 1966–86; 4027 (23%) lost to follow-up; death certificates coded to ICD-9	Pancreas (54) (after exclusion of 3 deaths) Stomach (75) Prostate (149)
Hsing *et al.* (1991); Zahm *et al.* (1992); Heineman *et al.* (1995), US Veterans' cohort	283 958 veterans who served in US Armed Forces during 1917–40 and who were aged 31–84 years in 1953 and held US government life insurance policies; 99.5% of policy holders were men, nearly all were white.	248 046 (84%) responded to the 1954 mailed questionnaire or the 1957 questionnaire mailed to 1954 non-respondents on use of chewing tobacco or snuff; 48 304 used smokeless tobacco, 2308 used smokeless tobacco only.	Follow-up 1954–80 (96% complete); death certificates coded according to ICD-7 (97% complete)	Oral cavity and pharynx (129 exposed) Colorectum (838) Prostate (1123) Soft-tissue sarcoma (119)
Putnam *et al.* (2000), Iowa cohort	1601 controls from a case–control study of cancer in Iowa, ascertained from 1986–89 via RDD and HCFA, resident in Iowa, aged 40–86 years, with no prior cancer; exclusion of 24 subjects with proxy respondents (n = 1577)	Mailed questionnaire supplemented by telephone interviews.	Vital status follow-up through to 1995 (3 subjects lost); follow-up for prostate cancer incidence through state cancer registry	Prostate (101), after exclusion of the cases diagnosed prior to return of questionnaire
Accort *et al.* (2002), NHANES I Follow-up cohort	Survey of the non-institutionalized US population who underwent a physical health examination in 1971–75, oversampling of the elderly, poor and women of childbearing age, aged 25–74 years (n = 14 407); analysis restricted to white and black subjects, aged 45–75 years at baseline (n = 6805)	In-person interviews of a random sample (n = 3847) on smokeless tobacco use at baseline or in first NHANES I epidemiological follow-up study (NHEFS)	13 861 persons (96%) successfully traced in at least one follow-up survey of the NHEFS in 1982–84, 1986, 1987 and 1992; death certificates available for 98% of the decedents; coded according to ICD-9	Oral cavity (19) Digestive system (NA) Lung (NA)

Table 69 (contd)

Location Reference, name of cohort	Cohort description	Assessment of smokeless tobacco use	Follow-up and outcome	Neoplasms reported (no.)
Chao *et al.* (2002); Henley *et al.* (2005), CPS-II	508 351 men and 676 306 women, aged ≥ 30 years, residing in a US household in which at least one member was 35 years or older (45 years or older for Chao *et al.*, 2002); analysis restricted to men without prior cancer (except non-melanoma skin cancer) at enrolment and with information on tobacco (*n* = 467 788) (Chao *et al.*, 2002) or restricted to men who never used any other tobacco (Henley *et al.*, 2005)	Questionnaire at enrolment; only men were asked about smokeless tobacco.	Vital status follow-up, 1982–2000 (1996 for Chao *et al.*, 2002); 0.2% lost to follow-up; death certificates coded to ICD-9 (98.9% complete)	Oral cavity and pharynx (46) Digestive system (1999) Stomach (996) Lung (418)
Henley *et al.* (2005), CPS-I and CPS-II	456 487 men and 594 544 women (CPS-I), aged ≥ 30 years, residing in a household in which at least one member was ≥ 35 years old; analysis restricted to men without prior cancer (except non-melanoma skin cancer) at enrolment and who never used any other tobacco.	Questionnaire at enrolment	Vital status follow-up, 1959–72; 6.7% lost to follow-up and 4.9% with follow-up truncated for logistic reasons in 1965; death certificates coded to ICD-7 (97% complete)	Oral cavity and pharynx (13) Digestive system (913) Lung (134)
Europe				
Heuch *et al.* (1983); Boffetta *et al.* (2005), Norwegian cohort	Probability sample of general adult population of Norway from 1960 census and relatives of migrants to the USA, alive on 1 January 1966 (*n* = 12 431)	Mailed questionnaires on lifestyle habits in 1964 and 1967; information on smokeless tobacco available for 10 136 men	Follow-up for cancer incidence via cancer registries, 1966–2001 (99.85% complete)	Oral cavity and pharynx (34) Oesophagus (27) Stomach (217) Pancreas (105) Lung (343) Kidney (88) Bladder (238)

CPS, Cancer Prevention Study; HCFA, Health Care Financing Administration; NA, not available; NHANES, National Health and Nutrition Examination Survey; RDD, random-digit dialling

Table 70. Results of cohort studies on use of smokeless tobacco and cancer of the oral cavity and pharynx

Location Reference, name of cohort	Use of smokeless tobacco	No. of cases	Relative risk (95% CI)	Adjustment for potential confounders; comments
North and South America				
Zahm *et al.* (1992), US Veterans cohort			Oral cavity (ICD-7 140–144)	
	Never used any tobacco products		1.0	
	Used chewing tobacco or snuff	74	3.0 (2.0–4.5)	
	Infrequent use		1.9 (1.0–3.5)	
	Frequent use		3.4 (2.1–5.6)	
			Pharynx	
	Never used any tobacco products		1.0	
	Used chewing tobacco or snuff	55	8.7 (4.1–18.3)	
	Infrequent use		4.5 (1.7–11.7)	
	Frequent use		11.2 (5.0–25.0)	
	Age at first use ≤ 14 years		20.7 (8.0–53.7)	
Accort *et al.* (2002), NHANES 1 Follow-up cohort			**SMR**	
	Ever smokeless tobacco use	2	107 (10–308)	
	Exclusive smokeless tobacco use	0	0 (0–580)	0.8 expected
Henley *et al.* (2005), CPS-I and CPS-II			Oral cavity and pharynx (ICD-7 140–148)	Multivariate, adjusted results for men who never used other tobacco products
		CPS-I		
	Never used smokeless tobacco	9	1.0	
	Current use of smokeless tobacco	4	2.0 (0.5–7.7)	
			Oral cavity and pharynx (ICD-9 140–148)	
		CPS-II		
	Never used smokeless tobacco	45	1.0	
	Current use of smokeless tobacco	1	0.9 (0.1–6.7)	
	Former use of smokeless tobacco	0		
Europe				
Boffetta *et al.* (2005), Norwegian cohort			Oral cavity and pharynx (ICD-7 141–148)	
	Never user	25	1.0	
	Ever used smokeless tobacco	9	1.1 (0.5–2.4)	Adjusted for age and smoking
	Current use of smokeless tobacco	6	1.1 (0.5–2.8)	
	Former use of smokeless tobacco	3	1.0 (0.3–3.5)	

CI, confidence interval; CPS, Cancer Prevention Study; NHANES, National Health and Nutrition Examination Survey; SMR, standardized mortality ratio

Table 71. Case–control studies on use of smokeless tobacco and cancer of the oral cavity and pharynx

Reference, study location, period	Organ site (ICD code)	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	No. of exposed cases	Relative risk (95% CI)	Adjustment for potential confounders	Comments
North and South America									
Williams & Horm (1977), USA, 1969–71		Cancer of the oral cavity from 7518 (57% of randomly selected) incident invasive cancers who participated in the population-based Third National Cancer Survey	Cancer at sites unrelated to tobacco	Personal interview	Smokeless tobacco	*Men*	*Cancer of gum and mouth*	Age, race, smoking	
					Moderate use	8	3.9 ($p < 0.01$)		
					Heavy use	3	6.7		
							Cancer of lip and tongue		
					Moderate use	1	0.4		
					Heavy use	2	1.9		
							Cancer of the pharynx		
					Moderate use	2	0.5		
					Heavy use	–	–		
Winn *et al.* (1981a,b, 1984); Blot *et al.* (1983); Winn (1986), North Carolina, USA, 1975–78	ICD-8 141, 143–146, 148	Oral and pharyngeal cancer from hospitals discharge diagnoses (156 women) or death certificates (99 women); response rate, 91%	410 (2 per case) matched by age, race, residence, source (hospital or death certificate); excluding mental disorders, cancer of the oesophagus or larynx and other oral or pharyngeal diseases; response rate, 82%	Self- and next-of-kin interviews	Snuff user, nonsmoker	*White women* 79	4.2 (2.6–6.7)	Poor dentition (Winn *et al.*, 1981b), use of mouthwashes (Blot *et al.*, 1983), fruit and vegetables (Winn *et al.*, 1984), type of respondent (Winn, 1986)	
						Black women 12	1.5 (0.5–4.8)		
					Years of snuff use in non-smokers		*Cancer of gum and buccal mucosa*		
					0	2	1.0		
					1–24	3	13.8 (1.9–98.0)		
					25–49	10	12.6 (2.7–58.3)		
					≥ 50	15	47.5 (9.1–249.5)		
							Cancer of other mouth and pharynx		
					0	22	1.0		
					1–24	3	1.7 (0.4–7.2)		
					25–49	14	3.8 (1.5–9.6)		
					≥ 50	8	1.3 (0.5–3.2)		

Table 71 (contd)

Reference, study location, period	Organ site (ICD code)	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	No. of exposed cases	Relative risk (95% CI)	Adjustment for potential confounders	Comments
Stockwell & Lyman (1986), Florida, USA, 1982	ICD-O 140–149	1920 incident cancers of the lip, tongue, salivary glands, gum, floor of mouth, other parts of mouth, oropharynx, hypopharynx, pharynx (unspec.), nasopharynx; from population-based Florida cancer registry; overall response rate in case group, 82%	6457 cancers of the colon or rectum, cutaneous melanoma, endocrine neoplasias from same source during same time period; response rate, 78%	Information on tobacco use was obtained by chart and histopathology review at reporting institutions; only primary type of tobacco used was recorded.	Unspecified		*Lip and tongue* 2.3 (0.2–12.9) *Salivary gland* 5.3 (1.2–23.4) *Mouth and gum* 11.2 (4.1–30.7) *Pharynx* 4.1 (0.9–18.0) *Nasopharynx* 5.3 (0.7–41.6)	Age, sex, race, tobacco use	
Blot *et al.* (1988), New Jersey, Atlanta metropolitan area, Santa Clara and San Mateo counties, Los Angeles, USA, 1984–85	ICD 141–149, excluding 142 and 147	1114 incident, pathologically confirmed from population-based cancer registries; all black and white cases; aged 18–79 years; response rate, 75%	1268; RDD for controls aged 64 and younger, HCFA for controls aged 65 and older; frequency-matched on age, sex, race; response rate, 76%	Structured questionnaire interview in home by trained interviewers; next of kin for 22% of cases and 2% of controls	Use of smokeless tobacco	*Men* 46 *Women* 11 *Nonsmoking women* 6	 [0.85] [3.4] 6.2 (1.9–19.8)	 Age, race, study location, respondent status	Nearly all male tobacco chewers were smokers. Female nonsmokers primarily used snuff rather than chewing tobacco. All six cases had oral cavity cancer.
Spitz *et al.* (1988), Houston, TX, USA, 1985–87		185 patients (131 men, 54 women), 19–95 years old, at MD Anderson Hospital; histologically confirmed squamous-cell carcinoma of the tongue (25), floor of mouth (14), other parts of the oral cavity (27), orohypopharynx (15), larynx (50); white US residents; response rate not stated	185 patients at MD Anderson Hospital during the same period, randomly selected, frequency-matched on age (± 5 years) and sex, excluding patients with squamous-cell carcinoma of any site; response rate not stated	Self-administered questionnaire as part of the registration procedure	Chewing tobacco Snuff use	23 9	[1.0] 3.4 (1.0–10.9)		All nine snuff dipping cases drank alcohol, seven also chewed tobacco, eight smoked cigarettes and one smoked cigars and pipes; three of four snuff dipping controls also smoked cigarettes.

Table 71 (contd)

Reference, study location, period	Organ site (ICD code)	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	No. of exposed cases	Relative risk (95% CI)	Adjustment for potential confounders	Comments
Franco *et al.* (1989), São Paulo, Curitiba and Goiânia, Brazil, 1986–88	ICD-9 141, 143–145	232 histologically confirmed, invasive carcinomas of the tongue, gum, floor of mouth and other parts of the oral cavity; from 3 hospitals in Sao Paulo, Curitiba and Goiânia; response rate, 98.3%	464 (2 per case) from same or neighbouring general hospitals; individually matched on sex, 5-year age group and trimester of hospital admission, excluding diagnoses of neoplasms or mental disorder	Cases interviewed using structured questionnaire in hospital, controls privately; no proxy respondents	Unspecified	9	[1.4]		Relative risk independent of tobacco smoking and alcohol drinking (data not shown)
Maden *et al.* (1992), Washington State, USA, 1985–89	ICD-O 141, 143–146	131 in-situ and invasive squamous-cell cancers of the lip (10), tongue (46), gum, floor of mouth (20), unspecified mouth and oropharynx (33); men aged 18–65 years; response rate, 54.4%	136 identified by RDD, frequency-matched on gender, year of diagnosis and age (5-year groups); response rate, 63%	In-person questionnaire interview at home or elsewhere	Unspecified	19	4.5 (1.5–14.3)	Age	
Marshall *et al.* (1992), New York counties, USA, 1975–83		290 histologically confirmed oral and pharyngeal cancer (tongue, 28%; floor of mouth, 14%; oropharynx, 22%; hypopharynx, 13%), excluding black race from 20 hospitals in three New York counties; 513 contacted, 290 (56%) participated.	290 individually matched on age (± 5 years), sex and neighbourhood; response rate, 41%	Interview	Snuff and chewing tobacco		'Increased risk (statistically non-significant)'	Matching variables	Data not shown

Table 71 (contd)

Reference, study location, period	Organ site (ICD code)	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	No. of exposed cases	Relative risk (95% CI)	Adjustment for potential confounders	Comments
Mashberg *et al.* (1993), New Jersey, USA, 1972–83		359 male black or white in-situ or invasive squamous-cell carcinoma of the oral cavity and oropharynx (histologically diagnosed) admitted to Veterans hospital in New Jersey; 94% of study subjects enroled between 1977 and 1982; response rate not stated	2280 from same series of patients with biopsied oral lesions without cancer or dysplasia of the oesophagus, pharynx, larynx, lung; response rate not stated	In-hospital questionnaire interview	Smokeless tobacco Chewing tobacco ever Snuff use ever	52	 1.0 (0.7–1.4) 0.8 (0.4–1.9)	Age, race, tobacco smoking, alcohol; further adjustments for religion, occupation, origin and interviewer did not 'modify materially' the odds ratio.	No dose-response by duration of use (data not shown)
Spitz *et al.* (1993), Houston, TX USA, 1987–91		108 white patients from MD Anderson Hospital with histologically confirmed cancers of the oral cavity (44), pharynx (31) and larynx (33); response rate not stated	108 blood and platelet donors; frequency-matched by age (± 5 years), sex, race and with no history of cancer; response rate not stated	Self-administered questionnaire in hospital	Chewing tobacco		1.2 'not statistically significant'		Data not shown
Kabat *et al.* (1994), USA, 1977–90		1560 cases from 28 hospitals in eight cities with incident, histologically confirmed cancers of the tongue, floor of mouth, gums, gingiva, buccal mucosa, palate, retromolar area, tonsil, other pharynx; response rate not stated	2948 individually matched on hospital, admission within 2 months after case, age, sex, race, with diseases not thought to be associated with tobacco or alcohol and no prior history of tobacco-related cancers; 50% cancers, 7% benign neoplasms, 43% non-neoplastic conditions; response rate not stated	In-hospital questionnaire interview	Chewing tobacco Snuff use	*Men* 4 *Women* 4	 2.3 (0.7–7.3) 34.5 (8.5–140.1)	Among never smokers Among never smokers	Less than 2% of women chewed. Among never-smoking women, there were no tobacco chewers; less than 2% of men and women used snuff. Among never-smoking men, 0 of 82 cases and 0.9% of 444 controls used snuff.

Table 71 (contd)

Reference, study location, period	Organ site (ICD code)	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	No. of exposed cases	Relative risk (95% CI)	Adjustment for potential confounders	Comments
Muscat *et al.* (1996), Illinois, Michigan, New York, Philadelphia, USA, 1981–90	ICD-9 141, 143–146, 148, 149	1009 (687 men, 322 women) hospital patients with incident, histologically confirmed cancers of oral cavity and pharynx; aged 21–80 years; response rate, 91%	923 (619 men, 304 women) hospital patients with conditions unrelated to tobacco use, matched by sex, age (± 5 years), race, date of admission (± 3 months); response rate, 97%	In-hospital questionnaire interview	Chewing tobacco: at least once a week for 1 year or more	*Men* 38 *Women* 0	[1.04]		
					Snuff use: at least once a week for 1 or more years	*Men* 9 *Women* 2	[0.81] [1.9]		
Schwartz *et al.* (1998), Seattle area counties, WA, USA, 1990–95		284 (165 men, 119 women) from population-based cancer registry with histologically confirmed incident in-situ or invasive (92%) squamous-cell cancers of the tongue, gum, floor of mouth, unspecefied mouth, tonsils, oropharynx; aged 18–65 years; response rate, 63.3%	477 (302 men, 175 women) from random digit dialling, frequency matched on sex and age, 3:2 ratio controls to cases; response rate, 60.9%	In-person questionnaire interview	Unspecified	*Men* 11	1.0 (0.4–2.3)		Only one female control used smokeless tobacco.
Europe									
Wynder & Wright (1957), Stockholm, Sweden, 1952–55		477 (265 men, 212 women) patients with squamous-cell cancer of lip (15), gingiva (36), tongue (70), buccal mucosa (18), maxillary sinus (45), nasopharynx (40), hypopharynx (116), oesophagus (74), larynx (63)	333 patients from same hospital with other cancers	Interview	Duration of snuff use		Gingiva, buccal mucosa, ~2* (non-significant)	Tobacco smoking similar to that in controls	*Ridit analysis Cancers often where quid was placed
							Other upper aerodigestive tract, 'no association'	Tobacco smoking higher than in controls	

Table 71 (contd)

Reference, study location, period	Organ site (ICD code)	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	No. of exposed cases	Relative risk (95% CI)	Adjustment for potential confounders	Comments
Blomqvist *et al.* (1991), Sweden		61 (57 men, four women) patients with squamous-cell cancer of the lower lip from one surgery department	61 age- and sex-matched hospital patients without prior diagnosis of cancer	Interview	Use of snuff	2*			*2 cases and 2 controls used snuff only. No details on mixed tobacco users provided
Lewin *et al.* (1998), Stockholm and southern Sweden, 1988–91		605 men from hospitals and cancer registries with head and neck cancer; oral cavity (128), pharynx (138), larynx (157), oesophagus (123); 40–79 years old; response rate, 90%	756 controls from the population registry; stratified by region and age; response rate, 85%	Personal interviews conducted by two specially trained nurses			*Head and neck*	Age, region, smoking, alcoholic beverage consumption	
					Current snuff use	43	1.0 (0.6–1.6)		
					Former snuff use	40	1.2 (0.7–1.9)		
					> 50 g/week	38	1.6 (0.9–2.6)		
					Never smokers	9			
					Current snuff use		3.3 (0.8–12.0)		
					Former snuff use		10.5 (1.4–117.8)		
							Oral cavity		
					Current snuff use	10	1.0 (0.5–2.2)		
					Former snuff use	15	1.8 (0.9–3.7)		
					> 50 g/week		1.7 (0.8–3.9)		
							Pharynx		
					Current snuff use	8	0.7 (0.3–1.5)		
					Former snuff use	7	0.8 (0.3–1.9)		
Schildt *et al.* (1998), northern Sweden, 1980–89	ICD-7 140, 141, 143–145	418 (175 alive; 235 deceased with relatives) reported to cancer registries with squamous-cell cancer; 354 matched pairs (237 men, 117 women) analysed	From population registry; matched by age, sex, county, vital status and year of death for deceased cases	Postal questionnaire			*Oral cancer*	Matching variables	'Ever use' also adjusted for smoking and alcoholic beverage consumption
					Ever use of snuff		0.8 (0.5–1.3)		
					Current snuff use	39	0.7 (0.4–1.1)		
					Former snuff use	28	1.5 (0.8–2.9)		
					Never smokers				
					Current snuff use	19	0.7 (0.4–1.2)		
					Former snuff use	9	1.8 (0.9–3.5)		
							Lip cancer		
					Current snuff use		'Close to unity'		
					Former snuff use		1.8 (0.9–3.7)		

Table 71 (contd)

Reference, study location, period	Organ site (ICD code)	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	No. of exposed cases	Relative risk (95% CI)	Adjustment for potential confounders	Comments
India and Pakistan									
Chandra (1962), India, 1955–59		450 cancers of the buccal mucosa registered in a hospital in Calcutta	500 friends or relatives who came to hospital with the patients, approximately age-matched	[Not reported]	Tobacco chewing No Yes No Yes	*Men* 18 *Women* 5	 [2.7] [2.5]		Not specified if tobacco product chewed was tobacco only or tobacco with lime
Wahi *et al.* (1968), India, 1964–66		346 oral and oro-pharyngeal cancers reported to temporary cancer registry in Uttar Pradesh	10% cluster sample of the district population	Interview	 Non-chewers of tobacco *Pattiwala** chewer	 84	**Period prevalence rate** 0.36/1000 1.17/1000		*Sun-cured tobacco leaf used with or without lime
Jafarey *et al.* (1977), Pakistan, 1967–72		1192 histologically diagnosed cancers of oral cavity or oropharynx	3562 controls matched for age, sex, place of birth	[Not reported]	Tobacco chewing No Yes No Yes	*Men* 27 *Women* 39	 [10.4] 13.7		
Goud *et al.* (1990), India, 1972–75		102 oral cancers from one hospital in Varanesi	102 age- and sex-matched patients from surgical and general wards of same hospital	Questionnaire	**Chewing tobacco** *Khaini* *Zarda* *Khaini* and *zarda*	 35 36 8	 [2.1] [3.7] [2.8]		Not clear whether *khaini* and *zarda* were chewed with or without betel quid
Wasnik *et al.* (1998), India [years of study not reported]		123 (73 men, 50 women) histologically confirmed ‘oro-pharyngeal’ cancers from three hospitals in Nagpur	246 pair-matched controls; 123 non-cancer patients and 123 patients with cancer at other sites; matched for age, sex	[Not reported]	Tobacco chewing Use of tobacco-containing material for cleaning teeth	24 33	11.4 (4.4–29.6) 4.1 (2.0–8.7)		Results refer to control group 1; in multivariate analysis, all types of tobacco chewing were combined.

Table 71 (contd)

Reference, study location, period	Organ site (ICD code)	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	No. of exposed cases	Relative risk (95% CI)	Adjustment for potential confounders	Comments
Merchant *et al*. (2000), Pakistan, 1996–98		79 (54 men and 25 women) histologically confirmed oral squamous-cell cancers (buccal, gingiva, floor of mouth, tongue, palate; fauces and others) from three hospitals	149 (94 men, 55 women) from orthopaedic and general surgical wards, with no past or present malignancy; individually matched on age, sex, hospital	Structured questionnaire, trained interviewer	Ever use of *naswar*	13	9.5 (1.7–53.5)	Cigarette smoking, alcoholic beverage consumption	
Africa									
Idris *et al*. (1995b), Sudan, 1970–85	ICD-0 141.5, 143.8, 144.9, 145.0 141.9, 145.5	(1) 375 squamous-cell cancer of the lip, buccal cavity, floor of mouth (sites of preference for placement of quid); (2) 271 squamous-cell cancer of the tongue, palate, maxillary sinus (sites with little or no contact with quid); both groups admitted to the Radiation and Isotope Center, Khartoum, Sudan	(1) 204 non-squamous-cell oral cancer and cancer of non-oral sites unrelated to tobacco, admitted to the same hospital during the same period; (2) 2820 volunteers attending oral health education programmes in various regions of Sudan	Questionnaire at registration in hospital; similar questionnaire administered by trained interviewers to volunteers	*Toombak* Never User < 10 years > 11 years Never User < 10 years > 11 years	 157 218 10 120	Hospital controls 1.0 7.3 (4.3–12.4) 0.7 (0.3–1.8) 11.0 (4.8–25.1) Population controls 1.0 3.9 (2.9–5.3) 0.2 (0.1–0.4) 4.3 (2.9–6.3)	Age, sex, tribe, residence	

CI, confidence interval; HCFA, Health Care Financing Administration; RDD, random-digit dialling

above for whom data on tobacco were available. Two oral cancers were observed in ever users of smokeless tobacco and 1.9 was expected based on US rates. No oral cancers were observed among exclusive users of smokeless tobacco, but only 0.8 were expected. [The Working Group noted that this study had limited power to examine use of smokeless tobacco and the risk for oral cancer.]

The cohorts of the American Cancer Society comprised volunteers, aged 30 years or above, who responded to a mailed questionnaire and resided in a household in which at least one member was aged 35 years or more (Chao *et al.*, 2002; Henley *et al.*, 2005). The CPS-I cohort included 456 487 men and 594 544 women, and the CPS-II cohort included 508 351 men and 676 306 women. At enrolment in 1959 (CPS-I) or 1982 (CPS-II), cohort members were questioned on use of smokeless tobacco. For CPS-I, vital status was followed-up through to 1972; 6.7% were lost to follow-up and follow-up was truncated for logistic reasons in 1965 for another 4.9%. Death certificates were 97% complete and were coded to ICD-7. For CPS-II, vital status was followed-up through to 1996 (Chao *et al.*, 2002) or 2000 (Henley *et al.*, 2005). Death certificates were 99.8% complete and were coded to ICD-9. Analyses were restricted to men who had had no previous cancer (except for non-melanoma skin cancer) at enrolment. Chao *et al.* (2002) further restricted the analysis to men for whom information on tobacco was available (n = 467 788) and Henley *et al.* (2005) restricted the analysis to men who had never used any other tobacco. In the CPS-I cohort, the hazard ratio for oral and pharyngeal cancers (ICD-7 140-148) for current users of smokeless tobacco was 2.02 (four deaths; 95% CI, 0.53–7.74), adjusted for potential confounders such as alcoholic beverage consumption and dietary intake. In the CPS-II cohort, the multivariate-adjusted hazard ratio for oral and pharyngeal cancers (ICD-9 140-148) was 0.9 (one death; 95% CI, 0.12–6.71) for current users of smokeless tobacco. No deaths occurred among former users of smokeless tobacco.

(ii) *Case–control studies*

A hospital-based case–control study in Atlanta, GA, USA (Vogler *et al.*, 1962), included four groups who were enroled over a 19-month period (1956–57): 333 white patients (235 men, 98 women) who had cancers of the oral cavity, pharynx or larynx, 214 patients who had other diseases of the mouth including leukoplakia, 584 patients who had other cancers and 787 patients who had no cancer and whose mouths were not examined. Use of smokeless tobacco was assessed by interview or questionnaire. Among 642 urban women, 40% of the 38 who had oral cavity cancers, but only 2%, 3% and 1% of the 57 who had other mouth diseases, 170 who had other cancers and 377 non-cancer controls, respectively, had used snuff. Similar findings were observed for the 371 rural women: 75% of the 55 cases of oral cavity cancer had used snuff orally in contrast to 11% of 37 who had other mouth diseases, 20% of 129 who had other cancers, and 11% of 150 non-cancer patients. Only 7% of female rural cases smoked. About 30–40% of urban women smoked cigarettes, but smoking habits were similar in each study group. The differences in snuff use between cases and controls were statistically significant for most of the age strata studied. In contrast to 53 (74%) women who had oral cavity cancer, one

of three female lip cancer patients and two (11%) women who had pharyngeal or laryngeal cancer had used snuff. [The Working Group noted that the reportedly similar proportions of smoking habits among urban women and the low proportion of smokers in the rural women indicate that the association between the use of snuff and cancer of the oral cavity was not confounded by smoking. Confounding by smoking could not be ruled out in men and results are not reported here.]

Williams and Horm (1977) conducted a population-based case–control study of the etiology of cancer at many different sites based on the interview responses of randomly selected incident cases of invasive cancer (n = 7518; 57% of those selected) from the Third National Cancer Survey (1969–71). Controls for smoking-related cancer case groups comprised men and women who had cancers that were unrelated to smoking. Among men, use of chewing tobacco and snuff was strongly associated with cancer of the gum or mouth, but not with cancer of the lip and tongue or pharynx; after controlling for age, race and smoking habits, relative risks were 3.9 (eight cases; $p < 0.01$) for moderate and 6.7 (three cases; non-significant) for heavy use of chewing tobacco or snuff. Among women, the relative risk for use of chewing tobacco or snuff for cancer of the gum or mouth was 4.9 (two cases; non-significant).

Winn *et al*. (1981a) conducted a case–control study of cancers of the oral cavity and pharynx among women in North Carolina, USA in 1975–78 to examine reasons for the exceptionally high rates of mortality from these cancers among white women throughout the southeastern USA. A total of 232 women (91% of eligible cases) who had been hospitalized with or who had died from cancers of the tongue (ICD-8 141), gum (ICD-8 143), floor of mouth (ICD-8 144), other mouth (ICD-8 145), oropharynx (ICD-8 146), hypopharynx (ICD-8 148) and pharynx unspecified (ICD-8 149) were included in the case group. Two age-, race- and region of residence-matched controls were obtained for each case; an interview was completed for 410 of the 502 eligible controls, excluding subjects with mental disorders or cancer of the oesophagus, larynx or other oral or pharyngeal diseases. Subjects or their next of kin were interviewed in their homes. Tobacco-related risks were estimated by using a common reference group: women who did not use tobacco. The relative risk for white women who used only oral snuff was 4.2 (79 cases; 95% CI, 2.6–6.7), while the relative risk associated with cigarette smoking among non-users of snuff was 2.9 (70 cases; 95% CI, 1.8–4.7). Among white women, the relative risk for those who both used oral snuff and smoked was 3.3 (11 cases; 95% CI, 1.4–7.8); these women had smoked fewer cigarettes and used snuff for fewer years than women who only smoked or used snuff. Risks for black women were somewhat lower, but they had used snuff for fewer years and had used fewer tins per week. Although 37 women had chewed tobacco, all but three were also oral snuff users. One-third of all oral snuff users had started the practice by the age of 10 years, and the average duration of use among white women was 48 years. For cancers of the gum and buccal mucosa, oral snuff use among nonsmokers was related to years of use, with relative risks of 13.8 (three cases; 95% CI, 1.9–98.0) for 1–24 years, 12.6 (10 cases; 95% CI, 2.7–58.3) for 25–49 years and 47.5 (15 cases; 95% CI, 9.1–249.5) for 50 or more years of use. For cancer at other sites

of the mouse and of the pharynx, the corresponding relative risks were 1.7, 3.8 and 1.3. The findings relating to oral snuff use could not be explained by poor dentition (Winn *et al*., 1981b) or by use of mouthwashes (Blot *et al*., 1983). The consumption of fruit and vegetables was associated with a reduction in risk in the study population, and was primarily evident in cigarette smokers but not among oral snuff users (Winn *et al*., 1984). A subsequent additional analysis compared the findings on snuff use and oral and pharyngeal cancer among study subjects who responded for themselves and those for whom next of kin responded to the questions on tobacco use (Winn 1986). Odds ratios by cancer site and race tended to be higher for self-interview versus next-of-kin data. Among non-smokers and non-alcoholic beverage drinkers, the odds ratio for oral and pharyngeal cancer was 3.8 (81 cases; 95% CI, 2.3–6.3) for snuff use.

Stockwell and Lyman (1986) ascertained cases and controls from the population-based cancer registry in the state of Florida, USA, over a 1-year period in 1982. Cases were persons who had incident cancers of the lip, tongue, salivary glands, gum, floor of mouth, other parts of mouth, oropharynx, hypopharynx, pharynx (unspecified) and nasopharynx (ICD-O 140-149). All cases of cancer of the colon and rectum, cutaneous melanoma and endocrine neoplasia from the same source during same period formed the control group. Data on tobacco use were obtained from clinical and registry records, and were available for 79% of the 2351 study subjects data (82% of cases, 78% of controls). Odds ratios, adjusted for age, sex, race and tobacco use by anatomical site were: tongue, 2.3 (95% CI, 0.2–12.9); salivary gland, 5.3 (95% CI, 1.2–23.4); mouth and gum, 11.2 (95% CI, 4.1–30.7); pharynx, 4.1 (95% CI, 0.9–18.0); and nasopharynx, 5.3 (95% CI, 0.7–41.6). [A limitation of this study is that information on tobacco use was obtained from medical records. It seems improbable that all hospitals in Florida captured this information uniformly and it is possible that clinicians may have been more careful in obtaining medical record information from persons who had these head and neck cancers compared with patients who had other forms of cancer.]

In a case–control study in the USA, 623 patients with head and neck cancer were recruited. Cancers of the oral cavity, the oropharynx and the hypopharynx were used as cases and controls were patients with cancer of the salivary gland, nasopharynx and paranasal sinuses. Among men, 3.5% had ever used snuff or chewed tobacco regularly. The authors reported that “there were no statistically significant differences between cancer site groups on these users of tobacco” (Young *et al.*, 1986) [data not shown].

The population-based case–control study of Blot *et al.* (1988) enroled subjects from cancer registries in New Jersey, Atlanta metropolitan area, Santa Clara and San Mateo counties, and Los Angeles, USA. Cases included all black and white persons aged 18–79 years with incident, pathologically confirmed cancer (coded ICD-9 141–149), excluding cancer of the salivary gland (ICD-9 142) and cancer of the nasopharynx (ICD-9 147) from 1 January 1984 through to 31 March 1985. Random-digit dialling was used to ascertain controls aged 64 years or younger and Health Care Financing Administration (HCFA) was used for controls aged 65 years and older; controls were frequency-matched on age, sex and race to the cases. Structured questionnaires were administered by trained

interviewers in homes and next of kin responded for 22% of cases and 2% of controls. The response rate was 75 and 76% for cases and controls, respectively, and a total of 1114 cases and 1268 controls were included in the analysis. Among men, 6% of 762 cases and 7% of 837 controls used smokeless tobacco, mostly chewing tobacco. Nearly all tobacco chewers were smokers. Among women, 3% of 352 cases and 1% of 431 controls used snuff [odds ratio, 3.44]. Among nonsmoking women, the odds ratio for snuff was 6.2 (95% CI, 1.9–19.8), based on six cases and four controls who used snuff. Nonsmoking women primarily used snuff rather than chewing tobacco. All six cases had oral cavity cancer.

Spitz *et al.* (1988) identified cases who had histologically confirmed squamous-cell carcinoma of the tongue, floor of the mouth, oral cavity, orohypopharynx and larynx in white US residents, at the MD Anderson Hospital, Houston, TX, USA, from January 1985 through to February 1987. Laryngeal cancer accounted for 38% of the 131 male cases. Controls were patients at MD Anderson Hospital during the same period, were randomly selected and were frequency-matched on age (± 5 years) and sex; patients who had squamous-cell carcinoma of any site were excluded. The study included 185 cases (131 men and 54 women) and 185 controls aged 29–95 years. Self-administered questionnaires were part of the registration procedure. The authors reported that there was 'no difference in distribution of sites of malignancy for snuff users compared to all other cases'. Among men, the crude odds ratio for chewing tobacco was [1.0]. For women, the odds ratio for snuff use was 3.4 (95% CI, 1.0–10.9). There was no adjustment for smoking. All nine snuff dipping cases drank alcoholic beverages, seven also chewed tobacco, eight smoked cigarettes and one smoked cigars and pipes. Three of four snuff dipping controls also smoked cigarettes.

Newly diagnosed cases were identified from three hospitals in São Paolo, Curitiba and Goiânia, Brazil, and comprised carcinomas of the tongue, gum, floor of the mouth and other oral cavity (ICD-9 141, 143-145) diagnosed from 1 February 1986 to 30 June 1988 (Franco *et al.*, 1989). Two controls per case were identified from same or neighbouring general hospitals, were individually matched on sex, 5-year age group and trimester of hospital admission and excluded diagnoses of neoplasms or mental disorder. Cases were interviewed using a structured questionnaire in hospital and controls were interviewed privately. Four per cent of 232 cases and 3% of 464 controls used smokeless tobacco. The authors reported that use of smokeless tobacco and oral cancer were 'not associated'. The crude odds ratio was [1.4]. They noted that the relative risk estimates were independent of tobacco smoking or alcoholic beverage drinking, sex or anatomical site. [The Working Group noted that data on the manner in which adjustment was carried out for these factors were not shown and that confidence intervals or statistical significance were not reported.]

A population-based case–control study by Maden *et al.* (1992) enroled subjects from three urban counties of western Washington State, USA. Cases were men aged 18–65 years with in-situ and invasive squamous-cell cancers of the lip, tongue, gum, floor of the mouth, unspecified mouth and oropharynx diagnosed during 1985–89. Controls ascertained by random-digit dialling were frequency-matched to cases on age (5-year groups),

sex and year of diagnosis; 131 cases (54.4%) and 136 controls (63%) completed in-person questionnaire interviews at home or elsewhere. Of 131 cases, 15% used smokeless tobacco in contrast to 4% of 136 controls, which yielded an age-adjusted odds ratio of 4.5 (95% CI, 1.5–14.3). [The Working Group noted that smoking was not controlled for.]

Histologically confirmed oral and pharyngeal cancers (including cancers of the tongue, floor of the mouth, oropharynx and hypopharynx) were identified in one study (Marshall *et al.*, 1992) from 20 hospitals in three New York counties, USA, during the period 1975–83. Cases of black ethnicity were excluded. Cases were individually matched on neighbourhood, age (± 5 years) and sex. Of 513 cases contacted, 290 (56%) participated and 290 controls were included. The authors noted that "there was a risk associated with chewing tobacco, but it was insignificant, with very few people exposed". [The data to support this statement were not shown.]

Mashberg *et al.* (1993) identified 359 cases among black or white men who had in-situ or invasive squamous-cell carcinoma of the oral cavity or oropharynx in a Veterans hospital in New Jersey, USA, during 1972–83. A total of 2280 patients from the same series of clinical examinations who had no cancer or dysplasia of the pharynx, larynx, lung or oesophagus were recruited and interviewed in hospital between 1977 and 1982 and served as controls; 94% of study subjects participated. Only 52 cases and 255 controls had ever used smokeless tobacco. Chewing tobacco (odds ratio, 1.0; 95% CI, 0.7–1.4) and snuff (odds ratio, 0.8; 95% CI, 0.4–1.9) were not associated with oral cancer. No trend by duration of tobacco chewing was observed [data not shown].

Spitz *et al.* (1993) identified 108 white cases who had histologically confirmed cancers of the oral cavity (44), pharynx (31) and larynx (33) at MD Anderson Hospital, Houston, TX, USA, from June 1987 to June 1991. Controls who had no history of cancer were ascertained from blood and platelet donors and were frequency-matched to cases by age (± 5 years), race and sex. Patients completed a self-administered questionnaire in the hospital. The odds ratio for chewing tobacco was 1.2. Smoking was not controlled for.

Kabat *et al.* (1994) ascertained cases from 28 hospitals in eight cities in the USA. Cases had histologically confirmed cancers of the tongue, floor of the mouth, gums, gingiva, buccal mucosa, palate, retromolar area, tonsil and other pharynx during 1977–90. Controls were individually matched to cases on hospital, admission within 2 months after the case, age, sex and race, and excluded persons with diseases thought to be associated with tobacco or alcoholic beverages or prior history of tobacco-related cancers. The conditions among the controls were: 50% cancers (also including cancer of the stomach, endometrium and leukaemia), 7% benign neoplasms and 43% other diseases. A total of 1560 cases and 2948 controls were included. In-hospital questionnaire interviews were conducted with the study subjects. Among men, 6.1% of 1097 cases and 5.1% of 2075 controls chewed tobacco. Among women, less than 2% of 1336 subjects chewed tobacco. Among never-smoking men, 4.9% of 82 cases were regular chewers as were 2.2% of 448 controls, yielding an odds ratio of 2.3 (95% CI, 0.7–7.3). Among never-smoking women, there were no tobacco chewers. Among never-smoking women, 3.5% of 113 used snuff in contrast to 0% of 470 controls (odds ratio, 34.5; 95% CI, 8.5–140.1). Among never-

smoking men, 0% of 82 cases and 0.9% of 444 controls were snuff users. [The estimate of the odds ratio of 34.5 used 0.5 snuff-using controls.]

Patients aged 21–80 years diagnosed with histologically confirmed cancer of oral cavity and pharynx (ICD-9 141, 143–146, 148, 149) were recruited between 1981 and 1990 from hospitals in Illinois, Michigan, New York and Philadelphia, USA (Muscat *et al.*, 1996). Hospital patients with conditions unrelated to tobacco use were matched to cases by sex, age (± 5 years), race and date of admission (± 3 months). Response rates were 91% for cases and 97% for controls to yield 1009 cases (687 men, 322 women) and 923 controls (619 men, 304 women). A questionnaire interview was conducted with cases and controls. Among men, 5.5% of 687 cases used chewing tobacco at least once a week for 1 year or more as did 5.3% of 619 controls [crude odds ratio, 1.04]. No women used chewing tobacco. Among men, 1.3% of cases and 1.6% of controls used snuff at least once a week for 1 or more years [crude odds ratio, 0.81]. For women, the crude odds ratio for snuff use was [1.9].

Muscat *et al.* (1998) reported a hospital-based case–control study on salivary gland cancer. One hundred and twenty-eight patients with newly diagnosed histologically confirmed salivary gland cancer and 114 age- and gender-matched controls were interviewed. One case reported using snuff, and three cases and three controls were tobacco chewers.

A population-based case–control study was conducted by Schwartz *et al.* (1998) of in-situ and invasive (92%) squamous-cell cancers of the tongue, gum, floor of mouth, unspecified mouth, tonsils and oropharynx in persons aged 18–65 years during 1990–95 in counties of Seattle area, WA, USA. Controls were ascertained by random-digit dialling and were frequency-matched to the cases on sex and age in a 3:2 ratio of controls to cases; 284 cases (165 men, 119 women) and 477 controls (302 men, 175 women) completed an in-person questionnaire interview; response rates among cases and controls were 63.3% and 60.9%, respectively. Among men, 6.7% of 165 cases and 5.6% of 302 controls used smokeless tobacco (odds ratio, 1.0; 95% CI, 0.4–2.3). Only one female control used smokeless tobacco. [The Working Group noted that smoking was not controlled for.]

(iii) *Cross-sectional study*

A cross-sectional study (Sterling *et al.*, 1992) used two nationally representative surveys to examine the relationship between smokeless tobacco use and cancer of the oral cavity and digestive organs: the 1986 National Mortality Follow-back Survey and the 1987 NHIS. The 1986 National Mortality Follow-back Survey was based on a stratified probability sample of 18 733 decedents in 1986 who were 25 years or older at time of death. A questionnaire sent to their next of kin also included questions on use of smokeless tobacco. Information was obtained for 16 598 decedents. The NHIS annually surveys samples of the non-institutionalized civilian population using a multistage, probability sampling design. Interviewers administered a questionnaire to sample persons in the household. The 1987 NHIS obtained data on the use of smokeless tobacco. Using a reference category of less than 100 times lifetime use of smokeless tobacco, the relative risks for cancers of the oral cavity and pharynx (ICD-9 140–149) for 100–9999 and 10 000 or

more lifetime use were 0.9 (95% CI, 0.3–3.4) and 1.2 (95% CI, 0.3–4.6), respectively, adjusted for sex, race, smoking, alcoholic beverage consumption and occupational group. [The Working Group noted concerns due to uncertainty of the comparability of the two surveys.]

(iv) *Characteristics of oral cancer in smokeless tobacco users*

Link *et al.* (1992) studied a series of 874 squamous-cell carcinomas and 129 verrucous carcinomas. Compared with the squamous-cell carcinomas in non-users of smokeless tobacco, those in the 12 users of smokeless tobacco developed later (mean age, 72.6 versus 61.5 years) and occurred in the buccal mucosa vestibule (33.3% versus 7.7%). Compared with the verrucous carcinomas in non-users of smokeless tobacco, those in the 10 users of smokeless tobacco developed later (mean age, 70.5 versus 64.2 years) and were more likely to occur in the buccal mucosa vestibule (80.0% versus 31.2%).

The Tumor Registry of Wake Forest University Medical Center, Winston-Salem, NC, USA, was used to identify all patients with oral cancer seen at this institution between 1977 and 1991 (Wray & McGuirt, 1993). Of 160 cases who used smokeless tobacco (primarily snuff), 128 (119 women, nine men; mean age, 73.3 years) used only snuff. Only 1.6% had used smokeless tobacco for less than 20 years and 78% had used smokeless tobacco for more than 40 years; 80% of the tumours were located where the smokeless tobacco was customarily held — between the cheek and the gum. Only one non-squamous-cell cancer was observed.

(*b*) *Europe*

(i) *Cohort study*

A Norwegian cohort was comprised of two samples; one was a probability sample of the general adult population of Norway identified from the 1960 census and the other consisted of relatives of Norwegian migrants to the USA. Information on snuff use and smoking was collected through mailed questionnaires in 1964 and 1967; response rates were 79% of the probability sample in 1964 and between 88 and 93% in 1997. Of the cohort, 12 431 men were alive on 1 January 1966 and information on snuff use was available for 10 136. Cohort members were followed until December 2001 for cancer incidence using national cancer registries, date of emigration or date of death. The follow-up was 99.9% complete. Cancer incidence was coded according to ICD-7 (see Table 69). After adjustment for age and smoking, the relative risk associated with ever using snuff was 1.10 (nine cases; 95% CI, 0.5–2.4) for oral and pharyngeal cancer (ICD-7 141–148) (see Table 70). The relative risks for former and current users were of the same order of magnitude but were based on smaller numbers (Heuch *et al.*, 1983; Boffetta *et al.*, 2005).

(ii) *Case–control studies*

In a study from Sweden, 477 patients with cancers of the lip, oral cavity, maxillary sinus, nasopharynx, hypopharynx, oesophagus and larynx were compared with 333 patients with other malignancies seen in a hospital in Stockholm, during 1952–55 (Wynder &

Wright, 1957). Cases and controls were interviewed and their medical records were reviewed. More of the patients who had buccal and gum cancer used snuff than controls. There was suggestive evidence by ridit analyses that snuff use was related to buccal mucosal cancer in men; nearly half of the patients were habitual users of snuff and the majority had tumours in the area of the mouth where the quid was held. Tobacco smoking among the cancer cases was similar to that in controls. Other upper aerodigestive tract cancers were not associated with snuff use. [The response rate and the number of snuff users were not reported.]

Blomqvist *et al.* (1991) investigated the role of different risk factors for squamous-cell carcinoma of the lower lip. Fifty-seven men and four women, all treated at the department of plastic surgery at a hospital in Sweden, were interviewed. Age- and sex-matched controls were selected among non-tumour patients without a prior diagnosis of cancer [no further details on the selection of controls were reported]. Two of the cases and two of the controls reported using snuff only [in all further analyses, all types of tobacco consumption were combined into one exposure factor].

In a population-based case–control study of 161 cases who had intra-oral squamous-cell carcinoma and 400 controls drawn from the Danish Central Population Register, matched on age and sex, eight patients and 14 controls were using or had used chewing tobacco (Bundgaard *et al.*, 1995). [No risk estimate reported.]

Lewin *et al.* (1998) studied squamous-cell carcinoma of the head and neck in Stockholm and the southern regions of Sweden. Cases included cancer of the oral cavity, pharynx, larynx and oesophagus and were identified through the hospital departments that treated the majority of these cases and the regional cancer registries in 1988–91. Controls were selected as a stratified random sample from the population registries that covered the source population. The number of cases identified was 605 and the number of controls selected was 756; the participation rates were 90 and 85%, respectively. Of the 605 cases, 128 were cancers of the oral cavity and 138 were cancers of the pharynx. Exposure data, including snuff use, were collected by personal interviews conducted by two specially trained nurses. The relative risk for the whole case group was 1.0 (43 cases; 95% CI, 0.6–1.6) for current snuff use, 1.2 (40 cases; 95% CI, 0.7–1.9) for former snuff use and 1.6 (38 cases; 95% CI, 0.9–2.6) for use of > 50 g/week, after adjustment for smoking and alcoholic beverage consumption. In the subgroup of never smokers, the relative risk in the whole case group for ever users of smokeless tobacco was 4.7 (nine cases; 95% CI, 1.6–13.8); the relative risk for current use was 3.3 (95% CI, 0.8–12.0) and that for former use was 10.5 (95% CI, 1.4–117.8). When the analysis was restricted to cancer of the oral cavity, the relative risk was 1.0 (10 cases; 95% CI, 0.5–2.2) among current users, 1.8 (15 cases; 95% CI, 0.9–3.7) among former users and 1.7 (95% CI, 0.8–3.9) among users of more than 50 g/week. For cancer of the pharynx, the relative risks for current and former snuff use were 0.7 (eight cases; 95% CI, 0.3–1.5) and 0.8 (seven cases; 95% CI, 0.3–1.9), respectively.

A study in the northern region of Sweden comprised cases of oral cancer (ICD-7 140, 141, 143, 143–145) diagnosed in 1980–89 (Schildt *et al.*, 1998) and identified through

cancer registries. Of the 418 cases, 175 were alive at the time of the study and 235 deceased had relatives. Controls were matched on age, sex, county and vital status. For each living case, one control was selected from the population registry; for each deceased case with relatives, one deceased control was selected from the Cause of Death Registry. Controls were further matched on age, sex, county and, for deceased cases, on year of death. Exposure, including use of snuff, was assessed based on a postal questionnaire sent to the living subjects and to the next of kin for the deceased; 354 matched pairs were analysed. The relative risk was estimated to be 0.7 (39 cases; 95% CI, 0.4–1.1) for current snuff users and 1.5 (28 cases; 95% CI, 0.8–2.9) for former snuff users. After restriction to never-smokers, the corresponding relative risks were 0.7 (19 cases; 95% CI, 0.4–1.2) and 1.8 (nine cases; 95% CI, 0.9–3.5), respectively. For lip cancer, the relative risk was 1.8 (95% CI, 0.9–3.7) for former snuff users and 'close to unity' for current snuff users. [The Working Group noted that the odds ratio in former snuff users increased from 1.5 (95% CI, 0.8–2.9) to 3.0 (95% CI, 0.9–9.4) in an analysis restricted to live subjects. Further, there was only a relative weak effect of smoking (relative risk, 1.1; 95% CI, 0.7–1.6) in an analysis with simultaneous adjustment for snuff and alcoholic beverage use.]

(*c*) *India and Pakistan*

Many studies from South-East Asia combined all smokeless tobacco use into one category, which was frequently termed tobacco chewing. In these studies, tobacco chewing often includes chewing of betel quid with tobacco. All such studies have been included in the monograph on betel-quid and areca-nut chewing (IARC, 2004a) and are not included here. Studies that have reported separate results for tobacco chewing without betel quid are reviewed here.

Chandra (1962) selected 450 cases of cancer of the buccal mucosa registered in a hospital in Calcutta, India, during 1955–59, and used 500 of the friends or relatives who came to the hospital with the patients as controls. Cases and controls were approximately age matched. Tobacco chewing was reported by 6.3% of 287 cases and 4.2% of 410 controls among men and 3.1% of 163 cases and 2.2% of 90 controls among women. Relative risks for tobacco chewing compared with no chewing or smoking were [2.7] for men and [2.5] for women. [The author did not clarify whether the chewing habit was tobacco only or tobacco plus lime.]

A population-based prospective study was reported by Wahi *et al.* (1968) from a temporary cancer registration system established in Uttar Pradesh (Mainpuri district). Over a period of 30 months (1964–66), a total of 346 oral- and oropharyngeal cancer cases were detected and confirmed. Exposure data were obtained by questionnaire, and a house-to-house interview survey was conducted on a 10% cluster sample of the district population. The numbers in various exposure categories were then extrapolated to the population as a whole and used as denominators to calculate oral cancer 'period prevalence rates' for different types of tobacco chewing. Prevalence rates among non-chewers of tobacco and chewers of *Pattiwala* (sun-cured tobacco leaf with or without lime) were 0.36/1000 and 1.17/1000 (based on 84 exposed cases), respectively. [The Working Group noted that

differences in age between cancer patients and the population sample do not seem to have been taken into account; and it is possible that the prevalence of chewing within the population was age-dependent.]

Jafarey *et al.* (1977) reported a hospital-based case–control study in Pakistan. The cases were 1192 histologically diagnosed oral cavity and oropharyngeal cancers. The 3562 controls were matched for age, sex and place of birth. Among men, 4% of 683 cases and 3% of 1978 controls and, among women, 7.7% of 509 cases and 3% of 1584 controls chewed tobacco, yielding relative risks of 10.4 and 13.7, respectively, compared with those who neither chewed nor smoked. [The Working Group considered that, although the chewing in this study is reported as 'tobacco' chewing, in view of other publications by the same authors, it was probably chewing of tobacco and lime.] Eighty-four patients and 114 controls used *naswar* (tobacco, slaked lime and indigo) and 88 patients and 1690 controls did not chew. The relative risk associated with *naswar* use was 14.2. [The Working Group noted that potential confounding due to other tobacco-related practices was not adjusted for.]

Goud *et al*. (1990) reported a case–control study of 102 oral cancer cases from a hospital in Varanesi, India, and an equal number of age- and sex-matched controls selected from general and surgical wards. The odds ratios were [2.1] for *khaini* use, 3.7 for *zarda* use and 2.8 for *khaini* plus *zarda*. [It was not clear whether *khaini* and *zarda* were chewed by themselves or in some cases as an ingredient of betel quid. There was no mention of control for smoking.]

Wasnik *et al*. (1998) reported a matched case–control study of 123 cases of histologically confirmed 'oropharyngeal' cancers [ICD codes not specified — probably included oral and pharyngeal cancers] selected from three hospitals in Nagpur, India. Two control groups were used: one of 123 non-cancer patients and another of 123 patients with cancer at other sites [not specified]. Controls were matched for age and sex. Of the cases, 24 were tobacco chewers (excluding those who chewed betel quid) and 33 reported using tobacco-containing material for cleaning teeth [these may include betel-quid chewers]. Unadjusted odds ratios for the two control groups were 11.4 (24 cases; 95% CI, 4.4–29.6) and 23.7 (95% CI, 7.7–72.4) for chewing tobacco without betel quid and 4.1 (33 cases; 95% CI, 2.0–8.7) and 8.7 (95% CI, 3.3–22.9) for using tobacco-containing material for cleaning teeth. In a multivariate analysis, tobacco chewing (19.5% of cases) was combined with betel-quid chewing (63.4% of cases) and the odds ratio was 8.0 (95% CI, 4.9–14.8) when smoking, alcoholic beverage consumption, occupation and the use of tobacco-containing cleaning material were included in an unconditional logistic regression model. In the same model, the odds ratio for using tobacco-containing material for teeth cleaning was 5.2 (95% CI, 2.5–11.8).

Merchant *et al*. (2000) conducted a case–control study of 79 histologically confirmed primary oral squamous-cell carcinomas from three hospitals in Karachi, Pakistan. The 149 controls were selected from orthopaedic and general surgical wards, had no history of malignancy and were individually matched on hospital, sex and age (± 5 years). Ever use of *naswar* was reported by 13 cases and 10 controls to yield an odds ratio (adjusted for

cigarette smoking and alcoholic beverage consumption) of 9.5 (13 cases; 95% CI, 1.7–52.5).

(*d*) *Other parts of Asia*

One case of oral cancer was reported among 289 *naswar* users in the Kazakh SSR who underwent oral examination; no oral cancer was seen in 243 smokers or in 1480 persons who neither smoked nor used *naswar* (Aleksandrova, 1970).

Nugmanov and Baimakanov (1970) carried out a study in the Kazakh SSR in which the practices of oral cancer patients were compared with those of controls in relation to use of *naswar.* Of 93 oral cancer patients, 30.1% used *naswar* compared with only 6.7% of 247 controls. Further comparisons that involved 28 *naswar* users with oral cancer and 19 *naswar*-using controls revealed that patients with oral cancer used *naswar* more frequently and kept it in the mouth longer than controls. [The Working Group noted that the sources of cases and controls were not reported; confounding due to other tobacco-related practices was not adjusted for; and no adequate statistical analysis was performed.]

In a study from Saudi Arabia (Amer *et al*., 1985), 49% of 68 patients with oral cancer reported using *shammah*. [The Working Group noted that the actual percentage may be higher, since *shammah* is illegal in Saudi Arabia and there may be some reluctance to admit to its use.]

Ibrahim *et al*. (1986) reported on the association between use of smokeless tobacco products and the risk for squamous-cell carcinoma of the head and neck. Between December 1981 and December 1983, 38 patients who had oral cancer and 26 patients who had pharyngeal or laryngeal cancer were seen at the King Faisal Hospital, Saudi Arabia. Based on information from the patients' files and further questioning during follow-up, all the 38 patients with oral cancer either used *al-shammah* alone (16) or used both *al-shammah* and *alquat* (22). Fourteen of the 26 patients with pharyngeal and laryngeal cancer used *al-shammah* alone.

From February 1982 to December 1989, a total of 65 patients who had squamous-cell carcinoma of head and neck (21 cancers of the oral cavity, 35 cancers of the pharynx including 28 cancers of the nasopharynx and nine cancers of the larynx) were seen at the King Faisal Hospital (Al-Idrissi, 1990); 17 of the 65 cases (26.2%) had chewed a mixture of tobacco, pepper and oil (*al-shammah*) for an average of about 10 years. [The Working Group noted that this case series probably overlapped with that reported by Ibrahim *et al.* (1986).]

The records from the Tumour Registry of the King Faisal Specialist Hospital and Research Center, Saudi Arabia, from 1976 to 1995 were reviewed (Allard *et al*., 1999). Among a total of 26 510 cancer patients, the frequency of oral cancer was investigated, specifically for those primary sites located near the habitual placement of *shammah* (mucosa of the lower lip, lower gum, tongue, floor of the mouth, cheek mucosa, vestibule of mouth and retromolar area). Of the 794 such oral cancers, 35.4% were referred from the province of Jizan. The percentage of such oral cancer cases from this province was substantially higher than that of other oral cancers (6.2%), total malignant cases referred

to the hospital from that province (5.6%) and the population of that province (6.0%) when compared with the whole of Saudi Arabia. [The Working Group noted that no information about the frequency of smokeless tobacco use in the province of Jizan was provided.]

(*e*) *Africa*

Elbeshir *et al*. (1989) interviewed 62 of 78 consecutive cases of oral cancer seen at the Department of Oral Surgery, Dental School, Khartoum, Sudan. Fifty (81%) patients (30 men, 20 women) who used oral snuff (*saffa*), five smoked cigarettes and a pipe, four only smoked cigarettes and eight patients reported no use of tobacco in any form. The mean duration of *saffa* use was 30 years (range, 10–45 years); 82% of the cases had used *saffa* for 20 years or more and one patient (aged 17 years) started using *saffa* at the age of 7 years.

During the period 1970–85, 850 cases of oral cancer (ICD 140–145), including 646 squamous-cell carcinomas and 204 tumours of other histology, were referred to the Radiation and Isotope Centre Khartoum, the only centre in Sudan that offered radiotherapy and chemotherapy to cancer patients (Idris *et al*., 1995a). The squamous cell-carcinomas were classified into sites that had direct contact with the *toombak* quid (lip, buccal mucosa, floor of the mouth) (n = 375) and sites with less or no contact (tongue, palate, maxillary sinus) (n = 271). Information on *toombak* use or cigarette smoking, age, sex, area of residence and tribal origin was obtained from the cases and controls through questionnaires routinely administered to all patients admitted to the Centre. Among the three groups who had squamous-cell carcinomas with direct contact, with less or no contact and tumours of other histologies, 218 (58%), 52 (19%) and 23 (11%) used *toombak*, respectively. The corresponding numbers for cigarette smokers were 46 (12%), 29 (11%) and 21 (10%).

Using the same data, Idris *et al*. (1995b) investigated the association between use of *toombak* and carcinoma of the oral cavity in a case–control study. Squamous-cell carcinomas at sites with direct contact or with less or no contact were defined as case group 1 or case group 2, respectively, and the non-squamous-cell cancers served as control group 1. In addition, a second control group of 2820 volunteers who attended oral health education programmes in various regions of Sudan was recruited. For the first case group compared with never users of *toombak*, the odds ratios adjusted for age, sex, tribe and area of residence for *toombak* use were 7.3 (218 cases; 95% CI, 4.3–12.4) and 3.9 (95% CI, 2.9–5.3) for hospital and volunteer controls, respectively. Among users of *toombak* for > 11 years, the corresponding odds ratios were 11.0 (120 cases; 95% CI, 4.8–25.1) and 4.3 (95% CI, 2.9–6.3), respectively. Corresponding odds ratios for the second case group were moderately and statistically non-significantly increased compared with hospital controls and not increased compared with the control group of volunteers.

2.2.2 *Precancerous lesions*

Studies on the natural history of oral cancer suggest that several potentially malignant lesions and conditions precede the development of cancer of the oral cavity. Oral precancerous lesions of relevance are leukoplakia and erythroplakia (Pindborg *et al.*, 1996).

(*a*) *North America*

(i) *Cross-sectional studies*

A number of cross-sectional studies or case series in the USA have reported prevalences of oral soft-tissue lesions among smokeless tobacco users (Greer & Poulson, 1983; Wolfe & Carlos, 1987; Creath *et al.*, 1988; Cummings *et al.*, 1989; Ernster *et al.*, 1990; Grady *et al.*, 1990; Creath *et al.*, 1991; Greene *et al.*, 1992; Kaugars *et al.*, 1992; Little *et al.*, 1992; Sinusas *et al.*, 1992; Grasser & Childers, 1997; Tomar *et al.*, 1997; Martin *et al.*, 1999) (Table 72).

All studies showed higher prevalences of oral soft-tissue lesions in smokeless tobacco users compared with tobacco non-users; in those studies that distinguished between chewing tobacco and snuff (Ernster *et al.*, 1990; Grady *et al.*, 1990; Greene *et al.*, 1992; Sinusas *et al.*, 1992; Tomar *et al.*, 1997), a higher prevalence was observed both with chewing tobacco and with snuff.

In those studies that controlled for smoking, the relative risks for oral leukoplakia in smokeless tobacco users exceeded those of non-users for smokeless tobacco overall (Ernster *et al.*, 1990; Tomar *et al.*, 1997; Martin *et al.*, 1999), for snuff (Ernster *et al.*, 1990; Tomar *et al.*, 1997; Martin *et al.*, 1999) and for chewing tobacco (Ernster *et al.*, 1990; Tomar *et al.*, 1997).

Strong dose–response relationships were observed between intensity and duration of use of smokeless tobacco, snuff or chewing tobacco. Increasing use of smokeless tobacco was associated with increasing prevalences of mucosal lesions whether measured by hours per day with tobacco in the mouth (Ernster *et al.*, 1990; Greene *et al.*, 1992; Tomar *et al.*, 1997), amounts used (Creath *et al.*, 1988; Ernster *et al.*, 1990; Greene *et al.*, 1992; Martin *et al.*, 1999), shorter time since last used (Ernster *et al.*, 1990; Greene *et al.*, 1992), duration of use in months or years (Creath *et al.*, 1988; Ernster *et al.*, 1990; Greene *et al.*, 1992; Tomar *et al.*, 1997; Martin *et al.*, 1999) or frequency of use in days per month (Tomar *et al.*, 1997). Dose–response relationships were reported separately for chewing tobacco (Ernster *et al.*, 1990; Tomar *et al.*, 1997; Martin *et al.*, 1999) and for snuff (Ernster *et al.*, 1990; Tomar *et al.*, 1997).

Prevalences or prevalence odds ratios for oral lesions were higher in current than in former users, and former users had higher prevalences or prevalence odds ratios than never users (Ernster *et al.*, 1990; Grady *et al.*, 1990; Creath *et al.*, 1991; Greene *et al.*, 1992; Sinusas *et al.*, 1992; Tomar e*t al.*, 1997).

Overall prevalence of lesions was higher among snuff users compared with tobacco chewers (Ernster *et al.*, 1990; Grady *et al.*, 1990; Greene *et al.*, 1992; Kaugars *et al.*, 1992; Sinusas *et al.*, 1992; Grasser & Childers, 1997; Tomar *et al.*, 1997; Martin *et al.*,

Table 72. Use of smokeless tobacco and prevalence of precancerous lesions in cross-sectional studies in the USA

Reference, study location, period	Study population	Prevalence of use; type of tobacco product	Type of lesions[a]	Exposure category	Prevalence of lesions (%)	Relative risk (95% CI or *p* value)	Adjustment for potential confounders; comments
Greer & Poulson (1983)[a], Denver, CO	1119 adolescents in grades 9–12	10.4% [current] users of smokeless tobacco	Mucosal alterations according to own classification (degree 1–3)	Non-user User Severity of lesions Degree 1 Degree 2 Degree 3	0 42.7 50 36 14		 Distribution of lesions among users with lesions (n = 50)
Wolfe & Carlos (1987), New Mexico [not reported]	226 Navajo American adolescents, aged 14–19 years	Use within last 7 months, 64.2%, of which 58.6% used snuff, 4.8% chewing tobacco, 36.6% both	Leukoplakia according to Greer & Poulson (1983) (degree 1–3)	Non-user User Degree 2 lesion Degree 3 lesion *Duration (years)* ≤ 1 2 3 4 ≥ 5 *Frequency of use* ≤ 1 day/week 1–2 days/week 3–4 days/week ≥ 5 days/week	3.7 25.5 4.1 8.3 13.3 15 38.5 62.5 21.1 11.9 33.3 42.9 40.0	1.0 8.9 (p = 0.001) 3.6 4.1 10.4 16.9 5.7	34/37 lesions coincided with the reported site of habitual quid placement.
Cummings *et al.* (1989), Buffalo, NY, 1985	25 professional baseball players, aged 22–44 years; participation rate, 93%	76% ever use; chewing or dipping tobacco	Soft-tissue lesion diagnosed by dental oncologist	Non-user Ever user	0 26.3		In 4/5 subjects, lesion occurred at the location where tobacco was held

Table 72 (contd)

Reference, study location, period	Study population	Prevalence of use; type of tobacco product	Type of lesions[a]	Exposure category	Prevalence of lesions (%)	Relative risk (95% CI or *p* value)	Adjustment for potential confounders; comments
Ernster *et al.* (1990); Grady *et al.* (1990), Countrywide, 1988	1109 professional baseball players; participation rate, 85%	42% current, 4% occasional, 13% former; among current users, 75% used snuff, 21% chewed tobacco	Oral leukoplakia /erythroplakia, diagnosed by specially trained dentist, graded 1-4 (categories similar to those of Greer & Poulson, 1983)	Non-user	1.4	1.0	Adjustment for age, race, cigarette smoking, alcoholic beverage consumption and dental hygiene did not change results significantly; no chewing tobacco user had a degree 3 or 4 lesion; histology of lesions described in Daniels *et al*. (1992); 94% of lesions located in the mandibular area, including 42% in the anterior area
				Former user	1.4	1.0 (0.2–5.0)	
				Occasional	2.5	1.8 (0.2–14.5)	
				Current	46.3	60.0 (27.8–129.5)	
				Chewing	17.2	14.5 (5.7–36.7)	
				Snuff	55.6	86.9 (39.9–189.5)	
				Amount used			
				Snuff (can/week)			
				< 1	36.4	39.8 (17.3–91.7)	
				2–3	69.2	156.2 (66.5–367.1)	
				> 4	83.6	354.1 (129.2–970.2)	
				Chew (pouches/week)			
				< 1	12.5	8.5 (3.0–32.9)	
				2–3	16.7	12.3 (3.8–51.3)	
				> 3	33.3	30.8 (9.4–128.3)	
				Duration of use (years)			
				≤ 3	32.4	33.2 (14.2–77.9)	
				4–6	52.0	75.1 (33.4–169)	
				7–9	52.7	77.4 (32.3–185)	
				≥ 10	50.0	69.4 (29.4–164)	
				Hours in mouth/day			
				0–0.5	24.5	22.6 (9.5–53.7)	
				> 0.5–1.0	42.8	52.1 (22.2–122)	
				> 1.0–1.5	53.6	80.1 (32.2–199)	
				> 1.5–2.0	67.5	144 (53–391)	
				> 2.0–4.0	62.5	115 (46.0–291)	
				> 4.0	83.8	361 (107–1215)	
				Time since last use (hours)			
				> 24	18.6	15.9 (5.9–42.9)	
				> 12–24	22.7	20.4 (8.4–49.3)	
				> 1–12	55.1	85.2 (37.1–195)	
				≤ 1	74.3	201 (84.9–475)	
				Type of snuff			
				Copenhagen	61.3	111 (50.1–246)	
				Skoal	54.0	81 (33–199)	
				Hawken	5.3	3.9 (0.5–33.0)	

Table 72 (contd)

Reference, study location, period	Study population	Prevalence of use; type of tobacco product	Type of lesions[a]	Exposure category	Prevalence of lesions (%)	Relative risk (95% CI or *p* value)	Adjustment for potential confounders; comments
Creath *et al*. (1988, 1991), Alabama [not reported]	1116 adolescent football players, aged 10–19 years	4.8% current, 30.2% former; among current users, 35 used snuff, 7 used chew and 12 used both.	Oral leukoplakia diagnosed by dentist, according to Axéll *et al*. (1984)	Non-user Ever user Skoal Copenhagen Former user Current user	0.5 3.0 1.2 13	1.0 6.3 ($p < 0.005$) 21.1 ($p < 0.01$) 5.8 ($p < 0.001$)	Current user = having used for at least 6 months and still using it; former user = stopped at least 1 month before study; 13/15 lesions in the mandibular vestibule retromolar areas
Greene *et al.* (1992), Countrywide, 1989–90	894 professional baseball players recruited in 1989–90	37% current users (within week of interview)	Oral leukoplakia/ erythroplakia, diagnosed by specially trained dentist, graded 1–4 (categories similar to those of Greer & Poulson, 1983)	Non-user Former Current Seasonal use Year-round use Snuff Copenhagen Skoal Hawken Chewing tobacco	2.9 3.5 51.7 32.1 66.7 61.2 72.3 42.6 11.1 14.8	1.0 36.0	Extension of studies by Ernster *et al.* (1990) and Grady *et al.* (1990); degree 3–4 lesions found only in current users; prevalence available by seasonality of use for numerous variables
Kaugars *et al.* (1992), Virginia [not reported]	347 users of smokeless tobacco recruited by advertisement; all white men aged 14–77 years	Use for at least 6 months	Epithelial dysplasia, graded 1–4 (focally mild-mild-moderate-severe)	All users Degree 3–4 lesions Snuff Chewing tobacco	[13] [0.9] [14.4] [8.3]		All lesions at the site of placement of the tobacco in the mouth

Table 72 (contd)

Reference, study location, period	Study population	Prevalence of use; type of tobacco product	Type of lesions[a]	Exposure category	Prevalence of lesions (%)	Relative risk (95% CI or *p* value)	Adjustment for potential confounders; comments
Little *et al.* (1992), Oregon, Washington	245 out-patients drawn from Kaiser Permanente Dental Care Program who used smokeless tobacco	100% user (use during the previous week)	Soft-tissue lesions diagnosed by dental hygienist according to Greer & Poulson (1983)	Overall	79		85% of lesions at the placement of tobacco
				Severity of lesions			*Read from graph
				Grade I	28		
				Grade II	27		
				Grade III	23		
				Grade III lesions			
				Frequency of use			
				≤ 2 days/week	7		
				3–6 days/week	7*		
				Daily	33		
				Times/day			*Read from graph
				< 2 (*n* = 24)	0		
				2–5	18		
				≥ 6 (*n* = 59)	37		
				Duration (years)			
				≤ 2	13		
				2–5	18*		
				6–10	18*		
				≥ 11	47		
Sinusas *et al.* (1992), 1990	2006 professional baseball players of major and minor leagues	42.7% current use, 16.5% former use; moist snuff, chewing tobacco, plug tobacco, exclusively or in combination	Leukoplakia diagnosed by specially trained doctor, according to modification of the classification system by Greer & Poulson (1983) (degree 1–3)	Never user	6		One degree 3 lesion present in a year-round user of chewing tobacco
				Former user	6		
				Seasonal user	8		
				Year-round user	37	9.35 (3.46–26.21)	
				Type of tobacco			
				Moist snuff	34.2		
				Chew or plug	16.7		
Grasser & Childers (1997), North Carolina, 1995	214 soldiers aged 18–47 years	7% current, 7% former; smokeless tobacco undefined	Oral leukoplakia, diagnosed by board-certified oral pathologist	Non-user	1 lesion		
				Chew	0 lesion		
				Snuff	4 lesions		

Table 72 (contd)

Reference, study location, period	Study population	Prevalence of use; type of tobacco product	Type of lesions[a]	Exposure category	Prevalence of lesions (%)	Relative risk (95% CI or *p* value)	Adjustment for potential confounders; comments
Tomar *et al*. (1997), Countrywide excluding Alaska, 1986–87	17 027 school children aged 12–17 years; response rate, 78%	3.1% smokeless tobacco (2.0% snuff, 1.5% chewing tobacco)	'Smokeless tobacco lesions' according to Greer & Poulson (1983) criteria, diagnosed by trained dental examiners	All subjects	1.5		Adjusted for age, cigarette smoking (current, former, never) and alcoholic beverage consumption (current, former, never); data on prevalence of lesions of different degrees (1–3) by duration of use, frequency of use and exposure time, for snuff and for chewing tobacco separately; 65% of lesions located in mandibular buccal vestibules, of which 24% in anterior labial vestibule or labial mucosa
					Snuff	*Chewing tobacco*	
				Current	18.4 (8.5–39.8)	2.5 (1.3–5.0)	
				Former	2.4 (1.0–6.1)	1.3 (0.7–2.2)	
				Duration (months)			
				< 1	1.0	1.0	
				1–12	8.1 (3.8–17.4)	2.0 (0.6–6.1)	
				13–24	23.3 (10.5–51.4)	6.6 (1.7–25.2)	
				> 24	58.9 (21.3–162)	13.4 (6.1–29.5)	
				Frequency (days/month)			
				0	1.0	1.0	
				1–14	4.2 (1.6–11.4)	2.9 (1.1–7.9)	
				15–29	7.9 (2.9–21.7)	4.8 (1.3–18.2)	
				30–31	51.4 (19.7–134)	12.1 (5.5–26.5)	
				Min/day in the mouth			
				< 1	1.0	1.0	
				1–30	9.5 (4.3–20.7)	2.8 (1.1–7.1)	
				31–105	14.6 (5.5–39.0)	6.3 (2.7–14.5)	
				> 105	26.7 (9.8–72.9)	11.1 (4.3–29.1)	
Martin *et al*. (1999), Texas, 1996	3051 male US Air Force trainees; participation rate, 99.97%	9.9% current users, of whom 93.4% used snuff, 6.6% chewed	Oral leukoplakia	*Duration of use* (*months*)			Percentage of severe lesions according to duration of use and amount used per day available; 97% of lesions found in the mandibular buccal or labial sulcus
				1–12	14.8	11.2 (5.5–22.6)	
				13–24	30.9	28.8 (15.1–54.1)	
				25–48	48.1	59.9 (34.0–105)	
				> 49	70.8	156 (81.0–303)	
				Cans snuff /day			
				< 1/2	29.6	24.0 (14.6–39.2)	
				1/2–1	44.8	46.0 (25.4–83.6)	
				> 1	63.0	108.2 (59.8–196.9)	
				Chewing tobacco	5.0	3.4 (0.08–22.3)	
				Type of snuff			
				Copenhagen	54.7	77.7 (43.4–139.6)	
				Skoal	38.3	40.0 (24.4–65.7)	
				Kodiak	36.2	36.5 (17.8–74.9)	

CI, confidence interval

[a] Greer and Poulson (1983) established a classification into three degrees of severity (instead of four degrees used previously) to be applied to persons who have used smokeless tobacco four years or less.

1999). In two studies (Ernster *et al.*, 1990; Martin *et al.*, 1999), no chewing tobacco user had a severe lesion.

Among snuff users, the prevalence of lesions and the relative risk varied depending on the brand used (Grady *et al.*, 1990; Greene *et al.*, 1992; Creath *et al.*, 1991; Martin *et al.*, 1999). Relative risks for chewing tobacco were much less variable (odds ratio range, 10–15) (Grady *et al.*, 1990; Greene *et al.*, 1992)

In those studies that noted where the tobacco was placed within the mouth (Greer & Poulson, 1983; Wolfe & Carlos, 1987; Cummings *et al.*, 1989; Creath *et al.*, 1991; Kaugars *et al.*, 1992; Little *et al.*, 1992), most or all lesions were at that site. Several other studies mentioned over 90% of the lesions in the mandibular area (Ernster *et al.*, 1990; Creath *et al.*, 1991; Tomar *et al.*, 1997; Martin *et al.*, 1999).

(ii) *Severity of lesions*

A few studies conducted analyses that distinguished between lesions of lower severity (degree ≤ 2) and lesions of higher severity (degree 3 and 4). The results of these studies are detailed below.

Wolfe and Carlos (1987) found no consistent relationship between any of the characteristics of smokeless tobacco use and the severity of leukoplakia [data not shown].

In a 3-year study in seven major league baseball teams and their associated minor league teams in the USA conducted in 1988–90, over 1000 players received an oral examination and completed a questionnaire on tobacco use (Ernster *et al.*, 1990; Grady *et al.*, 1990; Daniels *et al.*, 1992; Greene *et al.*, 1992). There was a significant increase in the percentage of more severe leukoplakia lesions (degree 3 and 4) with increasing amount of use, longer duration of use, shorter time since last use and use of snuff; no chewing tobacco user had a severe lesion. Histological examination of 142 lesions showed a prevalence of 4% of basal-cell hyperplasia in snuff users while none occurred in the users of chewing tobacco (Daniels *et al.*, 1992). In the study by Little *et al.* (1992), frequency and duration of smokeless tobacco use were strongly related to the severity of lesions.

In the study of the National Survey of Oral Health of US schoolchildren (Tomar *et al.*, 1997), conducted in 1986–87, schoolchildren in a probability sample of schools were examined and completed a questionnaire with an interviewer. Complete data on tobacco and alcoholic beverage consumption were available for 17 027 children aged 12–17 years. Degree 2 lesions were observed in 14.8% of current snuff users, 3.3% of former users and 0.8% of never users. The corresponding prevalences for degree 3 lesions were 3.0, 0.3 and 0.1%, respectively. The prevalences of degree 2 lesions in tobacco chewers were 7.9%, 2.2% and 1.3%; and those for degree 3 lesions were 2.6, 0.3 and 0.1%, respectively. The prevalence of degree 2 and degree 3 lesions increased with duration of use in months, frequency used per week and exposure time in the mouth for both snuff and chewing tobacco.

Martin *et al.* (1999) calculated the percentage of level I and that of level II or III lesions according to duration of use and amount used per day. They found a significant increase in the percentage of severe lesions with increased length of use and increased

amount used per day. In a logistic regression analysis, length of use was the only predictor of the severity of the lesions (odds ratio, 1.14; 95% CI, 1.01–1.29). The only lesion seen in a tobacco chewer was level I.

(iii) *Reversal of lesions*

Grady *et al.* (1991) examined 1031 male professional baseball players for oral lesions. Of these, 389 were current smokeless tobacco users and 185 had oral lesions. Those with leukoplakia were asked to return for biopsies 1–21 days after the initial examination, and 131 players complied. In the time between examinations, 15% of the lesions resolved and 18% improved by one degree. The lesions most likely to have resolved were smaller lesions in players who decreased or stopped smokeless tobacco use, among users of chewing tobacco compared with those of snuff, among light users and among seasonal users only. Duration of smokeless tobacco use and the number of days between the initial examination and follow-up examination were not associated with the disappearance or regression of lesions.

In a study at a US Air Force camp, male basic trainees were examined upon entry to camp, between 2 and 6 days after they had last used smokeless tobacco (Martin *et al.*, 1999). Of the 302 smokeless tobacco users, 119 had oral leukoplakia. At the end of the 6 weeks of cessation of tobacco use during training, 109 of the 119 were re-examined and 97% of the lesions had completely resolved.

(iv) *Progression of lesions*

Between 1988 and 1991, 70 patients with advanced oral leukoplakia were enroled in an intervention study to assess the efficacy of various chemopreventive treatments (all patients were treated). The relative risk for developing oral cancer in the one patient who chewed tobacco compared with the 21 who did not chew tobacco was 0.6 (95% CI, 0.2–1.6) (Lee *et al.*, 2000). [No information was given on the assessment of tobacco use or on the etiology of oral leukoplakia.]

(*b*) *Europe*

(i) *Prevalence of precancerous lesions*

A study of five coal mines in South Lancashire, United Kingdom (Tyldesley, 1971), revealed that, among 1490 miners, 1.7% of surface workers and 34.3% of underground workers chewed tobacco. Of these, 91.2% also smoked cigarettes. In a subanalysis of 280 chewers and 122 non-chewers, none of the non-users had leukoplakia compared with 3.6% of the chewers. In all cases, leukoplakia was observed at multiple sites. All patients with lesions were also smokers.

A randomly selected sample of 918 adults living in a Swedish county was examined for the presence of oral mucosal lesions. None of the women and 79 (17.6%) of the men dipped snuff. Among them, 58 used snuff only, 16 used snuff and smoked cigarettes and five used snuff and smoked a pipe. The prevalence of oral leukoplakia was 2.8% among

men and 1.1% among women; none of the lesions occurred among snuff users (Salonen *et al.*, 1990).

(ii) *Malignant transformation*

Among 450 patients with leukoplakia recorded between 1956 and 1970 at the University Hospital of Copenhagen, Denmark, 32 were snuff users. A 1-year follow-up was conducted for 394 patients. Among them, two of the snuff-induced lesions became malignant or dysplastic, which corresponded to a transformation rate of 6.2%. In contrast, 19.5% of the other leukoplakia patients developed carcinoma or showed dysplasia (Roed-Petersen & Pindborg, 1973).

(*c*) *India*

Because of the high prevalence of chewing betel quid with or without tobacco in South-East Asia, and particularly in India, many studies that investigated the prevalence of smokeless tobacco use did not dissociate the use of mixtures that included tobacco from those that did not. For this reason, it is difficult to assess precancerous lesions associated with smokeless tobacco only.

A case–control study design was applied to the baseline data of a cross-sectional study in Kerala, India, of a population screened by oral visual inspections that included 927 cases of oral leukoplakia (411 women, 516 men) and 47 773 population-based controls with no oral disease (29 876 women, 17 897 men). Interviews were conducted with structured questionnaires by health workers. Clinical diagnosis of oral precancers was confirmed by dentists and oncologists. For men and women who consumed only chewing tobacco combined, the odds ratio for leukoplakia adjusted for age, sex, education, body mass index, pack–years of smoking and years of alcoholic beverage drinking was 30.9 (eight cases; 95% CI, 13.7–69.7). For both sexes combined in an analysis restricted to nonsmokers and non-drinkers who consumed only chewing tobacco, the odds ratio for leukoplakia adjusted for age, sex, education and body mass index was 263.0 (three cases; 95% CI, 68.5–∞) (Jacob *et al.*, 2004). One tobacco-only chewer had multiple premalignant lesions (Thomas *et al.*, 2003).

(*d*) *Other parts of Asia*

A cross-sectional study on the prevalence of oral cancer and precancerous lesions among 674 consecutive dental patients carried out in Riyadh, Saudi Arabia, described 13 patients with oral leukoplakia, three of whom used *shammah*. The other lesions occurred among cigarette and *shisha* smokers (Mani, 1985).

A study conducted in Gizan province, Saudi Arabia, included 661 Saudi citizens aged ≥ 15 years. *Shammah* was used by 28% of the study population. Of the surveyed population, 129 (19.5%) had lesions of the oral mucosa diagnosed clinically as leukoplakia. All affected subjects reported the use of *shammah* for more than 5 years (Salem *et al.*, 1984). The lesions were almost always at the site where *shammah* was habitually held.

Male residents of nine villages in one local authority district in the Samarkand Oblast of Uzbekistan were invited to attend a medical examination, which included an interview concerning *naswar* use, smoking and alcoholic beverage consumption (Zaridze *et al.*, 1986). A total of 1569 residents were interviewed and had oral examination, of whom 42% reported using *naswar*. Oral leukoplakia was diagnosed in 127 (8%) individuals, with a total of 144 lesions. The most frequent sites of these lesions were the floor of the mouth, the lower surface of the tongue and the tip of the tongue (38%). [*Naswar* is usually placed under the tongue.] The prevalence of leukoplakia was highest among individuals who both smoked and used *naswar* (21%). Among nonsmokers, the prevalence of leukoplakia was 2.2% among *naswar* non-users, 11.5% among former users and 12% among current users.

A built-in case–control study was carried out to investigate the possible relationship between *naswar* use and practices and the risk for leukoplakia. A total of 191 cases were defined as having oral leukoplakia, while 466 controls were free of leukoplakia. Use of *naswar* was significantly associated with the risk for oral leukoplakia in ever users (3.8; 95% CI, 2.6–5.6), in former users (3.0; 95% CI, 1.1–8.3) and in current users (3.9; 95% CI, 2.6–5.5), adjusted for smoking, alcoholic beverage consumption and age. A significant dose–response relationship was observed with earlier age at start of *naswar* use ($p = 0.027$), duration of use ($p < 0.001$), daily frequency of use ($p < 0.001$) and lifetime intake of *naswar*, calculated as reported daily frequency at the time of interview multiplied by years of use ($p < 0.001$) (Evstifeeva & Zaridze, 1992).

(*e*) *Africa*

Idris *et al.* (1996) reported on 281 Sudanese subjects (229 men, 50 women and two sex not recorded) with distinctive *toombak*-associated oral lesions that were detected from a random population sample of 5500 persons during a house-to-house survey in northern Sudan. Subjects were interviewed regarding their tobacco habits. *Toombak*-related mucosal lesions were recorded according to a four-point scale proposed by Axéll *et al.* (1984). The majority of the cases had lesions in the anterior lower labial sulcus, the predominant site for snuff dipping among Sudanese. A strong association between the severity of the mucosal lesions and longer lifetime duration (> 10 years) of *toombak* use was found. None of the most severe lesions (degree 4) occurred among subjects with less than 10 years of use.

Ahmed *et al.* (2003) applied exfoliative cytology to 300 volunteers (100 exclusive *toombak* users, 100 exclusive cigarette smokers and 100 non-users of any form of tobacco) to assess the presence and severity of epithelial atypia. Cytological smears were obtained for all subjects from the buccal or labial mucosa, the sites where *toombak* quids are placed. Moderate and severe epithelial atypia was detected in seven of 300 study subjects, in five of 100 *toombak* dippers, in two of 100 cigarettes smokers and in none of the non-users of any form of tobacco.

2.2.3 *Cancer of the oesophagus*

Table 73 summarizes the case–control studies of smokeless tobacco and cancer of the oesophagus.

(*a*) *America*

The population-based case–control study by Williams and Horm (1977), described in Section 2.2.1, also reported on oesophageal cancer. Among men, the relative risk for moderate use of chewing tobacco or snuff based on two exposed cases was 0.9, adjusting for age, race and smoking.

Cases of oesophageal cancer, primarily (85%) squamous-cell carcinomas, ascertained in 1982–84 in selected hospitals in South Carolina, USA, were matched with two hospital controls per case by hospital, race and age (± 5 years). In addition, oesophageal cancer deaths among men who were residents of eight coastal counties of South Carolina were identified in 1977–81 and matched by race, age, county of residence and year of death to decedents who died from other causes. Controls with a diagnosis at admission or cause of death related to alcoholic beverages or diet were excluded. A total of 207 cases and 422 controls were included in the study. Users of smokeless tobacco were defined as those who had used at least one pouch or plug of chewing tobacco or a small can of snuff per week for at least 1 year. Relative to non-users of tobacco, the odds ratio for users of smokeless tobacco only was 1.7, and 1.2 (95% CI, 0.1–13.3) when adjusted for study series and alcoholic beverages (Brown *et al.*, 1988).

(*b*) *Europe*

(i) *Cohort study*

In the Norwegian cohort study (Boffetta *et al.*, 2005) described in Section 2.2.1, the relative risk for oesophageal cancer was 1.4 (nine cases; 95% CI, 0.6–3.2) for ever use compared with never use of snuff and adjusted for age and smoking (Table 74).

(ii) *Case–control studies*

The case–control study by Lewin *et al.* (1998) (see Section 2.2.1) reported results separately for oesophageal cancer. The relative risks for current and former versus never use of snuff were 1.1 (10 cases; 95% CI, 0.5–2.4) and 1.3 (nine cases; 95% CI, 0.6–3.1), respectively, after adjustment for age, smoking and alcoholic beverage intake. The relative risk for users of ≥ 50 g/week was 1.9 (95% CI, 0.8–3.9).

All patients with a new diagnosis of adenocarcinoma of the oesophagus or gastric cardia and half of the patients with oesophageal squamous-cell carcinoma were included in a population-based study that comprised the whole population of Sweden (< 80 years) during 1995 through to 1997 (Lagergren *et al.*, 2000). Cases were identified from all clinical departments in Sweden that were involved in the treatment of these diagnoses as well as from local tumour registries. Controls were randomly selected from the study population and frequency-matched for age and sex to the oesophageal adenocarcinoma

Table 73. Case–control studies of smokeless tobacco use and cancer of the oesophagus

Reference, study location, period	Characteristics of cases	Characteristics of controls	Exposure categories	No. of exposed cases	Relative risk (95% CI)	Adjustment for potential confounders	Comments
North America							
Williams & Horm (1977), USA, 1969–71	Cancer of the oesophagus from 7518 (57% of randomly selected) incident invasive cancers who participated in the population-based Third National Cancer Survey	Cancer at sites unrelated to tobacco	Moderate use Heavy use	2	0.9 –	Age, race, smoking	No exposed cases among women
Brown *et al.* (1988), USA, 1982–84 (cancer cases) 1977–81 (cancer deaths)	207 from selected hospitals in South Carolina; deaths in 8 coastal counties; 74 incident male oesophageal cancer cases (85% squamous-cell carcinoma), ≤ 143 male oesophageal cancer deaths, aged ≤ 79 years; response rate, 85% (incident cases), 94% (deceased cases and controls)	422; 157 hospital patients matched on hospital, race, age ± 5 years; ≤ 285 deaths, matched on race, age, county of residence, year of death; controls with diagnosis at admission or cause of death related to alcoholic beverages or diet excluded; response rate, 95% (hospital controls)	Non-user of tobacco Smokeless tobacco only		1.0 1.2 (0.1–13.3)	Study series and alcoholic beverages	Use defined as at least one pouch or plug of chewing tobacco or a small can of snuff per week for ≥ 1 year
Europe							
Lewin *et al.* (1998), Stockholm and southern Sweden, 1988–91	605 including 123 cancers of the oesophagus from hospitals and cancer registries, 40–79 years old; overall response rate, 90%	756 from the population registry; stratified by region and age; response rate, 85%	Current snuff use Former snuff use > 50 g/week	10 9	1.1 (0.5–2.4) 1.3 (0.6–3.1) 1.9 (0.8–3.9)	Age, region, smoking, alcoholic beverages	

Table 73 (contd)

Reference, study location, period	Characteristics of cases	Characteristics of controls	Exposure categories	No. of exposed cases	Relative risk (95% CI)	Adjustment for potential confounders	Comments
Lagergren *et al.* (2000), Sweden, 1995–97	Incident adeno-carcinomas of the oesophagus (189), 87% men; gastric cardia cancers (262), 85% men; squamous-cell cancers of the oesophagus (167), 72% men; < 80 years old; response rates, 87%, 83%, 73%, respectively	820 frequency-matched to adenocarcinoma of the oesophagus by age, sex; response rate, 73%	*Oesophagus (squamous-cell carcinoma)* Never use of snuff Ever use of snuff > 25 years of use *Oesophagus (adeno-carcinoma)* Never use of snuff Ever use of snuff > 25 years of use *Gastric cardia (adenocarcinoma)* Never use of snuff Ever use of snuff > 25 years of use	 134 33 14 154 35 15 209 53 15	 1.0 1.4 (0.9–2.3) 2.0 (0.9–4.1) 1.0 1.2 (0.7–2.0) 1.9 (0.9–4.0) 1.0 1.2 (0.8–1.8) 1.1 (0.6–2.2)	Age, sex, tobacco smoking, alcoholic beverages	Additional results by intensity of snuff use reported in text
Asia							
Phukan *et al.* (2001), India, 1997–98	502 (358 men, 144 women) histologically confirmed cancers of the oesophagus (predominantly squamous-cell cancer) from one hospital; response rate, 94%	Two visitors matched for age, sex	*Men* Non-chewer/nonsmoker **Chadha* chewer *Women* Non-chewer/nonsmoker **Chadha* chewer *Men* Non-chewer/non-alcoholic beverage drinker **Chadha* chewer *Women* Non-chewer/non-alcoholic beverage drinker **Chadha* chewer	 20 8 16 7	 1.0 3.2 (1.6–9.5) 1.0 6.2 (2.4–12.1) 1.0 3.8 (1.9–8.5) 1.0 5.8 (2.1–12.4)	Alcoholic beverage drinking Smoking	*Dried tobacco chewed alone

CI, confidence interval

Table 74. Results of cohort studies on use of smokeless tobacco and cancer of the oesophagus and pancreas

Reference, name of study	Exposure categories	No. of cases/ deaths	Relative risk (95% CI)	Adjustment for potential confounders; comments
Oesophagus				
Boffetta *et al.* (2005), Norwegian Cohort Study	Never user	18	1.0	Adjusted for age, smoking
	Ever user	9	1.40 (0.61–3.24)	
	Current user	4	1.06 (0.35–3.23)	
	Former user	5	1.90 (0.69–5.27)	
Pancreas				
Zheng *et al.* (1993), Lutheran Brotherhood Study	Ever users of ST	16	1.7 (0.9–3.1)	Adjusted for age, alcoholic beverages, smoking
Boffetta *et al.* (2005), Norwegian Cohort Study	Never user	60	1.0	Adjusted for age, smoking
	Ever user	45	1.67 (1.12–2.50)	
	Current user	27	1.60 (1.00–2.55)	
	Former user	18	1.80 (1.04–3.09)	

cases. Exposure data were collected through face-to-face interviews by professional interviewers. For oesophageal adenocarcinoma, the participation rate was 87% and the number of cases was 189; for gastric cardia cancer, the rate was 83% and the number of cases was 262; for squamous-cell carcinoma of the oesophagus, the participation rate was 73% and the number of participating cases was 167; 87%, 85% and 72% of the cases were men, respectively. The participation rate among the 820 controls who participated in the study was 73%. For gastric cardia adenocarcinoma, the odds ratio among ever users of snuff was 1.2 (53 cases; 95% CI, 0.8–1.8). For oesophageal adenocarcinoma, snuff users had a relative risk of 1.2 (35 cases; 95% CI, 0.7–2.0) compared with never users. Patients with more than 25 years of use had an adjusted relative risk of 1.9 (15 cases; 95% CI, 0.9–4.0) and those who used 15–35 quids per week had a relative risk of 2.0 (17 cases; 95% CI, 1.0–4.3). For the category of highest use (> 35 quids per week), no excess risk was seen. For oesophageal squamous-cell carcinoma, the relative risk was 1.4 (33 cases; 95% CI, 0.9–2.3) when ever users were compared with never users. Similarly to adenocarcinoma, for those with more than 25 years of use, the relative risk was 2.0 (14 cases; 95% CI, 0.9–4.1); those who used 15–35 quids per week had a relative risk of 2.1 (15 cases; 95% CI, 1.0–4.4) and those with highest intensity of use had no excess risk.

(*c*) *India*

Many studies from South-East Asia combined all smokeless tobacco use into one category, which was often termed tobacco chewing. In these studies, tobacco chewing

often included chewing of betel quid with tobacco. All such studies have been reviewed in the monograph on betel-quid and areca-nut chewing (IARC, 2004a) and are not included here. Only studies that reported separate results for tobacco chewing without betel quid are reviewed here.

A hospital-based case–control study was carried out in Assam, India, from 1997 to 1998, and recruited 502 (358 men, 144 women) histologically confirmed cases of oesophageal cancer (predominantly squamous-cell carcinomas), and two visitor controls per case group-matched for age and sex. Among nonsmokers compared with non-chewers (after adjusting for alcoholic beverage consumption), the odds ratio for developing oesophagal cancer associated with the use of dried tobacco leaf alone (locally known as *chada*) was 3.2 (20 cases; 95% CI, 1.6–9.5) and 6.2 (8 cases; 95% CI, 2.4–12.1) for men and women, respectively. Similarly, the risk for oesophageal cancer among non-alcoholic beverage drinkers for *chada* users compared with non-chewers (after adjusting for smoking) was 3.8 (16 cases; 95% CI, 1.9–8.5) among men and 5.8 (seven cases; 95% CI, 2.1–12.4) among women (Phukan *et al.*, 2001).

(*d*) *Africa*

Babekir *et al.* (1989) described the age, sex and geographical distribution of oesophageal cancers seen at the University Hospital of Khartoum, Sudan, in 1979–86. The annual crude incidence rates were 1.19/100 000 in the northern region and 0.17/100 000 or below in any of the other seven regions. Placing tobacco under the tongue or in the labiodental groove was discussed as a potential risk factor. No significant difference in the incidence was observed for the different tribes (Arab and Nuba) of the northern region. Alcoholic beverage drinking was excluded as a potential confounder since a similar difference in incidence rates was observed among women who rarely drink alcoholic beverages.

2.2.4 *Cancer of the pancreas*

Results of the cohort studies are presented in Table 74 and the case–control studies are summarized in Table 75.

(*a*) *North America*

(i) *Cohort study*

The Lutheran Brotherhood Insurance Society cohort comprised 17 818 (68.5%) of 26 030 white male policy holders, who responded to a mailed questionnaire in 1966 (Zheng *et al.*, 1993) (see Table 69). Cohort members were 30 years of age or older and lived in California, upper midwest or northeastern USA. After 20 years of follow-up for vital status in 1986, 4027 (23%) persons were lost to follow-up. At 11.5 years of follow-up, respondents, non-respondents and respondents lost to follow-up did not differ significantly with respect to demographic variables. Fifty-seven deaths from pancreatic cancer occurred during the 20-year follow-up period. For dietary reasons, 1656 respondents (including three pancreatic cancer deaths) were excluded from the analysis. The relative

Table 75. Case–control studies of smokeless tobacco use and cancer of the pancreas

Reference, study location, period	Characteristics of cases	Characteristics of controls	Exposure categories	No. of exposed cases	Relative risk (95% CI)	Adjustment for potential confounders	Comments
North America							
Williams & Horm (1977), USA, 1969–71	Cancer of the pancreas from 7518 (57% of randomly selected) incident invasive cancers who participated in the population based Third National Cancer Survey	Cancer at sites unrelated to tobacco	*Smokeless tobacco* Moderate use Heavy use	 2 1	 0.3 0.3	Age, race, smoking	No exposed cases among women
Farrow & Davis (1990), Washington, USA, 1982–86	148 married men from population-based cancer registry, aged 20–74 years; 46% histologically confirmed; interview with wife of patient	188 married men from same counties; frequency-matched on age (5-year categories); selected by RDD	Ever chewed tobacco	Prevalence among cases and controls, 6.9%	0.8 (non-significant)	Race, education	Further adjustment for age and dietary factors did not affect the odds ratio.
Muscat *et al.* (1997), New York, Pennsylvania, Michigan, Illinois, USA, 1985–93	484 incident histologically confirmed from daily hospital admission logs, aged 21–80 years; response rate, 51%	954 individually matched 2:1 on hospital, sex, age (± 5 years), race, year of diagnosis; patients without tobacco-related diseases; response rate, 63%	Never smoker and long-term (≥ 20 years) quitter Tobacco chewer ≥ 1 year and not current cigarette smoker Use of snuff ≥ 1 year	 6 2	1.0 3.6 (1.0–12.8) [Not reported]		Analysis restricted to men as no woman chewed tobacco or used snuff

Table 75 (contd)

Reference, study location, period	Characteristics of cases	Characteristics of controls	Exposure categories	No. of exposed cases	Relative risk (95% CI)	Adjustment for potential confounders	Comments
Alguacil & Silverman (2004), Atlanta, GA, Detroit, MI, New Jersey (USA), 1986–89	154 carcinoma of exocrine pancreas from population-based cancer registries, aged 30–79 years; lifelong non-smokers of cigarettes; of 1153 identified, 46.5% interviewed	844; RDD for cases ≤ 64 years; HCFA for cases aged ≥ 65 years; frequency-matched on age, race, sex, study site; lifelong non-smokers of cigarettes; 78% interviewed	Non-user of tobacco Chewing tobacco and/or snuff Ever used Only used *Tobacco type* Chewing tobacco Snuff *Ounces/week* ≤ 2.5 > 2.5 *Duration of use* ≤ 20 years > 20 years	 7 5	1.0 1.4 (0.5–3.6) 1.1 (0.4–3.1) 1.7 (0.6–4.5) 1.1 (0.4–3.5) 0.3 (0.04–2.5) 3.5 (1.1–10.6) *p* for trend = 0.04 1.1 (0.1–11.0) 1.5 (0.6–4.0) *p* for trend = 0.42	Race, sex, geographic site, cigar smoking, age	Tobacco chewers used more per week (7.2 oz.) than users of snuff (2.4 oz.). 1 can snuff = 1.2 oz.; 1 unit chewing tobacco = 3-oz pouches or 2.33-oz. plugs.

CI, confidence interval; HCFA, Health Care Financing Administration; RDD, random-digit dialling

risk for ever users of smokeless tobacco was 1.7 (16 deaths; 95% CI, 0.9–3.1), adjusted for age, alcoholic beverages and smoking.

(ii) *Case–control studies*

The population-based case–control study by Williams and Horm (1977) reported in Section 2.2.1 also reported on pancreatic cancer. Among men, the relative risks for cancer of the pancreas and for moderate or heavy use of chewing tobacco or snuff were 0.3 (two cases) and 0.3 (one case), respectively, adjusted for age, race and smoking. There were no exposed cases among women.

A population-based study included married men newly diagnosed with pancreatic cancer in the Seattle (USA) area and population-based controls frequency-matched on age (Farrow & Davis, 1990). A telephone interview with the wives was conducted between 2 and 4.5 years after diagnosis. Complete information was available for 148 cases and 188 controls. The odds ratio for chewing tobacco was 0.8 (overall prevalence, 6.9%) with a confidence interval that included 1.0 [smoking was not controlled for].

Muscat *et al.* (1997) conducted a hospital-based study in New York, Pennsylvania, Michigan and Illinois, USA. Of the 949 cases aged 20–81 years ascertained between 1985 and 1993 and the 1526 eligible controls, 484 cases and 949 controls were interviewed in the hospital. The controls did not have tobacco-related diseases, and were individually matched to cases on hospital, sex, age, race and year of diagnosis. The major reasons for non-interviews were that the patient was too ill or unable to communicate. Relative to never smokers and long-term quitters (≥ 20 years), the odds ratio for tobacco chewers who were not current cigarette smokers was 3.6 (95% CI, 1.0–12.8).

In a large population-based case–control study of incident cases of carcinoma of the exocrine pancreas in the Atlanta area, Detroit and New Jersey, USA, lifelong nonsmokers of cigarettes were examined (Alguacil & Silverman, 2004). Forty-one per cent of the cases died before interview, but response rates for the surviving cases and controls were 75% or better. Controls enroled by random-digit dialling (for cases ≤ 64 years) and HCFA (for cases ≥ 65 years) were frequency-matched to the cases on age, race, sex and study site. Persons were considered to be snuff users if they ever used snuff, whereas tobacco chewers were defined as those who used one pouch or plug per week for at least 6 months. Relative to non-users of tobacco, the odds ratio for ever having used smokeless tobacco was 1.4 (95% CI, 0.5–3.6) and that for having used smokeless tobacco only was 1.1 (95% CI, 0.4–3.1), adjusted for race, sex, geographic site, cigar smoking and age. In a statistical model with cigar smoking, chewing tobacco and snuff and pancreatic cancer as the outcome, the odds ratios were 1.7 (95% CI, 0.6–4.5) for chewing tobacco and 1.1 (95% CI, 0.4–3.5) for using snuff. Dose–response relationships were evaluated and adjusted for age, sex, race, cigar smoking and geographical region. Users of 2.5 oz or less of smokeless tobacco per week had an odds ratio of 0.3 (95% CI, 0.04–2.5) whereas users of more than 2.5 oz had an odds ratio of 3.5 (95% CI, 1.1–10.6; p for trend = 0.04). For 20 years or less of smokeless tobacco use, the odds ratio was 1.1 (95% CI, 0.1–11.0) and that for more than

20 years was 1.5 (95% CI, 0.6–4.0; *p* trend = 0.42). Tobacco chewers used more ounces of tobacco per week than users of snuff (7.2 versus 2.4 oz).

(*b*) *Europe*

In the Norwegian Cohort Study (Heuch *et al.*, 1983; Boffetta *et al.*, 2005), the relative risk for pancreatic cancer for ever use of smokeless tobacco was 1.7 (45 cases; 95% CI, 1.1–2.5); similar results were obtained for former and current users. After stratification on smoking status, the relative risks were 1.9 (28 cases, 95% CI, 1.1–3.1) among current smokers and 0.9 (three cases; 95% CI, 0.2–3.7) among never smokers. The results in current smokers were adjusted for amount of smoking. [The Working Group noted that never smokers were too few to give meaningful results and that the absence of an effect for lung cancer speaks against confounding by cigarette smoking.]

2.2.5 *Cancers at other sites*

The characteristics of cohort studies are presented in Table 69 and their results are summarized in Table 76. Case–control studies are summarized in Table 77.

(*a*) *Cancer of the stomach*

(i) *Cohort studies*

In the Lutheran Brotherhood cohort, white men aged 35 years and above were followed for vital status for 20 years (Kneller *et al.*, 1991). Relative to men who had never used tobacco, the relative risk for smokeless tobacco users was 2.3 (18 deaths; 95% CI, 0.98–5.2). Stratification by pack–years of smoking reduced this relative risk to 1.6 (95% CI, 0.6–4.5). Among nonsmokers who used smokeless tobacco, the relative risk was 3.8 (three deaths; 95% CI, 1.0–14.3).

Among men in the CPS-II cohort, and relative to having never used any type of tobacco, the relative risk for stomach cancer among current users of smokeless tobacco only was 1.6 (8 deaths; 95% CI, 0.8–3.3) adjusted for age, race, education, family history of stomach cancer, consumption of high-fiber grain foods, vegetables, citrus fruits or juices, use of vitamin C, multivitamins and aspirin. For former users of smokeless tobacco only, the relative risk was 1.1 (95% CI, 0.3–4.5) (Chao *et al.*, 2002).

In the cohort study from Norway, the relative risk for stomach cancer and for ever use of snuff was 1.1 (74 cases; 95% CI, 0.8–1.5) compared with never users. Results were similar for current and former users (Boffetta *et al*., 2005).

(ii) *Case–control studies*

The case–control study by Williams and Horm (1977) described in Section 2.2.1 also reported on stomach cancer. Among men, the relative risks for stomach cancer and for moderate or heavy use of chewing tobacco or snuff were 1.0 (6 cases) and 1.7 (6 cases), respectively, adjusted for age, race and smoking.

Table 76. Results of cohort studies on use of smokeless tobacco and cancer at other sites

Reference, name of study	Exposure categories	No. of cases/ deaths	Relative risk (95% CI)	Adjustment for potential confounders; comments
Stomach				
Kneller *et al.* (1991), Lutheran Brotherhood Study	Never used any tobacco		1.0	
	Smokeless tobacco users	18	2.3 (0.98–5.2)	
	Smokeless tobacco users (adjusted*)	18	1.6 (0.6–4.5)	*Stratified by pack–years of smoking
	Smokeless tobacco only users	3	3.8 (1.0–14.3)	
Chao *et al.* (2002), CPS-II	Never used any tobacco	169	1.0	Adjusted for age, race, education, family history of stomach cancer, aspirin use, dietary factors
	Current smokeless tobacco only	8	1.58 (0.76–3.28)	
	Former smokeless tobacco only	2	1.11 (0.27–4.50)	
Boffetta *et al.* (2005), Norwegian Cohort Study	Never user	143	1.0	Adjusted for age, smoking
	Ever user	74	1.11 (0.83–1.48)	
	Current user	42	1.00 (0.71–1.42)	
	Former user	32	1.29 (0.87–1.91)	
Colon and rectum				
Heineman *et al.* (1995), US Veterans Study	Never used any tobacco	782	1.0	Relative risks for smokeless tobacco users who never smoked cigarettes, pipes or cigars
	Smokeless tobacco user			
	Colon	39	1.2 (0.9–1.7	
	Rectum	17	1.9 (1.2–3.1)	
Digestive tract				
Accort *et al.* (2002), NHANES 1 Follow-up	No tobacco use		1.0	Adjusted for age, race, poverty index ratio, region of residence, alcoholic beverages, dietary fat intake
	Men			
	Ever smokeless tobacco user/never smoker		0.9 (0.3–2.3)	
	Ever smokeless tobacco user/ever smoker		0.7 (0.3–1.8)	
	Women			
	Ever smokeless tobacco user/never smoker		0.8 (0.3–2.7)	
	Ever smokeless tobacco user/ever smoker		0.2 (0.1–1.1)	

Table 76 (contd)

Reference, name of study	Exposure categories	No. of cases/ deaths	Relative risk (95% CI)	Adjustment for potential confounders; comments
Henley *et al.* (2005), CPS-I and CPS-II	*CPS-I*			Restricted to men who never used other tobacco products; adjusted for age, race, education, body mass index, exercise, aspirin use, alcoholic beverages, dietary factors; CPS-II also adjusted for status, type of employment
	Never use of smokeless tobacco	760	1.0	
	Current use of smokeless tobacco	153	1.26 (1.05–1.52)	
	CPS-II			
	Never use of smokeless tobacco	1932	1.0	
	Current use of smokeless tobacco	48	1.04 (0.77–1.38)	
	Former use of smokeless tobacco	19	0.99 (0.63–1.57)	
Lung				
Accort *et al.* (2002), NHANES 1 Follow-up	No tobacco use		1.0	Adjusted for age, race, poverty index ratio, region of residence, alcoholic beverages, recreational physical exercise, fruit/vegetable intake
	Men			
	Ever smokeless tobacco user/never smoker		–	
	Ever smokeless tobacco user/ever smoker		22.6 (6.4–80.3)	
	Women			
	Ever smokeless tobacco user/never smoker		9.1 (1.1–75.4)	
	Ever smokeless tobacco user/ever smoker		1.2 (0.2–8.9)	
Boffetta *et al.* (2005), Norwegian Cohort Study	Never user	39	1.0	Adjusted for age, smoking
	Ever user	72	0.80 (0.61–1.05)	
	Current user	44	0.80 (0.58–1.11)	
	Former user	28	0.80 (0.54–1.19)	
Henley *et al.* (2005), CPS-I and CPS-II	*CPS-I*			Restricted to men who never used other tobacco products; adjusted for age, race, education, body mass index, exercise, aspirin use, alcoholic beverages, dietary factors; CPS-II also adjusted for status, type of employment
	Never use of smokeless tobacco	116	1.0	
	Current use of smokeless tobacco	18	1.08 (0.64–1.83)	
	CPS-II			
	Never use of smokeless tobacco	378	1.0	
	Current use of smokeless tobacco	18	2.00 (1.23–3.24)	
	Former use of smokeless tobacco	4	1.17 (0.43–3.14)	

Table 76 (contd)

Reference, name of study	Exposure categories	No. of cases/ deaths	Relative risk (95% CI)	Adjustment for potential confounders; comments
Soft-tissue sarcoma				
Zahm *et al.* (1992), US Veterans Study	Never used any tobacco	20	1.0	No smokeless tobacco only users with soft-tissue sarcoma
	Used smokeless tobacco and other tobacco products	20	1.4 (0.8–2.6)	
Prostate				
Hsing *et al.* (1990), Lutheran Brotherhood Study	Never used any tobacco	19	1.0	Adjusted for cigarette smoking; similar results for 58 subjects for whom prostate cancer was not the underlying cause of death
	Ever used smokeless tobacco	42	2.1 (1.1–4.1)	
	Occasional	5	1.4 (0.5–3.9)	
	Former user	13	1.8 (0.8–3.9)	
	Regular	24	2.4 (1.3–4.9)	
	Smokeless tobacco only	10	4.5 (2.1–9.7)	
Hsing *et al.* (1991), US Veterans Study	Never used any tobacco	1075	1.0	
	Smokeless tobacco only	48	1.2 (0.9–1.6)	

CI, confidence interval; CPS, Cancer Prevention Study; NHANES, National Health and Nutrition Examination Survey

Table 77. Case–control studies of smokeless tobacco use and cancer at other sites

Reference, study location, period	Characteristics of cases	Characteristics of controls	Exposure categories	No. of exposed cases	Relative risk (95% CI)	Adjustment for potential confounders	Comments
Stomach							
Williams & Horm (1977), USA, 1969–71	Cancer of the stomach from 7518 (57% of randomly selected) incident invasive cancers who participated in the population-based Third National Cancer Survey	Cancer at sites unrelated to tobacco	Smokeless tobacco Moderate use Heavy use	*Men* 6 6	 1.0 1.7	Age, race, smoking	Personal interview
Hansson *et al.* (1994), Sweden, 1989–92	338 incident from population-based national cancer registry, aged 40–79 years; histologically confirmed; response rate, 74%	679, randomly selected from population registries, stratified by age and sex; response rate, 77%	Snuff dipping		0.7 (0.47–1.06)	Age, sex, socio-economic status, vegetable intake	All subjects are also included in Ye *et al.* (1999).
Ye *et al.* (1999), Sweden, 1989–95	561 incident from population-based national cancer registry, aged 40–79 years; histologically confirmed; response rate, 62%	1164, randomly selected from population registries, stratified by age and sex; response rate, 75.9%	Snuff dipping *Stomach cancer* Ever user among never smokers *Cardia* Current Former *Distal intestinal* Current Former *Distal diffuse* Current Former	 11 9 6 26 18 11 8	 0.5 (0.2–1.2) 0.5 (0.2–1.1) 0.8 (0.3–1.9) 0.8 (0.5–1.3) 0.9 (0.5–1.6) 0.6 (0.3–1.2) 0.7 (0.3–1.6)	Age, residence area, body-mass index, socio-economic status, smoking	Data available on age at start, duration and intensity of snuff dipping

Table 77 (contd)

Reference, study location, period	Characteristics of cases	Characteristics of controls	Exposure categories	No. of exposed cases	Relative risk (95% CI)	Adjustment for potential confounders	Comments
Extra-hepatic bile duct							
Chow *et al.* (1994), Los Angeles county, USA, 1985–89	64 incident cancers of the extrahepatic bile duct (ICD-O 156. 1), 41 of the ampulla of Vater (ICD-O 156.2), aged 30–84 years; histologically confirmed, white race; response rate, 76%; for the 58% of deceased cases, information obtained from next-of-kin	255; RDD for cases ≤ 64 years; HCFA for cases ≥ 65 years; matched on age (5-year age groups), sex; no history of cholecystectomy; response rate, 84% (RDD), 87% (HCFA)	Chewing tobacco [current]	Ampulla of Vater 3	18 (1.4–227.7)		Exposed cases also smoked cigarettes and 2 also used cigar/pipes.
Nasal cavities							
Brinton *et al.* (1984), USA, 1970–80	193 from four hospitals in North Carolina and Virginia, cancers of the nasal cavities and sinuses ICD-8 160.0, 160.2–160.5, 160.8–160.9 (86 squamous-cell carcinomas, 24 adenocarcinomas or adenoid cystic carcinomas, 36 other carcinomas, 14 other histologies), aged ≥ 18 years; response rate, 82.9%	*Live cases*: two hospital patients per case matched on hospital, year of admission, age, sex, race, excluding controls with admission diagnosis of other cancers or other diseases of the upper aero-digestive tract *Deceased cases*: one per case with similar criteria as above; one deceased identified from state vital statistics offices; response rate, 78.0%	Use of chewing tobacco Use of snuff	15 23	1.0 (ref.) 0.7 (0.4–1.5) 1.0 (ref.) 1.5 (0.8–2.8)	Sex	Similar results for matched analyses [data not shown]
Stockwell & Lyman (1986), Florida, USA, 1982	92 incident cancers of the nasal cavities and accessory sinuses from population-based Florida cancer registry; overall response rate, 82%	6457; all cancers of the colon or rectum, cutaneous melanoma, endocrine neoplasias from same source during same time period; response rate, 78%	Unspecified	1	3.3 (0.4–25.9)	Age, sex, race, tobacco use	Only primary type of tobacco used was obtained from chart and histopathology reviews.

Table 77 (contd)

Reference, study location, period	Characteristics of cases	Characteristics of controls	Exposure categories	No. of exposed cases	Relative risk (95% CI)	Adjustment for potential confounders	Comments
Laryngeal cancer							
Stockwell & Lyman (1986), Florida, USA, 1982	797 incident cancers of the larynx from population-based Florida cancer registry; overall response rate, 82%	6457; all cancers of the colon or rectum, cutaneous mealnoma, endocrine neoplasias from same source during same time period; response rate, 78%	Unspecified	5	7.3 (2.9–18.3)	Age, sex, race, tobacco use	Only primary type of tobacco used was obtained by chart and histopathology reviews.
Lewin *et al.* (1998), Stockholm and southern Sweden, 1988–91	605, including 157 cases of cancer of larynx, from hospitals and cancer registries, 40–79 years old; overall response rate, 90%	756 from the population registry; stratified by region, age; response rate, 85%	Current snuff use Former snuff use	15 9	1.0 (0.5–1.9) 0.8 (0.4–1.7)	Age, region, smoking, alcoholic beverages	
Lung							
Williams & Horm (1977), USA, 1969–71	Cancer of the lung from 7518 (57% of randomly selected) incident invasive cancers who participated in the population-based Third National Cancer Survey	Cancer at sites unrelated to tobacco	Smokeless tobacco Moderate use Heavy use	*Men* 26 10	 0.7 0.8	Age, race, smoking	Personal interview
Sarcoma							
Zahm *et al.* (1989), Kansas, USA, 1976–82	133 incident soft-tissue sarcomas from population-based registry considered 90% complete, histologically confirmed, white men, aged ≥ 21 years; 50% of interviews with next-of-kin; response rate for cases and controls, 93%	948; for living cases: white men selected through RDD and HCFA, frequency-matched by age (± 2 years); for deceased cases: decedents from Kansas, frequency-matched on age (± 2 years) and year of death, excluding lymphomas, sarcomas, ill-defined malignancies and homicide or suicide; 49% of interviews with next-of-kin	Ever use of smokeless tobacco *Location of tumour* Upper gastrointestinal Lung, pleura, thorax Head, neck, face Others *Cell type* Fibromatous Adipose Myomatous Others	28 4 5 3 16 7 3 7 11	1.8 (1.1–2.9) 3.3 (0.8–12.6) 3.1 (0.9–10.5) 2.4 (0.5–10.2) 1.4 (0.7–2.5) 1.8 (0.7–4.7) 1.1 (0.2–4.2) 2.1 (0.8–5.3) 1.9 (0.9–3.9)		

Table 77 (contd)

Reference, study location, period	Characteristics of cases	Characteristics of controls	Exposure categories	No. of exposed cases	Relative risk (95% CI)	Adjustment for potential confounders	Comments
Breast							
Spangler *et al.* (2001b), Spangler (2002), North Carolina, USA 1990–91	Eight Cherokee women with prevalent breast cancer (ascertained by personal history) from population-based survey in Cherokee tribal lands; age at diagnosis: three, < 55 years; five, ≥ 55 years; response rate, 81% (cases and controls combined)	962 other survey respondents	Ever use of smokeless tobacco Diagnosed at age < 55 years ≥ 55 years	 1 2	 1.3 (0.12–13.9) 1.2 (0.14–9.52)		Unknown whether smokeless tobacco use preceeded cancer diagnosis
Prostate							
Hayes *et al.* (1994), Georgia, Michigan, New Jersey, USA, 1986–89	981 men with incident pathologically confirmed prostate cancer from a population-based registry, aged 40–79 years; response rate, 76%	1315; RDD for cases ≤ 64 years, HCFA for cases ≥ 65 years; frequency-matched on age, sex, race; response rate, 74%	Never used tobacco *Tobacco chewing* Former Current *Snuff* Former Current	 56 14 10 10	1.0 1.0 (0.6–1.5) 0.5 (0.2–1.0) 0.6 (0.3–1.4) 5.5 (1.2–26.2)	Age, race, study site	
Urinary bladder							
Howe *et al.* (1980), Canada, 1974–76	632 (480 men, 152 women) newly diagnosed bladder cancers identified in three provinces	632 neighbours, individually matched by age (± 5 years), sex	Ever use of chewing tobacco, relative to never use	NR	0.9 (0.5–1.6)	Controlling for cigarette smoking did not affect the risk estimates.	61 discordant pairs

Table 77 (contd)

Reference, study location, period	Characteristics of cases	Characteristics of controls	Exposure categories	No. of exposed cases	Relative risk (95% CI)	Adjustment for potential confounders	Comments
Hartge *et al.* (1985), 10 areas in USA, 1977–78	2982 from population-based cancer registries, aged 21–84 years; response rate, 75%	2469 RDD and 3313 HCFA, frequency-matched by age, sex, geographic distribution of the cases; response rate, 82% (HCFA), 84% (RDD)	Snuff Chewing tobacco	11 40	0.8 (0.4–1.6) 1.02 (0.7–1.5)	Race, age, residence, pipe, cigars, chewing tobacco/snuff	Analysis restricted to men who never smoked cigarettes.
Slattery *et al.* (1988), Utah, USA, 1977–83	332 histologically confirmed from population-based Utah cancer registry; white men aged 21–84 years; response rate, 76.3%	686; RDD for cases ≤ 64 years, HCFA for cases ≥ 65 years; matched 2:1 on age, sex; response rate, 81.5%	Snuff Chewing tobacco *Snuff* Never smoker Smoker *Chewing tobacco* Never smoker Smoker	16 21	1.0 (0.5–1.9) 1.1 (0.6–1.9) 2.7 (0.5–15.6) 0.7 (0.4–1.4) 2.8 (0.4–20.2) 1.2 (0.7–2.2)	Crude, unmatched	
Burch *et al.* (1989), Alberta, Ontario, Canada, 1979–82	826 histologically confirmed population-based through cancer institute, tumour registry and hospitals, aged 35–79 years; response rate, 67%	792; randomly selected from province-wide annually updated listings, matched 1:1 on age (± 4 years), sex, area of residence; response rate, 53%	Ever snuff use Ever chewing tobacco	9 26	0.6 (0.3–1.1) 0.5 (0.2–1.1)	Age, lifetime cigarette consumption	Analysis restricted to 627 men

Table 77 (contd)

Reference, study location, period	Characteristics of cases	Characteristics of controls	Exposure categories	No. of exposed cases	Relative risk (95% CI)	Adjustment for potential confounders	Comments
Kidney							
Goodman *et al.* (1986), USA, 1977–83	267 incident primary adenocarcinomas of kidney, aged 20–80 years, from 18 hospitals in six US cities; response rate, 89%	267, individually matched on hospital, sex, race, age (± 5 years), time of admission; non-tobacco-, non-obesity related disease; response rate, 88%	Ever use* of chewing tobacco	13	4.0 (1.1–14.2)	Matched analysis	*At least once a day for 1 year or more
			Ever versus never use of chewing tobacco, among never users of cigarettes		0.9 (0.2–5.1)	Queteletet index, decaf-feinated coffee, pack-years, chewing tobacco (ever, never), pack-years × chewing tobacco.	Analysis restricted to 189 men
			Joint effect for smoking of 30 pack–years of cigarettes and tobacco chewing versus never use of any tobacco		26.00 (4.41–153.00)		
Asal *et al.* (1988), Oklahoma, USA, 1981–84	315 incident renal cell carcinomas from 29 hospitals; ascertained by tissue diagnosis (95%) or radiological examination (5%); response rate, 91%	313 hospital patients, individually matched by age (± 5 years), sex, race, hospital, time of interview; patients with kidney disease or pyshiatric diagnosis excluded; 336 RDD, frequency-matched by age (± 10 years), sex	Use of snuff		3.6 (1.2–13.3)		Among 209 men in matched-pair analysis with hospital controls

Table 77 (contd)

Reference, study location, period	Characteristics of cases	Characteristics of controls	Exposure categories	No. of exposed cases	Relative risk (95% CI)	Adjustment for potential confounders	Comments
McLaughlin *et al.* (1995), Australia, Denmark, Germany, Sweden, USA, 1989–92	1732 population-based (hospital-based in Germany) renal-cell adenocarcinomas, aged 20–79 years; histologically or cytologically confirmed; response rate, 72.3%	2309 from population registers, electoral rolls, residential lists, HCFA, RDD; response rate, 74.7%	Smokeless tobacco	11	1.3 (0.6–3.1)	Age, sex, centre, body mass index	Analysis restricted to men because no women used smokeless tobacco.
Muscat *et al.* (1995), Illinois, Michigan, New York, Pennsylvania, USA, 1977–93	788 renal-cell carcinomas, excluding renal pelvis; histologically confirmed from selected hospitals	779 patients with diseases unrelated to tobacco use, from daily admission lists; frequency-matched on age (± 5 years), sex, race, year of diagnosis	Ever chewing tobacco relative to non-users of smoklesss tobacco *Chews/week* ≤ 10 chews > 10 chews	2.6%	3.2 (1.1–8.7) 2.5 (1.0–6.1) 6.0 (1.9–18.7) *p* for trend < 0.05		Analysis restricted to men because no women used chewing tobacco. [Snuff was included in questionnaire but not mentioned in results]. Chewing tobacco defined as ever used regularly for at least 1 year
Brain							
Zheng *et al.* (2001), Iowa, USA, [not reported]	375 incident gliomas from population-based registry, 40–85 years of age; histologically confirmed; response rate, 91%	2434; drivers' licence records for cases aged ≤ 64 years, HCFA for cases ≥ 65 years; frequency-matched by age (5-year groups), sex; 6.5:1 ratio controls:cases; those with history of cancer excluded; response rate, 82% (licence), 80% (HCFA)	Chewing tobacco or use of snuff	Not reported	"Use of snuff or chewing tobacco was not associated with a significantly increased risk of brain cancer for either men or women."		

Table 77 (contd)

Reference, study location, period	Characteristics of cases	Characteristics of controls	Exposure categories	No. of exposed cases	Relative risk (95% CI)	Adjustment for potential confounders	Comments
Non-Hodgkin lymphoma							
Brown *et al.* (1992a), Iowa, Minnesota, USA, 1981–84	622 white men with incident histologically confirmed non-Hodgkin lymphoma, aged ≥ 30 years; population-based, outside of metropolitan areas; 89% interviewed	820 living; RDD for cases ≤ 64 years, HCFA for cases ≥ 65 years; frequency-matched on site, age (± 5 years); response rate, 77% (RDD), 79% (HCFA)	All lymphoma Follicular Diffuse Small lymphocytic	19 7 5 4	1.3 (0.7–2.5) 1.7 (0.7–4.3) 0.8 (0.3–2.3) 1.7 (0.5–5.4)	Age, state	Same subjects as study by Schroeder *et al.* (2002)
Schroeder *et al.* (2002), Iowa, Minnesota, 1980–82 (Minnesota), 1981–83 (Iowa)	622; 40% had archival tissue available.	1245 controls; 820 living controls and 425 deceased, from state death certificate files; interviews with next-of-kin of deceased subjects	*All cases* Chewing tobacco Snuff *t(14;18)-positive* Chewing Snuff *Age started chewing* > 18 years ≤ 18 years *t(14;18)-negative* Chewing Snuff *Age started chewing* > 18 years ≤ 18 years	 11% 10% 10 7 59 13 9 12 8 16	 1.3 (0.9–1.8) 1.0 (0.7–1.4) 1.7 (0.9–3.1) 1.0 (0.5–2.0) 1.3 (0.6–2.9) 2.5 (1.0–6.0) 1.0 (0.6–1.8) 0.9 (0.6–1.6) 1.2 (0.6–2.2) 1.0 (0.3–3.0)	Age, state, vital status	Same subjects as in study by Brown *et al.* (1992a). Ever used if used daily for at least 3 months

Table 77 (contd)

Reference, study location, period	Characteristics of cases	Characteristics of controls	Exposure categories	No. of exposed cases	Relative risk (95% CI)	Adjustment for potential confounders	Comments
Leukaemia							
Brown *et al.* (1992b), Iowa, Minnesota, USA, 1981–84	578 incident leukaemias, white men aged ≥ 30 years, from cancer registry in Iowa or 'special surveillance network of hospitals in Minnesota'; histologically confirmed; response rate, 86%; interviews with close relatives for deceased or too ill subjects	820 living; RDD for cases ≤ 64 years; HCFA for cases ≥ 65 years; frequency-matched on age (5-year groups), state; response rate, 77% (RDD), 79% (HCFA)	Non-user of tobacco		1.0	Age, state, alcoholic beverage use	
					All		
			Chewing tobacco/snuff only	24	1.8 (0.9–3.3)		
					Acute non-lymphocytic		
				3	0.9 (0.2–3.1)		
					Chronic myelogenous		
				2	2.1 (0.4–10.7)		
					Chronic lymphocytic		
				10	1.9 (0.8–4.3)		
					Myelodysplasia		
				4	2.7 (0.8–9.4)		
					Other		
				5	3.0 (0.9–9.2)		

CI, confidence interval; HCFA, Health Care Financing Administration; NR, not reported; RDD, random-digit dialling

Two case–control studies on stomach cancer were conducted in selected counties in central and northern Sweden with different rates of stomach cancer incidence (Hansson *et al.*, 1994; Ye *et al.*, 1999) (Table 77). Eligible cases were all patients with newly diagnosed and histologically confirmed stomach cancers between 1989 and 1992 or 1995, and were ascertained via personal contacts at all departments of surgery and pathology, supplemented by record linkages to the regional and national cancer registries. In the early study (Hansson *et al.*, 1994), the odds ratio for snuff dipping adjusted for age, sex, socioeconomic status and vegetable intake was 0.7 (95% CI, 0.5–1.1). In the later study (Ye *et al.*, 1999), the stomach cancers were classified as cancer of the cardia or of the distal stomach. About two controls per case were selected from the continuously updated population registry with stratification for age and sex. Face-to-face interviews were performed by specially trained personnel. The participation rates were 62% and 76% for cases and controls, respectively; the majority of the non-participants among the cases had died before the interview. For cardia cancer, the relative risk for current snuff use was 0.5 (95% CI, 0.2–1.1) and that for former users was 0.8 (95% CI, 0.3–1.9). For distal stomach cancer, the relative risks for current use were 0.8 (95% CI, 0.5–1.3) for the intestinal type and 0.6 (95% CI, 0.3–1.2) for the diffuse type. After restriction to never smokers and after combining all sites, the relative risk for ever using snuff was 0.5 (95% CI, 0.2–1.2). [The limitation is small numbers in the subgroups, which precludes e.g. site-specific analysis in never smokers with various categories of snuff use.]

(*b*) *Cancer of the colon and rectum*

Risk for colorectal cancer was examined in relationship to smokeless tobacco use among members of the US Veterans' cohort (Heineman *et al.*, 1995). Relative to those who had never used tobacco, smokeless tobacco users who had never smoked cigarettes, pipes or cigars had a relative risk of 1.2 (39 deaths; 95% CI, 0.9–1.7) for cancer of the colon and 1.9 (17 deaths; 95% CI, 1.2–3.1) for cancer of the rectum.

(*c*) *Cancer of the extra-hepatic bile duct*

A population-based case–control study in Los Angeles County, USA, included 64 cases of cancer of extra-hepatic bile duct, 41 cases of cancer of ampulla of Vater, and 255 controls (Chow *et al.*, 1994). In-person questionnaire interviews were conducted with the cases or their next-of-kin. Results obtained for the entire group of cases (personal and surrogate interviews) and for the subgroup with personal interviews only were consistent. An odds ratio of 18 (95% CI, 1.4–227.7) for chewing tobacco was observed for cancers of ampulla of Vater. [All cases of cancer of the ampulla of Vater who chewed tobacco also smoked.]

(*d*) *Cancer of the digestive system (combined)*

The case–control study by Sterling *et al.* (1992), described in Section 2.2.1, also reported results for cancers of the digestive organs (ICD-9 150–159). Using a reference

category of less than 100 lifetime uses of smokeless tobacco, the relative risks for 100–9999 and 10 000 or more lifetime uses were 0.2 (95% CI, 0.04–0.5) and 0.61 (95% CI, 0.3–1.1), respectively.

Gastrointestinal cancer deaths (ICD-9 150–159) were examined in the NHANES I follow-up study. Relative to non-users of tobacco, the hazard ratios for users of smokeless tobacco only for men and women were 0.9 (95% CI, 0.3–2.3) and 0.8 (95% CI, 0.3–2.7), respectively, adjusted for age, race, poverty index ratio, alcoholic beverage and dietary fat intake (Accortt *et al.*, 2002).

In the CPS-I cohort, men who reported current use of smokeless tobacco and never used other tobacco products had statistically significantly higher death rates than never users (153 deaths; hazard ratio, 1.3; 95% CI, 1.1–1.5) after adjustment for age, race, educational level, body mass index, exercise, alcoholic beverage consumption, fat consumption, fruit and vegetable intake and aspirin use. In the CPS-II cohort, compared with never users, the hazard ratio for men who reported current use of smokeless tobacco but never used any other tobacco products was 1.04 (48 deaths; 95% CI, 0.8–1.4) adjusted for the same variables and status and type of employment (Henley *et al.*, 2005).

(*e*) *Cancers of the respiratory tract*

(i) *Nasal cavities*

Brinton *et al.* (1984) performed a case–control study of risk factors for cancers of the nasal cavities and sinuses (ICD 8 160.0, 160.2–160.5, 160.8–160.9). Cases were selected from four hospitals in North Carolina and Virginia, USA, between 1 January 1970 and 31 December 1980. Cases were aged 18 years or older and were residents of the state in which the admitting hospital was located. For each case alive at the time of the interview, two hospital controls were selected and matched to the case on hospital, year of admission, age, sex, race and other factors. Controls with a primary diagnosis at admission of other cancers or other diseases of the upper aerodigestive tract were excluded. For deceased controls, two different controls were selected: a hospital control derived in the same manner as above and a deceased control identified through state vital statistics offices. A total of 193 cases, 232 hospital controls and 140 death certificate controls were identified and telephone interviews with study subjects or their next of kin were successfully conducted for 160 of the cases (82.9%) and 290 controls (78.0%). The cancers were 86 squamous-cell carcinomas, 24 adenocarcinomas or adenoid cystic carcinomas, 36 other carcinomas and 14 other histological types. Unmatched stratified analyses and logistic regression analyses for matched data were performed. Since the results were similar for the two analytical approaches [data not shown], only results that ignored the individual matching were presented. Sex-adjusted odds ratios for tobacco chewers or snuff users were 0.7 (15 cases; 95% CI, 0.4–1.5) and 1.5 (23 cases; 95% CI, 0.8–2.8), respectively. The odds ratio for snuff use and squamous-cell tumours was 1.9.

A case–control study in Florida assessed the association with use of smokeless tobacco among 92 cases of cancer of the nasal cavities and 6457 controls (Stockwell &

Lyman, 1986). Tobacco use was determined from medical and cancer registry records and was available for 79% of subjects. The odds ratio for smokeless tobacco was 3.3 (95% CI, 0.4–25.9), adjusted for age, race, sex and tobacco use. [The limitations of this study are presented in Section 2.2.1.]

(ii) *Larynx*

A case–control study in Florida assessed the association with use of smokeless tobacco among 797 cases of cancer of the larynx and 6457 controls (Stockwell & Lyman, 1986). Tobacco use was determined from medical and cancer registry records and was available for 79% of subjects. The odds ratio for smokeless tobacco was 7.3 (95% CI, 2.9–18.3), adjusted for age, race, sex and tobacco use. [The limitations of this study are presented in Section 2.2.1.]

The case–control study by Lewin *et al.* (1998, see Section 2.2.1) reported results separately for cancer of the larynx. Relative risks for curent and former use of snuff were 1.0 (95% CI, 0.5–1.9) and 0.8 (95% CI, 0.4–1.7), respectively, after adjustment for age, smoking and alcoholic beverages.

(iii) *Lung*

Lung cancer deaths were examined in the NHANES I follow-up study (Accortt *et al.*, 2002). In the multivariate analysis and relative to non-users of tobacco, the hazard ratio for women who used only smokeless tobacco was 9.1 (95% CI, 1.1–75.4), adjusted for age, race, poverty index ratio, region of residence, alcoholic beverages, recreational physical exercise and fruit/vegetable intake. No deaths from lung cancer occurred among men who used smokeless tobacco only.

In the CPS-I cohort, the hazard ratio for lung cancer for current smokeless tobacco users who never used other tobacco products was 1.1 (18 deaths; 95% CI, 0.6–1.8) after adjustment for age, race, level of education, body mass index, exercise, alcoholic beverage consumption, fat consumption, fruit and vegetable intake and aspirin use (Henley *et al.*, 2005). In the CPS-II cohort, the hazard ratio for men who reported current use of smokeless tobacco but never used any other tobacco products compared with never users was 2.0 (18 deaths; 95% CI, 1.2–3.2) adjusted for the same variables and status and type of employment. The hazard ratios were similar for those who chewed but never used snuff and for those who used snuff but never chewed.

In the Norwegian cohort study, the relative risk for lung cancer was 0.8 (72 cases; 95% CI, 0.6–1.1) in a comparison of ever users of smokeless tobacco with never users and adjusting for age and smoking. Results were similar for ever or current users of smokeless tobacco and when stratified by smoking status (Boffetta *et al.*, 2005).

The case–control study by Williams and Horm (1977) described in Section 2.2.1 also reported on lung cancer. Among men, the relative risks for lung cancer and for moderate or heavy use of chewing tobacco or snuff were 0.7 (26 cases) and 0.8 (10 cases), respectively, adjusted for age, race and smoking.

(*f*) *Sarcoma*

In the US Veterans' cohort, the relative risk for soft-tissue sarcomas associated with smokeless tobacco use relative to persons who never used tobacco products was 1.5 (95% CI, 0.8–2.7). None of the users of smokeless tobacco who never used other tobacco products developed a soft-tissue sarcoma (Zahm *et al.*, 1992).

A population-based registry in Kansas, USA, provided information on white men aged 21 years or older in 1976–82 who had soft-tissue sarcomas (Zahm *et al.*, 1989). Controls were recruited through RDD and HCFA and were frequency-matched to cases on age (± 2 years). In addition, decedents from Kansas during the same period were selected for deceased cases. Controls with lymphomas, sarcomas or ill-defined malignancies, or who were homicides or suicides were excluded. Telephone interviews were conducted with 133 cases and 948 controls. The odds ratio for ever use of smokeless tobacco was 1.8 (95% CI, 1.1–2.9). Odds ratios for smokeless tobacco use by anatomic site of the soft-tissue sarcoma were: upper gastrointestinal, 3.3 (95% CI, 0.8–12.6); lung, pleura and thorax, 3.1 (95% CI, 0.9–10.5); head, neck and face, 2.4 (95% CI, 0.5–10.2); and others, 1.4 (95% CI, 0.7–2.5). The odds ratios by cell type were: fibromatous, 1.8 (95% CI, 0.7–4.7); adipose, 1.1 (95% CI, 0.2–4.2), myomatous, 2.1 (95% CI, 0.8–5.3), and others, 1.9 (95% CI, 0.9–3.9). The relative risk was highest for those diagnosed at age 80 years or above (relative risk, 3.2; 95% CI, 1.0–10.1).

(*g*) *Breast*

In a study by Spangler *et al.* (2001b) and Spangler (2002), Cherokee Indian women were investigated over a 2-year period, and prevalent breast cancer cases were identified through medical histories from the women themselves, and other female survey respondents formed the control group; the women were interviewed in their homes. The odds ratio for use of smokeless tobacco in the women diagnosed at less than 55 years of age was 1.3 (one case; 95% CI, 0.1–13.9) and that in women diagnosed at more than 55 years was 1.2 (two cases; 95% CI, 0.1–9.5). [There are major limitations to this study. There was no medical verification of breast cancer and the time relationship between use of smokeless tobacco and breast cancer diagnosis was not reported.]

(*h*) *Cervix uteri*

The population-based case–control study of randomly selected patients from the Third National Cancer Survey (1969–71) also reported results on cervical cancer (Williams & Horm, 1977). Controls for the cervical cancer case group comprised patients with other cancers that were unrelated to smoking. The relative risks controlled for smoking, age and race were 4.7 (six cases: $p < 0.05$) for moderate and 3.6 (four cases; non-significant) for heavy use of chewing tobacco or snuff. [The Working Group noted that multiple comparisons were made of many risk factors and many cancer sites in this study and, therefore, that some positive findings may have been due to chance alone.]

(*i*) *Prostate*

The 1601 controls from a case–control study of cancer were used to form a historical cohort in Iowa, USA; they were ascertained in 1986–89 via RDD and US HCFA, had Iowa residency, were aged 40–86 years and had no prior cancer. Twenty-four subjects who required proxy respondents were excluded, which left 1577 in the cohort who answered a mailed questionnaire that was supplemented by telephone interviews. The cohort was followed for vital status, and three persons were lost to follow-up; 103 cases of prostate cancer were identified through the state cancer registry. Two cases were subsequently excluded due to diagnosis before the questionnaire was returned. The authors reported that 'no association was seen for [....] snuff and chewing tobacco' (Putnam *et al.*, 2000). [The Working Group noted that data were not presented to support this statement.]

The Lutheran Brotherhood cohort was examined for deaths from prostate cancer (Hsing *et al.*, 1990). Relative to never use of tobacco, the relative risk for users of smokeless tobacco only was 4.5 (10 deaths; 95% CI, 2.1–9.7) adjusted for age. Ever use of smokeless tobacco compared with never use of tobacco yielded a relative risk of 2.1 (42 deaths; 95% CI, 1.1–4.1), adjusted for age and cigarette smoking. Other relative risks were 1.8 (13 deaths; 95% CI, 0.8–3.9) for former users of smokeless tobacco, 1.4 (5 deaths; 95% CI, 0.5–3.9) for occasional users and 2.4 (24 deaths; 95% CI, 1.3–4.9) for regular users, adjusted for age and cigarette smoking. The relative risk for death from prostate cancer listed on the death certificate, but not as the underlying cause, was 2.3 (14 deaths; 95% CI, 1.0–5.2) for regular users of smokeless tobacco and 2.5 (eight deaths; 95% CI, 1.0–6.5) for smokeless tobacco only users.

In the US Veterans' cohort, the relative risk for prostate cancer of smokeless tobacco only users compared with those who never used any tobacco was 1.2 (48 deaths; 95% CI, 0.9–1.6) (Hsing *et al.*, 1991).

In-home interviews were conducted with population-based cases of prostate cancer and RDD and HCFA controls in the Atlanta metropolitan area, in Detroit and in 10 New Jersey counties, USA (Hayes *et al.*, 1994). Controls were frequency-matched on age and race. Interviews were completed for 981 cases and 1315 controls. Relative to those who had never used tobacco, the odds ratios for chewing tobacco were 1.0 (95% CI, 0.6–1.5) for former users and 0.5 (95% CI, 0.2–1.0) for current users. For snuff, the odds ratios were 0.6 (95% CI, 0.3–1.4) for former and 5.5 (95% CI, 1.2–26.2) for current users.

(*j*) *Penis*

In a case–control study in Chennai, India, in which 505 cases of squamous-cell carcinoma of the penis were identified over a period of 30 years (Harish & Ravi, 1995), the relative risk for snuff users was 4.2 (95% CI, 1.6–11.3) after adjustment for smoking, tobacco chewing and phimosis. [It was not clear whether snuff was used orally or nasally.]

(*k*) *Urinary bladder*

A population-based case–control study was conducted in three provinces of Canada (Howe *et al.*, 1980). Eligible cases were all patients who had recently been diagnosed with urinary bladder cancer; controls were matched individually for sex, age and neighbourhood. The study included 480 men and 152 women (cases), and the same number of controls. In a matched pair analysis, no association between chewing tobacco and bladder cancer was observed; the estimated relative risk was 0.9, based on 61 discordant pairs, and remained unchanged after controlling for smoking.

The study by Hartge *et al.* (1985) included 2982 patients with urinary bladder cancer who were identified from records of 10 large population-based cancer registries throughout the USA (1977–78) and who were interviewed to obtain information on tobacco use and other factors. A total of 5782 population-based controls were included: controls aged under 65 years were chosen by a RDD and those aged 65 years and older were selected from the HCFA. The analysis was restricted to men. Among men who never smoked cigarettes, the relative risk for bladder cancer was 1.0 for chewing tobacco and 0.8 for use of snuff, after controlling for age, race, residence and other non-cigarette tobacco practices.

In a population-based case–control study, urinary bladder cancer cases were identified from the Utah Cancer Registry between 1970 and 1983 in individuals aged 21–84 years (Slattery *et al.*, 1988). RDD and HCFA controls were frequency-matched to cases on age and sex. After exclusion of women and non-white subjects, 332 cases and 686 controls for whom information on tobacco was obtained through an interview at the participants' homes were analysed. The crude odds ratios for urinary bladder cancer were 1.03 for use of snuff and 0.96 for chewing tobacco. When never smokers and smokers were examined separately, the odds ratios for snuff use were 2.7 (95% CI, 0.5–15.6) among never smokers and 0.8 (95% CI, 0.4–1.4) among smokers. Corresponding estimates for tobacco chewing were 2.8 (95% CI, 0.4–20.2) and 1.2 (95% CI, 0.7–2.2).

A population-based case–control study of urinary bladder cancer was conducted in the Alberta and Ontario populations of Canada (Burch *et al.*, 1989). Province-wide annually updated listings were used to identify randomly selected controls who were matched to cases on age, sex and area of residence, and all participants completed a questionnaire. Response rates were 67% for cases and 53% for controls. The odds ratio for urinary bladder cancer was 0.6 (95% CI, 0.3–1.1) for ever versus never use of snuff, and 0.5 (95% CI, 0.2–1.1) for ever versus never chewing tobacco, adjusted for lifetime cigarette consumption. Analyses that were restricted to subjects who had never smoked cigarettes gave similar results [data not shown].

(*l*) *Kidney*

A hospital-based case–control study identified cases of renal cancer aged 20–80 years in 18 hospitals in six US cities in 1977–83 (Goodman *et al.*, 1986). A total of 267 controls were identified by RDD and were individually matched 1:1 on hospital, sex, race, age, time of admission and non-tobacco- and non-obesity-related diseases. The matched odds

ratio for chewing tobacco among men was 4.0 (95% CI, 1.1–14.2) compared with never users. The final logistic model included Quetelet index, consumption of decaffeinated coffee, pack–years of cigarette smoking and chewing tobacco (ever, never and an interaction term of pack–years × chewing tobacco). Based on this model, the odds ratio for chewing tobacco among never users of cigarettes was 0.9 (95% CI, 0.2–5.1).

In a case–control study in 29 hospitals in Oklahoma, USA (Asal *et al.*, 1988), 315 cases and 313 controls were individually matched on age, sex, race, hospital and time of interview. Controls with kidney disease or psychiatric diagnoses were excluded and interviews were conducted during hospitalization. Among men, snuff use was associated with a risk for renal-cell carcinoma to yield an odds ratio of 3.6 (95% CI, 1.2–13.3). [Smoking was not controlled for.]

A case–control study (McLaughlin *et al.*, 1995) that used cases from several countries was carried out in Europe, Australia and the USA. The main source of cases was population-based cancer registries, except in Germany, where cases were identified through hospital networks. Controls were selected from various sources, and interviews were completed for 1732 cases and 2309 controls. The odds ratio for use of smokeless tobacco only versus no use of tobacco was 1.3 (95% CI, 0.6–3.1), adjusted for age, sex, centre and body mass index.

In a case–control study in the USA, cases were ascertained from selected hospitals in the states of New York, Pennsylvania, Illinois and Michigan during 1977–93 (Muscat *et al.*, 1995). Controls who had conditions that were unrelated to tobacco use were selected from the same hospitals and were frequency-matched on age, sex, race, hospital and year of diagnosis. Questionnaires were administered by interviewers in the hospitals. A total of 788 cases and 779 controls were included in the analyses. Relative to men who had never chewed tobacco, the odds ratio for ever use of smokeless tobacco regularly for at least 1 year was 3.2 (95% CI, 1.1–8.7). A dose–response relationship was observed and yielded an odds ratio of 2.5 (95% CI, 1.0–6.1) for chewing 10 times or fewer per week and 6.0 (95% CI, 1.9–18.7) for chewing 11 or more times per week. [Smoking was not controlled for.]

(*m*) *Brain*

In a population-based case–control study in Iowa, USA, data from 375 brain cancer cases and 2434 controls from drivers licence records and HCFA were analysed (Zheng *et al.*, 2001). Cases were 40–85 years of age and controls were selected at a ratio to cases of 6.5:1. Information on tobacco use was obtained through a mailed questionnaire. Next of kin were used as respondents when the cases were deceased. Response rates were above 80% for both cases and controls. The authors reported that “use of [...] snuff or chewing tobacco was also not associated with a significantly increased risk of brain cancer for either men or women”. [Data to support this statement were not presented.]

(*n*) *Non-Hodgkin lymphoma*

Iowa and non-metropolitan areas in Minnesota, USA, were the sites of two population-based studies of non-Hodgkin lymphoma in men (Brown *et al.*, 1992a; Schroeder *et al.*,

2002). White male cases aged 30 years and older were identified in 1980–82. Living cases were matched to RDD and HCFA controls; state vital status lists provided controls for deceased cases. Controls were frequency-matched to cases on age, state of residence and vital status. In-person interviews were conducted for 622 cases and 1245 controls or their next of kin. Persons were considered to be smokeless tobacco users if they had used it daily for at least 3 months. In an analysis by lymphoma subtypes using cases and living controls only, odds ratios adjusted for age and state for users of smokeless tobacco only compared with never users of tobacco were: all lymphomas, 1.3 (95% CI, 0.7–2.5); follicular, 1.7 (95% CI, 0.7–4.3); diffuse, 0.8 (95% CI, 0.3–2.3); small lymphocytic, 1.7 (95% CI, 0.5–5.4); high-grade, 1.3 (95% CI, 0.1–10.8); and unclassified, 1.5 (95% CI, 0.3–7.4). For multiple myeloma, the odds ratio was 1.9 (95% CI, 0.5–6.6), adjusted for age. In a further analysis of lymphoma subtypes by t(14;18) positivity (Schroeder *et al.*, 2002), no consistent pattern emerged.

(*o*) *Leukaemia*

Brown *et al.* (1992b) conducted a case–control study of tobacco use and risk for leukaemia. Personal interviews were conducted with subjects or with close relatives for those who were deceased or too ill. Odds ratios adjusted for age, state and use of alcoholic beverages for users of smokeless tobacco only compared with non-users of tobacco were: all leukemias, 1.8 (95% CI, 0.9–3.3); acute non-lymphocytic, 0.9 (95% CI, 0.2–3.1); chronic myelogenous, 2.1 (95% CI, 0.4–10.7); chronic lymphocytic, 1.9 (95% CI, 0.8–4.3); myelodysplasia, 2.7 (95% CI, 0.8–9.4); other, 3.0 (95% CI, 0.9–9.2).

2.3 Nasal use

2.3.1 *Cancer of the oral cavity*

Three case–control studies from Kerala, India (Sankaranarayanan *et al.*, 1989a,b, 1990a) investigated the association between nasal snuff use and cancer of oral subsites among men (Table 78).

The first part of the study (Sankaranarayanan *et al.*, 1989b) focused on cancer of the anterior two-thirds of tongue and floor of the mouth and comprised 158 cases and 314 controls who were selected from a pool of 546 hospital controls with non-malignant conditions at sites other than the head and neck and were matched for age and religion. For cancer of the tongue and floor of the mouth, the age-adjusted odds ratio was 3.0 (95% CI, 0.9–9.6) for regular snuff users and 4.3 (95% CI, 1.2–14.7) for occasional snuff users. The odds ratio for < 100 unit years was 10.0 (95% CI, 1.2–86.1) and that for ≥ 100 unit years was 1.1 (95% CI, 0.2–6.2).

The second part of the study on cancer of the gingiva (Sankaranarayanan *et al.*, 1989a), comprised 109 cases, and the third part on cancer of buccal and labial mucosa comprised 250 cases (Sankaranarayanan *et al.*, 1990a). All 546 controls from the same pool as that in the first study were used for both the second and third studies. For gingival

Table 78. Case–control studies of nasal use of smokeless tobacco and oral cancer

Reference, study location, period	Organ site (ICD code)	Characteristics of cases	Characteristics of controls	Exposure categories	No. of exposed cases	Relative risk (95% CI)	Adjustment for potential confounders
Sankaranarayanan *et al.* (1989b), India, 1983–84	Tongue (ICD-9 141.1, 141.2, 141.3, 141.4), floor of mouth (ICD-9, 144)	158 men; biopsy-proved cases of cancer from one hospital	314 male patients with non-malignant conditions at sites other than head and neck; matched by age, religion	*Snuff inhalation* No Yes *Snuff inhalation* Never < 100 unit years ≥ 100 unit years	 142 8 142 6 2	 1.0 3.0 (0.9–9.6) 1.0 10.0 (1.2–86.1) 1.1 (0.2–6.2)	Age
Sankaranarayanan *et al.* (1989a), India, 1983–84	Gingiva (ICD-9, 143.0, 143.1)	109 men from one hospital	546 male patients with non-malignant conditions at sites other than head and neck	*Snuff inhalation* No Yes	 100 4	 1.0 3.0 (0.7–12.6)	*Bidis*, alcoholic beverages, betel quid
Sankaranarayanan *et al.* (1990a), India, 1983–84	Buccal mucosa (ICD-9, 145.0, 145.1, 145.6), labial mucosa (ICD-9, 140.3, 140.4)	250 men from one hospital	546 male patients with non-malignant conditions at sites other than head and neck	*Snuff inhalation* No Yes *Snuff inhalation* Never < 100 unit years ≥ 100 unit years	 232 12 232 7 5	 1.0 2.9 (0.98–8.8) 1.0 15.7 (2.0–125.3) 2.0 (0.6–6.6)	*Bidis*, alcoholic beverages, betel quid Age

cancer, the age-adjusted odds ratio for daily snuff use was 3.9 (95% CI, 1.2–12.7) and that for occasional use was 3.8 (95% CI, 1.1–13.5). The odds ratio for regular snuff use was 3.0 (95% CI, 0.7–12.7) after adjustment for daily frequency of use of betel quid, *bidi* smoking and alcoholic beverage use. For cancer of the buccal and labial mucosa, the age-adjusted odds ratio was 4.0 (95% CI, 1.5–10.3) for regular snuff users and 2.3 (95% CI, 0.8–7.0) for occasional snuff users. After adjusting for daily frequency of use of betel quid, *bidi* smoking and alcoholic beverage use, the odds ratio was 2.9 (95% CI, 0.98–8.8). The odds ratio for users of < 100 unit years was 15.7 (95% CI, 2.0–125.3) and that for users of ≥ 100 unit years was 2.0 (95% CI, 0.6–6.6).

2.3.2 *Cancer of the oesophagus*

The series of case–control studies from Kerala, India, also reported on 267 male patients with cancer of the oesophagus and the same 546 controls (Sankaranarayanan *et al.*, 1991). The age-adjusted odds ratio for daily snuff use was 2.4 (95% CI, 0.8–7.0) and that for occasional use was 3.6 (95% CI, 1.2–10.7) (Table 79). [The Working Group noted that effect estimates were not adjusted for smoking or betel quid chewing.]

2.3.3 *Cancer of the paranasal sinus*

Shapiro *et al.* (1955) studied 37 Bantu cases of cancer of the paranasal sinus from radiation therapy department records from 1949–51 of a group of hospitals in Johannesburg, South Africa. Cancer of the paranasal sinuses (22 men, five women) accounted for a high proportion of respiratory tract cancer (71% of men, 83% of women) in Bantu Africans. This was in sharp contrast to European cases seen in the Transvaal, where only seven (5%) of the respiratory tract cancers occurred in the nasal sinuses. Most of the cancers were in the maxillary antrum (28/34 studied) and were described typically as well-differentiated ‘squamous epitheliomata’. The authors noted that 80% of the 28 antral cancer cases reported ‘prolonged and heavy’ use of snuff in contrast to only 34% of Bantu men with cancer at other sites. According to Keen *et al.* (1955), the product snuffed by Bantus typically contained powdered tobacco leaves and an ash from aloe plants or other species, with the occasional addition of oil, lemon juice and herbs; typical use was ‘one teaspoonful’ per day. The authors stated that ‘there was no obvious correlation’ between cancer of the maxillary antrum and cigarette, pipe or *dagga* [marijuana] smoking. [The Working Group noted that the source and nature of the control group was not described.]

2.3.4 *Cancer of the larynx*

The series of case–control studies from Kerala, India, also reported on 191 male patients with biopsy-proved cancer of the larynx and used the same 546 controls (Sankaranarayanan *et al.*, 1990b). The age-adjusted odds ratio for daily snuff use was 1.2 (95% CI, 0.3–4.9) and that for occasional use was 2.8 (95% CI, 0.9–8.7) (Table 79). [The Working Group noted that effect estimates were not adjusted for smoking.]

Table 79. Case–control studies of nasal use of smokeless tobacco and cancer at other sites

Reference, study location, period	Characteristics of cases	Characteristics of controls	Exposure categories	No. of exposed cases	Relative risk (95% CI)	Adjustment for potential confounders
Oesophagus						
Sankaranarayanan *et al.* (1991), India, 1983–84	207 men from one hospital	546 male patients with non-malignant conditions at sites other than head and neck	Snuff inhalation No Yes	 192 7	 1.0 2.4 (0.8–7.0)	Age
Larynx						
Sankaranarayanan *et al.* (1990b), India, 1983–84	191 men biopsy-proved from one hospital	546 male patients with non-malignant conditions at sites other than head and neck	Snuff inhalation No Yes	 182 3	 1.0 1.2 (0.3–4.9)	Age
Lung						
Hsairi *et al.* (1993), Tunisia, 1988–89	110 (107 men, 3 women) from one hospital in Tunis; 77 histologically confirmed	110 men individually matched on age, sex, cigarettes/day (± 5)	Use of smokeless tobacco	20	2.2 (0.9–5.6)	Age, sex, number of cigarettes/day, water pipe, cannabis

2.3.5 *Cancer of the lung*

A case–control study was conducted by Hsairi *et al*. (1993) on 110 (107 men, three women) bronchial cancer patients and 110 controls individually matched for age, sex and number of cigarettes (± 5) smoked per day (Table 79). Cases were recruited from December 1988 to May 1989 in the Ariana Hospital that covered Tunis City and the suburban area; controls were chosen among residents of the same area. Twenty cases (18.2%) and eight controls (7.3%) had ever inhaled snuff, which yielded a crude odds ratio of 2.8 (95% CI, 1.2–6.8). The Cochrane Mantel-Haenzel method was used to adjust the association for age, sex, cigarette use (0, 1–10, 11–20, ≥ 20 per day), and water pipe and cannabis use. The adjusted odds ratio obtained was 2.2 (95% CI, 0.9–5.6). The authors indicated that no quantitative analyses were appropriated as the amounts used were 'relatively weak'. [The paper was written in French and the expression 'tabac à priser' was used for smokeless tobacco. The Working Group deduced that this represented nasal use of snuff according to the popular meaning of this expression. Nine interviewers were involved in the data collection. The control recruitment was not reported in detail.]

3. Studies of Cancer in Experimental Animals

The Working Group that evaluated smokeless tobacco previously noted that the majority of the early studies evaluated at that time (IARC, 1985) had various deficiencies, such as lack of quantitative and qualitative information on the nature of tobacco extracts and the degree of extraction, insufficient length of treatment, small group sizes and, in some cases, lack of appropriate controls. Since that time, new studies have been published and are included in this section. The cumulative published evidence for carcinogenicity of smokeless tobacco in experimental animals is summarized below and has also been reviewed recently (Hoffmann & Djordjevic, 1997; Grasso & Mann, 1998).

3.1 Tobacco

3.1.1 *Oral administration*

(*a*) *Mouse*

Groups [numbers unspecified] of male Swiss mice, 6–8 weeks of age, were administered a tobacco extract (ethanol extract from 50 g tobacco diluted in 10 mL distilled water) from a commercially available Indian chewing tobacco at a dilution of 1:25 or 1:50 [actual dose unspecified] by oral intubation for 15–20 months. A further group of mice was fed a diet that contained an extract of 10 g tobacco per 5 kg diet for up to 25 months. A group of 20 mice received distilled water only by intubation and served as controls. Administration of the 1:25 dilution was terminated at 18 weeks because of high mortality. Tumour incidences at 15–20 months were 0/4, 8/15 and 4/10 in the control, 1:50 dilution

and 1:25 dilution groups, respectively. At 21–25 months, 1/20 controls and 8/10 animals fed tobacco extract in the diet had developed tumours. The types of tumour observed were lung adenocarcinoma or hepatocellular carcinoma (Bhide *et al.*, 1984b). [The Working Group noted the incomplete reporting of the distribution of different types of neoplasm.]

(*b*) *Rat*

Weanling male Sprague Dawley rats were fed diets containing shark liver oil (sufficient in vitamin A, 60 rats) or without shark liver oil (vitamin A-deficient, 61 rats). Tobacco extract was prepared by extracting 100 g commercial tobacco with 1 L dichloromethane at room temperature for 72 h; the mixture was then filtered and dried under vacuum. Half of the rats in each group (29 vitamin A-sufficient, 31 vitamin A-deficient) received 3 mg tobacco extract dissolved in 0.05 mL dimethylsulfoxide (DMSO) by gavage five times per week for 21 months. The remaining (control) rats (31 vitamin A-sufficient, 30 vitamin A-deficient) received 0.05 mL DMSO by gavage five times per week for 21 months. All rats were necropsied, and all organs were examined for gross abnormalities; the liver, lung, stomach, brain and pituitary gland were examined histologically. No tumours were observed in control rats, irrespective of vitamin A status. Among vitamin A-sufficient rats given tobacco extract, 6/29 had single tumours: 3/29 had lung adenomas, 3/29 had forestomach papillomas, 0/29 had lung lymphomas or pituitary adenomas. Among vitamin A-deficient rats given tobacco extract, 29/31 had one or more tumours: 22/31 had lung lymphomas, 19/31 had pituitary adenomas, 28/31 had stomach papillomas and 0/31 had lung adenomas. The proportions of tumour-bearing rats were significantly greater in both tobacco extract-treated groups than in the corresponding control groups ($p < 0.001$, χ^2 test) (Bhide *et al.*, 1991). [The Working Group noted that primary lymphoma of the lung is extremely uncommon in rats. However, there was an increased incidence only of benign tumours in the vitamin-A sufficient rats.]

3.1.2 *Application to the oral mucosa or cheek pouch*

(*a*) *Mouse*

Groups of 9–16 male and female strain A (Strong) and Swiss mice, 2–3 months old, were administered different alkaloid-free extracts of an Indian chewing tobacco of the Vadakkan type (Meenampalayam variety). The extracts — a benzene extract and its neutral fraction, a water extract and four successive extracts (petroleum ether, benzene, chloroform and ethanol) — were applied by daily application to the oral mucosa for up to 18 months of age. No tumours were observed in mice exposed to the chewing tobacco extracts (Mody & Ranadive, 1959). [The Working Group noted the small number of animals used.]

(*b*) *Rat*

A group of 22 Wistar rats, 5 months of age, were painted on the oral mucosa with a 2% alkaloid-free extract of Vadakkan tobacco (Meenampalayam variety) in acetone twice

a week for life; 12 of these animals were also painted with a paste of lime (20% in distilled water) on the day after each treatment. Control groups of 10–14 rats received no treatment or were treated with lime only. No tumour was observed at the application site (Gothoskar *et al*., 1975).

(*c*) *Hamster*

A group of 50 young Syrian golden hamsters [age unspecified] received an implantation of a 2-cm^3 plug of chewing tobacco [unspecified] in the cheek pouch. The opening in the cheek pouch was ligated and the animals were followed for up to 30 months. Survival after 13 months was 21/50; eight were alive at 24 months, but none were alive at 30 months. No tumour was observed in any of the animals (Peacock & Brawley, 1959; Peacock *et al*., 1960).

A group of 34 male and female Syrian golden hamsters, 1–2 months of age received an implant into the cheek pouch of pellets of Philippine leaf tobacco with 10% lime mixed with beeswax. Animals were allowed to live their lifespan (up to 22 months) and were killed when moribund. No tumour at the implantation site was reported (Dunham & Herrold, 1962).

Groups of 11–12 male Syrian golden hamsters, 9 weeks of age, received topical applications on the cheek-pouch mucosa of a DMSO extract of cured Banarsi chewing tobacco or DMSO alone thrice weekly for 21 weeks, at which time all animals were killed. No tumour was seen in treated or control hamsters, but 8/12 treated animals had leukoplakia (Suri *et al*., 1971). [The Working Group noted the short duration of the experiment.]

A group of 12 male inbred Syrian golden hamsters, 2–3 months old, received topical applications to the cheek-pouch mucosa of DMSO extracts of an Indian chewing tobacco (Vadakkan) thrice weekly for life. A control group of seven animals received applications of DMSO alone. No local tumour but moderate hyperkeratosis was observed (Ranadive *et al*., 1976). However, one animal developed a stomach tumour [pathology is not described] after exposure to DMSO tobacco extract. [The Working Group noted that a similar stomach tumour developed in another experiment when a mixture of tobacco and areca nut DMSO extract was applied.]

Groups of 30–41 Syrian golden hamsters [sex unspecified], weighing 40–50 g, received an application of 60 g tobacco (‘Jada Jarda’) alone, in combination with lime or in combination with lime plus vitamin A in the cheek pouch thrice weekly for 100–110 weeks, at which time 24–32 animals were still alive. Moderate to severe keratotic and dysplastic changes developed in the mucosa, but no neoplastic change was observed (Kandarkar *et al*., 1981).

A group of 20 female Syrian golden hamsters, 6–7 weeks of age, received topical applications to the cheek-pouch mucosa of 1 mg lyophilized aqueous tobacco extract in 0.05 mL water twice daily for 6 months. Animals were observed for a further 6 months and were then killed. Squamous-cell papillomas and/or carcinomas occurred in 3/17 animals compared with none in 10 untreated and 10 vehicle (water) controls (Rao, 1984). [The findings were not statistically significant.]

Eighty male Syrian golden hamsters, 8 months of age, were divided into four treatment groups of 20 animals each: tobacco only, alcohol only, tobacco and alcohol and untreated controls. Smokeless tobacco (Skoal®, US Tobacco Co., Nashville, TN; 200 mg) was placed in each cheek pouch of hamsters in the tobacco groups five times a week. In the alcohol groups, 2 mL 15% ethanol was placed in each cheek pouch five times weekly. Hamsters in the negative control group received mechanical stimulation of the right cheek pouch to simulate the placement of the tobacco. After 26 weeks, the hamsters were killed and pouches and abdominal organs were examined. Acanthosis of the pouch epithelium was noted more frequently in the groups treated with tobacco (14/20; $p < 0.005$) and tobacco plus alcohol (12/20; $p < 0.025$), but no tumours were observed in the cheek pouches. Adenomas of the adrenal gland were noted in 2/20 hamsters in the tobacco-treated group and in 1/20 hamsters in each of the other three groups. Squamous-cell papillomas of the forestomach occurred in 2/20 hamsters in the tobacco-treated group, 3/20 hamsters in the alcohol-treated group, 4/20 hamsters in the alcohol plus tobacco-treated group and 0/20 hamsters in the control group. Incidences of forestomach tumours in the treated groups were not significantly elevated above the zero incidence in controls (Summerlin *et al.*, 1992). [The Working Group noted the short duration of the study and the advanced age of the animals at the beginning of the treatment.]

3.1.3 *Skin application*

Mouse

Groups of 40 CAF_1 (Jackson) and 40 Swiss (Millerton) mice [sex and age unspecified] received topical applications of a 50% methanol extract of unburnt cigarette tobacco on the skin three times a week for 24 months. Groups of 30 CAF_1 and 30 Swiss mice that similarly received whole-tar extract for 21–24 months served as controls. Among the CAF_1 mice exposed to the tobacco extract, 11 developed papillomas; among the Swiss mice, three treated mice developed papillomas compared with 16 papillomas that developed in each of the control groups. One papilloma later developed into cancer in the extract-treated Swiss mice compared with three that transformed in control Swiss mice and eight in control CAF_1 mice (Wynder & Wright, 1957).

Groups of 8–17 male and female strain A (Strong) and Swiss mice, 2–3 months of age, received skin applications of five different extracts (petroleum ether, benzene, chloroform, chloroform ether and ethanol) of an Indian chewing tobacco (Vadakkan type, Meenampalayam variety) up to 18 months of age; no tumour was observed at the site of application, and no excess incidence was reported at other sites (Mody & Ranadive, 1959). [The Working Group noted the small numbers of animals used.]

A group of 10 male and six female inbred strain C17 mice, 2–3 months of age, received thrice-weekly applications of a DMSO extract of an Indian chewing tobacco (Vadakkan type) on the skin of the interscapular region until 24 months of age. No skin tumour was observed (Ranadive *et al.*, 1976). [The Working Group noted the small number of animals used.]

3.1.4 *Other routes of administration*

(*a*) *Inhalation*

Mouse

Groups of 80 male strain A mice, 3 months of age, were exposed by inhalation to powdered tobacco leaf on alternate days for 30 months or served as untreated controls. The incidence of lung tumours (six alveologenic carcinomas, 35 squamous-cell carcinomas and three 'malignant adenomas'), leukaemia and hepatocellular carcinoma in animals surviving to 30 months was: 12/75 [$p < 0.001$; Fisher's exact test] and 1/80, 11/75 [$p < 0.01$; Fisher's exact test] and 2/80 and 3/75 and 0/80 in the treated and control groups, respectively (Hamazaki & Murao, 1969). [The Working Group noted that, while the incidence of lung tumours and leukaemia in treated animals was significantly increased, the incidence of lung and liver tumours in the untreated mice was unusually low.]

(*b*) *Subcutaneous administration*

Mouse

Two groups of 17 Paris albino XVII × C57 black mice [age and sex unspecified] received multiple subcutaneous injections of 0.1 mL of a 2% solution of 'partially or completely alkaloid-free' extract of tobacco (Vadakkan, Meenampalayam variety) once a month for 41–95 weeks. One squamous-cell carcinoma [site not specified] developed in an animal that received the partially alkaloid-free extract (Ranadive *et al.*, 1963). [The Working Group noted that the results were inconclusive.]

(*c*) *Intravesicular implantation*

Mouse

Groups of 5–12 male and female inbred strain C17 and Swiss mice, 2–3 months of age, received a single intravesicular implantation of paraffin pellets that contained chewing tobacco (Jarda), a mixture of chewing tobacco and lime or an alkaloid-free chewing tobacco extract or paraffin pellets alone, and were observed until 10–30 months of age. Among the C17 mice that received the alkaloid-free tobacco implantation, 2/12 developed transitional-cell tumours of the bladder and one female developed a tumour described as a 'myosarcoma of the cervix with metastasis to the kidney'. No tumour was observed in the controls or in the other treated groups (Randeria, 1972). [The Working Group noted the small group size and the potential carcinogenic effect of intravesicular foreign bodies in mice.]

(*d*) *Vaginal application*

Mouse

A group of four female inbred C17 strain mice and four female Swiss mice, 2–3 months of age, received daily vaginal applications of a fine mixture of (Jarda) tobacco dust that

contained lime derived from sea shells for 10–30 months: no vaginal tumour was observed (Randeria, 1972). [The Working Group noted that no control group was used in this study.]

3.1.5 *Skin application with known carcinogens or modifiers*

Mouse

Groups of 11–36 Paris albino XVII × C57 black (hybrid) or inbred Swiss mice [sex and age unspecified] received twice-weekly skin applications of 'total extract' plus 'partially alkaloid-free extract' or 'totally alkaloid-free extract' of 'Vaddakan' tobacco of Meenampalayam variety or acetone (control) for 95 weeks followed by weekly applications of croton oil. No control group of Swiss mice was included. Between 61 and 95 weeks after the start of treatment, the incidence of papillomas and of squamous-cell carcinomas at the site of application was: 10/21 and 6/21, 9/25 and 2/25, 22/35 and 10/35 and 3/19 and 0/19 in the hybrid mice, respectively. [The increases in the incidence of papillomas and carcinomas were statistically significant, except in the 'partially alcaloid-free extract'-treated group.] The incidence of papillomas in the Swiss mice was 2/9, 2/4 and 3/10, in the three tobacco-treated groups, respectively; no carcinoma was observed (Ranadive *et al*., 1963).

The co-carcinogenic [promoting] effect of the 'totally alkaloid free' extract of 'Vaddakan' tobacco of Meenampalayam variety was tested in a group of 16 Swiss albino and 13 hairless Swiss (Baldy) mice [sex and age unspecified] that received a single topical application of benzo[*a*]pyrene [dose unspecified] followed by twice-weekly applications of the extract for 80 weeks. A group of seven Swiss albino and 10 Swiss (Baldy) mice received the benzo[*a*]pyrene treatment only and served as controls. Two carcinomas and four papillomas were observed in Swiss (Baldy) mice treated with the tobacco extract and benzo[*a*]pyrene; no tumour was observed in benzo[*a*]pyrene-treated controls (Ranadive *et al*., 1963). [The Working Group noted the small number of animals and incomplete information concerning the initiating dose of benzo[*a*]pyrene.]

A total of six groups of 30 female ICR Swiss mice, 57 days of age, were untreated or received a single topical application of 125 µg 7,12-dimethylbenz[*a*]anthracene (DMBA) in 0.25 mL acetone. Twenty-one days later, mice received an application of 0.25 mL of either an acetone or 'concentrated' or 'dilute' barium hydroxide extract of unburnt commercial tobacco five times a week for 36 weeks. The amount of acetone extract was equivalent to 2.5 cigarettes per day. The barium hydroxide extract was prepared using two different extraction procedures (designated 'concentrated' and 'dilute') according to the yield: the 'concentrated' extract was equivalent to 0.5 cigarette per day and the 'dilute' extract was approximately 25% as strong as the 'concentrated' extract. Two control groups of 30 mice were untreated or received DMBA only. The incidence of tumours (all of which were small papillomas) was: acetone extract, 16 tumours in 7/30 mice (2.3 tumours per mouse); concentrated barium hydroxide extract, 18 tumours in 8/30 mice (2.2 tumours per mouse); and dilute barium hydroxide extract, six tumours in 2/30 mice (three tumours per mouse). No tumour was observed in either of the groups that received acetone or barium hydroxide

tobacco extract without DMBA pretreatment, in DMBA-treated or in untreated groups (Bock *et al*., 1964).

Groups of 30 female ICR Swiss mice, 55–60 days of age, were untreated or received a single topical application of 125 μg DMBA in 0.25 mL acetone. Three weeks later, mice were either untreated or received applications of different aqueous extracts (crude, acidic, neutral and basic fractions) of an unprocessed, commercial, flue-cured tobacco five times per week for 26 weeks. A total of 12 papillomas developed in 6/30 mice treated with crude tobacco extract (equivalent to 0.5 g tobacco daily) after DMBA initiation. One mouse developed a papilloma after treatment with the acidic fraction and DMBA. No skin tumour was found in animals treated with neutral or basic fractions after DMBA initiation, DMBA alone or with the various fractions of tobacco alone. After treatment with half the concentration (0.25 g tobacco), one mouse treated with the crude extract developed a papilloma and one mouse treated with the neutral fraction developed three papillomas after DMBA initiation (Bock *et al*., 1965).

Groups of 20 female Swiss ICR/Ha mice, 8 weeks of age, received a single application on the dorsal skin of 150 μg DMBA in 0.1 mL acetone followed 2–3 weeks later by thrice-weekly applications of solvent extracts (ether [25 mg], chloroform [1 mg], methanol [25 mg] or a reconstituted sample [25 mg]) of a flue-cured cigarette variety of tobacco leaf for 52 weeks. Groups of 20 mice that received DMBA alone or tobacco extracts alone served as controls. Two of 13 survivors in the DMBA/methanol extract group developed ‘cancers’. The numbers of mice with papillomas in the various groups were: 4/12 (ether extract), 1/10 (chloroform extract), 2/13 (methanol extract) and 5/14 (reconstituted extract). No tumour was observed in mice treated with DMBA or extracts alone (Van Duuren *et al*., 1966).

3.2 Snuff tobacco

[The Working Group noted that specific brands of snuff used in most studies was not specified by the investigators.]

3.2.1 *Oral administration*

Hamster

A total of 13 male and female Syrian golden hamsters, 1.5 months of age, were fed three different test substances for 16 months: group 1 (two males and two females) was fed 0.75 g scented snuff [type unspecified] per week; group 2 (two males and two females) was fed 0.75 g scented snuff [type unspecified] and 0.75 g calcium hydroxide per week; and group 3 (five animals) [sex distribution not specified] received calcium hydroxide alone. One male hamster in group 2, estimated to have consumed 52 g snuff and 52 g calcium hydroxide during the 16-month period, developed a pancreatic carcinoid 4.5 months after the termination of treatment. Another hamster that was not fed snuff developed a carcinoid of the glandular stomach at the age of 26 months. The tumour

incidence in the remaining groups and at other sites was not reported; however, the authors stated that no carcinoids had been found in more than 700 hamsters necropsied previously in that laboratory (Dunham *et al*., 1975). [The Working Group noted the relatively small group size used.]

Groups of 50 male BIO 15.16 and BIO 87.20 strain (carcinogen-susceptible) Syrian hamsters, 2–3 months of age, were fed one of the following five experimental diets for 2 years: diet containing 20% damp fresh US snuff; cellulose mixed with diet, such that the caloric content was reduced by 20% (negative control); control diet plus 50 treatments with 5 mg 20-methylcholanthrene per animal by stomach tube (positive control); cellulose diet plus 50 treatments with 0.5 mg 20-methylcholanthrene per animal by stomach tube; and snuff diet plus 50 treatments with 0.5 mg 20-methylcholanthrene per animal by stomach tube. The animals fed snuff diet alone showed a spectrum of tumours that was nearly identical to that of controls. No increased incidence of tumours was noted in animals administered snuff with 20-methylcholanthrene (Homburger *et al*., 1976).

3.2.2 *Application to the oral mucosa or cheek pouch*

(*a*) *Rat*

A group of 21 male and 21 female Sprague-Dawley rats, 3 months of age, was administered snuff into a surgically created canal in the lower lip. Approximately 0.2 g of a standard Swedish snuff (Röda Lacket; pH 8.3), was injected into the canals morning and night on 5 days per week for up to 22 months. The calculated daily dose (Hirsch & Thilander, 1981) was 1 g/kg bw and the mean retention time after each administration was 6 h (range, 5–8 h). The rats were killed at 9, 12 and 18–22 months. A second group of five male and five female rats was treated similarly with the same snuff but at pH 9.3 [produced by the addition of 50% sodium carbonate (1% of the total weight)] and was killed at between 18 and 22 months. Of 42 animals administered the snuff, one developed a squamous-cell carcinoma of the oral mucosa at 8.5 months. No tumour was seen in rats exposed to the alkaline snuff or in 15 rats that had surgically created canals but were not given snuff. Benign tumours outside the oral cavity were observed at approximately equal frequency in control and treated groups in both experiments (Hirsch & Johansson, 1983).

Fifteen male and 15 female HMT rats, 6 months of age, received weekly applications of smokeless tobacco to the buccal mucosa for 1 year and were followed for an additional observation period of 6 months. A commercially available snuff tobacco (0.4 g per pack) was moistened with distilled water and applied with a cotton swab to both sides of the mandibular mucobuccal fold of each rat. Tobacco was gradually swallowed by the rats and disappeared after several hours. Fifteen male and 15 female control rats received sham treatments with cotton swabs that were wetted with distilled water. No oral carcinomas were observed in treated or control rats (Chen, 1989).

Beginning at 10 weeks of age, the lips and oral cavities of male Fischer 344 rats were swabbed with 0.5 mL water (controls; 21 rats, group 1), aqueous snuff extract (30 rats, group 2), aqueous snuff extract enriched with 10-fold the natural concentrations of the

tobacco-specific nitrosamines *N′*-nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) (30 rats, group 3) or an aqueous solution of NNN plus NNK in concentrations equal to those in the *N*-nitroso compound-enriched snuff extract group (30 rats, group 4). The snuff used was a moist snuff product commercially available in the USA. Swabbing was performed once or twice daily during weeks 1–23 and twice daily during weeks 24–131. The rats were observed until moribund or until survivors were killed at the end of the study, when complete necropsies were performed. Oral cavity tumours (papillomas of the cheek, hard palate or tongue) developed in 0/21, 0/30, 3/30 and 8/30 rats in groups 1–4, respectively. Lung tumours developed in 1/21 (adenoma), 0/30, 2/30 (adenomas) and 5/30 (one adenoma, four adenocarcinomas) rats in groups 1–4, respectively. The incidence of oral tumours in group 4 was significantly greater than that in group 1 ($p < 0.05$; *t* test and χ^2 test). Tumours were also seen at various other sites in all groups but were not related to treatment. Snuff extract alone induced no tumours of either the oral cavity or lung (Hecht *et al.*, 1986).

Surgery to create a test canal in the lower lip was performed on 95 male Fischer 344 rats at 10 weeks of age. Animals were tested for 2 weeks for wound healing which ensured that the epithelium of the lip canal was intact. Beginning at 13 weeks of age, rats received no further treatment (controls; 10 rats, group 1) or received moist snuff (a brand commercially available in the USA; 32 rats, group 2), water-extracted snuff (21 rats, group 3) or snuff enriched with its own aqueous extract (32 rats, group 4). Snuff preparations (approximately 50 mg per rat) were inserted into the surgically created test canals on 5 days per week and were generally retained in the test canal for 24 h. The experiment was terminated after 116 weeks. Tumours developed in the test canal or the oral cavity in 0/10 control rats (group 1), 3/32 snuff-treated rats (group 2; one papilloma and one squamous-cell carcinoma of the test canal, one papilloma of the hard palate), 2/21 rats treated with water-extracted snuff (group 3; one papilloma of the tongue, one papilloma of the hard palate) and 1/32 rats treated with enriched snuff (group 4; papilloma of the floor of the mouth). One olfactory tumour (esthesioepithelioma) also occurred in group 4. None of these results was statistically significant (Hecht *et al*., 1986).

In an experiment of snuff-induced carcinogenesis, surgery to create a test canal in the lower lip was performed on male Sprague-Dawley rats at 8–9 weeks of age (see Table 80). Treatments were begun in groups of 30 rats 3–4 weeks after surgery and were continued for up to 108 weeks. One group received snuff (a brand available commercially in the USA), packed into the test canal with a spatula (at least 100 mg per application) twice daily on 5 days per week. (Snuff from the previous application was removed before the next treatment.) A second group of control rats received a cotton pellet dipped in saline twice daily on 5 days per week for 104 weeks. At the end of the study, complete necropsies were performed. Among the 29 rats of the snuff-treated group, five squamous-cell carcinomas (one lip, two hard palate, one nasal cavity, one forestomach), one squamous-cell carcinoma *in situ* (hard palate), three squamous-cell papillomas (one each of lip, hard palate and nasal cavity) and two undifferentiated lip sarcomas developed. No such tumours developed

Table 80. Tumour incidence in Sprague-Dawley rats following repeated treatment with snuff tobacco in the lip canal

Reference	Tumour site	Tumour type	Treated		Control
			Benign	Malignant	
Johansson *et al*. (1989)				*n* = 29	*n* = 29
	Lip	Sarcoma		2	0
		Squamous-cell papilloma	1		0
		Squamous-cell carcinoma		1	0
	Hard palate	Squamous-cell papilloma	1		0
		Squamous-cell carcinoma		3[a]	0
	Nasal cavity	Squamous-cell papilloma	1		0
		Squamous-cell carcinoma		1	0
	Forestomach	Squamous-cell carcinoma		1	0
	Total tumours		3	8*	0
Johansson *et al*. (1991)				*n* = 38	*n* = 30
	Lip	Squamous-cell papilloma	2		0
		Sarcoma		10	1
	Hard palate	Squamous-cell carcinoma		3	0
	Total tumours		2	13*	1

[a] Including one '*in situ*'
* $p < 0.01$

among the 29 control rats (all squamous-cell tumours, $p < 0.01$; malignant squamous-cell tumours, $p < 0.05$; Fisher's exact test) (Johansson *et al.*, 1989).

In an experiment designed to study the influence of 4-nitroquinoline *N*-oxide (4-NQO) and DMBA on snuff-induced carcinogenesis, male Sprague-Dawley rats underwent surgery to create a lip canal at 10 weeks of age (see Table 80). One group of 38 rats received 150–200 mg snuff (generic moist snuff type 1S3, University of Kentucky Research Center, USA) placed in the lip canal with a spatula twice daily on 5 days per week for 104 weeks. A second group of 30 control rats received cotton pellets dipped in saline once daily on 5 days per week for 100 weeks. Rats were killed when moribund, when they developed lip tumours or 104 weeks after the beginning of the study, and a complete necropsy was performed. Tumour incidences in different groups were compared by the Student *t* test and Fisher's exact test. Sarcomas of the lip occurred in 10/38 rats in the snuff-treated group ($p < 0.01$) and in 1/30 rats in the control group. Squamous-cell carcinomas and papillomas of the oral cavity (lip, palate and buccal mucosa) occurred in 3/38 rats in the snuff-treated group and in 0/30 rats in the control group. The incidence of epithelial tumours of the oral cavity of rats treated with snuff was not significantly different from that in controls. However, the combined incidence of malignant epithelial and mesenchymal tumours of lip and oral cavity was significantly greater in rats treated with snuff (three

squamous-cell carcinomas of the palate and 10 sarcomas of the lip; 13/38; $p < 0.01$) than in controls (one sarcoma of the lip; 1/30) (Johansson *et al*., 1991).

(*b*) *Hamster*

Groups of 50 young Syrian golden hamsters [age and sex unspecified] received an instillation into the left cheek pouch of 10 mL of a thick paste of snuff. The opening of the pouch was ligated, and the animals were followed for up to 30 months. The contralateral pouches of 25 of these animals were filled with sand and gum and served as controls. After 13 months, 21/50 hamsters were still alive; 10 were alive at 24 months, but none were alive at 30 months. No tumour was observed in control or treated pouches (Peacock & Brawley, 1959; Peacock *et al*., 1960).

A group of 35 male and female Syrian golden hamsters, 1–2 months of age, received a beeswax pellet that contained 20% snuff and 3% lime in the cheek pouch. A positive-control group of 71 hamsters was exposed to DMBA and 20-methylcholanthrene; a negative-control group of 36 animals was exposed to beeswax, which was used as a vehicle to prolong the retention time of the test substances. The animals were killed after 15–20 months or when moribund. Two of 35 animals exposed to snuff and lime and 2/36 exposed to beeswax only developed inflammatory lesions; among the positive controls, 23/56 developed malignant tumours (Dunham & Herrold, 1962).

Groups of four to seven male and female weanling Syrian golden hamsters [age unspecified] received twice-daily applications of 50 mg of a commercial US ‘Scotch’ (dry type) snuff, snuff and calcium hydroxide or calcium hydroxide alone into the cheek pouch on 5 days per week for up to 99 weeks. No local tumour was observed in any group (Dunham *et al*., 1966).

A group of 84 male and female Syrian golden hamsters (BIO hamsters of the RB strain), aged 3–4 months, was exposed to 0.5 g snuff placed in a stainless-steel webbing cartridge attached to the lower incisors for 30 min per day on 5 days a week for 51 weeks. A group of 84 hamsters exposed to dry cotton served as negative controls and two groups of 84 animals exposed to benzo[*a*]pyrene and 24 animals exposed to DMBA served as positive controls. No tumour was found in the oral mucosa, except in the positive controls (Homburger, 1971). [The Working Group noted the short duration of this study.]

3.2.3 *Subcutaneous administration*

Rat

A group of 82 male and female albino (Händler) rats, 100 days of age, was given subcutaneous injections of 0.15 mL (50 mg) of an ethanol extract of Swedish snuff (Ettan) in tri-*n*-caprylin once a week for 84 weeks. A group of 81 male and female rats received the same schedule of injections of ethanol and tri-*n*-caprylin and served as controls. Malignant tumours developed in equal numbers in both test and control rats, and were ‘retothelsarcomas’ (one in each group), one uterine carcinoma (in a test animal) and one ovarian carcinoma (in a control animal) (Schmähl, 1965).

3.2.4 *Administration with known carcinogens or modifiers*

(*a*) *Rat*

Four groups of 10 female Sprague-Dawley rats, 3 months of age, with surgically created canals in the lower lip received the following treatments: group 1 was infected with herpes simplex type 1 virus (HSV-1) by scarification and topical application on the inside of the lower lip, followed, 10 days later, by administration of a standard Swedish (Röda Lacket) snuff into the canal morning and night on 5 days per week; group 2 was infected with HSV-1 and received no other treatment; group 3 was sham-infected with sterile saline followed by snuff treatment; and group 4 was given neither HSV-1 nor snuff and served as controls. The HSV-1 infection was repeated once after a 1-month interval, and snuff was administered 10 days later as before. Snuff treatment was continued for 18 months, after which time all animals were killed. Three animals each in groups 1 and 2 died from encephalitis shortly after the second infection with HSV-1. In the group exposed to HSV-1 and snuff, squamous-cell carcinomas of the oral cavity developed in 2/7 rats and a retroperitoneal sarcoma occurred in 1/7 rats. In the group exposed to snuff alone, 1/10 animals developed a squamous-cell carcinoma of the anus and 1/10 developed a retroperitoneal sarcoma. No such tumours occurred in the HSV-1-infected (0/7) or control (0/10) groups (Hirsch *et al*., 1984a).

Surgery to create a test canal in the lower lip was performed on 150 male Sprague-Dawley rats at 8–9 weeks of age. Rats were randomized to five treatment groups initially of 30 rats each. Treatments were begun 3–4 weeks after surgery and continued for up to 108 weeks. Rats in group 1 received snuff (a brand available commercially in the USA) packed into the test canal with a spatula (at least 100 mg per application) twice daily on 5 days per week (snuff from the previous application was removed before the next treatment). Propylene glycol was applied three times a week for 4 weeks to the palate of each rat in group 2; no further treatment was given for the remainder of the study. 4-NQO dissolved in propylene glycol (approximately 0.13 mg/treatment) was applied three times per week for 4 weeks to the palate of each rat in group 3. Rats in group 4 received 4-NQO as for group 3 followed by snuff as for group 1. Group 5 (control) received a cotton pellet dipped in saline twice daily on 5 days per week for 104 weeks. At the end of the study, complete necropsies were performed; 28–29 rats in each group were evaluated. Squamous-cell papillomas and carcinomas of the lip, hard palate, tongue, nasal cavity, oesophagus and forestomach occurred only in groups 1, 3 and 4. Undifferentiated sarcomas of the lip occurred only in snuff-treated rats in groups 1 (two tumours) and 4 (three tumours). Among 29 rats in group 1 (snuff), five squamous-cell carcinomas (one lip, two hard palate, one nasal cavity, one forestomach), one squamous-cell carcinoma *in situ* (hard palate) and three squamous-cell papillomas (one each of the lip, hard palate and nasal cavity) developed. No such tumours developed among 29 rats in group 5 (all squamous-cell tumours, $p < 0.01$; malignant squamous-cell tumours, $p < 0.05$; Fisher's exact test) or among 28 rats in group 2 (propylene glycol control). At the sites specified above, a total of seven squamous-cell carcinomas and two squamous-cell papillomas occurred among 29 rats in group 3 (4-NQO) and eight squamous-cell carcinomas and

two squamous-cell papillomas among 28 rats in group 4 (4-NQO followed by snuff). Subsequent treatment with snuff did not enhance tumorigenesis by 4-NQO in the lip canal, oral cavity, nasal cavity, oesophagus or forestomach; the combined effects of 4-NQO and snuff were less than additive (Johansson *et al.*, 1989).

Male Sprague-Dawley rats underwent surgery to create a lip canal at 10 weeks of age. Group 1 (40 rats) was initiated with DMBA (0.1% in mineral oil) by placing cotton pellets containing approximately 70 mg of the solution in the lip canal three times per week for 4 weeks beginning at 12 weeks of age. Thereafter, the rats received a cotton pellet dipped in saline once daily on 5 days per week for 104 weeks. Group 2 was initiated with DMBA as for group 1 and subsequently received 150–200 mg snuff (generic moist snuff type 1S3, University of Kentucky Research Center, USA) placed in the lip canal with a spatula twice daily on 5 days per week (after removal of any material remaining from the previous application) for 104 weeks. Group 3 (38 rats) received snuff twice daily on 5 days per week for 104 weeks. Group 4 (40 rats) was initiated with 4-NQO dissolved (0.5 %) in propylene glycol; approximately 70 mg of solution on a cotton pellet was placed in the lip canal three times per week for 4 weeks, after which rats were treated with a cotton pellet dipped in saline once daily on 5 days per week for 100 weeks. Group 5 (38 rats) was initiated with 4-NQO as for group 4 three times per week for 4 weeks, followed by snuff twice daily for 100 weeks. Group 6 (30 rats) received cotton pellets dipped in saline once daily on 5 days per week for 100 weeks. Rats were killed when moribund, when they developed lip tumours or 104 weeks after the beginning of the study, and a complete necropsy was performed. Tumour incidences in different groups were compared by the Student *t* test and Fisher's exact test. Sarcomas of the lip occurred in 0/40 rats in group 1, 9/40 rats in group 2, 10/38 rats in group 3, 1/40 rats in group 4, 25/38 rats in group 5 and 1/30 rats in group 6. The incidence of lip sarcomas in rats treated with snuff only (group 3) was significantly greater ($p < 0.01$) than that in controls (group 6), and was significantly increased by pretreatment with 4-NQO (group 5) but not DMBA (group 2). Squamous-cell carcinomas and papillomas of the oral cavity (lip, palate and buccal mucosa) occurred in 0/40 rats in group 1, 3/40 rats in group 2, 3/38 rats in group 3, 9/40 rats in group 4, 8/38 rats in group 5 and 0/30 rats in group 6. The incidence of epithelial tumours of the oral cavity in rats treated with snuff alone (group 3) was not significantly different from that in controls (group 6) and was not significantly modified by pre-treatment with either DMBA (group 2) or 4-NQO (group 5). The combined incidence of malignant epithelial and mesenchymal tumours of lip and oral cavity was significantly greater in rats treated with snuff alone (group 3; three squamous-cell carcinomas of the palate and 10 sarcomas of the lip, 13/38; $p < 0.01$) than in controls (group 6; one sarcoma of the lip, 1/30) (Johansson *et al.*, 1991).

(*b*) *Hamster*

One hundred and twenty-five Syrian golden hamsters [age unspecified] were divided into seven groups of 15–20 animals, and the cheek pouches were inoculated with HSV-1, HSV-2 or culture medium. The mock and virus inoculations were performed once a

month for 6 consecutive months. In an effort to determine the effect of snuff on the mock- or virus-inoculated cheek pouches, a consistent amount (150 mg/pouch) of a commercially available snuff (USA) was placed into both the right and left pouches of half the animals twice daily on 5 days per week for 6 months. One group of animals was neither inoculated with HSV nor treated with snuff. At the end of the 6 months of simulated snuff dipping and 4 weeks after the final mock or virus inoculation, the hamsters were killed and the cheek pouches were removed for histopathological evaluation. Neither simulated snuff dipping nor HSV infection alone induced neoplastic changes in hamster cheek pouches (0/15 untreated controls, 0/15 mock inoculation, 0/15 mock inoculation plus simulated snuff dipping, 0/19 HSV-1 inoculation, 0/16 HSV-2 inoculation). HSV-1 or HSV-2 infection in combination with simulated snuff dipping resulted in epithelial dysplasia and invasive squamous-cell carcinoma in at least one cheek pouch in more than 50% of the animals (10/20 HSV-1 inoculation plus simulated snuff dipping; $p < 0.05$ (Fisher's exact test) versus untreated, mock inoculation or HSV-1-only groups; 11/20 HSV-2 inoculation plus simulated snuff dipping; $p < 0.05$ versus untreated, mock inoculation or HSV-2-treated groups) (Park *et al*., 1986).

As part of an experiment to evaluate the effects of various modulating agents including various snuffs on hamster cheek pouch carcinogenesis initiated by DMBA, 110 randomly bred male Syrian golden hamsters, 4–6 weeks of age and weighing 90–100 g, were divided into six groups. DMBA in liquid paraffin solution at a concentration of 0.25% was applied to each cheek pouch of 55 hamsters at a dose of 0.125 mg in 50 μL of oil twice a week for 1 month. Fifteen of these DMBA-treated hamsters served as positive controls. The snuffs studied included the Manglorian variety of ordinary (regular) snuff or scented snuff, both obtained from local markets. Snuff was suspended uniformly in liquid paraffin and applied to hamster cheek pouches at a dose of 20 mg per cheek pouch in a volume of 50 μL twice a week. Each kind of snuff was administered to a group of 20 DMBA-initiated hamsters, beginning 2 weeks after the last DMBA treatment and continuing until 6 months after the first DMBA treatment, for a total of 4.5 months of snuff administration; the same treatments were applied to groups of 20 hamsters that had received no DMBA for an equivalent period. Fifteen untreated hamsters served as controls. All hamsters were killed 6 months after the first DMBA treatment, at approximately 7 months of age. No tumours were observed in either cheek pouches or forestomach in the 15 untreated control hamsters. Cheek pouch tumours occurred in 10/15 hamsters given DMBA only, in 3/20 hamsters given DMBA followed by regular (Manglorian) snuff and in 2/20 hamsters given DMBA followed by scented snuff. Cheek pouch tumours did not occur in hamsters given regular snuff or scented snuff alone. Forestomach tumours occurred in 15/15 hamsters given DMBA only, in 20/20 hamsters given DMBA followed by regular snuff and in 19/20 hamsters given DMBA followed by scented snuff. Forestomach tumours also occurred in 17/20 hamsters given regular snuff alone and in 15/20 hamsters given scented snuff alone. DMBA and both kinds of snuff induced forestomach tumours in hamsters. Neither kind of snuff induced tumours in hamster cheek pouches and both kinds of snuff inhibited carcinogenesis in cheek pouches by DMBA (Gijare *et al*., 1990a).

3.3 *Bidi* tobacco, *mishri* and *naswar*

3.3.1 *Bidi* tobacco

Skin application

Mouse

In a study to determine the tumour initiation/promotion and complete carcinogenic potential of a processed blend of tobacco used for the manufacture of *bidis* [*bidi* tobacco is used for chewing by workers engaged in processing tobacco for the manufacture of *bidis*], groups of 15 female inbred hairless Swiss 'bare' mice (S/RVCri-ba strain), 6–7 weeks of age, were used. The supernatant of an aqueous extract of tobacco was lyophilized and dissolved in a minimal amount of DMSO; 1 µL DMSO contained 2.5 mg aqueous extract of tobacco. An appropriate volume of DMSO was made up to 100 µL with acetone to obtain the different doses. Doses of *bidi* tobacco extract corresponded to the original dry weight of tobacco. Eight groups of mice received topical applications on the back skin of 50 mg dried aqueous *bidi* tobacco extract in 100 µL acetone twice a week for 40 weeks (complete carcinogenesis experiment), a single topical application of 5 mg *bidi* tobacco extract in 100 µL acetone followed 1 week later by twice-weekly applications of 1.8 nmol 12-*O*-tetradecanoylphorbol-13-acetate (TPA) for 20 weeks (tumour initiation experiment) or a single topical application of 20 nmol DMBA followed by twice-weekly applications of acetone or 0.25, 2.5, 5 or 50 mg aqueous *bidi* tobacco extract or 1.8 nmol TPA (positive control) for 40 weeks (tumour promotion experiment). In order to determine the role of aqueous *bidi* tobacco extract in the progression of papillomas to carcinomas, skin papillomas were induced by initiation with 20 nmol DMBA and promotion with 1.8 nmol TPA for 20 weeks. TPA-dependent papillomas were allowed to regress during a 6-week treatment-free period and 50 mg aqueous *bidi* tobacco extract was applied twice weekly for 14 weeks. Aqueous extract of *bidi* tobacco did not exhibit skin tumour initiation, progression or complete carcinogenic activity. However, tumour promotion activity was observed with applications of 5 and 50 mg aqueous *bidi* tobacco extract after initiation with DMBA. The multiplicities of skin papillomas were significantly increased ($p < 0.01$) compared with DMBA-initiated controls. Tumour multiplicities were 9.69 ± 1.30 and 11.73 ± 1.38 tumours per mouse, respectively, versus 4.70 ± 1.01 tumours per mouse (control) (Bagwe *et al*., 1994).

3.3.2 *Mishri*

(*a*) *Oral administration*

(i) *Mouse*

Four groups of Swiss mice, 8 weeks of age, were fed brown (26 males and 26 females) and black *mishri* (24 males and 26 females) in the diet at 10% for 20 months and were then maintained on standard diet. Animals were killed at 25 months of age or when moribund. Control animals (27 males and 31 females) received standard diet only. The incidence of

forestomach papillomas was 46% in male mice fed black *mishri*, 54% in male mice fed brown *mishri*, and 42% in female mice treated with *mishri* of either variety, which was significantly higher than that in control males (11%; $p < 0.001$) and females (3%; $p < 0.001$) (Kulkarni *et al*., 1988).

(ii) *Rat*

Groups of 27 male and 24 female Sprague-Dawley rats, 8 weeks of age, were fed 10% brown *mishri* in the diet for 20 months. Animals were killed when moribund or at 25 months of age. Control animals (25 males and 30 females) received standard diet only. The incidence of forestomach papillomas was approximately 37% in both males (10/27) and females (9/24); no papillomas developed in control animals (0/25 males, $p < 0.001$; and 0/30 females, $p < 0.001$) (Kulkarni *et al*., 1988).

Two groups of 30–31 male rats and two groups of 30 male Sprague-Dawley rats, 19–21 days of age, were maintained on vitamin A-sufficient and vitamin A-deficient diets, respectively. In one group of vitamin A-sufficient and one group of vitamin A-deficient rats, a daily dose of 3 mg *mishri* extract was administered by gavage five times a week over a period of 21 months. The two remaining groups (controls) received 0.05 mL DMSO for the same period. Autopsies were performed on all animals killed after 12 or 21 months. Liver, lung and stomach tissues were fixed and processed for microscopic examination. Rats given vitamin A-sufficient diet and *mishri* developed lung adenomas and stomach papillomas. Tumour incidence at 9–15 months and 16–21 months was 58% (7/12) and 5.5% (1/18), respectively. At these time periods, tumour incidences in vitamin A-deficient rats that received *mishri* extract were 88% (8/9) and 95% (20/21), respectively. Sixteen rats in the latter group developed malignant lung tumours. No tumours appeared in control rats given DMSO. Total tumour incidence in both vitamin A-sufficient and vitamin A-deficient rats given *mishri* extract was significantly higher ($p < 0.001$) than that in corresponding controls (Ammigan *et al*., 1991).

(iii) *Hamster*

Two groups of 23 male and 26 female Syrian golden hamsters, 8 weeks of age, were fed a 10% black *mishri* diet and two groups of 28 males and 20 females were fed a brown *mishri* diet for 20 months. Twenty-three males and 23 females of the control groups were maintained on standard diet. Animals were killed at 25 months of age or when moribund. In male hamsters, the incidence forestomach papillomas was approximately 43% (12/28 brown *mishri* group and 10/23 black *mishri* group) and 25–27% in females (5/20 in the brown *mishri* group and 7/26 in the black *mishri* group), which was significantly higher than that in controls (9%, 2/23 males, $p < 0.01$; 4%, 1/23 females, $p < 0.02$). Forestomach carcinomas were observed in 2/23 male hamsters given the black *mishri* diet (Kulkarni *et al*., 1988).

(*b*) *Skin application*

Mouse

Groups of 'nude' Swiss mice [number, sex distribution and age unspecified] received topical applications of 20 μL of an acetone solution of the solid residue from a toluene extract of *mishri* [concentration not specified] on the midscapular region five times a week [duration of treatment not specified] or a single application of 200 nmol DMBA. The incidence of skin papillomas (~20%) was comparable in the two groups (Bhide *et al*., 1987b).

Brown and black varieties of *mishri* were tested for their carcinogenic or promoting potential on skin in several groups of 8-week-old male Swiss mice (hairy) and male and female hairless Swiss bare mice. Three groups of 29–30 male Swiss mice received a single initiating dose of 200 nmol DMBA on the back skin and four groups of 16–21 male Swiss bare mice were initiated with doses of 50 or 200 nmol DMBA. Black or brown *mishri* extract was applied to the skin of two of the three groups of DMBA-initiated male Swiss mice and two groups of 30 uninitiated male Swiss mice (black *mishri* extract only) at doses of 2.5 mg on 5 days a week for 20 months. Two of the four groups of DMBA-initiated male Swiss bare mice and four groups of 17–24 uninitiated male or female Swiss bare mice were treated similarly with 2.5 mg or 1 mg black *mishri* extract. All mice were killed when moribund or at 24 months. Tumours more than 1 mm in diameter and lung and liver tissues were fixed and examined microscopically. Tumour incidence was analysed statistically using Yate's modification of the chi-square test. Skin tumours did not appear in either group of male Swiss mice treated with black *mishri* (2.5 mg dose) extract only or one control group of 30 male Swiss mice treated with acetone for 20 months. At the 1-mg dose of black *mishri* extract only, 6/21 male (33%) and 5/24 female (21%) Swiss bare mice developed skin papillomas and one male mouse developed a skin carcinoma. Six of 17 (35%) male and 5/23 (22%) female bare mice treated with 2.5 mg black *mishri* developed papillomas. No skin papillomas were observed in 21 male and 23 female controls treated with acetone only for 20 months. Four of 30 and 4/29 male Swiss mice treated with brown or black *mishri*, respectively, after DMBA initiation developed skin papillomas, but no carcinomas were observed. No tumours were observed in one group of 30 male Swiss mice treated with DMBA only. Eight of 20 and 7/16 male Swiss bare mice initiated with 200 or 50 nmol DMBA and promoted with 1 mg or 2.5 mg black *mishri* extract, respectively, developed skin papillomas. Carcinomas were observed in 2/20 and 4/16 animals, respectively. Skin papillomas were observed in two groups of male Swiss bare mice treated with both DMBA doses only (9/21 and 7/17, respectively). Two carcinomas were also observed in each group. Promotion with brown or black *mishri* extract significantly ($p < 0.05$) increased the total tumour incidence in Swiss mice but not in Swiss bare mice. However, application of *mishri* extracts alone to the skin induced papillomas in male and female Swiss bare mice (Kulkarni *et al*., 1989).

3.3.3 Naswar

(*a*) *Application to the cheek pouch*

Hamster

In one experiment, a group of 28 female and 33 male Syrian hamsters [assumed to be 1–3 months of age] received applications of *naswar* (mixture of tobacco, lime, ash, plant oil and water) as a dry powder into the left cheek pouch for life; another group of 13 females and 24 males received *naswar* as a 50% suspension in refined sunflower oil in the cheek pouch (total dose per animal, 6.2–147.5 g; mean 53.8 ± 2.5 g). The animals were followed until death. No tumour was found at the site of *naswar* application. The average lifespan of animals that received *naswar* (50.8 weeks) was slightly shorter than that of untreated animals (57.3 weeks) or that of hamsters that received sunflower oil alone (57.6 weeks). Of 64 treated hamsters in both groups still alive at the time of appearance of the first tumour (17 and 37 weeks), 13 developed tumours: seven liver-cell tumours and one liver tumour of 'mixed structure', three tumours of the adrenal glands (described as a 'carcinoma of adrenal cortex' and as 'adenoma, chromaffinoma type' or 'carcinoma of adrenal cortex'), one forestomach papilloma, three uterine tumours (leiomyoma and/or fibromyoma and/or cysts), one skin melanoma, one benign skin tumour and one unspecified tumour of the large intestine. Among 110 untreated animals and 10 animals treated with sunflower oil, 53 survived to the appearance of the first tumour (59 weeks), and two developed tumours (one adrenal cortex neoplasm and one forestomach papilloma) (Kiseleva *et al.*, 1976).

In another experiment, *naswar* was introduced as a dry powder or as a 50% suspension in refined sunflower oil into the cheek pouch of 184 male and female hamsters, 1–3 months of age. *Naswar* was administered throughout life (total mean dose per animal, 53.8 ± 2.5 g). No tumour was found at the site of application. However, 26/138 hamsters that survived to the appearance of the first tumour (17 weeks after the experiment began) developed neoplasms at various sites: 13 tumours of the liver, six of the adrenal glands, five papillomas of the forestomach, four of the uterus and five other tumours. The mean survival time of the animals was 50.9 ± 1.9 weeks (Milievskaja & Kiseleva, 1976). [The Working Group noted deficiencies in reporting the number of males and females and that the incidences of different tumour types were not indicated.]

(*b*) *Skin application*

Hamster

A group of 19 female and 31 male Syrian hamsters [assumed to be 1–3 months of age] received topical applications of a suspension of *naswar* (45% tobacco, 8% lime, 30% ash, 12% plant oil and 5% water) on the dorsal skin. The average lifespan was 44.4 weeks. Three of nine animals still alive at the time of appearance of the first tumours (53 weeks) developed neoplasms: one liver 'lymphangioendothelioma', one adrenal gland tumour and one forestomach papilloma. No local tumour occurred. In the untreated control group

(69 females and 41 males), 2/45 hamsters that survived to the appearance of the first tumour (59 weeks) developed tumours: one adrenal cortex neoplasm and one forestomach papilloma (Kiseleva *et al*., 1976).

(*c*) *Administration with known carcinogens or modifiers*

Hamster

A group of 30 Syrian hamsters [age and sex unspecified] received a single application of 0.1 mg DMBA as a 0.1% solution in benzene in the cheek pouch. Another group of 30 hamsters received the same treatment, followed 7 weeks later by daily applications of *naswar* (composition as described above) as a dry powder in the cheek pouch; the total dose ranged from 11.2 to 102.5 g (mean, 38.9 ± 5.2 g). Three of 11 survivors at the time of appearance of the first tumour (23 weeks) that received DMBA alone developed tumours: one rhabdomyoblastoma of the cheek pouch and two papillomas of the forestomach. Six of 11 animals still alive at 50 weeks that received DMBA plus *naswar* had tumours: five papillomas of the forestomach and one cystic epithelioma of the skin of the jaw (Milievskaja & Kiseleva, 1976). [The Working Group noted the small number of animals that survived to the time of observation of the first tumour.]

[In consideration of the whole study by Kiseleva *et al.* (1976) and Milievskaja and Kiseleva (1976), the Working Group noted that the effective number, i.e. the number of animals that survived to the observation of the first tumour, was calculated separately for treated (number of survivors at 17 weeks with the dry powder) and control (59 weeks) animals. Therefore, the effective number of control animals should have been higher in the first experiment. High mortality of animals was noted, even in control groups, in the period preceding observation of the first tumour; average lifespan of untreated control animals was 57.3 weeks. The sex of animals in which liver tumours were found was not indicated.]

4. Other Data Relevant to an Evaluation of Carcinogenicity and its Mechanisms

4.1 Absorption, distribution, metabolism and excretion

4.1.1 *Humans*

(*a*) *Nicotine*

There are well-documented differences in the absorption of nicotine from smoked tobacco and that from smokeless tobacco products. However, once nicotine from smokeless tobacco has been absorbed into the systemic circulation, it is assumed that it is distributed, metabolized and excreted similarly, regardless of the route of administration. The experimental designs among the many pharmacokinetic studies of nicotine differ. Some experiments involved the intravenous administration of nicotine (occasionally as a radiolabelled

compound) and subsequent sampling of blood and urine at specified intervals over a fixed time period. In other studies, nicotine was delivered from cigarette smoking and, in relatively few studies, nicotine delivery from smokeless tobacco products was studied. The route of administration is regarded here as important when considering absorption characteristics of nicotine but after nicotine enters the circulation its distribution, metabolism and excretion are regarded as independent of the route of administration (see Table 81).

Table 81. Tobacco consumption and exposure to nicotine from smokeless tobacco use and cigarette smoking

Parameter	Oral snuff	Chewing tobacco	Cigarettes
Grams of smokeless tobacco or no. of cigarettes per day	15.6 ± 5.9[a] (6.8–22.0)[b]	72.9 ± 21.6 (33.7–103.7)	36.4 ± 10.4 (25.0–54.0)
Maximal plasma nicotine concentration (μmol/L)	0.20 ± 0.10 (0.07–0.38)	0.17 ± 0.07 (0.07–0.29)	0.19 ± 0.03 (0.14–0.22)
AUC_{nic} (μmol/L/h)	2.48 ± 1.13 (0.97–4.67)	2.06 ± 0.84 (0.83–3.69)	3.04 ± 0.69 (2.45–4.37)
Urine nicotine (μmol/24 h)	5.58 ± 5.06 (1.04–15.84)	6.45 ± 4.65 (1.29–13.68)	6.97 ± 2.87 (3.58–10.75)
AUC_{cot} (μmol/L/h)	48.50 ± 30.77 (24.79–118.26)	48.25 ± 29.66 (13.67–113.87)	46.17 ± 13.29 (29.19–64.87)
AUC_{cot}/AUC_{nic}	21.4 ± 6.2[c] (9.7–29.6)	23.9 ± 4.6[c] 17.8–33.6)	16.4 ± 3.0 (11.6–21.4)

Adapted from Benowitz *et al*. (1989)
AUC, area under the plasma concentration–time curve; $_{nic}$, nicotine; $_{cot}$, cotinine
[a] Mean ± standard deviation
[b] Range
[c] $p < 0.05$ compared with cigarettes

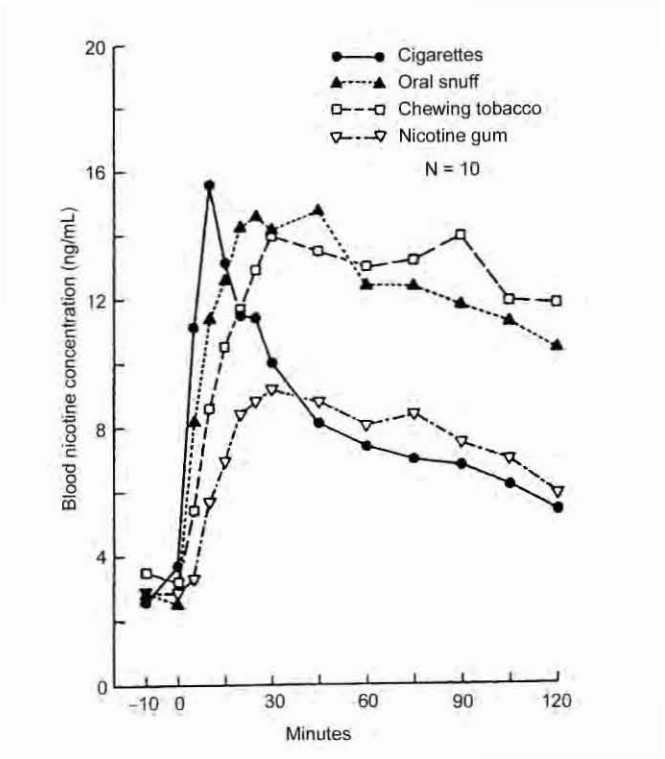
(i) *Absorption*

The absorption of nicotine (and possibly other components) from smokeless tobacco products is determined by several factors, such as the amount of the product used, the length of time it is kept in the mouth and the flux (movement of the product around the oral cavity) which are under the control of the consumer. Other factors, such as the concentration of nicotine in the tobacco product, the pH at the interface between the product and the buccal membrane and the particle size of the product, are not under the control of the consumer. However, as reviewed below, many studies have demonstrated the association between pH and nicotine absorption and some illustrated the importance of the particle size (cut) of the tobacco product.

Russell *et al.* (1981) demonstrated that moist snuff delivered measurable and significant quantities of nicotine to the systemic circulation. The time course of plasma nicotine levels was studied in three adults who were given 'Skoal Bandits' or nicotine chewing gum. The participants held the product in their mouths for 30 min, and blood was collected before and for up to 60 min after exposure. The peak increase in plasma levels of nicotine occurred after 30 min. Skoal Bandit increased plasma nicotine levels by 11 ng/mL over pretreatment levels, whereas 2 mg nicotine gum increased the levels by 6 ng/mL (Russel *et al.*, 1985).

In a study of 10 volunteers, Benowitz *et al.* (1988) measured plasma nicotine levels after use of moist (oral) snuff, chewing tobacco and nicotine chewing gum and cigarette smoking. After a single exposure to each product, maximal plasma levels were approximately equivalent for the cigarette and the smokeless tobacco products but, because of the prolonged exposure to the oral tobacco products, the overall amount of nicotine absorbed was twice as high as that after cigarette smoking. Nicotine gum delivered less nicotine than any of the other conditions (see Figure 6).

Figure 6. Blood nicotine concentrations during and after smoking cigarettes (average, 1 and 1/3 cigarettes), use of oral snuff (2.5 g), use of chewing tobacco (7.9 g) and chewing nicotine gum (two 2-mg pieces)



Adapted from Benowitz *et al.* (1988)

Many studies have demonstrated that the pH in the oral cavity is a major determinant of the absorption of nicotine from smokeless tobacco (Tomar & Henningfield, 1997). The unprotonated (free base) nicotine is most rapidly absorbed from the buccal cavity (Armitage & Turner, 1970), whereas the protonated molecule penetrates membranes very poorly. The percentage of unprotonated nicotine available is determined by use of the Henderson-Hasselbach equation which equates the ratio of the unprotonated and protonated molecules to the pH and the dissociation constant (pKa). Richter and Spierto (2003) compared the nicotine content, moisture and pH of 18 brands of smokeless tobacco products. The pH of the eight moist snuff products ranged from 5.35 to 8.28 and the nicotine content ranged from 4.28 to 13.54 mg/g. In the loose-leaf products (chewing tobacco), the pH ranged from 5.33 to 6.41 and the nicotine content ranged from 3.73 to 8.26 mg/g. The percentage of unprotonated nicotine in the moist snuff product ranged from 0.2 to 64.5%, whereas the loose-leaf product contained much less available nicotine (unprotonated form, 0.20–2.44%). The authors also noted that the moist snuff products with the highest levels of unprotonated nicotine were those that had the highest market share. The data suggest that the rapid availability of nicotine for absorption may be an important determinant of product appeal (discussed in the section on nicotine addiction) and supports the notion that smokeless tobacco products are used to obtain nicotine.

Henningfield *et al.* (1995) determined the aqueous pH of suspensions of four brands of moist snuff: Copenhagen, 8.6; Skoal Wintergreen, 7.6; Skoal Long Cut Cherry, 7.5; Skoal Bandits, 6.9. In a direct test of the proposal that pH determines nicotine absorption, Fant *et al.* (1999) administered these products to volunteers in a cross-over study. A different product was tested each day, and plasma nicotine levels were determined before administration, during the 30 min that the product was held in the mouth and at specified intervals for 90 min after it was removed. Plasma nicotine levels were directly related to pH. Specifically, 'Copenhagen', the product with the highest aqueous pH, delivered the highest peak levels of plasma nicotine, with an increase of 19.5 ng/mL above baseline. The products with intermediate pH ('Wintergreen' and 'Cherry') increased plasma nicotine levels by up to 12 ng/mL while 'Bandits' (lowest pH) induced an increase of 3 ng/mL only. Subjective ratings of preference and strength and increases in heart rate were also directly related to pH and plasma levels of nicotine.

It is recognized that both the saliva and the components of smokeless tobacco products have buffering capacity. These two buffering systems interact such that the pH of saliva at the buccal–product interface is maintained. Ciolino *et al*. (2001) investigated the relative buffering capacity of a series of six commercial smokeless tobacco products and 10 samples of unstimulated whole human saliva. The buffering capacity of the moist snuff products was 10–20 times greater than that of human saliva, which suggests that the pH at the buccal interface is almost entirely determined by the tobacco products.

The size of the tobacco cuttings in smokeless tobacco (fineness) also influences buccal nicotine absorption. More finely cut smokeless tobacco (smaller particle size) provides more surface area and a greater wetted surface which lead to the rapid absorption of nicotine (Connolly, 1995). Other additives that bind the tobacco cuttings together diminish absorp-

tion by decreasing the surface area. For example the 'long-cut' products use larger pieces of tobacco and a binding agent that allows the user to pack the tobacco tightly. These products release nicotine more slowly than the 'fine-cut' products (Connolly, 1995).

Another determinant of buccal nicotine absorption is the flux, i.e. the active process by the smokeless tobacco user of chewing, mastication and mixing the product with saliva. The extent to which the user 'works' the smokeless tobacco plug (quid) by chewing or moving it around the mouth may affect the speed and efficiency of nicotine absorption. As mentioned above, flux is a greater determinant of nicotine absorption from chewing tobacco than that from moist snuff products (Andersson *et al.*, 1994). Tomar and Henningfield (1997) concluded that the pH of moist snuff products are the main determinants of nicotine absorption and that rates of expectoration and oral manipulation probably have little effect on nicotine absorption.

Many moist snuff products are sold in small sachet pouches. This packaging appeals to consumers because the product is held closely together, it is provided in a unit dose and the tobacco particles do not migrate around the oral cavity. Connolly (1995) reported that the sachet packaging decreases nicotine absorption. The decrease may be due to less flux, slower and less saliva penetration (wetting) and the addition of another and limiting interface between the tobacco and the buccal membrane. The presence of the sachet seemed to slow down the release of nicotine from the tobacco to about 60% in the first minute (Nasr *et al*., 1998). Whether the sachet package decreases the absorption and penetration of other components of the smokeless tobacco has not been determined. Both in-vitro (Nasr *et al*., 1998) and in-vivo studies have reported that the release of nicotine from smokeless tobacco products is extremely fast. About 90% of the available nicotine was released within the first minute.

Although most absorption of nicotine from smokeless tobacco products is through the buccal mucosa and is pH-dependent, nicotine is absorbed from the intestine after oral administration (D'Orlando & Fox, 2004). Since swallowing the smokeless tobacco juice was documented in up to 48% of snuff users (Ebbert *et al*., 2004), it is possible that some nicotine absorption from smokeless tobacco products occurs in the intestine. Oral nicotine absorption is typically slower (peak, 1–2 h) than buccal absorption (D'Orlando & Fox, 2004), which may also contribute to the sustained plasma levels of nicotine observed after the use of snuff and chewing tobacco (Figure 6).

(ii) *Distribution*

After nicotine is absorbed into the systemic circulation, it is rapidly distributed to all areas of the body. At physiological pH (7.4), about 69% of the nicotine is protonated and 31% is unprotonated; less than 5% is bound to plasma proteins (Benowitz *et al*., 1982). The volume of distribution of nicotine averaged 180 L or about 2.5 times the body weight in kilograms. This means that, at steady state (equilibrium) levels, the amount of nicotine in the body tissue is 2.6 times that predicted by the product of blood concentration and body weight (DHHS, 1988).

Once nicotine enters the blood, it is rapidly distributed to body tissue and plasma levels fall very quickly after intravenous administration. Thus, immediately after intravenous administration, levels in the arterial blood, brain and lung are high whereas those in the muscle and adipose tissue (storage sites at steady-state concentrations) are quite low. As a result, the brain is immediately exposed to high levels of nicotine, and several animal studies have confirmed rapid uptake of nicotine into the brain (Schmiterlöw *et al*., 1967; Oldendorf, 1972; Maziere *et al*., 1976). When tobacco is smoked, the profile of nicotine distribution is very similar to that after intravenous administration: the concentration in the brain is influenced by distribution kinetics. The distribution half-life of nicotine is estimated to be 9 min (Feyerabend *et al*., 1985), and reflects the rapid nicotine uptake into the brain after tobacco has been smoked or after intravenous administration of nicotine. However, after administration of smokeless tobacco, nicotine absorption is slower and follows a more protracted time course. For example, Benowitz *et al.* (1988) measured plasma levels of nicotine in volunteers after smoking a cigarette, and after using chewing tobacco, moist oral snuff or nicotine gum. Plasma levels increased from 3 ng/mL to about 15 ng/mL after smoking the cigarette or use of the smokeless tobacco products, respectively (levels after the use of nicotine gum were lower). However, the cigarette peak occurred early (10 min) whereas the peak after the smokeless tobacco products was closer to 30 min (see Figure 6). Furthermore, there was a clear difference in the declining slope of the plasma levels; after cigarette smoking, two phases were apparent and represented tissue distribution for the first 15 min and a second slope that represented plasma elimination kinetics (half-life, 2 h). After administration of the smokeless tobacco products, the plasma levels of nicotine declined at a slow steady rate that was parallel to the slope of the elimination phase that followed intravenous administration. The plasma levels did not decline immediately after the product was removed from the mouth, which indicates that local tissue deposition of nicotine may contribute to systemic absorption for some time after the product is removed. As a consequence of the differences in absorption and distribution of nicotine after smoking or administration of smokeless tobacco, brain tissue is confronted with a steady rate of nicotine distribution after smokeless tobacco as opposed to the pulsed increases seen after each puff of a cigarette. It is generally recognized that the speed of delivery of psychoactive drugs is an important determinant of their abuse liability (DHHS, 1988).

Swallowing the juice was more prevalent among people who use moist snuff (48% always swallowed, 15% never swallowed) than those who use chewing tobacco (31% always swallowed, 41% never swallowed) (Ebbert *et al*., 2004). Furthermore, plasma cotinine analyses indicated that higher serum cotinine concentrations were associated with higher frequency of swallowing and the number of cans of product used per week. However, nearly 80% of nicotine that is absorbed from the intestine is metabolized (to cotinine) in the first pass through the liver and never reaches the systemic circulation. Thus, the level of plasma cotinine may not be as strong an index of consumption in users of smokeless tobacco as it is in cigarette smokers (Ebbert *et al.*, 2004).

(iii) *Metabolism*

In humans, 85–90% of a dose of nicotine is converted metabolically before its excretion, and only 5–10% is excreted unchanged in the urine. Nearly all nicotine metabolism occurs in the liver (Tricker, 2003), although one study (in dogs) showed that there is some metabolism in the lung (Turner *et al*., 1975). The rate of hepatic metabolism is very rapid. Non-renal clearance of nicotine averaged 1100 mL/min, and it has been estimated that about 70% of nicotine in the plasma is extracted in each pass through the liver (Benowitz *et al*., 1982; Table 82). More recent data discussed below suggest that some enzymes are capable of metabolizing nicotine in the brain (cytochrome-P450 [CYP] 2B6, CYP2E1) but it is uncertain to what extent such metabolism actually occurs.

Table 82. Pharmacokinetics of nicotine and cotinine in cigarette smokers

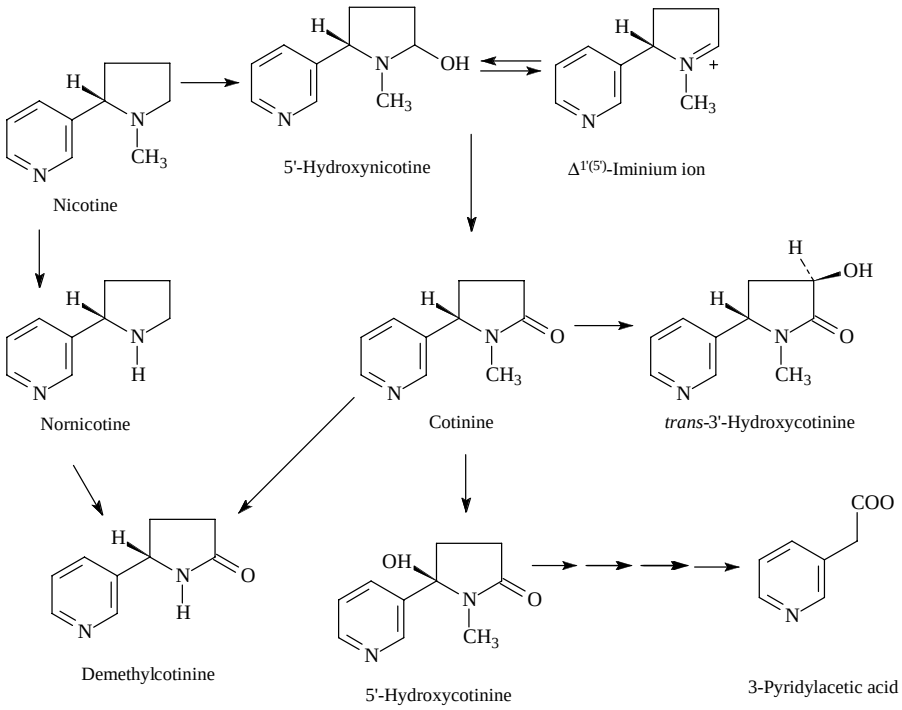
Parameter	Nicotine	Cotinine
Half-life	120 min	18 h
Volume of distribution	180 L	88 L
Total clearance	1300mL/min	72 mL/min
Renal clearance	200–600 mL/min (acid urine) 100 mL/min (pH 5.8)	12 mL/min
Non-renal clearance	1100 mL/min	60 mL/min

Adapted from Benowitz *et al*. (1982, 1983)

A recent review by Yildiz (2004) describes the metabolism of nicotine as a two-phase process that occurs in the liver. In the first stage (microsomal oxidation), nicotine is largely (about 80% of a given dose) converted to cotinine, the major hepatic metabolite of nicotine in humans. Cotinine is formed from the oxidation of nicotine at the 5 position of the pyrrolidine ring. As illustrated below (Figure 7), this is a two-step process with the formation of 5′-hydroxynicotine and the iminium ion which is mediated by the iso-enzymes (2A6) in the CYP system. In a second step, the nicotine iminium ion is converted to cotinine by aldehyde oxidase. Cotinine itself is further metabolized at a much slower rate than nicotine (plasma half life, 18 h) and only about 17% of cotinine is excreted unchanged in the urine (Benowitz *et al*., 1983). As shown below the major metabolites of cotinine include *trans*-3′-hydroxycotinine and 5′-hydroxycotinine and norcotinine.

In humans, the microsomal oxidation of nicotine to cotinine and from cotinine to *trans*-3′-hydroxycotinine are regulated by CYP enzymes. Several polymorphisms of these enzymes occur in humans, and some are more efficient than others at metabolizing nicotine. In the presence of less efficient enzymes, nicotine levels (after a single exposure) are higher and persist longer than when more efficient enzymes are present. Thus, polymorphism in the metabolizing enzymes may influence smoking behaviour (Nakajima *et al*., 2001; Sellers

Figure 7. C-Oxidation of nicotine



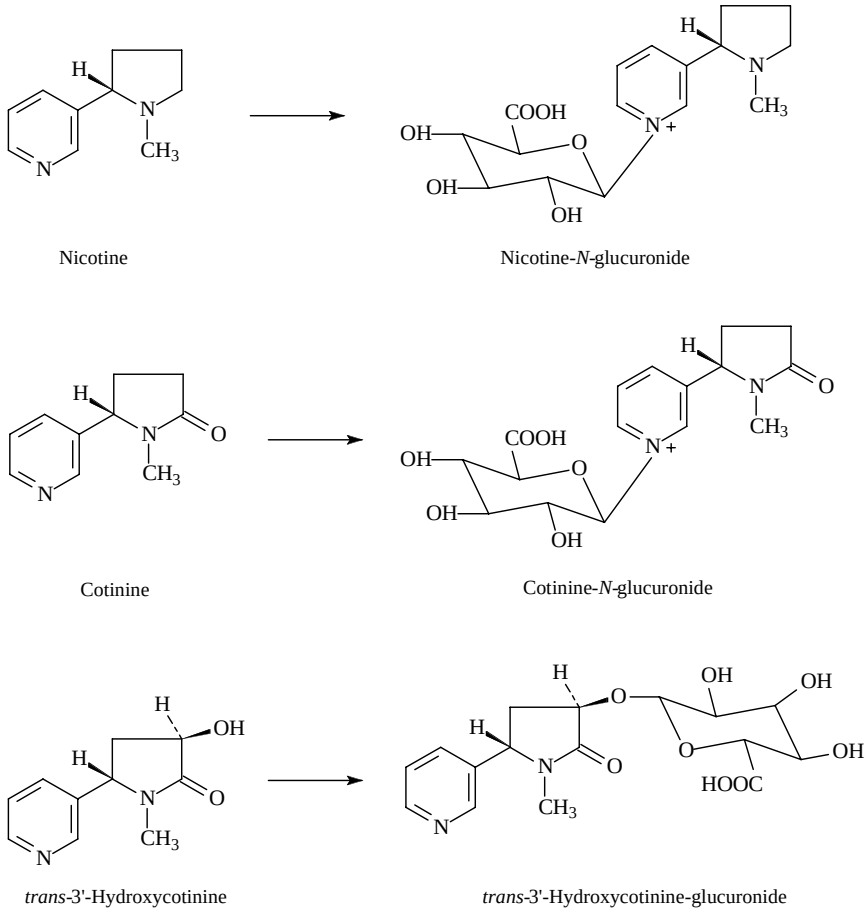
Modified from Yildiz (2004)

et al., 2003). However, contradictory studies do not support the notion that polymorphisms in the *CYP2A6* and other genes that encode enzymes in the metabolic pathway of nicotine influence smoking behaviour or the health consequences of smoking (Tricker, 2003; Carter *et al*., 2004). The influence of various polymorphisms of the *CYP2A6* gene on metabolism of nicotine from smokeless tobacco has not been studied.

The final step in nicotine metabolism is the formation of glucuronides. Glucuronidation of nicotine and cotinine results in compounds that are more water-soluble than the parent and more rapidly excreted in the urine. *N*- and *O*-Glucuronidation of nicotine and its metabolites (cotinine and *trans*-3′-hydroxycotinine) results in the formation of nicotine-*N*-glucuronide, cotinine-*N*-glucuronide and *trans*-3′-hydroxycotinine glucuronide (Figure 8 and Table 83) (Tricker, 2003; Yildiz, 2004).

Although most metabolism of nicotine occurs in the liver, enzymes present in the brain, lungs and elsewhere are also capable of metabolizing nicotine and may play a role in the health consequences of tobacco consumption. For example, CYP2B6 metabolizes nicotine and is present in the human brain. Expression of the gene in autopsy samples of the brains of nonsmokers, smokers, alcoholics and non-alcoholics were compared. Gene expression was specific for different brain regions and occurred in both neurons and astrocytes.

Figure 8. *N*- and *O*-Glucuronidation of nicotine and cotinine



Modified from Yildiz (2004)

CYP2B6 levels were higher in the brains of smokers and alcoholics, particularly in the hippocampus and the cerebellum. The authors suggested that higher brain levels of the enzyme may alter the sensitivity to psychoactive drugs, increase susceptibility to neurotoxins and carcinogenic xenobiotics and even play a role in the tolerance to nicotine (Miksys *et al*., 2003).

CYP2E1 is another enzyme that has been identified in the brain; it metabolizes alcohol and bioactivates tobacco-derived procarcinogens. Brain tissue from autopsied alcoholic smokers also revealed higher enzyme levels than tissue from non-alcoholic non-smokers (Howard *et al*., 2003). Finally, nicotine induced CYP2E1 in cell cultures of human neuroblastoma cells. The results of this study also indicated that administration of nicotine influences its own metabolism and also that of other drugs (Howard *et al*., 2003;

Table 83. Reported mean urinary excretion as a molar percentage (% ± standard deviation) of total recovered nicotine and metabolites in the urine of smokers, smokeless tobacco users and persons who received dermal nicotine

Nicotine and metabolites	Study 1 (11 subjects)	Study 2[a] (12 subjects)		Study 3 (54 subjects)	Study 4 (91 subjects)	Study 5 (12 subjects)	Study 6 (5 subjects)
	Smoking	Smoking	Transdermal nicotine	Smokeless tobacco	Smoking	Smoking	Smoking
Cotinine	13.2 ± 3.9	13.3 ± 3.1	14.9 ± 4.6	7.9 ± 2.2	9.2 ± 2.6	14.8 ± 5.9	15.2
Nicotine	10.4 ± 3.7	10.4 ± 4.4	11.1 ± 4.3	8.3 ± 5.7	9.4 ± 5.7	7.9 ± 4.6	9.5
trans-3′-Hydroxycotinine	35.2 ± 7.4	39.1 ± 12.5	37.0 ± 10.8	41.6 ± 10.6	36.1 ± 10.6	42.4 ± 12.8	34.1
Cotinine-*N*-glucuronide	17.5 ± 6.3	15.8 ± 7.8	15.4 ± 7.9	8.9 ± 4.6	14.0 ± 5.4	12.1 ± 6.0	20.1
Nicotine-*N*-glucuronide	2.8 ± 2.2	4.6 ± 2.9	5.3 ± 3.3	3.0 ± 1.8	4.5 ± 2.5	2.6 ± 2.1	3.7
trans-3′-Hydroxycotinine-*O*-glucuronide	8.5 ± 3.8	7.8 ± 5.9	7.9 ± 4.7	19.4 ± 11.0	22.8 ± 10.0	10.3 ± 7.6	7.4
Nicotine *N*-1′-oxide	6.8 ± 2.9	3.7 ± 0.9	2.7 ± 1.2	8.6 ± 6.9	3.0 ± 2.1	ND	6.7
Cotinine *N*-1-oxide	3.0 ± 1.9	4.5 ± 1.5	5.2 ± 1.5	2.5 ± 2.3	0.9 ± 0.9	ND	2.2
Nornicotine	–	0.6 ± 0.2	0.4 ± 0.1	–	–	–	–
Norcotinine	1.5 ± 0.5	ND		–	ND	ND	1.3
Others	–	–		–	–	10.1[b]	–
Total	99.8	99.8		100.2	99.9	100.2	100.2

Adapted from Tricker (2003)
ND, not determined
Study 1, Byrd *et al*. (1992); Study 2, Benowitz *et al*. (1994); Study 3, Andersson *et al*. (1994); Study 4, Andersson *et al*. (1997); Study 5, Hecht *et al*. (1999a); Study 6, Meger *et al*. (2002)
[a] In this study, 12 smokers were studied while smoking cigarettes and while receiving transdermal nicotine.
[b] Sum of 4-hydroxy-4-(3-pyridyl)butanoic acid and 4-oxo-4-(3-pyridyl)butanoic acid

Miksys *et al.*, 2003). The effects of nicotine delivered from smokeless tobacco products on metabolism have not been documented but they are probably similar to those of smoke-delivered nicotine. It is also possible that smokeless tobacco products affect local (buccal) disposition of drugs and carcinogens present in the tobacco products.

The metabolism of nicotine may be influenced by the actions of other drugs. In a recent study, the effects of menthol cigarette smoking on nicotine metabolism was investigated (Benowitz *et al*., 2004). Cigarette mentholation did not affect the intake of nicotine or carbon monoxide but nicotine metabolism was significantly slower after mentholated than after non-mentholated cigarettes. Menthol inhibited the metabolism of nicotine to cotinine and the formation of cotinine glucuronide. Menthol is a flavouring agent in many smokeless tobacco products. Although no studies are available, it is possible that menthol and other flavouring agents influence the metabolism of nicotine from smokeless tobacco products.

(iv) *Excretion*

Nicotine, cotinine and other metabolites are largely excreted in the urine. The excretion of unmetabolized nicotine (about 10% of a single dose) depends upon glomerular filtration rate and tubular secretion. The pH of the urine and urinary flow determine the amount of nicotine that is reabsorbed in the renal tubules. In an acidic pH, most of the nicotine is ionized and tubular reabsorption is low. Thus, an acidic urine increases the elimination of nicotine. Benowitz *et al*. (1983) determined that renal clearance of nicotine was 600 mL/min with acidic urine (pH 4.4) in which nicotine is largely protonated; conversely, when the urine is alkaline, nicotine is unprotonated and is reabsorbed into the circulation in the renal tubule and nicotine excretion is decreased. For example, when urine pH was adjusted to 7.0, the clearance of nicotine decreased to 17 mL/min; when urine pH was not controlled, pH averaged 5.8 and renal nicotine clearance was 100 mL/min, a rate that yielded an elimination of about 10–15% of the daily nicotine intake (Tables 82 and 83).

(*b*) *Smokeless tobacco constituents other than nicotine*

(i) *Absorption*

In several parts of the world, smokeless tobacco is invariably chewed with lime which is responsible for the highly alkaline pH (Nair *et al*., 1990, 1992). Almost all smokeless tobacco products contain additives, such as ammonia, carbonate or bicarbonate, to raise the pH (Nair *et al*., 2004). The pH of smokeless tobacco products is important, because nicotine most readily crosses the oral mucosa in the non-protonated form. Moist snuff products tested in volunteers were found to deliver high doses of nicotine to the bloodstream rapidly depending on the pH of the snuff product in aqueous solution (Fant *et al*., 1999 ; see also Section 4.1.1(*a*)).

_N_-Nitrosamines in saliva

Carcinogens derived from smokeless tobacco products have been detected in the saliva of users of these substances. The TSNA, NNN, NNK, NAT and NAB (see sections in the

monograph on Tobacco-specific *N*-nitrosamines for the structures), as well as the volatile nitrosamines, NDMA and *N*-nitrosodiethylamine (NDEA), were detected in the saliva of tobacco chewers and snuff dippers (see Table 84). Volatile nitrosamines are probably also tobacco-derived.

High levels of TSNA (NNN, NNK, NAB) and volatile nitrosamines were detected in saliva samples collected from India. The saliva of men who chewed tobacco with lime contained higher levels of TSNA than that of men who chewed betel quid with tobacco and lime (Bhide *et al*., 1986). NNN and NNK were also reported to be present in saliva in several other studies (Wenke *et al*., 1984; Nair *et al*., 1985, 1987). Volatile nitrosamines and TSNA in the saliva of chewers could derive from the leached nitrosamines present in the tobacco or could be formed endogenously from abundant precursors during chewing. The saliva of *mishri* users showed high levels of NNN (14–43 ppb [14–43 ng/mL]) and NPYR (2.2–8.3 ppb [2.2–8.3 ng/mL]) (Bhide *et al*., 1987b).

Levels of TSNA, nicotine and cotinine were measured in the saliva of 20 snuff dippers (Inuit, Northwest Territories, Canada). Levels of NNN, NNK and NAT plus NAB found in the saliva following a 15-min period of keeping 0.5–1.5 g moist snuff in the gingival groove were considerable: NNN, 115–2601 ppb [115–2601 ng/mL]; NAT plus NAB, 123–4560 ppb [123–4560 ng/mL]; and NNK, up to 201 ppb [201 ng/mL]. The salivary levels increase with the duration of keeping snuff in the mouth. The total amount of TSNA was estimated to be 444 μg per use, a large part of which could be swallowed (Brunnemann *et al*., 1987c).

Levels of TSNA were analysed every 10 min in the saliva of habitual snuff dippers. Detectable levels of at least two TSNA (NNN, NAT and NNK) were found in all samples collected between 10 and 30 min after the snuff had been placed in the mouth. The saliva of snuff dippers was reported to contain 57–420 ng/g NNN, up to 96 ng/g NNK and 7–470 ng/g NAT (Hoffmann & Adams, 1981). In a similar study, concentrations of 37–225, 0–61 and 48–555 ng/g NNN, NNK and NAT plus NAB were reported in snuff dippers' saliva, respectively (Palladino *et al*., 1986), and total concentrations of TSNA up to 241 ng/g were found. Trace levels of TSNA were still found in the saliva 20 min after the snuff had been removed (Österdahl & Slorach, 1988). Enzymatically active human saliva was found to liberate up to twice the amount of the NNK than heat-treated saliva (Prokopczyk *et al*., 1992b).

Salivary TSNA were measured in Sudanese oral snuff (*toombak*) users. NNN, NAT, NAB and NNK were measured before, during and after taking snuff. In addition, two other TSNA, NNAL and 4-(methylnitrosamino)-4-(3-pyridyl)-1-butanol (*iso*-NNAL), were detected in the saliva of tobacco chewers for the first time. Nine of 10 subjects had detectable levels of total salivary TSNA before chewing (0.01–1.0 μg/mL) and immediately following chewing (0.1–2.6 μg/mL). During dipping, TSNA concentrations in the saliva reached (μg/mL; range (number of subjects positive/total number)): NNN, 0.6–21 (12/12); NAT, 0.06–0.5 (2/12); NAB, 0.05–1.9 (12/12); NNK, 0.06–6.7 (11/12); NNAL, 0.05–3.3 (11/12); and *iso*-NNAL, 0.07–0.4 (8/12). These levels of salivary TSNA were 10–100 times

Table 84. Tobacco-specific *N*-nitrosamines (TSNA) in the saliva of tobacco chewers and snuff dippers

Smokeless tobacco	Country	No. of samples	TSNA (ng/mL saliva)			Reference
			NNN	NNK	NAT + NAB	
Snuff	USA	12	5–420	2–201	7–470	Hoffmann & Adams (1981)
	USA	30	37–225	ND–61	48–555	Palladino *et al*. (1986)
	Canada	20	115–2600	ND–201	123–4560	Brunnemann *et al*. (1987c)
	Sweden	4	3–140	ND–16	4–85	Österdahl & Slorach (1988)
Tobacco	India	7	36–130	ND	ND–380	Stich & Anders (1989)
	India	3	17–60	ND	14–52	Nair *et al*. (1985)
	India	10	10–430	ND–29	ND–133	Bhide *et al*. (1986)
Khaini	India	15	180–1580	ND–180	99–780	Stich *et al*. (1992)
Toothpaste containing tobacco	India	7	15–88	ND–10	10–70	Stich *et al*. (1992)
Mishri	India	9	14–44	ND	ND	Bhide *et al*. (1986)
Toombak[a]	Sudan	12	582–20 990	ND–6690	46–1940	Idris *et al*. (1992)

NAB, *N*′-nitrosoanabasine; NAT, *N*′-nitrosoanatabine; ND, not detected; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, *N*′-nitrosonornicotine

Note: Saliva samples were mostly collected a few minutes after beginning use of a new snuff dip or tobacco chew

[a] Saliva also contained ND–409 ng/mL 4-(methylnitrosamino)-4-(3-pyridyl)-1-butanol (*iso*-NNAL) and ND–3270 ng/mL 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL).

those reported previously and are consistent with the unusually high levels of TSNA in Sudanese *toombak* (Idris *et al*., 1992).

Levels of salivary TSNA were measured in Indian smokeless tobacco users, who place a mixture of *khaini* (tobacco and slaked lime) into the lower gingival groove, and users of tobacco-containing toothpaste (*gudhaku*) in Orissa, India. Among *khaini* tobacco chewers, up to 1580 ng/mL NNN, 690 ng/mL NAT, 90 ng/mL NAB and 180 ng/mL NNK were measured. Users of *gudhaku* showed much lower concentrations of these compounds, which may be due to the low amount of TSNA released from *gudhaku* and the short exposure time, which is restricted to the period of tooth brushing (Stich *et al*., 1992).

(ii) *Distribution*

NNAL and NNAL-glucuronides (NNAL-Gluc) have been detected in the plasma of smokeless tobacco users, and NNAL has been detected in the plasma of smokers (Hecht *et al*., 1999b; Hecht, 2002).

DNA and protein adducts

A ^{32}P-postlabelling assay has been explored to detect smokeless tobacco use-specific DNA adducts in the exfoliated oral mucosa cells of smokeless tobacco users. Adduct spots were detected in users as well as in non-users. ^{32}P-Postlabelling analysis of DNA from the oral cavity of these subjects did not demonstrate unique patterns or relative adduct level values. A lack of information on the structure of the majority of adducts was a serious limitation of these studies (Dunn & Stich, 1986; Chacko & Gupta, 1988).

Immunoassays for O^6-methyldeoxyguanosine, a DNA adduct that could arise from NNAL and NNK, have given negative results in exfoliated oral cells from snuff dippers (Hecht *et al*., 1987).

Haemoglobin adducts have been explored as biomarkers of exposure to and metabolic activation of TSNA. NNN and NNK form haemoglobin adducts in humans and experimental animals; these adducts release 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB) upon mild alkaline hydrolysis. HPB released from human haemoglobin can be quantified by gas chromatography–mass spectrometry (Hecht *et al*., 1991). Levels of HPB released from haemoglobin (fmol HPB/g haemoglobin) were 517 ± 538 (mean ± SD) in snuff dippers, 79.6 ± 189 in smokers and 29.3 ± 25.9 in nonsmokers (Carmella *et al*., 1990). In Sudanese snuff dippers, the levels of the HPB-releasing haemoglobin adduct ranged from 68 to 323 fmol/g haemoglobin (mean ± SD, 148 ± 104 fmol/g haemoglobin). The wide range of adduct levels observed suggests that, despite similar levels of exposure to NNK and NNN, significant differences exist in the ability of individuals in this population to activate these compounds (Murphy *et al*., 1994). Nasal snuff users also showed high levels of haemoglobin adducts; however, HPB-releasing adducts were not correlated with the amount or type of snuff used (Schäffler *et al*., 1993).

(iii) *Metabolism and excretion*

Urinary carcinogen biomarkers

All of studies reviewed in this section were carried out on smokeless tobacco users in the USA, unless otherwise specified.

The use of human urinary metabolites of carcinogens as biomarkers in tobacco carcinogenesis has been reviewed comprehensively (Hecht, 2002). NNK (see the monograph on Tobacco-specific nitrosamines) is metabolized to NNAL, which, similarly to NNK, is a potent pulmonary carcinogen in rodents. Glucuronidation of NNAL at the pyridine nitrogen yields NNAL-*N*-Gluc while conjugation at the carbinol oxygen yields NNAL-*O*-Gluc (Carmella *et al*., 2002). Both NNAL and NNAL-Gluc are excreted in human urine and are very useful biomarkers because they derive from NNK that is specific to tobacco products (Hecht, 2002). In all studies to date, these biomarkers have been found to be absolutely specific to exposure to tobacco and have not been detected in the urine of non-users of tobacco unless they had been exposed to secondhand tobacco smoke. Because NNAL is not usually present in tobacco, NNAL and NNAL-Gluc in urine originate largely from the metabolism of NNK. Most investigations to date have demonstrated a correlation between NNAL plus NNAL-Gluc and cotinine (Hecht, 2002).

Seven male Sudanese *toombak* (snuff) dippers excreted exceptionally high amounts of urinary NNAL and NNAL-Gluc (0.12–0.44 mg) daily. Therefore, assuming chronic *toombak* use, the minimum daily dose of NNK to which these users were exposed was 0.12–0.44 mg. This is the highest documented uptake of a non-occupational carcinogen. The (*S*)-NNAL-*O*-Gluc:(*R*)-NNAL-*O*-Gluc ratio was 1.9. The two diastereomers of NNAL-Gluc were present in all urine samples analysed (Murphy *et al*., 1994). The high systemic exposure to NNK suggests that these individuals may also be at risk for cancers other than those of the oral cavity.

The distribution half-lives of NNAL and NNAL-Gluc were determined in 13 male smokeless tobacco users. Baseline levels in urine as well as renal clearance of the NNK metabolites correlated with the number of tins or pouches of smokeless tobacco consumed. Ratios of (*S*)-NNAL:(*R*)-NNAL and (*S*)-NNAL-Gluc:(*R*)-NNAL-Gluc in urine were significantly higher 7 days after cessation than at baseline, which suggests a receptor site for the more carcinogenic NNAL enantiomer, (*S*)-NNAL. Urinary NNAL plus NNAL-Gluc also provides a good approximation of the dose of carcinogen taken in by snuff dippers. A correlation between the number of tins or pouches of smokeless tobacco consumed per week and NNAL plus NNAL-Gluc in the urine was observed, as well as a correlation between urinary cotinine and NNAL plus NNAL-Gluc in the urine of smokeless tobacco users (Hecht, 2002; Hecht *et al*., 2002).

Urinary NNAL and NNAL-Gluc levels in 39 male smokeless tobacco users were similar to those in smokers. The ratio of NNAL-Gluc:NNAL was higher in snuff dippers than in tobacco chewers. A significant association between levels of NNAL plus NNAL-Gluc in the urine of smokeless tobacco users and the presence of oral leukoplakia was

observed, which supports the potential role of NNK as a causative factor for this lesion (Kresty *et al*, 1996).

NNAL, NNAL-*N*-Gluc and NNAL-*O*-Gluc were analysed in the urine of 14 smokeless tobacco users. NNAL-*N*-Gluc in the urine comprised 24 ± 12% of total NNAL-Gluc and demonstrated that NNAL-*N*-Gluc contributes substantially to NNAL-Gluc in human urine (Carmella *et al*., 2002).

Pyridine-*N*-oxidation of NNK and its major metabolite, NNAL, produces NNK-*N*-oxide and NNAL-*N*-oxide, respectively, which are detoxification products of NNK metabolism and are excreted in the urine of rodents and primates. Analysis of the urine of smokeless tobacco users for NNAL-*N*-oxide showed its presence at lower concentrations than that of NNAL. Thus, pyridine-*N*-oxidation is a relatively minor detoxification pathway of NNK and NNAL in humans (Carmella *et al*., 1997).

Although tobacco products with reportedly reduced carcinogen content are on the market (see Section 1), carcinogen uptake in people who use these products has not been assessed systematically. In one study, 54 users of smokeless tobacco were randomly assigned to one of two groups. One switched to Swedish snuff (*snus*), while the other quit and used medicinal nicotine (the nicotine patch). All participants were assessed for urinary levels of total NNAL and NNAL-Gluc. Total NNAL levels were statistically significantly lower in users of smokeless tobacco after they had switched to Swedish moist snuff or to a nicotine patch than before the switch, although the overall mean total level of NNAL among subjects who used the nicotine patch was statistically significantly lower than that among those who used moist snuff (mean, 1.2 and 2.0 pmol NNAL/mg creatinine, respectively; mean difference, 0.9 pmol NNAL/mg creatinine; 95% CI, 0.2–1.5; $p = 0.008$) (Hatsukami *et al*., 2004a).

Absorption of NNN by smokeless tobacco users has been demonstrated by detection of NNN and NNN-*N*-glucuronide (NNN-*N*-Gluc) in urine. Levels in 11 users were 0.03–0.58 pmol/mg creatinine (mean ± SD, 0.25 ± 0.19 pmol/mg) NNN and 0.091–0.91 pmol/mg creatinine (mean ± SD, 0.39 ± 0.27 pmol/mg) NNN-*N*-Gluc (Stepanov & Hecht, 2005).

NAB and NAB-*N*-glucuronide were excreted in the urine of smokeless tobacco users. Levels in 11 users ranged from not detectable to 0.11 pmol/mg creatinine (mean ± SD, 0.037 ± 0.034 pmol/mg) NAB and 0.020–0.44 pmol/mg creatinine (mean ± SD, 0.19 ± 0.16 pmol/mg) NAB-*N*-glucuronide (Stepanov & Hecht, 2005).

Absorption of NAT by smokeless tobacco users and smokers has been demonstrated by detection of NAT and NAT-*N*-glucuronide in urine. Levels in 11 smokeless tobacco users were 0.020–0.15 pmol/mg creatinine (mean ± SD, 0.069 ± 0.046 pmol/mg) NAT and 0.08–2.78 pmol/mg creatinine (mean ± SD, 1.36 ± 1.06 pmol/mg) NAT-*N*-glucuronide (Stepanov & Hecht, 2005).

Endogenous nitrosation

Tobacco contains secondary and tertiary amines that can be nitrosated in the saliva during the chewing of tobacco when they react with available nitrite in the presence of

nitrosation catalysts such as thiocyanate. The NPRO test measures the potential for intragastric formation of carcinogenic nitrosamines in humans. Nitrate and L-proline are administered to volunteers; non-carcinogenic NPRO is produced by an acid-catalysed reaction of proline (a model for ingested amines) with nitrate-derived nitrite in the stomach. It is then absorbed and excreted in the urine, which is analysed for NPRO (Ohshima & Bartsch, 1981).

The role of poor oral hygiene in the formation of *N*-nitroso compounds was investigated by means of the NPRO assay. Endogenous nitrosation was significantly higher in tobacco chewers with poor oral hygiene (determined by dental plaque) compared with those with good oral hygiene (Nair *et al*., 1996). Nitrite found in human saliva is the product of the microbial reduction of nitrate released from the salivary glands. Salivary nitrite level and nitrate reductase activity, when detected, have been reported to be higher in tobacco chewers and *mishri* users from India than in controls (Murdia *et al*., 1982). Increased formation of nitrite and nitric oxide in the mouth was found in people with dental plaque (Carossa *et al*., 2001) and bacterial enzyme-mediated formation of nitrosamines has been reported (Calmels *et al*., 1996). Thus, dependent on the availability of nitrosatable amines from tobacco, the formation of nitrosamines is more extensive in subjects who have poor oral hygiene if they chew tobacco.

Among subjects dosed with proline, the level of NPRO was significantly elevated in the urine of individuals who chewed tobacco plus lime than in non-users (Chakradeo *et al*., 1994). Levels of other *N*-nitrosamino acids (NSAR, MNPA, MNBA, NTCA and MNTCA) were not significantly affected (Nair *et al*., 1985; Chakradeo *et al*., 1994).

Measurable concentrations of all tobacco alkaloids (nicotine, nornicotine, anabasine, and anatabine) were excreted in the urine of subjects who used smokeless tobacco. These compounds could be substrates for endogenous nitrosation in tobacco chewers (Jacob *et al*., 2002).

4.1.2 *Experimental systems*

(*a*) *Absorption*

Squier (1986) investigated the simultaneous penetration of nicotine and NNN across porcine skin and various regions of oral mucosa. Penetration by nicotine was as ‘rapid as water’. Non-keratinized regions were also permeable to NNN, a pattern that is mimicked by the locations of tumours in the oral cavity. A later study (Du *et al*., 2000) tested the influence of nicotine and alcohol on the permeability of the mucosal membranes to NNN *in vitro*. It was reported that alcohol and nicotine each increased the permeability of NNN and that, together, ethanol and nicotine increased the permeability to levels higher than that of ethanol alone.

(*b*) *Effect of smokeless tobacco on enzyme activities and metabolism*

(i) *Animal studies*

Most investigations of the effects of smokeless tobacco on enzyme activities in animals have measured changes in levels of phase I and phase II enzymes in the lung and liver of mice and rats.

While TSNA are the most abundant carcinogens in smokeless tobacco products, some forms of pyrolysed smokeless tobacco products also contain PAHs. The PAH profiles of *mishri* and snuff revealed significant amounts of carcinogenic PAH compounds. *Mishri* extract or snuff extract was given intraperitoneally to inbred male Swiss mice (eight animals per group) and the activities of hepatic microsomal cytochrome b5, CYP and benzo[*a*]pyrene hydroxylase were measured. A significant increase in levels of CYP and benzo[*a*]pyrene hydroxylase was observed as a result of both treatments (Bhide *et al*., 1984a, 1991).

Male Sprague-Dawley rats (12 weeks of age) were fed a standard diet and were given *mishri* extract and benzo[*a*]pyrene at 75% of the dose that causes 50% lethality (LD_{50}) by intraperitoneal injection three times at 24-h intervals. An increase in the levels of the hepatic phase I activating enzymes, CYP content, benzo[*a*]pyrene hydroxylase and benzphetamine demethylase and a significant decrease in glutathione-*S*-transferase (GST) activity were observed. Depletion in glutathione (GSH) content and hepatic vitamin A pool and a concomitant increase in vitamin C content were also noted (Ammigan *et al*., 1989a; Bhide *et al*., 1991).

Acute exposure to smokeless tobacco and nutritional deficiency

Malnutrition is a condition that affects the population in several countries where the use of smokeless tobacco is widespread. Altered metabolism as a result of vitamin deficiency and protein–calorie malnutrition may be an important factor in susceptibility to carcinogens. The following studies have investigated the effect of smokeless tobacco in the presence of some nutritional deficiencies (obtained using a well-defined semi-synthetic basal diet) on hepatic and pulmonary carcinogen-metabolizing enzymes in male Sprague-Dawley rats.

The effects of extracts of chewing tobacco and *mishri* were evaluated in a series of experiments on nutritional deficiency. NNN and benzo[*a*]pyrene were tested at the same time as standard carcinogens using the following basic experimental protocol.

Inbred male weanling Sprague-Dawley rats (19–21 days old and weighing 35–50 g) were randomly divided into three groups of eight animals each and were fed three different dietary regimens that consisted of standard diet, control semi-synthetic diet and semi-synthetic deficient diet. In each set of experiments, the semi-synthetic diets were either adequate (control) or deficient in vitamin A (Nair *et al*., 1991a), vitamin B complex (Ammigan *et al*., 1990a) or protein (Ammigan *et al*., 1989b, 1990b). At 12 weeks, tobacco extract, *mishri* extract, NNN or benzo[*a*]pyrene was administered intraperitoneally at 75% of the LD_{50} dose, divided in three equal doses at 24-h intervals. Twenty-four hours after the last

injection, animals overnight fasted were killed and the lung and liver were excised. The hepatic and pulmonary biotransformation enzymes, CYP, cytochrome b-5, benzo[*a*]pyrene hydroxylase, benzphetamine *N*-demethylase, GST and GSH content were determined. Vitamin A and C were also measured. Smokeless tobacco extracts were found to be more toxic to animals with nutritional deficiencies. The tolerance of animals on deficient diets was lower as seen by the increase in toxicity of the test substances. In vitamin A-, vitamin B complex- or protein-deficient rats, the LD_{50} for intraperitoneally injected tobacco extract was reduced by 32–40%, that of *mishri* extract by 43%, that of NNN by 20–24% and that of benzo[*a*]pyrene by 24% (Ammigan *et al.*, 1990b). These deficiencies also resulted in decreases in the basal levels of CYP, benzo[*a*]pyrene hydroxylase, benzphetamine demethylase, GST and GSH.

In vitamin A-sufficient and -deficient groups, treatment with *mishri* extract, benzo[*a*]-pyrene (Ammigan *et al*, 1990c), tobacco extract and NNN (Nair *et al*., 1991a) significantly increased the phase I activating enzymes in all groups. A greater increase in hepatic and pulmonary phase I activities was observed in deficient animals compared with sufficient groups. An increase in GSH and GST levels was observed in the sufficient group following treatment, but exposure to the test substances caused further suppression of the hepatic and pulmonary GSH/GST system in the deficient animals.

Groups of Sprague-Dawley rats fed low-protein (5% casein) or vitamin B complex-deficient diets were exposed to tobacco extract, *mishri* extract, NNN or benzo[*a*]pyrene according to the same protocol. All animals showed a significant increase in phase I enzymes with concurrent inhibition of GSH and GST levels compared with corresponding control groups on high-protein (20% casein) or vitamin B complex-sufficient diets (Ammigan *et al*., 1989b, 1990a).

Smokeless tobacco (50 or 100 mg/kg bw per day) was shown to modify the activity of phytic acid (1000 mg/kg bw per day by gavage) and butylated hydroxyanisole (1% w/w in diet) both directly and translactationally by significantly inhibiting the phytic acid-induced hepatic GST and GSH levels and further augmenting phytic acid- or butylated hydroxyanisole-induced microsomal cytochrome b5 and CYP in lactating dams and suckling pups of mice (Singh & Singh, 1998).

Chronic exposure to smokeless tobacco

The effects of chronic exposure to 10% *mishri* in a standard diet for 20 months on the activities of several activating enzymes, GST and GSH levels were measured in several organs of three rodent species: Swiss mice, Sprague-Dawley rats and Syrian golden hamsters. At 20 months, the upper alimentary tract, tongue, oesophagus, stomach, liver and lung were excised. Significant increases in activities of phase I activating enzymes and decreases in the phase II detoxification system were observed in most extrahepatic tissues of the treated animals of all three species. These observations suggest that prolonged exposure to smokeless tobacco extract affects the drug-metabolizing enzymes of the gastrointestinal tract, which may be an important factor that determines the susceptibility of different organs to exposure to carcinogens (Nair *et al*., 1991b). The base levels

of enzymes in proximal, medium and distal parts of the intestine in the three species were similar. However, the levels of CYP, benzo[*a*]pyrene hydroxylase and GST were highest in hamsters followed by rat and mice. In the exposed groups, a significant induction of CYP and benzo[*a*]pyrene hydroxylase and depletion of GSH and GST levels were observed only in the proximal and distal parts of the intestine of the three species, which suggests the importance of proximal and distal parts of the rodent intestine in metabolism and susceptibility to intestinal xenobiotic exposure (Nair *et al*., 1991c).

Chronic exposure to smokeless tobacco and vitamin A deficiency

Chronic exposure was investigated in two long-term studies in Sprague-Dawley rats that were fed standard vitamin A-sufficient and -deficient diets and were administered daily oral doses of 3 mg tobacco extract or *mishri* extract over a period of 21 months. Pulmonary and hepatic carcinogen-metabolizing enzymes, both phase I (CYP, cytochrome b5, benzo[*a*]pyrene hydroxylase, benzphetamine demethylase) and phase II (GST), as well as levels of vitamin A and C in plasma and liver were measured at 12 and 21 months. Overall, the phase I enzyme activities were significantly higher in vitamin A-sufficient than in vitamin A-deficient rats at both 12 months and 21 months. Treatment with tobacco extract increased the activity of hepatic and pulmonary phase I enzymes but decreased the GSH/GST system at both time points. The vitamin A-deficient *mishri*-treated animals also showed a decrease in the GSH/GST detoxification system after 12 and 21 months while the converse was observed in vitamin A-sufficient group after 21 months. Similar to short-term exposure studies, treatment with tobacco extract and *mishri* extract significantly lowered the hepatic and circulating levels of vitamin A, while a concurrent increase was observed in the level of vitamin C. The data showed that chronic exposure to smokeless tobacco together with vitamin A deficiency renders the rats more susceptible to smokeless tobacco (tumorigenicity data in Section 3.1), partly due to the augmented carcinogen activation together with depletion of the detoxifying GSH/GST system (Ammigan *et al*., 1991; Bhide *et al*., 1991).

(ii) *Cell culture systems*

Indian snuff extract showed an overall inhibitory effect on cell count, [^{3}H]thymidine uptake and ornithine decarboxylase and aryl hydrocarbon hydroxylase activities when incubated either alone or in combination with NNN or NNK in in-vitro cultures of embryonic mouse tongue primary epithelial cells (Gijare *et al*., 1989). Cultures treated with snuff extract in combination with DMBA also showed inhibition of cell proliferation and a decrease in ornithine decarboxylase and aryl hydrocarbon hydroxylase activities compared with control, DMBA- and DMBA plus TPA-treated cultures (Gijare *et al*., 1990b).

(*c*) *Smokeless tobacco and reactive oxygen species*

(i) *Animal studies*

Oxidative stress and reactive oxygen species can play a significant role in the cytotoxic effects induced by smokeless tobacco products. Acute and subchronic administra-

tion of smokeless tobacco extract in phosphate buffer to rats induced hepatic mitochondrial and microsomal lipid peroxidation, hepatic DNA single-strand breaks, significant increases in urinary excretion of malondialdehyde, formaldehyde, acetaldehyde and acetone, and significant increases of nitric oxide production in peritoneal macrophages, which suggest the involvement of oxidative stress in the toxicity of smokeless tobacco extract (Bagchi *et al*., 1994; Hassoun *et al.*, 1995; Bagchi *et al*., 1998).

(ii) *In-vitro studies*

Application of smokeless tobacco resulted in generation of reactive oxygen species in in-vitro experiments with peritoneal macrophages, and in hepatic mitochondria and microsomes from female Sprague-Dawley rats and that of nitric oxide in the peritoneal macrophage J774A.1 cells in culture (Bagchi *et al*., 1995; Hassoun *et al*, 1995; Bagchi *et al*., 1996).

Cultured human oral epithelial carcinoma cells produced reactive oxygen species following in-vitro incubation with an aqueous extract of smokeless tobacco (Bagchi *et al*, 1996). Smokeless tobacco extracts significantly induced the production of superoxide anion, and increased lipid peroxidation, DNA fragmentation and protein kinase C activity in primary cultures of human oral keratinocytes (Bagchi *et al*., 1997, 2002). Using flow cytometry with the fluorescent dye, propidium iodide, a dose-dependent increase in apoptotic cell death was observed following treatment with smokeless tobacco extract which was inhibited by several antioxidants including vitamin C and vitamin E (Bagchi *et al*., 1999).

Smokeless tobacco extract that contained an equivalent amount of nicotine was found to be more toxic than nicotine in the generation of reactive oxygen species, as assessed by the measurement of changes in GSH and malondialdehyde levels in Chinese hamster ovary cells (Yildiz *et al*., 1999).

In summary, nutritional deficiencies that are either already prevalent or caused by the use of smokeless tobacco are often observed in a large proportion of smokeless tobacco users. The situation is aggravated by further induction of phase I enzymes and suppression of antioxidant systems, such as the GSH/GST system. Reduced plasma levels of several antioxidant vitamins have also been reported in smokeless tobacco users. The generation of reactive oxygen species and lipid peroxidation due to smokeless tobacco extracts have been reported and can accelerate different stages of carcinogenesis.

(*d*) *Biomarkers (including adducts) of smokeless tobacco carcinogens*

(i) *In-vitro formation of TSNA from a variety of smokeless tobacco products*

The levels of the TSNA (NAB, NAT, NNN and NNK) were determined in a variety of chewing tobacco, oral snuff, *mishri* and *zarda* samples. The potential endogenous formation of TSNA was estimated by incubation of tobacco samples at pH 2.0 for 1 h at 37 °C and over the pH range 1.0–5.5 under conditions that simulated the normal fasting stomach, with a constant nitrite concentration of 25 μM. Under the simulated gastric

conditions, NAB, NAT and NNN were formed, and maximum formation of these TSNA occurred at pH 2.5. Under the acidic simulated gastric conditions (pH 2.0), slight decomposition of NNK via transnitrosation was observed (Tricker *et al.*, 1988).

(ii) *DNA adducts*

Male Fischer 344 rats were pretreated for 2 weeks with either a solution of a snuff extract or 0.002% nicotine in the drinking-water. Subsequently, the rats were given a single dose of NNK and the effects of snuff and nicotine on the methylation of guanine by NNK in the DNA of target organs were determined. Formation of 7-methylguanine in the liver, nasal mucosa and oral cavity and of O^6-methylguanine in the liver and oral cavity were much lower in the rats pretreated with snuff extract than in those that were not pretreated. In contrast, pretreatment of the rats with nicotine had no significant effect on the methylation of DNA by NNK nor on the elimination constants of NNK and its major metabolite NNAL (Prokopczyk *et al.*, 1987). The authors suggested that snuff extract contains one or more compounds other than nicotine that alter the methylation of guanine by NNK.

A ^{32}P-postlabelling assay to detect adducts in DNA from rat oral epithelial cells after their exposure *in vitro* to chewing tobacco extract in the presence of ethanol showed slightly higher adduct levels in treated cells than in control cells (Autrup *et al.*, 1992). However, following chronic exposure of rats to snuff in a surgically created canal in the lower lip, aromatic DNA adducts were not detected by ^{32}P-postlabelling. The adduction to DNA in organs of the gastrointestinal tract and the kidneys indicates that the use of snuff results in systemic exposure to carcinogens and may contribute to the incidence of neoplasms in organs outside the oral cavity (Smith *et al.*, 1997).

4.2 Toxic effects

4.2.1 *Humans*

(*a*) *Nicotine addiction*

At the Mayo Clinic, Morse *et al*. (1977) described a case of a 53-year-old man who requested treatment for dependence on chewing tobacco. The report was remarkable because the patient defined himself as addicted at a time when the medical community was reluctant to include tobacco as a dependence-producing substance. The patient exemplified the hallmarks of drug dependency — loss of control over consumption, compulsive use in the face of obvious harm, escalating patterns of use and symptoms of withdrawal on discontinuation of the drug. Despite this and other clinical cases, there was little systematic study of smokeless tobacco dependence and withdrawal until the 1980s. Nevertheless, on the basis of available evidence for all forms of tobacco use, the American Psychiatric Association included smokeless tobacco as a potential cause of dependence and withdrawal in its 1980 Diagnostic and Statistical Manual of Mental Disorders, Third Revision (American Psychiatric Association, 1980). The potential for smokeless tobacco to cause dependence and withdrawal was specifically reviewed by an advisory committee

to the US Surgeon General in 1986 (DHHS, 1986) which concluded that "The use of smokeless tobacco products can lead to nicotine dependence and addiction". The conclusion was based on evidence from clinical and animal studies that showed that exposure to nicotine from smokeless tobacco products, either through self administration of the products in people or from administration of nicotine to animals, induced psychoactive effects in both animals and people. These conclusions were reaffirmed in 1988 (DHHS, 1988) and later (DHHS, 1995, 1996).

It has now been recognized by several governing bodies that smokeless tobacco products initiate and sustain addiction — that is, they cause physical dependence (DHHS, 1986; Henningfield *et al*., 1997; Henningfield & Fant, 1999). The following section reviews characteristics that define drug addiction and dependence and gives information on how smokeless tobacco products fulfil these requirements.

(i) *'Addiction' versus 'dependence' and 'withdrawal'*

Technical scientific reports and medical diagnoses have used the terms 'dependence' and 'withdrawal' to define compulsive drug-seeking behaviour and the abstinence-associated behavioural and physiological disruptions (American Psychiatric Association, 1987, 1994; WHO, 2006). However, the term 'addiction' is more typically used as the most universally recognized term in general communications by major health organizations, and remains the term used to describe the phenomenon of compulsively driven drug-seeking behaviour.

The criteria for a drug to be identified as addictive have been delineated (DHHS, 1988), and include a pattern of use that is either highly controlled or compulsive, which entails psychoactive effects and drug-reinforced behaviour. Additional criteria include stereotypic use patterns, use despite harmful effects, relapse following abstinence and recurrent drug cravings. Physiological manifestations of dependence-producing drugs include the development of tolerance, physical dependence manifest by withdrawal signs and symptoms upon acute discontinuation of the drug and pleasurable or euphoriant acute effects.

Clinical criteria to determine whether a person is dependent upon a drug and/or if drug abstinence has produced a withdrawal syndrome are provided by the American Psychiatric Association (1987, 1994) and by the WHO (2006). In 1980, the American Psychiatric Association acknowledged 'tobacco dependence' and 'tobacco withdrawal' syndromes. The US National Institute on Drug Abuse came to similar conclusions and further concluded that nicotine met the same criteria as a dependence-producing drug such as cocaine and morphine (DHHS, 1988).

The determination of whether or not a substance meets criteria that can produce dependence and/or withdrawal has been elaborated through laboratory studies of the pharmacological actions of the drugs (United Nations Single Convention on Narcotic Drugs, 1961; United Nations Convention on Psychotropic Substances, 1971; Balster & Bigelow, 2003; Spillane & McAllister, 2003). In practice, both laboratory data and observations of clinical dependence in users are critical in determining whether a drug is appropriately categorized as 'dependence'-producing. It is important to note that the phenomenon of 'withdrawal'

can be determined independently in clinical evaluations and in laboratory studies and is neither necessary (e.g. many users of heroin and cocaine use the drugs intermittently and do not show signs of withdrawal upon discontinuation of use) nor sufficient (e.g. experimental production of physical dependence in laboratory studies and production of physical dependence in patients treated for pain with analgesics does not necessarily result in drug-seeking behaviour and dependence) (for reviews, see DHHS, 1988; Feinstein *et al.*, 2000).

(ii) *Tobacco versus nicotine*

Nicotine is the drug in tobacco that defines tobacco use as an addiction since it meets independent criteria for addiction and is delivered in sufficient quantities to produce physiological and behavioural effects that comprise addiction. However, nicotine alone does not fully explain all aspects of symptoms, clinical course or need for treatment that relate to addiction. As for other addictive drugs, the prevalence of use, risk of addiction and related consequences are linked to the formulation of the drug, cost, access and social image. Tobacco addiction, prognosis and treatment have been discussed in detail elsewhere (DHHS, 1988; Royal College of Physicians of London, 2001).

(iii) *Evaluation of potential dependence-producing effects of smokeless tobacco*

The majority of data that show that tobacco products are addictive derive from studies of cigarette smoking (DHHS, 1988; Royal College of Physicians of London, 2000). However, repeated demonstrations (Gritz *et al.*, 1981; Benowitz *et al*., 1983; Russell *et al*., 1985; Fant *et al*., 1999) that smokeless tobacco products rapidly deliver nicotine at levels equal to or greater than that of cigarette consumption imply that continual use of smokeless tobacco products initiates and sustains tobacco dependence. Demonstrable signs of withdrawal upon the discontinuation of regular smokeless tobacco use (Hatsukami *et al*., 1987; Keenan *et al*., 1989; Hatsukami *et al*., 1992) are further evidence that smokeless tobacco products produce dependence. Finally, the reports by smokeless tobacco users that they are addicted, have tried and failed to quit use on several occasions and that the smokeless tobacco products influence occupational and social behaviours (Hatsukami & Severson, 1999; Severson, 2003) also provide direct support for the addictive potential of smokeless tobacco products.

(iv) *Patterns of use*

Dependence-producing drugs typically cause a pattern of use that is characterized by strong, almost irresistible, urges and cravings to consume the drug. Consumption is continued even when the user acknowledges that the drug has the potential to or has caused physical harm or interferes with social or occupational pursuits. The use pattern persists even when there is a strong effort to stop drug consumption. The urge to use is enduring; even after long periods of drug abstinence, cravings for the drug are evident and re-initiation of drug use (relapse) is frequent. Reports have suggested patterns of escalating dosage with duration of smokeless tobacco use and age and have shown consi-

derable evidence for relapse and inability to maintain abstinence from smokeless tobacco (Hatsukami & Severson, 1999).

The pattern of compulsive use has been amply demonstrated for cigarette smoking (DHHS, 1988) and there is substantial evidence that the case is similar for smokeless tobacco. Many (74%) first-time users of smokeless tobacco found the experience unpleasant; when used a second time, 53% found the experience to be unpleasant (Ary *et al.*, 1989). These data suggest that some tolerance to the unpleasant effects of smokeless tobacco are apparent even after a single use. As users become more tolerant, the time interval between smokeless tobacco use decreases rapidly. For example, 20% of first-time users repeated the experience within 24 h and 33% used their second dip within 24 h. Consumption of smokeless tobacco tends to increase with age; adolescents that reported daily use consumed less smokeless tobacco than young adults who reported daily use (Ary *et al.*, 1987, 1989).

When daily smokeless tobacco use is established, levels of exposure to nicotine are comparable with those of daily cigarette smokers. Gritz *et al*. (1981) examined plasma nicotine levels over the course of a day in which participants were allowed to use smokeless tobacco *ad libitum*. After overnight abstinence, plasma nicotine levels averaged 2.9 ng/mL and increased to 21.5 ng/mL after 6 h of ad-libitum smokeless tobacco consumption. Eight of the 12 subjects had levels of nicotine similar to those of cigarette smokers. In other studies, Hatsumaki *et al*. (1987, 1988) reported that saliva cotinine levels among regular smokeless tobacco users ranged between 255 and 280 ng/mL, a level that was similar to those seen in daily cigarette smokers.

Daily use of smokeless tobacco was associated with brand switching to products that deliver more nicotine. Tomar *et al*. (1995) observed that smokeless tobacco users switch from low nicotine-delivery products to higher delivery products twice as frequently as they switch from higher to lower delivery products. Sales of Skoal Bandit and Hawken, the lowest nicotine delivery products, constitute 3% of the market but sales of Copenhagen and Kodiak (high nicotine-delivery products) constitute 43% of sales (Hoffmann *et al*., 1995). Similarly Henningfield *et al*. (1995) compared sales and nicotine delivery in three regions of the USA and reported that higher sales were associated with greater nicotine delivery. More recently, Richter and Spierto (2003) surveyed 18 brands of smokeless tobacco and concluded that those with the most available nicotine (as a function of pH and nicotine content) had the highest market share.

The duration of smokeless tobacco use appears to influence levels of perceived dependence. In a sample of youths, Riley *et al*. (1996) found that 37% of adolescents who used smokeless tobacco for longer than 1 year rated themselves as addicted and were 12 times more likely to report perceived addiction than adolescents who had used such products for less than 1 year.

When chronic smokeless tobacco users attempt to quit, they are frequently unsuccessful. As reviewed by Hatsukami and Severson (1999), between 75 and 100% of subjects in the control groups of seven treatment studies relapsed within 3–12 months. The studies reviewed by Hatsukami and Severson (1999) and Ebbert *et al*. (2003) suggest that,

even among volunteers in smokeless tobacco cessation treatment studies who are highly motivated to quit, successful and lasting cessation is very uncommon. These results concur with the clinical impressions that smokeless tobacco cessation is difficult — even more difficult than quitting cigarette smoking — as suggested by reports of subjects who have attempted to quit both substances (Severson, 2003).

It is clear that the risk and magnitude of dependence is directly related to the amount of nicotine ingested per day and probably to years of use (DHHS, 1988; Food and Drug Administration, 1995, 1996; Royal College of Physicians of London, 2001). It is not clear, however, what threshold dose produces dependence or, conversely, what dose would pose a negligible risk of dependence. Benowitz and Henningfield (1994) theorized that a threshold dose for nicotine dependence could be identified and that the level below which dependence was unlikely to be produced for cigarettes was 0.45 mg nicotine (less than 0.2 mg absorption per cigarette). Similar conclusions were drawn in a report commissioned and endorsed by the American Medical Association (Henningfield *et al.*, 1998). The Food and Drug Administration (1995, 1996) considered this theory and its implications for reducing the nicotine dose to a level that might be exempt from the label 'addictive'. They concluded, based in part on an advisory committee evaluation, that (*a*) a threshold for addiction probably exists but that the level is not known and (*b*) that low levels of nicotine that may not sustain addiction in adult users might pose a risk as a starter product for young people. It was concurred that even very low nicotine cigarettes should not be exempt from an addiction warning since they might promote the development of addiction (Henningfield *et al.*, 1998). The experience in the USA with very low-nicotine dose 'starter' smokeless tobacco products supports the extension of such a conclusion to smokeless tobacco products (DHHS, 1986; Connolly *et al.*, 1986).

Taken together, the data on patterns of use of smokeless tobacco support the conclusion drawn by Henningfield *et al.* (1997) that many users of smokeless tobacco are dependent but that the overall risk of dependence among users appears to be somewhat lower than that for cigarette smokers. This conclusion is consistent with pharmacokinetic data that compare cigarettes and smokeless tobacco (described in Section 4.1.1 and later in this section) and show that speed of nicotine absorption from smokeless tobacco is slower than that from inhaled cigarette smoke.

(v) *Psychoactive effects*

Dependence-producing drugs are psychoactive (i.e. they act on the brain to produce changes in mood, performance or thought). There is now abundant evidence from studies in animals and humans that administration of nicotine is associated with subjective and physiological changes due to actions on receptors in the brain (reviewed by DHHS, 1988; Henningfield *et al.*, 1993). Furthermore, there is a vast body of literature that documents the psychoactive effects of human cigarette smoking (DHHS, 1988). These studies have emphasized that the administration of intravenous or smoke-delivered nicotine is euphoric (and, in some subjects, is indistinguishable from cocaine or amphetamine). Laboratory studies have also shown that subjects will self-administer intravenous nicotine

(Henningfield *et al*., 1983, 1985). The administration of nicotine increases subjective measures of 'feeling good', appreciation of the drug and other measures that are typically associated with administration of psychostimulants (Jasinski *et al*., 1984).

A laboratory study of smokeless tobacco users (Fant *et al*., 1999) administered four smokeless tobacco products and a mint snuff that did not contain nicotine. Before administration, during the time (30 min) that the subjects held the product in their mouths and for up to 60 min after it was removed, the participants answered questions on subjective effects. The high nicotine-delivery product (Copenhagen snuff) yielded higher scores on scales of strength, head rush and feeling alert than the low nicotine-delivery product (Skoal Bandit) or the placebo mint snuff.

Surveys indicate that a significant number of adolescents and adults use smokeless tobacco products because they are relaxing and calming (Ary *et al*., 1989; Hatsukami & Severson, 1999). Gritz *et al.* (1981) studied the effects of smokeless tobacco in college students; few reported subjective effects — especially relaxation — but many reported stimulation related to the increase in plasma nicotine.

Another indicator of psychoactive effects is the circumstances that engender drug use. For example, cigarette smoking often occurs after meals, when alcohol or coffee is consumed, or in the presence of other smokers. Surveys of adolescent smokeless tobacco users demonstrate that its use is associated with situations of boredom, after meals and during sports (Gritz *et al*., 1981; Ary *et al*., 1989; Hatsukami & Severson, 1999). Although few data are available, one reason that smokeless tobacco products are consumed is to change mood or levels of arousal.

(vi) *Withdrawal signs and symptoms*

When psychoactive drugs are administered chronically, changes occur in brain structure and function that are referred to as neuroadaptation such that the individual functions in a relatively 'normal' state when the drug is present. When the drug is acutely withheld, a withdrawal syndrome occurs that is usually opposite to the acute drug effect. For example, withdrawal from sedatives/hypnotics is characterized by excitation and arousal whereas withdrawal from stimulants causes extended periods of lethargy. The presence of a withdrawal syndrome is neither necessary nor sufficient for a drug to be classified as dependence-producing. However, many studies have documented changes in mood or performance and physiological changes (electroencephalogram, heart rate) when chronic smokers abruptly quit smoking (DHHS, 1988). Similar changes occur when smokeless tobacco users stop using the product.

Hatsukami *et al*. (1999) reported that signs and syndromes of abstinence were regularly observed on discontinuation of smokeless tobacco. After a 24-h period of abstinence, smokeless tobacco users who volunteered for treatment experienced craving (95.7%), impatience (76.1%), irritability (73.8 %), increased eating (66.3%), restlessness (65.4%), anxiety (65%), difficulty in concentrating (60.2%), depressed mood (22.5%) and disrupted sleep (17%). Approximately 69% of the subjects reported four or more of the

symptoms, which is the number of symptoms required for a diagnosis of nicotine withdrawal (American Psychiatric Association, 1994).

In a study of the effects of 24-h abstinence from smokeless tobacco in 20 users, Keenan *et al*. (1989) reported behavioural, physiological and subjective signs typical of tobacco withdrawal. Specifically, heart rate decreased, reaction time on performance task increased, craving for smokeless tobacco increased and self-rated withdrawal symptoms increased.

The withdrawal effects of smokeless tobacco and cigarettes were compared in a prospective study of 16 smokeless tobacco users and 11 smokers. All subjects used tobacco products for 3 days and abstained for 3 days (5 days for tobacco smokers) (Hatsukami *et al*., 1987). Compared with baseline, smokeless tobacco abstinence was associated with significantly decreased heart rate and orthostatic pulse change, increased craving for tobacco, confusion, eating, number of awakenings, and total scores on both self-rated and observer-rated withdrawal checklists. The authors reported that withdrawal symptoms from cigarette smoking were more severe than those after discontinuation of smokeless tobacco.

Gire and Eissenberg (2000) examined the role of non-nicotine factors in smokeless tobacco withdrawal. Smokeless tobacco users administered their own brand of smokeless tobacco, an oral mint snuff (that contained no nicotine) or nothing each hour for 3 h. Subjects reported cravings and a desire to use smokeless tobacco; heart rate was recorded at intervals throughout the experimental session. Both the smokeless tobacco and the mint snuff significantly reduced cravings for smokeless tobacco compared with no use. Heart rate was significantly elevated in the smokeless tobacco users compared with the users of the mint snuff or no use. These data suggest that placebo smokeless tobacco can diminish some but not all signs of acute abstinence from smokeless tobacco. The duration of the effectiveness of the placebo smokeless tobacco to reduce cravings and other subjective signs of abstinence has not been determined. Similar results were observed in studies of nicotine-free cigarettes that reduced the symptoms of overnight tobacco abstinence in cigarette smokers (Robinson *et al*., 2000).

The probability and severity of withdrawal symptoms appears to be directly related to frequency of use; however, a threshold for the product of dependence has not been determined. A study by the CDC and an analysis by the Food and Drug Administration support these conclusions. The CDC study showed that increasing signs of withdrawal and dependence were associated with an increased frequency of use (MMWR, 1994). Similar conclusions relied upon the CDC data and those from other US national surveys (Food and Drug Administration, 1995, 1996).

(vii) *Measurement of tobacco dependence in smokeless tobacco users*

A number of approaches have been developed to assess quantitatively the level of physical dependence on tobacco products. Most research has focused on the quantification of dependence in cigarette smokers but some studies have specifically addressed

the issue in smokeless tobacco users. The level of dependence has been associated with severity of tobacco withdrawal and is predictive of success in attempts at smoking cessation. Levels of dependence are also used clinically to guide prescriptions and therapeutic interventions. Thus an accurate assessment of dependence has practical and theoretical importance (Severson, 2003).

In some studies, the emergence of tobacco withdrawal symptoms and signs have been used to define tobacco dependence in smokeless tobacco users. A systematic analysis of smokeless tobacco withdrawal symptoms was published by Hatsukami *et al*. (1992). Tobacco craving, difficulty in concentration, restlessness, excessive hunger and generalized withdrawal discomfort were identified as symptoms that reliably occurred during abstinence from smokeless tobacco products.

Cotinine is the major metabolite of nicotine and its levels in plasma, saliva or urine are a marker of nicotine ingestion. Some studies have used cotinine levels in smokeless tobacco users to define dependence (Gritz *et al*., 1981; Siegel *et al*., 1992). In these studies, it was observed that daily users of smokeless tobacco had cotinine levels in the same range as those of cigarette smokers, which suggests that similar levels of nicotine were absorbed. A relationship between plasma cotinine levels and tobacco dependence in smokeless tobacco users was described by Hatsukami *et al*. (1992).

A widely used and broadly accepted self-reported questionnaire, the Fagerström test for nicotine dependence (Fagerström, 1978; Heatherton *et al*., 1991), was adopted and verified for use among smokeless tobacco users (Boyle *et al*., 1995; see Table 85). Two groups of 100 and 121 smokeless tobacco users answered questions on their use of smokeless tobacco products and the responses were correlated with cotinine levels. As shown in Table 85, the questions included amount of use, type of product and pattern of use. Analyses of the individual items in each of the questionnaires indicated that there was a low correlation between items (indicating that the items indexed different domains) but a highly significant correlation between each of the items and the total score, which suggests that each of the items was related to the overall measure of dependence. Multiple regression analyses were used to determine which items were most predictive of cotinine levels. In group 1, use within 30 min of waking, experiencing strong cravings after 2 h of tobacco abstinence and the length of time a fresh chew is held in the mouth were identified as being particularly predictive. About 33% of the variability of the cotinine level could be predicted using the scores on only these three independent items. In group 2, the items that were significantly predictive of the cotinine levels were number of tins used per week, frequency of swallowing tobacco juice and time (≤ 30 min or > 30 min) from waking to using a chew. Scores on these three variables could predict about 15% of the variability of cotinine levels.

(viii) *Treatment of smokeless tobacco dependence*

Although numerous studies have assessed behavioural and pharmacological therapy for cigarette smoking cessation (DHHS, 1996; Fiore *et al*., 2000), relatively few studies have considered interventions for cessation of smokeless tobacco use. For example, a

Table 85. Items and scoring of dependence scale for sample 1 (100 subjects)

Questions	Answers	Points	Response (%)	r^a
1. After a normal sleeping period, do you use smokeless tobacco within 30 min of waking?	Yes No	1 0	68 32	0.43 $p < 0.0001$
2. Is it difficult for you not to use smokeless tobacco where its use would be unsuitable or restricted?	Yes No	1 0	53 47	
3. Do you use smokeless tobacco when you are sick or have mouth sores?	Yes No	1 0	63 37	0.21 $p < 0.04$
4. What brand of smokeless tobacco to you use?	L[b] M H	1 2 3	4 40 56	
5. How many days does a tin/can last you?	6–7 days 3–5 days < 3 days	1 2 3	11 21 68	
6. On average, how many minutes do you keep a fresh dip or chew in your mouth?	10–19 20–30 > 30	1 2 3	22 27 51	0.26 $p < 0.009$
7. Do you intentionally swallow tobacco juices?	Never Sometimes Always	0 1 2	33 47 20	0.25 $p < 0.01$
8. Do you keep a dip or chew in your mouth almost all the time?	Yes No	1 0	61 39	0.36 $p < 0.0004$
9. Do you experience strong cravings for a dip/chew when you go for more than 2 h without one?	Yes No	1 0	91 9	0.33 $p < 0.001$
10. On average, how many dips/chews do you take each day?	1–9 10–15 ≥ 16	1 2 3	39 46 15	0.21 $p < 0.04$

Adapted from Boyle *et al*. (1995)

[a] Significant correlations with the log of baseline salivary cotinine

[b] Scoring for question 4 is based on nicotine content. For example: a high (H) brand is Copenhagen, a medium (M) brand is Kodiak and a low (L) brand is Hawken/Skoal Bandits (see Tilashalski *et al*., 1994)

meta-analysis (Ebbert *et al*., 2003) noted only 20 published reports of cessation in smokeless tobacco users that met criteria for experimental rigor, of which 14 were randomized clinical trials: eight studies compared behavioural interventions and six investigated pharmacological interventions. Treatments that have demonstrable efficacy in the cessation of cigarette smoking have been evaluated for cessation of smokeless tobacco use. Specifically nicotine replacement products, bupropion and behavioural interventions show

varying degrees of efficacy to promote cessation of smokeless tobacco use. The overall conclusion of the meta-analyses was that behavioural therapies are effective, bupropion is probably effective and nicotine replacement therapy may be effective. Among behavioural interventions, those that involve an oral examination were consistently the most effective (see Table 86). Further comment on this study and a critical review of its results are provided elsewhere (Severson, 2003). The studies on treatment of smokeless tobacco dependence indicate that people who compulsively use smokeless tobacco regard themselves as dependent and actively seek treatment. Furthermore, as shown in Table 86, treatment is seldom effective. The demand for treatment and the high rates of relapse are characteristic of dependence-producing drugs.

Table 86. Smokeless tobacco cessation rates and relative risks by type of intervention

Channel of intervention Study	No. of subjects	Rates of quitting (%)		Relative risk (95% CI)	Months to assessment
		Treatment	Control		
Dental clinics					
Severson *et al*. (1998)	633	10.21[a]	3.3[a]	3.2 (1.5–7.0)	12
Stevens *et al*. (1995)	518	18.4[a]	12.5[a]	1.6 (1.0–2.6)	12
Sports					
Walsh *et al*. (1999)	365	34.5	15.9	2.8 (1.7–4.6)	12
Group support					
Glover *et al*. (1994)	23	55.5	7.1	12.1 (1.2–123.6)	1–2
Williams *et al*. (1995)	130	14.7	10.6	1.4 (0.5–4.0)	3
Hatsukami *et al*. (1996)	210	30.5	22.9	1.5 (0.8–2.7)	12
Self-help					
Severson *et al*. (2000a)	1069	12.9[a]	9.7[a]	1.4 (0.9–2.0)	12
Severson *et al.* (2000b)	172	24.0[a]	18.4[a]	1.4 (0.7–2.9)	6

Adapted from Severson (2003)
CI, confidence interval
[a] Sustained rates of quitting

(ix) *Conclusions*

As shown in the literature that directly tested the effects of smokeless tobacco products and from the inference of many studies on the effects of cigarette smoking, the conclusion that smokeless tobacco is an addictive and dependence-producing substance is warranted and justified.

(*b*) *Effects on hard and soft oral tissues*

The main categories of snuff- or smokeless tobacco-induced oral soft-tissue lesions are oral squamous-cell carcinoma, verrucous carcinoma, leukoplakia, erythroplakia, snuff dipper's lesion, tobacco and lime user's lesion, verrucous hyperplasia and snuff-induced submucosal deposits. These oral lesions are indicated by the authors' terminology and are discussed in Section 2. The pathological features of mucosal alterations due to the use of smokeless tobacco are described here. It should be noted that oral submucous fibrosis has not been found to be associated with the use of smokeless tobacco.

(i) *Pathology of leukoplakia and snuff dipper's lesions*

The histopathology of oral leukoplakia or snuff-induced lesions was reported by Greer *et al*. (1986) and Daniels *et al*. (1992) in cases from the USA, by Roed-Petersen and Pindborg (1973), Jungell and Malmström (1985) and Andersson *et al*. (1989) in cases from Scandinavia and by Idris *et al*. (1996) in cases from the Sudan.

Common epithelial changes noted were hyperorthokeratosis, hyperparakeratosis, chevron pattern keratinization, pale surface staining, koilocytosis-like changes with vacuolated cells and basal-cell hyperplasia. Dysplasia was uncommon in the Sudanese biopsies studied (Idris *et al*., 1996) and Larsson *et al*. (1991) noted that dysplasia may occur occasionally in snuff dipper's lesions. Although dysplasia was not found in 29 snuff dipper's lesions in moist snuff users in Sweden, increased mitotic rate was found in a large majority (Larsson *et al*., 1991). Kaugars *et al*. (1989) found that women were more liable to have moderate-to-severe epithelial dysplasia than men ($p = 0.02$), but this may be because their lesions were detected a decade or so later or because the women were older. Of all the pathological studies, that of Kaugars *et al*. (1989) recorded the highest prevalence of oral epithelial dysplasia (66.7% mild dysplasia, 5.4% severe dysplasia) but noted that 91% of these biopsies were taken from the site of tobacco quid placement. However, the majority of dysplastic changes were focal in nature. In a later study by the same group, 10 of 45 cases who had smokeless tobacco lesions were diagnosed with dysplasia (four cases were focally mild, three were mild, two were moderate and one was severe) (Kaugars *et al.*, 1992). In the USA, the use of snuff was more frequently associated with the development of oral mucosal lesions than that of chewing tobacco, and snuff appeared to cause a greater variety of epithelial changes than chewing tobacco (Daniels *et al*., 1992). In Sweden, loose snuff users had more increased thickening than sachet snuff users who had less pronounced epithelial changes (Andersson *et al*., 1989, 1994). In a study of biopsies from mucosal lesions in Sweden, Andersson *et al.* (1990) noted that the daily but intermittent use of snuff caused a mixed tissue reaction of injury and repair. Morphological koilocytic alterations noted in the epithelial cells in several studies (26/45 cases, Greer *et al*., 1986; 22/141 cases, Idris *et al*., 1996) suggested to the authors the presence of human papillomavirus (HPV) in smokeless tobacco-induced lesions (Greer *et al.*, 1986; Idris *et al.*, 1996).

(ii) *Changes in cell morphology*

Cellular atypia in buccal smears was more common in heavy users of *toombak* (≥ 11 quids per day) than in cigarette smokers with similar frequency of use (≥ 11 per day) but the authors remarked that the method is unreliable for the diagnosis of precancerous lesions, because cells are taken from the surface while abnormalities mostly occur at the base of the epithelium in the progenitor layers (Ahmed *et al*., 2003).

Ramaesh *et al*. (1999) reported variations in cell and nuclear diameters in Sri Lankan betel quid–tobacco chewers. While the nuclear diameter was increased, the cell diameter was reduced compared with normal buccal cells, to give an increased nucleus:cytoplasm ratio in chewers. In an electron microscopic examination, widening of intercellular spaces was noted in the spinous layer in Finnish snuff dippers (Jungell & Malmström, 1985).

(iii) *Effects on keratins*

Increased expression of keratins K13 and K14 in oral squamous-cell carcinomas of Sudanese snuff dippers has been reported (Ibrahim *et al*., 1998), which indicates dysregulation of keratinocyte maturation; a third of the lesions also expressed K19, a basal keratin, which suggests epithelial de-differentiation. Suprabasal expression of K19 was also reported by Luomanen *et al*. (1997a) in oral biopsies from snuff-affected mucosa of 11 snuff users from Sweden. The increased tenascin expression reported in biopsies of smokeless tobacco users was more manifest than that in smokers (Luomanen *et al*., 1997b). In normal oral mucosa, tenascin was seen to underlie the epithelium as a thin band. In the biopsies, tenascin was distributed as a broad band under the epithelium into the adjacent connective tissue and suggested a marked connective tissue reaction to snuff with an epithelial–mesenchymal interaction that was either inflammatory or preneoplastic in nature.

(iv) *Snuff-induced submucosal deposits*

An amorphous deposit in the lamina propria of the oral mucosa where the snuff is habitually placed was noted in users in Denmark 40 years ago (Pindborg & Poulsen, 1962). Several investigators have subsequently commented on the presence of a similar histological phenomenon that was initially regarded as being amyloid (Lyon *et al*., 1964) but was later thought to be non-amyloid (Archard & Tarpley, 1972; Hirsch *et al*., 1982) or collagen (Axéll *et al*., 1976). Idris *et al*. (1998c), using electron microscopy, later characterized an amorphous, eosinophilic, acellular deposit of varying size with a fibrillar texture at the margins in 25 oral snuff-induced lesions from the Sudan as being collagen.

(v) *Chronic oesophagitis*

Chronic oesophagitis was reported in 273 male *naswar* users from Uzbekistan following endoscopy. An increased risk was found in men who used *naswar* for more than 39 years (odds ratio, 1.6; 95% CI, 1.1–2.3), in men who started using *naswar* before the age of 24 years (odds ratio, 1.5; 95% CI, 1.03–2.1) and in men who used *naswar* 12–20 times/day (odds ratio, 1.5; 95% CI, 1.01–2.1) (Evstifeeva & Zaridze, 1992).

(vi) *Gingival recession/loss of periodontal attachment*

Gingival recessions are more common and are irreversible among users of loose snuff than among users of portion-bag packed snuff (Axéll, 1993). Robertson *et al*. (1990) reported that gingival sites adjacent to mucosal lesions in smokeless tobacco users showed significantly greater recession and loss of periodontal attachment than sites not adjacent to lesions in users or comparable sites in non-users. Recession increased by 0.36 mm within 1 year of use of smokeless tobacco. The odds of having gingival recession were estimated to be nine times higher among students who were smokeless tobacco users than among non-users, but only among those who had concurrent gingivitis (Offenbacher & Weathers, 1985).

Loss of periodontal attachment was measured over 3 years in older adults (Beck *et al*., 1995). Among a series of variables entered into the logistic regression in this study, smokeless tobacco use was highly significant (odds ratio, 3.0; $p = 0.001$) as a predictor of new periodontal lesions.

(vii) *Wear on teeth*

Snuff and smokeless tobacco have been shown to contribute to excessive incisal and occlusal wear (tooth wear) in an adult Swedish population of 220 subjects (Ekfeldt *et al*., 1990). This finding had been reported earlier in smokeless tobacco users in the USA (Christen *et al*., 1979). Silica compounds contained in snuff may contribute to the abrasive effect on teeth (Dahl *et al*., 1989).

(viii) *Dental caries*

The US Third National Health and Nutrition Examination Survey (NHANES III) reported a significantly increased incidence of root dental caries in adult tobacco chewers (odds ratio, 3.21; 95% CI, 2.0–4.98) compared with snuff users or smokers. Decayed dental surfaces matched sites of quid placement, which suggests cariogenicity of the tobacco (Tomar & Winn, 1999).

A higher prevalence of caries was found among adolescents in Göteborg, Sweden, who used smokeless tobacco (Hirsch *et al*, 1991).

(ix) *Effects at application sites: studies in human volunteers*

Inflammatory effects

Several studies have investigated the short-term application of smokeless tobacco in humans (Johnson *et al*., 1998; Payne *et al*., 1998). A group of 19 men (mean age, 25 ± 1.4 years) who were regular snuff users placed moist snuff on a new mucosal site during an experiment. The authors reported erythema, ulceration and white striae where the snuff was placed as early as 2–7 days after application. By 7 days, 56% of subjects displayed white striated lesions (Johnson *et al*., 1998). Significantly increased mucosal concentrations of interleukin (IL)-1 and prostaglandin E_2 (PGE_2), molecules that have immune and inflammatory functions, were also reported at new sites of snuff placement. These

data are similar to those reported earlier on 18 male smokeless tobacco users who exhibited increased gingival inflammation at new placement sites (Poore *et al*., 1995).

Effect of pH and nicotine content

When the pH and nicotine content of the snuff used by 20 regular healthy volunteers were reduced, significantly fewer pronounced clinical and histological changes were noted at the sites of placement (Andersson & Warfvinge, 2003).

Effect on buccal transport of smokeless tobacco substances

Exposure of human buccal mucosa to 1.5–2.5 g smokeless tobacco (in Ringer's solution) altered barrier function and caused dilatation of intercellular spaces of the epithelium, which suggests that smokeless tobacco may facilitate buccal transport of substances at application sites (Tobey *et al*., 1988).

Gingival blood flow was measured in 22 healthy snuff consumers from Norway (Mavropoulos *et al*., 2001). Unilateral application of commercial snuff (500 mg that contained ~1% nicotine) caused a markedly rapid increase in gingival blood flow on the exposed side as well as on the contralateral side.

(*c*) *Cardiovascular system*

(i) *Epidemiological studies of clinical cardiovascular disease outcomes*

Cohort studies

Four cohort studies have investigated deaths from cardiovascular disease and use of smokeless tobacco. Details of these studies are summarized in Table 87.

Bolinder *et al*. (1994) analysed mortality data from a large cohort of Swedish construction workers. Exclusive use of smokeless tobacco was associated with a significantly increased risk for mortality from all cardiovascular diseases in comparison with never use of tobacco (relative risk, 1.4; 95% CI, 1.2–1.6), adjusted for age and region. Relative risks for ischaemic heart disease, stroke and all cardiovascular disease were higher for workers aged 35–54 years at baseline than for workers aged 55–65 years. [This pattern of decreasing relative risk with increasing age is probably attributable to the rapid increase in background rates of cardiovascular disease with increasing age. A similar pattern is seen, for example, with coronary heart disease and active smoking (DHHS, 1983). Another possible factor that could contribute to lower relative risks in the older age group is that the older workers more probably quit tobacco use during follow-up than the younger workers.] No analysis was made for duration of use of smokeless tobacco; however, 87% of the deaths from cardiovascular disease among users of smokeless tobacco were reportedly in subjects who had ≥ 15 years of use at the time they entered the study. [The strengths of this study include the prospective design, the large number of exclusive users of smokeless tobacco in the cohort, the large number of deaths from cardiovascular disease and the availability of information on a number of important risk

Table 87. Cohort studies on use of smokeless tobacco and clinical cardiovascular disease

Reference, location, name of study	Cohort description	Exposure assessment	Cardiovascular disease outcome	Exposure categories	No. of deaths	Relative risk (95% CI)	Adjustment for potential confounders	Comments
Bolinder *et al*. (1994), Sweden, construction workers study	135 036 male construction workers ≤ 65 years old who underwent medical examinations in 1971–74 (75% response rate); 6297 exclusive smokeless tobacco users, 32 546 never users of tobacco; follow-up through to 1985	Self-reported current use assessed at baseline medical examination	Mortality from all cardiovascular diseases	Exclusive smokeless tobacco users versus never users of tobacco *Stratified by age at baseline (years)* 35–54 55–65	220 44 174	1.4 (1.2–1.6) 2.1 (1.5–2.9) 1.1 (1.0–1.4)	Age, region of origin	Further adjustment for body mass index, blood pressure, diabetes, history of heart symptoms and blood pressure medication reportedly did not change relative risks for cardiovascular disease.
			Mortality from ischaemic heart disease	35–54 55–65	35 137	2.0 (1.4–2.9) 1.2 (1.0–1.5)		
			Mortality from stroke	35–54 55–65	4 26	1.9 (0.6–5.7) 1.2 (0.7–1.8)		
Accortt *et al*. (2002), USA, NHANES I Epidemiological Follow-up Study (NHEFS)	6805 subjects aged ≥ 45 years at baseline (1971–75); 414 exclusive ever users of smokeless tobacco, 2986 non-users of tobacco; follow-up until 1992	Self-reported current use assessed at baseline interview for subsample; for the remainder, smokeless tobacco use inferred from 'ever use' in 1982–84 follow-up interview	All cardiovascular mortality	Exclusive ever users of smokeless tobacco versus non-users of tobacco	NR	*Men* 1.0 (0.7–1.5) *Women* 1.2 (0.7–1.9)	Age, race, poverty index ratio	Smokeless tobacco user category included former users; pipe or cigar use were not taken into account in tobacco use definitions.
			Mortality from ischaemic heart disease			*Men* 0.6 (0.3–1.2) *Women* 1.4 (0.8–2.2)	Age, race, poverty index ratio, alcohol, recreational physical exercise, fruit/vegetable intake, systolic blood pressure (ischaemic heart disease also adjusted for serum cholesterol, body mass index)	
			Mortality from stroke			*Men* 0.7 (0.2–2.0) *Women* 1.0 (0.3–2.9)		

Table 87 (contd)

Reference, location, name of study	Cohort description	Exposure assessment	Cardiovascular disease outcome	Exposure categories	No. of deaths	Relative risk (95% CI)	Adjustment for potential confounders	Comments
Henley *et al.* (2005), USA, CPS-I	Men aged ≥ 30 years enrolled in 1959; 7745 exclusive current smokeless tobacco users (median age at enrollment, 62 years); 69 662 never users of tobacco (median age, 53 years); 12-year follow-up (11 871 deaths)	Self-reported current use of smokeless tobacco assessed at baseline	Cardiovascular disease (ICD-7 330–468)	Never use	6378	1.00	Age, race, educational level, body mass index, physical activity, alcoholic beverage consumption, fat consumption, fruit/ vegetable intake, aspirin use	Demographically, people enrolled in CPS-I were more probably higher educated, married, middle class, and white than general US population; for full CPS-I cohort, 6.7% lost to follow-up; death certificate information obtained for 97% of known deaths; analyses of coronary heart disease excluded men with prevalent heart disease or diabetes; analyses of cerebro-vascular disease excluded men with prevalent stroke.
				Current use	1399	1.18 (1.11–1.26)		
			Coronary heart disease (ICD-7 420)	Never use	4035	1.00		
				Current use	809	1.12 (1.03–1.22)		
			Cerebro-vascular disease (ICD-7 330–334)	Never use	1451	1.00		
				Current use	460	1.46 (1.31–1.64)		
Henley *et al.* (2005), USA, CPS-II	Men aged ≥ 30 years enrolled in 1982; 2488 exclusive current smokeless tobacco users (median age at enrollment, 57 years); 839 exclu-sive former smoke-less tobacco users (median age, 62 years); 111 482 never users of tobacco (median age, 56 years); 18-year follow-up (19 588 deaths)	Self-reported current or former use of chewing tobacco or snuff, as well as frequency or duration of current use, assessed at baseline; current users: 74% used chewing tobacco only, 14% used snuff only, 12% used both	Cardiovascular disease (ICD-9 390–459)	Never use	8315	1.00	Age, race, educational level, body mass index, physical activity, alcoholic beverage consumption, fat consumption, fruit/ vegetable intake, aspirin use, employment status and type	Demographically, peopled enrolled in CPS-II were more probably higher educated, married, middle class, and white than general US population; for full CPS-II cohort, 0.2% lost to follow-up; death certificate information obtained for 98.9% of known deaths; analyses of coronary heart disease excluded men with prevalent heart disease or diabetes; analysis of cerebro-vascular disease excluded men with prevalent stroke; no clear dose–response trends observed by frequency or duration of current use.
				Current use	278	1.23 (1.09–1.39)		
				Chew/never snuff	186	1.26 (1.09–1.46)		
				Snuff/never chew	36	1.38 (0.99–1.92)		
				Both	37	1.26 (0.91–1.75)		
				Chew/former snuff	9	0.87 (0.45–1.70)		
				Snuff/former chew	10	0.64 (0.33–1.24)		
				Former use	96	0.92 (0.75–1.13)		
			Coronary heart disease (ICD-9 410–444)	Never use	4920	1.00		
				Current use	172	1.26 (1.08–1.47)		
				Chew/never snuff	111	1.25 (1.03–1.51)		
				Snuff/never chew	24	1.59 (1.06–2.39)		
				Both	23	1.31 (0.87–1.98)		
				Chew/former snuff	6	1.02 (0.45–2.30)		
				Snuff/former chew	8	0.80 (0.37–1.70)		
				Former use	44	0.70 (0.52–0.95)		
			Cerebro-vascular disease (ICD-9 430–438)	Never use	1858	1.00		
				Current use	71	1.40 (1.10–1.79)		
				Chew/never snuff	45	1.38 (1.02–1.86)		
				Snuff/never chew	4	0.62 (0.23–1.67)		
				Both	17	2.57 (1.59–4.17)		
				Chew/former snuff	3	1.24 (0.39–3.91)		
				Snuff/former chew	2	0.68 (0.17–2.75)		
				Former use	29	1.21 (0.83–1.76)		

CI, confidence interval; CPS-I, Cancer Prevention Study; CPS-II, Cancer Prevention Study II; NHANES I, US First National Health and Nutrition Examination Survey; NR, not reported

factors that was collected at baseline. Information on certain other potential confounders, such as dietary factors and family history, was not available; however, some of the main risk factors, such as age, body mass index, diabetes and blood pressure at baseline, were considered in the analyses. Furthermore, this cohort of construction workers was probably relatively homogeneous with respect to some lifestyle factors, and the relative risk analyses were based on internal comparisons. A possible upward bias of the relative risk estimates from the misclassification of smokers as exclusive users of smokeless tobacco is improbable because, as noted by the authors, an increased risk for lung cancer in smokeless tobacco users was not observed. In contrast, misclassification of tobacco users as never users of tobacco would tend to underestimate the relative risks for use of smokeless tobacco. Changes in tobacco use status after baseline would also tend to underestimate relative risks for current use, since people in these age groups would be more likely to quit using tobacco than to start or to change from smokeless tobacco use to smoking. It should be noted that follow-up was through to 1985; thus, it is possible that the results of this study may not reflect the risks of more contemporary Swedish snuff.]

Accortt *et al*. (2002) analysed mortality data from a follow-up study of a national survey in the USA. No significant increases in mortality from all cardiovascular disease, stroke or ischaemic heart disease were observed in exclusive smokeless tobacco users in comparison with non-users of tobacco. [Some of the strengths of this study include the prospective design, a high follow-up rate and the availability of information on a variety of risk factors. A limitation of the study is that current smokeless tobacco use at baseline was known only for a subset of participants and, for the remainder, use of smokeless tobacco was based on 'ever use' reported 10 years later. Thus, participants who were categorized as users of smokeless tobacco would include former users as well as current users, even at baseline; this would tend to underestimate any risk from current use of smokeless tobacco. Similarly, no information on quantity of smokeless tobacco consumed was available. An additional limitation was the inclusion of pipe or cigar smokers in the non-user of tobacco referent group. Since there was probably a greater proportion of participants who smoked pipes or cigars exclusively than of participants who smoked pipes or cigars and used smokeless tobacco but did not smoke cigarettes, this would tend to underestimate any risks from use of smokeless tobacco.]

The two most recent prospective studies of cardiovascular disease and smokeless tobacco use are the mortality analyses of the large US Cancer Prevention Study (CPS-I and CPS-II) cohorts by Henley *et al.* (2005). The CPS-I and CPS-II cohorts were recruited in a similar manner but at different periods of time. Statistically significant increases in mortality from all cardiovascular disease, coronary heart disease and stroke were observed in exclusive current (at baseline) smokeless tobacco users in comparison with never users of tobacco in both cohorts. In the CPS-II cohort, additional information was available on type of smokeless tobacco used, former use, and frequency and duration of current use. Results were not significantly different for use of chewing tobacco versus snuff; however, only 14% of the current users used snuff only, thus the results for use of snuff only are less stable. No clear dose–response trends by frequency or duration of

current use were observed. For stroke, however, 79% of the deaths in current users with information on duration of use were in the > 30 years of use group (hazard ratio, 1.7; 95% CI, 1.3–2.3). Cardiovascular diseases were not associated with former smokeless tobacco use. [The strengths of these studies include their prospective design, the large numbers of exclusive smokeless tobacco users, the large numbers of deaths available for analysis and the availability of information on a large number of potential confounders. A limitation of CPS-I is that the cohort was followed from 1959 through to 1972; therefore, the results might not represent current smokeless tobacco products. In addition, the current smokeless tobacco users in both cohorts differ substantially from the never users of tobacco in terms of education level and blue-collar employment (employment data for CPS-II only) and, although the results were adjusted for these covariates as well as a number of other important potential confounders, it is possible that uncontrolled confounding related to socioeconomic status or other factors could have influenced the results. The authors note, however, that significantly increased risks for mortality from cardiovascular disease were not observed for former smokeless tobacco users and that former users were more similar demographically to current users than to never users. In contrast, CPS-I also shows statistically significantly increased risks for mortality for a number of causes that were not expected to be causally related to smokeless tobacco use, such as non-cancer diseases of the respiratory system and digestive system, which similarly raised concerns that observed increases were the result of some factor(s) other than smokeless tobacco use. Increased risks for other causes of death are not observed in CPS-II. However, increased mortality from cirrhosis was observed for current smokeless tobacco users in CPS-II (hazard ratio, 3.0; 95% CI, 1.6–5.7; based on 11 deaths in current users), which suggests the possibility of residual confounding by alcoholic beverage consumption. In addition, the risk for mortality from lung cancer was significantly increased (hazard ratio, 2.0; 95% CI, 1.2–3.2; based on 18 deaths among current users), which raises the possibility that some smokers may have been included in the smokeless tobacco user group. Such misclassification of tobacco use is hypothetical and is not indicated in the CPS-I results. It is also possible that the association with lung cancer is due to chance (given the small number of cases) or is a true effect of smokeless tobacco (see the monograph on Tobacco-specific nitrosamines). It should also be noted that any misclassification of tobacco users as never users would tend to underestimate any risks for cardiovascular disease. Similarly, any cessation of smokeless tobacco use after the baseline survey would tend to underestimate risks associated with current use.]

Case–control studies

Three case–control studies of fatal and non-fatal cardiovascular disease outcomes have examined smokeless tobacco use. Details of these studies are summarized in Table 88.

Huhtasaari *et al*. (1992) conducted a case–control study of first-time acute cases of myocardial infarction in 35–64-year-old men in northern Sweden. No increased risk for myocardial infarction in nonsmoking snuff users versus current non-users of tobacco was observed. In a multiple logistic regression model that included current smoking habits and

Table 88. Case–control studies on use of smokeless tobacco and clinical cardiovascular disease outcomes

Reference, study, location, period	Cardiovascular disease outcome (ICD code)	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	Odds ratio (95% CI)	Adjustment for potential confounders	Comments
Huhtasaari *et al*. (1992), Northern Sweden MONICA Project, northern Sweden, 1989–91	First-time fatal or non-fatal acute myo-cardial infarction	585 men aged 35–64 years (93% of those identified in the two provinces)	589 of 750 men invited (250 in each 10-year age group; 609 participated; 20 excluded)	Self-reported; regular snuff use defined as at least once daily	Current snuff use, including former smokers, versus non-current users of tobacco, including former smokers and former smokeless tobacco users	0.89 (0.62–1.29)	Age	Only 146 nonsmoking current snuff users, including 70 former smokers; former smokers who did not use snuff had a significantly higher risk for myocardial infarction compared with snuff users who had never smoked (odds ratio, 1.8; 95% CI, 1.04–3.11).
Huhtasaari *et al*. (1999), Northern Sweden MONICA Project, northern Sweden, 1991–93	First-time fatal or non-fatal acute myo-cardial infarction or sudden death (ICD 410–414)	687 men identified in the northern Sweden MONICA register and validated using MONICA criteria; mean age, 55.6 years; 117 cases were fatal	687 men selected from population registers based on date of birth of index case, matched for county; mean age, 55.6 years	Self-reported in interview for live cases and matched controls or by questionnaire for next-of-kin of dead cases and matched controls; regular snuff use defined as at least once daily	Current snuff use, including former smokers; unadjusted comparison with never use of tobacco; adjusted comparisons include former smokers and former smokeless tobacco users as non-users.	*Fatal and non-fatal* 0.96 (0.65–1.41) *Fatal and non-fatal* 0.58 (0.35–0.94) *Fatal only* 1.50 (0.45, 5.03)	Matched on age, county Hypertension, education, marital status, diabetes, high cholesterol, family history	78% response rate; only 149 cases and controls were current smokeless tobacco users, including former smokers.
Asplund *et al*. (2003), nested in Northern Sweden MONICA Project and Vasterbotten Intervention Project, northern Sweden, 1985–2000	First occurrence of stroke (brain infarction or intracerebral haemorrhage; subarachnoid haemorrhage excluded), fatal or non-fatal	276 men identified in a northern Sweden MONICA register; 96% confirmed by CT scan or autopsy; mean age at risk factor survey, 54.8 years; mean age at stroke, 59.2 years; tobacco use data available for 89%; participation in MONICA, ~77%; participation in intervention project, ~60%	551 (2 per case), matched on sex, age (± 2 years), geographical area, year of baseline examination, cohort; mean age at survey, 54.7 years; tobacco use data available for 95%; partici-pation in MONICA, ~77%; participation in intervention project, about ~60%	Tobacco use ascertained at baseline survey within cohort study; regular smokeless tobacco use defined as use at least once daily	Exclusive smokeless tobacco use versus never use of tobacco. Smokeless tobacco use including former smokers versus never use of tobacco. Smokeless tobacco use including former smokers versus non-users including former smokers and former smokeless tobacco users	1.05 (0.37–2.94) 1.16 (0.60–2.22) 0.87 (0.41–1.83)	Taking matching into account Elevated blood pressure, education, marital status, diabetes, serum cholesterol	Collection of data on risk factors was prospective because nested within two cohort studies; average follow-up, 4.5 years; subarachnoid haemorrhage excluded, but this subtype has been associated with smoking; only 42 subjects were exclusive regular smokeless tobacco users; 53 former smokers were smokeless tobacco users at baseline.

CI, confidence interval; CT, computerized tomography; MONICA, WHO Monitoring Trends and Determinants in Cardiovascular Disease Project

the potential confounders age and education, snuff use was not a significant predictor of myocardial infarction. [A limitation of this study is that it did not examine exclusive use of smokeless tobacco. Former smokers were included in the group of non-users of tobacco as well as the group of smokeless tobacco users, despite the finding that non-snuff using former smokers had a significantly higher risk for myocardial infarction than snuff users who never smoked. This 'tobacco use misclassification' would create a downward bias on the risk estimate. A further limitation of the study is the low power to observe an effect of smokeless tobacco use because of the small number of current users of smokeless tobacco among the cases (59, including 33 former smokers) and in total (146, including 70 former smokers).]

Huhtasaari *et al*. (1999) conducted a case–control study of first-time acute myocardial infarction in men aged 25–64 years in northern Sweden. No increased risk for acute myocardial infarction was associated with regular snuff use. When the analysis was restricted to fatal cases, the adjusted odds ratio was 1.50 (95% CI, 0.45–5.03). [A limitation of this study is that it did not examine exclusive use of smokeless tobacco. In contrast, the inclusion of former smokers among the smokeless tobacco users did not yield an increased crude odds ratio for non-fatal or fatal acute myocardial infarction compared with never users of tobacco. However, for the adjusted results, former smokers and former smokeless tobacco users were apparently included in the group of non-users of tobacco and any risk in these former tobacco users would result in a downward bias on the odds ratio for current smokeless tobacco use. A further limitation of the study is the low power to observe an effect of smokeless tobacco use because of the small number of current smokeless tobacco users, including former smokers, among the cases (59) and in total (149). The authors further suggest the possibility of confounding by alcoholic beverage consumption, which is associated with a decreased risk for acute myocardial infarction, if alcoholic beverage consumption in snuff users was different from that of non-users of tobacco.]

Asplund *et al*. (2003) conducted a case–control study of stroke in men, nested within two cohort studies in northern Sweden. No significant increases in risk for stroke were associated with smokeless tobacco use. [A strength of this study was the prospective collection of information on a variety of risk factors for cardiovascular disease. The average time from baseline to a stroke event was 4.5 years, so it is improbable that many risk factors, including tobacco habits, changed greatly in such a short time. A limitation of the study was the low power to observe an effect of smokeless tobacco, because of the small number (42) of exclusive smokeless tobacco users in the study. In contrast, the inclusion of former smokers (an additional 53 smokeless tobacco users) did not yield an increased odds ratio for stroke in the adjusted analysis. However, former smokers and former smokeless tobacco users were apparently included in the group of non-users of tobacco in this analysis which would tend to underestimate the odds ratio for smokeless tobacco use. Another possible limitation is the definition of stroke that was used. It is unclear why cases of subarachnoid haemorrhage were excluded from the study, since subarachnoid haemorrhage has been associated with active smoking and exhibits some of the highest relative risks that relate to stroke and

smoking (DHHS, 2004b), and why cases of intracerebral haemorrhage, for which the association with smoking is less clear, were included.]

(ii) *Epidemiological and experimental studies of subclinical cardiovascular disease outcomes*

Atherosclerosis and thrombosis

Atherosclerosis, which is a thickening and hardening of the arteries, is a major risk factor for cardiovascular disease and plays a key role in the pathogenesis of coronary heart disease, stroke and peripheral artery disease. Smoking is associated with atherosclerosis and has been shown to affect a number of key processes in its development. Two cross-sectional studies investigated atherosclerosis and the use of smokeless tobacco. Both measured intima-media thickness in the carotid artery using ultrasound. Increased thickness of these inner layers of the artery wall is an indication of atherosclerosis.

Bolinder *et al*. (1997a) studied 143 healthy male firefighters, aged 35–60 years, in Sweden. Of these, 28 were exclusive users of smokeless tobacco (daily use for > 6 months; median, 25 years of use), 29 were smokers, 40 never used tobacco and the remainder had changed their tobacco habits at some time. Exclusive users of smokeless tobacco did not differ significantly from those who never used tobacco with regard to age, body mass index, blood pressure at rest, family history of myocardial infarction, alcoholic beverage and coffee consumption, cholesterol, triglycerides, apolipoproteins (Apo) A-1, Apo B or plasma fibrinogen, although all blood chemistry parameters had a tendency towards greater cardiovascular risk in the smokeless tobacco users. None of the measurements of intima-media thickness or lumen diameter differed significantly between smokeless tobacco users and those who never used tobacco. The largest difference was for the maximum carotid bulb thickness, which was 1.01 ± 0.18 mm in smokeless tobacco users and 0.95 ± 0.15 mm in those who never used tobacco, but this was not statistically significant. Atherosclerotic plaques were diagnosed in two smokeless tobacco users (7.1%) and none of those who never used tobacco. In smokers, the maximum carotid bulb thickness was 1.14 ± 0.34 mm ($p < 0.001$) and 11 (37.9%) had plaques. A statistically significant interaction was observed between smoking and serum cholesterol levels for increasing intima-media thickness; a similar pattern was observed for smokeless tobacco users but was not statistically significant. This study suggests that long-term smokeless tobacco use does not have a substantial impact on the progression of atherosclerosis; however, most of the measurements pointed to increased atherosclerosis in smokeless tobacco users compared with those who never used tobacco [and it is possible that a larger study might have found small but statistically significant differences].

Wallenfeldt *et al*. (2001) studied 391 healthy men, all aged 58 years, in Sweden, of whom 96 were current smokers,152 were former smokers, 139 had never used tobacco, 48 were current smokeless tobacco users and 33 were former smokeless tobacco users. Of the current and former smokeless tobacco users, only four had never smoked. The authors concluded that smokeless tobacco use is not associated with intima-media thickness of the carotid or femoral artery. However, most of the results presented were for ‘never snuff

user', 'ex-snuff user' or 'current snuff user' groups in which current and former smokers were included. Thus, in the 'never snuff user' referent group, only 45% of the men had never smoked. [The Working Group noted that this analysis is not useful to make conclusions about smokeless tobacco use and intima-media thickness, especially because these investigators reported significantly increased intima-media thickness in the femoral artery for both current and former smokers and in the carotid artery for current smokers. The groups os smokers also contained smokeless tobacco users; however, the referent group for these comparisons, i.e. 'never smokers', only contained 2/143 current smokeless tobacco users and 2/143 former smokeless tobacco users (< 3% of the total referent group) and more (171) current and former smokers were never smokeless tobacco users than current or former smokeless tobacco users who never smoked (four). Given these limitations, the multiple regression analyses that included both smoking and smokeless tobacco use would be expected to be of limited value to discern an independent effect of smokeless tobacco use.]

Two other cross-sectional studies have investigated the effects of smokeless tobacco use on endothelial function (Granberry *et al.*, 2003; Rohani & Agewall, 2004). Endothelial dysfunction is considered to be an important early event in atherosclerosis, and smoking has been linked to various adverse effects on the endothelium (DHHS, 2004b). Endothelial effects can also promote thrombus formation, and thrombosis is a key element in many cases of myocardial and cerebral infarction. Both studies measured endothelial-dependent flow-mediated dilation (FMD) in the brachial artery. Increases in blood flow were induced in the forearm by applying and then releasing a tourniquet. Normal endothelial cells react to increased blood flow by localized vasorelaxation. This response is reduced when endothelial cells are damaged. Impaired brachial artery FMD correlates with coronary artery endothelial dysfunction. Smoking is thought to affect FMD, at least in part, through the action of reactive oxygen species on nitric oxide, which is a major mediator of endothelial-dependent FMD. Impaired endothelial-dependent FMD is also considered to be a marker for other adverse changes in the endothelium. For example, endothelium-derived nitric oxide also has important anti-inflammatory and anti-thrombotic effects (Landmesser *et al.*, 2004).

Granberry *et al.* (2003) studied 17 healthy adult male volunteers in the USA; seven of the participants had not used tobacco for > 1 year (mean age, 25.6 years), five had used at least two containers of Skoal or Copenhagen smokeless tobacco per week for > 1 year (mean age, 28.8 years) and five had smoked ≥ 10 cigarettes per day for > 1 year (mean age, 21.2 years). Tobacco users were asked to abstain for 8 h before the procedure to reduce any acute effects of tobacco. Endothelial-dependent FMD was significantly impaired in both users of smokeless tobacco (4.1 ± 0.7% dilation) and tobacco smokers (3.9 ± 5.1%) compared with the non-users of tobacco (12.2 ± 5.7%); the magnitude of the effect was similar in the smokeless tobacco users and smokers. This similarity in effect suggests that endothelial dysfunction may be attributable to nicotine, since nicotine levels are similar for users of smokeless tobacco and smokers (Benowitz *et al.*, 1989). The authors cited several studies of transdermal nicotine, nicotine gum or nicotine nasal spray

and endothelial function as support for this hypothesis. Rohani and Agewall (2004) studied the acute effects of smokeless tobacco use on brachial artery FMD in 20 healthy adult users of smokeless tobacco (18 men and two women; mean age, 34 years). FMD decreased significantly ($p = 0.004$) from 3.4 ± 2.0% (baseline) to 2.3 ± 1.3% 35 min after administration of 1 g oral moist snuff, but did not change with an oral placebo.

A number of studies that investigated the effects of smokeless tobacco use also measured serum cholesterol levels. The incorporation of lipids into the arterial intima is an important feature of atherosclerosis and is directly related to high blood levels of low-density lipoprotein (LDL) cholesterol and low levels of high-density lipoprotein (HDL) cholesterol. Smoking is associated with adverse lipid profiles, especially reduced HDL, and the association is thought to be causal (DHHS, 1990, 2004b).

The results from studies on levels of cholesterol and smokeless tobacco are summarized in Table 89. In general, no effect of smokeless tobacco use on cholesterol levels was observed. Most of the studies of smokeless tobacco use that measured serum cholesterol levels did so as part of the collection of data on potentially confounding risk factors and not as an outcome measurement; thus, these data are not adjusted for age, diet, exercise or other potential confounders. An exception is the study of Tucker (1989) on tobacco use and hypercholesterolaemia, which examined serum cholesterol levels in adult male employees who had participated in a health examination programme in the USA. The estimated relative risk for hypercholesterolaemia in smokeless tobacco users was 2.51 (95% CI, 1.47–4.29) compared with current non-users of tobacco, adjusted for age, education, physical fitness and additional tobacco use. Control for body fat had little effect; dietary differences were not taken into account.

Another exception is the study of Ernster *et al*. (1990), who measured total cholesterol and HDL in professional baseball players in the USA. No differences in total cholesterol or HDL (adjusted for age, race, smoking and serum caffeine level) were observed between current, former and non-users of smokeless tobacco. [This study investigated a relatively young and physically fit population; therefore, the results may not be applicable to the general population.] A similar study of the same population by Siegel *et al*. (1992) 1 year later also reported no differences in cholesterol levels between smokeless tobacco users and non-users of tobacco.

Two studies of smokeless tobacco use in countries other than the USA and Sweden also provided measurements of cholesterol levels (Table 90). A study from India of subjects who ate a typical Indian diet measured increased levels of total cholesterol and LDL and significantly increased levels of very low-density lipoproteins (VLDL), together with significantly decreased levels of HDL, in tobacco chewers (Khurana *et al.*, 2000). A study from Turkey reported significant increases in total cholesterol and LDL and significant decreases in HDL with the use of *maras* powder (Güven *et al.*, 2003). [The stronger findings of adverse lipid profiles in both of these studies compared with the results generally seen in the US and Swedish studies could be a result of the different smokeless tobacco products that were consumed; however, they might reflect an increased ability to

Table 89. Measurements of cholesterol levels in epidemiological and experimental studies of smokeless tobacco

Reference, location, study	Description of study population	Comparison groups	Mean results (unless stated)	Comments
Tucker (1989), USA	2840 adult men (mean age, 40.7 years); 93 smokeless tobacco users, including 10 smokers; 2179 non-users of tobacco; 429 smokers of 1–20 cigarettes/day; 139 smokers of > 20 cigarettes/day		**Relative risk (95% CI)** *Hypercholesterolaemia*	Results adjusted for age, education, physical fitness, additional tobacco use; body fat had no effect; not adjusted for dietary factors; hypercholesterolaemia defined as total serum cholesterol ≥ 6.2 mmol/L
		Current non- users of tobacco	1.00	
		Smokeless tobacco users	2.51 (1.47–4.29)	
		Smokers 1–20 cigarettes/day	1.51 (1.14–2.00)	
		Smokers > 20 cigarettes/day	1.98 (1.29–3.03)	
			Serum cholesterol (mmol/L)	
		Current non-users of tobacco	5.29	
		Smokeless tobacco users	5.36	
		Smokers 1–20 cigarettes/day	5.38	
		Smokers > 20 cigarettes/day	5.52	
Ernster *et al.* (1990), USA	1109 professional baseball players (85.6% under 30 years of age); 463 current smokeless tobacco users; 4% of the subjects were current smokers, 9.1% were former smokers; these were included in the comparison groups.		**Cholesterol (mmol/L)** *Total* *HDL*	Adjusted for age, race, smoking, serum caffeine level; young, physically fit population; no association with duration or frequency of use, but inverse relation between levels of serum cotinine and HDL
		Non-user of tobacco	4.50 1.29	
		Former smokeless tobacco user	4.40 1.29	
		Current smokeless tobacco user		
		within a month	4.29 1.37	
		within a week	4.37 1.32	
Eliasson *et al.* (1991), Sweden	21 regular smokeless tobacco users; 18 never users of tobacco; 19 smokers; all healthy males ≤ 30 years old		*Serum cholesterol (mmol/L)* *LDL/HDL ratio*	Unadjusted, but of similar age and body mass index; young, healthy population
		Never users of tobacco	4.39 2.86	
		Smokeless tobacco users	4.45 2.87	
		Smokers	5.28* 3.16	

Table 89 (contd)

Reference, location, study	Description of study population	Comparison groups	Mean results (unless stated)	Comments
Huhtasaari *et al*. (1992), Sweden, case–control study within northern Sweden MONICA project	589 male controls aged 35–64 years; 114 non-smokeless tobacco-using smokers; 87 non-smoking snuff-users, including former smokers; participation rate, 81.2%	Snuff users Smokers	*Cholesterol (mmol/L)* 6.59 6.61	Age-adjusted; levels in non-users not reported
Siegel *et al*. (1992), USA	Follow-up of same professional baseball players as in Ernster *et al*. (1990) 1 year later; 477 current smokeless tobacco users, 584 non-users of tobacco; current smokers, former smokeless tobacco users and infrequent smokeless tobacco users excluded; former smokers included; 75% smokeless tobacco users used mainly oral snuff; remainder used chewing tobacco.	Non-user of tobacco Current smokeless tobacco user (within 1 week) Snuff user Tobacco chewer	**Cholesterol (mmol/L)** *Total* *HDL* 4.42 1.30 4.39 1.31 4.34 1.33 4.39 1.33	Adjusted for age, race, alcohol, caffeine; second set of results compared snuff and chewing tobacco and adjusted also for hours of smokeless tobacco use per day, time since last smokeless tobacco use, years of use; for subjects missing data in the follow-up, values from the earlier study were used; young, fit population; authors noted data on serum cotinine indicated these were relatively light smokeless tobacco users and 50% rarely used smokeless tobacco off-season; did not confirm earlier inverse relation between serum cotinine and HDL levels.

Table 89 (contd)

Reference, location, study	Description of study population	Comparison groups	Mean results (unless stated)			Comments
Allen *et al.* (1995), USA	56 male smokeless tobacco abstainers (mean age, 34 years) receiving nicotine gum or placebo gum in cessation study		**Cholesterol (mmol/L)**			Baseline measurements reflect period of regular smokeless tobacco use; later measurements are taken during period of smokeless tobacco abstention; only successful abstainers were included.
			Total	*LDL*	*HDL*	
		Nicotine gum group				
		Baseline	5.1	3.4	1.1	
		4 weeks	5.4	3.5	1.1	
		8 weeks	5.3	3.4	1.2	
		Placebo gum group				
		Baseline	5.1	3.2	1.2	
		4 weeks	5.2	3.4	1.3	
		8 weeks	5.2	3.4	1.3	
Eliasson *et al*. (1995), Sweden, northern Sweden MONICA study	Random sample of 2000 subjects aged 25–64 years, 250 men and 250 women from each 10-year age group in 1990; participation rate, 79%; smokeless tobacco analyses further restricted to 604 men; 220 never-users of tobacco, 130 former smokers (current non-users), 124 exclusive smokers, 92 snuff users (including former smokers of > 1 year) and 38 snuff and cigarette users		**Cholesterol (mmol/L)**			Unadjusted; mean ages (years): never-users of tobacco, 45.3; smokeless tobacco users, 42.0; former smokers, 49.9; smokers, 46.7; mean duration of smokeless tobacco use, 17 years
			Total	*HDL*		
		Never users of tobacco	6.2	1.28		
		Smokeless tobacco users	6.3	1.36		
		Former smokers	6.6	1.29		
		Smokers	6.2	1.24		

Table 89 (contd)

Reference, location, study	Description of study population	Comparison groups	Mean results (unless stated)	Comments
Bolinder *et al.* (1997a), Sweden	143 healthy male firemen, 35–60 years of age; 28 exclusive smokeless tobacco users (mean age, 44.4 years), 40 never users of tobacco (mean age, 43.1 years), 29 smokers (mean age, 48.0 years)		**Cholesterol (mmol/L)** *Total* *LDL* *HDL*	Age-adjusted
		Never users of tobacco	5.2 3.4 1.4	
		Smokeless tobacco users	5.3 3.5 1.3	
		Smokers	5.8* 3.8* 1.0*	
			Apolipoproteins (g/L) *Apo A-1* *Apo B*	
		Never users of tobacco	1.52 1.24	
		Smokeless tobacco users	1.41 1.34	
		Smokers	1.35* 1.60*	
Wallenfeldt *et al.* (2001), Sweden, Athero-sclerosis and Insulin Resistance study	391 healthy men, all 58 years old; 1728 invited, 69% participated, 818 eligible after exclusions for cardiovascular disease or medications, 391 after screening for different insulin sensitivity levels; 48 current and 33 former snuff-users, only 4 had never smoked; 96 current smokers; 139 never-users of tobacco		**Cholesterol (mmol/L)** *Total* *LDL* *HDL*	Unadjusted; smokers and exclusive smokeless tobacco users were not considered separately, i.e. all the smokeless tobacco comparison groups also contain current and former smokers, including the never users of tobacco referent group, in which only 45% had never smoked.
		Never users of smokeless tobacco	5.98 4.04 1.28	
		Former smokeless tobacco users	6.08 4.12 1.17	
		Current smokeless tobacco users	6.18 4.09 1.28	
			Apolipoproteins (g/L) *Apo A-1* *Apo B*	
		Never users of smokeless tobacco	1.43 1.21	
		Former smokeless tobacco users	1.37 1.24	
		Current smokeless tobacco users	1.43 1.25	

Table 89 (contd)

Reference, location, study	Description of study population	Comparison groups	Mean results (unless stated)	Comments
Accortt *et al*. (2002), USA, NHEFS	505 exclusive ever-smokeless tobacco users (mean age, 54.0 years), 5192 non-users of tobacco (mean age, 47.8 years), 5523 exclusive smokers (mean age, 44.9 years)		*Blood cholesterol (mg/dL)*	Unadjusted; smokeless tobacco users category includes former users; pipe or cigar use not taken into account
		Non-users of tobacco	237.8	
		Ever smokeless tobacco users	228.7	
		Smokers	235.1	
Granberry *et al*. (2003), USA	Healthy male volunteers; 5 regular smokeless tobacco users (mean age, 28.8 years), 7 current non-users of tobacco (mean age, 25.6 years), 5 smokers (mean age, 21.2 years)		*LDL (mg/dL)*	Unadjusted
		Non-users of tobacco	114.8	
		Smokeless tobacco users	99.1	
		Smokers	91.8	

Apo A-1, major protein in HDL; Apo B, main protein in LDL; HDL, high-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol; NHEFS, NHANES I Epidemiologic Follow-up Study
* $p < 0.05$, compared with non-users

Table 90. Measurements of various cardiac parameters and lipid profiles in epidemiological and experimental studies of smokeless tobacco products other than US and Swedish commercial brands

Reference, location	Characteristics of study population	Comparison groups	Mean results		Comments
Nanda & Sharma (1988), India	40 healthy men (mean age, 26 years) non-users of tobacco given *pan* with 200 mg tobacco to chew for 15–20 min; 24 age- and sex-matched controls given *pan* without tobacco to chew	*Baseline* *Pan* (control) *Pan* + tobacco *After chewing* *Pan* (control) *Pan* + tobacco	*SBP/DBP* 115.8/74.5 116.8/74.4 116.5/73.6 132.6*/86.2*	*HR* 72.3 72.1 72.9 84.3*	Unadjusted; similar results in 10 habitual tobacco chewers given *pan* + tobacco (data not shown); similar increases in subgroup of 10 subjects from the *pan* + tobacco group, who served as controls (each subject was given *pan* on two occasions: once without tobacco (self-control) and again *pan* + tobacco); changes in blood pressure and HR lasted 15–30 min after chewing.
Khurana *et al*. (2000), India	30 current tobacco chewers (> 10 years), 30 current smokers (> 10 years), 30 non-users of tobacco, all aged 20–60 years; subjects of normal body weight for height and eating average Indian diet; subjects with diseases and taking medication were not included.		*Cholesterol (mg %)* *Total* *HDL* 168.7 44.4 185.4 37.6* 181.4 39.8* *LDL* *VLDL* 86.7 20.5 99.1 34.1* 94.6 39.4*		Unadjusted; no significant difference in mean age of groups (data not shown); mean duration of smoking and chewing, 21 years and 16 years, respectively
		Non-users Chewers Smokers Non-users Chewers Smokers			

Table 90 (contd)

Reference, location	Characteristics of study population	Comparison groups	Mean results			Comments
Güven *et al*. (2003), Turkey	45 users of *maras* powder (mean age, 45 years), 32 smokers (mean age, 47 years), 30 non-users (mean age, 44 years); average duration of *maras* powder use, 15 years; average duration of smoking, 16 years		*SBP/DBP*	*HR*		Unadjusted; no statistically significant differences in ventricular repolarization parameters, left ventricular dimensions or systolic function parameters; significant differences in diastolic function parameters in both *maras* powder users and smokers: decreased left ventricular early filling velocity, E/A ratios; increased atrial filling velocity, deceleration time, isovolumetric relaxation time
		Non-users	125/70	78		
		Smokeless tobacco users	132/75	82		
		Smokers	130/77	85		
			Cholesterol (mg/dL)			
			Total	*LDL*	*HDL*	
		Non-users	175	100	42	
		Smokeless tobacco users	230*	150*	32*	
		Smokers	235*	155*	30*	
			Fibrinogen (mg/dL)			
		Non-users	330			
		Smokeless tobacco users	350*			
		Smokers	360*			

DBP, diastolic blood pressure (mm Hg); E/A ratios, left ventricular early filling velocity/atrial filling velocity; HDL, high-density lipoprotein cholesterol; HR, heart rate (beats/min); LDL, low-density lipoprotein cholesterol; SBP, systolic blood pressure (mm Hg); VLDL, very low-density lipoprotein cholesterol

* $p < 0.05$

observe effects of smokeless tobacco on cholesterol levels in people with different (probably lower fat, lower cholesterol) diets.]

Similarly, several studies on smokeless tobacco have measured other blood components that are thought to be related to the pathogenesis of atherosclerosis and thrombosis. Results for these components are presented in Tables 90 and 91. Fibrinogen is an acute-phase protein that increases blood coagulability and can be converted to fibrin, an essential component of blood clots. Smoking is strongly associated with increased plasma levels of fibrinogen, which is an independent risk factor for cardiovascular disease (DHHS, 2004b). Tissue plasminogen activator is a fibrinolytic protein, while plasminogen activator inhibitor has prothrombotic activity. Platelets are blood components that play a key role in blood coagulation and plaque formation, and it is well established that smoking increases platelet activation. Thromboxane A2 is an arachidonic acid derivative that is formed by activated platelets; it promotes platelet aggregation and is a vasoconstrictor. Leukocytosis, or an increased number of white blood cells, also increases blood coagulability, and leukocyte activation plays a role in atherogenesis. Smoking is associated with inflammation and increased leukocyte counts, and tobacco smoke induces leukocyte activation *in vitro*. Antioxidant vitamins may protect against smoking-related atherosclerosis, for example, by scavenging the reactive oxygen species thought to be responsible for endothelial dysfunction and by interfering with lipid peroxidation of LDL, which promotes its incorporation into foam cells and atherosclerotic plaques. Smoking is also associated with increased levels of C-reactive protein, a marker of inflammation that is also associated with cardiovascular disease.

Of the three US and Swedish studies that measured fibrinogen levels, one observed an increase of borderline significance in smokeless tobacco users, while the other two reported no increase. A Turkish study reported significantly increased fibrinogen levels in *maras* powder users (Table 90). Two studies reported no changes in tissue plasminogen activator or plasminogen activator inhibitor associated with smokeless tobacco use. Three studies observed no increase in white blood cell count, while one study measured a slight increase in platelet count in smokeless tobacco users that was not statistically significant. The one study that examined thromboxane A2 found no increase in smokeless tobacco users. In addition, one study that measured blood levels of antioxidant vitamins observed non-significant decreases in α- and β-carotene, but no decreases in α-tocopherol or ascorbate in smokeless tobacco users. Another study reported no significant change in levels of C-reactive protein in smokeless tobacco users. Interpretation of these results on blood factors is limited by the fact that many are unadjusted and are based on small numbers of subjects but, overall, they suggest that smokeless tobacco use does not cause significant changes in several known or suspected risk factors for cardiovascular disease related to atherosclerosis and/or hypercoagulability that are known or thought to be associated with smoking.

Table 91. Measurements of various blood components related to cardiovascular disease in epidemiological studies of smokeless tobacco

Reference, location, study	Characteristics of study population	Comparison groups	Mean results (unless stated)	Comments
Ernster *et al*. (1990), USA	1109 professional baseball players (85.6% under 30 years old); 463 current smokeless tobacco users; 4% were current smokers and 9.1% were former smokers, and these were included in the comparison groups.		*White blood cell count ($\times 10^9$/L)*	Adjusted for age, race, smoking, caffeine level; young, physically fit population
		Non-user of tobacco	6.8	
		Former smokeless tobacco user	6.3	
		Current smokeless tobacco user		
		within a month	6.9	
		within a week	6.3	
Eliasson *et al*. (1991), Sweden	21 regular smokeless tobacco users, 18 never users of tobacco, 19 cigarette smokers; all healthy males ≤ 30 years old		*Fibrinogen (g/L)*	Unadjusted, but of similar age and body mass index; young, healthy population; no consistent differences in PAI or tPA levels (data not shown)
		Never users of tobacco	1.78	
		Smokeless tobacco users	2.00 ($p = 0.07$)	
		Smokers	2.12*	
			Platelets ($\times 10^9$/L)	
		Never users of tobacco	246	
		Smokeless tobacco users	266	
		Smokers	264	
			White blood cell count ($\times 10^9$/L)	
		Never users of tobacco	4.7	
		Smokeless tobacco users	5.4	
		Smokers	7.2 ($p < 0.001$)	

Table 91 (contd)

Reference, location, study	Characteristics of study population	Comparison groups	Mean results (unless stated)	Comments
Wennmalm *et al*. (1991), Sweden	756 randomly selected 18–19-year-old men screened for compulsory military service; 577 responded and were not excluded for medication or health reasons.		*Median urinary thromboxane A2 metabolite (pg/mg creatinine)*	Unadjusted, but all men of same age
		Never users of tobacco (n = 344)	127	
		Former users of tobacco (n = 33)	132	
		Snuff only users (n = 127)	126	
		Smokers only (n = 43)	180 ($p < 0.001$)	
		Cigarettes + snuff (n = 30)	187 ($p < 0.001$)	
Siegel *et al*. (1992), USA	Follow-up 1 year later of same professional baseball players as Ernster *et al*. (1990); 477 current smokeless tobacco users, 584 non-users of tobacco; current smokers, former smokeless tobacco users and infrequent smokeless tobacco users excluded; former smokers included; 75% of smokeless tobacco users used mainly oral snuff, remainder used chewing tobacco.		*White blood cell count ($\times 10^9$/L)*	Adjusted for age, race, alcoholic beverages, caffeine; second set of results compared snuff and chewing tobacco and adjusted also for hours of smokeless tobacco use per day, time since last smokeless tobacco use, years of use. For subjects for whom data were missing in the follow-up, values from the earlier study were used; young, fit population; authors noted serum cotinine data indicated these were relatively light smokeless tobacco users and 50% rarely used smokeless tobacco off-season.
		Non-user of tobacco	6.6	
		Current smokeless tobacco user (within a week)	6.2*	
		Snuff user	6.1	
		Chewing tobacco user	6.2	

Table 91 (contd)

Reference, location, study	Characteristics of study population	Comparison groups	Mean results (unless stated)	Comments
Stegmayr *et al*. (1993), Sweden, Northern Sweden MONICA Project	243 40–49-year-old men; data on food intake available for 80.7%; 150 randomly selected for vitamin study; 116 participated; 54 non-users of tobacco (or < 1 cigarette/day), 17 regular smokeless tobacco users, 26 regular smokers		*α-Tocopherol (μmol/L)*	Unadjusted; no significant differences in intake of fruit and vegetables, but tended to be lower in smokers; estimated intake of ascorbic acid lower in smokers but also decreased in smokeless tobacco users; β-carotene lower in both smokers and smokeless tobacco users, but differences not significant; no effect on retinol or γ-tocopherol by smoking or smokeless tobacco use; only 17 smokeless tobacco users
		Non-users of tobacco	26.2	
		Snuff users	26.0	
		Smokers	23.9*	
			Ascorbate (μmol/L)	
		Non-users of tobacco	55.0	
		Snuff users	57.3	
		Smokers	38.3*	
			α-Carotene (μmol/L)	
		Non-users of tobacco	0.069	
		Snuff users	0.053	
		Smokers	0.032*	
			β-Carotene (μmol/L)	
		Non-users of tobacco	0.37	
		Snuff users	0.31	
		Smokers	0.26*	

Table 91 (contd)

Reference, location, study	Characteristics of study population	Comparison groups	Mean results (unless stated)	Comments
Eliasson *et al*. (1995), Sweden, Northern Sweden MONICA study	Random sample of 2000 subjects aged 25–64 years in 1990, 250 men and 250 women from each 10-year age group; participation rate, 79%; smokeless tobacco analyses restricted to the 604 men; 216 never users of tobacco, 129 former smokers, 162 smokers (including 38 who also use snuff), 90 snuff users (including former smokers of > 1 year)		*Fibrinogen (g/L)*	Unadjusted; mean ages (years): never-users of tobacco, 45.3; smokeless tobacco users, 42; former smokers, 50; smokers, 46.7; mean duration of smokeless tobacco use, 17 years; no measurement of smokeless tobacco use was related to fibrinogen, PAI or tPA levels in univariate analyses; similar results in multivariate (including age, body mass index, waist–hip ratio, height, cholesterol, triglycerides and blood pressure) analyses (data not shown).
		Never users of tobacco	3.24	
		Smokeless tobacco users	3.16	
		Former smokers	3.45*	
		Smokers	3.58 ($p < 0.001$)	
			tPA (IU/mL)	
		Never users of tobacco	0.81	
		Smokeless tobacco users	0.90	
		Former smokers	0.76	
		Smokers	0.78	
			PAI (U/mL)	
		Never users of tobacco	5.5	
		Smokeless tobacco users	5.4	
		Former smokers	6.7	
		Smokers	6.4	
Bolinder *et al*. (1997a), Sweden	143 healthy male firemen, 35–60 years of age; 28 exclusive smokeless tobacco users (mean age, 44.4 years), 40 never users of tobacco (mean age, 43.1 years), 29 smokers (mean age, 48.0 years)		*Fibrinogen (g/L)*	Age-adjusted
		Never users of tobacco	2.61	
		Smokeless tobacco users	2.73	
		Smokers	3.20*	

Table 91 (contd)

Reference, location, study	Characteristics of study population	Comparison groups	Mean results (unless stated)	Comments
Wallenfeldt *et al*. (2001), Sweden, Athero-sclerosis and Insulin Resistance study	391 healthy men, all 58 years old; 1728 invited, 69% participated, 818 eligible after exclusions for cardiovascular disease or medi-cations, 391 after screening for different insulin sensitivity levels; of 48 current and 33 former snuff users, only 4 had never smoked; 96 current smokers; 139 never-users of tobacco		*C-Reactive protein (mg/L)*	Unadjusted; smokers and exclusive smokeless tobacco users were not considered separately, i.e. all the smokeless tobacco comparison groups also contained current and former smokers, including the never user of tobacco referent group, in which only 45% had never smoked; significant increases reported in current and former smokers (including smokeless tobacco users)
		Never-user of tobacco	2.47	
		Former smokeless tobacco user	2.27	
		Current smokeless tobacco user	2.64	

PAI, plasminogen activator inhibitor; tPA, tissue plasminogen activator; MONICA: Monitoring of Trends and Determinants in Cardiovascular Disease Project
* $p < 0.05$, compared with non-users

Blood pressure, heart rate and exercise capacity

In addition to promoting atherosclerosis and hypercoagulability, the other major ways in which smoking is thought to increase the risk for cardiovascular disease are by its haemodynamic effects, e.g. by increasing myocardial workload, and by inducing arrhythmias. It is well established that both smoking and smokeless tobacco use cause acute increases in blood pressure and heart rate. These haemodynamic effects are thought to be mediated by nicotine, which causes the release of catecholamines and other neurotransmitters. Effects on hypertension are less clear. Numerous epidemiological studies have found lower blood pressure in smokers than in nonsmokers, while ambulatory studies generally find higher daytime blood pressure levels in smokers. Smoking has also been shown to reduce exercise capacity. Smoking-reduced exercise tolerance is believed to be attributable, at least in part, to carbon monoxide, which reduces oxygen delivery to the myocardium and other tissues. Studies of smokeless tobacco that examined blood pressure, heart rate and exercise capacity are summarized in Tables 90 and 92, except for two studies that were reviewed previously, both of which observed "a significant increase in pulse rate and blood pressure after tobacco chewing" (IARC, 1985).

The large cross-sectional study of Swedish construction workers by Bolinder *et al*. (1992) warrants a detailed discussion. Both smokers and smokeless tobacco users reported a greater prevalence of cardiovascular symptoms. Compared with those who never used tobacco, age-adjusted odds ratios in smokeless tobacco users were 1.4 (95% CI, 1.3–1.6) for breathlessness on slight effort, 1.2 (95% CI, 1.1–1.4) for chest pain walking uphill and 1.3 (95% CI, 1.1–1.5) for pain in the leg while walking, which could be a sign of peripheral vascular disease. In the tobacco groups analysed, 1370 were disability pensioners. Compared with those who never used tobacco, odds ratios in smokeless tobacco users were 1.6 (95% CI, 0.7–3.5) and 1.5 (95% CI, 1.1–1.9) for a cardiovascular diagnosis as the cause of disability pension in the 46–55-year age group and in the 56–65-year age group, respectively, and 3.0 (95% CI, 1.9–4.9) for hypertension as the cause of disability pension in the combined 46–65-year age group. For hypertension as the cause of disability pension, there was no excess risk for smokers.

Of the many studies of blood pressure, heart rate and/or exercise capacity summarized in Table 92, more than half a dozen examined the acute effects of smokeless tobacco use; subjects acted as their own controls. These studies consistently observed increased systolic and diastolic blood pressure and heart rate after consumption of smokeless tobacco. A study from India reported similar results from chewing *pan* plus tobacco (Nanda & Sharma, 1988) (Table 90). Two of the studies also measured stroke volume and reported significantly decreased levels following smokeless tobacco use (Hirsch *et al.*, 1992; van Duser & Raven, 1992).

Approximately 10 studies measured random resting or baseline blood pressure and/or heart rate levels. These generally showed no difference between smokeless tobacco users and non-users of tobacco. A study from Turkey reported similar results for users of *maras* powder (Table 90). The major exception is the large study of Bolinder *et al*. (1992), which reported significantly increased odds ratios for systolic blood pressure > 160 mm Hg,

Table 92. Measurements of blood pressure, heart rate and exercise capacity in epidemiological and experimental studies of smokeless tobacco

Reference, location, study	Description of study population	Comparison groups	Mean blood pressure (SBP/DPB)	Mean heart rate (beats/min)	Comments
Squires *et al*. (1984), USA	20 healthy male college students (mean age, 20 years); refrained from tobacco use for 72 h then used 2.5 g oral snuff for 20 min	Baseline Average over 20 min with oral snuff	118/72 129*/79*	69 89.3*	10 non-users of tobacco, 10 smokeless tobacco users; changes reported similar for both groups (data not shown); similar increases in 10 anaesthetized dogs treated with oral smokeless tobacco
Schroeder & Chen (1985), USA	1663 male and female volunteers, ≥ 18 years old	*Men, 18–25 years* Non-users of tobacco 19 smokeless tobacco users 23 smokers	 131.6/72.8 143.7/80.7 127.7/70.0	NR	Unadjusted; values only reported for 18–25-year-old men, the group with most pronounced results
Ksir *et al*. (1986), USA	5 male college baseball players, 18–22 years old, 160–202 lbs; all current users of 'Copenhagen' moist snuff; bicycle exercise test (4 min each at 3 increasing levels of intensity then 15 min recovery) for each subject on different days with and without smokeless tobacco (abstained during mornings of test)	*Before exercise* Without smokeless tobacco With smokeless tobacco *During exercise* Without smokeless tobacco With smokeless tobacco *After recovery* Without smokeless tobacco With smokeless tobacco	 – DBP: no change SBP: + 4 mm Hg* – DBP: no change SBP: higher* – SBP: NS change	 + 10* – Higher* at levels 1 and 2; + 6 (NS) at level 3 – + 20.9	Effects of exercise and smokeless tobacco use on heart rate appeared additive except at the highest level of exercise

Table 92 (contd)

Reference, location, study	Description of study population	Comparison groups	Mean blood pressure (SBP/DPB)	Mean heart rate (beats/min)	Comments
Bahrke & Baur (1988), USA	112 male soldiers, mean age, 28 years, 58 smokeless tobacco users (mostly chewing tobacco) and 54 non-users of tobacco; both groups of similar mean height and weight; performance on US Army physical fitness test was measured.		*Push-ups*	*Sit-ups*	Unadjusted; statistically significant ($p < 0.05$) correlation coefficients between years of smokeless tobacco use and poorer performance on push-ups, sit-ups and the 2-mile run
		Smokeless tobacco user	55.91	63.16	
		Non-user of tobacco	56.43	63.46	
			2-mile run (min:sec)		
		Smokeless tobacco user	14:04		
		Non-user of tobacco	14:40		
Benowitz *et al*. (1988), USA	10 healthy men, smokers with previous experience of oral snuff and chewing tobacco, aged 24–61 years; experiments on subsequent mornings after overnight abstinence	Average baseline for all tobacco users	121/70	58	The maximal increase in heart rate was similar for all forms of tobacco and significantly less for nicotine gum; responses to smoking were maximal when nicotine levels were maximal; peak responses to smokeless tobacco and gum preceded maximal blood nicotine levels; despite plateau or slight decline in nicotine, responses returned nearly to baseline after 120 min (i.e. tolerance acquired).
			Max. change	*Max. change*	
		9 min of smoking	18.6/12.2	26.0	
		2.5 g oral snuff for 30 min	15.6/11.4	18.2	
		7.9 g chewing tobacco for 30 min	18.6/14.4	19.0	
		2 × 2-mg pieces nicotine gum	16.0/10.4	13.6 ($p < 0.05$ compared with other forms of tobacco)	

Table 92 (contd)

Reference, location, study	Description of study population	Comparison groups	Mean blood pressure (SBP/DPB)	Mean heart rate (beats/min)	Comments
Benowitz *et al*. (1989), USA	8 healthy men, heavy smokers with previous experience of oral snuff and chewing tobacco, aged 27–61 years (mean, 49 years); 3–4 day experimental blocks in which subjects abstained or consumed cigarettes, chewing tobacco or oral snuff as desired		*Average*	*Average*	Heart rate and blood pressure measured every 4 h during the day; rate-pressure products reflect myocardial work and oxygen demand; subjects consumed hospital diet without additional salt; sodium absorption was 26 and 41 mmol/day from oral snuff and chewing tobacco, respectively, and may contribute to elevation of blood pressure.
		Abstinence	116.1/64.7	62.7	
		Smoking	121.4/67.7	69.9*	
		Chewing tobacco	119.2/67.2	70.9*	
		Oral snuff	118.9/66.7	66.7	
			Rate-pressure product (mmHg/min)		
		Abstinence	7285		
		Smoking	8480*		
		Chewing tobacco	8456*		
		Oral snuff	7965		
Ernster *et al*. (1990), USA	1109 professional baseball players (85.6% under 30 years old); 463 current smokeless tobacco users; 4% of the subjects were current smokers and 9.1% were former smokers, and these were included in the comparison groups.	Non-user of tobacco	118/73	67	Adjusted for age, race, smoking, caffeine level; to exclude effects of physical activity, only included subjects who did not come to examination directly after playing; young, physically fit population
		Former smokeless tobacco user	119/73	65	
		Current smokeless tobacco user			
		within a month	114/72	60	
		within a week	116/71	67	
Westman & Guthrie (1990), USA	74 men recruited at 3 county fairs in Kentucky; 27 non-users of tobacco, 25 leaf chewers (≤ 1 pouch/day), 7 chewers (> 1 pouch/day), 15 snuff users	Non-users	124.2/74.0	NR	Unadjusted; mean ages: 27.5, 30.3, 37.3 and 26.1 years, respectively
		≤ 1 pouch/day	122.9/79.3		
		> 1 pouch/day	139.3*/83.1		
		Snuff users	125/74.3		

Table 92 (contd)

Reference, location, study	Description of study population	Comparison groups	Mean blood pressure (SBP/DPB)	Mean heart rate (beats/min)	Comments
Eliasson *et al.* (1991), Sweden	18 never users of tobacco, 21 regular smokeless tobacco users, 19 cigarette smokers; all healthy males ≤ 30 years old		*DBP*		Unadjusted, but of similar age and body mass index; young, healthy population; reportedly no differences in pulse rate or SBP (data not shown)
		Never users of tobacco	72.8		
		Smokeless tobacco users	71.9		
		Smokers	77.5*		
Wennmalm *et al.* (1991), Sweden	756 randomly selected 18–19-year-old men screened for compulsory military service; 577 responded and were not excluded for medication or health reasons; 377 non-users of tobacco, 127 snuff users only, 43 smokers, 30 snuff users and smokers	Non-users of tobacco	122/66.4	NR	Unadjusted, but all men of same age; cardiovascular measurements were part of military examination; maximum heart rate measured at maximum work load
		Snuff users only	122/65.3		
		Smokers only	118*/67.8		
			Max. work load (W)	*Maximum*	
		Non-users of tobacco	300	192	
		Snuff users only	291	188	
		Smokers only	271*	190	
Bolinder *et al.* (1992), Sweden	97 586 male construction workers undergoing health examinations (75% response rate) in 1971–74; 5014 exclusive smokeless tobacco users, 8823 exclusive smokers of ≥ 15 cigarettes/day; 23 885 never users of tobacco	Smokeless tobacco users versus never users of tobacco (age in years):	*Odds ratio (95% CI) for DBP > 90 mm Hg*		Unadjusted; cardiovascular symptoms and disability pensions also higher in smokeless tobacco users (see text); for SBP and DBP, odds ratios < 1 for all age groups for smokers; for heart rate, odds ratios > 1 ($p < 0.05$) for both ≤ and > 45 year age groups for smokers; for DBP, odds ratios > 1 for all but one age-bodymass index stratification subgroups for smokeless tobacco users, but < 1 for all subgroups of smokers
		16–35	1.3 (1.0–1.7)		
		36–45	1.3 (1.0–1.6)		
		46–55	1.8 (1.5–2.1)		
		56–65	1.3 (1.1–1.4)		
			Odds ratio (95% CI) for SBP > 160 mm Hg		
		16–35	1.0 (0.5–1.7)		
		36–45	1.3 (0.8–2.1)		
		46–55	1.7 (1.3–2.1)		
		56–65	1.2 (1.1–1.4)		
				Odds ratio (95% CI) for > 80	
		> 45		1.4 (1.3–1.6)	

Table 92 (contd)

Reference, location, study	Description of study population	Comparison groups	Mean blood pressure (SBP/DPB)	Mean heart rate (beats/min)	Comments
van Duser & Raven (1992), USA	15 male volunteers, 18–31 years old, mean weight 81.8 kg; all habitual smokeless tobacco users; 12 h abstinence; 30 min smokeless tobacco (2.5 g) (or placebo) during seated rest, then removal of smokeless tobacco, 20-min on treadmill at 60% of pre-determined maximal steady state, then increasing incline until maximal exercise reached		Stroke volume (mL/beat) *Before* *After*	*Before* *After*	Effect of smokeless tobacco NS on oxygen uptake at rest, or at 60% or 85% or maximal exercise or on cardiac output at rest, at 60% or 85%; cardiac output (and stroke volume) could not be measured at maximal exercise; smokeless tobacco significantly increased plasma lactate at 60% and 85% levels but increase NS at maximal exercise; authors postulate greater demand for glycolytic energy due to decreased muscle blood flow; authors noted increased heart rate with presumed increased SBP implies increased cardiac work.
		Placebo	84 106	66 64	
		Smokeless tobacco	87 89*	66 82*	
			60% *85%*	*60%* *85%*	
		Placebo	126 127	153 171	
		Smokeless tobacco	119* 119*	164* 177*	
				no significant difference in maximum	
Hirsch *et al*. (1992), Sweden	9 healthy volunteers (8 men), 25–31 years old; 8 were habitual smokeless tobacco users; abstained 9 h before test. Phase 1: subjects rested (supine) 30 min after starting smokeless tobacco (2.5 g), then 3 min exercise test with handgrip, 30 min rest, 3 min cold pressor test, 30 min rest, oral cavity rinse; Phase 2: 1 h later started second smokeless tobacco dose, then workload test with bicycle, 15 min recovery. All experiments were carried out on two different experiment days separated in time by 2–3 weeks; one experimental day involved snuff intake whereas the alternative day served as a control day.	During rest periods of Phase 1: smokeless tobacco day versus control day	SBP and DBP increased after smokeless tobacco intake*; SBP remained elevated during 140 min of Phase 1*, DBP increase of shorter duration; cardiac output higher but NS; stroke volume significantly decreased at 30 and 110 min readings*.	Increased about 25%* 15–30 min after smokeless tobacco intake; increase remained during Phase 1	Handgrip test: blood pressure, but not heart rate higher* after tobacco smokeless at start (sitting) ($p < 0.05$, data not shown); heart rate response to exercise slightly higher after smokeless tobacco; differences in blood pressure tended to disappear. Cold pressor test: increased heart rate with smokeless tobacco remained (data not shown). Plasma epinephrine significantly increased at 30 min at 200 W workload but not at the lower workloads; no significant differences in norepinephrine or neuropeptide-Y. Authors suggest that pressor response, stroke volume and cardiac output response indicate that total peripheral resistance was increased.
		Phase 2: smokeless tobacco day versus control day	DBP, but not SBP, increased*, at low workloads only.	Increased* at low workloads; also higher* after recovery	

Table 92 (contd)

Reference, location, study	Description of study population	Comparison groups	Mean blood pressure (SBP/DPB)	Mean heart rate (beats/min)	Comments
Huhtasaari *et al*. (1992), Sweden, case–control study within Northern Sweden MONICA Project	589 control men aged 35–64 years; 114 smoking non-users of smokeless tobacco; 87 non-smoking snuff users, including former smokers; 177 never used tobacco.	Snuff users Smokers Non-tobacco users	133/86 131/83 NR		Age-adjusted
Siegel *et al*. (1992), USA	Follow-up 1 year later of same professional baseball players as Ernster *et al*. (1990); 477 current smokeless tobacco users, 584 non-users of tobacco; current smokers, former smokeless tobacco users and infrequent smokeless tobacco users excluded; former smokers included; 75% smokeless tobacco users used mainly oral snuff, remainder used chewing tobacco.	Non-user of tobacco Current smokeless tobacco user (within a week) Snuff user Chewing tobacco user	117.1/72.1 117.1/71.5 115.3/71.9 119.3/70.9	65.6 65.4 64.5 65.4	Adjusted for age, race, alcohol, caffeine; second set of results also adjusted for hours of use per day, time since last use, years of use; for subjects for whom data were missing in the follow-up, values from the earlier study were used; young, fit population; authors noted serum cotinine data indicated these were relatively light smokeless tobacco users and 50% rarely used smokeless tobacco off-season. Higher mean serum cotinine levels were associated with higher DBP (p = 0.02).

Table 92 (contd)

Reference, location, study	Description of study population	Comparison groups	Mean blood pressure (SBP/DPB)	Mean heart rate (beats/min)	Comments
Allen *et al*. (1995), USA	56 male smokeless tobacco abstainers (mean age, 34 years) received nicotine gum or placebo gum in cessation study.	*Nicotine gum* Baseline 4 weeks 8 weeks *Placebo gum* Baseline 4 weeks 8 weeks	 128.8/84.1 125.7/83.2 123.9/89.2 126.0/83.9 120.0/82.4 121.9/83.5	 76.5 70.6 66.6 73.8 71.5 73.4	Baseline measurements reflect period of regular smokeless tobacco use; later measurements were taken during period of smokeless tobacco abstention; only successful abstainers were included.
Eliasson *et al*. (1995), Sweden, Northern Sweden MONICA study	Random sample of 2000 subjects aged 25–64 years in 1990, 250 men and 250 women from each 10-year age group; participation rate, 79%; smokeless tobacco analyses further restricted to 604 men; 220 never users of tobacco, 130 former smokers (current non-users), 124 exclusive smokers, 92 snuff users (including former smokers of > 1 year) and 38 snuff users and smokers	Never users of tobacco Smokeless tobacco users Former smokers Smokers	130/82.4 129/82.9 132/84 130/82.1		Unadjusted; mean ages (years): never users of tobacco, 45.3; smokeless tobacco users, 42.0; former smokers, 49.9; smokers, 46.7; mean duration of smokeless tobacco use, 17 years; anti-hypertensive medication used by 12.2% of smokers and 4.5% of smokeless tobacco users and never users of tobacco
Bolinder *et al*. (1997a), Sweden	143 healthy male firefighters, 35–60 years of age; 28 exclusive smokeless tobacco users (mean age, 44.4 years), 40 never users of tobacco (mean age, 43.1 years), 29 smokers (mean age, 48.0 years)	Never users of tobacco Smokeless tobacco users Smokers	121/76 122/77 122/78	57 58 62	Unadjusted

Table 92 (contd)

Reference, location, study	Description of study population	Comparison groups	Mean blood pressure (SBP/DPB)	Mean heart rate (beats/min)	Comments
Bolinder *et al*. (1997b), Sweden	144 healthy male firefighters, 35–60 years of age; response rate, 75%; 48 smokeless tobacco users (median 25 years of use), 65 non-users of tobacco, 31 smokers; mean age about 45 years; underwent bicycle exercise test; 79% of smokeless tobacco users and 77% of smokers had consumed tobacco less than 2 h before the exercise test.	*At rest*			Appears to be the same population as that studied by Bolinder *et al*. (1997a); physically fit population; all results adjusted for age, body mass index, waist/hip ratio, alcohol consumption, physical demands of job, leisure-time exercise
		Non-users	124/79	57	
		Smokeless tobacco users	126/76	54	
		Smokeless tobacco > 2 h before test	116/68	52	
		Smokeless tobacco < 2 h before test	126[a]/75	56	
		Smokers	123/80	61	[a] $p < 0.05$ smokeless tobacco use < 2 h before test versus smokeless tobacco use > 2 h before test
		Exercise	*Max. O_2 uptake (mL/min/kg)*	*Max. workload (W)*	Smokeless tobacco user maximal workloads and maximal oxygen uptake similar to non-users in all age groups, but significantly lower for smokers in all age groups; in smokeless tobacco users, no correlation between maximal workload and amount of smokeless tobacco consumed. Electrocardiogram recordings were normal in 80% of non-users, 73% of smokeless tobacco users and 71% of smokers. Cardiac-ST-segment depressions < 1 mm were observed in 8% of non-users, 15% of smokeless tobacco users and 23% of smokers.
		Non-users	42.4	325	
		Smokeless tobacco users	43.9	320	
		Smokeless tobacco > 2 h before test	45.2	305	
		Smokeless tobacco < 2 h before test	45.1	310	
		Smokers	38.3 ($p < 0.001$)	266 ($p < 0.001$)	
		At 190 W	*SBP*		
		Non-users	184	130	
		Smokeless tobacco users	191	130	
		Smokeless tobacco > 2 h before test	178	126	
		Smokeless tobacco < 2 h before test	194	133	
		Smokers	198*	139*	
		10 min after work			
		Smokeless tobacco > 2 h before test	115/72	77	
		Smokeless tobacco < 2 h before test	124[a]/78[a]	85[a]	

Table 92 (contd)

Reference, location, study	Description of study population	Comparison groups	Mean blood pressure (SBP/DPB)	Mean heart rate (beats/min)	Comments
Bolinder & de Faire (1998), Sweden	135 healthy, normotensive male firefighters, aged 35–60 years; 47 smokeless tobacco users (median 25 years of use), 29 smokers, 59 non-users of tobacco; mean age about 45 years; none used hypertensive medication; underwent 24-h monitoring.	*Non-ambulatory baseline* Non-users Smokeless tobacco users Smokers Non-users Smokeless tobacco users Smokers	 124/78 123/78 119/78 *24 h average* 123/77 127*/79 128*/81	 57 60 58 *24 h average* 62 65* 69*	Appears to be the same population as that studied by Bolinder *et al*. (1997b); some subjects' measurements were of inadequate technical quality, leaving 135; adjusted for age, body mass index, waist–hip ratio, physical fitness level, alcoholic beverage intake; strong correlation between blood cotinine levels and 24-h SBP and DBP values in smokeless tobacco users, but not in smokers In smokeless tobacco users, all daytime 1-h mean heart rate values were significantly higher than in non-users; in smokeless tobacco users ≥ 45 years, all daytime DBP and most daytime SBP 3-h mean values were significantly elevated compared with non-users.
			Day *Night* 126/79 108/66 131*/81 106/67 131*/83* 110/68	*Day* *Night* 63 54 69* 56* 74* 58*	
Fant *et al*. (1999), USA	10 male volunteers, mean age 32 years, who were daily smokeless tobacco users (range of duration of use, 2–26 years), recruited to test effects of four commercial moist snuff brands; abstained 3 h before baseline, then took 2 g snuff for 30 min.	Baseline	124.4/72.9 *Max. increase* SBP 9.4–16.8 DBP 10.3–14.2	72.2 *Max. increase* 4.7–17.9	Unadjusted

Table 92 (contd)

Reference, location, study	Description of study population	Comparison groups	Mean blood pressure (SBP/DPB)	Mean heart rate (beats/min)	Comments
Mavropoulos *et al*. (2001), Norway	22 healthy volunteers (20 men), infrequent tobacco users, age range 19–39 years; ab-stained for at least 8 h before experiment; then 500 mg pouch of Swedish snuff kept in mouth 5 min; then 10 min recovery		*Arterial*		Unadjusted
		Baseline	91.2	61.0	
		Maximum	94.9*	66.4*	
Wallenfeldt *et al*. (2001), Sweden, Atherosclerosis and Insulin Resistance study	391 healthy men, all 58 years old; 1728 invited, 69% participated, 818 eligible after exclusions for cardiovascular disease or medications, 391 after screening for different insulin sensitivity levels; of 48 current and 33 former snuff users, only 4 had never smoked, 96 current smokers, 139 never users of tobacco		*Spearman's r-value for SBP*		Unadjusted; smokers and exclusive smokeless tobacco users were not considered separately, i.e. 'snuff–years' includes smokers and 'cigarette–years' includes smokeless tobacco users.
		Snuff–years	0.08		
		Cigarette–years	0.10*		
Accortt *et al*. (2002), USA, NHEFS	505 exclusive ever users of smokeless tobacco (mean age, 54.0 years), 5192 non-users of tobacco (mean age, 47.8 years), 5523 exclusive smokers (mean age, 44.9 years)		*SBP*		Unadjusted; smokeless tobacco user category includes former users; pipe or cigar use not taken into account
		Non-users of tobacco	142.3		
		Ever users of smokeless tobacco	147.8		
		Smokers	136.6		
Rohani & Agewell (2004), Sweden	20 healthy volunteers, 18 men and 2 women, mean age, 34 years; all smokeless tobacco users; measurements taken before and then 20 and 35 min after administration of 1 g oral snuff	Baseline	109/74	55	SBP, DBP and heart rate unchanged with placebo 'snuff'
		20 min after snuff	111*/78*	59*	
		35 min after snuff	110/76	58*	

CI, confidence interval; DBP, diastolic blood pressure; MONICA, WHO Monitoring of Trends and Determinants in Cardiovascular Disease Project; NHEFS, NHANES I Epidemiologic Followup Study; NR, not reported; NS, not statistically significant; SBP, systolic blood pressure

* $p < 0.05$, compared with non-users or baseline

diastolic blood pressure > 90 mm Hg and heart rate > 80 beats per min in the 45–65-year age groups, but not in the younger age groups. [Many of the studies for which no effects were observed evaluated primarily younger subjects and others did not stratify by age; thus, if there is an interaction with age, most of the studies were not designed to detect it. Alternatively, the results of Bolinder *et al*. (1992) may reflect the use of smokeless tobacco products from 30 years ago or uncontrolled confounding.]

In contrast to most of the data on resting blood pressure and heart rate, but consistent with similar studies of smokers, the two studies that took measurements throughout the day reported higher blood pressure and heart rate associated with smokeless tobacco use than with no tobacco use. Benowitz *et al*. (1989) measured blood pressure and heart rate every 4 h during the day in eight smokers who abstained, smoked or used chewing tobacco or snuff on different days. They observed higher average blood pressure, heart rate and heart rate × systolic blood pressure (cardiac work) associated with each type of tobacco use. The increases in heart rate and heart rate × systolic blood pressure were significant for smoking and use of chewing tobacco. Bolinder and de Faire (1998) monitored 24-h blood pressure and heart rate among non-users of tobacco, smokeless tobacco users and smokers. No significant differences were observed between the groups in the resting baseline measurements; however, 24-h average systolic blood pressure and heart rate were significantly higher in smokeless tobacco users and smokers. In addition, in smokeless tobacco users ≥ 45 years old, all 3-h mean values of daytime diastolic blood pressures and most of those for daytime systolic blood pressure were significantly higher than those of non-users of tobacco.

The four studies generally showed increased systolic blood pressure during exercise with smokeless tobacco use (Ksir *et al.*, 1986; Bahrke & Baur, 1988; Hirsch *et al.*, 1992; Bolinder *et al.*, 1997b). Heart rates were generally increased at the lower workloads with smokeless tobacco use, but the maximum heart rate did not typically differ from that in non-users of tobacco. One study that measured stroke volume found a significant decrease during exercise in smokeless tobacco users (Hirsch *et al.*, 1992). Increased heart rate with increased systolic blood pressure is an indication of increased cardiac work and oxygen demand. Some studies also noted that the pattern of responses observed suggested decreased blood flow to the muscle or increased total peripheral resistance (van Duser & Raven, 1992; Hirsch *et al.*, 1992).

It is apparent from the results of several studies that the effects of smokeless tobacco use on heart rate last longer than those on blood pressure (Hirsch *et al.*, 1992; Rohani & Agewell, 2004). In addition, the results of a few studies that compared users of chewing tobacco with users of snuff suggest that greater effects on blood pressure and heart rate are obtained from the use of chewing tobacco, although this is based on limited data (Benowitz *et al.*, 1989; Westman & Guthrie, 1990; Siegel *et al.*, 1992).

(iii) *Insulin resistance and diabetes as risk factors for cardiovascular disease*

Some additional risk factors for atherosclerotic cardiovascular disease that are associated with smoking are insulin resistance and diabetes. Moreover, smoking aggravates insulin resistance and further increases the risk for cardiovascular disease in people with diabetes (Targher *et al*., 1997; Eliasson, 2003). Smokeless tobacco use and insulin resistance/diabetes is covered in more detail below (Section 4.2.1(*d*)). The information is very limited, but an association is plausible. In addition, some evidence shows that exposure to nicotine may aggravate insulin resistance in people who already have diabetes (Axelsson *et al*., 2001), thus diabetics may be at particular risk for cardiovascular effects from smokeless tobacco use.

(iv) *Evidence from studies of nicotine or nicotine replacement therapy*

Because nicotine is presumably the major cardiovascular toxicant in smokeless tobacco, it may be informative to consider the results of studies of nicotine or nicotine replacement therapy on various cardiovascular outcomes. It must be noted, however, that the pharmacokinetics of nicotine delivery may not be the same for smokeless tobacco use and nicotine replacement therapy; for example, Benowitz *et al*. (1988) measured higher peak levels of blood nicotine from the use of oral snuff and chewing tobacco than from the use of nicotine gum. Therefore, while positive findings in the studies of nicotine replacement therapy would suggest that similar or greater risks from smokeless tobacco might be expected, negative findings in these studies would not necessarily exonerate smokeless tobacco. In addition, smokeless tobacco contains constituents other than nicotine that may contribute to cardiovascular risk.

The epidemiological studies of nicotine replacement therapy show little evidence of increased risk for morbidity or mortality from cardiovascular disease. However, these studies are somewhat limited to be able to draw inferences about smokeless tobacco use. For example, Greenland *et al*. (1998) conducted a meta-analysis of results reported from 34 clinical trials of nicotine patches that involved 5687 patch recipients and 3752 placebo recipients. However, most of these trials excluded subjects who had cardiovascular disease or major risk factors; thus, few cardiovascular disease outcomes were observed and the results might not be relevant to the general population. In addition, for a given cardiovascular disease outcome such as myocardial infarction or stroke, no more than four studies reported results for that outcome. Another issue with nicotine replacement therapy is that it is often for a short period of time for smoking cessation in contrast to smokeless tobacco use which is long-term. One large clinical trial of nicotine gum followed subjects for 5 years, at the end of which, 20% of them were still using the gum (Murray *et al*., 1996). Another clinical trial only studied a 10-week course of nicotine patch use (Joseph *et al*., 1996). Furthermore, these studies compared nicotine replacement therapy with non-nicotine replacement therapy in concomitant smokers and recent former smokers. Thus, the background rates for cardiovascular disease were relatively elevated and an effect of

nicotine, if one exists, would be more difficult to see than with background rates of cardiovascular disease for non-users of tobacco.

In experimental studies, nicotine impairs endothelial-dependent vasodilation in humans and may also have other effects on the endothelium, e.g. altering the release of various mediator substances (Institute of Medicine, 2001; Benowitz, 2003). Studies of the effects of nicotine on other cardiovascular end-points are less numerous. Those that investigated lipoprotein profiles generally showed improved profiles in smokers who switched to nicotine replacement therapy or no differences in subjects who were experimentally exposed to nicotine in comparison with controls, although this was not consistent with some animal models that showed adverse effects on lipid profiles in response to nicotine (Allen *et al.*, 1994; Kilaru *et al*., 2001). Similarly, studies that investigated risk factors for thrombogenesis typically reported no significant differences in platelet responses or fibrinogen levels (e.g. Benowitz *et al*., 2002). One small study suggested that long-term (> 11 months) use of nicotine gum is associated with insulin resistance, which is a risk factor for cardiovascular disease that is also associated with smoking (Eliasson *et al*., 1996). Finally, in addition to effects on blood pressure and heart rate, nicotine has been associated with a variety of cardiac arrhythmias, including ventricular arrhythmias (Benowitz & Gourlay, 1997). Wang *et al*. (2000) reported that nicotine may act directly on the potassium channels in the heart that maintain the hyperpolarization potential of the resting membrane, which may contribute to the ability of nicotine to promote cardiac arrhythmias independent of its role in the induction of catecholamine release.

(v) *Conclusions*

A limited number of epidemiological studies of clinical outcomes of cardiovascular disease, such as myocardial infarction or sudden cardiac death, is available to assess the potential risks for cardiovascular disease from the use of smokeless tobacco. The four cohort and three case–control studies from Sweden and the USA may not reflect the risks entailed by the use of other smokeless tobacco products in other countries. Of the seven studies, three cohort studies reported statistically significantly increased risks for cardiovascular death from smokeless tobacco use, while the other four reported no significantly increased risks for certain cardiovascular disease outcomes; however, most of these studies suffer from critical limitations that undermine their usefulness to address the issue. In the large Swedish cohort study of Bolinder *et al*. (1994), a statistically significant increased risk for mortality from cardiovascular disease was observed in male exclusive users of smokeless tobacco compared with those who never used tobacco (age-adjusted relative risk, 1.4; 95% CI, 1.2–1.6); adjustment for age, region, body mass index, blood pressure, diabetes, history of heart symptoms and medication for blood pressure reportedly did not change the relative risks. There is no apparent upward bias that would explain this observation, although one cannot rule out uncontrolled confounding. In two large US prospective studies based on the CPS-I and CPS-II cohorts (Henley *et al*., 2005), statistically significant increases in mortality from all cardiovascular disease, coronary heart disease and stroke were observed in male exclusive users of smokeless tobacco in

comparison with those who never used tobacco in both cohorts, with hazard ratios ranging from 1.12 to 1.46, adjusted for age, race, level of education, body mass index, physical activity, alcoholic beverage consumption, fat consumption and several other potential confounders. Information on frequency and duration of use was available for CPS-II, but no clear dose–response trends were observed.

The four studies that reported no significant increases for cardiovascular diseases were typically limited by several factors, such as the inclusion of former smokers and small numbers of smokeless tobacco users, which would undermine the ability to observe any effect of smokeless tobacco use. In one study (Huhtasaari *et al*., 1999), when the analysis was restricted to fatal cases, the adjusted odds ratio was 1.50 (95% CI, 0.45–5.03), which is consistent with the increased relative risk estimates for cardiovascular deaths observed by Bolinder *et al*. (1994) and Henley *et al*. (2005), although based on small numbers. It is plausible that an association between smokeless tobacco and cardiovascular disease may be primarily for fatal events because of the ability of nicotine to trigger catecholamine release, which could contribute to arrhythmias (Benowitz & Gourlay, 1997).

In terms of the subclinical effects on cardiovascular disease that have been investigated, increased carotid and femoral arterial intima-media thickness, a sign of atherosclerosis, is the outcome that represents the most advanced progression of chronic disease. In the study of Bolinder *et al*. (1997a), a suggestion of slightly increased atherosclerosis was observed in smokeless tobacco users, but none of the results were statistically significant in this small study (28 exclusive smokeless tobacco users). In the only other study of this end-point (Wallenfeldt *et al*., 2001), the results pertaining to smokeless tobacco use were largely uninformative because all but four of the smokeless tobacco users were also current or former smokers. Both studies observed strong evidence of an increased incidence of atherosclerosis in smokers, however, and, if there is an actual effect of smokeless tobacco on arterial thickening, it would appear to be of relatively small magnitude.

Endothelial dysfunction, which is thought to be an early event in atherogenesis, was found to be significantly increased in both studies of endothelial-dependent flow-mediated dilation in smokeless tobacco users. Impaired endothelial-dependent vasodilation has also been observed in experimental studies of nicotine administration. Correct endothelial function is also important for maintaining the haemostatic balance in the blood, e.g. to prevent hypercoagulation.

Several studies of smokeless tobacco measured cholesterol levels, the results of which generally showed little, if any, association between smokeless tobacco use and altered cholesterol levels, although some of these were not even age-adjusted, which limits their interpretation. An exception is the study of Tucker (1989), which reported a statistically significant increased risk for hypercholesterolaemia in smokeless tobacco users in the USA, after adjustment for several risk factors. It is unclear whether there is another explanation for this finding, although uncontrolled confounding, e.g. by dietary factors, is always a possibility. Other notable exceptions are an Indian study of smokeless tobacco chewers (Khurana *et al.*, 2000) and a Turkish study of *maras* powder users (Güven *et al.*, 2003), both of which reported significantly more adverse lipid profiles associated with smokeless

tobacco use. This could be a reflection of the different smokeless tobacco products or of the effects being more apparent in populations who have different basic diets.

With respect to acute cardiovascular events, smokeless tobacco use can potentially increase risks. It does not appear from the available studies that smokeless tobacco use has much impact on blood factors that increase hypercoagulation, which could result in thrombus formation; however, the well-established haemodynamic effects of smokeless tobacco could contribute to acute events. For example, recurrent episodes of coronary vasoconstriction and increases in blood pressure, which can occur from smokeless tobacco use, may augment haemodynamic stress and cause vulnerable plaques to rupture in people who have underlying atherosclerotic disease, and lead to emboli (Institute of Medicine, 2001). Use of smokeless tobacco has also been shown to increase the myocardial workload. Especially during exercise, this could raise the risk for cardiovascular disease in people who have pre-existing conditions, although, in the absence of carbon monoxide and the thrombogenic features of smoking, the impacts of increased cardiac work are less than those from smoking. In addition, because of the nicotine in smokeless tobacco, users may be at increased risk for sudden death from ventricular arrhythmias. Further studies to compare deaths from cardiovascular disease in long-term users of smokeless tobacco and never users of tobacco, or at least long-term non-users of tobacco, could be valuable to elucidate the risks for cardiovascular disease of smokeless tobacco use; however, such studies would have to be fairly large to be useful, and care would have to be taken to minimize potential confounding.

In summary, the evidence on the risk for cardiovascular disease from smokeless tobacco use is limited, although a small increase in risk is clearly possible. Because of the high background rates of cardiovascular disease, even a small increase in risk could represent a large public health impact in countries that have a high prevalence of smokeless tobacco use.

(*d*) *Diabetes*

Smoking is a risk factor for insulin resistance, which can lead to diabetes, and can aggravate insulin resistance in people with diabetes. Insulin resistance is a condition that is characterized by an inability of the body to use insulin correctly. As a consequence, the pancreas secretes additional insulin in an attempt to maintain normal glucose levels. Hyperinsulinaemia (e.g. high fasting levels of insulin) can be a marker for insulin resistance, the most accurate measurement of which is by the euglycaemic hyperinsulinaemic clamp technique. If normal glucose levels cannot be maintained, impaired glucose tolerance can result and is characterized by blood glucose levels that range between normal and diabetic. Impaired glucose tolerance is determined by an oral glucose tolerance test, which is considered to be a more reliable indicator of glucose intolerance than measurement of the fasting level of glucose. Both impaired fasting glucose and especially impaired glucose tolerance are signs of ‘pre-diabetes’ and are risk factors for the development of both type 2 diabetes and cardiovascular disease. Eventually, the pancreas can lose its ability to secrete enough insulin and result in type 2 diabetes, which is itself a major risk factor

for cardiovascular disease. Several prospective studies have found that smoking is an independent risk factor for diabetes (Will *et al*., 2001).

(i) *Studies of smokeless tobacco use and insulin resistance, glucose intolerance and diabetes*

Four studies investigated smokeless tobacco use and type 2 diabetes or glucose intolerance. Two were prospective studies from the USA (Henley *et al*., 2005), one was a cross-sectional study from Sweden (Persson *et al.*, 2000) and one was a Swedish cross-sectional study with a prospective component (Eliasson *et al.*, 2004); all four are summarized in Table 93. Henley *et al*. (2005) reported results from two prospective studies of the large US CPS-I and CPS-II cohorts that included analyses of mortality from diabetes. In CPS-I, no increased risk for mortality from diabetes was observed in current smokeless tobacco users compared with never users of tobacco. In CPS-II, no significantly increased risks were found for mortality from diabetes for either current or former users. [The strengths of these studies include their prospective design, the large numbers of exclusive smokeless tobacco users and the availability of information on a number of potential confounding factors, including body mass index and physical activity. A major limitation with respect to assessment of diabetes is that these were mortality studies and data on incidence were not available. Furthermore, prevalent cases were excluded at baseline (3.2% of current users and 2.3% of never users in CPS-I; 6% of current and former users and 5% of never users in CPS-II). Thus, the results on mortality were based on a small number of deaths attributed to diabetes on death certificates (20 in current users in CPS-I; eight in current users in CPS-II). It should also be noted that significantly increased risks for mortality from cardiovascular disease in current smokeless tobacco users were observed in both studies (Section 4.2.1(*c*)) and, since diabetes is a risk factor for cardiovascular disease, it could have been a contributing cause of death in some of those cases. An additional limitation is that any cessation of smokeless tobacco use after the baseline survey would tend to underestimate risks associated with current use.]

In the study of Persson *et al*. (2000), impaired glucose tolerance and type 2 diabetes were determined by an oral glucose tolerance test. Insulin resistance was based on homeostasis model assessment, a method to estimate insulin sensitivity from fasting blood sample results. The adjusted odds ratio for prevalence of type 2 diabetes was 2.7 (95% CI, 1.3–5.5) for the use of ≥ 3 boxes snuff per week in current users compared with never use of smokeless tobacco. For smokers, the odds ratio for diabetes was statistically significant only for the group who smoked ≥ 25 cigarettes per day (odds ratio, 2.6; 95% CI, 1.1–5.8). An interaction between smoking and family history of diabetes was apparent; no such interaction was seen for smokeless tobacco use. No significant increases in the prevalence of impaired glucose tolerance was observed in current or former smokeless tobacco users or in current or former smokers. In subjects with impaired glucose tolerance, no increased risk for insulin resistance was associated with smokeless tobacco use; the adjusted odds ratio for impaired insulin secretion was 1.2 (95% CI, 0.5–2.8) for current smokeless tobacco users and 2.2 (95% CI, 1.1–4.4) for former users. Insulin secretion was signifi-

Table 93. Epidemiological studies of diabetes, impaired glucose tolerance and insulin resistance in users of smokeless tobacco

Reference, location, name of study	Description of study population	Exposure assessment	Outcome	Exposure categories	No. of cases/No. of subjects	Relative risk estimate (95% CI)	Adjustment for potential confounders	Comments
Persson *et al.* (2000), Sweden	Cross-sectional; 3128 men, aged 35–56 years, 52% with a family history of diabetes (subjects were originally selected for a study focused on family history of diabetes) in 1992–94; known cases of diabetes excluded; participation rate, 70% among subjects selected based on a prescreening questionnaire; health examination after overnight fasting and abstention from smoking, including OGTT	Self-reported; smokeless tobacco use categories	Prevalence of impaired glucose tolerance	Never user of smokeless tobacco	121/2070	1.0	Age, body mass index, family history of diabetes, leisure physical activity, alcoholic beverage consumption	Smokeless tobacco use categories include current and former smokers; adjustment for smoking reportedly did not change results (not shown).
				Smokeless tobacco user	26/531	0.8 (0.5–1.3)		
				Former smokeless tobacco user	19/400	0.7 (0.4–1.2)		
			Prevalence of diabetes	Never user of smokeless tobacco	34/2070	1.0		
				Smokeless tobacco user	13/531	1.5 (0.8–3.0)		
				Former smokeless tobacco user	5/400	0.8 (0.3–2.0)		
				Smokeless tobacco user		1.0		
				≤ 2 boxes/week	1/246	0.2 (0.0–2.0)		
				≥ 3 boxes/week	12/283	2.7 (1.3–5.5)		
				Never user of tobacco	9/959	1.0		
				Exclusive smokeless tobacco user (current)	4/131	3.9 (1.1–14.3)		
			Prevalence of insulin resistance	Never user of smokeless tobacco	37	1.0		Insulin analyses restricted to subjects with impaired glucose tolerance; insulin resistance: highest tertile, homeostasis model assessment ≥ 161.1
				Current smokeless tobacco user	9	0.9 (0.4–2.0)		
				Former smokeless tobacco user	3	0.4 (0.1–1.3)		
			Prevalence of impaired insulin secretion	Never user of smokeless tobacco	28	1.0		Impaired insulin secretion: lowest tertile, 2-h insulin response ≤ 71.9 mU/L
				Current smokeless tobacco user	8	1.2 (0.5–2.8)		
				Former smokeless tobacco user	12	2.2 (1.1–4.4)		
				Ever smokeless tobacco user				
				≤ 2 boxes/week	13	2.1 (1.1–4.1)		
				≥ 3 boxes/week	7	1.2 (0.5–2.9)		

Table 93 (contd)

Reference, location, name of study	Description of study population	Exposure assessment	Outcome	Exposure categories	No. of cases/No. of subjects	Relative risk estimate (95% CI)	Adjustment for potential confounders	Comments
Eliasson *et al*. (2004), Sweden, Northern Sweden, MONICA study	Cross-sectional with follow-up; 3384 men, aged 25–64 years at 1986 or 1990 surveys or 25–74 years at 1994 or 1999 surveys; average survey response rate, 76%; OGTT results available for 1158 of the subjects without diagnosed diabetes at baseline; follow-up information in 1999 on 1275 men free from diabetes at baseline (from first 3 surveys; response rate, 69%); OGTT for 513 men with normal OGTT at baseline	Self-reported *snus* use at baseline survey and follow-up; 1201 never-users of tobacco, 314 exclusive current smokeless tobacco users, 161 exclusive former smokeless tobacco users	Prevalence of diabetes diagnosis	Never user Smokeless tobacco user Former smokeless tobacco user	29/1201 6/314 5/161	1.0 1.1 (0.4–2.6) 1.5 (0.5–3.9)	Age, waist circumference	Confirmed diagnoses of diabetes for incident cases; in 1990 survey, plasma nicotine and cotinine were measured in small subsample of participants to validate tobacco habit self-reports; further adjustment for alcoholic beverage consumption and leisure time physical activity reportedly did not substantially change results.
			Prevalence of impaired glucose tolerance or diabetes by OGTT	Never user Smokeless tobacco user Former smokeless tobacco user	35/429 5/100 6/59	1.0 1.1 (0.5–2.2) 1.48 (0.6–3.8)	Age, waist circumference	
			Incidence of diabetes diagnosis	Never user Smokeless tobacco user Former smokeless tobacco user	6/585 0/103 1/73	1.0 (0 cases) 1.7 (0.2– 14.8)	Age, follow-up, percentage weight gain	
			Impaired glucose tolerance by OGTT	Never user Smokeless tobacco user Former smokeless tobacco user	32/255 1/40 2/25	1.0 0.2 (0.03–1.8) 0.8 (0.2–3.6)	Age, waist circumference, follow-up	
			Diabetes by OGTT	Never user Smokeless tobacco user Former smokeless tobacco user	6/255 1/40 3/25	1.00 0.9 (0.1–8.0) 4.0 (0.9–18.3)	Age, waist circumference, follow-up	
Henley *et al*. (2005), USA, CPS-I	Prospective study; men aged ≥ 30 years enrolled in 1959; 7745 exclusive smokeless tobacco users (median age at enrolment, 62 years); 69 662 never users of tobacco (median age, 53 years); 12-year follow-up (11 871 deaths)	Self-reported current use of smokeless tobacco assessed at baseline	Diabetes death (ICD-7 code 260)	Never use Current use	97 20	1.0 0.9 (0.5–1.5)	Age, race, educational level, body mass index, physical activity, alcoholic beverage consumption, fat consumption, fruit/vegetable intake, aspirin use	Demographically, people enrolled in CPS-I more liable to be more educated, married, middle class and white than general US population; for full CPS-I cohort, 6.7% lost to follow-up; death certificate information obtained for 97% of known deaths; analyses of diabetes excluded men with prevalent diabetes at baseline.

Table 93 (contd)

Reference, location, name of study	Description of study population	Exposure assessment	Outcome	Exposure categories	No. of cases/No. of subjects	Relative risk estimate (95% CI)	Adjustment for potential confounders	Comments
Henley *et al.* (2005), USA, CPS-II	Prospective study; men aged ≥ 30 years enrolled in 1982; 2488 exclusive smokeless tobacco users (median age at enrolment, 57 years); 839 exclusive former smokeless tobacco users (median age, 62 years); 111 482 ever users of tobacco (median age, 56 years); 18-year follow-up (19 588 deaths)	Self-reported current or former use of chewing tobacco or snuff assessed at baseline; current users: 74% chewing tobacco only, 14% snuff only, 12% both	Diabetes death (ICD-9 code 250)	Never use Current use Former use	250 8 6	1.0 1.1 (0.6–2.3) 2.2 (0.95–4.9)	Age, race, educational level, body mass index, physical activity, alcoholic beverage consumption, fat consumption, fruit/vegetable intake, aspirin use, employment status and type	Demographically, people enrolled in CPS-II more liable to be more educated, married, middle class and white than general US population; for full CPS-II cohort, 0.2% lost to follow-up, death certificate information obtained for 98.9% of known deaths; analyses of diabetes excluded men with prevalent diabetes at baseline.

CI, confidence interval; CPS-I, Cancer Prevention Study I; CPS-II, Cancer Prevention Study II; MONICA, WHO Monitoring of Trends and Determinants in Cardiovascular Diseases project; OGTT, oral glucose tolerance test

cantly impaired in current smokers (odds ratio, 2.4; 95% CI, 1.1–5.2) and the odds ratio for insulin resistance was 1.5 (95% CI, 0.7–3.6) for smokers of ≥ 25 cigarettes per day. No significant associations were found between tobacco use and insulin resistance or impaired insulin secretion in subjects who had normal glucose tolerance (results not shown). [The strengths of this study include oral glucose tolerance test results, insulin resistance estimates and information on important potential confounders for such a large group of subjects. Major limitations of this study are the cross-sectional design and the inclusion of current and former smokers in the smokeless tobacco use categories. With such a design, there is uncertainty about the tobacco use status of the subjects prior to disease onset. At the time of the onset of disease, the men classified as current smokers or snuff users may have started using tobacco at the onset of glucose intolerance and some of those classified as former tobacco users may have been current smokers or snuff users at the time of the onset of glucose intolerance. In addition, the authors noted that their findings on insulin resistance and secretion are surprising. They reported that studies of acute smoking or nicotine administration observed insulin resistance without impaired insulin secretion. The impact of excluding people with known diabetes is unclear. This could create a bias, for example, if tobacco users were more liable to have a more severe, and thus more probably known disease. It is also unclear whether the over-sampling for subjects with a family history of diabetes impacted the results. Although no interaction between smokeless tobacco use and family history was observed, subjects with a strong family history of diabetes may be more susceptible to other risk factors for diabetes.]

Eliasson *et al*. (2004) investigated snuff use and the prevalence and incidence of type 2 diabetes and impaired glucose tolerance in men in northern Sweden. From the baseline information, no significant increases in the odds ratios for prevalent diagnosed diabetes or pathological glucose intolerance (i.e. diabetes or impaired glucose tolerance) were observed for current or former smokeless tobacco users compared with never users of tobacco; nor were dose–response relationships found for smokeless tobacco use (data not shown). For exclusive smokers, the adjusted odds ratio for prevalent diagnosed diabetes was 1.6 (95% CI, 0.9–3.1) for current smokers and 1.9 (95% CI, 1.1–3.2) for former smokers; the adjusted odds ratio for pathological glucose intolerance was 0.9 (95% CI, 0.5–1.9) for current smokers and 1.5 (95% CI, 0.8–2.6) for former smokers. For the 1275 subjects who had follow-up information (average duration of follow-up, 8.5 years), 27 incident cases of diagnosed diabetes occurred, none of which were in consistent exclusive smokeless tobacco users. For consistent exclusive smokers, the adjusted odds ratio for incident diagnosed diabetes was 4.6 (95% CI, 1.4–15.5). Based on the follow-up oral glucose tolerance test results in the 513 subjects with normal levels at baseline, the adjusted odds ratio for diabetes was 0.9 (95% CI, 0.1–8.0) for consistent exclusive users of smokeless tobacco and 4.0 (95% CI, 0.9–18.3) for former smokeless tobacco users. In consistent and former exclusive smokers, the adjusted odds ratios were 0.7 (95% CI, 0.1–5.6) and 1.3 (95% CI, 0.5–3.3), respectively. No increases in impaired glucose tolerance were found in current or former exclusive users of smokeless tobacco or in current or former exclusive smokers at follow-up. [Some of the strengths of this study were the

definitions of tobacco use, which considered exclusive smokeless tobacco use, the availability of information on a number of potential confounders and the availability of follow-up data to examine incident cases. As noted by the authors, cross-sectional studies can be prone to underestimate the risk for diabetes from tobacco use, because once people are diagnosed with diabetes, they will probably be encouraged to quit. In addition, this study used the oral glucose tolerance test to identify undiagnosed cases of diabetes and impaired glucose tolerance. A limitation of the study was the small number of incident cases that occurred during follow-up; additional follow-up time may help to accrue more cases. Furthermore, the number of subjects who used smokeless tobacco and had follow-up oral glucose tolerance test results was small (38 current, 20 former).]

Three other studies on smokeless tobacco, all from Sweden, measured serum insulin and/or blood glucose levels, and are summarized in Table 94. In a cross-sectional study, Eliasson *et al*. (1991) observed increased fasting levels of serum insulin in smokers and smokeless tobacco users; however, in their slightly larger study, Eliasson *et al*. (1995) did not replicate these findings. In a study of the acute effects of smoking or snuff use in a group of healthy smokers, Attvall *et al*. (1993) concluded that smoking, but not snuff use, acutely impairs the action of insulin. They further suggested that the increased levels of growth hormone (an insulin antagonist) observed during smoking could be a reason for the decreased sensitivity to insulin but that the smaller increase observed during snuff use may be inadequate to induce insulin resistance.

Several studies of nicotine replacement therapy or experimental exposure to nicotine suggested that nicotine may be associated with insulin resistance. For example, in a small cross-sectional study of healthy men, Eliasson *et al*. (1996) found that long-term use of nicotine gum was associated with hyperinsulinaemia and insulin resistance. In an experimental exposure study, Axelsson *et al*. (2001) observed that nicotine aggravated insulin resistance in type 2 diabetics but had no effect on insulin sensitivity in the age- and body mass index-matched non-diabetic subjects. Catecholamines and other hormones, the release of which is stimulated by nicotine, can act as antagonists to insulin (Eliasson, 2003).

(ii) *Conclusions*

The data on smokeless tobacco use and insulin resistance, glucose intolerance and diabetes are very limited and the results are inconsistent. Two prospective studies found no increased risks for mortality from diabetes associated with smokeless tobacco use; however, these were mortality studies that were not designed to investigate risks for diabetes and the number of deaths from diabetes was small. One cross-sectional study provided suggestive evidence of an increased prevalence of diabetes in heavy users of smokeless tobacco but not of impaired glucose tolerance or insulin resistance, while another cross-sectional study that included a follow-up component observed no significant increased risks for the prevalence or incidence of impaired glucose tolerance or diabetes. Both of the cross-sectional studies also suffer from critical limitations. Three other studies measured serum insulin and/or blood glucose levels. One of these observed a significant increase in mean serum insulin in smokeless tobacco users, while the other

Table 94. Measurements of serum insulin and blood glucose in epidemiological studies of smokeless tobacco users

Reference, location, study	Characteristics of study population	Comparison groups	Mean results	Comments
Eliasson *et al*. (1991), Sweden	21 regular smokeless tobacco users, 18 never users of tobacco, 19 cigarette smokers; all healthy men ≤ 30 years old; examination after overnight fasting and abstention from tobacco and 24-h abstention from alcoholic beverages		*Blood glucose*	Unadjusted, but of similar age and body mass index; young, healthy population
		Never users of tobacco	4.4 mmol/L	
		Smokeless tobacco users	4.3 mmol/L	
		Smokers	4.4 mmol/L	
			Serum insulin	
		Never users of tobacco	3.6 mU/L	
		Smokeless tobacco users	5.5 mU/L*	
		Smokers	8.6 mU/L*	
Attvall *et al*. (1993), Sweden	7 healthy smokers (4 women, 3 men), aged 24–35 years, no family history of diabetes or hypertension; each underwent 3 studies: (1) smoked 1 cigarette/h for 6 h, (2) 48 h abstinence, then 1 portion-bag of snuff/h for 6 h, (3) 48 h abstinence then abstinence during 6 h experiment; during the 6 h, insulin sensitivity measured by glucose-clamp technique		*Blood glucose during the clamps*	All subjects had moderate alcoholic beverage consumption; free fatty acid levels not significantly different before clamps and decreases were not significantly different during the clamps; rate of glucose appearance (mostly representing liver production) was similar before clamps, and decrease during clamps was similar (data not shown); basal glucose utilization similar before clamps, but increase was significant for abstinence (7.5 mg/kg/min) and smokeless tobacco use (7.7) versus smoking (6.9) for last 3 h of clamps.
		Abstinence	5.0 mmol/L	
		Smokeless tobacco use	4.9 mmol/L	
		Smoking	5.0 mmol/L	
			Fasting insulin before clamps	
		Abstinence	6.8 mU/L	
		Smokeless tobacco use	7.1 mU/L	
		Smoking	6.5 mU/L	
			Insulin AUC during clamps	
		Abstinence	76.1 mU/L/6 h	
		Smokeless tobacco use	79.6 mU/L/6 h	
		Smoking	79.6 mU/L/6 h	
			Mean growth hormone levels during clamps	
		Abstinence	2.4 nmol/L	
		Smokeless tobacco use	5.7 nmol/L*	
		Smoking	7.3 nmol/L*	

Table 94 (contd)

Reference, location, study	Characteristics of study population	Comparison groups	Mean results	Comments
Attvall *et al*. (1993) (contd)			*Mean glucose infusion rate during clamps*	
		Abstinence	6.6 mg/kg/min	
		Smokeless tobacco use	6.6 mg/kg/min	
		Smoking	5.9 mg/kg/min*	
Eliasson *et al*. (1995), Sweden, Northern Sweden MONICA study	Random sample of 2000 subjects aged 25–64 years in 1990, 250 men and 250 women from each 10-year age group; participation rate, 79%; 754 randomly selected for OGTT after overnight fasting; smokeless tobacco analyses restricted to men; of the men with OGTT: 125 never users of tobacco, 73 former smokers, 80 smokers (including snuff users), 42 snuff users (including former smokers of > 1 year)		*Fasting serum insulin (mU/L)*	Unadjusted; fasting plasma glucose and post-load glucose for men did not differ by tobacco use (data not shown).
		Never users of tobacco	6.2	
		Smokeless tobacco users	5.8	
		Smokers	6.1	
		Former smokers	6.5	
			Post-load insulin	
		Never users of tobacco	25.0	
		Smokeless tobacco users	20.6	
		Smokers	20.3	
		Former smokers	24.9	

AUC, area under the curve; MONICA, Monitoring of Trends and Determinants in Cardiovascular Disease Project; OGTT, oral glucose tolerance test
* $p < 0.05$, compared with non-users

two showed no effects. All seven studies were conducted in Sweden or the USA and no information on diabetes, glucose intolerance or insulin resistance in relation to smokeless tobacco use in other countries was available. In addition, several studies of nicotine replacement therapy or experimental exposure to nicotine suggested that nicotine may be associated with insulin resistance, and catecholamines and other hormones, the release of which is stimulated by nicotine, can act as antagonists to insulin. Thus, effects on insulin sensitivity and glucose tolerance and risk for diabetes from smokeless tobacco use are plausible, and diabetic smokeless tobacco users, in particular, may be at increased risk for aggravated insulin resistance.

(*e*) *Other effects*

(i) *Smokeless tobacco and inflammation*

Some constituents of tobacco are known to cause inflammation, DNA damage and cell death. The modulation of inflammatory mediators by smokeless tobacco has been purported to play a role in the development of oral cancer. Gingival recession and white mucosal lesions frequently occur at sites of placement of smokeless tobacco. The etiology of these alterations is presumably related to the effects of tobacco components. PGE2 and IL-1 are inflammatory mediators that are involved in periodontal destruction and keratinocyte proliferation.

(ii) *Effect on enzyme activities*

GST/GSH status

Ambient monitoring was undertaken to assess the extent of exposure to tobacco dust and biological alterations among *bidi* tobacco processing plant workers (Bhisey *et al*., 1999). GSH levels were significantly lower among the worker group who did not have any use of tobacco while GST activity was significantly lower in the lymphocytes of workers who did or did not use tobacco.

In 32 male tobacco chewers, a reduction in the GST activity of lymphocytes was observed although the levels of GSH were similar to those in controls. However, no correlation was observed between GST activity and *GSTM1* null genotype (Mahimkar *et al*., 2001).

Aldehyde dehydrogenase

The activity of aldehyde dehydrogenase (ALDH) in peripheral blood cells was found to be inhibited by 8% in Swedish moist snuff users compared with the value in non-users, although this difference did not reach statistical significance (Helander & Curvall, 1991).

4.2.2 *Experimental systems*

(*a*) *Human studies*

Exposure of keratinocyte cultures established from healthy tissues to low concentrations of smokeless tobacco extract did not affect cell numbers or viability, but significantly increased PGE2 and IL-1 levels (Johnson *et al*., 1996). IL-1 and PGE2 levels were determined by enzyme immunoassay in specimens from soft-tissue biopsies of white mucosal lesions at habitual placement sites, in normal alveolar mucosal tissue at non-placement sites of 18 smokeless tobacco users and in normal alveolar mucosal biopsies from 15 non-users. PGE2 levels were lower in both regions in the smokeless tobacco users compared with non-users of tobacco, but values did not vary significantly between the regions at placement and non-placement sites. Both IL-1α and IL-1β were significantly elevated in smokeless tobacco lesions compared with either non-placement sites in smokeless tobacco users or non-users of tobacco (Johnson *et al*., 1994). Thus, these mediators that are released as a result of smokeless tobacco-induced irritation may play a role in the development of oral mucosal lesions at habitual tobacco placement sites in smokeless tobacco users.

(*b*) *Animal studies*

(i) *Effects on oral mucosa*

Chen (1989) reported the effects of smokeless tobacco following weekly application to the buccal mucosa of rats for 1 year. No cancers were found but most epithelial changes noted were similar to snuff-induced lesions described earlier in humans. The subepithelial hyalinization noted in humans (Section 4.2.1(*b*)) was also found in rat mucosa. An interesting finding was a change of ploidy status; 25% of buccal epithelial cells of tobacco-treated rats were tetraploid and 5% were octaploid, which suggests that the mitotic process could be altered (Chen, 1989) (see also Section 4.4.2(*a*)). These results are of relevance because ploidy status has been reported (Sudbo *et al*., 2001) to be a significant putative marker for dysplasia with potential for malignant transformation.

Summerlin *et al*. (1992) examined the histological effects of smokeless tobacco and alcohol on the pouch mucosa and organs of Syrian hamsters. In the group treated with smokeless tobacco (20 animals) acanthosis (epithelial hyperplasia) was found after 26 weeks but no cancers developed at the test site. Numerous alterations were also found in organs, notably the forestomach, but the findings were not significantly different from those in other treatment groups (alcohol group or alcohol + smokeless tobacco group).

Cyclooxygenase-2 (COX-2), an inducible enzyme that is responsible for prostaglandin (PGE2 and 6-keto-PGF1a) synthesis, plays an important role in inflammatory diseases and carcinogenesis. It is upregulated in human squamous-cell carcinoma cells and primary tumour tissue from head and neck cancers (Chan *et al.*, 1999; Dannenberg *et al.*, 2001). Exposure to aqueous extract of smokeless tobacco (snuff) caused loss of the anti-inflammatory activity of annexin I in the golden Syrian hamster cheek pouch and up-regulation of the pro-inflammatory COX-2 in hamster cheek pouch carcinoma (HCPC-1)

cells (Vishwanatha *et al*., 2003) (see Section 4.2.2(*a*)). The dual effect of these regulatory events could lead the cells down the carcinogenic pathway.

(ii) *Cardiovascular effects*

Squires *et al*. (1984) studied haemodynamic parameters in 10 anaesthetized dogs that had 2.5 g US commercial moistened snuff (1.2% nicotine) placed in the buccal cavity for 20 min. They observed significant increases in heart rate, blood pressure, left ventricular pressure, left ventricular end diastolic pressure and dP/dt (first derivative of left ventricular pressure) and significant decreases in the coronary circumflex, renal and femoral arteries.

Suzuki *et al*. (1996) exposed hamster cheek pouch oral mucosa to an aqueous smokeless tobacco extract (US moist snuff) *in situ* and observed impairment of endothelium-dependent vasodilation elicited by two different agonists, acetylcholine and bradykinin.

(*c*) *Studies* in vitro

(i) *Effects on proliferation, differentiation and apoptosis*

Cell survival and DNA repair capacity

Significant O^6-methylguanine–DNA methyltransferase (MGMT) activities (which catalyse the repair of promutagenic O^6-methylguanine lesions in isolated DNA *in vitro*) were demonstrated in normal, non-tumorous human buccal mucosa, cultured buccal epithelial cells and fibroblasts from buccal tissue specimens. Lower MGMT activity than normal in two transformed buccal epithelial cell lines, SVpgC2a and SqCC/Y1, correlated with decreased MGMT mRNA and lack of functional p53 protein. Exposure of human buccal fibroblasts in culture to various organic or water-based extracts of products related to the use of tobacco and betel quid decreased both cell survival and MGMT activity. Organic extracts of *bidi*-smoke condensate and betel leaf showed higher potency than those of tobacco and snuff. An aqueous snuff extract decreased both parameters (Liu *et al.*, 1997).

Cell growth and differentiation

The effects of snuff extract on epithelial growth and differentiation were studied in HaCaT cells grown *in vitro* (Merne *et al*., 2004). Cultures exposed to snuff did not show any increase in cell proliferation as measured by Ki-67 staining but showed a disturbance in the differentiation process by a decrease of CK 10 and filaggrin expression. Murrah *et al*. (1993), however, demonstrated that smokeless tobacco extracts increase cell proliferation and growth effects of human oral epithelial cells in culture similar to the proliferative effects shown in human oral mucosa in tobacco users (Warnakulasuriya & MacDonald, 1995). Wang *et al*. (2001) reported increased proliferation of cultured human keratinocytes induced by low doses of smokeless tobacco and that of fibroblasts in organotypic culture induced by both low and high doses.

HCPC-1 cells treated with aqueous smokeless tobacco extract have shown significantly increased DNA synthesis as assessed by bromodeoxyuridine (BrdU) incorporation (Rubinstein, 2000).

Apoptosis

A dose-dependent induction of apoptosis mediated by nitric oxide was observed in HCPC-1 cells treated with smokeless tobacco extracts (Mangipudy & Vishwanatha, 1999).

Fox *et al*. (1995) demonstrated that cell death following long-term snuff exposure of human fibrosarcoma (HT-1080) cells *in vitro* is not a result of apoptosis but is related to epithelial–mesenchymal interactions that result in the loss of cell adhesion.

(ii) *Smokeless tobacco and inflammation*

Activation of complement was demonstrated *in vitro* using aqueous extracts of loose-leaf chewing tobacco, dry snuff and moist snuff (Chang *et al*., 1998). This may contribute to local inflammation at sites where snuff is placed and result in gingivitis, periodontitis or focal inflammation of mucosal tissue.

Furie *et al*. (2000) exposed cultured human umbilical vein endothelial cells to extracts of smokeless tobacco (US chewing tobacco, dry snuff and moist snuff) and observed increased production of compounds that promote the recruitment of leukocytes as well as increased migration of neutrophils across the endothelial cell monolayers. These investigators also reported that bacterial lipopolysaccharide in the smokeless tobacco extracts accounts for part, but not all, of the pro-inflammatory effect.

Increased PGE2 secretion was seen when peripheral blood mononuclear cells were cultured with 1% smokeless tobacco extracts (nicotine concentration, 117.5 μg/mL) (Bernzweig *et al*., 1998) relative to control cultures, although gingival mononuclear cells were not further activated. When subjected to 5 or 10% smokeless tobacco extracts (nicotine concentration, 560 or 1118 μg/mL), oral keratinocytes grown from healthy gingival sites were found to produce increased amounts of PGE2 and IL-1β (Johnson *et al*., 1996). The levels of IL-1 were not as high as those of PGE2. PGE2 is a regulator of keratinocyte proliferation and these experimental findings may also indicate host mechanisms for cell injury.

(iii) *Effects on collagen synthesis*

The effects of smokeless tobacco on bone glucose metabolism (oxygen consumption and lactate production) and collagen synthesis ([^{3}H]proline hydroxylation) were tested *in vitro* using cultures of tibiae from chick embryos. The smokeless tobacco extract contained 104–125 μg/mL nicotine. At concentrations found in the saliva of smokeless tobacco users, smokeless tobacco extract stimulated glycolysis and markedly inhibited bone collagen synthesis and mitochondrial activity (Galvin *et al*., 1988). Smokeless tobacco extract also inhibited the hydroxylation of proline and the synthesis of collagenase-digestible protein in isolated osteoblast-like cells (Galvin *et al*., 1991). Prolyl hydroxylase activity of chick embryos was inhibited by smokeless tobacco extract, but not by nicotine or anabasine (Galvin *et al*., 1992). Smokeless tobacco contains an inhibitor of prolyl hydroxylase activity which is present in methanol extracts. This was tested on several collagen-producing cells and tissues other than bone. Results revealed that inhibition of collagen synthesis by smokeless tobacco extract is not specific for bone,

that other collagen-producing cells are directly affected and that recovery is not immediate (Lenz *et al*, 1992). Thus, this phenomenon could contribute to the periodontal disease that is frequently seen in users of smokeless tobacco.

4.3 Reproductive, developmental and hormonal effects

4.3.1 *Humans*

(*a*) *Effects on pregnancy*

The rate of still births in Indian women who chewed tobacco (50–100 g per day) was 50 per 1000 live births (11/220) compared with only 17 per 1000 live births (20/1168) in women who did not chew tobacco. The mean birth weight of the offspring of tobacco chewers was approximately 100–200 g lower than that of offspring of non-chewers. This change was associated with a decrease in the mean gestation period. The sex ratio (male:female) of the offspring was 80:100 in the chewers compared with 108.5:100 in non-chewers (Krishna, 1978). The Council on Scientific Affairs of the American Medical Association reviewed the health effects of smokeless tobacco and, confirming the study of Krishna (1978), concluded that use of smokeless tobacco adversely affects pregnancy outcome [“babies of women who chewed tobacco during their pregnancies weighed an average of 100 to 200 g less at birth than did the babies of nonchewers”] (American Medical Association, 1986). It was noted in another review that this weight reduction at birth was mainly attributable to the proportion of chewers who delivered at 36 weeks or earlier, and that other potential confounders were not considered (Critchley & Unal, 2003).

The mean weight of the placenta from 48 Indian mothers who took tobacco (in 83% of the cases as a mixture of tobacco and lime) was 15% greater than that from 48 controls (Agrawal *et al*., 1983). Re-analysis of the 48 case–control pairs reported previously (Agrawal *et al*., 1983) with the paired *t* test showed an overall 65.4-g increase in the mean weight of placentas from smokeless tobacco users which was significantly different ($p < 0.001$) from those of non-users. The increase was 70 g in consumers of smokeless tobacco for 6 years or more compared with those who used it for a shorter period of time ($p < 0.001$) and in regular compared with intermittent and occasional users ($p < 0.001$), but the increase in placental weight was only significant in women who used tobacco with lime (Krishnamurthy, 1991). The mean weight of newborn babies of 70 Indian tobacco users (the tobacco was either chewed or ingested alone or mixed with betel leaf or with lime) was 14% less than that of the babies of 70 matched controls (Verma *et al*., 1983).

Birth weights, pre-term delivery and pre-eclampsia, pregnancy outcomes that have consistently been shown to be affected by cigarette smoking, were evaluated in Swedish women who used snuff and who delivered singleton, live-born infants without major congenital malformations from 1999 through to 2000. For each snuff user, 10 cigarette smokers and 10 non-users were randomly selected from the Swedish Birth Registry. After exclusions, 789 snuff users, 11 240 smokers and 11 495 non-users remained. Compared

with non-users, adjusted mean birth weight was reduced by 39 g (95% CI, 6–72 g) in snuff users, by 172 g (95% CI, 158–185 g) in light smokers and by 224 g (95% CI, 207–240 g) in moderate-to-heavy smokers. In women who were known to have continued using tobacco in late pregnancy, the adjusted mean birth weight was reduced by 93 g (95% CI, 38–147 g) in 268 snuff users, by 213 g (95% CI, 193–234 g) in 2821 light smokers and by 250 g (95% CI, 225–275g) in 1638 moderate-to-heavy smokers. Snuff use was not associated significantly with newborns that were small for gestational age. The risk for pre-eclampsia was reduced in smokers but increased in snuff users, who had a 60% increased risk (odds ratio, 1.6; 95% CI, 1.1–2.3) which was unchanged after stratification by parity. Both snuff use and cigarette smoking were associated with an approximately twofold increased risk for pre-term delivery. This risk was comparable in both snuff users and smokers. For 752 snuff users (versus 11 152 non-users), the odds ratio for pre-term delivery (after exclusion of women with pre-eclampsia) was 1.8 (95% CI, 1.3–2.5) (England *et al*., 2003).

A population-based cohort study was conducted in Mumbai, India, to determine the effect of using smokeless tobacco during pregnancy on birth weight and gestational age at birth for singleton infants. A total of 1217 women who were 3–7 months pregnant and who planned to deliver in the study area were identified, of whom 1167 (96%) were followed up. Individuals who used *mishri* (as a dentifrice) and/or chewed betel quid with tobacco, *gutka* or *pan masala* at least once daily were considered to be smokeless tobacco users. Smokeless tobacco use was associated with an average reduction of 105 g in birth weight (95% CI, 30–181 g) and a reduction in gestational age of 6.2 days (95% CI, 3.0–9.4 days). The odds ratio for low birth weight was 1.6 (95% CI, 1.1–2.4) adjusted by logistic regression for maternal age, education, socioeconomic status, weight, anaemia, antenatal care and gestational age. The adjusted odds ratio for pre-term delivery (< 37 weeks) was 1.4 (95% CI, 1.0–2.1). The odds ratios increased for delivery before 32 weeks (4.9; 95% CI, 2.1–11.8) and before 28 weeks (8.0; 95% CI, 2.6–27.2) (Gupta & Sreevidya, 2004).

(*b*) *Effects on male fertility*

Semen samples were collected from 626 men, 20–35 years of age, in Ahmedabad, India, who atttended a clinic for idiopathic infertility and had no history of systemic disease, genital tract disorder, varicocele, genital infections or genital surgical operations, hormonal abnormalities or treatments, exposure to radiation or alcoholism or drug abuse. These included 288 non-users of any form of tobacco (mean age, 26.5 years), 119 addicted tobacco chewers (> 10 helpings per day; mean age, 26.2 years) and 219 cigarette smokers (> 10 cigarettes per day; mean age, 26.7 years). Mild or occasional tobacco users and former users were not included in the study. Both tobacco smokers and chewers had a slightly smaller ejaculate volume than non-users and a non-significant decrease in sperm density and total sperm count (Student *t* test, $p > 0.05$). No significant difference was observed among any of the groups for sperm motility or proportion of morphologically normal spermatozoa (Dikshit *et al*., 1987).

Semen samples were obtained over a 1-year period from 165 men, aged 27–44 years, in Calcutta, India. Samples from men with a history of systemic disorders, genital tract infections, operations, varicocele, drug or hormone treatment, exposure to radiation, heavy alcoholic beverage drinking or sperm density below 1 000 000/mL were excluded. The remaining men included 21 never users of tobacco, 29 tobacco (*zarda*) chewers, 40 cigarette smokers and eight who were addicted to multiple forms of tobacco use. The mean age of never users was 36.2 years and that of all users combined was 34.3 years. Semen volume, sperm density, total sperm count and motility were significantly ($p < 0.05$, ANOVA) reduced in tobacco users (all groups combined) and the frequency of abnormal spermatozoa was significantly greater ($p < 0.05$) than in never users. Sperm density and total sperm count were more significantly reduced among tobacco chewers than in all other groups ($p < 0.05$). Sperm density and motility were not significantly lower among cigarette smokers than among never users, but total sperm count was significantly reduced and the frequency of abnormal sperm was higher ($p < 0.01$) in this group than in either tobacco chewers or never users. The frequency of abnormal spermatozoa was related to the level of tobacco consumption and was highest in the group with multiple addictions (Banerjee *et al.*, 1993).

4.3.2 *Experimental systems*

(*a*) *Reproductive toxicity*

Female CD-1 mice received an aqueous extract of standard reference moist snuff tobacco (University of Kentucky Tobacco and Health Research Institute, USA) at dose levels that supplied either 12 or 20 mg/kg bw nicotine at each application. Tobacco extract was administered by gavage thrice daily for 2 weeks before breeding, during breeding and during gestational days 0–16. These doses resulted in plasma nicotine levels of 363 and 481 ng/mL and 9.6 and 28% mortality among the dams, respectively. Controls received distilled water instead of tobacco extract; none of the control dams died during treatment. Surviving mice were killed on gestational day 17. No significant differences in litter sizes or in the ratios of total implantation sites to live fetuses were observed. Placental weights were increased ($p < 0.05$) in mice that had received tobacco extract at the lower dose; at the higher dose, resorptions were increased, fetal weights were reduced and fetal skeletal ossification was decreased; the lower dose had a negligible effect on maternal weight gain and fetal weights. The higher dose resulted in fetal growth retardation ($p < 0.05$), increased embryotoxicity and decreased fetal skeletal ossification ($p < 0.05$) (Paulson *et al.*, 1991).

(*b*) *Prenatal developmental toxicity*

Aqueous extracts of a commercial brand of moist snuff (USA) were administered to pregnant CD-1 mice by subcutaneously implanted minipumps at doses that maintained plasma nicotine levels in the range of 29–44 ng/mL during gestation days 7–14 or, using double the concentration of tobacco extract, in a higher range of 34–75 ng/mL. Dams were

killed on day 17. The main effect on fetuses was reduction of body weight; body weight was significantly lower than that of controls at the higher dose (8.6% reduction, $p < 0.0001$) and was accompanied by an increase in the number of fetal deaths ($p < 0.03$). The lower dose produced an increase in the incidence of haemorrhages and supernumerary ribs in fetal mice and a significant delay ($p < 0.05$) in ossification of the supraoccipital bone, the sacrococcygeal vertebrae and the bones of the feet. Weights of dams were significantly reduced only at the higher dose level (Paulson *et al*., 1988).

Aqueous extracts of a reference standard moist snuff (University of Kentucky Tobacco and Health Research Institute, USA) were administered by gavage to pregnant CD-1 mice thrice daily on days 1–16 of gestation, in weight-adjusted volumes that contained 4, 12 or 20 mg/kg bw nicotine and generated plasma nicotine concentrations of 99, 398 and 623 ng/mL, respectively. Solvent controls received distilled water. Mice were killed on day 17 of gestation. The number of resorptions increased in a dose-related manner and resorptions were more frequent in all treated groups than in solvent controls. The two higher doses caused increasing numbers of maternal deaths (31% at the highest dose). Fetal weights were reduced only in the highest-dose group. Placental weights were not affected. Internal malformations included a significant increase in the incidence of minor heart defects (Paulson *et al*., 1989).

Pregnant CD-1 mice received an aqueous extract of ethanol (1.8 g/kg bw), an aqueous solution of D-glucose of equal caloric value or an aqueous extract of standard moist snuff tobacco (University of Kentucky Tobacco and Health Research Institute, USA) equivalent to 8 mg/kg bw nicotine plus either ethanol or D-glucose by gavage thrice daily on days 6–15 of gestation and were killed on day 17 of gestation. No significant differences were observed in maternal weight gain, litter size or incidence of resorptions, fetal deaths or malformations. Fetal weights were reduced in all treatment groups, with the greatest reduction in the tobacco extract-treated group followed by the ethanol-treated group and finally the combined tobacco extract and ethanol-treated group. Placentas of the tobacco extract-treated group weighed significantly less than those of controls. Ossification of the fetal skeleton was affected to the greatest extent in the tobacco extract-treated group, followed by the ethanol-treated and combined tobacco extract and ethanol-treated groups. Craniofacial measurements were significantly affected in all three treatment groups. No interactive effect of ethanol and tobacco extract was observed on fetal growth or development (Paulson *et al*., 1992).

Pregnant Sprague-Dawley rats received aqueous extracts of reference standard moist snuff (Code 1S3, University of Kentucky Tobacco and Health Research Institute, USA) by gavage thrice daily during days 6–18 of gestation at doses that provided 1.33 or 6 mg/kg bw nicotine and generated mean plasma nicotine levels of 283 and 846 ng/mL, respectively. Controls received distilled water. Rats were killed on gestational day 19. Weight gain of dams was reduced in both treatment groups in comparison with controls ($p < 0.05$) but fetal weights were reduced only at the higher dose. Placental weights, litter size, resorptions, deaths and malformations were not significantly affected by treatment

with tobacco extract. Decreased levels of ossification in the fetal skeletons were seen at the higher-dose level ($p < 0.05$) (Paulson *et al*., 1994a).

(*c*) *Postnatal developmental toxicity*

Lactating random-bred Swiss albino mice received 100 μL freshly prepared aqueous extract of smokeless tobacco powder (Vadakkan variety, Mysore, India) by gavage daily on days 1–21 after delivery. Litters were normalized to five pups by redistribution before treatments began. Dams were distributed to groups that received extracts of 50 or 100 mg/kg bw tobacco per day, alone or together with 1% *tert*-butylated hydroxyanisole (BHA) in the diet or phytic acid (1000 mg/kg bw per day) by gavage throughout lactation. Dams and pups were then killed and livers processed for enzyme and thiol measurements. Hepatic GST levels and thiol content were depressed in dams and pups by the higher dose of tobacco extract, but the increased levels of GST and thiol content induced by BHA or phytic acid alone were only slightly reduced in dams and pups that received combined treatment with one of these substances plus tobacco extract. Hepatic cytochrome b_5 and CYP levels were increased in dams at both doses of smokeless tobacco extract and in pups at the higher-dose level only. Combined exposures to tobacco extracts and either BHA or phytic acid resulted in increased cytochrome b_5 and CYP levels that were comparable with or higher than those in dams or pups given BHA or phytic acid alone (Singh & Singh, 1998).

Pregnant Sprague-Dawley rats received aqueous extracts of reference standard moist snuff (Code 1S3, University of Kentucky Tobacco and Health Research Institute, USA) by gavage thrice daily on days 6–20 of gestation at doses that provided 1.33 or 4 mg/kg bw nicotine. Controls received distilled water. Immediately after parturition, offspring were fostered to control mothers and litters were culled to 4 ± 1 male and 4 ± 1 female offspring. Preweaning behavioural tests, including surface righting, swimming development, negative geotaxis and open-field activity, were conducted during postnatal days 1–21 and post-weaning tests, including open-field activity, active avoidance shuttle box and Cincinnati water maze, were conducted during postnatal days 22–67. Maternal weight gain and mean pup weights at birth were lower at the higher-dose level. No significant treatment-related differences were observed in postnatal development of physical landmarks, activity levels or learning development (Paulson *et al*., 1993).

Pregnant Sprague-Dawley rats received aqueous extracts of reference standard moist snuff (Code 1S3, University of Kentucky Tobacco and Health Research Institute, USA) by gavage thrice daily on days 6–20 of gestation at doses that provided 1.33, 4 or 6 mg/kg bw nicotine. Controls received distilled water. Litters were culled to 4 ± 1 male and 4 ± 1 female offspring and raised by their natural mothers. Weights, physical landmark development and behavioural performance of pups were monitored during pre- and post-weaning periods. Behavioural tests included surface righting, negative geotaxis, swimming development, open-field activity and active avoidance in a shuttle box. The two higher doses caused reduced maternal weight gain and significant reductions in pup weight that persisted during the early postnatal period, and infant mortality increased with increasing

exposure to tobacco extract in a dose-dependent manner. A decreased success rate for surface righting was noted for pups exposed to tobacco extract. Variable results were obtained with other measures of behavioural development: pups exposed to the intermediate dose of tobacco extract performed best in swimming development; highest-dose pups were most active during the pre-weaning period while lowest-dose pups were most active during the post-weaning period; no treatment-related differences were noted in the active avoidance shuttle box (Paulson *et al*., 1994b).

4.4 Genetic and related effects

4.4.1 *Humans*

In the previous monograph on 'Betel-quid and areca-nut chewing' (IARC, 2004a), the following studies on the genetic and related effects in humans exposed to these substances with or without tobacco were included: Saranath *et al.*, 1991; Kaur *et al.*, 1994; Kuttan *et al.*, 1995; Heinzel *et al.*, 1996; Munirajan *et al.*, 1996; Baral *et al.*, 1998; Kaur *et al.*, 1998; Munirajan *et al.*, 1998; Murti *et al.*, 1998; Pande *et al.*, 1998; Agarwal *et al.*, 1999; Patnaik *et al.*, 1999; Ravi *et al.*, 1999; Saranath *et al.*, 1999; Chakravarti *et al.*, 2001; Pande *et al.*, 2001; Ralhan *et al.*, 2001; Tandle *et al.*, 2001; Nagpal *et al.*, 2002a,b; Chakravarti *et al.*, 2003). [The Working Group noted that, in the absence of detailed information on the smokeless tobacco consumption of oral squamous-cell carcinoma patients, the data from these studies and others (Lazarus *et al.*, 1996a; Ralhan *et al.*, 1998) are not included here.]

(*a*) *Mutagenicity and genotoxicity*

(i) *Mutagenicity*

The mutagenicity of urine samples from six *mishri* users and six non-users was similar, when tested in the *Salmonella*/microsome assay in the presence of β-glucuronidase or nitrite plus metabolic activation. However, the samples from *mishri* users showed stronger mutagenicity in TA98 in the presence of β-glucuronidase plus metabolic activation ($0.82 \pm 0.41 \times 10^6$ revertants per mol creatinine) compared with those from non-users ($0.11 \pm 0.11 \times 10^6$ rev/mol creatinine) (Govekar & Bhisey, 1993).

Mutagen levels in concentrates of urine from Swedish wet snuff users, cigarette smokers and non-tobacco users were compared. The concentrates were assayed for mutagenicity towards *S. typhimurium* TA98, both in the presence and absence of a metabolic activation system (the postmitochondrial liver fraction from Aroclor-1254-induced rats). The mean mutagenic activity in urine concentrates from smokers, found only in the presence of metabolic activation (8.6×10^3 revertants per 24 h), was significantly higher than that in the urine from snuff users (1.3×10^3 revertants per 24 h), abstinent snuff users (1.3×10^3 revertants per 24 h) and non-users (0.9×10^3 revertants per 24 h). No significant difference in mutagenic activity was found between the urine from snuff users, whether using or abstaining from snuff use, and that from non-tobacco users (Curvall *et al*., 1987).

Niphadkar *et al* (1994) determined the bacterial mutagenicity of gastric fluid samples from chewers of tobacco and from non-users in *Salmonella* strains TA98, TA100 and TA102. While all six gastric fluid samples from non-chewers were not mutagenic, samples from nine chewers were mutagenic, either directly or upon nitrosation, in the three tester strains and in TA102 in the presence of activating enzymes.

(ii) *Micronuclei, chromosomal aberrations and sister chromatid exchange*

Several studies have shown a relationship between snuff user's hyperkeratosis and elevated frequencies of micronucleated cells and/or chromosomal aberrations and sister chromatid exchange in snuff users compared with non-user controls (Livingston *et al*., 1990; Tolbert *et al*., 1991, 1992; Roberts, 1997). These studies are summarized in Table 95. Swedish moist snuff users showed increased mitotic rate, increased cell density and loss of cell cohesion (Larsson *et al*., 1991).

Higher frequencies of micronucleated cells and/or chromosomal aberrations and/or sister chromatid exchange were also reported in smokeless tobacco consumers and in patients with oral squamous-cell carcinoma, in comparison with non-user controls (see Table 95; Stich *et al.*, 1982a,b; Nair *et al*., 1991; Das & Dash, 1992; Kayal *et al*., 1993; Trivedi et *al*., 1995; Ozkul *et al*., 1997).

Elevated levels of micronucleated cells (%, with range) were found in the oral mucosa of *khaini* chewers from Bihar, India (2.1%; 0.8-4.9%), *gudhaku* chewers from Orissa, India (0.7%; 0.3-1.8%) and *naswar* users from Uzbekistan (former Soviet Union) (4.1%; 2.7–5.7%) compared with controls (non-chewers, nonsmokers) from various locations (0.5%; 0.0–1.0) (Stich & Anders, 1989). Localized micronucleus formation in the oral mucosa was described in *khaini* chewers; 2% of the cells in the gingival groove showed micronuclei (Stich *et al*., 1992). In another study from India, 6.3% of the cells were micronucleated in chewers of tobacco with lime (Ghose & Parida, 1995).

In a study designed to monitor genotoxicity in the *bidi* industry that included tobacco-processing plant workers and *bidi* rollers who did not use tobacco, the mean frequency of micronucleated cells in the buccal epithelium was significantly higher among *bidi* rollers and plant workers than among non-exposed controls (Bagwe & Bhisey, 1993).

(iii) *Genomic instability*

Genomic instability, as reflected by microsatellite alterations in specific target regions in tobacco chewers with primary oral squamous-cell carcinoma (77 cases), was analysed by Mahale and Saranath (2000). Using a panel of 11 microsatellite markers on chromosome 9, 48/77 (62%) patients demonstrated microsatellite alterations of which 27% had microsatellite instability and 52% had loss of heterozygosity. A majority of the alterations occurred on the p arm at 9p21-23 that may be indicative of involvement of the multiple tumour-suppressor 1 (*MTS1)* p16 *(CDKN2)* gene on chromosome 9p21 in a subset of chewing tobacco-induced oral cancers. [The Working Group noted the lack of information on the consumption pattern of tobacco in this study.]

Table 95. Micronucleated cells and sister chromatid exchange in oral mucosa of non-users and users of smokeless tobacco products

Study location	No. and definition of non-users	No. of users and type of smokeless tobacco used	Micronucleated cell frequency (mean/100 or 1000 cells)		Sister chromatid exchange (mean frequency/cell ± SE)		Reference
			Non-user	User	Non-user	User	
India			*Mean per 100 cells*		NT	NT	Stitch *et al.* (1982a,b)
	10 non-chewers	27 *khaini* (tobacco + lime) users	0.49	2.2 (front) 2.7 (right) 3.04 (left)			
USA	24 non-users	24 snuff users	0.27	2.22	6.78 ± 0.15	6.70 ± 0.18	Livingston *et al.* (1990)
India			*Mean per 1000 cells*				Nair *et al.* (1991d)
	27 non-users	35 tobacco + lime users	2.59 ± 0.37	5.20 ± 0.66	NT	NT	
USA	15 female non-users	38 female snuff users	1.58 ± 0.58	3.79 ± 0.56	NT	NT	Tolbert *et al.* (1991, 1992)
India			*Mean per 100 cells*				Das & Dash (1992)
	102 non-users	120 *gudhaku* (tobacco paste) users	0.35	2.06 (users for more than 20 years)	NT	NT	

Table 95 (contd)

Study location	No. and definition of non-users	No. of users and type of smokeless tobacco used	Micronucleated cell frequency (mean/100 or 1000 cells)		Sister chromatid exchange (mean frequency/cell ± SE)		Reference
			Non-user	User	Non-user	User	
India			*Mean per 1000 cells*				Kayal *et al.*
(Gujarat, Maharastra)	**Gujarat**	20 *mawa* (healthy) users	1.90 ± 0.19	6.9 ± 0.54	NT	NT	(1993)
	15 non-users	21 *mawa* (OSMF) users		7.05 ± 0.75			
		14 tobacco + lime (healthy) users		5.9 ± 0.49			
		12 dry snuff users (healthy)		5.66 ± 0.39			
	Maharashtra						
	13 non-users	16 *mishri* users (healthy)	1.00 ± 0.32	3.19 ± 0.63	NT	NT	
India					Mitomycin C-induced		Trivedi *et al.*
	40 non-users	40 chewing tobacco (healthy)	NT	NT	20.29 ± 0.519	21.29 ± 0.587	(1995)
		40 chewing tobacco (oral cancer)				23.14 ± 0.428	
Turkey			*Mean per 100 cells*				Ozkul *et al.*
	15 non-users nonsmokers	25 maras powder (smokeless tobacco) users	0.84 ± 0.22	1.86 ± 0.26	NT	NT	(1997)
USA			*Mean per 1000 cells*				Roberts
	19 non-users	22 snuff users	1.8	2.52	NT	NT	(1997)

NT, not tested; OSMF, oral submucosal fibrosis; SE, standard error

(*b*) *Alterations in* TP53, K-RAS *and related genes* (Figure 9)

(i) TP53, K-RAS *and* H-RAS

[The Working Group noted that details on (smokeless) tobacco use are often not or inadequately provided in the studies that were reviewed. Tobacco chewing in combination with betel quid is a common habit in the Indian population. As detailed data on these mixed exposures were not given in Saranath *et al.* (1992), Kannan *et al.* (2000), Tandle *et al.* (2000), Krishnamurthy *et al.* (2001) or Teni *et al.* (2002), these studies are not included in the review below.]

Analyses of *TP53* mutations in oral carcinomas associated with only tobacco chewing are limited. These studies report *TP53* mutations in oral cancers in the USA, Sudan, Norway and Sweden and are summarized in Table 96 (Lazarus *et al.*, 1995, 1996a,b; Xu *et al.*, 1998; Ibrahim *et al.*, 1999; Kannan *et al.*, 1999; Saranath *et al.*, 1999; Schildt *et al.*, 2003). In a limited study of *toombak* users, four head and neck squamous-cell carcinomas from three patients who used *toombak* and one patient who did not use *toombak* were screened for *TP53* mutations (Lazarus *et al.*, 1996a,b). Mutations were found in tumours resected from two of three *toombak* users, one at codon 282 (C→T) and the other in intron 6 (AT→GC). No K-*RAS* (codons 12 and 13) or H-*RAS* (codon 12) mutations were found in tumours that harboured *TP53* mutations and the other tumours.

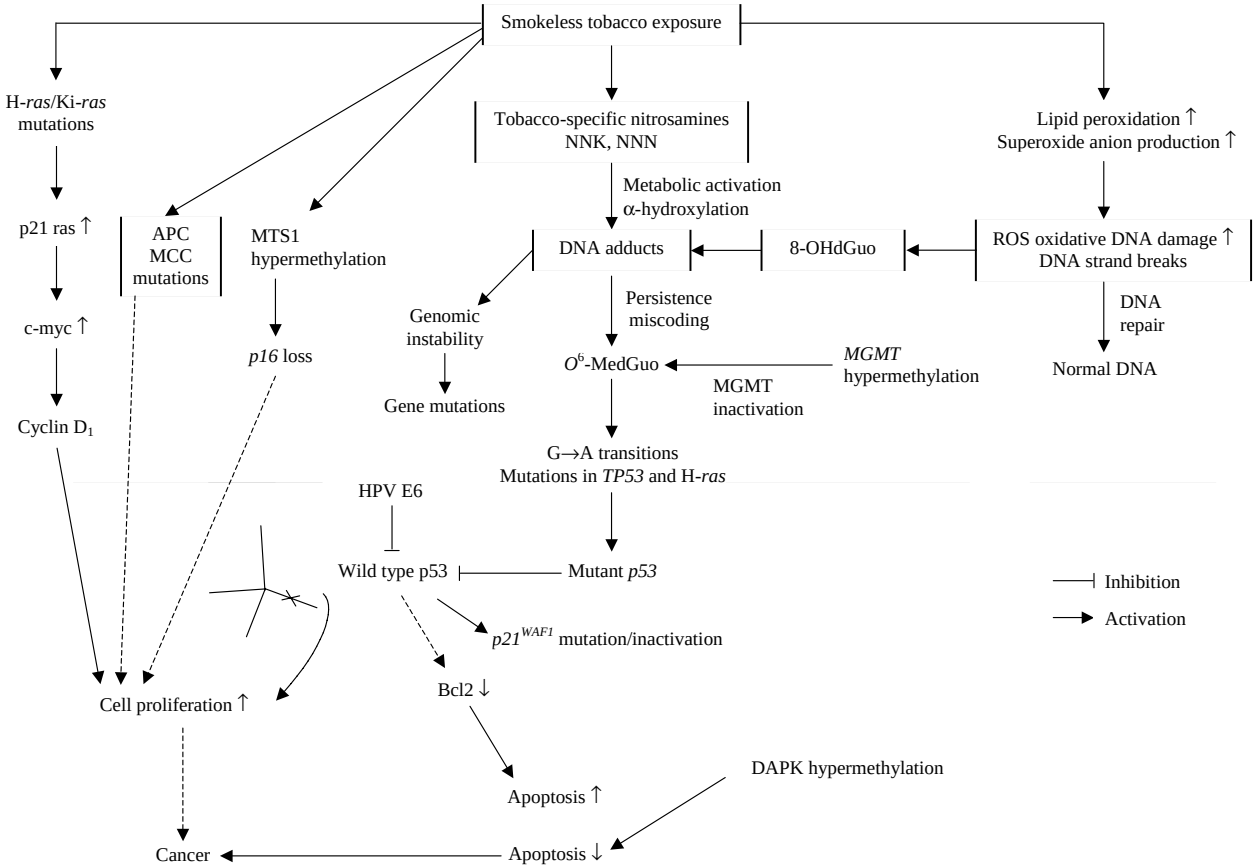
A high incidence of H-*RAS* mutations (codons 12, 13 or 61) was reported in oral cancers from India, the majority of which were in tobacco chewers (Saranath *et al.*, 1991) (see Table 97). [The Working Group noted that details on tobacco chewing were not mentioned in this study.]

Xu *et al*. (1998) analysed four oral squamous-cell carcinomas from users of snuff or chewing tobacco and 16 oral squamous-cell carcinomas from smokers only. Two of the tumours from users of snuff or chewing tobacco showed *TP53* mutations, while p53 protein accumulation was observed in all four tumours. No H-*RAS* mutation was observed in any of the tumours. No differences were observed in the p53, cyclin D1 and Rb profiles of users of smokeless tobacco and cigarette smokers.

TP53 mutations in 56 oral squamous-cell carcinomas from Sudanese *toombak* dippers and non-dippers and from Scandinavian non-dippers were analysed (Ibrahim *et al.*, 1999). No *TP53* mutations were found in non-malignant oral lesions from *toombak* dippers or non-dippers from Sudan. *TP53* mutations in exons 5–9 were found in 13/14 (93%) *toombak* dippers compared with 8/14 (57%) non-dippers from Sudan and 17/28 (61%) non-dippers from Scandinavia. Mutations G:C→A:T; C:G→T:A; G:C→T:A which are known to be associated with TSNA were found to be most common in oral squamous-cell carcinomas from *toombak* dippers which suggests a possible role of TSNA in the induction of *TP53* mutations in these tumours. [The Working Group noted that the information on cigarette smoking in Sudanese non-dippers and on alcohol drinking in 19 of the 28 Scandinavian patients were not available in this study.]

Saranath *et al.* (1999) reported *TP53* mutations in 14/83 (17%) oral squamous-cell carcinoma patients from India, the majority of whom were tobacco chewers, and multiple

Figure 9. Molecular targets affected by smokeless tobacco



APC, adenomatous polyposis coli; DAPK, death-associated protein kinase; HPV, human papillomavirus; MCC, mutated in colon cancer; MGMT, O^6-methylguanine–DNA methyltransferase; MTS, methyl transferase; NNK, 4-methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, *N*′-nitrosonornicotine; O^6-MedGuo, O^6-methyldeoxyguanosine; 8-OHdGuo, 8-hydroxydeoxyguanosine; ROS, reactive oxygen species

Table 96. Use of smokeless tobacco and *TP53* mutations in human oral premalignant and malignant lesions

Study location	Total no. of cases	Exons analysed	Total no. of lesions with *TP53* mutations	Chewing tobacco/snuff		*TP53* mutational analysis Codon/mutations	Reference
				Total cases	No. of cases with *TP53* mutation		
USA	14 oral cavity premalignant lesions (dysplasia)	5–9	4 premalignant lesions	1 dysplasia (snuff)	1	**Exon 5** 163/A:T→G:C and 155/A:T→C:G (same case)	Lazarus *et al.* (1995)
Sudan	4 HNSCCs	5–9	2	3 HNSCCs (*toombak* dipper) 1 HNSCC (non-dipper)	2 0	Intron 6, nt 553/A:T→G:C (1 case) Exon 8 codon 282/C:G→T:A (1 case)	Lazarus *et al.* (1996)
USA	29 oral cancers	5–8	17	4 oral cancers (chewing tobacco/snuff)	2	**Exon 6** +220/TAT→TGT (1 case)[s] 191/CCT→ACT and 192/CAG→CAT (1 case)	Xu *et al.* (1998)
Sudan, Norway, Sweden	56 OSCCs	5–9	38	14 (*toombak* dipper)	13	**Exon 5** **Sudanese *toombak* dippers** 130/CTC→CTT (1 case)[s] 166/TCA→GCA* (1 case) 132/AAG→ATG* 133/ATG→GATG[i] 148/GAT→TGA*. (All 3 from 1 case) 139/AAG→GAG 142/CCT→CTT (1 case) **Sudanese non-dippers** 132^/AAG→ATG* (2 cases) **Scandinavian non-dippers** 130^/CTC→CTT (2 cases)[s], 136^/CAA→CCA* (1 case), 146^/TGG→TAG (stop) (1 case)	Ibrahim *et al.* (1999)

Table 96 (contd)

Study location	Total no. of cases	Exons analysed	Total no. of lesions with *TP53* mutations	Chewing tobacco/snuff		*TP53* mutational analysis Codon/mutations	Reference
				Total cases	No. of cases with *TP53* mutation		
Sudan, Norway, Sweden (contd)				14 (Sudanese non-dippers) No data on cigarette smoking for Sudanese non-dippers	8	**Exon 6** **Sudanese *toombak* dippers** 190/CCT→CCG* (1 case)[s], 216^/GTG→GGG* (1 case) **Exon 7** **Sudanese *toombak* dippers** 229/TGT→TGG* (1 case), 229/TGT→TA del (1 case), 237/ATG→ATA (1 case), 239/AAC→TAA* (stop) (1 case), 242/TGC→TAC (1 case), 249/AGG→AAG (2 cases), 245/GGC→GAA (1 case), 252/CTC→CAC* (2 cases) **Sudanese non-dippers** 240^/AGT→CCC* and 249/AGG→AAG (both in 1 case), 244/GGC→TGA* (2 cases), 254^/ATC→GTC (1 case)	Ibrahim *et al.* (1999) (contd)

Table 96 (contd)

Study location	Total no. of cases	Exons analysed	Total no. of lesions with *TP53* mutations	Chewing tobacco/snuff		*TP53* mutational analysis Codon/mutations	Reference
				Total cases	No. of cases with *TP53* mutation		
Sudan, Norway, Sweden (contd)				28 (Scandinavian non-dippers) 11/17 Norwegian and 8/11 Swedish non-dippers reported cigarette smoking.	17	**Scandinavian non-dippers** 238/TGT→CGT (1 case) 246/ATG→ATA (3 cases), 248/CGG→CGA (2 cases), 248/CGG→CAA (1 case), 244/GGC→GAA (1 case) 239^/AAC→ACC (1 case), 246^/ATG→ATA (1 case), 249^/AGG→AAG (1 case) **Exon 8** **Sudanese *toombak* dippers** 299/CTG→CTTG (1 case)[i], 305/AAG→TAA* (stop) (1 case) **Scandinavian non-dippers** 279/GGG→GAG (1 case), 281^/GAC-GCC* (1 case), 299^/CTG→CTTG[i] and 305^/AAG-TAA* (stop) (2 cases), 306^/CGA→TGA (stop) (1 case)	Ibrahim *et al.* (1999) (contd)

Table 96 (contd)

Study location	Total no. of cases	Exons analysed	Total no. of lesions with *TP53* mutations	Chewing tobacco/snuff		*TP53* mutational analysis Codon/mutations	Reference
				Total cases	No. of cases with *TP53* mutation		
Sudan, Norway, Sweden (contd)						**Exon 9** **Sudanese *toombak* dippers** 310/AAC→TTG* (1 case), 310/AAC→ACC* (2 cases), 312/ACC→AGC* (1 case) **Sudanese non-dippers** 322^/CCA→CGA and 323^/CTG→CG del (1 case), 323^/CTG→GGG* (1 case) **Scandinavian non-dippers** 308/CTG→CTA 315/TCT→TGT* 308/CTG→CTC^{s} (1 case), 309^/CCC→TCC and 323^/CTG→CGG* (1 case), 322^/CCA→CGA (1 case)	Ibrahim *et al.* (1999) (contd)

Table 96 (contd)

Study location	Total no. of cases	Exons analysed	Total no. of lesions with *TP53* mutations	Chewing tobacco/snuff		*TP53* mutational analysis Codon/mutations	Reference
				Total cases	No. of cases with *TP53* mutation		
India	72 OSCCs	4–9	15	72 (All patients were tobacco chewers for > 10 years.)		**Exon 5** 139/AAG→AAT (1 case) 141/TGC→TAC (1 case) 179/CAT→TAT (1 case) **Exon 7** 237/ATG→ATA (1 case) 248/CGG→CAG (1 case) **Exon 6** 213/CGA→CGG (1 case) 213/CGA→CGG (1 case) **Exon 8** 266/GGA→GAA (1 case) 272/GTG→TTG (1 case) 272/GTG→TTG (1 case) 273/CGT→CAT (1 case) 273/CGT→TGT (2 cases) 282/CGG→TGG (1 case) **Exon 9** 307/GCA→GCG (1 case) **Exon 5** 176/TGC→TTC and (**Exon 8**) 266/GGA→GTA (1 case) **Exon 6** 190/CCT→CTT and 213/CGA→CGG (1 case) **Exon 6** 213/CGA→CGG and **Exon 7** 237/ATG→ATA (1 case)	Kannan *et al.* (1999)

Table 96 (contd)

Study location	Total no. of cases	Exons analysed	Total no. of lesions with *TP53* mutations	Chewing tobacco/snuff		*TP53* mutational analysis Codon/mutations	Reference
				Total cases	No. of cases with *TP53* mutation		
India	83 OSCCs 22 leukoplakias	5–9	14 and p53 protein over-expression in 23/62 OSCCs and in 6/22 leukoplakias	105 (All patients of OSCCs/leukoplakia were habitual tobacco chewers for a minimum duration of 10 years.)		**Exon 5** 146/TGG→TAG (1 case) 154^/GGC→GTT (1 case) 141^/TGC→TGT and 175/CGC→CAC (1 case) 152^/CCG→CTG (1 case) 153^/CCC→CCT (1 case) 175^/CGC→CAC (1case) **Exon 6** 188/GTC→GTA and 205/TAT→TAC (1 case) 205^/TAT→TGT (1 case) 212^/TTT→TTG (1 case) 194^/CTT→ATT (1 case) 205^/TAT→TGT (1 case) **Exon 7** 239/AAC→GAC (1case) 231/ACC→ACA (1 case) 248/CGG→TGG (2 cases) 249^/del G and 290/CGC→TGC (1 case) all the above in OSCCs. Leukoplakias NA for *p53* point mutations	Saranath *et al.* (1999)
Sweden	114 OSCCs	5–9	41 and p53 protein over-expression in 72 tumours	12 (never smokers)	2 (9 patients with p53 positive by IHC)	**Exon 8** (details on mutations not reported)	Schildt *et al.* (2003)

HNSCCs: head and neck squamous-cell carcinomas; IHC, immunohistochemistry; NA, not analysed due to insufficient DNA; OSCCs, oral squamous-cell carcinomas; nt, nucleotide
del, deletions; * transversions
^p53 positive by IHC
[i] Insertions leading to frame shift and stop codon
[s] Silent mutation

Table 97. Analysis of cancer-related gene and protein alterations in oral premalignant and malignant lesions of tobacco users

Study location	Gene/ protein	Total no. of cases	Tobacco (chewers/smokers)		Tobacco (without betel quid and smokers)		Alterations Codon/mutation	Reference
			Total no. of cases	No. of cases altered	Total no. of cases	No. of cases altered		
India	H-*RAS*	57 OSCCs	–	–	57 chewers	20	12.2/GGC→GTC (5 cases) 12.2/GGC→GTC and 61.2/CAG→CGG (2 cases) 12.1/GGC→AGC (1 case) 13.2/GGC→GAC (1 case) 61.2/CAG→CGG (7 cases) 61.2/CAG→CTG (1 case) 61.3/CAG→CAT (3 cases)	Saranath *et al.* (1991)
USA	Cyclin D1 Rb	29 oral cancers			4 chewers 4 chewers	1 1	Overexpression Absence of expression	Xu *et al.* (1998)
Sudan, Scandinavia, USA/UK	$p21^{WAF1}$ (Exon 2)	90 OSCCs	14 Sudanese *toombak* dippers 14 Sudanese non-dippers 27 Scandinavian non-dippers 35 US/UK non-dippers	6 2 6 5	–	–	**Sudanese *toombak* dippers** 6/GGG→GAG 7/GAT→GGT 35/GAT→TGA* (stop codon) All from 1 case 30/CTG→TTG^{s} (1 case) 10/CAG→CAA^{s} (1 case) 68/GTG→GTA^{s} 83/CGG→CGA^{s} Both from 1 case 138/CAG→TAG (stop codon) 144/CAG→TAG (stop codon) Both from 1 case 84/CGA→TGA (stop codon) 98/TCA→TAA* (stop codon) 106/GCA→GTA 107/GAG→CAG* All from 1 case	Ibrahim *et al.* (2002)

Table 97 (contd)

Study location	Gene/ protein	Total no. of cases	Tobacco (chewers/smokers)		Tobacco (without betel quid and smokers)		Alterations Codon/mutation	Reference
			Total no. of cases	No. of cases altered	Total no. of cases	No. of cases altered		
Sudan, Scandinavia, USA/UK (contd)							**Sudanese non-dippers** 107/GAG→CAG* 114/TCA→CCA 116/TCT→CCT and 125/GAG→AGA All from 1 case 20/CGC→CGT[s] 92/GGC→GGT[s] 94/CGG→CGTG* (1) 95/CCT→GTC* and 117/TGT→TAG (stop codon) All from 1 case **Scandinavian non-dippers** 138/CAG→TAG (stop codon) 31/AGC→AGA* Both from 1 case 31/AGC→AGA* (1 case) 8/GTC→ATC 62/GAC→AAC 117/TGT→TAT 137/TCT→TGT* All from 1 case 126/CAG→CAC* 140/CGA→CGC*[s] 142/CGG→ACG* All from 1 case 29/CAG→CAC* 52/GAC→CAC* 88/GAG→TGA* (stop codon) All from 1 case	Ibrahim *et al.* (2002) (contd)

Table 97 (contd)

Study location	Gene/ protein	Total no. of cases	Tobacco (chewers/smokers)		Tobacco (without betel quid and smokers)		Alterations Codon/mutation	Reference
			Total no. of cases	No. of cases altered	Total no. of cases	No. of cases altered		
Sudan, Scandinavia, USA/UK (contd)							**US/UK non-dippers** 32/CGC→ACGC*[i] 35/GAT→TGA* (stop codon) Both from 1 case 19/CGC→CGA*[s] 20/CGC→CGT[s] 64/GCC→GTC 79/CCC→ACC* All from 1 case 95/CCT→ACT* 98/CGC→TGA (stop codon) Both from 1 case 95/CCT→TCT 98/TCA→TAA* (stop codon) Both from 1 case 31/AGC→AGA* (1 case)	Ibrahim *et al.* (2002) (contd)

OSCC, oral squamous-cell carcinoma
*Tranversions
[s], Silent mutation
[i], Insertion leading to frameshift resulting in stop codon

mutations were seen in 5/14 (35%) cancer tissues. Ten of 14 mutations were at cytosines. TP53 expression was found in 23/62 (37%) oral squamous-cell carcinomas. TP53 inactivations that included point mutations, protein overexpression and/or presence of HPV were observed in 38/83 (46%) patients with oral cancer; 17/38 (45%) patients showed mutation/overexpression of TP53 (altered TP53) and no detectable HPV, and 21/38 (55%) were HPV 16-positive; while 13/38 (34%) HPV 16-positive patients had no mutation/overexpression of TP53 (unaltered TP53) and 8/38 (21%) HPV 16-positive patients had mutation/overexpression of TP53 (altered TP53). HPV 18 was not detected in any of the samples. [The Working Group noted that the details of tobacco chewing consumption were not mentioned in this study.]

Discordant results on TP53 immunopositivity, assessed by immunohistochemistry, have been reported in studies of snuff-induced oral lesions. Low expression levels of p53 protein were reported by Ibrahim *et al.* (1996) and Merne *et al.* (2002), while Wood *et al.* (1994) and Wedenberg *et al.* (1996) observed elevated expression of p53 in snuff-induced lesions. The accumulation of p53 protein in leukoplakia of snuff users was higher than that in mucosa that appeared normal from snuff users or from non-user controls (Wood *et al.*, 1994).

Oral squamous-cell carcinomas from Sudanese snuff dippers showed p53 expression in 3/14 (21%) patients while 9/14 (64%), 39/60 (65%) and 28/41 (68%) oral squamous-cell carcinomas from non-snuff users from Sudan, Sweden and Norway expressed p53 protein, respectively (Ibrahim *et al*., 1996). [The non-user patients from Sudan did not use any other form of tobacco and only eight patients from Sweden and 11 patients from Norway reported cigarette smoking. No data on alcoholic beverage consumption were available.]

In a Swedish study, Schildt *et al.* (2003) reported *p53* mutations in 41/114 (36%) oral squamous-cell carcinomas, 34 of which were localized in exon 8, and p53 protein expression in 72/114 (63%) tumours. No clear pattern in relation to the expression of the biological markers p53, PCNA, Ki-67 and bcl-2 emerged in oral squamous-cell carcinomas from snuff users; however, very few snuff users (12) were included in this study.

Studies on genetic alterations in other cancer-related genes in users of smokeless tobacco are summarized in Table 97 and Figure 9.

(ii) p21^{WAF1} *and* S100A4

Mutations in exon 2 of *p21^{WAF1}*, the cyclin-dependent kinase inhibitor gene, were found in oral squamous-cell carcinomas in 6/14 (43%) *toombak* users compared with 13/76 (17%) non-users of snuff, the majority of whom were smokers (Ibrahim *et al.*, 2002). Nucleotide changes differed in *toombak* dippers [codons 10, 30, 68 and 83 in 3/14 (21%) cases] versus non-users of snuff [codons 19, 20, 92 and 140 in 6/76 (8%) cases]. These differences were not statistically significant. In the appropriate oral squamous-cell carcinomas, the presence of *p21^{WAF1}* exon 2 mutation coincided with the detection of a mutation in the *TP53* gene exon 5 to 9 (Ibrahim *et al.*, 1999) (see Table 96).

Mutations in the metastasis-inducing *S100A4* gene, a member of the S100 family of the calcium binding proteins, were found in three oral squamous-cell carcinomas (one

from a *toombak* dipper and two from non-users of snuff). The oral squamous-cell carcinoma from the *toombak* dipper had four mutations (one transition, three transversions), while those from non-users of snuff showed three mutations each (one transition, two transversions). All of these three cases were negative for mutations in $p21^{WAF1}$ and *TP53* genes (Ibrahim *et al.*, 2002). No mutations were found in the non-malignant oral lesions from snuff-dippers/non-users. No correlation was found between *S100A4* mutations and $p21^{WAF1}$ and/or *TP53* mutations. [The Working Group noted that the sample size in this study was small and that information on alcohol drinking was unavailable.]

(iii) *Adenomatous polyposis coli (APC) and mutated in colon cancer (MCC) genes*

Loss of heterozygosity (LOH) at *APC* and *MCC* genes was studied in 40 oral squamous-cell carcinomas and 57 leukoplakia patients from eastern India (Sikdar *et al*., 2003). Among the oral squamous-cell carcinomas, 58% were tobacco chewers, while only 10% of leukoplakia patients were tobacco chewers. Four of 16 (25%) oral squamous-cell carcinomas, three of which were from tobacco chewers, and 1/29 (3%) leukoplakias, also from a tobacco chewer, demonstrated LOH at *APC* and were positive for HPV 16. None of the oral squamous-cell carcinomas or leukoplakias showed any LOH at the *MCC* gene.

(iv) p16, death-associated protein kinase (DAPK), MGMT *and* GSTP1 *genes*

Kulkarni and Saranath (2004) studied concurrent hypermethylation of tumour-suppressor gene *p16*, *DAPK*, *MGMT* and *GSTP1* genes in 60 primary oral tumours from habitual tobacco chewers and corresponding adjacent clinically and histopathologically normal mucosa as well as buccal epithelial scrapings from normal mucosa of 20 healthy non-users of tobacco. Fifty-two of 60 (86.7%) oral squamous-cell carcinomas and 46/60 (76.7%) adjacent mucosa showed hypermethylation in the promoter regions of *p16*, *MGMT* and *DAPK* genes. However, none of the tissues analysed showed promoter hypermethylation in the *GSTP1* gene. None of the tissues from tobacco non-user controls showed any hypermethylation. Promoter hypermethylation was observed in 40/60 (66.7%), 41/60 (68.3%) and 31/60 (51.7%) oral squamous-cell carcinomas in *p16*, *DAPK* and *MGMT* genes, respectively. Among the samples of adjacent mucosa analysed, 30/60 (50%), 36/60 (60%) and 16/60 (26.7%) tissues demonstrated promoter hypermethylation in *p16*, *DAPK* and *MGMT* genes, respectively. Thus the percentage of hypermethylation of *p16* and *MGMT* genes was significantly higher in tumour tissues than in corresponding adjacent mucosa. The *DAPK* methylation profile in both kinds of tissue was similar. No correlation could be established between hypermethylation of these genes and clinicopathological parameters in patients.

(v) *BAX/BCL2/Ki-67*

Loro *et al.* (2000) reported a higher rate of apoptosis and a higher expression of BAX in oral squamous-cell carcinomas from Norway compared with those from Sudan. No

significant differences were observed in apoptosis, BAX, BCL-2 or Ki-67 in oral squamous-cell carcinomas from Sudan in relation to *toombak* use or *TP53* gene status.

(c) *Polymorphisms in carcinogen-metabolizing enzymes*

Several isozymes of CYP, e.g. CYP1A1, CYP2D6, CYP2E1, CYP2A6 and CYP2A13, are involved in the metabolic activation of the carcinogens present in smokeless tobacco — TSNA (major class) and PAHs (in some products). The initial steps usually carried out by CYP enzymes involve oxygenation of the carcinogens. Other enzymes such as lipoxygenases, cyclooxygenases, myeloperoxidase and monoamine oxidases may also be involved, but less commonly. When the oxygenated intermediates formed in these initial reactions are electrophilic, they may react with DNA or other macromolecules to form covalent binding adducts. Alternatively, these metabolites may undergo further transformations that are catalysed by GST, uridine-5-diphosphate-glucuronosyltransferases, epoxide hydrolase, *N*-acetyltransferases, sulfotransferases and other enzymes. Such reactions frequently, but not always, result in detoxification. The balance between activation and detoxification can affect the development of cancer. Numerous alleles that cause extinguished, defective, qualitatively altered, diminished or enhanced rates of metabolism have been identified for several phase I and phase II enzymes and can result in marked interindividual differences in carcinogen metabolism (reviewed in Vineis *et al*., 1999; Nair & Bartsch, 2001; Wu *et al*., 2004). An interaction between genetic polymorphisms and smokeless tobacco in the causation of cancer in humans is plausible: possible mechanisms of interaction include the activation or detoxification of carcinogens present in the tobacco. However, the effects are probably complex as frequently simultaneous exposure to smokeless tobacco with cigarette smoking and/or alcoholic beverage consumption occurs.

The GSTs are a large family of enzymes that protect DNA against damage and adduct formation by the conjugation of GSH to electrophilic substances, which predominantly creates hydrophilic, less reactive metabolites that can be excreted. Several GST families (alpha, mu, pi, theta) exist and show genetic polymorphisms associated with large variations in enzyme activities (Hayes & Pulford, 1995).

The impact of *GST* genotypes on the risks for pre-cancer and cancer have been investigated in Indian users of smokeless tobacco quids.

The influence of *GSTM1* and *GSTT1* genotypes on risk for oral leukoplakia was investigated using genomic DNA from biopsies from 98 oral leukoplakia patients and exfoliated cells from 82 healthy controls from India (Nair *et al*., 1999). Most cases of leukoplakia were heavy chewers (15–20 quids of tobacco with or without betel quid per day), whereas the chewers among controls were regular but light chewers (1–2 quids per day). A significantly increased risk for oral leukoplakia was associated with *GSTM1* null (odds ratio, 22; 95% CI, 10–47) or *GSTT1* null (odds ratio, 11; 95% CI, 5–22) genotypes. Combined null genotypes of *GSTM1* and *GSTT1* prevailed in 60.2% of the cases but none were detected in controls.

The impact of *GSTM1* null genotype on the risk for oral cancer was also analysed in separate groups of individuals from India who used different types of tobacco (297 cancer patients and 450 healthy controls). The odds ratios associated with *GSTM1* null genotype was 3.7 (95% CI, 2.0–7.1) in chewers of tobacco with lime or with betel quid. Increased lifetime exposure to tobacco chewing was associated with a twofold increase in the risk for oral cancer in *GSTM1* null individuals. The risk for cancer associated with *GSTM1* null genotype increased from 2.5 (95% CI, 0.9–7.1) among chewers with less than median lifetime exposure to 4.6 (95% CI, 1.9–11.4) in chewers with more than median lifetime exposure (Buch *et al*., 2002). [The gene–environment interaction was not estimated for chewing betel quid with or without tobacco.]

In a study of 211 cases of oral cancer and 160 controls from India, no significant differences in the distributions of *GSTM1*, *GSTM3* or *GSTT1* genotypes was observed between cases and controls. However, an increased risk for oral cancer was reported for heavy chewers with the *GSTT1* null genotype (odds ratio, 3.0; 95% CI, 1.0–9.8) and for light chewers who had *GSTP1 ile*/*ile* at codon105 (odds ratio, 2.0; 95% CI, 1.0–3.7) (Sikdar *et al*., 2004). [The Working Group noted that 30% of chewers also smoked tobacco.]

Few studies are available that looked specifically into host genotype and exposure to smokeless tobacco, and were too small to provide clear evidence. Carefully designed studies of sufficient size targeted specifically on smokeless tobacco users need to be conducted to enable a clear picture of the relationship between genotype, smokeless tobacco use and cancer risk to be formed. At present, due to the incomplete nature of the literature, firm conclusions cannot be drawn regarding the modulating effects of polymorphisms on smokeless tobacco-associated cancers.

4.4.2 *Experimental systems*

(*a*) *Mutagenicity and genotoxicity of various types of smokeless tobacco*

(i) *In-vivo studies*

Urine samples were collected from groups of Sprague-Dawley rats that were maintained on semi-synthetic diets sufficient or deficient in vitamin A, B complex or protein. Each dietary group was exposed to tobacco extract, *mishri* extract, benzo[*a*]pyrene, NNN or NDEA (see Section 4.1.2). Urine was tested for mutagenic activity using the *Salmonella*/microsome (metabolic activation) assay. Higher mutagenic activity of urine was observed in all exposed groups. The order of mutagenicity of all treatments was deficient diet > standard laboratory diet > nutritionally sufficient diet (Ammigan *et al.*, 1990c).

As noted in Section 4.2.2(*a*), a change of ploidy status has been shown in buccal epithelial cells of rats treated with smokeless tobacco by application to the buccal mucosa weekly for 1 year: 25% of buccal epithelial cells were tetraploid and 5% were octaploid which suggests that the mitotic process could be altered (Chen, 1989).

[These results are relevant because ploidy status has been reported (Sudbo *et al*., 2001) to be a significant marker for dysplasia with potential for malignant transformation.]

(ii) *In-vitro studies*

Hannan *et al*. (1986) showed the mutagenic potential of *shammah* (Yemeni snuff used in Yemen and some parts of Saudi Arabia) by use of the *S. typhimurium*/microsome mutagenicity assay (Ames test), aberrant colony formation and mitogenic gene conversion in yeast, tryptophan gene conversion in the D7 diploid strain of *Saccharomyces cerevisiae* and in-vitro oncogenic transformation in C3H mouse embryo 10T1/2 cells.

Jansson *et al*. (1991) investigated the genotoxicity of aqueous and methylene chloride extracts of Swedish moist oral snuff. Methylene chloride extract contained much more nicotine (9.1 mg/mL) than the aqueous extract (2.4 mg/mL). The aqueous extract was found to induce sister chromatid exchange in human lymphocytes *in vitro* and chromosomal aberrations in V79 Chinese hamster ovary cells *in vitro* (both with and without metabolic activation). However, no induction of mutation was observed in *Salmonella* or V79 cells, and no micronuclei were found in mouse bone marrow cells. The authors speculated that the induction of chromosomal aberrations without metabolic activation may have been due to a high concentration of salt in the extract, and that the potentially clastogenic agent(s) in the extract required metabolic activation. Methylene chloride extract gave positive results in the Ames test, and induced chromosomal aberrations and sister chromatid exchange (only in the presence of a metabolic activation system). However, no induction of mutation was observed in V79 Chinese hamster ovary cells. The in-vivo administration of methylene chloride extract did not cause micronucleus formation in mouse bone marrow cells or sex-linked recessive lethal mutations in *Drosophila melanogaster*.

The mutagenicity was determined of extracts of two leading brands of American chewing tobacco, treated with or without sodium nitrite under acidic conditions. Mutagenic activity was found only for nitrite-treated extract in *S. typhimurium* tester strains TA98 and TA100, independently of metabolic activation (Whong *et al.*, 1985). However, in a previous study, these authors had also reported the mutagenic activity of tobacco snuff treated under acidic conditions in *Salmonella* with and without a microsomal activation system (Whong *et al.*, 1984).

The mutagenic potential of an aqueous extract of *mishri*, chewing tobacco alone and a mixture of chewing tobacco with lime was tested in the *Salmonella* mutagenicity assay. *Mishri* extract was mutagenic in strain TA98 with metabolic activation by Arochlor 1254-induced rat liver microsomes. A nitrosated aqueous extract of *mishri* was mutagenic in strains TA100 and TA102. While the aqueous extract of 'Pandharpuri' — a brand of chewing tobacco — was mutagenic to TA98 and TA100 with nitrosation only, the aqueous extract of the same tobacco with lime was directly mutagenic in strains TA98, TA100 and TA102 (Niphodkar *et al.*, 1996).

Aqueous extracts of two commercial brands of smokeless tobacco were found to be clearly mutagenic (dose range, 1–3 mg/mL extractable solids) in human lymphoblastoid TK-6 cells, which do not express CYP. In human lymphoblastoid AHH-1 cells, which constitutively express CYP1A1, a similar result was found for both products tested. The mutagenicity of both extracts in TK-6 and AHH-1 cells was markedly decreased by treatment at neutral pH with sodium nitrite (0.25 mM) or by acid treatment (2 h; pH 3.0).

Since these two cell lines were found to be equally sensitive to the induction of mutation by aqueous tobacco extracts, the authors concluded that these brands of tobacco contain precursors for the formation of mutagens, the activity of which is not CYP-mediated (Shirnamé-Moré, 1991).

(*b*) *Effects on p53 and other genes*

Bagchi *et al.* (2001) demonstrated that treatment of human oral keratinocytes with smokeless tobacco extract (200 mg/mL) resulted in increased *TP53* mRNA expression and decreased *Bcl-2* mRNA expression. At higher concentrations, the expression of *TP53* mRNA decreased confirming an increase in apoptotic cell death as reported earlier (Bagchi *et al.*, 1999).

The human oral squamous carcinoma cell line (Amos III) established from a smokeless tobacco consumer was shown to harbour a *TP53* mutation (an insertion of C at nucleotide 13197, resulting in a termination codon at 180) and accumulation of p53 protein. Accumulation of cyclin D1, bcl-2, $p21^{waf1}$ and mdm2 and no detectable expression of p16, pRb and RARβ proteins suggested deregulation of cell cycle and apoptotic pathways (Kaur & Ralhan, 2003).

(*c*) *Effect on viruses*

Smokeless tobacco extracts and TSNA have been shown to enhance cell transformation by HSV-1 (Park *et al.*, 1991), increase cell longevity (Murrah *et al.*, 1993) and to inhibit replication of virus in the oral cavity (Hirsch *et al.*, 1984b; Park *et al.*, 1988). Smokeless tobacco extracts also increased survival of ultraviolet-irradiated HSV in monkey kidney CV-1 cell cultures (Dokko *et al.*, 1991).

Smokeless tobacco extracts did not activate latent Epstein-Barr virus into lytic replication (Jenson *et al.*, 1999a), but decreased the rate of lymphocyte population doubling (Jenson *et al.*, 1999b).

The inhibitory effects of snuff extract and the TSNA NNN and NNK on HSV-1 replication *in vitro* and on HSV-1 protein synthesis in infected cells were analysed. Addition of snuff extract after adsorption of the virus to cell membranes resulted in significantly reduced production of the virus at low multiplicities of infection, but, at high multiplicities of infection the inhibitory effect was less pronounced. Smokeless tobacco extracts increased the production of immediate-early (α) infected cell proteins (ICPs) 4 and 27, and early (β) ICPs 6 and 8 and decreased production of late (γ) ICPs 5, 11 and 29. Snuff extract has been proposed to block the replicative cycle of HSV at an early stage, which results in enhanced production of early ICPs and in prolonged maintenance of cellular functions that may be important for HSV-induced cell transformation (Larsson *et al.*, 1992).

Demirci *et al.* (2000) showed that smokeless tobacco extracts modulate exogenous reporter gene expression under control of the cytomegalovirus immediate enhancer and promoter in early passage transfected and cultured human oral epithelial cells, which suggests an influence of smokeless tobacco extracts on viral enhancers/promoters.

4.5 Mechanistic considerations

TSNA such as NNK and NNN are the most abundant strong carcinogens in smokeless tobacco producs. Data on biomarkers such as measurements of NNK and NNN in the saliva and of the NNK metabolites NNAL and NNAL-Gluc in urine clearly demonstrate the uptake and metabolism of these carcinogens in smokeless tobacco users. The levels reported are sometimes extraordinarily high; for example, *toombak* users represent the highest reported human exposure to a non-occupational *N*-nitrosamine carcinogen. NNK and NNN are metabolically activated by α-hydroxylation in rodent and human tissues. This process leads to DNA and haemoglobin adducts. The DNA adducts are critical in the carcinogenesis of NNK and NNN. The haemoglobin adducts of NNK and/or NNN have been detected in the blood of smokeless tobacco users in three studies, which demonstrates the metabolic activation of NNK and/or NNN in these humans. Although NNK and/or NNN DNA adducts have been detected in tissues of smokers, no studies have been reported in smokeless tobacco users.

Elevated micronucleus formation and chromosome breaks have been reported in oral exfoliated cells in smokeless tobacco users. The frequency of cytogenetic alterations was significantly elevated in peripheral blood lymphocytes in smokeless tobacco users compared with those of non-users.

Smokeless tobacco-associated oral premalignant and malignant lesions have been shown to harbour mutations in various genes that play a pivotal role in carcinogenesis. Accumulation of p53 protein and *TP53* mutations G:C→A:T, C:G→T:A and G:C→T:A have been reported in oral squamous-cell carcinomas from *toombak* dippers and tobacco chewers. A high incidence of G→T and G→A mutations has been observed in oral squamous-cell carcinomas from a tobacco-chewing Indian population. Inactivation of the *p16* tumour-suppressor gene and of *MGMT* and *DAPK* by promoter hypermethylation has been observed in oral carcinomas from tobacco chewers. Aberrant levels of expression of bcl-2 and bax, as well as mutations in the $p21^{waf1}$ gene have been observed in oral cancers in chewers.

In summary, multiple features of the carcinogenic process have been observed to occur *in vitro* and *in situ* in the oral cavity of smokeless tobacco chewers and in experimental animals treated with smokeless tobacco. Collectively, the available data on biomarkers provide convincing evidence that carcinogen uptake, activation and binding to cellular macromolecules are higher in smokeless tobacco users than in non-users. Smokeless tobacco is genotoxic in humans and in experimental animals. Tumours of smokeless tobacco users contain mutations in oncogenes and tumour-suppressor genes. Most of the genetic effects seen in smokeless tobacco users are also observed in cultured cells or in experimental animals exposed to smokeless tobacco.

5. Summary of Data Reported and Evaluation

5.1 Exposure data

The use of tobacco dates back to at least 5000 years in the Americas, and, by the 1700s, had spread throughout the world. The major tobacco products are made primarily from the *Nicotiana tabacum* species, but those in Asia and Africa are frequently also made from *N. rustica* and other species. Globally, smokeless tobacco includes a wide variety of commercially and non-commercially prepared products that are used either orally or nasally and are not burned when used. They are consumed by hundreds of millions of people in many regions of the world. The largest number of users of smokeless tobacco live in South-East Asia, particularly in India and Bangladesh. Consumption appears to be increasing in many populations.

Smokeless tobacco products differ in their composition and chemical profile, but they all contain nicotine, which is an addictive substance. The doses of nicotine in smokeless tobacco products, particularly in moist snuff, are manipulated by commercial manufacturers, and levels vary among product types and brands as a result of tobacco-processing techniques. In addition, all smokeless tobacco products expose users to a number of identified carcinogens that arise mainly during post-harvest processing of the tobacco; the most abundant in smokeless tobacco products are tobacco-specific *N*-nitrosamines, *N*-nitrosoamino acids, volatile *N*-nitrosamines and aldehydes. The amounts of *N*-nitrosamine in smokeless tobacco exceed those found in food and cosmetic products by several orders of magnitude. Some smokeless tobacco products also contain high levels of carcinogenic polycyclic aromatic hydrocarbons.

Some health scientists have suggested that smokeless tobacco should be used in smoking cessation programmes and have made implicit or explicit claims that its use would partly reduce the exposure of smokers to carcinogens and the risk for cancer. They also attribute declines in smoking in Sweden to increased consumption of moist snuff; these claims, however, are not supported by the available evidence.

Particular types of smokeless tobacco are banned or regulated in parts of the world, but, in many areas, either product regulation is non-existent or the degree of enforcement of established regulations is uncertain.

5.2 Human carcinogenicity data

Oral cancer

Several studies in various countries have identified the use of smokeless tobacco as a cause of oral cancer. A case–control study in the USA (North Carolina) that investigated large numbers of smokeless tobacco users who did not smoke found that the risk for oral

cancer was strongly associated with the use of snuff among nonsmokers who did not drink alcoholic beverages. A dose–response relationship was observed between increasing duration of use and the risk for cancers of the gum and buccal mucosa.

Additional strong evidence is available from two large case–control studies — one from India in 1962 and one from Pakistan in 1977 — that reported two- to 14-fold increases in the risk for oral cancer among chewers of tobacco (or tobacco plus lime) who were not betel-quid chewers and were also nonsmokers. Another case–control study from Pakistan in 2000 on users of *naswar* reported a nearly 10-fold increase in the risk for oral cancer after adjusting for tobacco smoking and alcoholic beverage consumption. An additional case–control study from Nagpur, India, reported an eightfold increase in risk for smokeless tobacco use among nonsmokers and also reported a 15-fold increase in risk for all oral cancers combined for those who use materials that contain tobacco to clean their teeth, after adjusting for tobacco smoking, alcoholic beverage consumption, occupation and tobacco chewing.

Two population-based case–control studies on snuff use — one on head and neck cancer and one on oral cancer — were conducted in southern and northern parts of Sweden. The study from southern Sweden found no significant association between snuff use and the risk for head and neck cancer, either for all sites combined or when restricted to cancers of the oral cavity. When the analysis was restricted to men with no history of smoking, there was a nearly fivefold elevated risk for head and neck cancer associated with snuff use. The small sample size precluded a separate site-specific analysis for oral cancer among those who never smoked. The study conducted in northern Sweden investigated cancer of the oral cavity in relation to snuff use and controlled for alcoholic beverage use and tobacco smoking. Overall, this study did not suggest an association between snuff use and oral cancer. However, some relevant subgroups (e.g. those who never smoked, cases of lip cancer) had increased relative risks that were of borderline statistical significance. In both studies, the risks for oral cancer among former snuff users were increased with borderline statistical significance.

One cohort study from the USA reported a non-significant increased risk for oral cancer among those who never smoked but used smokeless tobacco. Less confidence can be placed on two other cohort studies from the USA and one from Norway that did not report an increase in risk, because the number of cases was small or the effect estimates were not controlled for tobacco smoking.

Additional support for a causal association derives from four case–control studies in North America that also showed a relationship between the use of smokeless tobacco and oral or oral and pharyngeal cancer. These studies addressed potential confounding by tobacco smoking through stratification by examining nonsmokers only or by statistical adjustment. However, they were based on small numbers and internal consistency in the results was not assessed.

In a number of regions across the world, supporting evidence for an association between the oral use of tobacco and increased risk for oral cancer is based on studies that have reported high prevalences of users of these products in case series of oral cancer and reports

of cancers that developed at anatomical sites where the tobacco was placed. In studies that had some methodological limitations, high rates of oral cancer have been reported in regions that had high prevalences of smokeless tobacco use, e.g. among *toombak* users in Sudan and among *naswar* and *shammah* users in central Asia and Saudi Arabia.

Cross-sectional studies in many countries have demonstrated strong associations between smokeless tobacco use (after accounting for confounding factors) and precancerous lesions such as oral leukoplakia.

The studies from the USA, Asia and Africa — in particular, one study from the USA and four studies from South Asia — provide sufficient evidence for a causal association of smokeless tobacco use with oral cancer. The Swedish studies are not inconsistent with positive studies in other regions for various reasons. First, variations in magnitudes of risk across studies may be due to differences in tobacco species and tobacco processing or in practices that include amounts used, years of use or keeping the tobacco in the mouth for long periods; in addition, variations in oral hygiene status or individual susceptibility factors may also play a role. Second, in one Swedish study, positive findings were observed in the subgroup of those who had never smoked and, in both Swedish studies, risks were elevated in former users, which might be expected if the presence of oral precancerous lesions led to cessation of the use of smokeless tobacco.

Oesophageal cancer

A fivefold increase in risk for oesophageal cancer among chewers of tobacco leaves (locally called *chada*) was reported among nonsmokers (adjusted for alcoholic beverage use) and among non-alcoholic beverage drinkers (adjusted for smoking) in a case–control study from Assam, India. Similar levels of risk were observed among men and women when they were analysed separately.

In a Swedish case–control study, only a modest increase in risk was observed overall, but a higher increase in risk was found for long-term users. This study also reported a dose–response with intensity of use, although there was no increased risk in the highest category. A cohort study from Norway found a modest, statistically non-significant increase in risk. Another Swedish case–control study of head and neck cancer reported only a very modest increase in risk for oesophageal cancer and a case–control study in the USA reported no effect.

Pancreatic cancer

Two case–control studies from the USA and two cohort studies, one from the USA and one from Norway, have reported positive associations between the use of smokeless tobacco and pancreatic cancer. In one case–control study in the USA, a statistically significant elevated risk for pancreatic cancer was observed among those who had never smoked and long-term quitters. In the other case–control study among lifelong nonsmokers in the USA, an elevated risk among users of more than 2.5 ounces [~70 g] per week was reported. One cohort study of men in Norway found an excess risk for pancreatic cancer among those who had ever used smokeless tobacco after controlling for smoking; however,

in a stratified analysis, the excess risk was confined to smokers. The Lutheran Brotherhood cohort study found an excess risk of borderline significance in those who had ever used smokeless tobacco, taking into account smoking and alcoholic beverage consumption. The evidence on dose–response relationships is restricted to one study from the USA which found an increased risk only among heavy users of smokeless tobacco.

Other cancers

Studies on cancers at other sites did not provide conclusive evidence of a relationship with smokeless tobacco use.

Nasal use

Studies on nasal use of snuff did not provide conclusive evidence of a relationship with cancer.

5.3 Animal carcinogenicity data

In two studies, squamous-cell carcinomas and papillomas of the oral and nasal cavities and forestomach and undifferentiated sarcomas of the lip developed with a significantly increased incidence in rats that had received moist snuff tobacco repeatedly applied to a surgically created oral canal. When snuff-treated rats were pretreated with 4-nitroquinoline *N*-oxide, an increased incidence of sarcomas of the lip was observed. In another experiment, benign and malignant epithelial tumours of the oral cavity developed in rats when snuff tobacco, water-extracted snuff tobacco or snuff tobacco enriched with its own aqueous extract was applied to a surgically created oral canal. However, the increase in tumour incidence did not achieve statistical significance. In addition, snuff tobacco was tested for carcinogenicity in rats by topical administration in a surgically-created oral canal alone or in combination with herpes simplex virus type 1 infection. Squamous-cell carcinomas of the oral cavity were observed in the group that received both treatments, but this result was not statistically significant.

Rats given tobacco extracts by gavage showed a statistically non-significantly increased incidence of forestomach papillomas and lung adenomas, and rats on a vitamin A-deficient diet given the same tobacco extract developed a high incidence of forestomach papillomas and pituitary adenomas. Weekly applications of snuff tobacco to the oral mucosa caused no tumours in rats of either sex. Subcutaneous injection of ethanol extracts of snuff tobacco to rats did not produce an increase in tumour incidence. Aqueous snuff tobacco extracts and snuff tobacco extracts enriched to 10-fold their natural concentrations of tobacco-specific nitrosamines were tested by repeatedly swabbing the lips and oral cavities of rats. A small, statistically non-significant increase in the incidence of lung adenomas and papillomas of the oral cavity occurred in rats treated with preparations enriched in tobacco-specific nitrosamines, but non-enriched snuff tobacco extracts alone produced no tumours of either the oral cavity or lung.

In one experiment, inoculation of herpes simplex virus-1 or -2 into the cheek pouches of hamsters followed by repeated application of snuff tobacco into the cheek pouches resulted in a high incidence of invasive squamous-cell carcinomas at the site of application. No tumours developed in cheek pouches treated with inoculations of virus alone or in those treated with snuff alone or in controls. In one experiment, snuff tobacco suspended in liquid paraffin and administered repeatedly to hamster cheek pouches caused forestomach papillomas but no tumours at the site of application. Hamsters given tobacco alone or in combination with alcohol into the cheek pouch developed a low and statistically non-significantly increased incidence of forestomach papillomas, but no tumours developed in the treated cheek pouches. Several studies in hamsters in which snuff tobacco alone or in combination with calcium hydroxide was administered as single or repeated applications into the cheek pouch or fed in the diet gave negative results or yielded inadequate data for evaluation.

In a study in which *mishri* was fed in the diet, an increase in the incidence of forestomach papillomas was observed in mice, hamsters and rats. Malignant tumours of the lung and stomach papillomas developed in rats maintained on a vitamin A-deficient diet and given *mishri* by gavage. In one study, repeated application of *mishri* extract to mouse skin resulted in the development of skin papillomas in some mice and one squamous-cell carcinoma of the skin.

In a two-stage mouse skin assay, applications of tobacco extract followed by promotion with croton oil induced papillomas and squamous-cell carcinomas of the skin. In another two-stage mouse skin assay, application of *bidi* tobacco extracts following initiation by 7,12-dimethylbenz[*a*]anthracene resulted in papillomas.

Available studies on *naswar* were inadequate for evaluation.

5.4 Other relevant data

Tobacco-specific nitrosamines, the most abundant strong carcinogens in smokeless tobacco products, nicotine and cotinine have been detected in the saliva of snuff dippers and tobacco chewers in many studies around the world. Levels of tobacco-specific nitrosamines in saliva are remarkably high in Sudanese users of oral snuff (*toombak*). Adducts of tobacco-specific nitrosamines to haemoglobin — via analysis of an alkaline hydrolysis product — have been explored as biomarkers of exposure to smokeless tobacco and were found in snuff dippers and nasal snuff users in several studies. 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol, a metabolic product of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, and a glucuronidation product of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol are very useful and specific biomarkers of tobacco use and provide a good approximation of carcinogenic dose. These substances have been found in the urine of smokeless tobacco users in numerous studies, and the *toombak* users in the Sudan showed exceptionally high concentrations (excretion of up to 0.4 mg daily).

N′-Nitrosonornicotine, *N*′-nitrosoanabasine and *N*′-nitrosoanatabine and their respective glucuronides have been detected in the urine of smokeless tobacco users at significantly higher levels than in non-users.

In addition to tobacco-specific nitrosamines, tobacco also contains secondary and tertiary amines that can be nitrosated by reaction with available nitrite in the saliva or in the stomach of tobacco chewers (endogenous nitrosation). This process is enhanced by bacteria in dental plaque and by the acidic environment in the stomach (many chewers swallow the chewed tobacco).

In humans, the absorption of nicotine from smokeless tobacco products is slower than that from tobacco products used for smoking, but overall equivalent plasma levels of nicotine are achieved. The absorption of nicotine is largely dependent on the pH of the product–buccal interface. Other factors, such as the quantity of smokeless tobacco used, product flux, nicotine content of the product and the length of time that the product is in contact with the buccal membrane also determine the extent and rapidity of absorption. Once nicotine is absorbed at the buccal membrane, it enters the systemic circulation (avoiding first-pass hepatic metabolism) and is rapidly distributed throughout the body. Nicotine is cleared from the blood by hepatic metabolism to cotinine, *trans*-3′-hydroxycotinine and other products that are excreted in the urine. Considerable quantities of nicotine can be absorbed rapidly from smokeless tobacco products, which leads to reinforced feelings of euphoria, re-administration, neuroadaptation and compulsive use that are the hallmark characteristics of drugs that produce dependence.

Since malnutrition is a problem in many countries where the use of smokeless tobacco is highly prevalent, experimental studies in rats have focused on dietary modulation of the effects of smokeless tobacco and have shown that smokeless tobacco is more toxic to rats fed vitamin- or protein-deficient diets than to animals fed healthy diets. Remarkably, the activity of phase I enzymes involved in the bioactivation of xenobiotics was increased, while that of detoxification enzymes was decreased after chronic exposure to smokeless tobacco products in the diet.

In experimental systems, exposure to smokeless tobacco products was associated with the generation of reactive oxygen species, modulation of inflammatory mediators, inhibition of collagen synthesis and impairment of DNA repair capacity.

Smokeless tobacco products deliver nicotine in quantities and at rates that cause psychoactive effects, which eventually lead to tolerance and addiction. All of the currently recognized criteria to establish that a drug produces dependence are fulfilled in the case of smokeless tobacco products, which are psychoactive and induce a compulsive pattern of use. On discontinuation of use, drug craving and other signs of drug withdrawal are evident. Furthermore, there is a high rate of relapse among people who attempt to quit smokeless tobacco products. The effects of the use and discontinuation of use of smokeless tobacco products are similar to those of nicotine delivered through cigarette smoking. It was concluded that addiction to smokeless tobacco is analogous to addiction to nicotine.

The pathology of soft-tissue lesions in the mouth associated with the use of smokeless tobacco indicates features of premalignancy and neoplasia at the site of application. In

studies in human volunteers, application of smokeless tobacco products produced morphological changes, and white and erythematous lesions of oral mucosa. In experimental systems *in vitro*, smokeless tobacco products have been shown to affect inflammatory mediators, cell proliferation and apoptosis.

The evidence on the risk for cardiovascular disease from smokeless tobacco use is limited. Three cohort studies observed statistically significant increased risks for mortality from cardiovascular disease, with increased risks for both coronary heart disease and stroke, while four other cohort and case–control studies observed no significant increased risks for particular cardiovascular disease outcomes. Most of these studies suffer from important limitations. Evidence on most subclinical cardiovascular end-points is similarly inconclusive, although smokeless tobacco clearly causes acute increases in blood pressure and heart rate. A small increase in the risk for cardiovascular disease from smokeless tobacco use is certainly possible and, because of the high background rates of cardiovascular disease, even a small increase in relative risk could represent a large public health impact in countries that have a high prevalence of smokeless tobacco use.

The data on smokeless tobacco use and insulin resistance, glucose intolerance and diabetes are limited and the results are inconsistent. Effects on insulin sensitivity, glucose tolerance and the risk for diabetes from smokeless tobacco use are plausible, however, based on some positive results seen in the available studies of smokeless tobacco and nicotine. Diabetic smokeless tobacco users, in particular, may be at increased risk for aggravated insulin resistance.

The use of smokeless tobacco causes reproductive and developmental toxicity. In humans, the use of smokeless tobacco during pregnancy increases the risks for pre-eclampsia and premature birth, causes increased placental weight and reduces mean birth weight. Smokeless tobacco use by men causes reduced semen volume, reduced sperm count, reduced sperm motility and an increased frequency of abnormal spermatozoa.

In pregnant mice, extracts of moist snuff caused increased placental weights, reduced fetal weights, retarded fetal skeletal ossification and increased the rate of fetal resorption. Infant mice exposed translactationally to smokeless tobacco extracts had depressed levels of hepatic glutathione *S*-transferase, depressed hepatic thiol content and increased cytochrome P450 levels.

Elevated micronucleus formation, sister chromatid exchange and chromosomal aberrations have been reported in the oral exfoliated cells of consumers of smokeless tobacco, and TP53 protein accumulation and mutations have been reported in their oral premalignant lesions and squamous-cell carcinomas. These mutations include G→A and C→T transitions and G→T transversions. Mutations in *H-RAS* and $p21^{waf1}$ and other alterations in gene expression were also observed in oral premalignant lesions and squamous-cell carcinomas of smokeless tobacco consumers.

In a study of dietary modulation, the urine of rats that received an intraperitoneal dose of smokeless tobacco extract was mutagenic in *Salmonella typhimurium*. The level of urinary mutagenicity was higher in rats fed vitamin- and protein-deficient diets than in animals fed a normal diet.

Numerous studies in different types of prokaryotic and eukaryotic cells *in vitro* have reported the mutagenicity and clastogenicity of aqueous and organic extracts of a variety of smokeless tobacco products, including Yemeni snuff, Swedish moist oral snuff and various types of American and Indian chewing tobacco.

A few studies have examined the effects of smokeless tobacco and tobacco-specific nitrosamines on viral infection. These substances enhanced cell transformation by herpes simplex virus type 1 and inhibited replication of the virus in the oral cavity.

5.5 Evaluation

There is *sufficient evidence* in humans for the carcinogenicity of smokeless tobacco. Smokeless tobacco causes cancers of the oral cavity and pancreas.

There is *sufficient evidence* in experimental animals for the carcinogenicity of moist snuff.

Overall evaluation

Smokeless tobacco is *carcinogenic to humans (Group 1).*

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SOME TOBACCO-SPECIFIC *N*-NITROSAMINES

SOME TOBACCO-SPECIFIC *N*-NITROSAMINES

Four tobacco-specific *N*-nitrosamines (TSNA), namely, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), *N*′-nitrosonornicotine (NNN), *N*′-nitrosoanabasine (NAB) and *N*′-nitrosoanatabine (NAT) were considered by a previous Working Group in October 1984 (IARC, 1985). Since that time, new data have become available and are presented in this monograph.

In addition to these compounds, new TSNA have been identified (Figure 1) and their concentrations in tobacco and tobacco smoke have been assessed. The occurrence of 4-(methylnitrosamino)-4-(3-pyridyl)butyric acid (*iso*-NNAC), 4-(methylnitrosamino)-4-(3-pyridyl)-1-butanol (*iso*-NNAL) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) in tobacco is reported in the monograph on Smokeless Tobacco; however, as a result of the limited data available, these TSNA have not been considered in the present evaluation.

This monograph does not consider the exposure of workers involved in the production of these compounds, which are used solely for laboratory research purposes.

1. Exposure Data

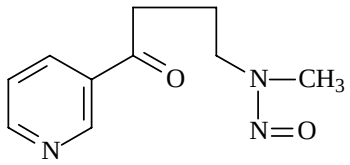
4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)

1.1 Chemical and physical data

1.1.1 *Synonyms and trade names*

Chem. Abstr. Services Reg. No.: 64091-91-4
Chem. Abstr. Name: 1-Butanone, 4-(methylnitrosoamino)-1-(3-pyridinyl)-
IUPAC Systematic Name: 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone
Synonym: 4-(*N*-Methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone

1.1.2 *Structural and molecular formulae and relative molecular mass*



$C_{10}H_{13}N_3O_2$ Relative molecular mass: 207.2

1.1.3 *Chemical and physical properties of the pure substance*

From Toronto Research Chemicals (2005, 2006), unless otherwise specified

(*a*) *Description:* Light-yellow crystalline solid
(*b*) *Melting-point:* 61–63 °C
(*c*) *Spectroscopy data*: Infrared, nuclear magnetic resonance and mass spectra have been reported (IARC, 1985).
(*d*) *Solubility*: Soluble in dichloromethane, dimethyl sulfoxide (DMSO), dimethylfuran, ethyl acetate and methanol
(*e*) *Stability*: Sensitive to light

N′-Nitrosonornicotine (NNN)

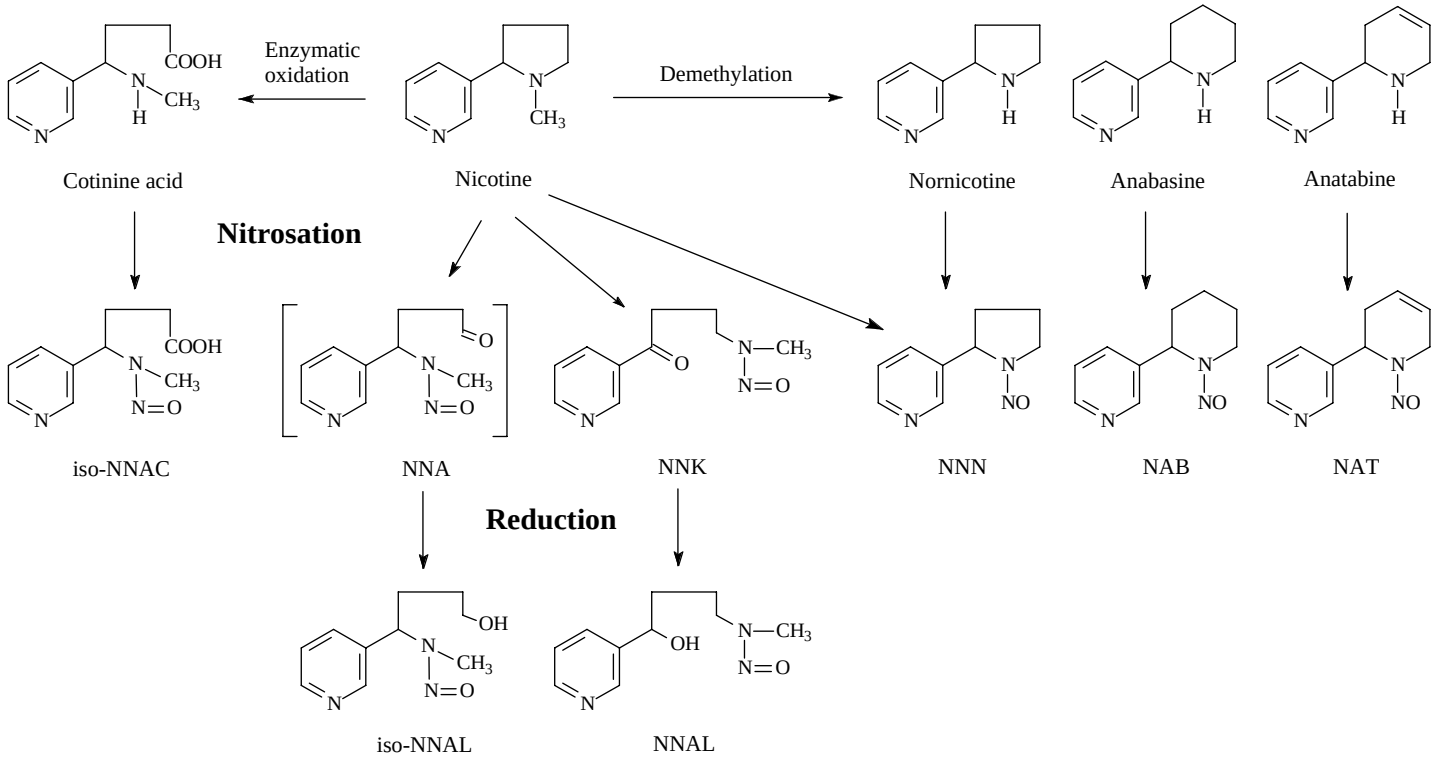
1.1 Chemical and physical data

1.1.1 *Synonyms and trade names*

Chem. Abstr. Services Reg. Nos: 80508-23-2; 16543-55-8[1]; 84237-38-7[2]
Chem. Abstr. Names: Pyridine, 3-(1-nitroso-2-pyrrolidinyl)-; pyridine, 3-(1-nitroso-2-pyrrolidinyl)-,(S)-[1]; pyridine, 3-(1-nitroso-2-pyrrolidinyl)-, (+,–)-[2]
IUPAC Systematic Name: 1′-Demethyl-1′-nitrosonicotine
Synonyms: 1′-Demethyl-1′-nitrosonicotine; 1′-desmethyl-1′-nitrosonicotine; 1′-nitroso-1′-demethylnicotine; nitrosonornicotine; *N*-nitrosonornicotine; 1′-nitrosonornicotine; 1-nitroso-2-(3-pyridyl)pyrrolidine; 3-(1-nitroso-2-pyrrolidinyl)pyridine

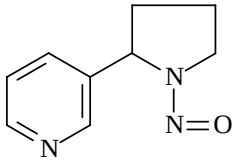
[1] The Chemical Abstracts Services Registry Number and Name refer to the (S) stereoisomer.
[2] The Chemical Abstracts Services Registry Number and Name refer to the racemic mixture that was synthesized and used in the biological studies reported in this monograph.

Figure 1. Formation of tobacco-specific *N*-nitrosamines



From Hoffmann *et al.* (1995)
iso-NNAC, 4-(methylnitrosamino)-4-(3-pyridyl)butyric acid; *iso*-NNAL, 4-(methylnitrosamino)-4-(3-pyridyl)-1-butanol; NAB, *N*′-nitrosoanabasine; NAT, *N*′-nitrosoanatabine; NNA, 4-(methylnitrosamino)-4-(3-pyridyl)butanal; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, *N*′-nitrosonornicotine
Note: NNA is a very reactive aldehyde and has therefore never been quantified in tobacco or tobacco smoke.

1.1.2 *Structural and molecular formulae and relative molecular mass*



$C_9H_{11}N_3O$ Relative molecular mass: 177.2

1.1.3 *Chemical and physical properties of the pure substance*

From Toronto Research Chemicals (2005, 2006), unless otherwise specified
(*a*) *Description*: Light-yellow oil
(*b*) *Boiling-point*: 154 °C at 0.2 mm (IARC, 1985)
(*c*) *Melting-point*: 47 °C (IARC, 1985); 42–45 °C
(*d*) *Spectroscopy data*: Mass, ultraviolet, infrared and nuclear magnetic resonance spectra have been reported (IARC, 1985).
(*e*) *Solubility*: Soluble in acetone and chloroform
(*f*) *Stability*: Hygroscopic

N′-Nitrosoanabasine (NAB)

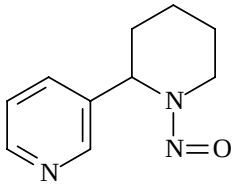
1.1 Chemical and physical data

1.1.1 *Synonyms and trade names*

Chem. Abstr. Services Reg. Nos: 37620-20-5; 1133-64-8[1]; 84237-39-8[2]
Chem. Abstr. Names: Pyridine, 3-(1-nitroso-2-piperidinyl)-; pyridine, 3-(1-nitroso-2-piperidinyl)-,(S)-[1]; pyridine, 3-(1-nitroso-2-piperidinyl), (+,–)-[2]
UPAC Systematic Name: 1-Nitrosoanabasine
Synonym: N-Nitrosoanabasine

[1] The Chemical Abstracts Services Registry Number and Name refer to the (S) stereoisomer.
[2] The Chemical Abstracts Services Registry Number and Name refer to the racemic mixture that was synthesized and used in the biological studies reported in this monograph.

1.1.2 *Structural and molecular formulae and relative molecular mass*



$C_{10}H_{13}N_3O$ Relative molecular mass: 191.2

1.1.3 *Chemical and physical properties of the pure substance*

From Toronto Research Chemicals (2005, 2006), unless otherwise specified

(*a*) *Description*: Yellow oil
(*b*) *Boiling-point*: 162 °C at 1 mm Hg (IARC, 1985); 165–167 °C at 0.25 mm Hg
(*c*) *Optical rotation*: The specific rotation of NAB has been reported (IARC, 1985)
(*d*) *Spectroscopy data*: Infrared, ultraviolet, nuclear magnetic resonance and mass spectra have been reported (IARC, 1985).
(*e*) *Solubility*: Soluble in chloroform and dichloromethane

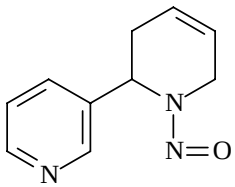
N′-Nitrosoanatabine (NAT)

1.1 Chemical and physical data

1.1.1 *Synonyms and trade names*

Chem. Abstr. Services Reg. No.: 71267-22-6
Chem. Abstr. Name: 2,3′-Bipyridine, 1,2,3,6-tetrahydro-1-nitroso-
IUPAC Systematic Name: 1,2,3,6-Tetrahydro-1-nitroso-2,3-bipyridine
Synonym: NAtB

1.1.2 *Structural and molecular formulae and relative molecular mass*



$C_{10}H_{11}N_3O$ Relative molecular mass: 189.2

1.1.3 *Chemical and physical properties of the pure substance*

From Toronto Research Chemicals (2005, 2006), unless otherwise specified

(*a*) *Description:* Pale-yellow oil
(*b*) *Boiling-point*: 176 °C at 0.5 mm Hg (IARC, 1985)
(*c*) *Spectroscopy data*: Infrared, mass and nuclear magnetic resonance spectra have been reported (IARC, 1985).
(*d*) *Solubility*: Soluble in chloroform, ethanol and methanol
(*e*) *Stability*: Sensitive to light

1.2 Technical products and impurities, analysis, production and use

1.2.1 *Technical products and impurities*

NNK, NNN, NAB and NAT are available commercially at a purity of 98%, in units of up to 1 g (Toronto Research Chemicals, 2006). NNK and NNN isolated from tobacco are mixtures of 72.7% E-isomer and 27.3% Z-isomer (Hecht *et al.*, 1977; Hoffmann *et al.*, 1980).

1.2.2 *Analysis*

Standard methods for the analysis of NNK, NNN, NAB and NAT have been described previously (Egan *et al*., 1983). Since that time, numerous studies on the levels of TSNA in tobacco and tobacco smoke have included descriptions of methods for the extraction and quantification of TSNA (Andersen *et al*., 1989; Djordjevic *et al*., 1989a; Fischer & Spiegelhalder, 1989; Fischer *et al*., 1989a; Spiegelhalder *et al*., 1989; Sharma *et al*., 1991; Stepanov *et al*., 2002; Ashley *et al*., 2003; Jansson *et al*., 2003; Wu *et al*., 2003, 2004; Stepanov *et al*., 2005; Wu *et al*., 2005; Stepanov *et al*., 2006a).

Risner *et al*. (2001) reported a collaborative investigation of methods for the determination of TSNA in tobacco. Seventeen laboratories around the world participated in this study and used seven different methods or variations thereof. The methods varied in sample preparation, conditions of analysis, compound detection and quantification of results. Morgan *et al*. (2004) reported the results from a collaborative study carried out by 15 different laboratories that used two different methods to determine TSNA. Both methods proved to be efficient for the determination of TSNA in a variety of tobacco types.

Methods for the assessment of NNAL, *iso*-NNAL and *iso*-NNAC in tobacco have also been described (Brunnemann *et al*., 1987a; Djordjevic *et al*., 1989a; Djordjevic *et al*., 1993a).

Methods for determination of TSNA in mainstream tobacco smoke are also available on various websites, such as those from Health Canada (http://www.hc-sc.gc.ca/hl-vs/tobac-tabac/legislation/reg/indust/method/index_e.html) or from the United Kingdom Government Benchmark Study (http://www.the-tma.org.uk/benchmark/).

1.2.3 *Production*

NNK was first prepared by the reaction of sodium hydroxide and sodium nitrite with 4-(*N*-methyl)-1-(3-pyridyl)-1-butanone dihydrochloride (IARC, 1985). NNK is currently produced commercially (Toronto Research Chemicals, 2005, 2006).

NNN was first prepared by treating nornicotine with sodium nitrite in dilute hydrochloric acid (IARC, 1985). NNN is currently produced commercially (Toronto Research Chemicals, 2005, 2006).

NAB was first prepared by treating anabasine with sodium nitrite in dilute hydrochloric acid solution (IARC, 1985). NAB is currently produced commercially (Toronto Research Chemicals, 2005, 2006).

NAT was first prepared by the reaction of sodium nitrite with a solution of anatabine in hydrochloric acid (IARC, 1985). NAT is currently produced commercially (Toronto Research Chemicals, 2005, 2006).

iso-NNAC was synthesized from cotinine *via* 4-(methylamino)-4-(3-pyridyl)butyric acid and *N*-nitrosated. Currently, *iso*-NNAC, *iso*-NNAL and NNAL are also produced commercially as well (Toronto Research Chemicals, 2005, 2006).

1.2.4 *Use*

No evidence was found that NNK, NNN, NAB or NAT have ever been used other than in laboratory research that included animal studies (Hoffmann *et al*., 1993a; Hecht, 1998).

1.3 Occurrence

The wide disparity in levels of TNSA in the mainstream smoke of cigarettes (Counts *et al*., 2005; Gregg *et al.*, 2005; King *et al*., 2007) and in smokeless tobacco products (Hoffmann *et al*., 1995; Stepanov *et al*., 2006a) is largely due to differences in the tobacco types used in any given product, agricultural practices, curing methods and manufacturing processes (IARC, 2004). While trace amounts of TSNA were measured in green tobacco leaves (Bhide *et al*., 1987; Djordjevic *et al*., 1989b), these compounds are formed from their alkaloid precursors and from nitrite or nitrate predominantly during tobacco curing, fermentation and ageing (Burton *et al*., 1989a,b; Djordjevic *et al*., 1993a). The type of tobacco (e.g., Burley, Bright, Virginia or Oriental), its nitrate and nitrite content, the mode of curing (e.g. air-, flue- or sun-curing) and the various steps of processing used are therefore the determining factors for the yields of TSNA in tobacco (Fischer *et al.*, 1989a; Bush *et al*., 2001; Peele *et al*., 2001). NNN, NAB and NAT are formed primarily from their corresponding secondary amines (namely nornicotine, anatabine and anabasine) in the early stages of tobacco curing and processing whereas the majority of NNK and some NNN are formed from the tertiary amine nicotine at the later stages of tobacco curing and fermentation (Spiegelhalder & Fischer, 1991).

Levels of NNN and other TSNA are consistently higher in Burley than in Bright tobacco, regardless of the curing method (Chamberlain & Chortyk, 1992; Morgan *et al*., 2004). However, flue-curing of Bright tobacco produces nearly three times more TSNA than air-curing of the same tobacco. TSNA content varies between the parts of the plant. For example, NNK and NNN contents are higher in the midrib than in the lamina in air-cured tobacco, whereas the converse is observed in flue-cured tobacco. Flue-curing of Burley tobacco reduces the alkaloid content but greatly increases TSNA content in the lamina. Midribs from air-cured Burley leaves contain three times the TSNA concentrations of the lamina (Chamberlain & Chortyk, 1992). Analyses of 41 leaf segments from a dark air-cured tobacco variety (KY 171) revealed that concentrations of the individual nitrosamines were lowest at the tip and the periphery of the leaf (Burton *et al*., 1992). The midvein contained the lowest concentrations of NNK, NNN and NAT at two-thirds of the length of the leaf; the highest concentrations were found at the base of the leaf. The correlation between nitrite nitrogen and TSNA was higher than that between alkaloid content and TSNA in the leaf.

DeRoton *et al*. (2005) summarized the major factors involved in the formation of TSNA in dark air-cured and Burley tobacco during curing and post-curing treatment. The main genetic trait involved in the formation of TSNA is the propensity of a variety of tobacco to convert nicotine to nornicotine. In addition, the ability of a variety to lose water rapidly limits the formation of nitrite and hence also the formation of TSNA. Since TSNA are derived from tobacco alkaloids, agricultural practices that increase alkaloid concentrations in the tobacco leaves favour their formation. Air flow at the site of curing also influences the levels of TSNA: leaves that are cured in well-ventilated curing structures, such as plastic sheds, generally contain lower amounts of TSNA than those cured in barns. Levels of TSNA may increase after curing if tobacco leaves are stored under humid conditions (Staaf *et al*., 2005) or in bales. The biological mechanism for TSNA formation in air-cured tobacco relates to the breakdown of plant cell membranes due to moisture loss, which makes the cell contents available to microorganisms that produce nitrite. That is, microbes generate nitrite as a by-product, and this becomes available to react with alkaloids to form TSNA. When curing is begun with relatively high humidity at the yellowing phase followed by a pronounced decline as cell breakdown begins, the TSNA content in tobacco will be lower. A uniform air-flow increases the rate and amount of moisture loss from the tobacco, and also reduces possible gas-phase reactions between alkaloids and gaseous nitric oxides.

Microorganisms appear to play a lesser role in TSNA formation in flue-cured tobacco. Morin *et al*. (2004) found that microbial populations were inversely correlated with concentrations of TSNA and with temperature. However, TSNA concentrations increased as temperature increased during curing. This finding supports the hypothesis that TSNA could result from the reaction of combustion gases (e.g. nitrogen oxides) with tobacco alkaloids during flue-curing. Removal of heating with propane as part of the curing process has been shown to reduce the levels of NNK and NNN substantially (Peele *et al*., 2001; IARC, 2004).

In summary, a lesser degree of fertilization, particularly with products that contain nitrates, and careful manipulation of curing parameters and tobacco blending can lower the level of nitrosamines in tobacco products.

1.3.1 *Fresh tobacco*

The mean concentrations of NNK, NNN and NAT in the green leaves harvested at all stalk positions from the flue-cured tobacco plant NC-95 were 280, 260 and 790 ng/g dry tobacco, respectively (Djordjevic *et al.*, 1989b), and were six times higher in cured tobacco (namely 1810, 1560 and 6670 ng/g dry tobacco, respectively).

Bhide *et al.* (1987) reported the presence of NNK and NNN in green leaves of *N. tabacum* and *N. rustica* species grown in India in two different seasons. In one season, mature green leaves of *N. rustica* contained up to 2340, 46 100, 5200 and 23 700 ng/g tobacco (dry wt) NNK, NNN, NAB and NAT, respectively. One year later, tobacco harvested at the same location contained 352 ng/g tobacco NNK and 5730 ng/g tobacco NNN. These levels rose to 25 800 ng/g tobacco NNK and 15 000 ng/g tobacco NNN in sun-dried tobacco. In comparison, the levels of TSNA in sun-dried *N. tabacum* species grown in the same area during the same seasons were 37 ng/g tobacco NNK and 49 ng/g tobacco NNN.

1.3.2 *Cured tobacco*

A wide range of TSNA concentrations are found in cured tobacco, regardless of the type (Table 1). In each category of tobacco type, the range reflects the diversity of the tobacco variety, production year, climate, country of origin, agricultural practices including fertilization, post-harvesting and curing technologies, post-curing handling and storage conditions, as well as the analytical methods used and the reporting of the analytical results (e.g. ng/g dry tobacco wt versus ng/g wet tobacco wt).

The levels of total TSNA are highest in air-cured Burley tobacco and lowest in sun-cured Oriental (Turkish) tobacco. The highest levels of NNN were reported in Burley laminae and midribs (up to 8620 and 9080 ng/g dry tobacco, respectively). The highest reported concentrations of NNN were 1700 ng/g dry wt in flue-cured Bright tobacco and 420 ng/g dry wt in sun-cured Oriental tobacco. MacKown *et al*. (1988) also reported levels of NNN up to 3400 ng/g dry wt in reconstituted tobacco sheets that are used in cigarette blends.

The highest levels of NNK were reported in midribs of Burley tobacco (6660 ng/g dry tobacco) and laminae of Bright (Virginia) tobacco (2690 ng/g dry tobacco). It should be noted that levels of NNK in Burley midribs exceed those in the laminae (6600 versus 1370 ng/g dry wt). It should also be noted that NNK is a predominant TSNA in Bright tobacco (2690 ng NNK compared with 1370 ng NNN) while NNN is predominant in Burley tobacco (1370 ng NNK compared with 8620 ng NNN). Similarly to NNN, NAB and NAT are formed by nitrosation of a respective secondary amine alkaloid precursor

Table 1. The concentration ranges of tobacco-specific *N*-nitrosamines in cured tobacco produced worldwide

Tobacco type	Reported as[a]	NNK	NNN	NAB	NAT	NAB + NAT	Reference
Oriental (Turkish)	Dry	ND–83[b]	20–420	50	20–170		Djordjevic *et al.* (1991); Morgan *et al.* (2004)
	Wet	ND–70[c]	20–460			20–200	Fischer *et al.* (1989a)
Bright (Virginia)	Dry	160–2690	240–1700	20–150	280–6670		Djordjevic *et al.* (1989b); Risner *et al.* (2001); Morgan *et al.* (2004)
	Wet	30–1100	10–600			30–950	Fischer *et al.* (1989a)
Burley (laminae)	Dry	ND–1370	1070–8620	200–223	4270–19 700		MacKown *et al.* (1988); Burton *et al.* (1989a); Risner *et al.* (2001); Morgan *et al.* (2004)
	Wet	100–1400	1300–8850			500–3600	Fischer *et al.* (1989a)
Burley (stems/midrib)	Dry	ND–6660	1500–9080	155	1800–20 940		MacKown *et al.* (1988); Burton *et al.* (1989a); Morgan *et al.* (2004)
Reconstituted tobacco sheets	Dry	490	3400		2500		MacKown *et al.* (1988)

NAB, *N*′-nitrosoanabasine; NAT, *N*′-nitrosoanatabine; ND, not detected; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, *N*′-nitrosonornicotine

[a] Dry, ng/g dry wt; wet, ng/g wet wt

[b] Detection limit for NNK, < 10 ng/g tobacco

[c] Detection limit for NNK, < 50 ng/g tobacco

(Figure 1), and their accumulation in tobacco follows that of NNN: the highest concentrations were reported in Burley tobacco and the lowest in Oriental tobacco.

Some authors did not report concentrations of NAB and NAT separately but as a sum (Fischer *et al*., 1989a). In some reports, TSNA values are expressed per dry tobacco wt while values are not adjusted for the moisture content in others. The variability of the data thus reflects not only diversity of tobacco specimens but also of analytical methods and instrumentation. There is an urgent need for standardization and validation of analytical methods for measurements of TSNA in tobacco, including reporting of results, to permit a meaningful comparison of data.

1.3.3 *Cigarette tobacco*

Table 2 presents a comparison of concentrations of NNK, NNN, NAB and NAT in tobacco from commercial cigarettes sold worldwide. NNK ranged from not detected to 10 745 ng/cigarette in cigarettes sold in Italy (Fischer *et al*., 1990a). NNK was not detected in some cigarettes sold in the Central Europe, Middle East and Africa region, the European Union and the former USSR (Fischer *et al.*, 1990a; Djordjevic *et al.*, 1991; Counts *et al*., 2005). High concentrations of NNK were also reported for cigarettes sold in India (4800 ng/g dry tobacco), the USA (1760 ng/g wet tobacco) and France (1530 ng/g dry tobacco).

NNN ranged from 20 ng/g dry tobacco for cigarettes sold in the former USSR (Djordjevic *et al*., 1991) to 58 000 ng/g dry tobacco for cigarettes from India (Nair *et al*., 1989). High concentrations of NNN were also reported in cigarettes sold in France (18 600 ng/g dry tobacco), the USA (up to 7900 ng/g dry tobacco), Germany (up to 5340 ng/cigarette), Poland (up to 4870 ng/g dry tobacco), Japan (up to 3892 ng/g dry tobacco), Norway (3736 ng/g dry tobacco) and Malaysia (up to 3350 ng/g dry tobacco).

NAB ranged from not detectable levels to 322 ng/g dry tobacco. The highest amount was measured in Philip Morris cigarettes marketed in the Central Europe, Middle East and Africa region (Counts *et al*., 2005).

NAT ranged from 20 to 15 100 ng/g dry tobacco. The lowest concentrations were reported for cigarettes from the former USSR (Fisher *et al*., 1990a; Djordjevic *et al*., 1991) and the highest concentration for an Indian cigarette (Nair *et al*., 1989). High levels of NAT were also reported in cigarettes from France, the Central Europe, Middle East and Africa region, the European Union, Japan, Taiwan (China) and the USA.

Ashley *et al*. (2003) measured TSNA in tobacco from cigarettes purchased in 21 countries. US brands of cigarettes marketed worldwide generally had higher levels than popular local cigarettes in many countries.

Higher TSNA concentrations were generally measured in the tobacco from non-filter cigarettes, especially those made of dark tobacco (Fischer *et al*., 1989a; Tricker *et al*., 1991). Among the 55 brands sold in Germany in 1987 (Table 3), the lowest amounts of NNK and NNN were measured in cigarettes made from Oriental tobacco (NNK, not detectable to 177 ng/cigarette; NNN, 45–432 ng/cigarette), followed by cigarettes made with Virginia tobacco (NNK, 170–580 ng/cigarette; NNN, 133–330 ng/cigarette) and

Table 2. International comparison of the concentration ranges for preformed tobacco-specific *N*-nitrosamines in tobacco from commercial cigarettes

Country	Reported as[a]	NNK	NNN	NAB	NAT	NAB + NAT	Reference
Argentina	Dry	812	1866	357	1559		Counts *et al.* (2005)
Australia	Dry	490–1193	420–2888	NQ[b]–207	715–2366		Counts *et al.* (2005)
Austria	ng/cig	92–310	306–1122				Fischer *et al.* (1990a)
Belgium	ng/cig	219–594	504–1939				Fischer *et al.* (1990a)
Canada	ng/cig	447–884	259–982			564–1017	Fischer *et al.* (1990b)
CEMA	Dry	NQ[b]–1127	1094–3739	NQ[b]–322	1014–2989		Counts *et al.* (2005)
European Union	Dry	NQ[b]–860	332–2736	NQ[b]–262	423–2253		Counts *et al.* (2005)
France	Dry ng/cig	260–1530 57–990	4770–18 600 120–6019	100	1200–9970		Djordjevic *et al.* (1989a); Ohshima *et al.* (1985); Fischer *et al.* (1990a)
Germany	ng/cig Dry	ND–1120[c] 445–469	45–5340 1355–1361	 NQ[b]	 1142–1207	ND–2490[c]	Fischer *et al.* (1989a, 1990a); Tricker *et al.* (1991); Counts *et al.* (2005)
India	Dry ng/cig	40–4800 19–174	1300–58 000 68–730		800–15 100	 98–519[c]	Nair *et al*. (1989); Kumar *et al.* (1991)
Italy	ng/cig	153–10 745	632–12 454				Fischer *et al.* (1990a)
Japan	Dry	190–1171	360–3892	NQ[b]–320	300–3139		Djordjevic *et al.* (2000a); Counts *et al.* (2005)
Malaysia	Dry	434–923	2223–3350	NQ[b]–176	1302–2170		Counts *et al.* (2005)
Moldova	Wet	104–942	93–2090	ND–75	55–1290		Stepanov *et al.* (2002)
Netherlands	ng/cig	105–587	58–1647				Fischer *et al.* (1990a)
Norway	Dry	1124	3736	379	2945		Counts *et al.* (2005)

Table 2 (contd)

Country	Reported as[a]	NNK	NNN	NAB	NAT	NAB + NAT	Reference
Poland	Dry ng/cig	70–660 140–450	670–4870 870–2760				Fischer *et al.* (1990a); Djordjevic *et al.* (2000b)
Sweden	ng/cig	192–569	544–1511				Fischer *et al.* (1990a)
Switzerland	ng/cig	450–554	1280–2208				Fischer *et al.* (1990a)
Taiwan	Dry	1194	3769	195	2883		Counts *et al.* (2005)
United Kingdom	ng/cig	92–433	140–1218				Fischer *et al.* (1990a)
USA	Dry Wet ng/cig	420–1270 1410–1760 433–733	880–7900 2590–4300 993–1947	ND–212 100–140	880–5810 1610–2660		Ohshima *et al.* (1985); Djordjevic *et al.* (1990); Fischer *et al.* (1990a); Djordjevic *et al.* (2000a); Stepanov *et al.* (2002); Counts *et al.* (2005)
Former USSR	Dry ng/cig	ND–40[d] ND–150[c]	20–420 60–850		20–170		Fischer *et al.* (1990a); Djordjevic *et al.* (1991)
Venezuela	Dry	591	2170	244	1339		Counts *et al.* (2005)

Adapted from IARC (2004)

CEMA, Central Europe, Middle East, Africa; NAB, *N*′-nitrosoanabasine; NAT, *N*′-nitrosoanatabine; ND, not detected; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, *N*′-nitrosonornicotine; NQ, not quantifiable

[a] Dry, ng/g dry tobacco; ng/cig, ng/cigarette; wet, ng/g wet tobacco

[b] Limit for quantitation; NNK, 272 ng/g; NNN, 180 ng/g; NAB, 103 ng/g; NAT, 213 ng/g

[c] Detection limit for NNK, < 50 ng/cigarette; for NNN and for NAB + NAT < 25 ng/cigarette

[d] Detection limit for NNK, < 10 ng/g tobacco

Table 3. International comparison of the concentration ranges of preformed tobacco-specific *N*-nitrosamines in tobacco from commercial cigarettes with a wide range of ISO/FTC nicotine and 'tar' mainstream smoke yields[a]

Country (total no. of cigarette brands in the study)	Reported as[b]	Tobacco filler	F/NF	NNK	NNN	NAB	NAT	NAB + NAT	Reference
Canada (25)	ng/cig	Ultra-low yield (V)	F	447–785	288–982			666–1017	Fischer *et al.* (1990b)
		Low yield (V)	F	510–884	292–527			586–978	
		Moderate yield (V)	F	569–705	337–407			666–779	
		High yield (V)	F	495–663	259–381			564–758	
Germany (20)	ng/cig	Blend	F	100–410	400–1390			220–1340	Tricker *et al.* (1991)
		Blend	NF	270–500	660–2670			460–1110	
		Dark	NF	800–960	4500–5340			1650–2330	
(55)		Oriental	F + NF	ND–177	45–432			ND–575	Fischer *et al.* (1989a,b)
		Virginia	F + NF	170–580	133–330			253–630	
		American blend	F	160–696	500–2534			440–2490	
		Dark	NF	370–1120	3660–5316			266–315	
Japan (6)	Dry	Low yield	F	190–330	810–1110	30–60	410–660		Djordjevic *et al.* (2000a)
		Medium yield	F	200–320	360–1040	30–70	300–620		
USA (13)	Dry	Ultra-low yield (AB)	F	500–580	1750–1980	ND	970–1080		Djordjevic *et al.* (1990, 2000a)
		Low yield (AB)	F	490–800	1900–3050	90–120	1030–1670		
		Moderate yield (AB)	F	420–890	1780–2890	70–110	1030–1680		
		High yield (AB)	NF	770–920	1290–2160	40–110	920–1170		
(11)	Wet	Quest 1,2,3[c]	F	54–190	820–930	3–13	43–310		Stepanov *et al.* (2006a)
		Ultra-low yield (AB)	F	750–770	2800–2900	55–58	1100–1200		
		Low yield (AB)	F	550–680	2700–2800	51–61	1100–1300		
		Moderate yield (AB)	F	580–960	1100–2900	25–100	560–2300		

Table 3 (contd)

Country (total no. of cigarette brands in the study)	Reported as[b]	Tobacco filler	F/NF	NNK	NNN	NAB	NAT	NAB + NAT	Reference
Philip Morris commercial brands (39)	Dry	Ultra-low yield (AB)	F	NQ[d]–1171	420–3892	NQ[d]–262	715–3139		Counts *et al.* (2005)
		Low yield (AB)	F	NQ[d]–1270	332–3438	NQ[d]–322	423–2676		
		Moderate yield (AB)	F	NQ[d]–1194	868–3769	NQ[d]–379	778–2945		

AB, American blend cigarettes; F, filter-tipped cigarettes; NAB, *N*′-nitrosoanabasine; NAT, *N*′-nitrosoanatabine; ND, not detected; NF, non-filtered cigarettes; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, *N*′-nitrosonornicotine; NQ, not quantifiable; V, Virginia-type cigarettes

[a] Cigarettes were designated into classes based either on ISO/FTC nicotine smoke yields (Djordjevic *et al.*, 2000a) or ISO/FTC 'tar' smoke yields (IARC, 1986).

[b] Dry, ng/g dry tobacco; ng/cig, ng/cigarette; wet, ng/g wet tobacco

[c] Nicotine-reduced cigarettes

[d] Limit for quantitation: NNK, 272 ng/g; NAB, 103 ng/g

American blend cigarettes (NNK, 160–696 ng/cigarette; NNN, 500–2534 ng/cigarette). The highest TSNA levels were reported in the dark tobacco cigarettes (NNK, 370–1120 ng/cigarette; NNN, 3660–5316 ng/cigarette) (Fischer *et al*., 1989a,b; Tricker *et al*., 1991).

Tobacco from ultra-low-, low-, medium- and high-yield cigarettes sold internationally, as ranked based on data obtained by the ISO/FTC (International Standardization Organization/Federal Trade Commission) machine-smoking method (IARC, 2004), contain similar amounts of preformed NNK and NNN regardless of the type of product (Table 3; Djordjevic *et al.*, 1990; Fischer *et al*., 1990b; Djordjevic *et al.*, 2000a; Counts *et al*., 2005; Stepanov *et al*., 2006a). For example, levels of NNK and NNN in the tobacco of Canadian ultra-low-yield cigarettes ranged from 447 to 785 ng/cigarette and from 288 to 982 ng/cigarette, respectively, whereas high-yield cigarettes contained 495–663 ng/cigarette NNK and 259–381 ng/cigarette NNN (Fischer *et al*., 1990b). Similar observation was reported for American blend cigarettes sampled in the USA (Djordjevic *et al*., 1990, 2000a; Stepanov *et al*., 2006a) or internationally (Counts *et al*., 2005). Tobacco from Canadian brands with a wide range of nicotine and tar yields as measured by the ISO/FTC method contained lower amounts of preformed NNN (up to 982 ng/cigarette; Fischer *et al*., 1990b) than US brands sold domestically or internationally (up to 3050 and 3892 ng/g dry tobacco, respectively; Djordjevic *et al*., 1990, 2000a; Counts *et al*., 2005). In contrast, NNK content in cigarette tobacco was of the same order of magnitude in the two countries (up to 884 ng/cigarette in Canadian cigarettes and up to 920 ng/cigarette in cigarettes sold in the USA). However, Philip Morris American blend cigarettes marketed worldwide contained the highest quantities of both NNK and NNN (up to 1270 and 3892 ng/g dry tobacco, respectively). Japanese and German cigarettes contained the lowest concentrations of preformed NNK (up to 330 ng/g dry tobacco).

Typically, levels of NNK in a given cigarette blend are lower than those of NNN except in cigarettes manufactured in Canada, the United Kingdom and Australia that use Bright Virginia flue-cured tobacco as a filler; in the latter brands, NNK is the dominant TSNA (Fischer *et al*., 1989a,b, 1990b).

Cigarettes manufactured and marketed globally contain a wide range of TSNA. The dose of TSNA delivered in mainstream smoke is largely determined by their levels in the tobacco blend (Fischer *et al*., 1990c; d'Andres *et al*., 2003). In addition, exposure to TSNA depends on the relationship between the product and how it is used, that is, on smoking patterns and intensity (Djordjevic *et al*., 2000c; Hecht *et al.*, 2005; Melikian *at al*., 2007a,b).

1.3.4 *Mainstream cigarette smoke*

Monitoring of tobacco smoke has historically been limited to the measurement of carbon monoxide emissions from cigarettes using the ISO/FTC machine-smoking protocol, which does not reflect the characteristics of human smoking. During the past two decades, many studies have reported the occurence of TSNA in mainstream smoke. Table 4

Table 4. International comparison of the concentration ranges (ng/cigarette) of tobacco-specific *N*-nitrosamines in the mainstream smoke of commercial cigarettes (ISO/FTC machine-smoking method)

Country	NNK	NNN	NAB	NAT	NAB + NAT	Reference
Argentina	79.5	99.6	14.5	106.9		Counts *et al.* (2005)
Australia	12.4–106.1 25.7[a,b]	5.0–151.8 22.4[a,b]	2.0–22.3	8.0–134.7		Counts *et al.* (2005) King *et al.* (2007)
Austria	12–100	42–172				Fischer *et al.* (1990a)
Belgium	29–150	38–203				Fischer *et al.* (1990a)
Canada	6–97 50.9[a,c]	4–37 25.6[a,c]			9–82	Fischer *et al.* (1990b) King *et al.* (2007)
CEMA	18.0–75.0	42.5–147.5	6.0–18.5	38.6–129.2		Counts *et al.* (2005)
European Union	12.7–78.4	14.8–126.0	2.0–17.9	18.0–106.8		Counts *et al.* (2005)
Germany	ND[d]–470	5–855	6.6–9.0	39.9–52.6	6.6–520	Fischer *et al.* (1989b, 1990a); Tricker *et al.* (1991); Counts *et al.* (2005)
France	18.3–498	11–1000	15.4–18.7	127–182		Djordjevic *et al.* (1989a); Fischer *et al.* (1990a)
India	TR–73	6–401		3.8–99.4	18–146	Nair *et al.* (1989); Kumar *et al.* (1991)
Italy	8–1749	21–1353				Fischer *et al.* (1990a)
Japan	18.6–87.1	36–146	6.8–21	40.3–138		Djordjevic *et al.* (1996); Counts *et al.* (2005)
Malaysia	45.0–87.3	63.4–195.3	7.8–28.5	55.7–153.3		Counts *et al.* (2005)
Netherlands	5–102	9–163				Fischer *et al.* (1990a)
Norway	103.9	189.4	18.6	160.4		Counts *et al.* (2005)
Poland	36–990	68–2830				Fischer *et al.* (1990a); Gray *et al.* (1998); Djordjevic *et al.* (2000b)

Table 4 (contd)

Country	NNK	NNN	NAB	NAT	NAB + NAT	Reference
Sweden	27–84	44–141				Fischer *et al.* (1990a)
Switzerland	69–124	121–226				Fischer *et al.* (1990a)
Taiwan (China)	96.2	161.2	24.7	142.8		Counts *et al.* (2005)
Thailand	16–369	28–730			43.5–483	Brunnemann *et al.* (1996)
United Kingdom	5.2–500	6.5–257.6	1.1–44.2	7.8–148		Fischer *et al.* (1990a); Gregg *et al*. (2005)
USA	3–425	6–1007	ND–34.7	6–250	102–744	Adams *et al*. (1987a); Fischer *et al*. (1990a); Djordjevic *et al.* (1990); Brunnemann *et al.* (1994); Djordjevic *et al*. (1996)
Former USSR	4–55	23–389		71–196		Fischer *et al.* (1990a); Djordjevic *et al.* (1991)
Venezuela	64.2	100.1	13.3	78.6		Counts *et al.* (2005)

Adapted from IARC (2004)

CEMA, Central Europe, Middle East, Africa; NAB, *N*′-nitrosoanabasine; NAT, *N*′-nitrosoanatabine; ND, not detected; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, *N*′-nitrosonornicotine; TR, trace amounts

[a] Reported as ng/mg tobacco

[b] Mean of 15 brands

[c] Mean of 21 brands

[d] Limit of detection for NNK, 40 ng/cigarette

presents an international comparison of TSNA concentration ranges in mainstream smoke of commercial cigarettes using the ISO/FTC machine-smoking method.

Similarly to cigarette tobacco filler, the levels of individual TSNA in mainstream smoke vary dramatically among products regardless of the country of origin. The emissions of NNK ranged from undetected to 1749 ng/cigarette (Italy; Fischer *et al*., 1990a). The concentrations of NNN ranged from 4 ng/cigarette (Canada) to 2830 ng/cigarette (Poland; Gray *et al*., 1998). The highest emissions of NNN were reported in the mainstream smoke of cigarettes sold in Germany (855 ng/cigarette), France (1000 ng/cigarette), the USA (1007 ng/cigarette), Italy (1353 ng/cigarette) and Poland (2830 ng/cigarette). The concentrations of NAB ranged from undetected to 44.2 ng/cigarette and those of NAT from 6 to 250 ng/cigarette.

The lowest emissions of TSNA were measured in the mainstream smoke from blended cigarettes sold in Australia, Austria, Canada, Japan, the Netherlands and Sweden, with upper values of 66–106 ng NNK. Surprisingly, levels of NNK and NNN in the mainstream smoke of two cigarette brands from India were very low despite the extremely high levels of preformed nitrosamines in tobacco (Nair *et al*., 1989).

A comparative assessment of the composition of mainstream smoke from three popular brands of filter-tipped cigarette from the USA that were purchased on the open market in 29 countries worldwide showed from three- to ninefold differences in the yields of NNK and NNN within each brand. Yields of NNK and NNN were highly correlated (r = 0.88; Gray *et al*., 2000).

The parameters that affect smoke yields and compositions have recently been reviewed (Hoffman *et al*., 2001; Borgerding & Klus, 2005). Measurements of smoke yields using the ISO/FTC machine-smoking method do not provide information that is representative of the exposure of a smoker. Machine-smoking protocols other than the ISO/ FTC protocol have been examined, particularly those that have more intense puffing parameters which block some or all of the ventilation holes in cigarette filters. Examples include those developed by the Massachusetts Department of Public Health (MDPH) and by Health Canada (IARC, 2004). Table 5 depicts the differences in TSNA emissions in the mainstream smoke generated by machine smoking using the ISO/FTC, MDPH and Health Canada protocols. Thirty-nine Phillip Morris cigarettes marketed globally (Counts *et al*., 2005) were grouped into three categories (moderate-yield, low-yield and very low-yield) based on the ISO/FTC emissions of tar, nicotine and carbon monoxide (IARC, 1986, 2004). The range of levels of NNK, NNN, NAB, NAT and total TSNA in the emissions of each product category was wide regardless of the machine-smoking protocol. The levels of emissions within each protocol increased from very low-yield cigarettes to moderate-yield cigarettes. Since the tobaccos in each product category contain similar amounts of preformed TSNA per gram of tobacco (Table 3), the differences in emission levels are due solely to the characteristics of the cigarette design such as the amount of tobacco, length and circumference of a cigarette, perforation of the cigarette and filter paper, and the type of material used for filtration (Hoffmann & Hoffmann, 2001; Hoffmann *et al*., 2001; IARC, 2004; Borgerding & Klus, 2005; Counts *et al*., 2005). It was calculated that, compared with the ISO/FTC conditions,

the MDPH method produces up to 2.2-fold higher yields of total TSNA for moderate-yield cigarettes, 2.7-fold higher yields for low-yield brands and threefold higher yields for ultra-low-yield brands. The ratios for the emissions using the Health Canada protocol follow the same trend but are somewhat higher (up to 2.4-, 3.4- and 5.2-fold, respectively) because of complete blockage of filter ventilation holes. Overall, MDPH and Health Canada protocols generally produce higher yields per cigarette and reduce the differences between brands in the yields.

Table 5. Comparison of the concentration ranges[a] of tobacco-specific *N*-nitrosamines (TSNA) in the mainstream smoke of 39 Philip Morris commercial brands marketed globally

Measurement conditions[b]	Cigarette type[c]		
	Moderate-yield	Low-yield	Very low-yield
ISO-condition			
NNK (ng/cig)	27.7–107.8	12.7–75	18–53.9
NNN (ng/cig)	48.1–195.3	14.8–147.5	5–103.5
NAB (ng/cig)	6–28.5	2–18.5	2–16.3
NAT (ng/cig)	44.1–160.4	18–129.2	8–91.2
Total TSNA (ng/cig)	127.1–472.3	47.5–370.2	27.4–264.9
MDPH-condition			
NNK (ng/cig)	60.6–208.6	36.2–150.4	25.8–130.8
NNN (ng/cig)	100.4–374.2	26.5–262.4	16.3–219.7
NAB (ng/cig)	10.5–46.9	4–27.7	5.8–33
NAT (ng/cig)	91.8–295.3	37.5–204.7	31.9–196.3
Total TSNA (ng/cig)	273.5–892.1	104.2–643.4	88.6–571.1
Health Canada-condition			
NNK (ng/cig)	73–263	44.7–171.1	39.1–157.5
NNN (ng/cig)	114.5–410.6	30.6–359.1	20.6–277.8
NAB (ng/cig)	13.8–50.1	5.3–42.8	4.3–33.1
NAT (ng/cig)	105.3–345.1	43.5–283.9	49.3–240.3
Total TSNA (ng/cig)	312.4–1049.8	124.1–852.8	113.3–708.7
Total TSNA ratios			
MDPH/ISO	1.7–2.2	1.7–2.7	2.1–3.0
Health Canada/ISO	1.9–2.5	1.9–3.4	2.7–5.2

From Counts *et al.* (2005)

NAB, *N*′-nitrosoanabasine; NAT, *N*′-nitrosoanatabine; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, *N*′-nitrosonornicotine

[a] The measurements are the averages of seven replicates within each machine-smoking method.

[b] Health Canada machine-smoking method; ISO, International Standardization Organization machine-smoking method; MDPH, Massachusetts Department of Public Health machine-smoking method

[c] Cigarettes were designated into classes based on ISO/FTC 'tar' smoke yields (IARC, 1986).

Table 6 presents an overview of the range of TSNA concentrations in mainstream and sidestream smoke of 26 major US cigarette brands as determined using the MDPH machine-smoking protocol. The concentrations of each individual TSNA vary substantially between cigarette brands marketed in the USA; these values are comparable to those reported for Philip Morris American blended cigarettes marketed globally (Table 5; Counts *et al*., 2005).

Table 6. Range of concentration of tobacco-specific *N*-nitrosamines in the mainstream and sidestream smoke of 26 US commercial cigarette brands (MDPH machine-smoking method)

	Concentration (ng/cigarette)		
	Mainstream	Sidestream	Sidestream/mainstream ratio
NNK	53.5–220.7	50.7–96.7	0.40
NNN	99.9–317.3	69.8–115.2	0.43
NAB	14.2–45.3	11.9–17.8	0.55
NAT	95.2–298.6	38.4–73.4	0.26

From Borgerding *et al.* (2000)
MDPH, Massachusetts Department of Public Health; NAB, *N*′-nitrosoanabasine; NAT, *N*′-nitrosoanatabine; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, *N*′-nitrosonornicotine

One study assessed the concentration of NNK in mainstream smoke using the smoking pattern of smokers. Among 133 smokers of low- or medium-yield cigarettes (Djordjevic *et al*., 2000c), smokers of low-yield brands drew somewhat larger puffs (48.6 versus 44.1 mL) and more smoke both per cigarette (615 mL versus 523 mL) and daily (9.5 L versus 8.2 L) compared with smokers of medium-yield cigarettes; however, concentrations of NNK in mainstream smoke were marginally higher among smokers of medium-yield cigarettes compared with smokers of low-yield brands (250.9 versus 186.5 ng/cigarette).

The most recent report examined whether differences in gender and ethnicity exist in relation to exposure to selected mainstream cigarette smoke constituents as a result of variations in smoking behaviour or type of cigarettes smoked among 129 female and 128 male smokers (Melikian *et al*., 2007a). Compared with men, women took smaller puffs (37.6 versus 45.8-mL puff; $p = 0.0001$) of shorter duration (1.33 versus 1.48-s puff; $p = 0.002$) but drew more puffs from their cigarettes (13.5 versus 12.0; $p = 0.001$) and smoked less of their cigarette (left 36.3-mm butts or 40.2% of cigarette length versus 34.3-mm butts or 39.2% of cigarette length; $p = 0.01$). The daily dose of smoke was significantly higher in men (9.3 versus 8.0 L; $p = 0.02$). When data were stratified by ethnicity, no difference was found in puffing characteristics between Caucasian American and African–American smokers, except that women and men in the latter group smoked their cigarettes

to an equal length (butt lengths, 34.5 versus 33.9 mm; $p = 0.93$). However, because African–Americans smoked fewer cigarettes, the daily smoke volume was significantly higher among Caucasian American smokers (8.61 versus 7.45 L for women; 10.6 versus 7.8 L for men). The emissions of selected compounds per cigarette, as determined by mimicking human smoking behaviours, were greater among the male smokers than among the female smokers and correlated significantly with delivered smoke volume per cigarette. Cigarettes smoked by women yielded 139.5 ng/cigarette NNK compared with 170.3 ng for men ($p = 0.0007$). The gender differences with regard to cigarette smoke delivery were more profound in Caucasian Americans than in African–Americans. On average, the smoking behaviour of African–American men produced the highest emissions and that of Caucasian American women produced the lowest.

1.3.5 *Sidestream cigarette smoke*

Adams *et al*. (1987a) determined the levels of TNSA in the mainstream and sidestream smoke of four different types of US commercial cigarette brands (untipped and filter-tipped, with or without filter perforation) with a wide range of ISO/FTC yields. The highest levels of NNK and NNN were measured in the sidestream smoke of untipped cigarettes (1444 and 857 ng/cigarette, respectively). The amounts of NAB and NAT ranged from 125 to 783 ng/cigarette. It was noted that, with the ISO/FTC machine-smoking method, NNK was the predominant TSNA in sidestream smoke, while NNN was predominant in mainstream smoke. The data obtained in the Massachusetts Benchmark Study (Borgerding *et al*., 2000) showed that, under more intense smoking patterns that included partial vent blocking, American blend cigarettes released 50.7–96.7 ng/cigarette NNK, 69.8–115.2 ng/cigarette NNN, 11.9–17.8 ng/cigarette NAB and 38.4–73.4 ng/cigarette NAT (Table 6). With more intense puffing and shorter smouldering time, lower amounts of NNK than NNN were released into the ambient air.

Emissions of TSNA from sidestream smoke are significantly lower when cigarettes are smoked using a more intense machine-smoking protocol compared with the standard ISO/FTC method. The explanation for this is that the physical and chemical processes that occur in a burning cigarette change with changing puffing intensity. Moreover, there is less time between puffs to allow for tobacco combustion in the smoldering phase and release of TSNA in ambient air.

For NNK only, the yields in sidestream smoke measured by the Health Canada method were up to 2.6-fold higher than those measured by the ISO method, but only for the ‘extra light’ and ‘ultra light’ brands (Government of British Columbia, 2002).

1.3.6 *Other smoked tobacco products*

(*a*) *Cigars*

Levels of NNK, NNN and NAT in cigars from the Netherlands were 2850–4250, 6750–53 000 and 4560–20 400 ng/g dry tobacco wt, respectively (Ohshima *et al*., 1985).

One Indian cigar brand contained 8900 ng/g NNK, 25 000 ng/g NNN and 13 700 ng/g NAT fresh tobacco wt (Nair *et al.*, 1989).

Under standard ISO/FTC machine-smoking conditions, the levels of NNK in the mainstream smoke of premium cigars were 17 times higher than those of medium-yield cigarettes; NNN levels were 22.4 times higher (931 versus 41.5 ng/unit) (Rickert & Kaiserman, 1999).

Djordjevic *et al.* (1997) also reported high emissions of TSNA in the mainstream smoke of small, large and premium cigars generated by a standard ICCSS (International Committee for Cigar Smoke Study) machine-smoking method developed for cigars. Concentrations in the mainstream smoke ranged from 290 to 2490 ng/unit NNK, from 595 to 1225 ng/unit NNN and from 310 to 1145 ng/unit NAT. The NNK emissions from premium cigars exceeded the highest levels reported for cigarettes (see Table 4). Djordjevic *et al.* (1997) also explored the levels of TSNA in the mainstream smoke of small cigars using methods that mimic human smoking patterns and compared the data with those obtained by standard machine-smoking methods. Under human smoking conditions, the emissions of NNK, NNN and NAT were 1.7-fold, 2.1-fold and 1.8-fold higher, respectively, than those obtained under standard protocols.

(*b*) Bidis

Bidis originated in India and have been gaining popularity in the USA during the last decade, particularly among adolescents. The levels of preformed NNK, NNN and NAT in tobacco from *bidis* sold in India ranged from 400 to 1400 ng/g, from 6200 to 12 000 ng/g and from 9000 to 12 500 ng/g, respectively, reported as wet tobacco wt (Nair *et al*., 1989). In the mainstream smoke of these *bidis*, the levels of NNK ranged from not detected to 40 ng/cigarette, those of NNN from 11.6 to 250 ng/cigarette and those of NAT from 9.9 to 175 ng/cigarette. These concentrations were comparable with those measured in the mainstream smoke of Indian cigarettes (Nair *et al*., 1989; Pakhale & Maru, 1998).

Wu *et al.* (2004) analysed the TSNA content of tobacco filler and mainstream smoke from 14 brands of *bidis* purchased in Atlanta, GA (USA). In *bidi* tobacco filler, the levels of NNK ranged from 90 to 850 ng/g and those of NNN ranged from 150 to 1440 ng/g tobacco. These levels are slightly lower than those in typical American blended cigarettes (see Table 2) and substantially lower than those in *bidis* sold in India. In mainstream smoke from these *bidis*, the levels of NNK ranged from 8.56 to 62.3 ng/cigarette and those of NNK ranged from 2.13–25.9 ng/cigarette.

The wide variation in the TSNA levels most probably reflects the hand-rolled nature of *bidis*, which results in products that have a less homogeneous tobacco content and a wider variation in overall cigarette quality. Since *bidis* contain on average 215.3 mg tobacco versus 738 mg in commercial US cigarettes (Malson *et al*., 2001), normalization of mainstream smoke emissions of TSNA per milligram of nicotine would give more realistic information on the ultimate levels of exposure to carcinogens (King *et al*., 2007).

(*c*) Chutta *tobacco*

The levels of NNK, NNN and NAT in *chutta* tobacco were reported to be extremely high: from 12 600 to 210 300 ng/g, from 21 100 to 295 800 ng/g and from 89 200 to 686 800 ng/g, respectively, reported as fresh tobacco wt. The reverse smoker inhales both the mainstream and sidestream smoke. NNK, NNN and NAT levels in the mainstream smoke of *chutta* ranged from 150 to 2651 ng, from 289 to 1260 ng and from 431 to 1722 ng/*chutta*, respectively (Nair *et al*., 1989). Stich *et al*. (1992) reported comparable levels of NNK, NNN and NAT in the mainstream smoke of *chutta*: 274–2520, 925–3910 and 141–1300 ng/cigarette, respectively.

(*d*) *Pipe tobacco*

Ohshima *et al*. (1985) analysed the TSNA content of pipe tobacco from France, the Netherlands and the United Kingdom. The concentrations of NNK ranged from not detected to 1130 ng/g, those of NNN from 3000 ng/g to 6880 ng/g and those of NAT from 1990 to 4850 ng/g dry tobacco wt, respectively. Chamberlain *et al*. (1988) reported levels of 300 ng/g tobacco NNK and 1800 ng/g tobacco NNN in pipe tobacco from the USA. TSNA emissions in the mainstream and sidestream smoke generated by pipe smoking have not been reported to date.

1.3.7 *Secondhand tobacco smoke*

The NNN concentrations measured in a poorly ventilated office where heavy smoking of cigarettes, cigars and pipes took place ranged from not detected to 6 ng/m^3 and those of NNK from not detected to 13.5 ng/m^3 (Klus *et al*., 1992). The upper levels reported by Klus *et al*. (1992) and by Adlkofer *et al*. (1990) for ‘heavily smoked rooms’ (11 cigarettes smoked in 2 h in a 84-m^2 office) were lower than those measured inside bars, restaurants, trains, a car, an office and a smoker’s home: NNN concentrations ranged from not detected to 22.8 ng/m^3 and NNK concentrations ranged between 1.4 and 29.3 ng/m^3 (Brunnemann *et al*., 1992).

1.3.8 *Smokeless tobacco products*

The occurrence of TSNA in smokeless tobacco products is discussed extensively in the monograph on Smokeless tobacco. An international comparison of the concentration ranges in a variety of smokeless tobacco products is presented in Table 7. [TSNA concentrations are customarily expressed in micrograms per gram of dry (or wet) tobacco. To enable the comparison of the TSNA levels in cigarette and smokeless tobacco, the values in this table are expressed in nanograms per gram of dry (or wet) tobacco.] There is a very wide range of concentrations of TSNA in smokeless tobacco, which reflects the product category (e.g. chewing tobacco, moist snuff, dry snuff, new low-TSNA products), product characteristics in each category (e.g. short cut or long cut tobacco, flavourings), tobacco

Table 7. International comparison of the concentration ranges of tobacco-specific *N*-nitrosamines in smokeless tobacco products (ng/g tobacco)

Country	Type of product	Reported as[a]	NNK	NNN	NAB	NAT	References
Belgium	Chewing tobacco	Dry	130	7380	NR[b]	970[c]	Ohshima *et al*. (1985)
Canada	Moist snuff	Dry	3200–5800	50 400–79 100	4000–4800	152 000–170 000	Brunnemann *et al*. (1985)
	Chewing tobacco	Dry	240	2090	100	1580	
Denmark	Chewing tobacco	Wet	19–1900	80–1600	30	180–2900	Österdahl *et al*. (2004)
Germany	Chewing tobacco	Dry	30–300	1420–2300	30–50	330–3700[b]	Brunnemann *et al*. (1985); Tricker *et al*. (1988)
	Dry snuff	Dry	580–6430	2390–18 750	NR	1030–7830	Tricker & Preussmann (1991);
		Wet	100	680	NR	310	Österdahl *et al*. (2004)
India	Moist snuff	Wet	240	560	20	380	Stepanov *et al*. (2005)
	Chewing tobacco	Dry	130–600	470–850	30–70	300–500[b]	Brunnemann *et al*. (1985); Tricker *et al*. (1988)
		Wet	2700–6500	15 300–24 400	NR	10 000–44 600	Nair *et al*. (1989)
	Zarda	Dry	220–24 100	400–79 000	NR[b]	780–99 100[c]	Tricker & Preussmann (1988); Tricker *et al*. (1988)
		Wet	1070–3090	4810–19 900	190–1190	640–1980	Stepanov *et al*. (2005)
		NR		6550–7360			Gupta (2004)
	Mishri	Dry	294–1100	300–6995	NR	488–14 151	Tricker *et al*. (1988); Nair *et al*. (1987)
		Wet	870	4210	150	2550	Stepanov *et al*. (2005)
		NR		4020–4470			Gupta (2004)
	Khiwam	Dry	100–1030	2500–8950	NR[b]	1830–10 360[c]	Tricker & Preussmann (1989)
	Khaini	Dry	110–5290	25 800–40 000	1240–2480	660–18 800	Stich *et al*. (1992)
		Wet	2340–28 400	39 400–76 900	3870–8830	4830–13 800	Stepanov *et al*. (2005)
	Gutka	Wet	40–430	90–1090	ND–50	10–80	Stepanov *et al*. (2005)
		NR	10 680–11 510	1870–5730	5750–6890	6270–6530	Gupta (2004)
	Supari	Wet	ND	ND	ND	ND	Stepanov *et al*. (2005)
		NR	4900–11 580	1920–2450	11 580–12 580	3600–4570	Gupta (2004)

Table 7 (contd)

Country	Type of product	Reported as[a]	NNK	NNN	NAB	NAT	References
India (contd)	Creamy snuff/ toothpaste	Wet	1310–12 500 4380–4880	2520–48 700	70–110	530–26 600	Stepanov *et al*. (2005); Nair *et al.* (1989) Gupta (2004)
	Snuff for inhalation	Wet	245 500	1 356 000	NR	1 857 000	Nair *et al.* (1989)
	Toothpowder	Wet	ND	ND–40	ND	ND	Stepanov *et al*. (2005)
	Tuibur	NR		19 650–20 120			Gupta (2004)
	Other tobacco	Wet	80–2610	1740–19 200	12–1570	350–11 900	Stepanov *et al*. (2005)
Norway	Moist snuff	Wet	3300	21 000	1700	13 000	Österdahl *et al.* (2004)
South Africa	Low-TSNA moist snuff	Dry	270–290	1050–2070	90–110	890–1520	Brunnemann *et al.* (2004)
Sudan	*Toombak*	Dry	188 000–7 870 000	141 000–3 080 000	13 900–2 370 000	20 000–290 000	Idris *et al.* (1991); Prokopczyk *et al.* (1995)
Sweden	Moist snuff	Dry	190–2950	1120–154 000	40–1700	900–21 400[c]	Brunnemann *et al.* (1985); Ohshima *et al.* (1985); Tricker *et al.* (1988); Hoffmann *et al*. (1991); Tricker & Preussmann (1991); Brunnemann & Hoffmann (1992); Djordjevic *et al.* (1993a); Connolly (2001)
		Wet	190–1300	490–4400	30–170	320–3500	Österdahl & Slorach (1988); Österdahl *et al.* (2004)
	Low-TSNA moist snuff	Wet	30–360	150–2300	10–130	60–980	Österdahl *et al.* (2004); Stepanov *et al*. (2006a)
	Chewing tobacco	Wet	10–460	700–1700	ND	1100–2100	Österdahl *et al.* (2004)
Thailand	Chewing tobacco	Dry	100	500	NR[b]	500[c]	Tricker *et al*. (1988)

Table 7 (contd)

Country	Type of product	Reported as[a]	NNK	NNN	NAB	NAT	References
United Kingdom	Moist snuff	Dry	400–13 000	1100–52 000	86	2000–6500[c]	Hoffmann *et al*. (1988); Brunnemann & Hoffmann (1992)
	Chewing tobacco	Dry	300	900	NR	1500[c]	Tricker *et al*. (1988)
	Dry snuff	Dry	580–4300	2390–16 000	NR	1030–7830	Tricker & Preussmann (1991); Brunnemann & Hoffmann (1992)
		Wet	260	1800	NR	820	Österdahl *et al*. (2004)
USA	Moist snuff	Dry	ND–18 000	ND–147 000	20–10 670	240–339 000	Brunneman *et al*. (1985); Ohshima *et al*., 1985; Adams *et al*. (1987b); Brunnemann *et al*. (1987a,b); Chamberlain *et al*. (1988); Tricker *et al*. (1988); Andersen *et al*. (1989); Djordejvic *et al*. (1989a); Hoffmann *et al*. (1991); Brunnemann & Hoffmann (1992); Prokopczyk *et al*. (1992); Djordjevic *et al*. (1993a); Hoffmann *et al*. (1995); Prokopczyk *et al*. (1995); Connolly (2001); Brunnemann *et al*. (2002, 2004)
		Wet	60–13 000	710–63 000	14–2800	240–83 000	Österdahl *et al*. (2004); Stepanov *et al*. (2006a)
	Low-TSNA moist snuff	Wet	32–33	620–640	17–18	310–320	Stepanov *et al*. (2006a)

Table 7 (contd)

Country	Type of product	Reported as[a]	NNK	NNN	NAB	NAT	References
USA (contd)	Chewing tobacco	Dry	ND–1100	670–6500	20–140	670–12 400	Brunnemann *et al*. (1985); Chamberlain *et al*. (1988); Andersen *et al*. (1989); Djordjevic *et al*. (1989a); Brunnemann & Hoffmann (1992)
		Wet	80–110	250–1100	20	150–940	Österdahl *et al*. (2004)
	Dry snuff	Dry	880–84 400	9370–116 100	520–1530	11 200–238 800[c]	Adams *et al*. (1987); Brunnemann *et al*. (1987a); Andersen *et al*. (1989); Djordjevic *et al*. (1989a)
	Compressed tobacco lozenges	NR	37–43	19–56	7–8	12–17	Stepanov *et al*. (2006a)
Uzbekistan	*Naswar*	Dry	20–130	120–520	8–30	32–300	Brunnemann *et al*. (1985)

NAB, *N*′-nitrosoanabasine; NAT, *N*′-nitrosoanatabine; ND, not detected; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, *N*′-nitrosonornicotine; NR, not reported

[a] Reported as ng/g dry wt (dry) or wet wt (wet) tobacco

[b] The upper value reported as NAT also includes NAB.

[c] The upper value includes NAB + NAT.

blend, tobacco curing and manufacturing technologies, ageing, country of origin, year of production and analytical methods.

Because of the prohibition of the marketing of moist snuff in the European Union except in Sweden and in the EFTA (European Free Trade Association) country Norway (see the monograph on Smokeless tobacco, Section 1.5.3), data on the TSNA content of smokeless tobacco products marketed in the European countries beyond 1992 are limited. Chewing tobacco contained the lowest amounts of TSNA: NNK ranged from undetectable levels (USA) to 1900 ng/g wet wt (Denmark); NNN ranged from 80 ng/g wet wt (Denmark; Österdahl *et al.*, 2004) to 7380 ng/g dry wt (Belgium; Ohshima *et al.*, 1985); NAB ranged from undetectable levels to 140 ng/g dry wt (USA) and NAT ranged from 150 ng/g wet wt to 12 400 ng/g dry wt (USA).

The levels of NNK, NNN, NAB and NAT in moist snuff were, in general, substantially higher than those in chewing tobacco; upper concentrations reached 18 000 ng/g dry tobacco NNK (USA; Connolly, 2001), 154 000 ng/g dry tobacco NNN (Sweden; Ohshima *et al.*, 1985), 10 670 ng/g dry tobacco NAB (USA; Brunnemann & Hoffmann, 1992) and 339 000 ng/g dry tobacco NAT (USA; Ohshima *et al.*, 1985). Levels in Indian moist snuff were surprising low (Stepanov *et al.*, 2005).

High levels of TSNA in dry snuff were also measured in the USA: NNK, up to 84 400 ng/g dry tobacco; NNN, up to 116 100 ng/g dry tobacco; NAB, up to 1530 ng/g dry tobacco and NAT, up to 238 000 ng/g dry tobacco. In Indian snuff used for inhalation, TSNA levels were even higher: 245 000 ng/g fresh tobacco NNK, 1 356 000 ng/g fresh tobacco NNN and 1 875 000 ng/g fresh tobacco NAT (Nair *et al.*, 1989).

Although there has been a decline in the concentrations of TSNA in smokeless tobacco products in Sweden and the USA since the 1980s (Djordjevic *et al.*, 1993b; Brunnemann *et al*., 2004; Österdahl *et al*., 2004), the trend may not apply to other products and countries. For example extremely high levels (in milligrams) of TSNA were measured in *toombak* (Idris *et al*., 1991; Prokopczyk *et al*., 1995; Idris *et al*., 1998): NNK, up to 7 870 000 ng/g dry tobacco; NNN, up to 3 085 000 ng/g dry tobacco; NAB, up to 237 000 ng/g dry tobacco and NAT, up to 290 000 ng/g dry tobacco. In contrast, very low levels of TSNA have been reported in *naswar*: NNK, 130 ng/g dry tobacco; NNN, up to 520 ng/g dry tobacco; NAB, 30 ng/g dry tobacco; and NAT, 300 ng/g dry tobacco.

In recent years, some manufacturers of smokeless tobacco products have used novel tobacco curing and processing technologies to produce moist snuff with lower TSNA. These products are marketed in Norway, South Africa, Sweden, the USA and other countries (Connolly, 2001; Brunnemann *et al*., 2004; Österdahl *et al*., 2004; Stepanov *et al*., 2006a; McNeill *et al*., 2007). Levels of NNK and NNN in moist snuff produced by the new manufacturing process (Gothia, 2004) have been reported to be up to 45 times lower than those in leading products manufactured under standard process (Connolly, 2001). In Sweden, all moist snuff brands on the market in 2002 contained low levels of TSNA. NNN concentrations in moist snuff have decreased consistently from 1983 to 2002 in this country (Österdahl *et al*., 2004). However, low-TSNA Swedish *snus*, purchased both in Sweden and the USA between 2003 and 2005, contained up to 360 ng/g wet

tobacco NNK, up to 2300 ng/g wet tobacco NNN, up to 130 ng/g wet tobacco NAB and up to 980 ng/g wet tobacco NAT (Stepanov *et al*., 2006a). Since the values were not adjusted for the moisture content, the concentrations may be even higher. Other new smokeless tobacco products (e.g. compressed tobacco lozenges such as Ariva®, Stonewall, Hard Snuff) contain even lower concentrations of NNK, NNN, NAB and NAT (Table 7).

In India, smokeless tobacco products account for over one-third of all tobacco used (see the monograph on Smokeless tobacco). Traditional forms of smokeless tobacco include betel quid with tobacco, tobacco with lime and tobacco tooth powder but a variety of new products are gaining popularity (Gupta & Ray, 2003). Table 7 presents an overview of TSNA concentrations in a variety of Indian products. The highest levels of NNK, NNN, NAB and NAT were reported in *zarda* and *khaini* (Tricker & Preussmann, 1988; Tricker *et al*., 1988; Stepanov *et al*., 2005). Other products also contain high levels of TSNA (Gupta, 2004; Stepanov *et al*., 2005). In contrast, *gutka*, moist snuff and chewing tobacco contain significantly lower levels of TSNA (Stepanov *et al*., 2005). Some tobacco products consumed in India, such as *supari* and toothpowder, did not contain quantifiable levels of TSNA (Stepanov *et al*., 2005). Brown and black *mishri*, a half-burnt tobacco product commonly used as a dentifrice in some parts of India, were reported to contain significantly higher amounts of TSNA compared with unburnt tobacco: NNK, up to 1100 ng/g dry wt; NNN, up to 6995 ng/g dry wt; and NAT, up to 14 150 ng/g dry wt (Nair *et al*., 1987; Tricker *et al*., 1988). In addition to TSNA, *mishri* contains high concentrations of benzo[*a*]pyrene (from 27 to 119 μg/g; Nair *et al*., 1987).

Hoffmann *et al*. (1995) compared the concentrations of TSNA between two major categories of moist snuff that contain high and low levels of unprotonated nicotin (Table 8). In the high-level unprotonated nicotine brands, concentrations were in the range of: NNK,

Table 8. The levels of tobacco-specific *N*-nitrosamines (TSNA) in five leading brands of moist snuff purchased in the USA, 1994

Product	TSNA (μg/g dry wt)				
	NNK	NNN	NAB	NAT	Total TSNA
Skoal Bandits Straight[a]	0.92 ± 0.26	5.09 ± 1.03	0.13 ± 0.03	2.05 ± 0.60	8.19 ± 1.72
Hawken Wintergreen[a]	0.23 ± 0.04	3.07 ± 0.3	0.13 ± 0.02	0.63 ± 0.15	4.08 ± 0.44
Skoal Original Fine Cut Wintergreen[b]	1.25 ± 0.13	8.18 ± 1.33	0.37 ± 0.09	5.10 ± 1.01	14.90 ± 2.50
Copenhagen Snuff[b]	1.89 ± 0.62	8.73 ± 1.44	0.50 ± 0.12	6.13 ± 1.02	17.24 ± 2.97
Kodiak Wintergreen[b]	0.55 ± 0.15	6.3 ± 1.06	0.32 ± 0.10	3.79 ± 1.25	10.96 ± 2.44

From Hoffmann *et al.* (1995)

NAB, *N*′-nitrosoanabasine; NAT, *N*′-nitrosoanatabine; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, *N*′-nitrosonornicotine

[a] Brands with low levels of unprotonated nicotine

[b] Brands with high levels of unprotonated nicotine

550–1890 ng/g dry tobacco; NNN, 6300–8730 ng/g dry tobacco; NAB, 320–500 ng/g dry tobacco; and NAT, 3790–6130 ng/g dry tobacco. In the low-level unprotonated nicotine brands, concentrations were in the range of: NNK, 230–920 ng/g dry tobacco; NNN, 3070–5090 ng/g dry tobacco; NAB, 130 ng/g dry tobacco; and NAT, 630–2050 ng/g dry tobacco. Thus, the three leading snuff brands in the USA (Copenhagen, Skoal Fine Cut and Kodiak) that made up 92% of the market in 1995 not only had a high pH and contained high levels of nicotine and unprotonated (free) nicotine, but also contained high concentrations of carcinogenic TSNA in comparison with the moist snuff brands that ranked fourth and fifth — Hawken and Skoal Bandits (3% of the US market).

A high-pH (7.99) and high-moisture (52.7%) moist snuff brand purchased in the USA in 2000 contained 2500 ng/g NNK, 15 400 ng/g NNN, 1200 ng/g NAB and 18 500 ng/g NAT, reported as dry tobacco wt; in contrast, a low-pH (5.84) and low-moisture (24%) moist snuff brand contained 500 ng/g NNK, 3100 ng/g NNN, 200 ng/g NAB and 800 ng/g NAT (Brunnemann *et al.*, 2002). TSNA levels in low-moisture brands are generally more consistent over time while they fluctuate dramatically in high-moisture brands.

In general, TSNA levels per unit dose are higher in smokeless tobacco compared with the levels in the mainstream cigarette smoke as determined by the ISO/FTC machine-smoking method (see Tables 4 and 7). Even in Swedish *snus*, levels of TSNA are comparable with those in cigarette smoke (e.g. the highest reported ISO/FTC NNN yield in the mainstream smoke was 1353 ng/cigarette versus 2300 ng/g *snus*; Stepanov *et al*., 2006a).

1.4 Biomonitoring in saliva, urine and other tissues

Analysis of TSNA and their metabolites in saliva, urine and blood has proven to be extremely useful in estimating human exposure to these carcinogens. While studies of levels in saliva and blood have been somewhat limited, there is extensive literature of urinary NNAL and NNAL-glucuronides (NNAL-Gluc), metabolites of NNK (see Section 4.1), in smokers, smokeless tobacco users and nonsmokers exposed to secondhand smoke. Studies relevant to the toxicokinetics (absorption, distribution, metabolism and excretion) of TSNA in humans and to the mechanisms of carcinogenesis by these compounds are discussed in Section 4. This section presents data that relate tobacco exposure to TSNA and their metabolites. Levels of NNK, NNN, NAB and NAT in the saliva of tobacco users are summarized in Table 9. Only studies published since the previous evaluation (IARC, 1985) have been included.

1.4.1 *4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and its metabolites*

(*a*) *NNK and its metabolites in saliva*

NNK has been detected in the saliva of snuff dippers, users of *khaini*, of *gudhaku* chewers of betel quid with tobacco and users of *toombak* (Table 9; Nair *et al.*, 1985; Bhide *et al.*, 1986; Österdahl & Slorach, 1988; Idris *et al.*, 1992; Hoffmann *et al.*, 1994). Levels in snuff-dippers ranged from not detected to 201 ng/g saliva and those in *khaini* users were

Table 9. Concentrations of NNK, NNN, NAB and NAT in the saliva of users of various forms of tobacco

Reference	Country of study	Tobacco product	Unit	Mean concentration (range) or range			
				NNK	NNN	NAB	NAT
Nair *et al.* (1985)	India	Betel quid with tobacco	ng/mL saliva	0.34 (0–2.3)	7.5 (1.6–14.7)	–	4.8 (1.0–10.9)
		Chewing tobacco		ND	33.4 (16.5–59.7)		29.8 (13.5–51.7)
		Cigarette		ND	ND	–	ND
Bhide *et al.* (1986)	India	Betel quid with tobacco	ng/g saliva	–	3–85.7	ND–40	–
		Mishri		ND	23.7 (14.3–43.5)	ND	–
		Khaini		ND–28.5	91.9 (10.0–430)	–	ND–133.0
Brunnemann *et al.* (1987c)	Canadian Inuits	Snuff	ng/g saliva	56 (ND–201)	980 (115–2601)	–	1318 (123–4560)
Österdahl & Slorach (1988)	Sweden	Snuff	ng/g saliva	ND–16	3–140	–	4–85
Idris *et al.* (1992)	Sudan	*Toombak*	ng/mL saliva	ND–6689	582–20988	46–1944	ND–471
Stich *et al.* (1992)	India	*Khaini*	ng/mL saliva	ND–180	150–1580	13–90	86–690
		Gudhaku	ng/mL saliva	ND–10	15–88	1–15	9–55
		Chutta (reverse smoking)	ng/g saliva	–	45–5890	–	ND–1880

NAB, *N*′-nitrosoanabasine; NAT, *N*′-nitrosoanatabine; ND, not detected; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, *N*′-nitrosonornicotine

up to 180 ng/g saliva. The highest levels were found in *toombak* users (up to 6690 ng/g saliva). Levels of NNK were generally 10–100 times greater in the saliva of *toombak* users than in users of other types of smokeless tobacco (Idris *et al.*, 1992; Hoffmann *et al.*, 1994).

NNAL, a metabolite of NNK, was also detected in the saliva of *toombak* users at levels as high as 3270 ng/g saliva (Idris *et al.*, 1992).

(*b*) *NNK and its metabolites in cervical mucus and pancreactic juice*

NNK was detected in 16 samples of cervical mucus from 15 women who were smokers at concentrations of 11.9–115 ng/g mucus (two samples were collected from one smoker at different times) and in nine of 10 samples from nonsmokers at concentrations of 4.1–30.8 ng/g mucus; the concentrations of NNK in specimens from cigarette smokers were significantly higher than in those obtained from nonsmokers (Prokopczyk *et al*., 1997).

NNK was detected in 15/18 samples of pancreatic juice from smokers at concentrations of 1.4–604 ng/mL and in six of nine samples from nonsmokers (range of concentrations, 1.13–97 ng/mL); the levels were significantly higher in smokers than in nonsmokers. NNAL was present in 11/17 samples from smokers and in three of nine samples from nonsmokers (Prokopczyk *et al*., 2002).

(*c*) *NNAL and NNAL-Gluc in urine*

Unchanged NNK is not detected in urine (Hecht *et al.*, 1999a).

Several studies have quantified NNAL and NNAL-Gluc in human urine. Levels of total NNAL (NNAL plus NNAL-Gluc) are generally 2–4 pmol/mg creatinine in smokeless tobacco users, 1–4 pmol/mg creatinine in smokers and 0.02–0.07 pmol/mg creatinine in nonsmokers exposed to secondhand smoke. Several studies have quantified these metabolites in 24-h urine. Baseline levels of excreted NNAL and NNAL-Gluc are typically about 1 nmol NNAL/24 h and 2.2 nmol NNAL-Gluc/24 h (Hecht *et al.*, 1999a); levels of total NNAL generally reported were 6.6 nmol/24 h in smokeless tobacco users, 3–4 nmol/24 h in smokers and 0.03–0.13 nmol/24 h in nonsmokers exposed to secondhand tobacco smoke. Occasionally, however, as in the case of *toombak* users, far higher levels of total NNAL in urine have been observed.

(i) *Smokers*

The earlier literature on NNAL and NNAL-Gluc in the urine of smokers has been reviewed (Hecht, 2002; IARC, 2004). In a study of 274 smokers of cigarettes in the contemporary yield ranges of the German market and 100 nonsmokers, total NNAL levels were significantly higher in smokers than in nonsmokers (Scherer *et al.*, 2007).

Total NNAL has been shown to correlate with number of cigarettes smoked per day but the increase was not linear (Joseph *et al*., 2005). No statistically significant differences in urinary levels of total NNAL were observed in urine samples from 175 smokers of regular, light or ultra-light cigarettes (Burns *et al.*, 2001; Harris *et al.*, 2004; Bernert *et al.*, 2005; Hecht *et al.*, 2005).

Two studies investigated the effects of reducing the number of cigarettes smoked per day on levels of NNAL and NNAL-Gluc. Decreases in levels of NNAL, NNAL-Gluc and total NNAL were observed, but these were generally modest, and were always proportionally less than the reduction in the number of cigarettes smoked per day (Hurt *et al.*, 2000; Hecht *et al.*, 2004a).

Levels of total NNAL were significantly reduced in smokers who switched from their customary brand to the Omni cigarette (a cigarette with reduced levels of NNK) and were significantly lower than those in smokers who stopped with the aid of a nicotine patch (Hatsukami *et al.*, 2004).

A comparison of total NNAL in the urine of Caucasians and African–Americans demonstrated that mean concentrations were greater in African–American men for each cigarette smoked; no difference was seen in women (Muscat *et al.*, 2005).

(ii) *Nonsmokers exposed to secondhand tobacco smoke*

Levels of total NNAL in the urine of nonsmokers exposed to secondhand tobacco smoke are typically about 1–5% of those in smokers (Hecht, 2002; IARC, 2004). In more recent studies, total NNAL was quantified before and after a 4-h visit to a gambling casino where smoking was allowed (Anderson *et al.*, 2003) and in nonsmokers who worked in restaurants and bars that allow smoking (Tulunay *et al.*, 2005). Both studies showed significant increases in urinary levels of total NNAL after exposure to secondhand smoke.

Levels of total NNAL in the urine of 144 infants (< 1 year old) averaged 0.083 ± 0.200 pmol/mL urine; the mean number of cigarettes smoked per week by any family member in the home or car when the infant was present was significantly higher in the families of children with detectable levels of NNAL compared to those with undetectable levels. The levels of NNAL detected in the urine of these infants was higher than those in most other field studies of exposure to secondhand tobacco smoke (Hecht *et al.*, 2006).

In another study, total NNAL was detected in 69/80 urine samples from Moldovan children. The mean level (0.09 ± 0.077 pmol/mL) was comparable with those observed in previous studies of secondhand smoke exposure (Stepanov *et al.*, 2006b).

(iii) *Smokeless tobacco users*

Snuff dippers/tobacco chewers in the USA excreted 6.6 nmol/24 h total NNAL (NNAL plus NNAL-Gluc) in urine (Hecht *et al.*, 2002). In one study of snuff dippers and tobacco chewers, urinary excretion of total NNAL averaged 4.4 pmol/mg creatinine. Levels in 23 snuff-dippers (5.9 pmol/mg creatinine) were significantly higher than those in 13 tobacco chewers (2.1 pmol/mg creatinine) (Kresty *et al.*, 1996).

Seven *toombak* users excreted an average of 1270 pmol/mL urine total NNAL, which was approximately 300 times that excreted by snuff-dippers/chewers (Murphy *et al.*, 1994).

Among snuff dippers in the USA, total daily dip duration, total daily dipping time and number of dips per day were significantly correlated with levels of total NNAL (Lemmonds *et al.*, 2005). Levels of total NNAL correlated with the number of tins used per day in one study (Hecht *et al.*, 2002) but not in another (Lemmonds *et al.*, 2005). Total levels of NNAL

were significantly lower in users of smokeless tobacco after they switched to Swedish snuff or to nicotine patch; the overall mean level of total NNAL among subjects who used a nicotine patch was significantly lower than that among those who used snuff (Hatsukami *et al.*, 2004).

Levels of NNAL and NNAL-Gluc were quantified in the urine of 420 smokers and 182 smokeless tobacco users (Hecht *et al.*, 2007). Levels of total NNAL/mL urine and levels of total NNAL/mg creatinine, adjusted for age and sex, were significantly higher in smokeless tobacco users than in smokers ($p < 0.001$).

(*d*) *Total NNAL in blood*

A liquid chromatography–electrospray ionization tandem mass spectrometry method was developed for the analysis of total NNAL in plasma. Levels averaged 42 ± 22 fmol/mL in 16 smokers; total NNAL was not detected in the plasma of nonsmokers. Levels were 1–2% of those found in urine (Carmella *et al.*, 2005, 2006).

(*e*) *Total NNAL in toenails*

Total NNAL, nicotine and cotinine were analysed in human toenails by liquid chromatography–electrospray ionization tandem mass spectrometry in 35 smokers. Mean total NNAL was 0.41 ± 0.67 pg/mg toenail (Stepanov *et al.*, 2006c).

1.4.2 N′-*Nitrosonornicotine (NNN)*

(*a*) *Saliva*

Formation of additional quantities of NNN by the reaction of salivary nitrite with nicotine or nornicotine during the oral use of snuff or during tobacco chewing has been implied from in-vitro studies (Hoffmann & Adams, 1981).

NNN has been detected in the saliva of snuff dippers (Brunnemann *et al.*, 1987c; Österdahl & Slorach, 1988; Hoffmann *et al.*, 1994), chewers of betel quid with tobacco (Nair *et al.*, 1985; Bhide *et al.*, 1986), users of *khaini* (Bhide *et al.*, 1986; Stich *et al.*, 1992), users of *gudakhu* (Stich *et al.*, 1992), users of *mishri* (Bhide *et al.*, 1986), reverse smokers of *chutta* (Stich *et al.*, 1992) and users of *toombak* (Idris *et al.*, 1992) (Table 9). The exceptionally high levels of NNN in the saliva of *toombak* users reflects the unusually high concentrations of NNN (mg/g) in this product (Idris *et al*., 1991).

(*b*) *Urine*

Levels of NNN in the urine of 14 smokers ranged from not detected to 0.43 pmol/mg creatinine (mean ± standard deviation (SD), 0.086 ± 0.12 pmol/mg) and those of NNN-*N*-glucuronide (NNN-*N*-Gluc) ranged from not detected to 0.36 pmol/mL creatinine (mean ± SD, 0.096 ± 0.11 pmol/mL). The corresponding values in 11 smokeless tobacco users were 0.03–0.58 pmol/mg creatinine (mean ± SD, 0.25 ± 0.19 pmol/mg) and 0.091–0.91 pmol/mg creatinine (mean ± SD, 0.39 ± 0.27 pmol/mg) (Stepanov & Hecht, 2005).

(*c*) *Pancreatic juice*

NNN was found in two of 17 samples of pancreatic juice from smokers (68 and 242 ng/mL) (Prokopczyk *et al*., 2002).

1.4.3 N′-*Nitrosoanabasine (NAB)*

(*a*) *Saliva*

NAB was detected in the saliva of chewers of betel quid with tobacco (Bhide *et al*., 1986), *toombak* (Idris *et al.*, 1992), *khaini* (Stich *et al.*, 1992) and *gudhaku* (Stich *et al.*, 1992) (Table 9).

(*b*) *Urine*

NAB and NAB-*N*-glucuronide (NAB-*N*-Gluc) have been found in the urine of smokers and smokeless tobacco users. Levels in 14 smokers ranged from not detected to 0.019 pmol/mg creatinine (mean ± SD, 0.003 ± 0.006 pmol/mg) NAB and from not detected to 0.14 pmol/mg creatinine (mean ± SD, 0.038 ± 0.039 pmol/mg) NAB-*N*-Gluc. Corresponding levels in 11 smokeless tobacco users ranged from not detected to 0.11 pmol/mg creatinine (mean ± SD, 0.037 ± 0.034 pmol/mg) and 0.028–0.44 pmol/mg creatinine (mean ± SD, 0.19 ± 0.16 pmol/mg), respectively (Stepanov & Hecht, 2005).

1.4.4 N′-*Nitrosoanatabine (NAT)*

(*a*) *Saliva*

NAT has been detected in the saliva of snuff dippers (Brunnemann *et al.*, 1987c; Österdahl & Slorach, 1988; Hoffmann *et al.*, 1994), *toombak* users (Idris *et al.*, 1992), *khaini* tobacco users (Bhide *et al.*, 1986; Stich *et al.*, 1992), *gudhaku* users (Stich *et al.*, 1992) and users of betel quid with tobacco (Nair *et al.*, 1985) (Table 9).

(*b*) *Urine*

NAT and NAT-*N*-glucuronide (NAT-*N*-Gluc) were detected in the urine of smokers and smokeless tobacco users. Levels in 14 smokers ranged from not detected to 0.31 pmol/mg creatinine (mean ± SD, 0.067 ± 0.104 pmol/mg) NAT and from not detected to 0.43 pmol/mg creatinine (mean ± SD, 0.12 ± 0.11 pmol/mg) NAT-*N*-Gluc. Corresponding levels in 11 smokeless tobacco users were 0.020–0.15 pmol/mg creatinine (mean ± SD, 0.069 ± 0.046 pmol/mg) and 0.08–2.78 pmol/mg creatinine (mean ± SD, 1.36 ± 1.06 pmol/mg), respectively (Stepanov & Hecht, 2005).

2. Studies of Cancer in Humans

No data were available to the Working Group.

3. Studies of Cancer in Experimental Animals

Volumes of data exist on tumour development in various animal models that have been exposed to TSNA by various modes of administration. Not all of these studies are included in the monograph. Studies presented here are considered to be pivotal for the establishment of the carcinogenicity of NNK, NNN and NNAL. Animals studies on these substances have been reviewed comprehensively (Hecht, 1998). Studies of NAB and NAT are also reviewed although the number of studies are fewer.

3.1 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) (Table 10)

The carcinogenicity of NNK in experimental animals has been evaluated previously (IARC, 1985).

3.1.1 *Intraperitoneal administration*

Mouse

Groups of 25 female strain A/J mice, 6–8 weeks of age, received thrice-weekly intraperitoneal injections of 0.1 mL of a 1% solution of NNK [purity not specified] in trioctanoin for a total of 22 injections (total dose, 22 mg or 0.11 mmol per mouse) and were observed for 30 weeks after the final injection. Controls consisted of groups of 25 of untreated mice, vehicle controls and positive (urethane-treated) controls. When animals were killed, lungs were examined macroscopically for total lesions and microscopically for histological type. The Student's *t*-test was used to determine statistical significance. In untreated controls, 1/25 animals developed lung tumours at a multiplicity of 0.04 ± 0.20 tumours per mouse; in the vehicle controls, 5/24 animals developed lung tumours at a multiplicity of 0.2 ± 0.41 tumours per mouse. Of the NNK-treated animals, 20/23 developed lung tumours with a multiplicity of 2.61 ± 1.85 tumours per mouse ($p < 0.05$ compared with vehicle controls). Tumours were described as adenomas (Hecht *et al*., 1978).

Groups of 25 female strain A/J mice, 6–8 weeks of age, received thrice-weekly intraperitoneal injections of 0.1 mL of a 1% solution of NNK (> 99% pure) suspended in trioctanoin for 7.3 weeks (22 injections; total dose, 0.11 mmol/mouse) and were held for 30 weeks after the last injections. Untreated and vehicle-treated animals served as controls.

Table 10. Summary of reports of tumours induced in experimental animals by NNK and NNN

Compound/ species	Lung	Nasal cavity	Oral cavity	Trachea	Oeso-phagus	Fore-stomach	Pancreas	Liver	Adrenal gland	Skin
NNK										
Mouse	x					x		x		x
Rat	x	x	x[a]				x	x		
Hamster	x	x		x			x[c]		x[c]	
Mink	x	(x)								
NNN										
Mouse	x					x				
Rat		x	x[b]		x					
Hamster		x		x						
Mink		(x)								

NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, *N*′-nitrosonornicotine
[a] In combination with NNN
[b] In combination with NNK
[c] In progeny

Lung tumours were counted macroscopically at the time of sacrifice and were fixed in 10% formalin for histological evaluation. The Student's *t*-test was used to determine statistical significance. Of the NNK-treated mice, 23/23 developed 865 lung tumours (412 carcinomas) with a multiplicity of 37.6 ± 11.8 tumours per mouse ($p < 0.0001$ compared with vehicle controls). Six treated mice had tumours other than lung adenomas: three hepatocellular adenomas, two hepatocellular carcinomas and one squamous-cell papilloma of the nasal cavity. In untreated animals, 10/25 animals developed lung adenomas with a multiplicity of 0.6 ± 0.9 adenomas per surviving mouse whereas 4/25 trioctanoin controls developed lung adenomas with a multiplicity of 0.2 ± 0.5 adenomas per mouse (Castonguay *et al.*, 1983a).

In a study in which NNK and several structural analogues of NNK and NNN were examined, groups of 30 female strain A/J mice, 6–8 weeks of age, received thrice-weekly intraperitoneal injections of NNK (total dose, 20 μmol/mouse) in 0.2 mL saline for 7 weeks. NNK was synthesized and considered pure by high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC). Mice were held for 30 weeks after the last injection. The Student's *t*-test was used to determine statistical significance. Saline control mice had a lung tumour incidence of 40% (12/30), with a tumour multiplicity of 0.5 ± 0.7. NNK induced a 100% (30/30) incidence of lung tumours with a multiplicity of 7.2 ± 3.4 tumours per mouse ($p < 0.0001$) (Hecht *et al.*, 1988a). [The Working Group noted that the number of surviving animals was not listed, only macroscopic examination was carried out and that histological confirmation was not presented.]

Groups of 25 female strain A/J mice mice [age unspecified] received thrice-weekly intraperitoneal injections of NNK (99% pure by HPLC and gas chromatography–mass spectrometry) in 0.1 mL saline for a total of 20 injections (total dose, 20 μmol/mouse). Animals were held for 30 weeks after the final injection and statistical significance was

evaluated using Student's *t*-test. Controls consisted of animals injected over the same schedule with saline only. NNK induced a lung tumour incidence of 100% (25/25) and a multiplicity of 15.7 ± 4.1 tumours per mouse (saline control: 20% incidence (5/25); tumour multiplicity of 0.20 ± 0.40 tumours per mouse; [$p < 0.0001$]). Twenty-three treated mice had adenomas only and two had both adenomas and adenocarcinomas (Rivenson *et al*., 1989).

Female strain A/J mice, 5 weeks of age, were maintained on an AIN-76A or NIH-07 diet. At 7 weeks of age, groups of 15 mice (weighing 18.7 ± 0.07 g) received a single intraperitoneal injection of 0 (saline control), 2.5, 5 or 10 μmol NNK (> 99% pure) in 0.1 mL saline and were killed at semi-monthly intervals between 3 and 7 months after injection. Lung tumours were enumerated for each period and statistical significance was evaluated by ANOVA followed by Newman-Kuels' range test and chi-squared test. Selected tumours were confirmed by histopathology. Lung tumour incidence eventually reached 100% in nearly all NNK-treated groups. Lung tumour multiplicity became maximal at 3.5 months, with no significant increase between 3.5 and 7 months. A dose–response was seen for tumour multiplicity at 3.5 months: 0 tumour per mouse with saline alone, 1.0 ± 0.2 tumours per mouse with 2.5 μmol NNK, 3.7 ± 0.7 tumours per mouse with 5 μmol and 9.6 ± 0.8 tumours per mouse with 10 μmol. In mice given 10 μmol NNK, lung tumour multiplicity at 7 months did not increase over that found at 3.5 months. Final overall tumour multiplicities with 5 μmol and 2.5 μmol NNK were significantly lower than those with 10 μmol (Hecht *et al*., 1989).

To characterize and quantify lung lesions and their progression, groups of 15 female strain A/J mice, 6 weeks of age, received a single intraperitoneal injection of 100 mg/kg bw NNK (99% pure) suspended in trioctanoin and were maintained on an NIH-07 diet. Mice were killed starting at 14 weeks after injection and every 4 weeks thereafter up to 54 weeks. At 14 weeks, 100% of the lesions were hyperplasias; at 34 weeks the types and frequencies of lesions ranged from hyperplasia (57%), adenoma from hyperplasia (18%), adenoma (14%), carcinoma from adenoma (0%), carcinoma (8%) to microcarcinoma (3%). By 54 weeks, 13% of hyperplasia, 4% of adenoma from hyperplasia, 29% of adenoma, 18% of carcinoma from adenoma, 38% of carcinoma and 0% of microcarcinoma were observed (Belinsky *et al*., 1992). [The Working Group noted that the percentages at 54 weeks added up to 102%.]

Two groups of female strain A/J mice, 6–8 weeks of age, received thrice weekly intraperitoneal injection of a total dose of 5 μmol (30 mice) or 20 μmol (20 mice) NNK in 0.1 mL saline for 7 weeks. After 30 weeks, animals were killed and lung adenomas were enumerated. Statistical analysis of variance (nested model) followed by Scheffe's test for multiple comparison of means was conducted. A group of 30 negative control mice was injected with saline alone. In controls, tumour incidence was 26.7% (8/30) and tumour multiplicity was 0.27 ± 0.58 tumours per mouse; in NNK-treated mice, the incidence of tumours was 76.7% (23/30; $p < 0.001$) and 100% (20/20; $p < 0.001$) and tumour multiplicity was 1.6 ± 1.2 and 9.2 ± 6.3 tumours per mouse, in the low- and high-dose groups, respectively (Amin *et al*., 1996).

3.1.2 *Intravesicular administration*

Rat

Groups of 12 female Fischer 344 rats, 10 weeks of age, received twice-weekly instillations of 0.2 mL of a solution of 11 mg NNK (> 98% purity) dissolved in ethanol and diluted in sterile water for 30 weeks (total dose, 1.5 mmol) into the urinary bladder after excreting the residual urine. At a median of 70 weeks, no bladder tumours were observed in rats of either control or experimental groups, but 33% (4/12) of the rats exposed intravesically to NNK had liver tumours and 42% (5/12) had lung tumours [no further histopathological details were provided]. No liver or lung tumours were reported in controls (Lijinsky *et al*., 1991).

3.1.3 *Administration in the drinking-water*

(*a*) *Mouse*

Groups of male BALB/c mice [initial number unspecified], 8 weeks of age, were untreated or received 1 mg NNK in distilled water deposited on the tongue thrice weekly (total dose, 22 mg NNK); groups of male Swiss mice [initial number unspecified], 8 weeks of age, were untreated or received 0 (vehicle control) or 1 mg NNK in distilled water deposited on the tongue thrice weekly (total dose, 22 mg NNK). Animals were killed at 22 months or when moribund. Tumours were observed in 10/13 (77%) NNK-treated Swiss mice (nine lung adenomas, one forestomach papilloma and one hepatoma) and in 11/11 (100%) NNK-treated BALB/c mice (four lung adenomas, six forestomach papillomas and one hepatoma) [the Working Group noted that these tumours were reported only for nine of the 11 mice]). Tumours [not specified] developed in 2/19 (11%) untreated Swiss mice and no tumours were observed in 14 untreated BALB/c mice or 11 Swiss mice given distilled water only (Padma *et al*., 1989a).

(*b*) *Rat*

Male Fischer 344 rats, 8 weeks of age, were given 0.0 ppm [mg/mL] (80 rats), 0.5 ppm (80 rats), 1.0 ppm (80 rats) or 5.0 ppm (30 rats) NNK (> 99% pure) in the drinking-water from 8 weeks of age until the animals were killed at 128 weeks (0.5 ppm and control) or when moribund (108–120 weeks for 1.0 and 5.0 ppm NNK). A group of 80 rats (water only) served as controls. The incidence of lung tumours in controls and at 0.5-ppm, 1.0-ppm and 5.0-ppm NNK was 6/80, 9/80, 20/80 ($p < 0.01$) and 27/30 ($p < 0.01$), respectively. In the 1.0-ppm group, most of the tumours were adenomas, whereas in the 5.0-ppm groups, most were adenocarcinomas (13/27) and adenosquamous carcinomas (9/27). The incidence of exocrine pancreatic tumours in the groups treated with 0.0, 0.5, 1.0 and 5.0 ppm NNK was 1/80, 5/80, 9/80 ($p < 0.05$) and 2/80, respectively. Tumours in the 1.0-ppm group were eight acinar adenomas and one acinar or ductal adenocarcinoma. The authors speculated that the low incidence of pancreatic tumours in the 5.0-ppm group was due to the high incidence of tumours of the lung, nasal cavity and liver, which shortened survival (Rivenson *et al*., 1988).

3.1.4 *Oral cavity swabbing*

Rat

Groups of male Fischer 344 rats, 10 weeks of age, were swabbed in the oral cavity and lips with 0.3 mL of a 0- (control) or 15-mmol solution of NNK (purity, > 99%) using a cotton swab dipped into the solution until the entire 0.3 mL was used; the cotton swab was then rinsed with 0.1 mL water and this solution was also applied. Rats were treated three times during the first week, five times over 5 days for 3 weeks and, from the 5th week onwards, twice a day for 5 days per week until termination at 71 weeks of age. The approximate total dose of NNK was 539 mg or 2.60 μmol. Statistical analysis was by Student's *t*-test. This protocol produced only one papilloma in the oral cavity of 29 rats and no tumours were observed in the oesophagus. However, significant tumour formation was found in the lungs (5/29 adenomas; 19/29 adenocarcinoma; and 4/29 adenosquamous carcinoma), the nasal cavity (13/29 papilloma or adenoma; and 2/29 carcinoma) and liver (9/29 adenoma; 3/29 carcinoma). No tumours were observed in control animals (Prokopczyk *et al*., 1991).

3.1.5 *Cheek pouch application*

Hamster

Groups of male and female Syrian golden hamsters, 8 weeks of age, received applications of 0 or 1 mg NNK in distilled water on the cheek pouch three times a week (total dose, 120 mg). Animals were killed at 22 months or when moribund. Tumours occurred in 5/9 treated hamsters (two lung adenomas, four forestomach papillomas and one hepatoma); no tumours occurred in 11 untreated hamsters (Padma *et al*., 1989a).

3.1.6 *Subcutaneous administration*

(*a*) *Rat*

Groups of male and female Fischer 344 rats, 9 weeks of age, received thrice weekly subcutaneous injections of NNK in trioctanoin for 20 weeks to give total doses of 0 (26 males and 26 females), 1.0 (27 males and 27 females), 3.0 (15 males and 15 females) or 9.0 (15 males and 15 females) mmol/kg bw. After 7 weeks, injections were interrupted for 2 weeks because of weight loss in the high-dose group. Animals were killed when moribund or when only 20% of the group were alive. Major organs were fixed and examined microscopically. The high dose of NNK resulted in the deaths of all rats by 60–70 weeks; the animals in the mid-dose group survived to approximately 110 weeks. Survival in low-dose NNK-treated rats was comparable with that of trioctanoin controls. Trioctanoin control rats did not develop lung tumours except for one adenoma in a female rat. Lung tumour incidence in male rats given 0, 1.0, 3.0 and 9.0 mmol/kg bw was 0/26, 23/27 ($p < 0.01$), 13/15 ($p < 0.01$) and 14/15 ($p < 0.01$), respectively; that in female rats was 1/26, 8/27 ($p < 0.05$), 7/15 ($p < 0.01$) and 8/15 ($p < 0.01$), respectively. Lung tumours were adenomas or adenocarcinomas; five male rats had also squamous-cell carcinomas. Nasal tumour inci-

dence in male control, low-, mid- and high-dose rats was 0/26, 20/27 (19 benign, one malignant; $p < 0.01$), 13/15 (six benign, seven malignant; $p < 0.01$) and 14/15 (four benign, 10 malignant; $p < 0.01$), respectively; that in female rats was 0/26, 10/27 (10 benign; $p < 0.01$), 12/15 (9 benign, three malignant; $p < 0.01$) and 14/15 (four benign and 10 malignant; $p < 0.01$), respectively. Liver tumour incidence in the four groups of male rats was 3/26 (three benign), 3/27 (two benign, one malignant), 4/15 (one benign, three malignant) and 6/15 (two benign, four malignant; $p < 0.05$), respectively; and that in female rats was 1/26 (one benign), 4/27 (three benign, one malignant), 4/15 (two benign, two malignant) and 5/15 (two benign, three malignant; $p < 0.05$), respectively (Hoffmann *et al*., 1984).

Groups of male Fischer 344 rats, 8 weeks of age, received thrice-weekly subcutaneous injections of 0.0055 mmol/kg bw NNK in trioctanoin or trioctanoin alone (control) for 20 weeks. Surviving animals were killed after 104 weeks and major organs were examined for the presence of tumours. Of rats injected with NNK, 13/27 had lung tumours, four of which were adenocarcinomas and nine were adenomas, versus 1/26 (adenoma) trioctanoin controls; 6/27 had nasal tumours, one of which was a squamous-cell carcinoma and five of which were squamous-cell papillomas, and 10/27 had liver tumours, two of which were hepatocellular carcinomas and eight of which were adenomas. None of the 26 trioctanoin control rats developed nasal or liver tumours (Hecht *et al*., 1986a).

Groups of male Fischer 344 rats [initial number and age unspecified] (weighing 175–200 g) received thrice-weekly subcutaneous injections of 0, 0.03, 0.1, 0.3, 1.0, 10 or 50 mg/kg bw NNK in trioctanoin for 20 weeks. Animals were killed over a 100-week period and were examined for tumours. Rats were also killed when they had a weight loss of 10–15% over a 2-week period compared with controls. Tumour incidence increased in a dose–responsive manner from 0.03 mg/kg to 50 mg/kg. At the highest dose of NNK, the incidence of lung hyperplasia was 93.5% (58/62) and that of benign and malignant lung tumours was 87.1% (54/62). The incidence of lung tumours increased with increasing dose: 2.5% of 40 rats at 0 mg/kg, 6.7% of 60 rats at 0.03 mg/kg, 10.0% of 60 rats at 0.1 mg/kg, 13.3% of 60 rats at 0.3 mg/kg, 53.3% of 30 rats at 1.0 mg/kg, 73.3% of 30 rats at 10 mg/kg and 87.1% of rats at 50 mg/kg. The majority of benign lung tumours were classified as solid adenomas (72 tumours), papillary adenomas (seven tumours) or mixed (five tumours). Among the malignant tumours, 11 were solid carcinomas, 46 were papillary carcinomas, three were mixed carcinomas and 16 were squamous-cell carcinomas. In the highest-dose group, a 34% (21/62) incidence of tumours was found in the respiratory region and a 50% (31/62) incidence of tumours was observed in the olfactory region. In the respiratory region, 18 of the tumours were benign and three were malignant. At 10 mg/kg, tumour incidence was 53% (16/30) in the respiratory region and 26% (8/30) in the olfactory region. The types of tumours found were: 15 benign and one malignant in the respiratory region and four benign and four malignant in the olfactory region. The remainder of the lower doses did not induce tumours in the nasal cavity (Belinsky *et al*., 1990).

(*b*) *Hamster*

Groups of 15 male and 15 female Syrian golden hamsters, 8–10 weeks of age, received thrice-weekly subcutaneous injections of 0 (control) or 10 mg NNK (99% pure) in 0.3 mL trioctanoin (total dose, 0.91 mmol per hamster). In a second experiment, groups of 10 male and 10 female hamsters received thrice-weekly subcutaneous injections of 2.5 mg NNK in 0.3 mL (total of 75 injections; total dose, 0.91 mmol per hamster). In the first experiment, only 50% of the hamsters survived after 10 weeks; by 14 weeks, survival was only 26%. In the second experiment, all animals were alive after 4 months, 80% after 7 months, 75% after 10 months and 30% after 13 months. The first experiment was terminated after 16 months and the second experiment after 17 months. In the first experiment, 8/15 males and 11/15 females had lung tumours. In the second experiment, 10/10 males and 6/10 females developed lung tumours. In the first experiment most of the tumours were adenomas; in the second experiment, 6/10 tumours in males were adenocarcinomas and 4/6 tumours in females were adenocarcinomas. No lung tumours were observed in the control hamsters (Hoffmann *et al*., 1981).

In a study that examined the effect of smoke inhalation on lung tumour formation, groups of 10 male and 10 female Syrian golden hamsters, 8 weeks of age, received a single subcutaneous injection of 0 (vehicle control), 1.0, 3.3 or 10 mg NNK in 0.3 mL trioctanoin. The experiment was terminated after 72 weeks. Statistical significance was determined using the χ^2 test. NNK (10 mg) plus sham smoking produced three lung adenomas in males and one lung adenoma in a female. At the mid-dose, NNK induced two lung adenomas in males and none in females; the low dose of NNK induced two lung adenomas in males and two in females; trioctanoin alone induced no tumours. When NNK was followed twice daily by an exposure period to tobacco smoke for 69 weeks, the incidence of lung tumours in females treated with 3.3 mg rose from 0/10 to 6/10 ($p < 0.01$) (Hecht *et al*., 1983a).

In a study to evaluate the effect of hyperoxia on lung tumour development produced by NNK, four groups of 15 male Syrian golden hamsters each received twice-weekly subcutaneous injections of 0 (two control groups) or 1.25 mg/kg bw NNK (two experimental groups) in 0.15 mL trioctanoin, and were maintained under either ambient air conditions or in hyperoxia chambers (oxygen concentration, 70%) for periods of 8–12 months (ambient air) or 12–16 weeks (70% oxygen). Animals were killed at intervals of 4 weeks and three hamsters per time-point were evaluated for tumour formation. The cumulative incidence of lung tumours (adenomas and mixed adenosquamous carcinomas) in animals treated with NNK alone was 80% (12/15). When animals were maintained in an atmosphere of 70% oxygen, 70% (10/14) of animals developed neuroendocrine or mixed neuroendocrine and squamous-cell tumours. Under hyperoxia, the latent period was reduced from 16 weeks to 8 weeks. No tumours were observed in control animals (Schuller *et al*., 1990).

(*c*) *Mink*

Groups of random-bred mink (originated from the breeding farm of the Norwegian College of Veterinary Medicine), 3 months of age, received twice-weekly subcutaneous injections of NNK (purity, > 99%) for 28 weeks (four females; total dose, 6.3 mM) or NNN + NNK (two males and four females; total doses, 11.9 mM + 6.3 mM). Survival ranged from 56 to 136 weeks for mink injected with NNK and from 16 to 130 weeks for mink injected with NNN + NNK. Control animals were killed at 156 weeks (one male and four females). NNK alone induced malignant tumours in the nasal cavity (mainly esthesioneuroepithelioma) with invasion into the forebrain in all four females; one of them also developed multiple lung tumours (adenomas and/or adenocarcinomas). Time to tumour was 77 ± 39 weeks. Following the combined treatment with NNK + NNN, all males developed tumours in the nasal cavity (esthesioneuroepithelioma) and invasion into the forebrain was observed at 39 and 40 weeks. Nasal cavity tumours (mainly esthesioneuroepithelioma) were induced in three females with invasion into the forebrain. Of the four females, one developed multiple lung tumours (adenomas and adenocarcinomas) and one a liver tumour (bile duct adenoma). Time to tumour was 58 ± 44 weeks. No tumours were observed in the control minks (Koppang *et al*., 1997).

3.1.7 *Transplacental or neonatal exposure*

(*a*) *Mouse*

Groups of male and female neonatal Cr:NIH (S) mice from 15 litters [initial number unspecified] received intraperitoneal injections of 50 mg/kg bw NNK in saline on days 1, 3, 5, 7 and 10. Controls consisted of eight litters that were injected with saline alone. Mice were killed at 15 months or when they showed signs of illness. Representative tumours were stained and classified as adenomas or carcinomas following microscopic examination. Statistical analysis was conducted using the Fisher's exact test for tumour incidence and Student's *t*-test for number of tumours per mouse. In male mice, 30/55 animals developed liver tumours (including four carcinomas) with an average of 1.15 ± 1.4 tumours per mouse, whereas no liver tumours were seen in the 33 control males. In females, 8/57 mice developed liver tumours (including two carcinomas) with a multiplicity of 0.14 ± 0.35 tumours per mouse and no liver tumours were observed in control females. Lung tumours [no histopathological details provided] were found in 56.6% of treated males (30/55) ($p < 10^{-7}$) with a multiplicity of 0.74 ± 0.9 versus 0.3 ± 0.6 tumours per mouse in 21% (7/33) of saline controls ($p < 0.025$, *t*-test). In females, lung tumours [no histopathological details provided] were observed in 36.8% (21/57) of NNK-treated mice versus 22% (7/32) of saline controls. The average number of tumours per lung in treated females was 0.51 ± 0.75 versus 0.25 ± 0.5 in saline controls ($p < 0.1$, *t*-test) (Anderson *et al*., 1991).

Pregnant Swiss (Cr:NIH) mice were treated either with 0 (untreated mothers) or with a close to maximum tolerated dose of 100 mg/kg bw NNK by intraperitoneal injection in

saline on gestation days 15, 17 and 19. Infant mice of untreated mothers received an intraperitoneal injection of either 50 mg/kg bw NNK in saline or saline alone on day 4. The number of animals in each group ranged from 27 to 30 and comprised male and female progeny from at least 10 litters. All animals were killed at 52 weeks. Statistical significance of tumour multiplicities was performed using the Kruskal-Walls ranking procedure for differences among treatment groups for organ/sex combinations and Wilcoxon rank-sum to make pairwise comparisons between treatment groups. NNK did not induce tumours transplacentally in male or female offspring. Infant male mice treated with NNK on postnatal day 4 developed both lung (incidence, 8/30 versus 2/27 controls; multiplicity, 0.27 ± 0.45 versus 0.07 ± 0.27 in controls) and liver tumours (mainly adenomas) (incidence, 7/30 versus 1/27 controls; multiplicity, 0.23 ± 0.50 versus 0.03 ± 0.18 in controls, $p = 0.035$). In postnatally treated females, the incidence of lung tumours was not significantly increased and no liver tumours occurred (Beebe *et al*., 1993). [The Working Group noted that the control group for transplacental treatment was used to perform statistics for the postnatal experiment.]

(*b*) *Hamster*

Groups of five pregnant Syrian golden hamsters received either a single subcutaneous injection of 50, 100 or 200 mg/kg bw NNK (> 98% pure) in trioctanoin on day 15 of gestation or multiple subcutaneous injections of 50 or 100 mg/kg bw NNK in trioctanoin on days 13, 14 and 15 of gestation. Tumour incidence was analysed by the paired *t*-test. No tumours were observed in 82 and 83 offspring of animals treated with a single or multiple injections of trioctanoin alone. After single injections of 50, 100 or 200 mg/kg bw NNK, tumours (all sites combined) were observed in 29% (11/38), 56% (20/36) and 76% (19/25) of male and 51% (17/35), 56% (20/36) and 61% (25/41) of female offspring ($p < 0.01$ versus controls for all six groups). After multiple injections of 50 and 100 mg/kg bw NNK, tumours (all sites combined) were observed in 49% (19/39) and 73% (29/40) and 63% (25/40) and 62% (23/37) of male and female offspring, respectively ($p < 0.01$ versus controls for all 4 groups). The incidence of respiratory tract tumours (nasal cavity, larynx, trachea) in offspring that received single injections of 50 mg/kg bw NNK was 21% (8/38) in males and 26% (9/35) in females; in those treated with 200 mg/kg bw, the incidence was 24% (6/25) in males and 61% (25/41) in females ($p < 0.05$ versus controls for all four groups). After multiple injections of NNK, the frequency of respiratory tract tumours in offspring treated with 50 mg/kg bw was 19% (7/37) in males and 33% (12/36) in females; in those treated with 100 mg/kg bw, the incidence was 38% (15/39) in males and 24% (9/38) in females ($p < 0.05$ versus controls for all four groups) (Correa *et al*., 1990).

Groups of four pregnant female Sendai virus-free Syrian golden hamsters were given 0 or 10% ethanol in the drinking-water from day 5 to day 16 of gestation and received a single intratracheal instillation of 50 mg/kg bw NNK (> 98% pure) in distilled water on day 15 of gestation. Controls were treated with either water alone or ethanol alone. No tumours were observed in the offspring of females treated with distilled water alone (0/28). Tumours developed in two offspring of mothers treated with ethanol alone (1/17 males, pancreas; and 1/23

females, lymphoma). Two adenocarcinomas of the olfactory region and one adrenal pheochromocytoma developed in 3/9 (33.3%) male offspring transplacentally exposed to NNK alone. In six female offspring, five adrenal pheochromocytomas, two colonic polyps, one liver tumour and three lymphomas were observed. In the offspring of mothers exposed to ethanol followed by NNK, tumours developed in 8/16 males and 13/17 females; adenocarcinomas of the nasal cavity were found in two males and two females ($p < 0.01$) [the Working Group calculated that this was not significant; $p = 0.11$, Fisher's exact test], ductular adenocarcinomas in the pancreas were observed in four males and 10 females ($p < 0.01$ compared with NNK alone), pheochromocytomas in the adrenals developed in three males and seven females ($p < 0.01$ compared with NNK alone) and one tumour in the colon occurred in one male and one female. No lymphomas were observed in this group (Schüller *et al.*, 1993).

Groups of pregnant Syrian golden hamsters received a single subcutaneous injection of 1, 5, 10 or 20 mg/kg bw NNK (> 98% pure) in trioctanoin on day 15 of gestation. Other groups of pregnant hamsters were given 0.05, 5 or 50 mg/kg bw NNK in distilled water by intratracheal instillation on day 15 of gestation. All control animals were given trioctanoin (15 males, 21 females) or distilled water alone (12 males, 15 females) and were killed at 59 weeks when the last NNK-treated hamsters were killed. Statistical analysis was performed by the paired *t*-test. Tumours were observed in NNK-exposed offspring at multiple sites including nasal cavity, adrenal glands, colon, pancreas and lymphoma. Total tumour incidence in the male and female offspring of mothers that received a subcutaneous injection was: 1 mg/kg bw, 27.3% in males (3/11) and 16.7% in females (3/18), 5 mg/kg bw, 27.3% in males (3/11) and 21.4% in females 3/14); 10 mg/kg bw, 33.3% in males (4/12) and 28.6% in females (2/7); and 20 mg/kg bw, 50% in males (3/6) and 57.2% in females (8/14). No tumours were observed in the trioctanoin controls. In offspring of mothers treated by intratracheal instillation, tumour incidence was: 0.05 mg/kg bw, 33.3% in males (2/6) and 50% in females (10/20); 5 mg/kg bw, 28.6% in males (4/14) and 42.1% in females (8/19); and 50 mg/kg bw, 33.3% in males (3/9) and 40% in females (6/15). No tumour occurred in distilled water controls. Tumours in NNK-exposed offspring were predominantly found in the nasal cavity and adrenal glands. The total tumour incidence in all NNK-exposed offspring was significantly increased ($p < 0.01$) with no significant difference between the routes of administration (Schüller *et al.*, 1994).

Groups of outbred female Syrian golden hamsters, 8 weeks of age, were given ethanol (10% v/v) in the drinking-water from day 5 through to day 15 of pregnancy. Some females were also instilled intratracheally with 50 mg/kg NNK on day 15 of pregnancy. Offspring were born on the evening of day 16 and were observed until clinical symptoms of pancreatic disease occurred. Groups of offspring were given either the cyclooxygenase inhibitor ibuprofen (infant Motrin oral suspension diluted with sterile water to yield 2.86 mg/kg given orally three times a week for life) or the 5-lipoxygenase-activating protein inhibitor MK886 (10 mg/kg dissolved in 0.25% carboxymethylcellulose in sterile water given orally thriceweekly for life). Ten of 16 (62%) offspring of the hamsters given ethanol and NNK alone developed pancreatic ductal adenocarcinoma compared with 5% of controls. Significant

reductions (p = 0.0026) were observed in ibuprofen- (6/24) and MK886- (8/19) treated offspring (Schuller *et al.*, 2002).

3.1.8 *Administration with known carcinogens or modifying factors*

These studies have been reviewed comprehensively (Hecht, 1998). One study in mice and two studies in rats that were not included in this review are summarized below.

(*a*) *Mouse*

The NNK/mouse lung model has been used extensively by numerous investigators to determine factors, conditions, drugs or chemopreventive compounds that can modulate the formation of lung tumours in mice. One of these studies is summarized below.

A study was conducted to determine the capacity of cigarette smoke to induce lung tumours and promote lung tumorigenesis induced by NNK. Groups of 20 female A/J mice, 7 weeks of age, were exposed for 6 h per day on 5 days per week for 26 weeks to filtered air (FA), cigarette smoke (CS; diluted mainstream smoke (target concentration, 250 mg total particulate matter/m^3) from IR3 research cigarettes), NNK or NNK plus CS. Mice were exposed for 3 days to 50% of the target concentration of CS and for 4 days to 75% of the target concentration of CS before full exposure. Three days before CS exposure, mice received an intraperitoneal injection of 100 mg/kg bw NNK in 0.1 mL saline. Mice were killed 5 weeks after the exposures were terminated. Total tumours were enumerated macroscopically and characterized microscopically. Differences in survival were analysed by Breslow statistics in a Kaplan-Meier survival analysis. Student's *t*-test with a Bonferroni multiple comparisons correction was used to examine group differences in lung weight, tumour multiplicity for all animals and tumour multiplicity in tumour-bearing animals, with significance set at the $p < 0.05$ level. The lung tumour incidence among the four groups was: FA, 5/19 (26%); CS, 0/19 (0%); FA + NNK, 19/20 (95%); and CS + NNK, 13/16 (81%). The lung tumour multiplicities (total tumours/animal at risk) were: FA, 0.32 ± 0.58 tumours per animal; FA + NNK, 2.50 ± 1.67 tumours per animal; and CS + NNK, 2.50 ± 1.97. Those among tumour-bearing animals were: FA, 1.20 ± 0.44 tumours per animal; FA + NNK, 2.63 ± 1.61 tumours per animal; and CS + NNK, 3.08 ± 1.71. CS exposure decreased both body weights and lung weights, but treatment with NNK had no additional effect. Tumour multiplicity was greater in the FA + NNK- and the CS + NNK-treated groups compared with the FA- and CS-treated groups ($p < 0.05$) among all animals, but tumour multiplicity in the tumour-bearing animals did not differ between the FA-, FA + NNK- or CS + NNK-treated groups (Finch *et al.*, 1996). [The Working Group noted that animals were held for a relatively short period of exposure to let tumours develop].

(*b*) *Rat*

Groups of 30 male Fischer 344 rats, 10 weeks of age, were treated with a mixture of 0.5 mL NNK + NNN (total dose, 14 + 68 μg) dissolved in water by swabbing the oral cavity and lips of the animals with a cotton swab dipped into the solution. A group of 21

rats was used as water controls. Application was performed as follows: once a day for 7 days, twice a day for 5 days per week, once a day for 2 days in weeks 2–23 and twice a day from week 24 to 131. The mean approximate total dose of NNK was 19 mg per rat and that of NNN was 97 mg. The experiment was terminated at 131 weeks at which time survival was 14%. The incidence of oral tumours was 8/30 (six cheek papillomas, one hard palate papilloma and two tongue papillomas) in NNK + NNN-treated animals and 0/21 in controls ($p < 0.05$). In addition to oral tumours, four lung adenocarcinomas and one lung adenoma were found in five treated animals, and one lung adenoma developed in one control animal. The incidence of tumours in the prostate and mammary glands and that of leukaemia/lymphoma in treated animals did not differ from that in controls (Hecht *et al*., 1986b).

Groups of male Fischer 344 rats were maintained on a high-fat (HF) diet (23.5% corn oil) or on a low-fat (LF) diet (5.0% corn oil). NNK was administered in the drinking-water at a concentration of 0 or 2.0 ppm [mg/L]. The number of animals in the treatment groups was: NNK–HF, 60 rats; NNK–LF, 60 rats; tap-water–HF, 20 rats; tap-water–LF, 20 rats. The experiment was terminated after 95–105 weeks. Incidences of lung tumours at termination were: NNK–HF, 30/60; NNK–LF, 27/60; HF, 1/20; LF, 1/20. Lung tumours were mainly adenomas or adenocarcinomas. There was no significant difference in the final incidence of lung tumours between NNK–HF and NNK–LF groups, but significantly survival was shorter in the NNK–HF than in the NNK–LF group. The incidence of pancreatic tumours was: NNK–HF, 28/60 ($p < 0.05$); NNK–LF, 19/60; HF, 6/20; LF, 6/20. In the NNK–HF group, 18 rats had benign and malignant tumours of the exocrine pancreas; in addition, 10 islet-cell tumours were observed. In the NNK–LF group, the corresponding numbers were 14 and five (Hoffmann *et al*., 1993a).

3.1.9 *Carcinogenicity of NNK metabolites*

Mouse

Groups of 25 female strain A/J mice received thrice weekly intraperitoneal injections of 0.01 mL of a 1% solution of NNK-*N*-oxide suspended in trioctanoin for 7.3 weeks (22 injections; total dose, 0.11 mmol/mouse) and were held for 30 weeks after the last injection. Twenty-four of 25 mice treated with NNK-*N*-oxide developed lung tumours (24 carcinomas/90 tumours) at a multiplicity of 3.6 ± 2.7 tumours per mouse. One NNK-*N*-oxide-treated mouse had a leiomyoma of the uterus. In untreated animals, 10/25 animals developed lung tumours with a multiplicity of 0.6 ± 0.9 tumours per surviving mouse whereas 4/25 trioctanoin controls developed lung tumours with a multiplicity of 0.2 ± 0.5 tumours per mouse (Castonguay *et al*., 1983b).

3.2 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL)

The carcinogenicity of NNAL in experimental animals has been evaluated previously (IARC, 1985).

3.2.1 *Intraperitoneal administration*

Mouse

A group of 25 female strain A/J mice, 6–8 weeks of age, received thrice-weekly intraperitoneal injections of 0.2 mL of a 0.5% solution of NNAL (purity > 99%) in saline for 7 weeks (total of 22 injections; total dose, 22 mg [0.11 mmol]) and were held without further treatment for an additional 30 weeks. Further groups of 25 female mice served as untreated and vehicle controls. Histological examination of lung and other organs that showed macroscopic lesions revealed lung adenomas in 1/25 untreated controls, 3/25 vehicle controls and 9/25 NNAL-treated mice (vehicle controls compared with treated mice, $p = 0.047$). No malignant tumours were observed in the lung or at other sites in any of the groups (Hecht *et al.*, 1978).

Groups of 25 female strain A/J mice, 6–8 weeks of age, received thrice-weekly intraperitoneal injections of a 1% NNAL (> 99% pure) suspension in 0.1 mL trioctanoin for 7.3 weeks (22 injections; total dose, 0.11 mmol/mouse). Treated mice were held for 30 weeks after the last injection. Twenty-five untreated and 15 vehicle-treated female mice served as controls. Lung tumours were examined macroscopically for total lesions and microscopically for histological evaluation. The Student's *t*-test was used to determine statistical significance. Of the NNAL-treated mice, 25/25 developed 658 lung tumours (243 carcinomas) at a multiplicity of 26.3 ± 11.7 tumours per surviving mouse ($p < 0.0001$ compared with vehicle controls). Two NNAL-treated mice developed extrapulmonary tumours: a squamous-cell papilloma of the nasal cavity and a papilloma of the tongue. In untreated animals, 10/25 developed tumours with a multiplicity of 0.6 ± 0.9 tumours per surviving mouse. In trioctanoin controls, 4/25 animals had tumours with a multiplicity of 0.2 ± 0.5 tumours per surviving mouse (Castonguay *et al.*, 1983a).

Two groups of 30 and 20 female strain A/J mice, 6–8 weeks of age, received thrice-weekly intraperitoneal injections of a total dose of 10 μmol and 50 μmol NNAL in 0.1 mL saline for 7 weeks. After 30 weeks, animals were killed and lung adenomas were enumerated. Statistical analysis was conducted by analysis of variance (nested model) followed by Scheffe's test for multiple comparison of means. Controls were 30 mice injected with saline alone (negative controls), 30 mice injected with 5 μmol NNK and 20 mice injected with 20 μmol NNK (positive controls). In NNAL-treated mice, 22/30 low-dose females had tumours with a tumour multiplicity of 1.5 ± 1.4 tumours per mouse and 20/20 high-dose females had tumours with a multiplicity of 9.7 ± 6.4 tumours per mouse. In the saline controls, 8/30 animals had tumours with a tumour multiplicity of 0.27 ± 0.58 tumours per mouse. In the NNK controls, the tumour incidence was 23/30 in low-dose females with a tumour multiplicity of 1.6 ± 1.2 tumours per mouse and 20/20 in high-dose females with a tumour multiplicity of 9.2 ± 6.3 tumours per mouse (Amin *et al.*, 1996).

Groups of 14–20 female strain A/J mice, 7 weeks of age, received a single intraperitoneal injection of 20 μmol NNAL or a metabolite of NNAL [4-(methylnitrosamino)-1-(3-pyridyl)but-(*S*)-1-yl] β-*O*-D-glucosiduronic acid, 4-(methylnitrosamino)-1-(3-pyridyl-*N*-oxide)-1-butanol, 5-(3-pyridyl)-2-hydroxytetrahydrofuran, 4-(3-pyridyl)butane-1,4-diol or

2-(3-pyridyl)tetrahydrofuran) in 0.2 mL of saline. A control group of 20 female mice was injected with saline only. Mice were killed after 16 weeks and lung tumours enumerated. Statistical analysis was carried out using ANOVA and χ^2. In saline controls, 2/20 animals developed lung tumours (adenomas) with a tumour multiplicity of 0.1 ± 0.3 tumours per mouse. None of the five NNAL metabolites was tumorigenic in the mouse lung; 20/20 NNAL-treated mice developed lung adenomas ($p < 0.001$) with a tumour multiplicity of 12.1 ± 5.6 tumours per mouse ($p < 0.0001$) (Upadhyaya *et al*., 1999).

3.2.2 *Administration in the drinking-water*

Rat

A group of 30 male Fischer 344 rats, 8 weeks of age, was given 5.0 ppm NNAL (> 99% pure) in the drinking-water throughout the experimental period. Animals were killed at 112 weeks. A group of 80 male rats (drinking-water only) served as controls. Lung tumours developed in 26/30 NNAL-treated rats; five rats had adenomas, 12 rats had adenocarcinomas ($p < 0.01$) and nine rats had adenosquamous carcinomas ($p < 0.01$). Lung tumours developed in 6/80 drinking-water controls: three adenomas, two adenocarcinomas and one adenosquamous carcinoma. The NNAL-treated rats also developed pancreatic tumours (8/30; $p < 0.01$). Three rats had acinar adenomas, four had ductal adenocarcinomas and one had an acinar adenocarcinoma. One control rat had an acinar adenoma (Rivenson *et al*., 1988).

3.2.3 *Administration with known carcinogens or modifying factors*

(*a*) *Mouse*

Female Hid:SENCAR BR mice, 50–55 days of age, received topical applications of 2.8 μmol/mouse NNAL in 100 μL acetone every other day (10 doses; total dose, 28 μmol/mouse). Twice weekly applications of 2.0 μg TPA began 10 days after the last NNAL treatment for 20 weeks. Control mice were treated with acetone followed by TPA. Statistical analysis was by χ^2 test. All mice were examined macroscopically for skin tumours and for tumours in lung and liver. Skin tumours developed in 2/29 mice given NNAL. No tumours were observed in the 29 controls and none of the NNAL-treated animals developed lung or liver tumours (LaVoie *et al*., 1987).

(*b*) *Hamster*

The effects of administration of low doses of NNAL were investigated in Syrian golden hamsters treated with *N*-nitrosobis(2-oxopropyl)amine (BOP). Three groups of 30 female Syrian golden hamsters, 5 weeks of age, were given a single subcutaneous injection of 10 mg/kg bw BOP. After this treatment, animals were given drinking-water alone, or drinking-water supplemented with 2 ppm or 5 ppm NNAL during weeks 2–53. Three additional groups of 10, 20 and 20 hamsters were given tap-water alone, 2 ppm NNAL or 5 ppm NNAL, respectively. NNAL did not influence the incidence of pancreatic adenocarcinomas

or dysplastic lesions. However, the total incidence of pancreatic adenocarcinomas and dysplastic lesions was significantly higher ($p < 0.05$) in the BOP/high-dose NNAL group (14/30) than in the groups treated with BOP alone (5/27) or BOP/low-dose NNAL (4/29). NNAL itself did not induce any proliferative lesions of the exocrine pancreas. No effects were found on the incidence or multiplicity of pancreatic islet-cell proliferative lesions. (Furukawa *et al*., 1997).

3.3 *N*′-Nitrosonornicotine (NNN) (Table 10)

The carcinogenicity of NNN in experimental animals has been evaluated previously (IARC, 1985).

3.3.1 *Intraperitoneal administration*

(*a*) *Mouse*

A group of 20 male and 20 female Chester Beatty mice, 6 weeks of age, received weekly intraperitoneal injections of 0.1 mL 2% NNN dissolved in arachis oil for 41 weeks. Controls were 15 male and 15 female mice that received arachis oil only. During the first 7 months of NNK treatment, 14 males and 11 females died with no evidence of tumours. However, after the 8th month, eight animals died. Seven of these (five females and two males) were autopsied and were found to have multiple pulmonary adenomas that were confirmed histologically. In five animals, the number of pulmonary lesions was greater than 30. In addition, one of the males had a lymphosarcoma in the kidney (Boyland *et al*., 1964a).

Groups of 25 strain A/J female mice, 6–8 weeks of age, received a total of 22 intraperitoneal injections of 0.5% NNN in 0.2 mL saline or 1% NNN in 0.1 mL trioctanoin over a period of 7 weeks (total dose, 22 mg per mouse). After the final injection, mice were held for an additional 30 weeks. Controls comprised untreated animals and vehicle controls (saline and trioctanoin). Statistical comparisons were made using the Student's *t*-test. Animals injected with NNN in saline had lung tumour incidence of 76% (16/21) with a tumour multiplicity of 1.74 ± 1.37 tumours per mouse compared with a tumour incidence of 12% (3/25) and a multiplicity of 0.24 ± 0.72 tumours per mouse in the saline controls. Animals injected with NNN in trioctanoin had a lung tumour incidence of 57% (12/23) and a tumour multiplicity of 0.87 ± 1.01 tumours per mouse compared with a 21% (5/24) incidence and 0.2 ± 0.41 tumours per mouse multiplicity in vehicle controls. NNN induced significant increases in tumour incidence over vehicle controls ($p < 0.05$) (Hecht *et al*., 1978).

Groups of 25 female strain A/J mice, 6–8 weeks of age, received thrice-weekly intraperitoneal injections for 7.3 weeks (22 injections) of NNN (> 99% pure) suspended in 0.1 mL saline (total dose, 0.12 mmol/mouse). Mice were held for 30 weeks after the last injection. Untreated and vehicle-treated animals served as controls. Lung tumours were counted macroscopically when animals were killed and fixed in 10% formalin for histo-

logical evaluation. The Student's *t*-test was used to determine statistical significance. In NNN-treated animals, 16/24 had lung tumours with a multiplicity of 1.2 ± 1.3 tumours per surviving mouse ($p < 0.05$ compared with vehicle controls). In untreated animals, 10/25 developed lung tumours with a multiplicity of 0.6 ± 0.9 tumours per surviving mouse; in saline controls, 7/24 animals had lung tumours with a multiplicity of 0.4 ± 0.6 tumours per surviving mouse. Most of the tumours were classified as lung adenomas, except for 10 carcinomas in NNN-treated animals (Castonguay *et al*., 1983a).

Groups of 30 female strain A/J mice, 6–8 weeks of age, received thrice-weekly intraperitoneal injections of NNN (considered pure by HPLC and TLC) in 0.2 mL saline for 7 weeks (total dose, 100 μmol/mouse). Mice were held for 30 weeks after the last injection. The Student's *t*-test was used to determine statistical significance. NNN induced an 83% (25/30) ($p < 0.0001$) incidence of lung tumours with a multiplicity of 1.8 ± 1.4 tumours per mouse; the saline controls had a tumour incidence of 40% (12/30) with a tumour multiplicity of 0.5 ± 0.7 tumours per mouse (Hecht *et al*., 1988a).

Two groups of 30 female strain A/J mice, 6–8 weeks of age, received thrice-weekly intraperitoneal injections of a total dose of 40 μmol or 200 μmol NNN in 0.1 mL saline for 7 weeks. After 30 weeks, animals were killed and lung adenomas were enumerated. Statistical analysis was conducted by analysis of variance (nested model) followed by Scheffe's test for multiple comparison of means. Controls were 30 mice injected with saline alone (negative controls), 30 mice injected with 5 μmol NNK and 20 mice injected with 20 μmol NNK (positive controls). In NNN-treated mice, the incidence of tumours was 43.3% [13/30] and 80% [24/30] ($p < 0.001$); tumour multiplicity was 0.7 ± 1.0 and 2.3 ± 2.1 tumours per mouse in the low- and high-dose group, respectively. In the saline controls, tumour incidence was 26.7% [8/30] and tumour multiplicity was 0.27 ± 0.58 tumours per mouse. In the NNK controls, tumour incidence was 76.7% [23/30] and 100% [20/20] and tumour multiplicity was 1.6 ± 1.2 and 9.2 ± 6.3 tumours per mouse in animals treated with 5 and 20 μmol, respectively (Amin *et al*., 1996).

(*b*) *Hamster*

Male Syrian golden hamsters, 8 weeks of age, were placed on a liquid diet (#711, Bio-Serv, Frenchtown, NJ). At 9 weeks of age, animals were divided into two groups of 105 each; one of these groups was placed on a liquid diet that contained ethanol (6% w/v). At 13 weeks of age, each group was subdivided into five groups of 21 animals; two groups received thrice-weekly intraperitoneal injections of 1 mmol (2.37 mg) or 2 mmol (4.75 mg) NNN in saline for 25 weeks (total doses, 1 mmol and 2 mmol per hamster). After 16 weeks on the liquid diets, a significant decrease in weight was observed; all animals were placed on an NIH-07 diet for 1 month and injections were suspended. Liquid diets were reinstated and, after 1 week, injections were resumed without further interruption. Animals were killed and autopsied when moribund and all remaining animals were killed 15 months after the first injection. None of the 19 ethanol-treated or 21 diet control animals developed tumours in the nasal cavity or trachea. Approximately 50% of the control animals and NNN-treated animals developed adrenal tumours. Five of 21 NNN-

treated (1 mmol) animals had tumours (one nasal cavity and four tracheal; $p < 0.05$). When the diet was supplemented with ethanol, 6/17 NNN-treated (1 mmol) animals had tumours (one nasal cavity and five tracheal). After treatment with 2 mmol, 13/21 animals had tumours (five nasal cavity and nine tracheal) ($p < 0.001$); in the ethanol-supplemented group, 10/21 had tumours (four nasal cavity and seven tracheal) (McCoy *et al*., 1981).

3.3.2 *Skin application*

Mouse

NNN (purity, > 98.5%) was applied twice a week to the interscapular region of female CFLP mice at doses of 12.5, 50 and 200 μg/mouse in 200 μL acetone for 104 weeks. No skin tumours appeared in 65 acetone-treated controls. The 65 mice treated with 12.5 μg developed three skin carcinomas; in 65 mice treated with 50 μg, two skin papillomas and one skin carcinoma developed. In 64 mice treated with 200 μg, three skin carcinomas were observed (Deutsch-Wenzel *et al*., 1985).

3.3.3 *Oral administration*

(*a*) *Mouse*

Groups of male BALB/c mice, 8 weeks of age, were untreated or received 1 mg NNN in distilled water deposited on the tongue thrice weekly (total dose, 72 mg NNN) and groups of male Swiss mice, 8 weeks of age, were untreated or received 0 (vehicle control) or 1 mg NNN in distilled water deposited on the tongue thrice weekly (total doses, 22 or 72 mg NNN). Animals were killed at 22 months or when moribund. Tumours were observed in 13/19 (68%) Swiss mice treated with the low dose of NNN (eight lung adenomas and seven forestomach papillomas) and in 5/10 (50%) Swiss mice treated with the high dose of NNN (three lung adenomas, two forestomach papillomas and two hepatomas) and 6/6 (100%) NNN-treated BALB/c mice (four lung adenomas, two forestomach papillomas and two hepatomas). Tumours [not specified] developed in 2/18 (11%) untreated Swiss mice and no tumours were observed in 14 untreated BALB/c mice or 11 Swiss mice given distilled water only (Padma *et al*., 1989a).

(*b*) *Rat*

A group of 20 male Fischer 344 rats [age unspecified] was given 0.02% NNN in the drinking-water on 5 days per week and tap-water on weekends for a period of 30 weeks (total dose, 630 mg). Moribund animals were killed and autopsied and the remaining animals were killed after 11 months. A group of 19 control rats given drinking-water only did not develop tumours in any major organs. Rats treated with NNN developed oesophageal tumours (12/20; 11 papillomas and three carcinomas) [$p < 0.0001$, Fisher's exact test]. Three of 20 rats developed nasal cavity carcinomas and one rat had a pharyngeal papilloma (Hoffmann *et al*., 1975).

Groups of 12 male and 12 female Fischer 344 rats, 6–8 weeks of age, were given NNN in the drinking-water at a concentration of 0 (control) and 0.012% (total dose, 3.6 mmol for males and 3.3 mmol for females) for a period of 36 weeks after which time animals received tap-water. The experiment was terminated after 104 weeks. Statistical significance was analysed using the χ^2 test. In males, NNN induced a total of 12 papillomas and three squamous-cell carcinomas in the oesophagus in 12/12 animals. In females, NNN induced a total of 11 papillomas and three squamous-cell carcinomas in 11/12 animals. In the nasal cavity, NNN induced eight papillomas and six malignant tumours in 10/12 males and seven papillomas and nine malignant tumours in 11/12 females. No oesophageal or nasal tumours were observed in 12 male or 12 female controls. The incidence of Leydig-cell tumours in males or mammary tumours in females was not increased in treated rats (Hecht *et al*., 1983b).

Groups of male Fischer 344 rats, 9 weeks of age, were given an ethanol diet (Groups 2 and 6) or a control liquid diet (#711, Bioserv, Frenchtown, NJ; Groups 1 and 5). At 13 weeks of age, animals in Groups 1 and 2 (26 rats each) received thrice-weekly subcutaneous injections of 0.3–0.5 mL saline. Groups 5 and 6 (30 rats each) began a liquid diet that contained NNN (17.5 mg/L) and ethanol and NNN, respectively. After 27 weeks, Groups 5 and 6 were placed on standard diet until they were killed at 98 weeks of age. Animals treated with saline developed no nasal cavity tumours. In Groups 5 and 6, most of the tumours observed in the nasal cavity were benign (11 and 20 benign and seven and six malignant, respectively). Significant numbers of benign and malignant tumours (squamous-cell carcinomas) were found in the oesophagus (16 and 13 benign and nine and seven malignant, respectively) (Castonguay *et al*., 1984a).

(*c*) *Hamster*

Groups of 10 male and 10 female Syrian golden hamsters, 6–7 weeks of age, were given 0 (control) and 0.016% NNN (purity, > 98%) in the drinking-water for 31 weeks, after which animals received tap-water. Total doses of NNN were estimated to be 1.9 mmol for males and 2.8 mmol for females. The experiment was terminated after 96 weeks. Statistical significance was analysed using the χ^2 test. NNN induced two nasal cavity tumours and one tracheal tumour in both males and females (papillomas). A lymphoma in the caecum and a liver angiosarcoma were observed in NNN-treated males. No tracheal or nasal tumours were observed in 10 male or 10 female control hamsters, although one female developed a lymphoma (Hecht *et al*., 1983b).

3.3.4 *Cheek pouch application*

Hamster

A group of 36 male and female [assumed to be equally distributed] Syrian golden hamsters [age unspecified] had one cheek pouch painted with 10 mg NNN (98% pure) in mineral oil five times a week for 24 weeks; in 16 control male and female [assumed to be equally distributed] hamsters, one buccal pouch was treated with mineral oil alone. Each

dose of NNN was approximately 10 mg. After 24 weeks, none of the animals had developed tumours (Papageorge *et al*., 1996).

3.3.5 *Subcutaneous administration*

(*a*) *Rat*

Groups of 26 and 30 male Fischer 344 rats, 9 weeks of age, were given an ethanol diet (Groups 2 and 6) or a liquid diet (#711, Bio-Serv, Frenchtown, NJ; Groups 1 and 3). At 13 weeks of age, animals in Groups 1 and 2 (26 rats each) received thrice-weekly subcutaneous injections of 0.3–0.5 mL saline. Groups 3 and 4 (30 rats each) received thrice-weekly subcutaneous injections of 10 mg/kg bw NNN in saline (56–66 injections; total dose, 1 mmol/rat, respectively). Animals were killed at 98 weeks of age. Animals treated with saline developed no nasal cavity tumours. Most of the tumours in the nasal cavity were malignant in Groups 3 (20/24) and 4 (20/22). Only two benign and one malignant tumours were found in the oesophagus in Group 4 (Castonguay *et al*., 1984a).

Groups of male and female Fischer 344 rats, 9 weeks of age, received thrice-weekly subcutaneous injections of NNN in trioctanoin for 20 weeks (total doses, 1.0, 3.0 or 9.0 mmol/kg bw) and were killed when moribund or when only 20% of the animals were alive. A control group was injected with trioctanoin only. Major organs were fixed and examined microscopically. The high dose of NNN resulted in the deaths of all rats (15 males and 15 females) by 60–70 weeks; rats treated with the mid-dose (15 males, 15 females) survived to approximately 110 weeks. Survival of low-dose NNN-treated rats (27 males, 27 females) was comparable with that of trioctanoin controls. The group of 26 male and 26 female trioctanoin controls did not develop any tumours. The incidence of benign and malignant nasal cavity tumours in male and female rats was 56% (15/27) and 44% (12/27) in the low-dose group, 73% (11/15) and 60% (9/15) in the mid-dose group and 86% (12/14) and 100% (15/15) in the high-dose group, respectively. At the high dose, all of the nasal cavity tumours were malignant (Hoffmann *et al*., 1984).

(*b*) *Hamster*

Groups of 10 female and 10 male Syrian golden hamsters, 8–10 weeks old, received thrice-weekly subcutaneous injections of 5 mg NNN in 0.5 mL saline for 25 weeks (total dose, 375 mg). Controls were treated with saline only. Moribund animals were killed and autopsied; the remaining animals were killed after 83 weeks. Papillary tumours of the trachea occurred in 7/9 treated females and 5/10 treated males. Nasal cavity tumours were found in 0/10 females and 1/10 males (one adenocarcinoma). No nasal or tracheal tumours developed in the controls (Hilfrich *et al*., 1977).

Groups of 15 male and 15 female Syrian golden hamsters, 8–10 weeks of age, received thrice-weekly subcutaneous injections of 8.6 mg NNN (> 99% pure) in 0.3 mL trioctanoin, (total dose, 0.91 mmol/hamster). In a second experiment, groups of 10 male and 10 female hamsters received a total of 75 subcutaneous injections of 2.15 mg NNN in 0.3 mL trioctanoin (total dose, 0.91 mmol/hamster). In the first experiment, treatment with NNN

did not result in early mortality. In the second experiment, all animals were alive after 4 months, 90% after 7 months, 80% after 10 months and 60% after 13 months. The first experiment was terminated after 16 months and the second experiment after 17 months. In the first experiment, 3/15 males and 2/15 females developed tracheal tumours (papillomas) and 1/15 females developed an adenoma of the lung. In the second experiment, 0/10 males and 2/10 females developed respiratory tumours (one lung adenocarcinoma and one tracheal tumour) (Hoffmann *et al*., 1981).

(*c*) *Mink*

Thirteen female and seven male random-bred mink (originated from the breeding farm of the Norwegian College of Veterinary Medicine), 3 weeks of age, received twice-weekly subcutaneous injections of NNN in sterile water for 38 weeks. Injections from the beginning to week 7 contained 1.5 mg NNN/mink and the dose was increased to 30 mg at week 8. The total dose was 2130 mg for both males and females, although the dose per kilogram body weight was twofold for females. Animals were killed when moribund and autopsies were performed. Among females, 13/13 developed nasal tumours (mainly esthesioneuroepithelioma) that invaded into the forebrain. In males, 1/7 developed a nasal tumour that also invaded the forebrain and five developed localized nasal tumours. Time to tumour was 128 ± 23 weeks after the first NNN injections. No tumours were observed in four control female animals (Koppang *et al*., 1992).

Random-bred mink (originated from the breeding farm of the Norwegian College of Veterinary Medicine), 3 months of age, received twice-weekly subcutaneous injections of NNN (purity, > 99%) for 28 weeks (total dose, 11.9 mM). Two males and four females received injections of NNN + NNK (total doses, 11.9 mM + 6.3 mM). Survival ranged from 69 to 156 weeks for mink injected with NNN and from 16 to 130 weeks for mink inoculated with the combination. Control animals were killed at 156 weeks. In the two males, NNN alone induced two malignant tumours (esthesioneuroepitheliomas) in the nasal cavity, one of which invaded the forebrain. In three females, three nasal tumours (esthesioneuroepitheliomas) developed with two invading the forebrain. In both males that received NNN + NNK, tumours were induced in the nasal cavity (esthesioneuroepitheliomas) and invasion into the forebrain was observed at 39 and 40 weeks. In the females, nasal cavity tumours (esthesioneuroepitheliomas) were induced with invasion into the forebrain; in addition, multiple lung tumours (mainly esthesioneuroepitheliomas) developed in one female and a liver tumour (bile duct adenoma) in another. Time to tumour was 58 ± 44 weeks. No tumours were observed in the control minks (Koppang *et al*., 1997).

3.3.6 *Administration with known carcinogens or modifying factors*

(*a*) *Mouse*

Female Hcfd:SENCAR BR mice, 50–55 days of age, received topical applications of 2.8 μmol NNN in 100 μL acetone every other day (total dose, 28 μmol/mouse). Twice-weekly applications of 2.0 μg 12-*O*-tetradecanoylphorbol-13-acetate (TPA) began 10 days

after the last NNN treatment and were continued for 20 weeks. Controls consisted of mice treated with acetone followed by TPA . Significance was analysed using χ^2 test. All mice were examined macroscopically for skin tumours and for tumours in the lung and liver. Mice initiated with NNN developed 0.07 skin tumours per mouse (tumour incidence, 2/27), an incidence that was twofold lower than that in the acetone controls (0.14 skin tumour/mouse; tumour incidence, 4/28) (LaVoie *et al*., 1987).

(*b*) *Rat*

Groups of 30 male Fischer 344 rats, 10 weeks of age, were treated with a mixture of 0.5 mL NNK + NNN (total dose, 14 + 68 μg) dissolved in water by swabbing the oral cavity and lips of the animals with a cotton swab dipped into the solution. A group of 21 rats was used as water controls. Application was performed once a day for 7 days, twice a day for 5 days per week, once a day for 2 days in weeks 2–23 and twice a day from week 24 to week 131. The mean approximate total dose of NNK was 19 mg per rat and that of NNN was 97 mg. The experiment was terminated at 131 weeks at which time survival was 14%. The incidence of oral tumours was 8/30 (six cheek papillomas, one hard palate papilloma and two tongue papillomas) in NNK + NNN-treated animals and 0/21 in controls ($p < 0.05$). In addition to the oral tumours, four lung adenocarcinomas and one lung adenoma were found in five treated animals. One lung adenoma was found in one control animal. The incidence of tumours in the prostate and mammary glands and that of leukaemia/lymphoma did not differ between treated animals and controls (Hecht *et al*., 1986b).

3.3.7 *Carcinogenicity of NNN metabolites*

(*a*) *Intraperitoneal injection*

Mouse

Groups of 25 female strain A/J mice, 6–8 weeks of age, received thrice-weekly intraperitoneal injections for 7.3 weeks (22 injections) of 3′-hydroxy-NNN, 4′-hydroxy-NNN or NNN-1-*N*-oxide suspended in 0.1 mL saline (total dose, 0.12 mmol/mouse) Mice were held for 30 weeks after the last injection. Untreated and vehicle-treated animals served as controls. Lung tumours were counted macroscopically when animals were killed and fixed in 10% formalin for histological evaluation. The Student's *t*-test was used to determine statistical significance. In 3′-hydroxy-NNN-, 4′-hydroxy-NNN- and NNN-1-*N*-oxide-treated mice, tumour multiplicities were 0.9 ± 1.4, 1.6 ± 1.5 ($p < 0.05$) and 0.8 ± 0.7 tumours per surviving mouse, respectively. Tumour incidences were 12/25, 19/25 and 16/25, respectively. In untreated animals, 10/25 developed lung tumours with a multiplicity of 0.6 ± 0.9 tumours per surviving mouse; in saline controls, 7/24 animals had lung tumours with a multiplicity of 0.4 ± 0.6 tumours per surviving mouse (Castonguay *et al*., 1983a).

(*b*) *Administration in drinking-water*

(i) *Rat*

Groups of 12 male and 12 female Fischer 344 rats, 6–8 weeks of age, were given NNN-1-*N*-oxide in the drinking-water at a concentration of 0.012% for a period of 36 weeks, after which time animals received tap-water (total estimated doses, 3.9 mmol for males, 2.9 mmol for females). The experiment was terminated after 104 weeks. Statistical significance was analysed using the χ^2 test. In males, NNN-1-*N*-oxide induced a total of five papillomas and three squamous-cell carcinomas in the oesophagus in 7/12 animals. In females, NNN-1-*N*-oxide induced a total of three carcinomas in the oesophagus in 3/12 animals. In the nasal cavity, NNN-1-*N*-oxide induced a total of five papillomas and seven malignant tumours in 11/12 males and a total of three papillomas and four malignant tumours in 7/12 females. No oesophageal or nasal tumours were observed in 12 male or 12 female controls. The incidence of Leydig-cell tumours in males or mammary tumours in females was not increased in treated rats (Hecht *et al*., 1983b).

(ii) *Hamster*

Groups of 10 male and 10 female Syrian golden hamsters, 6–7 weeks of age, were given NNN-1-*N*-oxide (purity, > 98%) in the drinking-water for 31 weeks, after which animals received tap-water. Total doses were estimated as 2.1 mmol for males and 2.3 mmol for females. The experiment was terminated after 96 weeks. Statistical significance was analysed using the χ^2 test. NNN-1-*N*-oxide failed to induce either nasal cavity or tracheal tumours in males or females, although one caecum adenoma in males and two colon adenomas and five malignant tumours of different sites in females were observed in animals treated with NNN-1-*N*-oxide. No tracheal or nasal tumours were observed in 10 male or 10 female control hamsters, although one female developed a lymphoma (Hecht *et al*., 1983b).

3.4 *N*′-Nitrosoanabasine (NAB)

The carcinogenicity of NAB in experimental animals has been evaluated previously (IARC, 1985).

3.4.1 *Intraperitoneal administration*

Mouse

A group of 31 female strain A/J mice, 6–8 weeks of age, received thrice-weekly intraperitoneal injections of a total dose of 100 μmol NAB in 0.1 mL saline for 7 weeks. After 30 weeks, animals were killed and lung adenomas were enumerated. Statistical analysis was conducted by analysis of variance (nested model) followed by Scheffe's test for multiple comparison of means. Control mice were injected with saline alone (negative controls; 30 mice) or with 5 (30 mice) or 20 (20 mice) μmol NNK (positive controls). In NAB-treated mice, the incidence of tumours was 90.3% [28/31] and tumour multiplicity was 1.8 ± 1.1

tumours per mouse. In the negative controls, tumour incidence was 26.7% [8/30] with a tumour multiplicity of 0.27 ± 0.58 tumours per mouse. In the positive controls, the tumour incidence was 76.7% [23/30] with a tumour multiplicity of 1.6 ± 1.2 tumours per mouse (5 μmol NNK) and 100% [20/20] with a tumour multiplicity of 9.2 ± 6.3 tumours per mouse (20 μmol NNK) (Amin *et al*., 1996).

3.4.2 *Administration in the drinking-water*

Rat

Groups of 16 male and 16 female Chester Beatty strain albino rats, approximately 7 weeks of age, were given drinking-water that contained 0.2% NAB [purity not specified] *ad libitum* on 6 days per week [presumably continuously]. The estimated daily dose was 5 mg/day and animals were killed when moribund or sick, at various intervals between 251 and 550 days of study. A group of 16 males and 16 females served as untreated controls. All but two female rats in the treated group were subjected to post-mortem examination. Of the 16 treated males, four had oesophageal carcinomas, nine had oesophageal papillomas and three had no tumour; of the 14 treated females, one had an oesophageal carcinoma, 11 had oesophageal papillomas and two had no tumour. No oesophageal tumour was reported in the control rats (Boyland *et al.*, 1964b).

A group of 20 male Fischer 344 rats, 7 weeks of age, was given 0.02% NAB in the drinking-water for 5 days a week and tap-water on weekends for a period of 30 weeks (total dose, 630 mg). Moribund animals were killed and autopsied and the remaining animals were killed after 11 months. A group of 19 control rats that was given drinking-water only did not develop tumours in any major organ. Rats treated with NAB developed one oesophageal papilloma (1/17) and one pharyngeal papilloma (1/20). No tumours were observed in the nasal cavity (Hoffmann *et al*., 1975).

3.4.3 *Subcutaneous administration*

Hamster

Groups of 10 male and 10 female Syrian golden hamsters, 8–10 weeks of age, received thrice-weekly subcutaneous injections of 5 mg NAB in 0.5 mL saline for 25 weeks (total dose, 375 mg). Controls (10 males and 10 females) were treated with saline only. Moribund animals were killed and autopsied; the remaining animals were killed after 83 weeks. No tumours were found in the NAB-treated animals. In control rats, no nasal or tracheal tumours developed; one female had a fibrovascular polyp in the uterus (Hilfrich *et al*., 1977).

3.5 *N′*-Nitrosoanatabine (NAT)

The carcinogenicity of NAT in experimental animals has been evaluated previously (IARC, 1985).

Subcutaneous injection

Rat

Groups of male and female Fischer 344 rats, 9 weeks of age, received thrice-weekly subcutaneous injections of NAT in trioctanoin for 20 weeks (total doses, 1.0, 3.0 and 9.0 mmol/kg bw). Animals were killed when moribund or when only 20% of the animals were alive. A control group was injected with trioctanoin only. Major organs were fixed and examined microscopically. After treatment with NAT, 70–84% of the animals survived to 100 weeks. Lung adenocarcinomas developed in 1/21 male and 0/19 female rats injected with 1.0 mmol NAT; no primary lung tumours developed in rats injected with 3.0 or 9.0 mmol/kg NAT. At the 3.0 mmol/kg dose, 2/12 males had benign nasal cavity tumours. No nasal cavity tumours occurred at other doses. In trioctanoin controls, lung adenomas developed in 0/26 male and in 1/26 female rats (Hoffmann *et al.*, 1984).

4. Other Data Relevant to an Evaluation of Carcinogenicity and its Mechanisms

4.1 Absorption, distribution, metabolism and excretion

4.1.1 *Humans*

(*a*) *4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL)*

(i) *Absorption*

NNK has been detected in the saliva of snuff dippers, users of *khaini*, chewers of betel quid with tobacco and users of *toombak* (see Section 1.4.1(*a*)).

Absorption of NNK in smokeless tobacco users was demonstrated by the detection of its metabolites, NNAL and NNAL-Gluc, in plasma and urine and of NNAL-*N*-oxide in urine (Kresty *et al.*, 1996; Carmella *et al.*, 1997; Hecht, 2002; Hecht *et al.*, 2002). Similar results have been obtained in smokers, although only NNAL has been reported in plasma (Hecht *et al.*, 1999a; Hecht, 2002). NNAL-*N*-Gluc comprised 50 ± 25% of total NNAL-Gluc in the urine of smokers and 24 ± 12% in snuff-dippers (Carmella *et al.*, 2002). *Toombak* users excreted exceptionally high levels of NNAL and NNAL-Gluc (0.12–0.14 mg per day), which demonstrated a higher uptake of NNK by humans than of any other non-occupational carcinogen (Murphy *et al.*, 1994). Further information on NNAL and NNAL-Gluc in urine is presented in Section 4.1.1(*c*).

Absorption of NNK by nonsmokers exposed to secondhand cigarette smoke has been demonstrated by detection of NNAL and NNAL-Gluc in urine in several studies (Hecht, 2002).

NNAL, but not NNAL-Gluc, was detected in the amniotic fluid of mothers who smoked (Milunsky *et al.*, 2000). Both NNAL and NNAL-Gluc were detected in the urine of neonates born to mothers who smoked, but not in the urine of newborns of nonsmoking mothers (Lackmann *et al.*, 1999). These results indicate that NNK is converted to NNAL in the mother, and that NNAL crosses the placental barrier and is absorbed and metabolized to NNAL-Gluc in the late stages of fetal development.

(ii) *Distribution*

As noted above, NNAL and NNAL-Gluc have been detected in the plasma of smokeless tobacco users, and NNAL has been detected in plasma of smokers (Hecht *et al.*, 1999a, 2002). Pyridyloxobutyl–haemoglobin adducts derived from NNK and/or NNN have been detected in the blood of smokers, snuff-dippers, *toombak* users and nasal snuff users (Hecht, 1998). NNK was detected in the cervical mucus of smokers and its levels were significantly higher than those in nonsmokers (Prokopczyk *et al.*, 1997). NNK and NNAL were detected in pancreatic juice of smokers. Levels of NNK were significantly higher than in pancreatic juice from nonsmokers, and NNAL was detected more frequently in smokers than in nonsmokers (Prokopczyk *et al.*, 2002). DNA adducts of NNK and/or NNN have been detected in the lung tissue of smokers (Hecht, 1998). Haemoglobin and DNA adducts are discussed in more detail in Section 4.1.1(*c*).

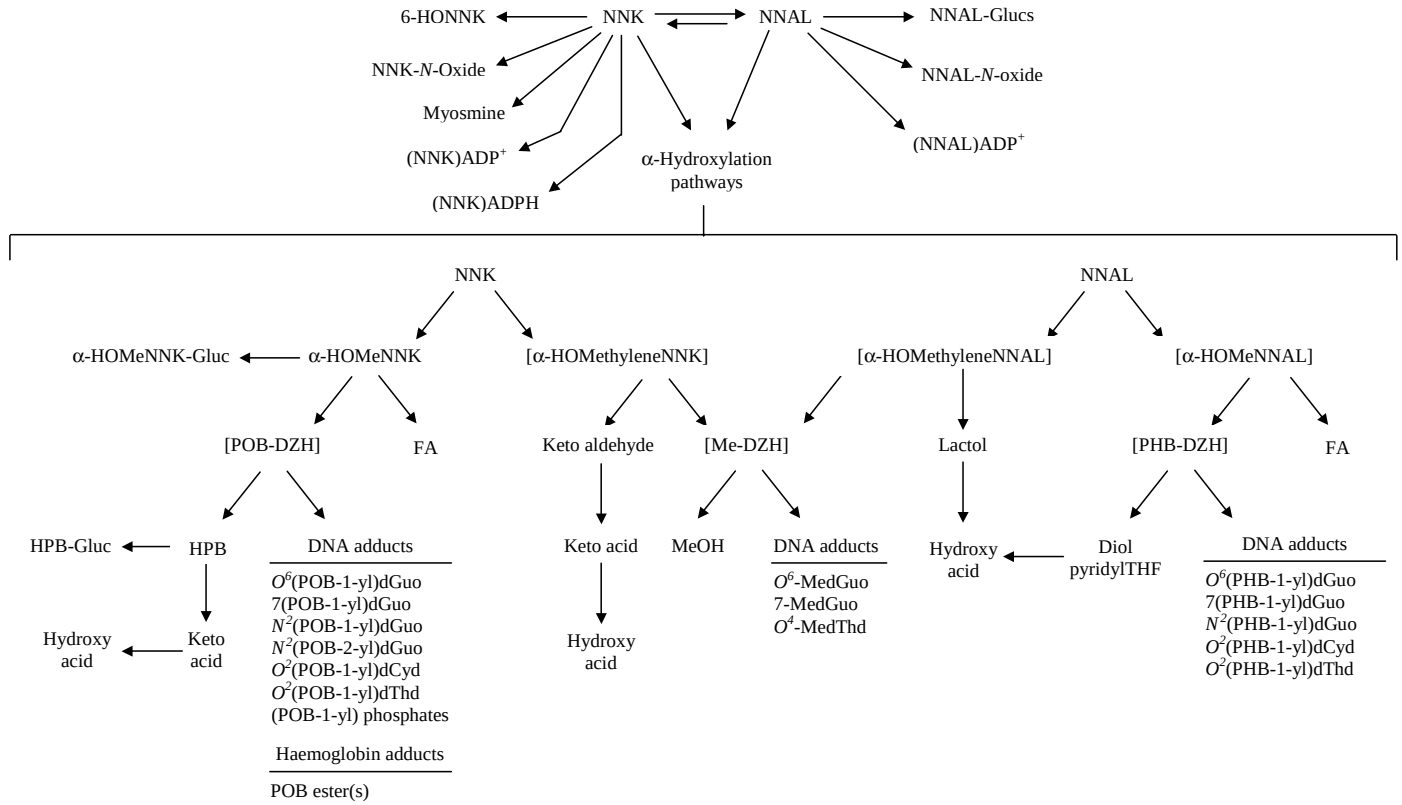
NNAL and NNAL-Gluc are excreted in the urine more slowly than would be expected, based on their structures, after cessation of smoking or smokeless tobacco use (Hecht *et al.*, 1999a, 2002). One week after smoking cessation, 34.5% of baseline NNAL plus NNAL-Gluc was detected in urine, whereas the corresponding values for the structurally related compounds cotinine and nicotine were 1.1 and 0.5%, respectively. Even 6 weeks after cessation, 7.6% of the original levels of NNAL plus NNAL-Gluc remained. The distribution half-life of NNAL and NNAL-Gluc was 3–4 days, while the elimination half-life was 40–45 days. Total body clearance of NNAL was estimated to be 61.4 ± 35.4 mL/min, and the volume of distribution in the β-phase was estimated to be 3800 ± 2100 L, which indicates substantial distribution into tissues (Hecht *et al.*, 1999a). After cessation of smokeless tobacco use, the distribution half-lives of NNAL (1.32 ± 0.85 versus 3.35 ± 1.86 days) and NNAL-Gluc (1.53 ± 1.22 versus 3.89 ± 2.43 days) were significantly shorter than those in smokers. There were no significant differences in the terminal half-lives. Ratios of (*S*)-NNAL:(*R*)-NNAL and (*S*)-NNAL-Gluc:(*R*)-NNAL-Gluc in urine were significantly (3.1–5.7 times) higher 7 days after cessation than at baseline in both smokeless tobacco users and smokers, which indicates stereoselective retention of (*S*)-NNAL in humans. From these results, the authors suggest that there is a receptor in the body, possibly in the lung, for (*S*)-NNAL (Hecht *et al.*, 2002).

(iii) *Metabolism*

Introduction

The metabolic pathways of NNK and NNAL and the modes of formation of their adducts are summarized in Figure 2. Structures of the NNK and NNAL metabolites are

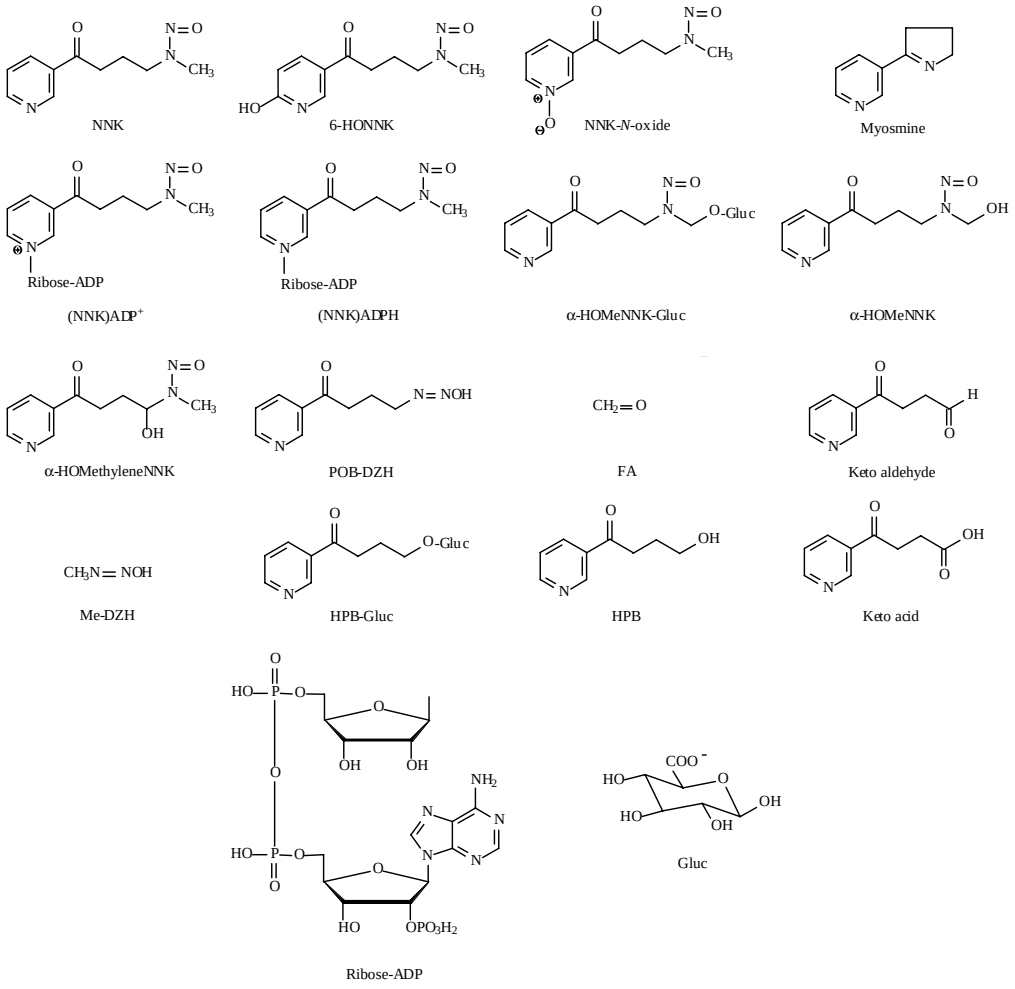
Figure 2. Metabolism of NNK and NNAL and formation of adducts, based on studies in laboratory animals and humans



ADP+, adenosine dinucleotide phosphate; ADPH, adenosine dinucleotide phosphate (reduced form); dCyd, deoxycytidine; dGuo, deoxyguanosine; dThd, deoxythymidine; DZH, diazohydroxide; Gluc, glucuronide; FA, formaldehyde; HO, hydroxy; HOMe, hydroxymethyl; HOMethylene, hydroxymethylene; HPB, 4-hydroxy-1-(3-pyridyl)-1-butanone or keto alcohol; MedGuo, methyldeoxyguanosine; Me-DZH, methaneDZH; MedThd, methyldeoxythymidine; MeOH, methanol; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; PHB, 4-hydroxy-4-(3-pyridyl)-1-butyl; PHB-DZH, 4-hydroxy-4-(3-pyridyl)-1-butaneDZH; POB, 4-oxo-4-(3-pyridyl)-1-butyl or pyridyloxobutyl; POB-DZH, 4-oxo-4-(3-pyridyl)-1-butaneDZH; THF, tetrahydrofuran

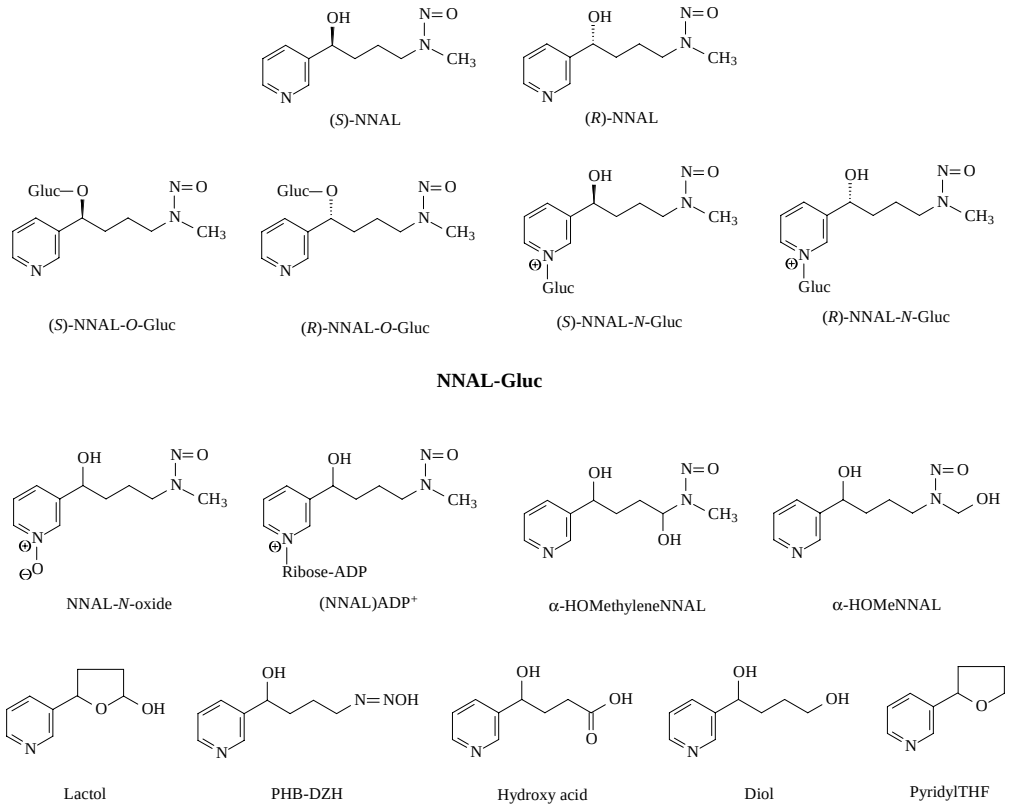
illustrated in Figures 3 and 4, and structures of the adducts are shown in Figure 5. This information is based on studies *in vitro*, in laboratory animals and in humans. Specific pathways that have been observed in in-vitro studies with human tissues or enzymes or in humans are discussed below (see Hecht, 1998).

Figure 3. Structures of NNK and metabolites



DZH, diazohydroxide; FA, formaldehyde; Gluc, glucuronide; HO, hydroxy; HOMe, hydroxymethyl; HOMethylene, hydroxymethylene; HPB, 4-hydroxy-1-(3-pyridyl)-1-butanone or keto alcohol; Me-DZH, methaneDZH; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; (NNK)ADP+, (NNK)adenosine dinucleotide phosphate; (NNK)ADPH, (NNK)adenosine dinucleotide phosphate (reduced form); POB-DZH, 4-oxo-4-(3-pyridyl)-1-butaneDZH

Figure 4. Structures of NNAL and metabolites



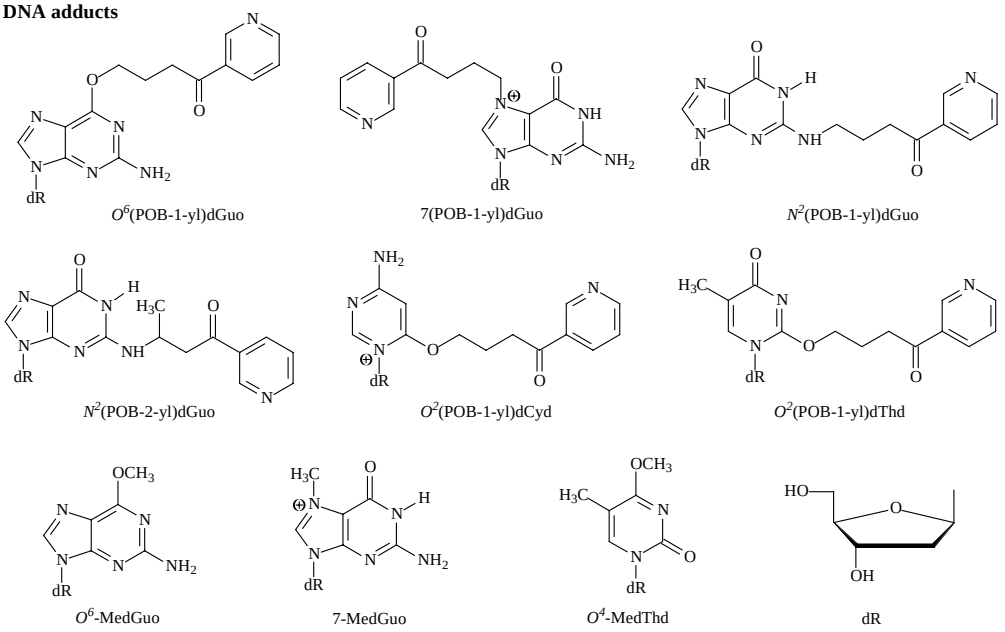
DZH, diazohydroxide; Gluc, glucuronide; HOMe, hydroxymethyl; HOMethylene, hydroxymethylene; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; (NNAL)ADP$^+$, (NNAL)adenosine dinucleotide phosphate; PHB-DZH, 4-hydroxy-4-(3-pyridyl)-1-butaneDZH; THF, tetrahydrofuran
See Figure 3 for structures of Gluc and ribose-ADP.

NNK can be converted to the pyridine oxidation products 4-(methylnitrosamino)-1-[3-(6-hydroxypyridyl)]-1-butanone (6-HONNK) and NNK-*N*-oxide. Denitrosation of NNK followed by oxidation produces myosmine. NNK can replace nicotinamide in NADP$^+$ or NADPH, to yield NNK adenosine dinucleotide phosphate ((NNK)ADP$^+$) and (NNK)ADPH (reduced form). Carbonyl reduction of NNK produces NNAL which can be conjugated by glucuronidation giving four diastereomers of NNAL-Gluc (Figure 4): two isomers of 4-(methylnitrosamino)-1-(3-pyridyl)-1-(*O*-β-D-glucopyranuronosyl)butane (NNAL-*O*-Gluc) and two isomers of 4-(methylnitrosamino)-1-(3-pyridyl-*N*-β-D-glucopyranuronosyl)-1-butanolonium inner salt (NNAL-*N*-Gluc). NNAL is also converted to NNAL-*N*-oxide and NNAL(ADP$^+$).

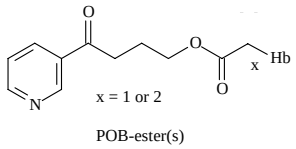
α-Hydroxylation of NNK and NNAL leads to DNA and haemoglobin adducts. Hydroxylation of the NNK methyl group gives 4-(hydroxymethylnitrosamino)-1-(3-pyridyl)-1-

Figure 5. Structures of DNA adducts and haemoglobin adducts derived from NNK

DNA adducts



Haemoglobin adducts



dCyd, deoxycytidine; dGuo, deoxyguanosine; dThd, deoxythymidine; dR, deoxyribose; Me, methyl; POB, 4-oxo-4-(3-pyridyl)-1-butyl or pyridyloxobutyl

Adducts derived from NNAL have the same structures except that an hydroxy group replaces the carbonyl group in the adducted portion of the molecule.

butanone (α-HOMeNNK) which can be conjugated as a glucuronide, α-HOMeNNK-Gluc. α-HOMeNNK spontaneously decomposes to 4-oxo-4-(3-pyridyl)-1-butanediazohydroxide (POB-DZH) and formaldehyde. POB-DZH reacts with water to give 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB) which can be conjugated as its glucuronide, HPB-Gluc. POB-DZH also reacts with DNA and haemoglobin to produce a series of adducts (Figures 2 and 5). α-Hydroxylation of the NNK methylene group produces 4-(methylnitrosamino)-1-(3-pyridyl)-1-(4-hydroxy)butanone (α-HOMethyleneNNK). This metabolite spontaneously decomposes to 4-(3-pyridyl)-4-oxobutanal (keto aldehyde) and methanediazohydroxide (Me-DZH). Keto aldehyde is further metabolized to 4-(3-pyridyl)-4-oxobutanoic acid (keto acid) which in turn can be converted to 4-hydroxy-4-(3-pyridyl)butanoic acid (hydroxy acid).

Me-DZH reacts with water to yield methanol and with DNA to produce methyl adducts as shown in Figures 2 and 5. NNAL similarly undergoes α-hydroxylation at its methylene group to yield 4-(methylnitrosamino)-1-(3-pyridyl)-1-(4-hydroxy)butanol (α-HOMethyleneNNAL) and at its methyl group to yield 4-(hydroxymethylnitrosamino)-1-(3-pyridyl)-1-butanol (α-HOMeNNAL). α-HOMethyleneNNAL spontaneously decomposes to Me-DZH and 5-(3-pyridyl)-2-hydroxytetrahydrofuran (lactol), which can be converted to hydroxy acid. α-HOMeNNAL spontaneously decomposes to 4-hydroxy-4-(3-pyridyl)-1-butanediazohydroxide (PHB-DZH) and formaldehyde. PHB-DZH reacts with water to yield 4-(3-pyridyl)butane-1,4-diol (diol), cyclizes to 2-(3-pyridyl)tetrahydrofuran (pyridylTHF) and reacts with DNA and haemoglobin to produce the adducts shown in Figures 2 and 5.

In-vitro studies in human tissues and cells

NNK

A variety of human tissues that includes cultured bronchus, peripheral lung, trachea, buccal mucosa, oesophagus and bladder metabolize NNK (Castonguay *et al.*, 1983c; Hecht, 1998), as do microsomes from human tissues such as liver, lung, cervix, placenta and pancreas (Hecht, 1998; Jalas *et al.*, 2005). A common observation in all of these studies is extensive conversion to NNAL. NNK is converted to NNAL in cultured human tissues (50–80%) (Castonguay *et al.*, 1983c), human red blood cells (Murphy & Coletta, 1993) and human liver microsomes and cytosol (18 and 25%, respectively). The enantiomeric composition in the liver was 64% (*S*)-NNAL in microsomes and 90% (*S*)-NNAL in cytosol (Upadhyaya *et al.*, 2000). Human cervical cells and human cervical microsomes formed (*R*)-NNAL while human cervical cytosol produced mainly (*S*)-NNAL (Prokopczyk *et al.*, 2001). Five enzymes that mediate conversion of NNK to NNAL in humans have been identified to date: microsomal 11β-hydroxysteroid dehydrogenase type 1 and cytosolic carbonyl reductase, which both belong to the superfamily of the short-chain dehydrogenases/reductases, and three members of the aldo-keto reductase superfamily (Maser, 2004).

Microsomes from human liver catalyse the α-hydroxylation of NNK to keto alcohol and keto aldehyde and the pyridine-*N*-oxidation to NNK-*N*-oxide. The rates of these reactions are lower than that of carbonyl reduction to NNAL (Smith *et al.*, 1992a; Patten *et al.*, 1996; Staretz *et al.*, 1997a; Jalas *et al.*, 2005) and reported Michaelis constant (K_m) values are relatively high. Correlation studies and the use of chemical inhibitors and inhibitory antibodies have suggested a role for cytochrome P450s (CYPs) 1A2, 2A6 and 3A4 in the formation of keto alcohol or keto aldehyde (Smith *et al.*, 1992a; Patten *et al.*, 1996; Hecht, 1998; Jalas *et al.*, 2005). Microsomes from human lung catalyse the conversion of NNK to keto aldehyde, hydroxy acid and NNK-*N*-oxide. The rate of metabolism of NNK to keto aldehyde was very low (less than 0.05 pmol/mg/min) whereas the rate in liver microsomes was 1–5 pmol/mg/min (Smith *et al.*, 1992a). One study suggested that lipoxygenase may be involved in the metabolism of NNK by human lung, but another study demonstrated the contrary (Smith *et al.*, 1995; Bedard *et al.*, 2002). The contribution of CYP2A6 and/or CYP2A13, as well as CYP2B6 to the α-hydroxylation of NNK and NNAL by human lung samples were suggested by selected chemical and antibody inhibi-

tion in some subjects (Jalas *et al.*, 2003a; Smith *et al.*, 2003). One of the difficulties in studying xenobiotic metabolism in human lung microsomes is the low level of CYP activity. CYP levels in lung microsomes are reported to be between 1 and 10 pmol/mg microsomal protein (Shimada *et al.*, 1992; Guengerich, 1993); the average concentration in 60 liver microsomal samples was 344 ± 167 pmol/mg hepatic microsomal protein (Shimada *et al.*, 1994). CYP-catalysed activity levels and mRNA levels are also much lower in the lung than in the liver (Shimada *et al.*, 1996; Ding & Kaminsky, 2003). However, CYPs have been reported to be expressed in particular regions of the lung which may result in higher localized concentrations (Anttila *et al.*, 1997; Hukkanen *et al.*, 2002). A diffusible inhibitor of CYP activity has been reported to be present in human lung microsome preparations; it was also reported that 7-ethoxycoumarin-*O*-deethylase activity in rat lung microsomes was inhibited by pre-incubation with human lung microsomes (Lorenz *et al.*, 1979).

Human fetal nasal microsomes metabolized NNK by α-hydroxylation to HPB and keto aldehyde with low K_m (6.7 ± 0.8 μM and 6.5 ± 1.1 μM, respectively) and antibody inhibition studies indicated that CYP2A13 (K_m, 2.8–11.3 μM; Table 11) was involved (Wong *et al.*, 2005a).

CYPs are the major catalysts of NNK α-hydroxylation in humans and rodents. Table 11 summarizes the steady-state kinetic parameters for CYP-mediated NNK metabolism (Jalas *et al.*, 2005). Based on the K_m data in Table 11, the relative efficiencies in NNK metabolism by human CYP are (from greatest catalyst to least): 2A13 > 2B6 > 2A6 > 1A2 ~ 1A1 > 2D6 ~ 2E1 ~ 3A4. Similar results are obtained when the ratio maximum velocity (V_{max})/K_m is considered. The actual involvement of these enzymes in NNK metabolism *in vivo* depends on many factors that include relative expression levels, the amount of CYP oxido-reductase expressed in a given tissue, tissue localization and inducibility of individual CYPs, and the concentration of NNK in human tissues. Among hepatic CYPs, 2B6 has the highest affinity for NNK. However, low levels of this enzyme are present in most liver samples (Gervot *et al.*, 1999). CYP2A6 is also present at relatively low levels, and accounts for < 1% to 4% of the total CYP content (Shimada *et al.*, 1996). Levels of CYP1A2 are four- to 20-fold higher than those of CYP2A6 (Shimada *et al.*, 1996). Therefore, despite its somewhat higher K_m and lower V_{max}/K_m, CYP1A2 is most probably as important a catalyst of NNK α-hydroxylation in human liver as CYP2A6. CYP3A4 may also play a role in hepatic NNK α-hydroxylation, since it is often present at concentrations that are 10–50 times greater than those of CYP2A6 (Shimada *et al.*, 1994). It is not possible to rule out completely the presence of CYP2A13, the best known human catalyst of NNK metabolism, in the liver. However, the very low hepatic mRNA levels of CYP2A13 relative to CYP2A6 suggest that, if this enzyme is present, it is so at very low levels. Results to date do not identify that any single CYP in the liver is a key player in NNK activation. Several enzymes, including CYP1A2, CYP2A6, CYP2B6 and CYP3A4, clearly play a role. The relative contribution of any one of these CYPs varies among individuals, and their relative abundance and catalytic efficiencies suggest that rarely, if ever, is one of them the dominant catalyst (Jalas *et al.*, 2005).

Table 11. Steady-state kinetic parameters for cytochrome P450 (CYP)-mediated NNK metabolism

Species/enzyme	NNK concentration range (μM)	Metabolite	Kinetic parameters			Expression system	Reference
			V_{max}[a]	K_m (μM)	V_{max}/K_m[b]		
Human CYP1A1	1–500	Keto aldehyde	4.44 ± 0.41	1400 ± 148	$3.2 \times 10^{-3} \pm 5 \times 10^{-4}$	Gentest Supersomes[c]	Smith *et al.* (1995)
		HPB	0.824 ± 0.118	371 ± 6	$2.2 \times 10^{-3} \pm 3 \times 10^{-4}$		
Human CYP1A2	1–1000	Keto aldehyde	0.51 ± 0.04	1180 ± 60	$4.3 \times 10^{-4} \pm 4 \times 10^{-5}$	Purified, reconstituted protein	Smith *et al.* (1996)
		HPB	1.7 ± 0.05	380 ± 30	$4.5 \times 10^{-3} \pm 4 \times 10^{-4}$		
		HPB	1.96 ± 0.07	400 ± 10	$4.9 \times 10^{-3} \pm 2 \times 10^{-4}$	as above, DMSO control	
			2.09 ± 0.20	760 ± 10	$2.8 \times 10^{-3} \pm 3 \times 10^{-4}$	as above, but with 50 nM PEITC added	
			2.06 ± 0.21	820 ± 20	$2.5 \times 10^{-3} \pm 3 \times 10^{-4}$	as above, but with 100 nM PEITC added	
			2.05 ± 0.25	1240 ± 70	$1.7 \times 10^{-3} \pm 2 \times 10^{-4}$	as above, but with 200 nM PEITC added	
Human CYP1A2[d]	10–350	HPB	4.2 ± 0.2	309 ± 16	0.014 ± 0.001	Hep G2 cell lysate	Smith *et al.* (1992a)
Human CYP2A6	5–2000	Keto aldehyde	0.437	392	1.11×10^{-3}	Baculovirus-infected *Spodoptera frugiperda* (Sf9) cells	Patten *et al.* (1996)
		HPB	0.163	349	4.67×10^{-4}		
Human CYP2A6 (+ b_5)[e]	5–2000	Keto aldehyde	1.03	118	8.73×10^{-3}	Baculovirus-infected Sf9 cells	Patten *et al.* (1996)
		HPB	0.419	141	2.97×10^{-3}		
Human CYP2A13	2–160	Keto aldehyde	4.1 ± 0.6	11.3 ± 3.5	0.36 ± 0.12	Baculovirus-infected Sf9 cells	Su *et al.* (2000)
		HPB	1.2 ± 0.2	13.1 ± 5.1	0.092 ± 0.039		
Human CYP2A13	2–100	Keto aldehyde	14.5 ± 0.8	4.6 ± 0.4	3.2 ± 0.3	Purified, reconstituted protein	Zhang *et al.* (2002)
		HPB	5.7 ± 0.7	2.8 ± 0.5	2.0 ± 0.4		
Human CYP2A13 (Arg 257 Cys)	2–100	Keto aldehyde	8.4 ± 0.8	6.2 ± 0.7	1.4 ± 0.2	Purified, reconstituted protein	Zhang *et al.* (2002)
		HPB	3.2 ± 0.5	4.8 ± 1.0	0.67 ± 0.17		
Human CYP2A13	0.25–50	Keto aldehyde	13.8 ± 0.8	3.6 ± 0.7	3.9 ± 0.8	Baculovirus-infected Sf9 cells	Jalas *et al.* (2003a)
		HPB	4.6 ± 0.2	3.2 ± 0.5	1.4 ± 0.2		

Table 11 (contd)

Species/enzyme	NNK concentration range (μM)	Metabolite	Kinetic parameters			Expression system	Reference
			V_{max}[a]	K_m (μM)	V_{max}/K_m[b]		
Human CYP2B6	2.5–150	Keto aldehyde and HPB[f]	0.18 ± 0.01	33 ± 0.7	$5.5 \times 10^{-3} \pm 3 \times 10^{-4}$	BD Biosciences Discovery Labware Supersomes	Dicke *et al.* (2005)
Human CYP2D6	5–2000	Keto aldehyde HPB	0.105 4.01	1061 5525	9.9×10^{-5} 7.3×10^{-4}	Baculovirus-infected Sf9 cells	Patten *et al.* (1996)
Human CYP2D6	5–2000	Keto aldehyde HPB	0.13 6.04	1075 5632	2×10^{-4} 1×10^{-3}	Chinese hamster ovary cells	Patten *et al.* (1996)
Human CYP2E1 (+ b_5)[e]	5–2000	Keto aldehyde HPB	0.026 1.17	720 3334	3.6×10^{-5} 3.5×10^{-4}	Baculovirus-infected Sf9 cells	Patten *et al.* (1996)
Human CYP3A4	5–8000	Keto aldehyde HPB	0.787 0.086	3091 1125	2.6×10^{-4} 7.6×10^{-5}	Chinese hamster ovary cells	Patten *et al.* (1996)
Rabbit CYP2A10/2A11	2.9–154	Keto aldehyde HPB	1.38 1.30	15 9.0	0.092 0.14	Purified, reconstituted protein	Hong *et al.* (1992)
Rabbit CYP2A10/2A11 (+ b_5)[e]	2.9–154	Keto aldehyde HPB	0.849 0.575	28.6 16.3	0.0297 0.0353	Purified, reconstituted protein	Hong *et al.* (1992)
Rabbit CYP2A10/2A11 (+ 80 μM nicotine)	2.9–154	Keto aldehyde HPB	1.33 1.26	40.2 29.5	0.033 0.043	Purified, reconstituted protein	Hong *et al.* (1992)
Rabbit CYP2G1	2.9–154	Keto aldehyde HPB	0.735 ND	186	3.95×10^{-3}	Purified, reconstituted protein	Hong *et al.* (1992)
Rat CYP1A1	1–5000	Keto aldehyde HPB	2.2 ± 0.1 0.68 ± 0.03	180 ± 34 140 ± 26	0.013 ± 0.002 $4.9 \times 10^{-3} \pm 9 \times 10^{-4}$	Gentest Supersomes	Jalas *et al.* (2005)
Rat CYP1A2	1–5000	Keto aldehyde HPB	5.0 ± 0.1 6.1 ± 0.09	180 ± 22 200 ± 11	0.028 ± 0.003 0.034 ± 0.002	Gentest Supersomes	Jalas *et al.* (2005)
Rat CYP2A3	0.25–50	Keto aldehyde HPB	10.8 ± 0.4 8.2 ± 0.3	4.6 ± 0.5 4.9 ± 0.5	2.3 ± 0.3 1.7 ± 0.2	Baculovirus-infected Sf9 cells	Jalas *et al.* (2003a)

Table 11 (contd)

Species/enzyme	NNK concentration range (μM)	Metabolite	Kinetic parameters			Expression system	Reference
			V_{max}[a]	K_m (μM)	V_{max}/K_m[b]		
Rat CYP2B1	10–1300	Keto aldehyde HPB NNK-*N*-oxide	0.0897 ± 0.003 0.333 ± 0.016 0.295 ± 0.006	191 ± 20 318 ± 5 131 ± 10	$4.70 \times 10^{-4} \pm 5.2 \times 10^{-5}$ $1.05 \times 10^{-3} \pm 5 \times 10^{-5}$ $2.25 \times 10^{-3} \pm 1.7 \times 10^{-4}$	Purified, reconstituted protein	Guo *et al.* (1991a)
Rat CYP2C6	1–5000	Keto aldehyde HPB NNK-*N*-oxide	2.5 ± 0.1 16 ± 0.5 1.5 ± 0.06	1300 ± 130 1400 ± 89 1100 ± 110	$1.9 \times 10^{-3} \pm 2 \times 10^{-4}$ 0.011 ± 0.0008 $1.4 \times 10^{-3} \pm 4 \times 10^{-4}$	Gentest Supersomes	Jalas *et al.* (2005)
Mouse CYP2A4	1–5000	Keto aldehyde	190 ± 23	3900 ± 995	0.049 ± 0.014	Baculovirus-infected Sf9 cells	Felicia *et al.* (2000)
Mouse CYP2A4	0.5–3000	Keto aldehyde HPB	7.3 ± 0.4 1.8 ± 0.1	97 ± 19 67 ± 17	0.075 ± 0.015 0.027 ± 0.007	Baculovirus-infected Sf9 cells	Jalas *et al.* (2003b)
Mouse CYP2A5	0.25–100	HPB	4.0 ± 0.4	1.5 ± 0.6	2.7 ± 1.8	Baculovirus-infected Sf9 cells	Felicia *et al.* (2000)
Mouse CYP2A5	0.25–50	Keto aldehyde HPB	2.0 ± 0.1 6.5 ± 0.2	4.3 ± 0.8 4.5 ± 0.4	0.47 ± 0.09 1.4 ± 0.1	Baculovirus-infected Sf9 cells	Jalas *et al.* (2003b)

Adapted from Jalas *et al.* (2005)

DMSO, dimethyl sulfoxide; HPB, 4-hydroxy-1-(3-pyridyl)-1-butanone; K_m, Michaelis constant; NNK, 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone; PEITC, phenethyl isothiocyanate; ND, not detected; OPB, 4-oxo-4-(3-pyridyl)butanol or keto aldehyde; V_{max}, maximum velocity

[a] Units are pmol product/min/(pmol CYP).

[b] Units are pmol product/min/(pmol CYP)/μM.

[c] V_{max} units were converted using the published CYP concentration of 34 pmol CYP/(mg protein) (Smith *et al.*, 1995). Supersomes that contain co-expressed CYP and oxidoreductase were purchased from Gentest (Woburn, MA).

[d] The V_{max} value was computed based on a CYP1A2 concentration of 13 pmol/(mg protein); this is an upper limit, the lowest possible V_{max} value is 3.1 ± 0.1 pmol/min/(pmol CYP) based on 18 pmol/(mg protein). See Aoyama *et al.* (1990).

[e] Cytochrome b_5 was added at a 3:1 b_5:CYP molar ratio. b5:CYP molar ratio is corrected according to the original reference (Patten *et al.*, 1996).

[f] HBP:keto aldehyde (OPB) ratio ≈ 10:1

In the lung, CYP2A13 may be the key catalyst of NNK α-hydroxylation (Su *et al.*, 2000; Jalas *et al.*, 2003a). However, the level of CYP2A13 protein in the lung is unknown. The relative levels of human lung CYPs are not well characterized. CYP1A1 (when induced in smokers) and CYP1B1 are probably present at relatively high levels in this tissue (Shimada *et al.*, 1992; Spivack *et al.*, 2001). CYP1A1 is a catalyst of NNK metabolism but is much less efficient than CYP2A13 (Table 11). Metabolism of NNK by CYP1B1 has not been studied. Based on reported expression levels, other enzymes that may contribute to NNK activation in the lung are CYP2B6 and CYP3A5 (Table 11; Hukkanen *et al.*, 2002; Smith *et al.*, 2003).The kinetic parameters for CYP3A5-catalysed NNK metabolism have not been determined.

NNAL

Human liver microsomes convert NNAL to NNAL-*O*-Gluc and NNAL-*N*-Gluc (Wiener *et al.*, 2004a). The hepatic enzymes UGT2B7 and UGT1A9 appear to be important catalysts for conversion of NNAL to NNAL-*O*-Gluc while UGT1A4 plays a significant role in the formation of NNAL-*N*-Gluc (Ren *et al.*, 2000; Wiener *et al.*, 2004a). Large variations in the ability to glucuronidate NNAL have been observed among liver microsomal specimens from different humans. Polymorphisms in the *UGT1A4* and *UGT2B7* genes were associated with altered levels of NNAL glucuronidation in these specimens (Wiener *et al.*, 2004b). Thus, interindividual differences in the conversion of NNK to NNAL and of NNAL to NNAL-Gluc could be important in determining individual susceptibility to the carcinogenic effects of NNK.

Human liver microsomes convert NNAL to lactol, diol, hydroxy acid, NNAL-*N*-oxide and NNK. Conversion to NNK occurs at the fastest rate (Staretz *et al.*, 1997b). There was no significant difference in the rates of metabolism of (*S*)- and (*R*)-NNAL by human liver microsomes (Upadhyaya *et al.*, 2000). Human CYP2A13 catalyzed the conversion of racemic NNAL to lactol, diol and NNAL-*N*-oxide with K_m values of 36 ± 3, 40 ± 3 and 30 ± 7 μM, respectively (Jalas *et al.*, 2003a).

In-vivo studies

Overview

The metabolism of NNK in humans was definitively established by studies that demonstrated the presence of its metabolites in the urine of smokeless tobacco users, smokers and nonsmokers exposed to secondhand smoke. NNK metabolites identified and quantified to date are NNAL, NNAL-*O*-Gluc, NNAL-*N*-Gluc and NNAL-*N*-oxide. NNAL and NNAL-Gluc have also been detected in plasma. These metabolites have been reported only in individuals exposed to tobacco or tobacco smoke, and their levels are too high for them to derive from the small amounts of NNAL in tobacco products. The only possible source is NNK. In addition, haemoglobin and DNA adducts that could arise from either NNK or NNN have been quantified in human blood and tissues.

NNAL and NNAL-Gluc

Studies on NNAL and NNAL-Gluc in human urine have been reviewed (Hecht, 2002; IARC, 2004) (see also Section 1.4.1(*c*)). Mean levels reported in the urine of smokeless tobacco users, smokers and nonsmokers exposed to secondhand tobacco smoke are summarized in Table 12.

Table 12. Total NNAL in the urine of smokeless tobacco users, smokers and nonsmokers exposed to secondhand tobacco smoke

No. of subjects	pmol/mL	pmol/mg creatinine	nmol/24 h	Reference
Smokeless tobacco users				
7 *toombak* users	1270	NR	NR	Murphy *et al.* (1994)
39 smokeless tobacco users	NR	4.4	NR	Kresty *et al.* (1996)
13 smokeless tobacco users	4.22	3.55	6.60	Hecht *et al.* (2002)
10 snuff dippers	4.20	NR	NR	Carmella *et al.* (2002)
55 snuff dippers	NR	3.25	NR	Carmella *et al.* (2003)
41 snuff dippers	NR	3.0	NR	Hatsukami *et al.* (2004)
54 snuff dippers	NR	3.3	NR	Lemmonds *et al.* (2005)
Smokers				
11 smokers	NR	NR	11.4	Carmella *et al.* (1993)
61 smokers	NR	3.76	NR	Carmella *et al.* (1995)
11 smokers	NR	NR	3.28	Hecht *et al.* (1995)
20 smokers	NR	NR	3.22	Meger *et al.* (2000)
13 smokers	NR	3.90	NR	Taioli *et al.* (1997)
27 smokers	1.95	2.70	3.14	Hecht *et al.* (1999a)
18 smokers	NR	3.69	NR	Hurt *et al.* (2000)
10 smokers	1.22	NR	NR	Carmella *et al.* (2002)
99 smokers	NR	2.07	NR	Hecht *et al.* (2004a)
41 smokers	NR	2.60	NR	Carmella *et al.* (2003)
34 smokers	NR	3.5	NR	Hughes *et al.* (2004)
38 smokers	NR	2.3	NR	Hatsukami *et al.* (2004)
84 smokers	NR	1.6	NR	Hecht *et al.* (2004b)
7 smokers	2.36	NR	5.32	Byrd & Ogden (2003)
12 smokers	2.8	NR	NR	Breland *et al.* (2003)
11 smokers	NR	1.34	NR	Sellers *et al.* (2003)
Nonsmokers exposed to secondhand tobacco smoke				
5 exposed men	NR	NR	0.127	Hecht *et al.* (1993a)
9 exposed hospital workers	0.059	NR	NR	Parsons *et al.* (1998)
29 exposed nonsmokers	NR	NR	0.027	Meger *et al.* (2000)
23 exposed women	0.050	NR	NR	Anderson *et al.* (2001)
74 exposed children	0.056	NR	NR	Hecht *et al.* (2001)
16 casino patrons	0.033	0.037	NR	Anderson *et al.* (2003)
19 restaurant workers	0.066	0.070	0.107	Tulunay *et al.* (2005)
27 newborns of smoking mothers	0.14	NR	NR	Lackmann *et al.* (1999)

NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL); NR, not reported

Smokeless tobacco users

Snuff dippers/tobacco chewers in the USA excreted 6.6 nmol/24 h total NNAL (NNAL plus NNAL-Gluc) in urine (Hecht *et al.*, 2002) (Table 12). Swedish snuff users excreted 48% less total NNAL than snuff dippers who used products marketed in the USA (Hatsukami *et al.*, 2004). Assuming that total NNAL represents 20% of the NNK dose, uptake of NNK amounts to 33 nmol per day in snuff dippers/chewers and 17 nmol per day in Swedish snuff users. The total dose of NNK would be 4.8 μmol/kg bw over 30 years of snuff dipping/chewing and 2.5 μmol/kg bw over 30 years of *snus* use. Rats treated with 0.5 ppm of NNK in the drinking-water for 2 years (total dose, 73 μmol/kg bw) had an increased incidence of exocrine pancreatic tumours (Rivenson *et al.*, 1988) (see Section 3.1.4(*b*)). The total dose in the rat study was about 15–29 times higher than the estimated total human dose achieved over 30 years of snuff dipping/chewing or *snus* use. Lower doses of NNK have not been tested for induction of pancreatic tumours, but tumorigenicity of NNK in the rat pancreas is enhanced by a high-fat diet (Hoffmann *et al.*, 1993a).

Toombak users excreted an average of 1270 pmol/mL urine total NNAL, which was approximately 300 times higher than that excreted by snuff dippers/chewers or Swedish snuff users (4.2 pmol/mL) (Murphy *et al.*, 1994; Carmella *et al.*, 2002). Applying the above calculation, the 30-year total dose of NNK in *toombak* users would be about 1440 μmol/kg bw (298 mg/kg bw). NNN levels in the saliva of *toombak* users were approximately 30 times greater than those of NNK (Idris *et al.*, 1992). Thus, the corresponding NNN dose could be estimated as 43 200 μmol/kg bw (9.04 g/kg bw). These estimated total doses of NNK (1440 μmol/kg bw) and NNN (43 200 μmol/kg bw) can be compared with the total doses of NNK (240 μmol/kg bw) and NNN (1400 μmol/kg bw) which induced a significantly increased incidence of oral cavity tumours when swabbed repeatedly in the oral cavity of Fischer 344 rats (Hecht *et al.*, 1986b). Thus, 30-year *toombak* users are exposed to total doses of NNK and NNN that are approximately six and 31 times higher than those required to induce oral tumours in rats.

The ratio of (*S*)-NNAL-*O*-Gluc:(*R*)-NNAL-*O*-Gluc was 1.9 and that of NNAL-*O*-Gluc:NNAL-*N*-Gluc was 7.1 in the urine of *toombak* users (Murphy *et al.*, 1994; Carmella *et al.*, 2002) and 3.6 in the urine of snuff dippers (Carmella *et al.*, 2002). Free NNAL comprised 31% of total NNAL in the urine of *toombak* users (Murphy *et al.*, 1994) and 35% of total NNAL in the urine of snuff dippers (Carmella *et al.*, 2002).

When snuff dippers who used products marketed in the USA switched to a brand of Swedish snuff over a 4-week period, a statistically significant average reduction of about 48% in total urinary NNAL was observed. However, when snuff dippers switched to a nicotine patch for 4 weeks, there was a significantly greater reduction of about 90% in total NNAL, which showed that a switch to *snus* is inferior to abstention from smokeless tobacco with respect to NNK uptake (Hatsukami *et al.*, 2004).

In one study of snuff dippers and tobacco chewers, a significant association between urinary levels of total NNAL and the presence of oral leukoplakia was observed (Kresty *et al.*, 1996).

Smokers

Smokers excrete about 3.2 nmol/24 h total NNAL (Table 12). Assuming that total NNAL represents 20% of NNK dose, daily uptake of NNK would be 16.5 nmol, or 180 μmol (2.4 μmol/kg bw; 0.5 mg/kg bw) over 30 years of smoking. The lowest total dose of NNK shown to induce a significantly increased incidence of lung tumours in rats, as part of a dose–response relationship, was 1.8 mg/kg bw (8.7 μmol/kg bw), 3.6 times higher than the dose of a smoker (Belinsky *et al.*, 1990) (see Section 3.1.2(*a*)).

The enantiomeric distribution of NNAL in urine was 54% (*R*)-NNAL and 46% (*S*)-NNAL whereas the diastereomeric distribution of NNAL-Gluc was 68% (*R*)-NNAL-Gluc and 32% (*S*)-NNAL-Gluc (Carmella *et al.*, 1999). (*R*)-NNAL is the more tumorigenic NNAL enantiomer in A/J mice (Upadhyaya *et al.*, 1999). The ratio of NNAL-*O*-Gluc:NNAL-*N*-Gluc was 1.3 in smokers (Carmella *et al.*, 2002). Free NNAL comprised a mean of 38% of total NNAL in smokers, and gave an NNAL-Gluc:NNAL ratio of 1.6 (Carmella *et al.*, 2002). A wide range of NNAL-Gluc:NNAL ratios has been observed and there is evidence for a high ratio (6–11) phenotype in a minority of smokers (Carmella *et al.*, 1995).

Consistently, total NNAL correlates with total cotinine in smokers (Hecht, 2002), number of cigarettes per day and 1-hydroxypyrene in urine. The increase in total NNAL with number of cigarettes per day was not linear (Joseph *et al.*, 2005). As cotinine is a marker for the uptake of nicotine, total NNAL is a marker for the uptake of NNK.

Five recent studies have employed NNAL and NNAL-Gluc, as well as other biomarkers, to investigate approaches to tobacco harm reduction. Two concerned the effects of reducing numbers of cigarettes smoked per day and whether this would have a significant effect on the uptake of NNK. One study of 23 subjects found a moderate reduction of NNAL-Gluc and total NNAL (Hurt *et al.*, 2000). A second study of 102 smokers examined NNAL and NNAL-Gluc in the urine of smokers who reduced their smoking by up to 75% over a 6-week period. Statistically significant reductions in NNAL, NNAL-Gluc and total NNAL were observed at most intervals, but the observed decreases were generally modest, were always proportionally lower than the reductions in the number of cigarettes smoked per day and were sometimes transient (Hecht *et al.*, 2004a). Apparently, smokers compensate in their smoking behaviour when they smoke fewer cigarettes per day, and thereby decrease the expected reduction in carcinogen uptake. Three studies examined the effects of switching to brands with lower NNK delivery, as measured by standardized machine methods. In one study of the Omni cigarette, which, according to machine measurement, delivers less NNK than traditional brands, no significant decrease in the level of total NNAL was observed (Hughes *et al.*, 2004). A second study of Omni did demonstrate a significant reduction in total NNAL compared with the smokers' usual brand, but less than that achieved by cessation with medicinal nicotine (Hatsukami *et al.*, 2004). A significant reduction in total NNAL was also observed in smokers who switched to the Advance cigarette (Breland *et al.*, 2003).

Levels of NNAL and NNAL-Gluc were measured in the urine of 84 Singapore Chinese smokers, who were interviewed about their intake of cruciferous vegetables (Hecht *et al.*,

2004b). There was a significant correlation between increased consumption of glucobrassicins (precursors of indole-3-carbinols) from these vegetables and decreased levels of NNAL in the urine, after adjustment for number of cigarettes smoked per day; similar trends were observed for NNAL-Gluc and total NNAL. These results are consistent with those of previous studies that demonstrated that indole-3-carbinol, an in-vivo hydrolysis product of glucobrassicins, decreased the levels of urinary NNAL, probably by inducing hepatic metabolism of NNK (Morse *et al.*, 1990a; Taioli *et al.*, 1997).

Urine samples from 175 smokers of regular, light or ultra-light cigarettes were analysed for total NNAL (Hecht *et al.*, 2005). There were no statistically significant differences in urinary levels of total NNAL among smokers of these types of cigarettes, and no correlation between levels of tar and total NNAL. These results are consistent with epidemiological studies that showed no difference in the risk for lung cancer among smokers of different types of cigarettes (Burns *et al.*, 2001; Harris *et al.*, 2004).

Nonsmokers exposed to secondhand tobacco smoke

Levels of total NNAL in the urine of nonsmokers exposed to secondhand tobacco smoke are typically about 1–5% of those in smokers (Table 12). Correlations between levels of urinary cotinine and total NNAL have consistently been observed in studies of nonsmokers exposed to secondhand tobacco smoke (Hecht, 2002; IARC, 2004). Most studies on NNAL in the urine of nonsmokers have been reviewed in a previous monograph which concluded: “The data demonstrating uptake of NNK, a lung carcinogen in rodents, by nonsmokers are supportive of a causal link between exposure to secondhand tobacco smoke and development of lung cancer” (IARC, 2004). Two studies have appeared since that time. In one, total NNAL was quantified before and after a 4-h visit to a gambling casino where smoking was allowed. Paired samples showed statistically significant mean increases in total cotinine (cotinine plus its glucuronide) and total NNAL in urine after the visit (Anderson *et al.*, 2003). A second study examined the uptake of total nicotine, total cotinine and total NNAL in nonsmokers who worked in restaurants and bars that permit smoking (Tulunay *et al.*, 2005). Urine samples were collected for 24 h on working and non-working days. The results showed significant increases in urinary levels of total nicotine, total cotinine and total NNAL on working days compared with non-working days.

NNAL-N-*oxide*

Levels of NNAL-*N*-oxide ranged from 0.06 to 1.4 pmol/mg creatinine (mean, 0.53 pmol/mg creatinine) in the urine of smokers and from 0.02 to 1.2 pmol/mg creatinine (mean, 0.41 pmol/mg creatinine) in the urine of smokeless tobacco users. NNK-*N*-oxide was not detected in the urine. The amounts of NNAL-*N*-oxide in urine were about 20 and 50% of the amounts of NNAL-Gluc and NNAL, respectively. Thus, pyridine-*N*-oxidation is less important than glucuronidation as a detoxification pathway for NNK and NNAL in humans (Carmella *et al.*, 1997).

Haemoglobin adducts

Haemoglobin adducts of NNK and NNN are formed upon reaction of a common intermediate, POB-DZH, with aspartate or glutamate in haemoglobin. In the case of NNK, POB-DZH is generated by CYP-mediated hydroxylation of the methyl group to give α-HOMeNNK (Figures 2 and 5). The POB-aspartate and -glutamate esters in haemoglobin can readily be hydrolysed by base treatment to release HPB, which can be quantified by gas chromatography–mass spectrometry (Carmella *et al.*, 1990a; Hecht, 1998). The presence of HPB-releasing haemoglobin adducts in humans provides strong evidence for the metabolic activation of NNK and/or NNN, although another possible source — nitrosation of myosmine — has been proposed (Wilp *et al.*, 2002).

The highest levels of HPB-releasing haemoglobin adducts have been found in smokeless tobacco users. Mean levels (fmol/g haemoglobin) were 517 in snuff-dippers, 236 in nasal snuff users and 148 in *toombak* users (Carmella *et al.*, 1990a; Falter *et al.*, 1994; Murphy *et al.*, 1994). Lower levels were reported in smokers. Mean levels (fmol/g haemoglobin) in smokers and nonsmokers in four studies were 79.6 and 29.3 (Carmella *et al.*, 1990a), 54.7 and 26.7 (Branner *et al.*, 1998), 61 and 34 (Falter *et al.*, 1994) and 26 and 19 (Atawodi *et al.*, 1998). Levels of HPB-releasing haemoglobin adducts were not higher in nonsmokers exposed to secondhand tobacco smoke than in non-exposed nonsmokers (Branner *et al.*, 1998).

DNA adducts

NNK can form adducts by two α-hydroxylation pathways (Figure 2). α-Methyl hydroxylation produces α-HOMeNNK, which spontaneously decomposes to POB-DZH. POB-DZH can also be formed by 2′-hydroxylation of NNN. POB-DZH reacts with DNA to give a variety of adducts (Figures 2 and 5), some of which release HPB upon acid or neutral thermal hydrolysis. POB-DZH-derived adducts in DNA can be quantified by treating the DNA with acid and measuring released HPB by gas chromatography–mass spectrometry. α-Methylene hydroxylation of NNK or NNAL ultimately produces Me-DZH, which can react with DNA to produce O^6-methyldeoxyguanosine (O^6-MedGuo), 7-MedGuo, O^4-methyldeoxythymidine (O^4-MedThd) and other adducts (Figure 2).

HPB-releasing DNA adducts were detected in human lung (Foiles *et al.*, 1991). Mean levels (fmol/mg DNA) were 11 ± 16 in nine smokers and 0.9 ± 2.3 in eight nonsmokers. Mean adduct levels in tracheobronchus were 16 ± 18 in four smokers and 0.9 ± 1.7 in four nonsmokers. In another study, HPB-releasing adducts were not detected in lung samples from four individuals, two of whom were confirmed smokers (detection limit reported as 8–50 fmol/mg DNA) (Blömeke *et al.*, 1996).

Methyl DNA adducts have been detected in tissues or cells of smokers and nonsmokers in several studies. Levels of 7-MedGuo were higher in pulmonary alveolar cells or bronchial tissues of smokers than nonsmokers (Mustonen *et al.*, 1993; Petruzzelli *et al.*, 1996) in two studies, while a third showed no difference (Kato *et al.*, 1995). Sample sizes and their origins were insufficient to judge the effects of smoking in four other studies (Wilson *et al.*, 1989; Shields *et al.*, 1990; Kato *et al.*, 1993; Blömeke *et al.*, 1996). Levels

of 7-alkylguanines in DNA from larynx tissue increased with the amount of smoking (Szyfter *et al.*, 1996). Levels of 7-MedGuo plus 7-(2-hydroxyethyldeoxyguanosine) were higher in lymphocytes of smokers compared with nonsmokers (Kumar & Hemminki, 1996). There was no effect of smoking or exposure to secondhand tobacco smoke on levels of O^6-MedGuo in human peripheral and cord blood DNA (Georgiadis *et al.*, 2000), and no difference in O^6-MedGuo levels in placenta from smokers and nonsmokers (Foiles *et al.*, 1988). Some of the methyl DNA adducts detected in these studies may have originated from NNK, but there are other sources of methyl DNA adducts in cigarette smoke, notably *N*-nitrosodimethylamine.

(iv) *Excretion*

Urine is the only route of excretion of NNK metabolites for which data from studies with humans are currently available (see Section on metabolism above and Table 12). Studies in laboratory animals indicate that urine is the major route of excretion of NNK metabolites (see Section 4.1.2(*d*)).

(*b*) N′-*Nitrosonornicotine (NNN)*

(i) *Absorption*

NNN has been detected in the saliva of snuff dippers, chewers of betel quid with tobacco, users of *khaini*, *gudhaku*, *toombak* and *mishri* and reverse smokers. The data are presented in Section 1.4.2(*a*).

Absorption of NNN has been demonstrated by detection of NNN and NNN-*N*-Gluc in the urine in smokeless tobacco users and smokers (Stepanov & Hecht, 2005; see Section 4.1.1(*b*)(iv)).

(ii) *Distribution*

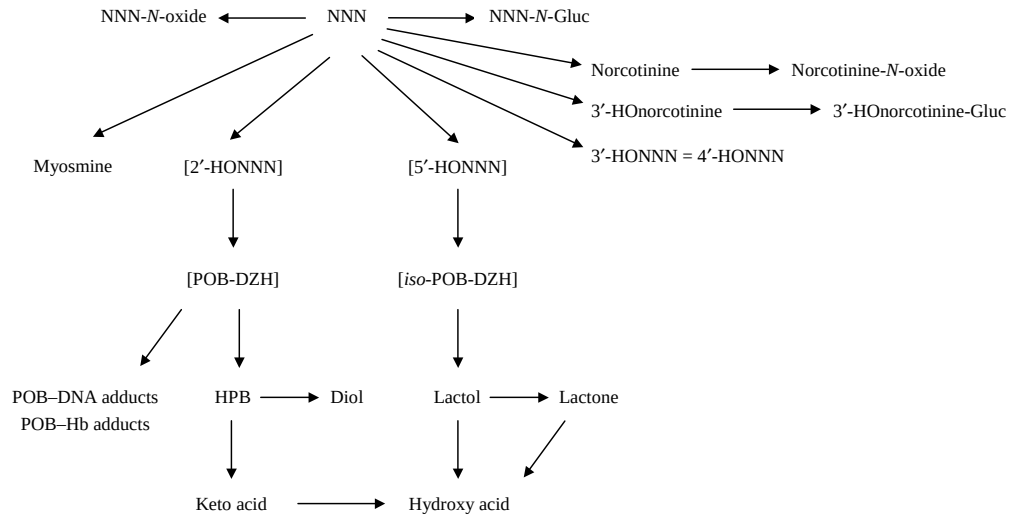
NNN was detected in two of 17 samples of pancreatic juice from smokers at levels of 68.1 and 242 ng/mL juice. It was not detected in the pancreatic juice from nine nonsmokers (Prokopczyk *et al.*, 2002). Pyridyloxobutyl–haemoglobin adducts have been detected in smokeless tobacco users and smokers, and pyridyloxobutyl–DNA adducts have been detected in smokers. These adducts could arise from NNN or NNK and are discussed in the section on NNK.

(iii) *Metabolism*

Introduction

The metabolic pathways of NNN and modes of DNA adduct formation are shown in Figure 6, and structures of the metabolites are illustrated in Figure 7. This information is based on studies *in vitro*, in laboratory animals and in humans. Specific pathways that have been observed in in-vitro studies with human tissues or enzymes or *in vivo* in humans are discussed below.

Figure 6. Metabolism of NNN and formation of adducts, based on studies in laboratory animals and humans

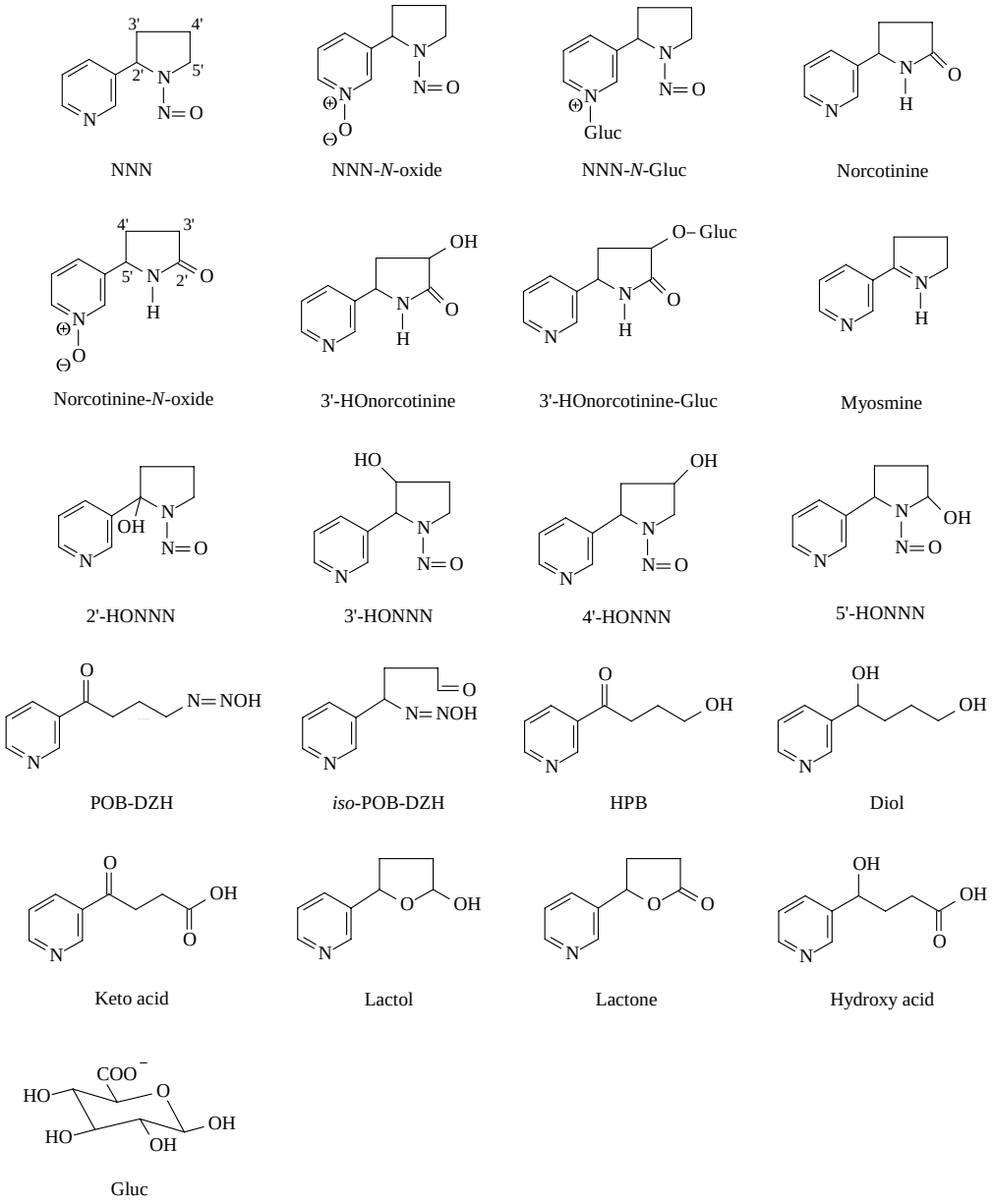


DZH, diazohydroxide; Gluc, glucuronide; Hb, haemoglobin; HO, hydroxy; HPB, 4-hydroxy-1-(3-pyridyl)-1-butanone or keto alcohol; NNN, *N*′-nitrosonornicotine; POB, 4-oxo-4-(3-pyridyl)-1-butyl or pyridyloxobutyl

NNN can undergo pyridine *N*-oxidation to yield NNN-*N*-oxide and pyridine *N*-glucuronidation to yield NNN-*N*-Gluc. Denitrosation and oxidation of NNN yields myosmine or norcotinine, and the latter can undergo pyridine *N*-oxidation to yield norcotinine-*N*-oxide. NNN also is metabolized to 3′-hydroxynorcotinine (3′-HOnorcotinine) but it is not clear whether this proceeds via norcotinine or by an independent pathway. 3′-HOnorcotinine undergoes glucuronidation on its hydroxyl group to yield 3′-HOnorcotinine-Gluc.

Hydroxylation of the pyrrolidine ring of NNN produces 2′-hydroxy-NNN (2′-HONNN), 3′-HONNN, 4′-HONNN and 5′-HONNN. While 3′-HONNN and 4′-HONNN are stable metabolites, 2′-HONNN and 5′-HONNN, which are α-hydroxynitrosamines, are unstable. 2′-HONNN spontaneously undergoes ring opening to yield POB-DZH. POB-DZH reacts with water to form HPB. POB-DZH also reacts with DNA and haemoglobin to form adducts that are believed to have structures identical to those formed by the POB-DZH pathway in NNK metabolism. HPB can be further oxidized to keto acid or reduced to diol. 5′-HONNN spontaneously undergoes ring opening to produce 1-(3-pyridyl)-4-oxo-1-butanediazohydroxide (*iso*-POB-DZH), which reacts with water to form 5-(3-pyridyl)-2-hydroxytetrahydrofuran (lactol). Lactol can be oxidized to 5-(3-pyridyl)tetrahydrofuran-2-one (lactone) or 4-hydroxy-4-(3-pyridyl)butanoic acid (hydroxy acid). Thus, HPB and keto acid are indicative of the metabolic activation of NNN by 2′-hydroxylation whereas the lactol and the hydroxy acid are indicative of the metabolic activation of NNN by 5′-hydroxylation.

Figure 7. Structures of NNN and metabolites



DZH, diazohydroxide; Gluc, glucuronide; HO, hydroxy; HPB, 4-hydroxy-1-(3-pyridyl)-1-butanone or keto alcohol; NNN, *N*′-nitrosonornicotine; POB, 4-oxo-4-(3-pyridyl)-1-butyl or pyridyloxobutyl

In-vitro studies in human tissues and cells

Metabolism of NNN by human tissues *in vitro* has been reviewed (Hecht, 1998). NNN-*N*-oxide and hydroxy acid (from 5′-hydroxylation) have been detected after incubation of cultured human tissues — buccal mucosa, trachea, oesophagus, bronchus, peripheral lung and urinary bladder — with NNN (Castonguay *et al.*, 1983c). Smaller amounts of keto acid (from 2′-hydroxylation) were also observed (Castonguay *et al.*, 1983c). Adult and fetal human oesophageal cultures metabolized NNN to hydroxy acid (5′-hydroxylation), keto acid (2′-hydroxylation) and NNN-*N*-oxide (Chakradeo *et al.*, 1995). Human oesophageal microsomes also metabolized NNN by 5′-hydroxylation and 2′-hydroxylation; and the latter was partially inhibited by troleandomycin, an inhibitor of CYP3A (Smith *et al.*, 1998).

Human liver microsomes metabolically activate NNN by 5′-hydroxylation to yield lactol and by 2′-hydroxylation to yield keto alcohol. Consistently, 5′-hydroxylation predominates (Hecht, 1998). Overall rates of metabolic activation of NNN were greater than those of NNK, NNAL or benzo[*a*]pyrene in human liver microsomes (Staretz *et al.*, 1997a). At concentrations of 0.8–1 μM NNN, metabolism by human liver microsomes was mainly by 5′-hydroxylation and the reaction was strongly correlated with coumarin 7-hydroxylation, which suggests the involvement of CYP2A6 (Patten *et al.*, 1997). 2′-Hydroxylation correlated with 6β-hydroxylation of testosterone, a CYP3A4-specific activity (Patten *et al.*, 1997).

CYPs have been identified as the major catalysts of NNN metabolic activation by α-hydroxylation. Human CYP2A6 metabolized NNN exclusively by 5′-hydroxylation, with a K_m of 2.1 μM (Patten *et al.*, 1997). The K_ms for 5′-hydroxylation of (*R*)-NNN and (*S*)-NNN were 22 μM and 2.3 μM, respectively (Wong *et al.*, 2005b). Rates of metabolism of NNN by CYP2E1 and 2D6 were much lower (Patten *et al.*, 1997). Metabolism of NNN by expressed human CYP3A4 was specific for 2′-hydroxylation (HPB formation) with a K_m of 304 μM (Patten *et al.*, 1997). Human CYP2A13 metabolized both (*R*)- and (*S*)-NNN by 5′-hydroxylation to lactol, with K_ms of 24 and 23 μM, respectively, and metabolized (*R*)-NNN to HPB with a K_m of 21 μM (Wong *et al.*, 2005b). Studies with *Salmonella typhimurium* strains that co-express human CYPs demonstrated that CYP2A6 was the most effective catalyst of NNN metabolic activation among 11 CYPs tested (Fujita & Kamataki, 2001).

In summary, studies with human tissues clearly demonstrate the metabolic activation of NNN; 5′-hydroxylation predominates in liver microsomes and both 2′-hydroxylation and 5′-hydroxylation are observed to comparable extents in oesophageal microsomes. NNN metabolism has also been observed in a variety of cultured tissues. Human CYP2A6 and CYP2A13 are effective catalysts of NNN α-hydroxylation.

In-vivo studies

NNN-*N*-Gluc has been detected in the urine of smokeless tobacco users and smokers (Stepanov & Hecht, 2005). Keto acid and the enantiomers of hydroxy acid have been quantified in the urine of smokers and abstinent smokers who used nicotine replacement therapy to test the hypothesis that (*S*)-hydroxy acid could be a biomarker of metabolic

activation of NNK and NNN while (*R*)-hydroxy acid would be formed predominantly from nicotine as indicated by studies with rats (Hecht *et al.*, 1999b; Trushin & Hecht, 1999). (*R*)-Hydroxy acid was the major enantiomer in human urine. The amount of (*S*)-hydroxy acid was far greater than that which could be formed from NNK and NNN. Both (*S*)-hydroxy acid and keto acid were formed in substantial amounts from nicotine, which precludes their use as biomarkers of NNN uptake in smokers.

HPB-releasing haemoglobin adducts and DNA adducts have been detected in tobacco users. These adducts can be formed either from NNK or from 2′-hydroxylation of NNN, and are discussed in section 4.1.1(*a*)(iii).

(iv) *Excretion*

NNN and NNN-*N*-Gluc have been detected in the urine of smokeless tobacco users and smokers (Stepanov & Hecht, 2005). The data are presented in Section 1.4.2(*b*).

(*c*) N′-*Nitrosoanabasine (NAB)*

(i) *Absorption*

NAB has been detected in the saliva of users of *toombak*, *khaini* tobacco and *gudhaku***.** The data are presented in Section 1.4.3(*a*).

Absorption of NAB by smokeless tobacco users and smokers has been demonstrated by detection of NAB and NAB-*N*-Gluc in urine (Stepanov & Hecht, 2005).

(ii) *Distribution*

No data were available to the Working Group

(iii) *Metabolism*

In experiments with 11 strains of *S. typhimurium* YG7108, each of which co-expresses a form of human CYPs, CYP 3A4, 2A6, 1A1 and 3A5 were capable of converting NAB to mutagenic products. CYP3A4 was the best catalyst (Fujita & Kamataki, 2001).

(iv) *Excretion*

NAB and NAB-*N*-Gluc have been detected in the urine of smokers and smokeless tobacco users (Stepanov & Hecht, 2005). The data are presented in Section 1.4.3(*b*).

(*d*) N′-*Nitrosoanatabine (NAT)*

(i) *Absorption*

NAB has been detected in the saliva of users of *toombak*, *khaini* and *gudhaku*. The data are presented in Section 1.4.4(*a*).

Absorption of NAT has been demonstrated by the detection of NAT and NAT-*N*-Gluc in the urine of smokeless tobacco users and smokers (Stepanov & Hecht, 2005).

(ii) *Distribution*

No data were available to the Working Group.

(iii) *Metabolism*

In experiments with 11 strains of *S. typhimurium* YG7108, each of which co-expresses a form of human CYPs, CYP2A6 was capable of converting NAT to mutagenic products (Fujita & Kamataki, 2001).

(iv) *Excretion*

NAT and NAT-*N*-Gluc have been detected in the urine of smokers and smokeless tobacco users (Stepanov & Hecht, 2005). The data are presented in Section 1.4.4(*b*).

4.1.2 *Experimental systems*

(*a*) *NNK and NNAL*

Studies on the absorption, distribution, metabolism and excretion of NNK in experimental systems have been comprehensively reviewed (Hecht, 1998). The reader is referred to that review for detailed coverage of the literature. Selected studies from the previous review that illustrate important points as well as more recent studies pertinent to the evaluation are presented below.

(i) *Absorption*

Beagle dogs were exposed to a single spray bolus of dissolved NNK in the distal trachea (0.48 nmol/dog) (Gerde *et al.*, 1998). NNK was rapidly absorbed and extensively metabolized in the tracheal mucosa. Most NNK appeared rapidly in the blood that drains the airway mucosa, but a phase of slow clearance was also observed. During absorption, NNK was distributed within the entire depth of the mucosa to the tracheal cartilage. A portion was bound to the mucin component of the mucous lining. First-pass metabolism and activation of NNK in the airway mucosa were sufficiently rapid to cause levels of binding at the site of absorption that were 20-fold those at distal tissues, which indicates a mechanism by which NNK could act as a carcinogen at the site of entry. In a comparison of NNK and benzo[*a*]pyrene, it was concluded that NNK is diffused into the mucosa sufficiently rapidly for blood perfusion to limit clearance, whereas benzo[*a*]pyrene is diffused into the mucosa more slowly. NNK passes into the blood about 100 times more rapidly than benzo[*a*]pyrene. NNK and its metabolites swiftly disperse thoughout the mucosa, whereas benzo[*a*]pyrene and its activated metabolites are confined to the target epithelium. NNK was metabolized in the airway mucosa at least 15 times faster than benzo[*a*]-pyrene, but NNK metabolites bound less effectively than those of benzo[*a*]pyrene at the site of entry. The rapid absorption of NNK at the site of entry could lead to accumulation at peripheral sites in the lung, which is consistent with its ability to induce adenocarcinoma (Gerde *et al.*, 1998). Rapid absorption of NNK has also been observed after admi-

nistration to rodents and monkeys by various routes (Castonguay *et al.*, 1983b; Tjälve & Castonguay, 1983; Castonguay *et al.*, 1985a; Tjälve, 1991).

(ii) *Distribution*

Audoradiographic studies demonstrate that, 1 min after intravenous administration of [carbonyl-^{14}C]NNK to rats, radioactivity was homogeneously distributed in most tissues of the body at a level similar to that in the blood (Castonguay *et al.*, 1983b). At later time intervals, accumulation of bound radioactivity was observed in tissues such as the lung and nasal mucosa, which are targets of NNK carcinogenicity. Relatively large amounts of unbound radioactivity are also observed in the stomach contents and melanin-containing tissues, due to the basicity of NNK and NNAL (Castonguay *et al.*, 1983b; Tjälve & Castonguay, 1983; Castonguay *et al.*, 1985a; Hecht, 1998). These studies show that NNK is distributed rapidly and homogeneously throughout the body and has the ability to cross cellular membranes freely and partition evenly in the intra- and extracellular tissue water (Castonguay *et al.*, 1983b). Initially, strong labelling was observed in parts of the nasal mucosa, in the liver, bronchial mucosa, adrenal cortex, preputial gland, salivary gland and stomach contents. At later time points, radioactivity persisted in certain tissues and was seen in the kidney, urinary bladder and gastrointestinal contents (Castonguay *et al.*, 1983b). Similar results have been obtained in autoradiographic and other studies carried out in Syrian golden hamsters, dogs and marmoset monkeys by various routes of administration (Tjälve & Castonguay, 1983; Castonguay *et al.*, 1985a; Gerde *et al.*, 1998; Tjälve, 1991).

Micro-autoradiographic studies in Fischer 344 rats injected with [CH_3-^{3}H]NNK showed that the highest degree of labelling in the lung was in the Clara cells (Belinsky *et al.*, 1987a); in the nasal passages, the highest degree of labelling was in Bowman's glands, Steno's gland and serous glands of the respiratory mucosa, with a lower degree of labelling in the respiratory and olfactory epithelia (Belinsky *et al.*, 1987b). Similar results were obtained with [carbonyl-^{14}C]NNK (Tjälve *et al.*, 1985; Tjälve, 1991).

Pharmacokinetic studies of NNK and NNAL in rats demonstrated large volumes of distribution of NNK (321 ± 137 mL) and NNAL (2772 ± 1423 mL) (Wu *et al.*, 2002). The enantiomers of NNAL appear to be distributed differently in the body, as indicated by the apparent volumes of distribution: 1792 ± 570 mL for (*S*)-NNAL and 645 ± 230 mL for (*R*)-NNAL, a difference that was significant (Zimmerman *et al.*, 2004). These data suggest extensive tissue binding that is greater for (*S*)-NNAL. Tissue distribution studies demonstrated that (*S*)-NNAL was retained in the lung 24 h after administration; the (*S*):(*R*) ratio increased from 1.1 1 h after administration to 4.23 24 h after administration (Zimmerman *et al.*, 2004). An increase of this magnitude was observed only in the lung, which suggests that (*S*)-NNAL is stereoselectively retained in the rat lung, possibly at a receptor site. This tissue distribution of NNAL may partially explain the initial accumulation of radioactivity in certain rat tissues, as seen by autoradiography, as well as the relatively long retention of (*S*)-NNAL seen in smokers and smokeless tobacco users.

(iii) *Metabolism*

Extensive studies of the metabolism of NNK have been carried out *in vitro* and *in vivo* in a variety of species including rats, hamsters, mice, rabbits, pigs and monkeys (reviewed in Hecht, 1998). Figure 2 summarizes the metabolism of NNK determined from these investigations. Virtually all systems examined conform to this general scheme, with major pathways of metabolism generally being reduction to NNAL and α-hydroxylation of NNK and NNAL. In the conversion of NNK to NNAL, (*S*)-NNAL is the predominant enantiomer formed in rat and mouse liver and lung microsomes and cytosol, as well as red blood cells (Upadhyaya *et al.*, 2000).

In-vitro studies of NNK metabolism

In-vitro studies of NNK metabolism have been comprehensively reviewed (Hecht, 1998). Studies of the kinetic parameters for NNK metabolism mediated by microsomal preparations from tissues of laboratory animals (Table 13) and relevant CYP enzymes (Table 11) are discussed below. Other selected studies of NNK metabolism *in vitro* are also included.

Monkey

Kinetic parameters for NNK metabolism have been reported for patas monkey lung and liver microsomes (Table 13; Smith *et al.*, 1997). In the lung, HPB is formed with the greatest efficiency, followed by NNK-*N*-oxide, the keto aldehyde and NNAL (Table 13; Smith *et al.*, 1997). K_m values for keto aldehyde, HPB and NNK-*N*-oxide formation were about 5–10 μM. The K_m for NNAL formation was much higher, however, which is consistent with the hypothesis that this metabolite is not formed by the same enzymes that catalyse α-hydroxylation (Maser *et al.*, 1996; Maser, 1998; Maser *et al.*, 2000; Finckh *et al.*, 2001). Similar results were obtained in patas monkey liver microsomes (Table 13). The kinetic parameters for NNK metabolism by expressed monkey CYPs have not been reported, but antibody and chemical inhibition studies imply that members of the CYP1A and 2A subfamilies are important catalysts of NNK metabolism (Smith *et al.*, 1997). The metabolism of NNK was also studied in short-term cultures of patas and cynomolgus monkey lung, and in Fischer 344 rat lung for comparison. Substantial amounts of metabolites from the α-hydroxylation pathway of metabolic activation were observed, together with the formation of NNK-*N*-oxide and NNAL. The metabolism of NNK by cultured monkey lung was generally similar to that observed in rat lung, which indicates that there are no major species differences between rodents and non-human primates in the pulmonary metabolism of NNK (Hecht *et al.*, 2000).

Rabbit

NNK metabolism by rabbit nasal, but not liver or lung, microsomes has been studied; kinetic parameters have not been reported (Hong *et al.*, 1992; Hecht, 1998). Kinetic parameters for CYPs purified from rabbit nasal microsomes — CYP2A10/2A11 (a mixture of two CYPs previously referred to as NMa) and CYP2G1 (previously NMb) — have been

Table 13. Apparent steady-state kinetic parameters for microsome-mediated NNK metabolism

Species/tissue	Metabolite	Kinetic parameters			Experimental conditions	Reference
		V_{max}[a]	K_m (μM)	V_{max}/K_m[b]		
Female Patas monkey lung	Keto aldehyde	5.3 ± 0.4	10.3 ± 0.8	0.51 ± 0.06	1–20 μM NNK; 30-min incubation, 0.5 mg/mL microsomal protein.	Smith *et al.* (1997)
	HPB	19.1 ± 0.8	4.9 ± 0.2	3.9 ± 0.2		
	NNK-*N*-oxide	11.0 ± 0.3	5.4 ± 0.2	2.0 ± 0.01		
	NNAL[c]	479 ± 35	902 ± 21	0.53 ± 0.04		
Female Patas monkey liver	Keto aldehyde	37.7 ± 1.9	8.2 ± 0.3	4.6 ± 0.3	1–50 μM NNK; 20-min incubation, 0.25 mg/mL microsomal protein.	
	HPB	37.4 ± 1.0	8.1 ± 0.2	4.6 ± 0.2		
	NNAL[c]	3470 ± 103	474 ± 28	7.3 ± 0.5		
Male Sprague-Dawley rat lung	Keto aldehyde	11.7 ± 1	28.9 ± 1.2	0.40 ± 0.04	1–50 μM NNK, 30-min incubation, 0.25 mg/mL microsomal protein.	Smith *et al.* (1992a)
	HPB	14.6 ± 1.0	7.0 ± 0.5	2.1 ± 0.2		
	NNK-*N*-oxide	35.1 ± 2.3	10.4 ± 0.9	3.4 ± 0.4		
	NNAL	195.3 ± 9	178 ± 10	1.1 ± 0.1		
Male Fischer 344 rat liver	Formaldehyde	1478	5	296	12.5–4000 μM NNK, 0.55 mg/mL microsomal protein.	Castonguay *et al.* (1991)
	Formaldehyde	197	534	0.37		
Male Sprague-Dawley rat liver	Keto aldehyde	153 ± 16	234 ± 38	0.65 ± 0.13	5–200 μM NNK, 5-min incubation, 0.75 mg/mL microsomal protein.	Guo *et al.* (1992)
	HPB	156 ± 8	211 ± 20	0.74 ± 0.08		
	Keto aldehyde	381 ± 51	149 ± 32	2.6 ± 0.6	As above, but animals were treated with 3-methylcholanthrene	
	HPB	270 ± 43	246 ± 54	1 ± 0.3		
	Keto aldehyde	329 ± 35	119 ± 22	2.8 ± 0.6	As above, but animals were treated with phenobarbital.	
	HPB	358 ± 27	177 ± 20	2.0 ± 0.3		
	NNK-*N*-oxide	140 ± 10	57 ± 9	2.5 ± 0.4		
	Keto aldehyde	550 ± 44	133 ± 21	4.1 ± 0.7	As above, but animals were treated with pregnenolone 16α-carbonitrile.	
	HPB	247 ± 18	187 ± 24	1.3 ± 0.2		
	NNK-*N*-oxide	167 ± 15	103 ± 21	1.6 ± 0.4		
Male Sprague-Dawley rat nasal mucosa	Keto aldehyde	2833 ± 81	9.6 ± 0.3	295 ± 12	1–100 μM NNK, 10-min incubation, 0.013 mg/mL microsomal protein.	Smith *et al.* (1992a)
	HPB	3275 ± 60	10.1 ± 0.2	324 ± 9		

Table 13 (contd)

Species/tissue	Metabolite	Kinetic parameters			Experimental conditions	Reference
		V_{max}[a]	K_m (μM)	V_{max}/K_m[b]		
Female A/J mouse lung	Formaldehyde	57.2 ± 2.2	5.6 ± 0.9	10.2 ± 1.7	1–20 μM NNK, 30-min incubation, 0.25 mg/mL microsomal protein.	Smith *et al.* (1990)
	HPB	56.0 ± 3.8	5.6 ± 0.9	10.0 ± 1.7		
	Keto acid	4.2 ± 0.5	9.2 ± 1.0	0.456 ± 0.074		
	NNK-*N*-oxide	54.2 ± 1.3	4.7 ± 0.9	11.5 ± 2.2		
	NNAL[c]	1322 ± 10	2541 ± 15	0.52 ± 0.005		
	Keto aldehyde	58.9 ± 2.6	23.7 ± 2.6	2.5 ± 0.3	0.5–100 μM NNK, 30-min incubation, 0.25 mg/mL microsomal protein.	Peterson *et al.* (1991a)
	HPB	32.5 ± 2.5	3.6 ± 0.9	9.0 ± 2.4		
Female A/J mouse lung	Keto aldehyde	34.0	4.9	6.9	1–10 μM NNK, 30-min incubation, 0.25 mg/mL microsomal protein.	Smith *et al.* (1993)
	HPB	38.1	2.6	15		
	NNK-*N*-oxide	60.0	1.8	33		
	Keto aldehyde	31.8	5.0	6.4	As above, but animals were treated with PEITC (1 μmol/g diet).	
	HPB	35.1	2.9	12		
	NNK-*N*-oxide	51.0	1.8	28		
	Keto aldehyde	25.7	4.7	5.5	As above, but animals were treated with PEITC (3 μmol/g diet).	
	HPB	23.0	2.4	9.6		
	NNK-*N*-oxide	36.7	1.6	23		
	Keto aldehyde	84.7 ± 2.8	4.5 ± 0.4	19 ± 2	0.25–20 μM NNK, 30-min incubation, 0.25 mg/mL microsomal protein.	
	HPB	62.8 ± 1.3	1.9 ± 0.2	33 ± 4		
	NNK-*N*-oxide	83.3 ± 3.6	2.0 ± 0.3	42 ± 7		
	Keto aldehyde[d]	89.2 ± 6.3	24.0 ± 2.7	3.7 ± 0.5	As above, but with PEITC (400 nM) added to the incubation mixtures.	Smith *et al.* (1993)
	HPB[d]	60.4 ± 1.8	14.9 ± 0.8	4.1 ± 0.2		
	NNK-*N*-oxide[d]	85.8 ± 4.6	17.9 ± 1.7	4.8 ± 0.5		
	Keto aldehyde	71 ± 3	4.8 ± 0.7	15 ± 2	0.25–50 μM NNK, 15-min. incubation, 0.25 mg/mL microsomal protein.	Jalas *et al.* (2003b)
	HPB	93 ± 3	3.0 ± 0.4	31 ± 4		
	NNK-*N*-oxide	109 ± 4	2.1 ± 0.3	52 ± 8		

Table 13 (contd)

Species/tissue	Metabolite	Kinetic parameters			Experimental conditions	Reference
		V_{max}[a]	K_m (μM)	V_{max}/K_m[b]		
Female A/J mouse liver	Keto aldehyde	245 ± 17	24 ± 4	10 ± 2	0.5–100 μM NNK, 15-min incubation, 0.25 mg/mL microsomal protein.	Nunes *et al.* (1998)
	HPB	100 ± 7	18 ± 4	5.6 ± 1.3		
	Keto aldehyde[d]	213 ± 41	23 ± 12	9.3 ± 5.2	As above, but with 2.5 μM 4-HPO added	
	HPB[d]	77 ± 13	17 ± 8	4.5 ± 2.3		
	Keto aldehyde[d]	210 ± 16	24 ± 4	8.8 ± 1.6	As above, but with 5.0 μM 4-HPO added	
	HPB[d]	69 ± 11	17 ± 8	4.1 ± 2.0		
	Keto aldehyde[d]	170 ± 14	22 ± 5	7.7 ± 1.9	As above, but with 10 μM 4-HPO added	
	HPB[d]	71 ± 4	18 ± 3	3.9 ± 0.7		
	Keto aldehyde[d]	78 ± 1	22 ± 8	3.5 ± 1.3	As above, but with 20 μM 4-HPO added	
	HPB[d]	44 ± 5	18 ± 5	2.4 ± 0.7		
	Keto aldehyde	173 ± 6	19.1 ± 2	9.1 ± 0.7	1–100 μM NNK, 10-min incubation, 0.25 mg/mL microsomal protein.	Peterson *et al.* (1991a)
	HPB	239 ± 11	73.8 ± 6.8	3.2 ± 0.3		
	Keto aldehyde	132 ± 11	5.5 ± 0.3	24 ± 2	1–10 μM NNK, 10-min incubation, 0.50 mg/mL microsomal protein.	Smith *et al.* (1993)
	HPB	60.4 ± 3.1	5.1 ± 0.2	11.8 ± 0.8		
	NNK-*N*-oxide	8.0 ± 0.6	8.8 ± 0.5	0.91 ± 0.09		
	Keto aldehyde	77.0 ± 9.3	5.4 ± 0.4	14.3 ± 2.0	As above, but animals were treated with PEITC (3 μmol/g diet).	
	HPB	39.3 ± 2.5	5.3 ± 0.3	7.4 ± 0.6		
	NNK-*N*-oxide	5.6 ± 0.7	9.1 ± 0.3	0.62 ± 0.08		

Adapted from Jalas *et al.* (2005)

HPB, 4-hydroxy-1-(3-pyridyl)-1-butanone; 4-HPO, 4-hydroxy-1-phenyl-1-octanone; K_m, Michaelis constant; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; NNK, 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone; PEITC, phenethyl isothiocyanate; V_{max}, maximum velocity

[a] Units are pmol/mg/ min.

[b] Units are pmol/mg/min/μM.

[c] NNK concentrations up to 1000 μM were used to determine kinetic parameters for NNAL formation.

[d] Values are V_{max}', K_m', and V_{max}'/K_m', respectively, due to the presence of an inhibitor in the incubation mixtures.

determined (Hong *et al.*, 1992). In the absence of exogenous cytochrome b_5, reconstituted CYPs 2A10/2A11 exhibited K_m values for NNK α-hydroxylation that were similar to those for human CYP2A13 (Table 12; Hong *et al.*, 1992; Su *et al.*, 2000; Zhang *et al.*, 2002). CYPs 2A10/2A11 exhibited lower K_m and higher V_{max}/K_m values for the keto aldehyde formation than those determined using CYP2G1 (Table 11; Hong *et al.*, 1992). Nicotine was a competitive inhibitor of CYP2A10/2A11-mediated NNK metabolism (Table 11; Hong *et al.*, 1992).

Rat

Studies in isolated perfused liver and lung demonstrated that, at a concentration of 35 nM, NNK was rapidly eliminated (Schrader *et al.*, 1998). The clearance was almost exclusively mediated by metabolism. The kinetics of NNK metabolism in the liver was substantially faster than that in the lung but, on a per gram basis, lung clearance was faster. Products of α-hydroxylation were the major metabolites in the liver and NNK-*N*-oxide was the major metabolite in the lung, followed by α-hydroxylation products. Studies in rat lung and liver cells gave similar results and indicated a correlation between metabolite profiles in lung cells and urinary metabolites obtained after treatment of rats with NNK and modifiers (Schneider *et al.*, 1999). A study of NNK metabolism in rat alveolar type II cells demonstrated substantial metabolism by α-hydroxylation at low concentrations of NNK (50 nM), a concentration probably relevant to human exposure (Schrader *et al.*, 2000).

The kinetics of NNK metabolism have been studied extensively using microsomes prepared from Sprague-Dawley and Fischer rat lung, liver and nasal mucosa (Table 13). In rat lung, NNK-*N*-oxide is the major NNK metabolite, followed by HPB, NNAL and the keto aldehyde (Hecht, 1998). K_m values for HPB and NNK-*N*-oxide formation were similar to those in patas monkey lung (Table 13). The kinetic parameters of NNK metabolism in rat liver and the effects of various inducers of CYP on these parameters have been evaluated in several studies. The K_m values for the keto aldehyde and HPB formation by rat liver microsomes were higher than those in human or patas monkey liver microsomes (Table 13; Guo *et al.*, 1992; Patten *et al.*, 1996; Smith *et al.*, 1997). Treatment of rats with phenobarbital, which induces expression of CYP2B enzymes (Soucek & Gut, 1992; Whitlock & Denison, 1995), led to detectable levels of NNK-*N*-oxide and slightly increased the efficiency of POB and HPB formation (Table 13; Guo *et al.*, 1992). Treatment of rats with pregnenolone 16α-carbonitrile, an inducer of CYP3A enzymes (Heuman *et al.*, 1982; Soucek & Gut, 1992; Whitlock & Denison, 1995), also led to enhanced efficiency of keto aldehyde and HPB formation, as well as to the formation of NNK-*N*-oxide (Table 13; Guo *et al.*, 1992).

Microsomes prepared from rat nasal mucosa are better catalysts of NNK bioactivation than any other microsomal system investigated to date (Table 13). The K_m values for HPB formation were comparable in rat lung and nasal mucosa, whereas the K_m for the keto aldehyde formation by rat nasal mucosal microsomes was threefold lower than that in lung microsomes (Table 13). The much higher catalytic efficiency of NNK metabolism in rat nasal mucosal microsomes than lung or liver was clearly due to the larger V_{max} values in

the nasal mucosa (Table 13). These data are consistent with a role for CYP2A3 (discussed below) as an important catalyst of NNK α-hydroxylation in lung and nasal mucosa.

The kinetic parameters for NNK metabolism by rat CYPs have been studied only for two enzymes — CYP2A3 and 2B1 (Table 12; Guo *et al.*, 1991a; Jalas *et al.*, 2003a). CYP2A3 is expressed in both the nasal mucosa and lung, but not the liver (Su *et al.*, 1996). Both protein and mRNA levels are much greater in the nasal mucosa than in the lung (Su *et al.*, 1996; Gopalakrishnan *et al.*, 1999). CYP2A3 catalyses α-hydroxylation of NNK much more efficiently than CYP2B1. A comparison of the primary sequence of rat and human CYP2A enzymes showed that rat CYP2A3 was 85 and 87% identical to human CYPs 2A6 and 2A13, respectively. CYP2A3 catalyses NNK metabolism with an efficiency similar to that of human CYP2A13 (Honkakoski & Negishi, 1997; Jalas *et al.*, 2003a). The high catalytic efficiency of CYP2A3 for NNK α-hydroxylation most probably plays a role in the carcinogenicity of NNK to the rat lung and nasal mucosa (Jalas *et al.*, 2005).

CYP2B1 also catalyses the α-hydroxylation of NNK, but with much higher K_m and lower V_{max} values than CYP2A3 (Table 11; Guo *et al.*, 1991a). CYP2B1, unlike CYP2A3, also catalyses NNK-*N*-oxide formation (Table 11; Guo *et al.*, 1991a). Rat CYP2B1 and CYP2C6, together with human CYP2C8, are the only CYP enzymes reported to catalyse the *N*-oxidation of NNK (Guo *et al.*, 1991a; Smith *et al.*, 1992a; Lacroix *et al.*, 1993; Jalas *et al.*, 2005). Because NNK-*N*-oxide formation represents quantitatively the major CYP-catalysed pathway of NNK metabolism in the rat lung, studies have examined the enzyme(s) responsible for this reaction (Smith *et al.*, 1992b). Anti-2B1 antibodies do not inhibit the formation of this metabolite by rat lung microsomes (Smith *et al.*, 1992b). Furthermore, phenobarbital treatment of rats induced the formation of NNK-*N*-oxide catalysed by rat liver microsomes (Table 13), but did not significantly enhance the rate of formation of this metabolite catalysed by rat lung microsomes (Guo *et al.*, 1992).

Kinetic parameters for NNK metabolism for rat CYPs 1A1, 1A2, and 2C6 are also presented in Table 11 (Jalas *et al.*, 2005). Rat CYP1A1, similarly to human CYP1A1 (with which it shares 78% primary sequence identity; Soucek & Gut, 1992) preferentially catalyses keto aldehyde formation, but with much lower K_m values (Table 11; Smith *et al.*, 1995; Jalas *et al.*, 2005). The V_{max}/K_m values for keto aldehyde formation were an order of magnitude higher for the rat enzyme compared with the human enzyme, but these values were similar for HPB formation (Table 11; Smith *et al.*, 1995; Jalas *et al.*, 2005). In contrast to rat CYP1A1, rat CYP1A2 does not exhibit the regioselectivity observed with the orthologous human enzyme (primary sequence identity, 70%; Soucek & Gut, 1992) (Table 11; Smith *et al.*, 1992a, 1996; Jalas *et al.*, 2005).

Rat CYP2C6, similarly to rat CYP2B1 and human CYP2C8, catalyses the *N*-oxidation in addition to α-hydroxylation of NNK (Table 11; Guo *et al.*, 1991a; Smith *et al.*, 1992b; Lacroix *et al.*, 1993; Jalas *et al.*, 2005). The K_m values for CYP2C6 were much higher than those for CYP2B1, but higher V_{max} values led to V_{max}/K_m values that were similar between the two enzymes (except in the case of HPB formation) (Table 11; Guo *et al.*, 1991a; Jalas *et al.*, 2005). Rat CYP2C6 catalyses HPB formation about 10-fold more efficiently than

CYP2B1 (Table 11; Guo *et al.*, 1991a; Jalas *et al.*, 2005). Rat CYPs 2D1, 2D2, 3A1 and 3A2 do not metabolize NNK (Jalas *et al.*, 2005).

Mouse

Lung microsomes from female A/J mice have been used by several laboratories to determine the kinetic parameters of NNK metabolism (Table 13). The reported K_m values were consistently in the 1–10 μM range for keto aldehyde, HPB and NNK-*N*-oxide (Smith *et al.*, 1990; Peterson *et al.*, 1991a; Smith *et al.*, 1993; Jalas *et al.*, 2003b). Similarly to results with rat lung microsomes, NNK-*N*-oxide was the major metabolite (Table 13). In female A/J mouse liver microsomes, NNK was converted to keto aldehyde, HPB and NNK-*N*-oxide (Table 13). The K_m values were similar in mouse lung and liver microsomes, but the V_{max} values were generally higher for liver microsome-mediated formation of keto aldehyde and HPB (Table 13).

Kinetic parameters for NNK metabolism have been determined for mouse CYPs 2A4 and 2A5 (Felicia *et al.*, 2000; Jalas *et al.*, 2003b). CYP2A4 and CYP2A5 are more than 90% identical to human CYPs 2A6 and 2A13 and are expressed in many mouse tissues, including the liver and lung (Honkakoski & Negishi, 1997). These two mouse CYP2A enzymes differ in primary sequence by only 11 amino acids (of 494), but exhibit strikingly different substrate specificities (Lindberg & Negishi, 1989; Honkakoski & Negishi, 1997). Mutation of only one amino acid residue (Phe 209) in CYP2A5 to the corresponding residue in CYP2A4 (Leu 209) is sufficient to convert the substrate specificity of CYP2A5 to that of a 2A4-like enzyme (Lindberg & Negishi, 1989). Investigation of the kinetic parameters of NNK metabolism by these two highly similar CYPs revealed significant differences in their ability to catalyse NNK α-hydroxylation (Table 11; Felicia *et al.*, 2000). CYP2A5 exhibits a much lower K_m value and preferentially hydroxylates the α-methyl carbon of NNK, whereas CYP2A4 exhibits a much higher K_m value and preferentially catalyses the α-methylene hydroxylation of NNK (Table 11; Felicia *et al.*, 2000; Jalas *et al.*, 2003b). The K_m value for CYP2A5-mediated α-methyl hydroxylation of NNK is the lowest among those of the CYPs listed in Table 11.

Hamster

Kinetic parameters for NNK metabolism were determined in tissue slices from the lung, liver and kidney of female Syrian golden hamsters (Richter *et al.*, 2000). High and low K_m and V_{max} values were observed in the lung and liver for the formation of most NNK metabolites including hydroxy acid, keto acid, HPB, diol, NNK-*N*-oxide, NNAL-*N*-oxide and NNAL. K_m values ranged from 0.04 to 1952 μM. In the lung, α-hydroxylation accounted for 13–31% of metabolism. The liver showed the highest capacity for NNK metabolism, and α-hydroxylation accounted for 12–25% of the metabolites. The kidney showed a low capacity for NNK metabolism with α-hydroxylation accounting for < 3% of the metabolites. Conversion of NNK to NNAL was greatest in the kidney, followed by the liver and lung.

Summary

In the species that have been studied, a number of CYP2A enzymes are excellent catalysts of NNK α-hydroxylation, but more research is needed to clarify the contribution of individual CYPs to microsome-mediated NNK metabolism in animals. The K_m values for NNK α-hydroxylation in patas monkey lung and liver microsomes are consistent with the involvement of a CYP2A13 orthologue, but further study is required. The K_m values observed using rat lung microsomes are consistent with the involvement of CYP2A3 which is closely related to human CYP2A13 or mouse CYP2A5. The rat liver does not express a CYP2A6 or 2A13 orthologue (Honkakoski & Negishi, 1997; Gopalakrishnan *et al.*, 1999) and the K_m values for NNK metabolism by liver microsomes are consistent with the involvement of CYP2B1. Kinetic data on NNK metabolism by other rat CYPs is needed to assess better the involvement of individual enzymes in this species. The K_m values for NNK metabolism in mouse lung and liver microsomes are similar to those determined for CYP2A5. It seems probable that CYP2A5 plays a role in NNK bioactivation in mice; however, additional studies are needed to define better the contribution of CYP2A5 in both the lung and liver. Table 14 summarizes K_m values and regioselectivity in NNK metabolism for both laboratory animal and human CYPs.

In-vitro studies of NNAL metabolism

Rat liver microsomes convert NNAL to lactol and hydroxy acid (products of α-methylene hydroxylation), to diol and pyridylTHF (products of α-methyl hydroxylation), as well as to NNAL-*N*-oxide, $NNAL(ADP)^+$ and NNK (Peterson *et al.*, 1994; Staretz *et al.*, 1997b; Upadhyaya *et al.*, 2000). In rat pancreatic microsomes, only $NNAL(ADP)^+$ was observed (Peterson *et al.*, 1994). Rat liver microsomes and co-factors convert NNAL predominantly to (*R*)-NNAL-*O*-Gluc; the uridine diphospho (UDP)-glucuronosyl transferase, UGT2B1, is an important catalyst of this reaction (Ren *et al.*, 1999).

Kinetic parameters for NNAL metabolism catalysed by microsomes have only been reported for A/J mouse lung microsomes (Table 15). K_m values for NNAL and NNK metabolism were similar (Tables 13 and 15), but V_{max} values for NNAL were almost an order of magnitude lower. Thus, *in vivo*, metabolic activation of NNAL may be a less important source of DNA-reactive electrophiles than metabolic activation of NNK.

NNAL-*N*-oxide was the major metabolite formed from (±)-, (*R*)- and (*S*)-NNAL in mouse lung microsomes (Table 15; Jalas & Hecht, 2003). NNAL-*N*-oxide was formed from (*S*)-NNAL at much greater maximal rates than from the other two substrates, but the K_m value was also higher for (*S*)-NNAL; this resulted in relatively similar V_{max}/K_m values for NNAL-*N*-oxide formation among all three substrates (Table 15; Jalas & Hecht, 2003).

Kinetic parameters for NNAL metabolism have also been determined with hamster liver, lung and kidney tissue slices (Richter *et al.*, 2000). NNAL was converted to hydroxy acid, NNAL-*N*-oxide and NNK in all three tissue preparations, as well as to NNK-*N*-oxide in the liver and to diol in the lung (Richter *et al.*, 2000). K_m values for these metabolites ranged from 1.6 μM for NNK formation in the lung to 1624 μM for NNAL-*N*-oxide formation in the same tissue (Richter *et al.*, 2000).

Table 14. Summary of K_m values and regioselectivity for cytochrome P450-mediated NNK metabolism

Metabolic pathway	Low K_m (< 50 μM)	Intermediate K_m (50–500 μM)	High K_m (> 500 μM)	Regioselectivity
α-Methylene hydroxylation	Human 2A13 Rabbit 2A10/2A11 Rat 2A3 Mouse 2A5	Human 2A6 Rabbit 2G1 Rat 1A1 Rat 1A2 Rat 2B1 Mouse 2A4	Human 1A1 Human 1A2 Human 2D6 Human 2E1 Human 3A4 Rat 2C6	α-Methylene > α-methyl Human 1A1 Human 2A13 Human 3A4 Rabbit 2G1 Rat 1A1
α-Methyl hydroxylation	Human 2B6 Human 2A13 Rabbit 2A10/2A11 Rat 2A3 Mouse 2A5	Human 1A1 Human 1A2 Human 2A6 Rat 1A1 Rat 1A2 Rat 2B1 Mouse 2A4	Human 2D6 Human 2E1 Human 3A4 Rat 2C6	α-Methylene ≅ α-methyl Human 2A6 Rabbit 2A10/2A11 Rat 1A2 Rat 2A3
N-Oxidation		Rat 2B1	Rat 2C6	α-Methylene < α-methyl Human 1A2 Human 2B6 Human 2D6 Human 2E1 Rat 2B1 Rat 2C6 Mouse 2A5

Adapted from Jalas *et al.* (2005)
K_m, Michaelis constant

Table 15. Apparent steady-state kinetic parameters for A/J mouse lung microsome-mediated metabolism of NNAL

Substrate	Metabolite	Kinetic parameters			Experimental conditions	Reference
		V_{max} [a]	K_m (μM)	V_{max}/K_m [b]		
(±)-NNAL	Lactol	6.6 ± 0.3	5.7 ± 0.9	1.2 ± 0.2	0.25–50 μM NNAL; 45-min incubation; 0.3 mg/mL microsomal protein.	Jalas & Hecht (2003)
	Diol	6.0 ± 0.3	3.7 ± 0.7	1.6 ± 0.3		
	NNAL-*N*-oxide	20 ± 0.4	2.2 ± 0.2	9.1 ± 0.8		
(*R*)-NNAL	Lactol	6.5 ± 0.4	5.7 ± 1	1.1 ± 0.2		
	Diol	5.8 ± 0.3	5.0 ± 0.9	1.2 ± 0.2		
	NNAL-*N*-oxide	13 ± 0.4	2.0 ± 0.2	6.5 ± 0.7		
(*S*)-NNAL	Lactol	14 ± 0.8	9.9 ± 1.5	1.4 ± 0.2		
	Diol	15 ± 0.6	7.8 ± 1	1.9 ± 0.3		
	NNAL-*N*-oxide	103 ± 2	9.4 ± 0.5	11 ± 0.6		

K_m, Michaelis constant; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; V_{max}, maximum velocity
[a] Units are pmol/mg/min.
[b] Units are pmol/mg/min/μM.

The kinetic parameters for CYP-mediated NNAL metabolism have been reported only for three enzymes — mouse CYP2A5, rat CYP2A3 and human CYP2A13 (Table 16; Jalas & Hecht, 2003; Jalas *et al.*, 2003a). Similarly to results with mouse lung microsomes, the K_m values for CYP2A5-mediated NNAL metabolism were similar to those for CYP2A5-mediated NNK metabolism and were largely independent of substrate stereochemistry (Tables 11 and 16). In contrast, the V_{max} values were generally more than an order of magnitude lower for the NNAL substrates (Tables 12 and 16). Thus, NNK is clearly a much better substrate for CYP2A5 than NNAL (or the individual enantiomers) (Tables 11 and 16). Rat CYP2A3 metabolizes NNAL with an efficiency similar to mouse CYP2A5 and human CYP2A13, but the K_m values are slightly higher for the human enzyme (Table 16; Jalas *et al.*, 2003a).

In-vivo studies of NNK metabolism

The literature on in-vivo studies of NNK metabolism has been reviewed comprehensively (Hecht, 1998). Only selected studies are discussed below.

Monkey

Intravenous injection of NNK into patas monkeys resulted in rapid and extensive metabolism by α-hydroxylation and the formation of keto acid and hydroxy acid (Hecht *et al.*, 1993b). These metabolites accounted for a relatively large proportion of serum and urinary metabolites at all time-points. The other major metabolic pathway was reduction to NNAL, which was detected both unconjugated and as NNAL-*O*-Gluc, of which (*S*)-NNAL-*O*-Gluc predominated. NNAL-*O*-Gluc accounted for 15–20% of the urinary metabolites in monkeys given 0.1 μg/kg bw NNK, a dose similar to that of a smoker. Pharmacokinetic parameters for NNK metabolism were similar to those observed in baboons, which indicates rapid metabolism of NNK in primates (Adams *et al.*, 1985a). A comparison of pharmacokinetic parameters in rats, mice and hamsters showed that clearance of NNK varied predictably with body weight, similar to observations with NDMA, which implies that common phenomena govern the pharamacokinetics of these nitrosamines (Hecht *et al.*, 1993b).

Rat

Studies of the metabolism of NNK in rats consistently demonstrate rapid and extensive conversion of NNK to products of α-hydroxylation, pyridine-*N*-oxidation and carbonyl reduction. Less than 1% of the dose is excreted unchanged in the urine. Rapid metabolism of NNK is observed in all tissues examined; the highest amounts of α-hydroxylation are observed in the nasal mucosa, liver and lung, which are target tissues of NNK carcinogenesis (reviewed in Hecht, 1998).

The pharmacokinetics and metabolism of NNK (8.4 μmol/kg bw intravenously) were studied in bile duct-cannulated Fischer 344 rats (Wu *et al.*, 2002). After 24 h, approximately 85% of NNK was recovered (17.5% from bile and 67.6% from urine), which is consistent with previous studies (Schulze *et al.*, 1992; Hecht, 1998). Pharmacokinetic

Table 16. Steady-state kinetic parameters for human cytochrome P450 (CYP) 2A13-, rat CYP2A3- and mouse CYP2A5-mediated metabolism of NNAL

Species/ enzyme	Substrate	Metabolite	Kinetic parameters			Expression system	Reference
			V_{max} [a]	K_m (μM)	V_{max}/K_m [b]		
Human CYP2A13	(±)-NNAL	Lactol	1.50 ± 0.05	36 ± 3	0.042 ± 0.004	Baculovirus-infected Sf9 cells	Jalas *et al.* (2003a)
		Diol	0.79 ± 0.02	40 ± 3	0.020 ± 0.002		
		NNAL-*N*-oxide	0.12 ± 0.01	30 ± 7	0.0040 ± 0.0010		
Rat CYP2A3	(±)-NNAL	Lactol	0.41 ± 0.01	3.8 ± 0.5	0.11 ± 0.01		
		Diol	0.98 ± 0.02	16 ± 1	0.061 ± 0.004		
		NNAL-*N*-oxide	0.046 ± 0.003	2.6 ± 0.7	0.018 ± 0.005		
Mouse CYP2A5	(±)-NNAL	Lactol	0.55 ± 0.02	1.7 ± 0.3	0.32 ± 0.06	Baculovirus-infected Sf9 cells	Jalas & Hecht (2003)
		Diol	0.47 ± 0.01	5.1 ± 0.5	0.092 ± 0.009		
		NNAL-*N*-oxide	0.17 ± 0.01	4.7 ± 1.1	0.036 ± 0.009		
		NNK	0.78 ± 0.04	14 ± 1.8	0.056 ± 0.008		
	(*R*)-NNAL	Lactol	0.42 ± 0.02	2.1 ± 0.5	0.20 ± 0.05		
		Diol	1.2 ± 0.03	11 ± 0.8	0.11 ± 0.008		
		NNAL-*N*-oxide	ND				
		NNK	0.31 ± 0.03	16 ± 3.9	0.019 ± 0.005		
	(*S*)-NNAL	Lactol	0.45 ± 0.03	0.78 ± 0.26	0.58 ± 0.22		
		Diol	0.17 ± 0.01	1.3 ± 0.4	0.13 ± 0.04		
		NNAL-*N*-oxide	0.16 ± 0.01	1.1 ± 0.4	0.15 ± 0.05		
		NNK	0.78 ± 0.03	4.2 ± 0.6	0.19 ± 0.03		

K_m, Michaelis constant; ND, not detected; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; NNK, 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone; Sf9, *Spodoptera frugiperda*; V_{max}, maximum velocity

[a] Units are pmol/min/pmol CYP.

[b] Units are pmol/min/pmol CYP/μM.

analysis indicated that NNK had a short urinary half-life (2.6 h), a large volume of distribution (321 ± 137 mL) and a total body clearance of 12.8 ± 2.0 mL/min. (*R*)-NNAL-Gluc was the major metabolite in bile and represented approximately 14% of the total NNK dose. Nearly all NNAL-Gluc were excreted as the (*R*)-diastereomer. The major metabolite in urine was keto acid (26% of the dose). Urinary excretion of NNAL and hydroxy acid comprised about 30% of the dose. Metabolism of NNK to (*S*)-NNAL appeared to favour α-hydroxylation of (*S*)-NNAL and stereoselective localization in the lung while metabolism of NNK to (*R*)-NNAL appeared to lead to detoxification through glucuronidation and biliary excretion (Wu *et al.*, 2002).

In a study designed to probe the effects of nicotine on NNK metabolism, Holzman rats received acute (36 h) or chronic (2 week) subcutaneous infusions of nicotine at rates that produced serum nicotine concentrations that were two to three times the venous nicotine concentrations usually measured in smokers (Keyler *et al.*, 2003). A single intraperitoneal dose of NNK (39 nmol/kg bw) was administered 24 h before the end of each infusion. Neither acute nor chronic nicotine infusion had any effect on the extent of NNK metabolism by α-hydroxylation; some small effects on pyridine-*N*-oxidation were observed. The results indicate that nicotine infusion has no effect on the carcinogenic pathways of NNK metabolism in the rat.

Mouse

A/J mice treated intraperitoneally with NNK (0.005–500 μmol/kg bw) excreted hydroxy acid (18–37% of urinary metabolites), keto acid (11–27%), (*R*)-NNAL-*O*-Gluc (ND–22%), NNAL-*N*-oxide (6–14%), NNK-*N*-oxide (ND–10%), NNAL (ND–29%) and 6-hydroxy-NNK (1%) in the 48-h urine, similar to results obtained in rats (Morse *et al.*, 1990b; Desai *et al.*, 1993; Hecht, 1998). At lower doses of NNK, levels of α-hydroxylation products increased while the levels of NNAL and NNAL-*O*-Gluc decreased. NNAL glucuronidation is quantitatively an unimportant metabolic pathway at low doses of NNK (Morse *et al.*, 1990b).

Following a 2-h nose-only exposure to mainstream cigarette smoke, mice were administered NNK intraperitoneally (7.5 μmol). Control mice were sham-exposed and treated with the same dose of NNK (Brown *et al.*, 2001a). The pattern of urinary metabolites was affected by smoke exposure. Mice exposed to smoke excreted 25% more NNAL, 15% less hydroxy acid and 42% less keto acid than control mice. Other metabolites (NNAL-Gluc and NNAL-*N*-oxide) were not affected. These results indicate that mainstream cigarette smoke causes a shift in metabolism which leads to lesser α-hydroxylation and greater excretion of NNAL.

Hamster

As in rats and mice, NNK is extensively metabolized by α-hydroxylation and other pathways in Syrian golden hamsters (Hecht, 1998). Female Syrian golden hamsters treated subcutaneously with NNK (80 nmol/kg) excreted 96% of the dose as urinary metabolites within 24 h of treatment (Richter & Tricker, 2002). α-Hydroxylation to keto acid (30.7% of

radioactivity in urine) and hydroxy acid (22.3%), and detoxification to NNAL-*N*-oxide (24.2%) accounted for almost 80% of the NNK metabolites in the 24-h urine. Smaller amounts of diol, HPB, NNAL-*O*-Gluc, NNK-*N*-oxide and NNAL were observed. Concurrent treatment with nicotine, cotinine or 2-phenethyl isothiocyanate (PEITC) decreased total α-hydroxylation from 58.1% (control) to 49.6% (nicotine), 41.2% (cotinine) and 54.6% (PEITC), with concomitant increases in NNAL (Richter & Tricker, 2002).

In-vivo studies of NNAL metabolism

Earlier studies demonstrate that the NNK/NNAL equilibrium favours NNAL in rodents and primates, and report some pharmacokinetic parameters for NNAL metabolism. Conversion of (*R*)-NNAL-*O*-Gluc to metabolites of NNAL and NNK was also observed (reviewed in Hecht, 1998).

Rat

Male Fischer 344 rats were treated intravenously with racemic [5-^{3}H] NNAL (8.5 μmol/kg) (Wu *et al.*, 2002). After 24 h, 43% of the dose was recovered from urine and 20% from bile. Urinary elimination half-lives were 9.5 h for NNAL, 7.8 h for hydroxy acid and 8 h for keto acid. Total body clearance of NNAL was 8.65 ± 2.6 mL/min and volume of distribution was 2772 ± 1423 mL. (*R*)-NNAL-Gluc was the major metabolite in bile and accounted for 16% of the NNAL dose. The urinary excretion profile following NNAL administration was similar to that of NNK except that less keto acid was excreted. Enantiomeric ratios for NNAL were evaluated in plasma, urine and bile. (*R*)-NNAL was the predominant form excreted. In the lung, 1 h after administration of racemic NNAL, 49% of the total metabolites was NNAL, with lesser amounts of NNAL-*N*-oxide (19%), hydroxy acid (10%) and keto acid (7%). NNAL levels in the liver were comparable with those in the lung; significant amounts of (*R*)-NNAL-Gluc and hydroxy acid were also observed. NNK was also identified in the lung and liver samples. Four hours after administration, NNAL remained the most abundant metabolite in the lung, but lower amounts were found in the liver and kidney. Enantiomeric ratios demonstrated that (*S*)-NNAL predominated in the lung and liver 4 h after administration. At 24 h after administration, the (*S*):(*R*) ratio was 57 in the lung, 1.2 in the liver and 3.4 in the kidney. NNAL made up 75% of total metabolites in the lung 24 h after administration. Collectively, the results demonstrated stereoselective excretion of (*R*)-NNAL-*O*-Gluc and retention of (*S*)-NNAL in the rat lung (Wu *et al.*, 2002).

Male Fischer 344 rats were treated intravenously with [5-^{3}H] (*R*)-NNAL (0.11–0.65 mg/kg) or (*S*)-NNAL (0.198–1.07 mg/kg) (Zimmerman *et al.*, 2004). After 24 h, 65% of the dose of (*S*)-NNAL was recovered (15% from the bile and 48% from urine), while almost 90% of the (*R*)-NNAL dose was recovered (44% from the bile and 45% from urine). Thus, excretion of (*S*)-NNAL was substantially less than that of (*R*)-NNAL, principally due to the difference in biliary excretion of (*R*)-NNAL-Gluc. The volume of distribution of (*S*)-NNAL (1792 ± 570 mL) was significantly greater than that of (*R*)-NNAL (645 ± 230 mL). Urinary elimination half-lives of metabolites were significantly shorter

after administration of (*R*)-NNAL (4.2 h) than after that of (*S*)-NNAL (6.3 h). Urinary metabolite profiles of the two enantiomers were markedly different. For (*R*)-NNAL, major metabolites consisted largely of unchanged NNAL, hydroxy acid and (*R*)-NNAL-Gluc. For (*S*)-NNAL, more keto acid and less hydroxy acid, more NNK-*N*-oxide and less NNAL were formed than in the case of (*R*)-NNAL; NNK was also observed. Tissue retention of metabolites demonstrated that a larger proportion of the dose was retained in the liver, lung and kidney 24 h after administration of (*S*)-NNAL compared with (*R*)-NNAL. There was significant and rapid conversion of (*S*)-NNAL to NNK in the tissues. At 24 h after administration of (*S*)-NNAL, 78% of lung metabolites was NNAL, with an (*S*):(*R*) ratio of 4.2, a shift from a ratio of 1.1 at 1 h after dosing, which indicated selective retention of (*S*)-NNAL in the lung. At 24 h after administration of (*R*)-NNAL, no quantifiable metabolites were found in the lung. These results demonstrate that (*R*)-NNAL, the less carcinogenic NNAL enantiomer (in mice), is excreted more rapidly than (*S*)-NNAL, while (*S*)-NNAL is selectively retained in the lung and is extensively converted to NNK.

Mouse

Racemic and enantiomeric NNAL as well as (*R*)-NNAL-Gluc (20 μmol) were administered intraperitoneally to female A/J mice and urine was collected (Upadhyaya *et al.*, 1999). The metabolites of racemic NNAL were hydroxy acid (10% of the dose), NNAL-*N*-oxide (3.1%), NNAL (20%) and (*S*)-NNAL-Gluc (16%). The profile of metabolites from the enantiomers was quite similar to that of racemic NNAL, except that (*S*)-NNAL was metabolized less effectively to (*R*)-NNAL-Gluc than (*R*)-NNAL. The formation of (*S*)-NNAL-Gluc from (*R*)-NNAL indicates that (*R*)-NNAL was converted to NNK *in vivo*. Minor amounts of urinary metabolites other than (*S*)-NNAL-Gluc were detected when (*S*)-NNAL-Gluc was administered to mice.

Haemoglobin adducts of NNK and NNAL

Adducts with globin are formed in rats by both the α-methylene and α-methyl hydroxylation pathways of NNK (Carmella & Hecht, 1987; Hecht, 1998). Depending on dose, species and protocol employed, approximately 15–40% of the pyridyloxobutyl adducts are released as HPB upon mild base hydrolysis (Carmella & Hecht, 1987; Murphy *et al.*, 1990a; Peterson *et al.*, 1990; Murphy & Coletta, 1993). The HPB-releasing adducts are esters, most probably with aspartate, glutamate or the terminal carboxy groups of globin (Carmella *et al.*, 1992). Pyridyloxobutyl cysteine adducts do not appear to be formed in measurable amounts in rats (Carmella *et al.*, 1990b). Co-incubation of rat hepatocytes and human red blood cells results in the formation of HPB-releasing adducts, as seen *in vivo* (Murphy & Coletta, 1993). Therefore, α-HOMeNNK or its glucuronide are sufficiently stable to migrate out of the hepatocyte and into the red blood cell. Human red blood cells alone can activate NNK but not to HPB-releasing adducts (Murphy & Coletta, 1993). In rats, both methyl and pyridyloxobutyl haemoglobin adducts increase linearly with the dose over a more than 3000-fold range (Murphy *et al.*, 1990a). DNA adducts in the liver and lung also increase over this range but the relationship with dose is non-linear.

The utility of HPB-releasing haemoglobin adducts as a biomarker of the overall carcinogenic effect of NNK was demonstrated in studies in which treatment of rats with PEITC or 6-phenylhexyl isothiocyanate resulted in a significant decrease in HPB-releasing adducts over an 18-month period compared with rats treated with NNK only; concurrently, a significant decrease in lung tumour induction was observed in the rats treated with isothiocyanates and NNK (Hecht *et al.*, 1996a,b). In rats treated with a mixture of benzo[*a*]pyrene (2 ppm in diet; 2 mg/kg) and NNK (2 ppm in drinking-water; 2 μg/mL), HPB-releasing haemoglobin adducts were significantly inhibited by concurrent treatment with dietary PEITC, or a mixture of PEITC and benzyl isothiocyanate, but not with benzyl isothiocyanate alone. Concurrently with the inhibition of HPB-releasing haemoglobin adducts, a decrease in HPB-releasing DNA adducts was observed in the rat lung, but not in the liver, as was an increase in the levels of NNAL and NNAL-Gluc excreted in urine (Boysen *et al.*, 2003). HPB-releasing haemoglobin adducts are also formed upon treatment of rats with NNAL (Hecht & Trushin, 1988).

DNA adducts of NNK and NNAL

In experimental systems, DNA adducts have been measured by high-performance liquid chromatography–radioflow detection after administration of [^{3}H]-labelled compounds, or by gas chromatography–mass spectrometry of released HPB. The latter technique has also been used in human studies (see Section 4.1.1(*a*)(iii)).

Studies of DNA adduct formation by NNK and NNAL have been extensively reviewed (Hecht, 1998). Highlights and recent studies are presented below.

In-vitro studies

All data indicate that there are two major types of NNK–DNA adducts: methyl adducts formed by α-methylene hydroxylation and pyridyloxobutyl adducts formed by α-methyl hydroxylation. Adduct formation is summarized in Figures 2 and 5. α-Methylene hydroxylation of NNK leads to the formation of Me-DZH and/or the methyldiazonium ion, which react with DNA to form 7-MedGuo, O^6-MedGuo and O^4-MedThd. Other adducts are probably also produced, based on studies of other methylating nitrosamines and nitrosoureas (Singer & Grunberger, 1983). DNA methylation by NNK is observed in a number of in-vitro studies with different systems that are capable of activating its metabolism, including rat lung cells and lung, liver or nasal mucosal microsomes (with added DNA), rat oral tissue and hamster lung (Devereux *et al.*, 1988; Rossignol *et al.*, 1989; Murphy *et al.*, 1990a; Guo *et al.*, 1991b; Hecht, 1998).

The chemistry of the intermediates that result from α-methyl hydroxylation of NNK has been studied in detail. Since α-HOMeNNK (Figure 2) is not very stable, this metabolite has been generated *in situ* by solvolysis of 4-(acetoxymethylnitrosamino)-1-(3-pyridyl)-1-butanone (NNKOAc) and 4-(carbethoxynitrosamino)-1-(3-pyridyl)-1-butanone. Extensive studies conclusively demonstrate that the major DNA adducts formed by this pathway *in vitro* and *in vivo*, which account for at least 50% of the DNA binding, release HPB (Figure 3) upon acid or neutral thermal hydrolysis (Hecht & Trushin, 1988;

Hecht *et al.*, 1988b; Spratt *et al.*, 1989; Murphy *et al.*, 1990a; Peterson *et al.*, 1990; Peterson & Hecht, 1991; Foiles *et al.*, 1992; Trushin *et al.*, 1994; Staretz *et al.*, 1997c; Hecht, 1998). These adducts are produced via POB-DZH but not by HPB itself (Hecht *et al.*, 1988). The HPB-releasing adducts have different stabilities in DNA, and are released in a triphasic manner (Peterson *et al.*, 1991b). Adducts that release HPB upon neutral thermal hydrolysis have recently been identified as 7(POB-1-yl)dGuo and O^2(POB-1-yl)dCyd (Wang *et al.*, 2003; Hecht *et al.*, 2004c). Other adducts formed by this pathway that have been identified are shown in Figures 2 and 5 (Haglund *et al.*, 2002; Wang *et al.*, 2003; Hecht *et al.*, 2004c). A similar group of adducts is formed upon methyl hydroxylation of NNAL (Figures 2 and 5; Upadhyaya *et al.*, 2003; Hecht *et al.*, 2004c).

Pyridyloxobutyl adducts inhibit O^6-alkylguanine–DNA alkyltransferase (AGT), the enzyme responsible for the repair of O^6-MedGuo. Since O^6-MedGuo is also formed by metabolic activation of NNK, this phenomenon is probably important in the persistence of O^6-MedGuo in NNK-exposed tissues (Peterson *et al.*, 1993).

In-vivo studies

Since O^6-methylguanine (O^6-MeGua) and 7-MeGua were first detected in the liver and lung of NNK-treated Fischer 344 rats (Castonguay *et al.*, 1984b), substantial research has been carried out on the occurrence and biological significance of methyl and pyridyloxobutyl adducts that result from the metabolic activation of NNK (Hecht, 1998).

Rat

Levels of 7-MeGua are 7.5–25 times higher than those of HPB released in the whole lung, depending on dose, with lower ratios at lower doses (Murphy *et al.*, 1990b). Levels of HPB-releasing adducts are about twice those of O^6-MeGua, which are about 10 times greater than those of O^4-MedThd (Belinsky *et al.*, 1986; Staretz *et al.*, 1997c). Consistently, the highest levels of O^6-MeGua and HPB-releasing adducts are found in Clara cells of the rat lung, with lower amounts in type II cells, macrophages and small cells (Belinsky *et al.*, 1987a; Belinsky *et al.*, 1988; Devereux *et al.*, 1988; Staretz *et al.*, 1997c). The dose–response relationship for adduct formation in whole lung and lung cell types is non-linear. Adduct levels at the lowest doses are higher than would be expected by linear extrapolation from higher doses, e.g. efficiency of alkylation increases considerably at low doses (Belinsky *et al.*, 1987a; Devereux *et al.*, 1988; Belinsky *et al.*, 1990; Murphy *et al.*, 1990b; Staretz *et al.*, 1997c). One interpretation of these data is the presence of CYPs in the rat lung, such as CYP2A3, which efficiently catalyse α-hydroxylation at low concentrations of NNK. A second interpretation relates specifically to O^6-MeGua. Pyridyloxobutyl adduct concentrations increase at low doses of NNK, which may lead to greater inhibition of AGT and consequent higher levels of O^6-MeGua (Peterson *et al.*, 1993).

During chronic treatment with high doses of NNK, O^6-MeGua increases consistently in the rat lung (Belinsky *et al.*, 1986). At lower doses over a 4-day period, O^6-MeGua persists to a greater extent in Clara cells than in other cell types (Belinsky *et al.*, 1988). This is partly because of lower levels of AGT in Clara cells than in other cell types after treatment with

NNK. Treatment with NNK inhibits AGT, which is probably due to DNA pyridyloxobutylation. However, when NNK was administered over the full 20-week course to induce lung tumours in rats, O^6-MeGua levels decreased by 82% in the Clara cells during the treatment period and were lower than those in the macrophages at 20 weeks (Staretz *et al.*, 1997c). The decrease is due to inhibition of CYP-catalysed α-methylene hydroxylation by NNK, as demonstrated in a study of α-methylene hydroxylation by isolated rat pulmonary microsomes after 20 weeks of treatment with NNK (Staretz *et al.*, 1997b).

Structure–activity studies suggest that both DNA methylation and pyridyloxobutylation are important in NNK-induced lung tumorigenesis in rats. Neither NDMA, which only methylates DNA, nor NNN, which pyridyloxobutylates but does not methylate DNA, is an effective lung carcinogen in rats (Hecht *et al.*, 1980a; Hoffmann *et al.*, 1984; Hecht *et al.*, 1986b). NNK yields greater amounts of O^6-MeGua than NDMA in Clara cells, but not in alveolar type II cells, and is metabolized somewhat more effectively to a pyridyloxobutylating agent than NNN in rat lung cells (Devereux *et al.*, 1988; Belinsky *et al.*, 1989a). Such differences in binding and metabolism may partially account for the distinctly greater pulmonary carcinogenicity of NNK than that of NDMA or NNN. However, only NNK provides the combination of DNA methylation and pyridyloxobutylation that appears to be critical for rat lung tumorigenesis. NNK-induced rat lung tumours arise in type II cells (Belinsky *et al.*, 1990). Levels of HPB-releasing adducts in type II cells of NNK-treated rats correlate with lung tumour incidence over a range of doses, which suggests that pyridyloxobutylation is an important pathway (Staretz *et al.*, 1997c). Levels of O^6-MeGua in Clara cells also correlate with lung tumour incidence over a wide dose range, which suggests some role of this adduct in spite of the fact that the Clara cell is probably not the cell of origin of the tumours (Belinsky *et al.*, 1990). The effects of PEITC on levels of HPB-releasing adducts in type II cells and other cell types of the lung correlate well with inhibition of NNK-induced lung tumorigenesis by PEITC, which provides further evidence for the importance of these adducts (Staretz *et al.*, 1997c). PEITC also inhibits O^6-MeGua levels in Clara cells to the same extent that it inhibits lung tumorigenesis, but this is not seen in other cell types (Staretz *et al.*, 1997c). PEITC selectively inhibits HPB-releasing DNA adducts in the lung, but not in the liver of NNK-treated rats (Boysen *et al.*, 2003). Collectively, the available data indicate that HPB-releasing adducts and O^6-MeGua are both important in lung tumour induction by NNK in rats.

Levels of DNA methylation in rat nasal mucosa are frequently higher than those in other tissues of NNK-treated animals (Belinsky *et al.*, 1986; Hecht *et al.*, 1986b; Belinsky *et al.*, 1987b). This is a consequence of the high NNK α-hydroxylation activity mediated by CYPs of the nasal mucosa. Although both α-methylene and α-methyl hydroxylation of NNK occur at similar rates in nasal mucosa microsomes (Smith *et al.*, 1992b), DNA methylation is greater than pyridyloxobutylation (Trushin *et al.*, 1994). These data indicate that, in rat nasal mucosa, α-HOMethyleneNNK is more effective as a DNA methylating agent than α-HOMeNNK is as a pyridyloxobutylating agent, possibly due to differences in the reactivity of the resulting alkylating agents, or to other factors such as glucuronidation of α-HOMeNNK (Hecht, 1998).

In spite of the relatively low levels of HPB-releasing adducts formed in rat nasal mucosa after treatment with NNK, these adducts appear to be important in tumour induction. NNK and NNN have similar carcinogenic activities toward the nasal mucosa, but NDMA has little activity (Hecht *et al.*, 1986b; Trushin *et al.*, 1994). NNK and NNN both pyridyloxobutylate nasal mucosa DNA to form HPB-releasing adducts (Trushin *et al.*, 1994). NNN does not methylate DNA whereas NDMA methylates but does not pyridyloxobutylate nasal DNA. These results support the role of DNA pyridyloxobutylation in rat nasal tumorigenesis. Studies with deuterated analogues of NNK further support this conclusion. Thus, [methylene-D_2]NNK is a stronger nasal carcinogen than either [methyl-D_3]NNK or NNK (Hecht *et al.*, 1987; Trushin *et al.*, 1994). Moreover, DNA pyridyloxobutylation by [methylene-D_2]NNK exceeds that by NNK while levels of *O*^{6}-MedGuo from [methylene-D_2]NNK are significantly lower than those from NNK or [methyl-D_3]NNK (Trushin *et al.*, 1994). Collectively, these data provide strong support for the proposal that DNA pyridyloxobutylation is critical in rat nasal carcinogenesis by NNK (Hecht, 1998).

Levels of 7-MeGua are 13–49 times greater than those of HPB-releasing adducts in rat liver, and levels of the latter are generally greater than those of *O*^{6}-MeGua (Murphy *et al.*, 1990b). The 7-MeGua:HPB-releasing adduct ratio at lower doses is lower in the liver than in the lung (Murphy *et al.*, 1990b). At low doses, levels of HPB-releasing adducts are lower in the liver than in the lung (Murphy *et al.*, 1990b; Boysen *et al.*, 2003). At high doses, the formation of 7-MeGua is saturated in the lung, but not in the liver, whereas the formation of HPB-releasing adducts is saturated in both tissues (Murphy *et al.*, 1990b). The higher levels of HPB-releasing adducts than of *O*^{6}-MeGua are probably due to differences in repair (Belinsky *et al.*, 1986, 1990; Peterson *et al.*, 1991b). During chronic NNK treatment, *O*^{6}-MeGua reaches a maximum in both hepatocytes and non-parenchymal cells, then declines rapidly due to induction of AGT (Belinsky *et al.*, 1986). The removal of HPB-releasing adducts from hepatic DNA appears to be slower than that of *O*^{6}-MeGua (Belinsky *et al.*, 1986; Peterson *et al.*, 1991b).

Treatment of rats with a single dose of NNK or NNAL resulted in the formation of 7-MeGua and *O*^{6}-MeGua (Hecht & Trushin, 1988). In hepatic DNA, levels of these adducts were similar 1–48 h after treatment with NNK or NNAL, while adduct levels in nasal mucosa and lung were somewhat higher after treatment with NNK than with NNAL. HPB-releasing adducts were formed with both NNK and NNAL in the liver; levels of the NNK-derived adducts were somewhat higher (Hecht & Trushin, 1998).

Mouse

Lung tumours are induced rapidly by a single dose of 10 μmol NNK in A/J mice (Hecht *et al.*, 1989). This model has been used extensively to examine mechanistic phenomena as well as the modifying effects of chemopreventive agents (Hecht, 1998). Levels of 7-MeGua are greater than those of *O*^{6}-MeGua, which in turn exceed those of HPB-releasing adducts (Peterson & Hecht, 1991). Levels of 7-MeGua and *O*^{6}-MeGua reached a maximum 4 h after injection of 10 μmol NNK, whereas the levels of released HPB were maximal after 24 h (Peterson & Hecht, 1991). Levels of 7-MeGua and released HPB

decrease with time, but O^6-MeGua is persistent, such that its levels exceed those of 7-MeGua 15 days after treatment (Peterson & Hecht, 1991). Levels of O^6-MedGuo were highest in type II cells and Clara cells, followed by small cells and whole lung (Devereux *et al.*, 1993).

Persistent O^6-MeGua is the critical determinant of lung tumour induction in A/J mice (Peterson & Hecht, 1991), but does not account for differences in sensitivity to NNK-induced lung tumorigenesis between A/J and C57BL/6 mice (Devereux *et al.*, 1993). In A/J mice, acetoxymethylmethylnitrosamine (AMMN), which can only methylate DNA, is highly tumorigenic whereas NNKOAc and NNN, which only pyridyloxobutylate DNA, are weakly active (Peterson & Hecht, 1991). [Methylene-D_2]NNK is significantly less tumorigenic than NNK or [methyl-D_3]NNK and also forms significantly less O^6-MeGua (Hecht *et al.*, 1990). Similarly, (4*R*)[4D]NNK is significantly less tumorigenic than either NNK or (4*S*)[4D]NNK, and also leads to lower levels of persistent O^6-MeGua (Jalas *et al.*, 2003b). There is an inflection in the dose–response curve for lung tumour induction by NNK in A/J mice, with an increase above a dose of 2–3 μmol NNK, at which persistent O^6-MeGua begins to be measurable (Peterson & Hecht, 1991). Evidently, AGT activity is saturated above this dose. Levels of O^6-MedGuo measured 96 h after treatment of A/J mice correlate strongly with tumour multiplicity, independent of the source of the methylating agent, e.g. NNK, AMMN or AMMN plus NNKOAc (Peterson & Hecht, 1991). In addition, GC→AT transitions in codon 12 of the K-*ras* gene are observed in a high percentage of lung tumours induced by NNK in A/J mice (see Section 4.4.2(*a*)(ii)), consistent with the importance of O^6-MeGua (Belinsky *et al.*, 1989b). Human methylguanine–DNA methyltransferase transgenic mice, which express high levels of AGT in the lung, were crossbred with A/J mice. Human AGT was expressed throughout the lung and, after treatment with NNK, these mice had lower levels of O^6-MeGua, lower tumour multiplicity and size of tumours in the lung and a lower frequency of K-*ras* mutations in the lung tumours than non-transgenic mice (Liu *et al.*, 1999).

The pyridyloxobutylation pathway is important in increasing the activity of the methylation pathway in A/J mouse lung tumorigenesis since NNKOAc markedly increases the tumorigenicity of AMMN over a wide dose range (Peterson & Hecht, 1991). NNKOAc enhances the persistence of O^6-MeGua in the lung of AMMN-treated mice due to the ability of HPB-releasing adducts to inhibit AGT (Peterson *et al.*, 1993; Liu *et al.*, 1996; Peterson *et al.*, 2001). The ability of NNKOAc to enhance the persistence of O^6-MeGua in the lung was similar to that of O^6-benzylguanine, a known inhibitor of AGT (Peterson *et al.*, 2001). The pyridyloxobutyl adduct, O^6(POB-1-yl)Gua, was detected in the liver, but not in the lung, of A/J mice treated with NNK (Thomson *et al.*, 2003). This adduct was also detected in the lung and liver of mice treated with NNKOAc, in the presence but not absence of O^6-benzylguanine, which indicates that O^6(POB-1-yl)Gua is repaired in part by AGT (Thomson *et al.*, 2003). Further studies demonstrated that O^6(POB-1-yl)Gua is repaired by mammalian AGT and that the rate of repair is highly dependent on protein structure (Mijal *et al.*, 2004). Inefficient repair of O^6(POB-1-yl)Gua by bacterial AGT explains the high mutagenic activity of this adduct in bacterial systems (Pauly *et al.*, 2002).

Adduct measurements have been made in A/J mouse liver after treatment with NNK, although tumour induction in this tissue is infrequent (Belinsky *et al.*, 1989b). As in the lung, the relative levels of adduct formation are 7-MeGua > O^6-MeGua > HPB-releasing adducts (Morse *et al.*, 1990a; Peterson *et al.*, 1990; Morse *et al.*, 1991). Levels of HPB-releasing adducts are higher in liver than in the lung, as are levels of 7-MeGua and O^6-MeGua (Morse *et al.*, 1990a; Peterson *et al.*, 1990; Morse *et al.*, 1991; Peterson & Hecht, 1991). The relatively high level of adducts in the liver is consistent with metabolic studies that show efficient α-hydroxylation with little or no pyridine-*N*-oxidation in this tissue, in contrast to the lung where pyridine-*N*-oxidation is a major competing detoxification pathway. Despite the high DNA adduct levels in A/J mouse liver, tumours are observed in the lung and this is clearly related to susceptibility factors inherent in this mouse strain (Hecht, 1998).

Hamster

Initial levels of 7-MeGua and O^6-MeGua are similar in rat and hamster liver after a single dose of NNK (Liu *et al.*, 1992). However, O^6-MeGua is repaired more rapidly in rats (half-time, 12 h), while only 14% of the initial O^6-MeGua is repaired 72 h after treatment in hamsters. 7-MeGua also persists longer in hamster than in rat liver. NNK rapidly depletes AGT in both rat and hamster liver, but AGT recovers in rats and not in hamsters. These results do not correlate with tumour induction by NNK. Whereas NNK is a weak hepatocarcinogen in rats, it does not induce liver tumours in hamsters. The results suggest that O^6-MeGua is not important in the hepatocarcinogenesis of NNK.

Other types of DNA damage

Single-strand breaks are observed in hepatocytes incubated with NNK and in the livers of NNK-treated animals (Hecht, 1998), and are probably produced by spontaneous or enzymatic depurination of adducts such as 7-MeGua or 7(POB-1-yl)Gua. Among the metabolites of NNK, keto aldehyde has received the most attention as a source of single-strand breaks. However, the single-strand breaks induced by keto aldehyde appear to have different properties (for example, pH dependence) from those caused by NNK, which indicates that NNK single-strand breaks do not result from keto aldehyde (Demkowicz-Dobrzanski & Castonguay, 1991). NNK single-strand breaks are repaired slowly and damage persists for 2–3 weeks after a single treatment with NNK in rats and hamsters (Jorquera *et al.*, 1994). One study demonstrated an increase in NNK-induced single-strand breaks in human lung cells after generation of superoxide by hypoxanthine/xanthine oxidase (Weitberg & Corvese, 1993).

Treatment with NNK causes increases in levels of the promutagenic adduct 8-oxodeoxyguanosine in mouse and rat lung, and in fetal liver following transplacental exposure of mice to NNK (Chung & Xu, 1992; Sipowicz *et al.*, 1997).

(iv) *Excretion*

Urine was the major route of excretion of NNK and NNAL metabolites with a pyridine ring in all studies with rodents and primates (Hecht, 1998).

In rats, 47% of a dose of [^{14}C-methyl]NNK was excreted in the expired air (Castonguay *et al.*, 1983b). From 7 to 17% of an NNK dose was excreted in the bile of rats, mainly as (*R*)-NNAL-*O*-Gluc (Schulze *et al.*, 1992; Wu *et al.*, 2002). After intravenous administration of individual NNAL enantiomers, metabolites of (*S*)-NNAL (15% of the total dose) and (*R*)-NNAL (44% of the total dose) were excreted in the bile of rats (Zimmerman *et al.*, 2004).

Male rhesus monkeys received a single intravenous dose of radioactive NNK (4.6–9.8 μg/kg) and urine was collected for 10 days (Meger *et al.*, 1999). Within the first 24 h, 86% of the dose was excreted. NNK-derived radioactivity was still detectable in urine 10 days after treatment. Patterns of metabolites in the urine during the first 6 h closely resembled those seen in patas monkeys (Hecht *et al.*, 1993b); end-products of NNK metabolic activation represented more than 50% of total radioactivity. At later time-points, the pattern shifted toward NNAL and NNAL-*O*-Gluc. There was no preferential biliary excretion of NNAL-Gluc compared with rats.

(*b*) *NNN*

(i) *Absorption*

Studies of the absorption, distribution, metabolism and excretion of NNN in experimental systems have been comprehensively reviewed (Hecht, 1998), and the reader is referred to that review for detailed coverage of the literature. Selected studies from this review which illustrate important points as well as more recent studies that are pertinent to the evaluation are presented below.

The penetration of NNN across porcine skin and various regions of the oral mucosa was determined. Specimens of porcine skin, keratinized gingival and non-keratinized mucosa from the floor of the mouth and cheek were studied. Skin showed a lower permeability than the oral regions, and the floor of the mouth was generally the most permeable site. The non-keratinized oral regions were most permeable to NNN (Squier, 1986). Concentrations of 25% ethanol and above significantly increased the permeability of oral mucosa to NNN, but this increase ceased with 50% ethanol. The permeability of oral mucosa to NNN was also increased by nicotine (0.2–2%). Combined use of nicotine and ethanol significantly increased the penetration of NNN across oral mucosa compared with ethanol alone (Du *et al.*, 2000). Permeability of rat skin and buccal mucosa to NNN was significantly increased in rats maintained on a diet that contained 6.7% ethanol compared with rats kept on an isocaloric diet without ethanol (Squier *et al.*, 2003).

(ii) *Distribution*

Whole-body and micro-autoradiographic studies of the distribution of NNN in rats, mice, marmoset monkeys and mini-pigs have been reviewed (Tjälve, 1991). In general, NNN is rapidly distributed throughout the body, which reflects an ability of this

compound to pass freely across biological membranes and distribute evenly in the intra- and extracellular tissue water. However, accumulation of radioactivity has been observed in certain tissues. In rats, 5 min after intravenous injection of [2′-^{14}C]NNN, the radioactivity was distributed homogeneously throughout most of the body and levels in tissues did not exceed the level in blood. However, high uptake was observed in a few tissues including the mucosa of the ethmoturbinates and the mucosa that covers the naso- and maxillo-turbinates. High radioactivity was also present in the submaxillary salivary glands, lacrimal glands, Zymbal glands, tarsal glands of the eyelids, preputial glands and stomach contents. The radioactivity in the nasal and tracheo-bronchial mucosa and the mucosa of the oesophagus and tongue was non-extractable, and a low level of non-extractable radioactivity was found in the liver, whereas that in other tissues was extractable (Brittebo & Tjälve, 1981). Whole-body autoradiography of mice treated intravenously with [2′-^{14}C]NNN showed a similar spectrum of tissue localization of bound metabolites as that in rats; the only difference was in the salivary glands which accumulated radioactivity in mice but not in rats (Brittebo & Tjälve, 1980; Waddell & Marlowe, 1980; Tjälve, 1991). Whole-body autoradiograms taken 15–220 min after intracardiac administration of [5-^{3}H]NNN to miniature pigs showed high levels of radioactivity in the mandibular and parotid salivary glands, Harder's gland, lacrimal glands, glands of the snout and respiratory part of the nasal cavity and melanin of the eyes and skin. Bound radioactivity was most abundant in the nasal mucosa and liver (Domellöf *et al.*, 1987). Autoradiograms obtained 4 h after intravenous injection of [2′-^{14}C]NNN into a marmoset monkey showed the highest levels of radioactivity in the liver, nasal mucosa, melanin of the eyes, hair follicles of the skin and ceruminous ear glands. Bound radioactivity was observed in the liver and nasal mucosa (Castonguay *et al.*, 1985a).

(iii) *Metabolism*

In-vitro studies

The in-vitro metabolism of NNN has been studied in the rat liver, oesophagus, nasal mucosa, oral tissue and lung, in hamster liver and oesophagus and in mouse lung; these studies have been reviewed (Hecht, 1998), and only selected studies and more recent investigations are discussed below.

Rat liver metabolizes NNN by hydroxylation at each position of the pyrrolidine ring, which results in the formation of HPB, lactol and other secondary metabolites, 3′-HONNN and 4′-HONNN. Myosmine and NNN-*N*-oxide have also been observed (Chen *et al.*, 1978; Hecht *et al.*, 1980b; Hecht, 1998).

In cultured rat oesophagus, which is a target tissue for NNN carcinogenesis, metabolites that result from the 2′-hydroxylation pathway of metabolic activation exceed those that result from the 5′-hydroxylation pathway of metabolic activation (Hecht *et al.*, 1982). DNA isolated from rat oesophagus cultured with [5-^{3}H]NNN contained HPB-releasing adducts (Murphy *et al.*, 1990a). Oesophageal microsomes metabolized NNN to HPB via 2′-hydroxylation and to lactol via 5′-hydroxylation. These reactions were probably mediated by a CYP enzyme, which had an apparent K_m of 49 μM (Murphy & Spina, 1994).

Products of 2′-hydroxylation exceeded those of 5′-hydroxlation by threefold, as in cultured rat oesophagus; however, in liver microsomes, the ratio of 2′:5′-hydroxylation varied between 0.23 and 0.71 depending on the concentration of NNN (Murphy & Spina, 1994). Cultured rat oesophagus metabolized (*S*)-NNN predominantly to products of 2′-hydroxylation while these products were significantly less prevalent in incubations with (*R*)-NNN. The 2′:5′-hydroxylation ratio ranged from 6.22 to 8.06 at various time intervals in the incubations with (*S*)-NNN, while the corresponding ratios were 1.22–1.33 in experiments with (*R*)-NNN (McIntee & Hecht, 2000). The CYP enzyme that is responsible for the metabolic activation of NNN in rat oesophagus has not been identified. One candidate was thought to be CYP2A3, which has been identified in small amounts in the rat oesophagus (Gopalakrishnan *et al.*, 2002). CYP2A3 is an efficient catalyst of NNN α-hydroxylation (K_m, 13 μM). However, metabolism of (*R*)- and (*S*)-NNN gives results that contrast to those observed in cultured rat oesophagus, which indicates that CYP2A3 is not involved in the catalysis of NNN α-hydroxylation in this tissue (Murphy *et al.*, 2000).

Cultured rat nasal mucosa, which is another target tissue of NNN carcinogenesis, metabolized NNN extensively by α-hydroxylation; pyridine *N*-oxidation was not observed (Brittebo *et al.*, 1983). HPB-releasing DNA adducts of NNN were detected in rat nasal mucosa cultured with NNN (Spratt *et al.*, 1989). Rat nasal mucosal microsomes catalysed both 2′- and 5′-hydroxylation of NNN, with low K_m values of 2–3 μM. NNN inhibited coumarin 7-hydroxylation, which suggests the involvement of a CYP2A enzyme (Patten *et al.*, 1998).

Cultured rat oral tissue metabolized NNN in a fashion similar to that observed with cultured rat oesophagus, but HPB-releasing DNA adducts were not observed (Murphy *et al.*, 1990a). NNN metabolism was inhibited by nicotine and, to a lesser extent, by NNK (Murphy & Heiblum, 1990).

There is some consistency between the ratios of 2′:5′-hydroxylation in different rodent tissues and their susceptibility to carcinogenesis by NNN (Hecht, 1998). The 2′:5′-hydroxylation ratio is typically 2–4 in rat oesophagus and nasal mucosa, which are the main target tissues of NNN in the rat. In the liver, which is a non-target tissue, 2′:5′-hydroxylation ratio is 0.3–1.4. Hamster oesophagus, which is a non-target tissue, predominantly 5′-hydroxylates NNN (Hecht *et al.*, 1982). These results are consistent with a role for 2′-hydroxylation in tumour induction by NNN and for 5′-hydroxylation in detoxification. In contrast, 5′-hydroxylation and 2′-hydroxylation occur to equal extents in hamster trachea (McCoy *et al.*, 1982) and 5′-hydroxylation exceeds 2′-hydroxylation in A/J mouse lung (Castonguay *et al.*, 1983a), both of which are target tissues of NNN carcinogenesis (Hecht, 1998).

Rat CYP2A3 and mouse CYP2A5 catalyse 5′-hydroxylation of both enantiomers of NNN with low K_m values (0.74–3.35 μM). Mouse CYP2A4 is a poorer catalyst with K_m values of 54.1–68.5 μM. Rat CYP2A3 and mouse CYP2A5 catalyse 2′-hydroxylation of (*R*)-NNN with K_m values of 0.73–1.64 μM, while the K_m for mouse CYP2A4 is 66 μM. 2′-Hydroxylation of (*S*)-NNN was not observed in studies with these enzymes (Wong *et al.*, 2005b).

2′- and 5′-Hydroxylation of NNN can lead to DNA damage. 2′-Hydroxylation generates the same intermediate — POB-DZH (Figure 6) — as methyl hydroxylation of NNK (Hecht, 1998; Wang *et al.*, 2003; Hecht *et al.*, 2004c). Formation of HPB-releasing DNA adducts is therefore expected following 2′-hydroxylation of NNN and this been observed in cultured rat oesophagus and nasal mucosa. Adducts could also be formed from 5′-HONNN via *iso*-POB-DZH, but this has not been reported (Hecht, 1998).

In-vivo studies

The effect of NNN on hepatic and pulmonary carcinogen metabolizing enzymes in male Sprague-Dawley rats was evaluated in a series of studies that used the following basic experimental protocol. Inbred male weanling Sprague-Dawley rats (19–21 days of age and weighing 35–50 g) were randomly divided into three groups of eight animals each and were placed on three different dietary regimens that consisted of a standard diet, a control semisynthetic diet and a semisynthetic deficient diet. In each set of experiments, the semisynthetic diets were either adequate (control) or deficient in vitamin A (Nair *et al.*, 1991), vitamin B complex (Ammigan *et al.*, 1990a) or protein (Ammigan *et al.*, 1989). At 12 weeks, 75% of the dose that causes 50% lethality (LD_{50}) was divided into three equal doses and was given intraperitoneally at 24-h intervals. Twenty-four hours after the last injection, overnight fasted animals were killed and the lung and liver were excised. Hepatic and pulmonary biotransformation enzymes, CYPs, cytochrome b-5, benzo[*a*]-pyrene hydroxylase, benzphetamine *N*-demethylase, glutathione *S*-transferase (GST) and glutathione (GSH) content were determined. Vitamin A and C were also determined.

NNN was more toxic to animals with nutritional deficiencies. In the vitamin A-, B complex- or protein-deficient rats, the LD_{50} of NNN was reduced by 20–24% (Ammigan *et al.*, 1990b). These deficiencies resulted in a decrease in the basal levels of CYPs, benzo[*a*]-pyrene hydroxylase, benzphetamine demethylase, GST and GSH.

In vitamin A-sufficient and -deficient groups, treatment with NNN significantly increased the levels of phase I-activating enzymes in all treatment groups. A higher increase in hepatic and pulmonary phase I activities was observed in the deficient animals compared with the sufficient groups. An increase in the GSH/GST system was observed in the sufficient group following treatment; however, in the deficient animals, exposure to the NNN caused suppression of the hepatic and pulmonary GSH/GST systems (Nair *et al.*, 1991).

When groups of Sprague-Dawley rats fed low-protein (5% casein) or vitamin B complex-deficient diets were exposed to NNN by the same protocol, a significant increase in phase I enzymes with concurrent inhibition of the GSH/GST levels was observed compared with the corresponding control groups fed high-protein (20% casein) or vitamin B complex-sufficient diets (Ammigan *et al.*, 1989, 1990a). The hepatic pool of vitamin A was depleted while that of vitamin C was increased in Sprague-Dawley rats fed low-protein diet and exposed to NNN. Altered metabolism resulting from vitamin deficiency and/or protein–calorie malnutrition may be an important factor in the modulation of the metabolism of NNN.

The metabolism of NNN has been studied in rats, hamsters, mice, monkeys and mini-pigs (reviewed in Hecht, 1998). NNN is rapidly metabolized and eliminated primarily in urine. Hydroxy acid via 5′-hydroxylation and keto acid via 2′-hydroxylation are the major urinary metabolites of NNN in rodents, marmoset monkeys and mini-pigs (Hecht, 1998). Other metabolites that are consistently observed in the urine are NNN-*N*-oxide and norcotinine (Hecht, 1998). In patas monkeys, the major urinary metabolites are hydroxy acid, 3′-Honorcotinine, 3′-HOnorcotinine-Gluc, norcotinine-*N*-oxide and norcotinine (Upadhyaya *et al.*, 2002). Small amounts of unchanged NNN are also observed in the urine of treated animals (Hecht, 1998).

In rats treated with racemic NNN, (*S*)-hydroxy acid and (*R*)-hydroxy acid represented 36% and 64% of total hydroxy acid in the urine, respectively (Trushin & Hecht, 1999). Products of 2′-hydroxylation predominated in the urine of rats treated with (*S*)-NNN while products of 5′-hydroxylation were more prevalent in the rats treated with (*R*)-NNN (McIntee & Hecht, 2000).

Haemoglobin adducts

HPB-releasing haemoglobin adducts are formed in rats treated with NNN. The adduct levels are only about 16% of those induced by NNK (Carmella & Hecht, 1987).

DNA adducts

HPB-releasing adducts are present in acid or enzyme hydrolysates of hepatic DNA from NNN-treated rats, in acid hydrolysates of pulmonary DNA from NNN-treated mice and in acid hydrolysates of DNA from the respiratory and olfactory parts of the nasal mucosa of rats treated with NNN; the levels in the respiratory mucosa are higher (reviewed in Hecht, 1998). As may be expected, O^6-MeGua is not detected in the nasal mucosa or liver of rats treated with NNN (Castonguay *et al.*, 1985b).

(iv) *Excretion*

Urine is the major route of excretion of NNN and metabolites in rodents and accounts for 60–80% of the dose (Hecht, 1998).

Urine samples were collected from seven groups of eight Sprague-Dawley rats that were maintained on semisynthetic diets sufficient or deficient in vitamin A, sufficient or deficient in vitamin B complex and sufficient or deficient in protein, or on standard control diet. All groups were exposed to NNN. Urine was tested for mutagenic activity using the *Salmonella*/microsome assay. A higher mutagenic activity of urine was observed in the exposed groups on each of the deficient diets. The order of mutagenicity of all treatments was deficient diet > standard diet > nutritionally sufficient diet (Ammigan *et al.*, 1990b). Thus, NNN-exposed animals probably have greater exposure to mutagenic metabolites, which are generated by increased phase I enzymes and decreased detoxification system.

(*c*) *NAB*

(i) *Absorption*

No data were available to the Working Group

(ii) *Distribution*

No data were available to the Working Group

(iii) *Metabolism*

In rats treated by gavage with [2′-^{14}C]NAB, 68.5% of the dose was excreted in the urine (Hecht & Young, 1982). 5-Hydroxy-5-(3-pyridyl)pentanoic acid, formed by 6′-hydroxylation, and NAB-*N*-oxide were detected as urinary metabolites. In cultured rat oesophagus treated with [2′-^{14}C]NAB , 5-hydroxy-5-(3-pyridyl)pentanoic acid, formed by 6′-hydroxylation, was a major metabolite and 5-oxo-5-(3-pyridyl)pentanoic acid, formed by 2′-hydroxylation, was a minor metabolite at all time-points examined. These results contrasted with those obtained by rat oesophageal metabolism of NNN, in which 2′-hydroxylation predominated (Hecht & Young, 1982).

(iv) *Excretion*

Urine was the major route of excretion of NAB metabolites in the rat (Hecht & Young, 1982).

(*d*) *NAT*

(i) *Absorption*

No data were available to the Working Group.

(ii) *Distribution*

No data were available to the Working Group.

(iii) *Metabolism*

In Fischer 344 rats, the pharmacokinetics of NAT fit a two compartment model. Pharmacokinetic parameters were: half-life, 540 min; blood clearance, 128 mL/h; and apparent volume of distribution, 695 mL (Adams *et al.*, 1985b).

(iv) *Excretion*

No data were available to the Working Group.

4.2 Toxic effects

4.2.1 *Humans*

No data were available to the Working Group

4.2.2 *Experimental systems*

(*a*) *NNK and NNAL*

(i) *Animals*

NNK induced cytotoxicity in the nasal passage of male Fischer 344 rats: it damaged Steno's and Bowmans's glands and caused degeneration of the olfactory epithelium at intraperitoneal doses of 4.8–48 μmol/kg bw daily for up to 12 days (Belinsky *et al.*, 1987b). NNK also induced mild centrilobular necrosis in the liver, which progressed to collapse of the centrilobular architecture at higher doses (Belinsky *et al*., 1986). An intraperitoneal dose of 0.39 mmol/kg bw induced an increase in levels of alanine transaminase, aspartate transaminase and lactate dehydrogenase in male Syrian golden hamsters over 2–3 weeks (Jorquera *et al.*, 1994).

(ii) *In-vitro cellular systems*

NNK induced cytotoxicity in rat tracheal epithelial (RTE) cells at concentrations of 100–200 μg/mL (Zhu *et al*, 1991) and in human–hamster hybrid A_L cells at a dose of 500 μg/mL (Zhou *et al.*, 1999). NNK caused dose- and time-dependent toxicity in hamster pancreatic duct cells *in vitro* (Baskaran *et al.*, 1994).

No data on NNAL were available to the Working Group.

(*b*) *NNN*

The subcutaneous LD_{50} of NNN in male rats observed for 8 days was > 1000 mg/kg bw. In rats that died, haemorrhages were observed in the lungs and abdominal organs and epithelial-cell necrosis in the posterior nasal cavities and liver (Hoffmann *et al*., 1975). The LD_{50} determined in 12-week-old Sprague-Dawley rats was 200 mg/kg bw with a standard diet and 190 mg/kg bw for animals fed vitamine B- and protein-deficient diets (Ammigan *et al.*, 1990b).

(*c*) *NAB*

The subcutaneous LD_{50} of NAB in Fischer rats was > 1000 mg/kg bw (Hoffmann *et al*., 1975).

(*d*) *NAT*

No data were available to the Working Group.

4.3 Reproductive and developmental effects

No data were available to the Working Group.

4.4 Genetic and related effects

4.4.1 *Humans*

DNA adducts as biomarkers of exposure to NNK and NNN are discussed in Section 4.1. No data were available for NNAL, NAB or NAT.

4.4.2 *Experimental systems*

(*a*) *NNK and NNAL*

(i) *NNK*

DNA adducts as biomarkers of exposure to NNK have been discussed in Section 4.1.2.(*a*)(iii).

Mutagenicity, clastogenicity, cell transformation and other effects

Mutagenicity and allied effects (for details and references, see Table 17)

In-vitro studies

NNK caused a dose-dependent increase in mutations in *S. typhimurium* strains TA100 and TA1535 in the presence of a liver microsomal preparation from Aroclor-1254-induced rats.

Primary hepatocytes, a liver metabolic activation system (S9 fraction), and tracheal epithelial cells from normal and Aroclor-1254-induced rats were compared for bio-activation of NNK in the *Salmonella* mutagenicity assay. Without activation, NNK was not mutagenic in *S. typhimurium* TA1535. The bioactivation of NNK to a mutagenic metabolite was achieved by incubation with the liver S9 metabolic activation system from Aroclor-1254-induced rats or with primary hepatocytes from both untreated and Aroclor-1254-pretreated rats. In contrast, NNK incubated with rat tracheal epithelial cells from both uninduced or Aroclor-1254-induced rats produced no measurable mutagenic activity in strain TA1535 (Zhu *et al*., 1991).

NNK was mutagenic in a *Salmonella* tester strain that carries the human *CYP2A6* and human NADPH-*CYP reductase* (YG7108 *2A6*/*OR*) in the absence of an exogenous metabolic activation system. In another report, NNK was shown to be mutagenic in strain TA7004 with rat and hamster metabolic activation systems, but not in TA100. It was also mutagenic in *S. typhimurium* TA98.

NNK has been used as a model mutagen in several studies that employed the *S. typhimurium* mutagenicity assay to determine the anti-mutagenic properties of various compounds (reviewed in Hecht, 1998).

NNK was mutagenic in the Mutatox test using the dark mutant M-169 of *Vibrio fischeri* (Yim & Hee, 2001).

In primary rat hepatocytes and rabbit lung cells, NNK induced DNA strand breaks without exogenous activation and unscheduled DNA synthesis.

Table 17. Genetic and related effects of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)

Test system	Result[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Salmonella typhimurium TA100, TA1535, reverse mutation	NT	+	2 µmol/plate [414 µg/plate]	Hecht *et al.* (1983c)
Salmonella typhimurium TA100, TA1535, reverse mutation	NT	+	2 µmol/plate[c] [418 µg/plate]	Hecht *et al.* (1983c)
Salmonella typhimurium TA100, TA1535, reverse mutation	NT	–	4 µmol/plate[d] [840 µg/plate]	Hecht *et al.* (1983c)
Salmonella typhimurium TA100, reverse mutation	–	+	1000 µg/plate	Padma *et al.* (1989b)
Salmonella typhimurium TA100, reverse mutation	–	–	2000 µg/plate	Yim & Hee (2001)
Salmonella typhimurium TA1535, reverse mutation	–	+	1000 µg/mL	Padma *et al.* (1989b)
Salmonella typhimurium TA1535, reverse mutation	–		2500 µg/plate	Zhu *et al.* (1991)
Salmonella typhimurium TA1535, reverse mutation		+	500 µg/plate	Zhu *et al.* (1991)
Salmonella typhimurium TA1538, reverse mutation	–	–	1000 µg/mL	Padma *et al.* (1989b)
Salmonella typhimurium TA98, reverse mutation	–	–	1000 µg/plate	Padma *et al.* (1989b)
Salmonella typhimurium TA98, reverse mutation	-	+	20 µM [4.2 µg/mL]	Kolar & Lawson (1997)
Salmonella typhimurium YG7108, reverse mutation	–	NT	0.7 mM [145 µg/mL]	Kushida *et al.* (2000a,b)
Salmonella typhimurium YG7108 -2A6/OR[e], reverse mutation	+	NT	0.1 mM [20.7 µg/mL]	Kushida *et al.* (2000a,b)
Salmonella typhimurium YG7108 -2E1/OR[f], reverse mutation	–	NT	0.7 mM [145 µg/mL]	Kushida *et al.* (2000a,b)
Salmonella typhimurium TA7004, reverse mutation	–	+	2000 µg/plate	Yim & Hee (2001)
DNA strand breaks, primary rat hepatocytes *in vitro*	+	NT	5 mM [1035 µg/mL]	Liu *et al.* (1990)
DNA strand breaks, primary rat hepatocytes *in vitro*	+	NT	6.25 µmol/mL [1297 µg/mL]	Pool-Zobel *et al.* (1992)
DNA strand breaks, isolated rabbit lung cells (Type II and Clara) *in vitro*	+	NT	30 µM [6.22 µg/mL]	Becher *et al.* (1993)
DNA strand breaks, isolated rabbit alveolar macrophages *in vitro*	–	NT	300 µM [62.2 µg/mL]	Becher *et al.* (1993)
Unscheduled DNA synthesis, freshly isolated rat hepatocytes *in vitro*	+	NT	1 mM [207.5 µg/mL]	Williams & Laspia, 1979
Unscheduled DNA synthesis, rabbit lung cells *in vitro*	+	NT	2 mM [414.5 µg/mL]	Dahl *et al.* (1990)

Table 17 (contd)

Test system	Result[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Gene mutation, Chinese hamster lung fibroblastic V79 cells, *Hprt* locus, *in vitro*	–	+	10 mM [2072 µg/mL]	Swedmark *et al.* (1994)
Sister chromatid exchange, Chinese hamster lung fibroblastic V79 cells *in vitro*	–	+	20 µg/mL	Zimonjic *et al.* (1989)
Sister chromatid exchange, Chinese hamster lung fibroblastic V79 cells *in vitro*	–	+	20 mM [4145 µg/mL]	Alaoui Jamali *et al.* (1988)
Sister chromatid exchange, Chinese hamster ovary (CHO) cells *in vitro*	NT	+	0.1 mM [20.7 µg/mL]	Lee *et al.* (1996)
Micronucleus formation, Chinese hamster lung fibroblastic V79 cells *in vitro*	–	+	5 mM [1036 µg/mL]	Alaoui Jamali *et al.* (1988)
Micronucleus formation, rat tracheal epithelial cells *in vitro*	+	NT	50 µg/mL	Zhu *et al.* (1991)
Chromosomal aberrations, Chinese hamster lung fibroblastic V79 cells *in vitro*	+	–	20 mM [4145 µg/mL]	Alaoui Jamali *et al.* (1988)
DNA strand breaks, human MRC-5 fetal lung cells *in vitro*	+	NT	5 mM [1035 µg/mL]	Weitberg & Corvese (1993, 1997)
Gene mutation, human lymphoblastoid cells, *HPRT* locus, *in vitro*	+	NT	1 µg/mL	Krause *et al*. (1999)
Sister chromatid exchange, human peripheral blood lymphocytes *in vitro*	+	NT	[0.48 mM] 100 µg/mL	Padma *et al.* (1989b)
Sister chromatid exchange, human peripheral blood lymphocytes *in vitro*	+	+	[0.096 mM] 20 µg/mL	Zimonjic *et al.* (1989)
Micronucleus formation, human AGT repair-deficient fibroblasts *in vitro*	+	NT	0.05 mM [10.4 µg/mL]	Pohlmann *et al.* (1992)
Micronucleus formation, human AGT repair-proficient fibroblasts *in vitro*	–	NT	1 mM [207.5 µg/mL]	Pohlmann *et al.* (1992)
Micronucleus formation, human-derived hepatoma HepG2 and Hep3B cells *in vitro*	–	NT	5 mM [1036 µg/mL]	Majer *et al.* (2004)
Chromosomal aberrations, human peripheral blood lymphocytes *in vitro*	+	NT	0.48 mM [100 µg/mL]	Padma *et al.* (1989b)

Table 17 (contd)

Test system	Result[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
DNA strand breaks, rat hepatocytes *in vivo*	+		12.5 mg/kg bw po 1× [60 µmol/kg bw]	Pool-Zobel *et al.* (1992)
DNA strand breaks, rat and hamster hepatocytes *in vivo*	+		0.39 mmol/kg bw [81 mg/kg bw] ip 1×	Jorquera *et al*. (1994)
Gene mutation, rat splenic T lymphocytes, *Hprt* locus, *in vivo*	–		150 mg/kg bw ip 1×	Jansen *et al*. (1996)
Micronucleus formation, Swiss male mice bone marrow *in vivo*	+		250 mg/kg bw/d ip 2×	Padma *et al.* (1989b)
Micronucleus formation, rat tracheal epithelial cells *in vivo*	+		150 mg/kg bw/d ip 3×	Zhu *et al.* (1991)

[a] +, positive;–, negative; NT, not tested
[b] LED, lowest effective dose; HID, highest ineffective dose; d, day; ip, intraperitoneal injection
[c] [4,4-dideutero]NNK
[d] [4-trideutero]NNK
[e] Co-expressing human CYP 2A6 together with human NADPH-CYP reductase (OR)
[f] Co-expressing human CYP 2E1 together with human NADPH-CYP reductase (OR)

NNK was mutagenic in V79 hamster cells when assayed with a metabolic activation system obtained from ethanol-treated pancreatic duct epithelial cells from humans and Syrian hamsters and from ethanol-treated CK cells (immortalized hamster pancreatic duct epithelial cells) (Kolar & Lawson, 1997). It induced mutations at the *Hprt* locus in V79 Chinese hamster cells co-cultivated with rat hepatocytes that provided metabolic activation.

NNK caused sister chromatid exchange in Chinese hamster ovary cells in the presence of metabolic activation in three studies.

NNK induced micronucleus formation in Chinese hamster lung fibroblastic V79 cells in the presence of a metabolic activation system. Micronuclei were also induced in V79 cells by NNK activated by fetal liver and lung homogenates from rat fetuses on the 15th day of the gestation (Alaoui Jamali *et al.*, 1998). In rat tracheal epithelial cells treated with NNK, micronuclei were formed without a metabolic activation system.

NNK induced chromosomal aberrations without exogenous bio-activation in Chinese hamster lung fibroblastic V79 cells.

NNK induced a dose-dependent toxicity in human–hamster hybrid A_L cell assay. Treatment with NNK at a low dose, when combined with radon α-particles, resulted in a combined mutagenic effect that was consistent with an additive model, but less than additive at higher concentrations of NNK (Zhou *et al.*, 1999). In mammalian cells, NNK induced mostly deletions (Zhou *et al*., 1999).

NNK induced DNA strand breaks in fetal human lung cells. NNK induced mutations at the *HPRT* locus in human lymphoblastoid MCL-5 cells without metabolic activation. In human lymphocytes, NNK induced sister chromatid exchange without exogenous bioactivation in two studies. Micronucleus formation was observed in AGT repair-deficient, but not in repair-proficient human fibroblasts in the absence of exogenous activation. NNK did not induce micronuclei in human hepatoma cell lines HepG2 and Hep3B. NNK induced chromosomal aberrations without exogenous bioactivation in human lymphocytes.

In-vivo studies

NNK did not induce mutations in rat splenic T-lymphocytes *in vivo*. NNK induced DNA strand breaks in the hepatocytes of rats and hamsters (see Section 4.1.2(*a*)(iii)), and micronucleus formation in the bone marrow of male Swiss mice.

Mutations in transgenic systems

In vitro, human CYP2A6 was lipofected via a retroviral vector in AS52 Chinese hamster ovary cells, which contain the bacterial *gpt* gene that can be mutated to 6-thioguanine resistance. At the highest dose of NNK (1200 μg/mL), a 14-fold (339×10^{-6}) increase in mutant frequency was observed in AS52-E8 cells compared with the spontaneous frequency of 24×10^{-6} (Tiano *et al.*, 1994).

In vivo, NNK was administered intraperitoneally to *lacZ* transgenic mice (Muta^{TM}Mouse) at 125 and 250 mg/kg bw once a week for 4 weeks. The mutant frequencies in the *lacZ* and *cII* genes from lung and liver increased dose-dependently up to 10-fold compared with the controls. The proportion of G:C→A:T transition mutations in

the total number of mutants was less than the number of A:T→T:A and A:T→C:G transversions (Hashimoto *et al*., 2004). NNK was also mutagenic in a mixture of pooled oral tissues (gingival, buccal, pharyngeal and sublingual), and in tongue and lung tissue of *lacZ* transgenic mice (Muta™ Mouse) (von Pressentin *et al*., 1999).

K-*ras* and *TP53* mutations

DNA isolated from 20 lung hyperplasias obtained after treatment of adult A/J mice with NNK was screened for the presence of activated K-*ras*. This gene was activated in 17/20 lesions; 85% of the mutations were a G:C→A:T transition within codon 12 (GGT→GAT), a mutation that is consistent with base mispairing produced by the formation of the O^6-MeGua adduct (Belinsky *et al*., 1992).

Activated K-*ras* gene was detected in 100% of lung tumours induced in C3H mice by treatment with NNK (50 mg/kg bw); the activating mutation detected in all samples was a G:C→A:T transition (GGT→GAT) in codon 12 (Devereux *et al*., 1991). NNK caused GGT→GAT mutations in codon 12 of K-*ras* gene in lung tumours induced in A/J mice (Chen *et al*., 1993; Ronai *et al*., 1993).

The relationship between the development of peripheral lung lesions induced by NNK and K-*ras* gene mutation, and the correlations between histological alterations and the course of lung lesion development after treatment with NNK and K-*ras* gene mutation were investigated in A/J mice. K-*ras* gene mutations were identified in seven of 12 (58.3%) hyperplasias, in 42/56 (75.0%) adenomas and in three of four (75.0%) adenocarcinomas. The most frequent K-*ras* gene mutation was a G→A transition at the second base of codon 12, which accounted for 86.5% of all the mutations detected (Kawano *et al*., 1996).

Analysis of lung tumour DNA from A/J mice treated with NNK indicated that 15/17 (88%) samples contained G→A transitions at the second base of codon 12 in the K-*ras* gene. Similarly, in lung tumours from (A/J × TSG-*p53*)F_1 hybrid mice treated with NNK, 29/30 (97%) contained G→A transitions at the second base of codon 12 of the K-*ras* gene. No mutations of the *p53* gene were found in any of the tumours analysed, which suggests minimal involvement of this gene in the development of lung adenomas. The *p53* allele in (A/J × TSG-*p53*)F_1 mice does not alter the incidence or multiplicity of NNK-induced lung tumours (Matzinger *et al*., 1995).

Lung tumours induced by subcutaneous injection of NNK into Syrian golden hamsters were examined for mutations in the K-*ras* oncogene and the *TP53* tumour-suppressor gene by direct sequencing. The K-*ras* mutation frequency in RNA isolated from pooled tumours and that in DNA isolated from individual tumours were found to be identical. Activated K-*ras* alleles were detected in 77–94% of tumours. All mutations observed except one (from a total of 65), at either codon 12 or 13, were G:C→A:T. No mutations were detected at codon 61. Examination of the same tumours for *TP53* mutations showed only one point mutation. Treatment of Syrian golden hamsters with NNK resulted in a distinct mutation pattern in the K-*ras* gene whereas *TP53* gene mutations may not play a major role at this stage in hamster lung tumorigenesis (Oreffo *et al*., 1993).

Gene expression profile

Characteristic expression profiles induced by NNK at a dose of 20 mg/kg bw per day were investigated in rat liver for 14 days. Fourteen genes that are involved in DNA-damage response (five), detoxification response (six) and cell survival/proliferation (three) were up-regulated and one gene each that is involved in mitochondrial damage and dedifferentiation were down-regulated (more than two-fold). O^6-MeGua–DNA methyltransferase was among one of the genes that were up-regulated. Increased expression profiles were weakly detectable at day 1 and then increased with time (Ellinger-Ziegelbauer, 2004).

Cytotoxicity and cell transformation

The cytotoxicity and transforming activity of NNK was studied by the assays of colony-forming efficiency, micronucleus formation and cell transformation in rat tracheal epithelial cells both *in vitro* and *in vivo*. Results from the in-vitro experiments indicated that low concentrations of NNK (0.01–25 μg/mL) caused increases in colony-forming efficiency of rat tracheal epithelial cells from 15% to more than 100%. At higher concentrations (100–200 μg/mL), NNK was significantly toxic to these cells. Treatment with NNK *in vitro* (50–200 μg/mL) significantly increased the transformation frequency in four of five (50 μg/mL) and six of eight (100 μg/mL) experiments. The in-vivo exposure of rats to NNK (150–450 mg/kg intraperitoneally) resulted in a 60–85% reduction in colony-forming efficiency in rat tracheal epithelial cells (Zhu *et al*., 1991).

Immortalized human bronchial epithelial cells (BEAS-2B cells) grown in de-epithelialed rat tracheas were exposed to NNK and subcutaneously transplanted into athymic nude mice. The cells were neoplastically transformed to produce invasive adenocarcinoma with phenotypic changes similar to the progressive changes that occur during human lung carcinogenesis (Klein-Szanto *et al.*, 1992).

In-vitro transformation of spontaneously immortal hamster pancreatic duct cells has been described following exposure to 20 mM NNK for 1, 3, 5 and 7 days. Cells treated with NNK grew as a monolayer with numerous mitotic figures and multinucleated large cells. One- and 3-day NNK-treated cells grown in complete duct medium produced well-differentiated, mucinous tumours after their injection in nude mice. Analysis of DNA from these tumours for K-*ras* mutation at codons 12, 13 and 61 showed a G→A transition at codon 12 of the K-*ras* oncogene in tumour cells after 1 and 3 days of NNK treatment (Baskaran *et al*., 1994).

In-vivo treatment with cumulative doses of 150 and 300 mg/kg bw NNK produced significant increases in transformation frequency of tracheal cells in three of three and two of three rats, respectively (Zhu *et al.*, 1991).

Yoo *et al.* (2000) showed that normal human gingival keratinocytes immortalized with human papillomavirus 16 (IHGK) were transformed by NNK to IHGKN cells. Transformation of IHGK cells resulted in the activation of vascular endothelial growth factor associated with angiogenesis. Inactivation of the G1 phase of cell-cycle regulation occurred during immortalization before cell transformation, and was sustained after carcinogen exposure.

Other effects

In cultured human oral epithelial cells, treatment with NNK resulted in increased longevity and a sustained differentiated phenotype for 8.5–10 weeks. The treated cells displayed focal growth and morphological changes suggestive of early stages of cell transformation as compared with controls in which cells were terminally differentiated (Murrah *et al*., 1993).

A higher frequency of hyperplasia with hyperkeratosis was observed in the forestomach of hamster treated with a combination of nicotine and NNK as compared with either NNK or nicotine treatment alone. Squamous-cell papillomas were evident in the forestomach of animals treated with both NNK and nicotine (Chen *et al*., 1994).

NNK was shown to inhibit the production of IL-12 and TNF, key molecules of immune response in rat alveolar macrophages and stimulate the production of IL-10 and prostaglandin E_2 (Therriault *et al.*, 2003). Using model compounds NNKOAc and *N*-nitro-(acetoxymethyl)methylamine (NDMAOAc), it has been demonstrated that the above effect of NNK is mediated by α-methyl hydroxylation of NNK, the same pathway that induces DNA pyridyloxobutylation (Proulx *et al*., 2004).

(ii) *NNAL*

NNAL was mutagenic in *S. typhimurium* TA1535 in the presence of a liver metabolic activation system from Aroclor-1254-induced rats or hamsters in the range of 0.025–0.2 μmol/plate [5.5–42 μg/plate] (maximum tested dose) (Brown *et al*., 2001b).

(*b*) *NNN*

(i) *Mutagenic and cytogenetic effects, DNA damage* (for details and references, see Table 18)

NNN was mutagenic in *S. typhimurium* strain TA100 in three of four assays (two with and one without exogenous metabolic activation), in strain TA1535 (with activation) and in TA1530 and TA7004 (without activation). NNN was also mutagenic in *Salmonella* tester strains YG7108 that carry the human *CYPs 2A6*, *1A1*, *3A4* and *3A5* in the absence of an exogenous activation system, but was not mutagenic in YG7108 itself or in YG7108 that carries *CYP2E1* (Kushida *et al.*, 2000a; Fujita & Kamataki, 2001). NNN was not mutagenic in *Salmonella* strains TA98 or TA1538 (single tests only).

NNN was a direct-acting mutagen in the Mutatox test using dark mutant M-169 of *Vibrio fischeri*, but was not mutagenic in this assay in the presence of a metabolic activation system from rat or hamster (Yim & Hee, 2001).

Using model compounds, it was shown that the putative diazohydroxide formed by 2′-hydroxylation of NNN has higher inherent mutagenicity toward *S. typhimurium* than the corresponding diazohydroxide formed by 5′-hydroxylation (Hecht & Lin, 1986).

NNN induced DNA strand breaks in primary rat hepatocytes but not in lung cells or alveolar macrophages of rabbits.

Table 18. Genetic and related effects of N′-nitrosonornicotine (NNN)

Test system	Result[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Salmonella typhimurium TA100, reverse mutation	–	+	2.5 µmol/plate [443 µg/plate]	Bartsch *et al.* (1980)
Salmonella typhimurium TA100, reverse mutation	–	+	250 µg/plate	Padma *et al.* (1989b)
Salmonella typhimurium, TA100, TA7004, reverse mutation	+	–	2000 µg/plate	Yim & Hee (2001)
Salmonella typhimurium TA100, TA7004, reverse mutation	+	–	500 µg/plate	Yim & Hee (2001)
Salmonella typhimurium TA1530, reverse mutation	+	NT	1000 µg/plate	Andrews *et al.* (1978)
Salmonella typhimurium TA1535, reverse mutation	–	+	1000 µg/plate	Padma *et al.* (1989b)
Salmonella typhimurium TA1538, TA 98, reverse mutation	–	–	1000 µg/plate	Padma *et al.* (1989b)
Salmonella typhimurium YG7108, reverse mutation	–	NT	0.7 mM [124 µg/mL]	Kushida *et al.* (2000a); Fujita & Kamataki (2001)
Salmonella typhimurium YG7108 -2A6/OR[c], reverse mutation	+	NT	0.2 mM [35.5 µg/mL]	Kushida *et al.* (2000a); Fujita & Kamataki (2001)
Salmonella typhimurium YG7108 -2E1/OR[d], reverse mutation	–	NT	0.7 mM [124 µg/mL]	Kushida *et al.* (2000a); Fujita & Kamataki (2001)
DNA strand breaks, primary rat hepatocytes *in vitro*	+	NT	5 mM [886 µg/mL]	Liu *et al.* (1990)
DNA strand breaks, isolated rabbit lung cells (Type II and Clara) and alveolar macrophages *in vitro*	–	NT	3 mM [531 µg/mL]	Becher *et al.* (1993)
DNA strand breaks, rat hepatocytes *in vitro*	–	NT	25 mM [4430 µg/mL]	Pool-Zobel *et al.* (1992)
Unscheduled DNA synthesis, freshly isolated rat hepatocytes *in vitro*	+	NT	1 mM [177 µg/mL]	Williams & Laspia (1979)
Unscheduled DNA synthesis, rabbit lung cells *in vitro*	–	NT	2 mM [354 µg/mL]	Dahl *et al.* (1990)
Gene mutation, Chinese hamster V79 cells, Hprt locus, *in vitro*	–	+	10 mM [1770 µg/mL]	Swedmark *et al.* (1994)
DNA strand breaks, human MRC-5 fetal lung cells *in vitro*	+	NT	5 mM [886 µg/mL]	Weitberg & Corvese (1993, 1997)
Sister chromatid exchange, human peripheral blood lymphocytes *in vitro*	–	NT	100 µg/mL	Padma *et al.* (1989b)

Table 18 (contd)

Test system	Result[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Chromosome aberrations, human peripheral blood lymphocyte *in vitro*	–	NT	100 µg/mL	Padma *et al.* (1989b)
DNA strand breaks, Sprague-Dawley rat liver *in vivo*	+		100 mg/kg bw po × 1 [565 µmol/kg bw]	Pool-Zobel *et al.* (1992)
Micronucleus formation, Swiss male mice bone marrow *in vivo*	+		250 mg/kg bw ip ×2	Padma *et al.* (1989b)

[a] +, positive;–, negative; NT, not tested
[b] LED, lowest effective dose; HID, highest ineffective dose; ip, intraperitoneal injection
[c] Co-expressing human CYP 2A6 together with human NADPH-cytochrome P450 reductase (OR)
[d] Co-expressing human CYP 2E1 together with human NADPH-cytochrome P450 reductase (OR)

NNN induced DNA strand breaks in MRC-5 human fetal lung cells. When these MRC-5 cells were treated with NNN in combination with enzymatically generated oxygen radicals, DNA strand-breakage increased by approximately 50%, while oxygen-radical scavengers (superoxide dismutase, catalase, mannitol) significantly reduced the DNA damage caused by NNN (Weitberg & Corvese, 1993).

NNN induced DNA single-strand breaks in cultured primary rat hepatocytes (as measured by the alkaline elution assay). It did not cause any marked DNA damage (as measured by the alkaline elution assay) in isolated Clara and type II cells from rabbit lung and in isolated rabbit alveolar macrophages. NNN induced unscheduled DNA synthesis in freshly isolated hepatocytes from adult rats. NNN had genotoxic effects in primary rat hepatocytes *in vitro*, measured as DNA damage by alkaline elution and nick translation (Pool-Zobel *et al.*, 1992). NNN-induced mutations were not observed at the *Hprt* locus of V79 Chinese hamster cells after S9 metabolic activation in a co-cultivation system that used either freshly isolated rat hepatocytes or H4IIE rat hepatoma cells (Swedmark *et al.*, 1994).

NNN was mutagenic in a mixture of pooled oral tissues (gingiva, buccal cavity, pharynx and sublingua), and in tongue and oesophageal tissue in *lacZ*-transgenic mice (Muta^{TM}Mouse) (von Pressentin *et al.*, 1999).

NNN did not induce cytogenetic effects (sister chromatid exchange or chromosomal aberrations) in human peripheral blood lymphocytes.

NNN had genotoxic effects in Sprague-Dawley rat liver *in* vivo, measured as DNA damage by alkaline elution and nick translation (Pool-Zobel *et al.*, 1992). *In vivo*, NNN induced micronuclei in the bone marrow of Swiss mice.

In animal-mediated DNA-repair assays with *Escherichia coli* K-12 strains (injected intravenously just before nitrosamine treatment), intraperitoneal administration of NNN to mice caused dose-dependent genotoxic effects in indicator bacteria recovered from various organs of the treated animals. The genotoxic effect was enhanced by ethanol treatment prior to carcinogen treatment (Knasmüller *et al.*, 1994).

(ii) *Other effects*

In cultured human oral epithelial cells, treatment with NNN resulted in increased longevity and a sustained differentiated phenotype for 8.5–10 weeks. The treated cells displayed focal growth and morphological changes that were suggestive of early stages of cell transformation in comparison with control cells which were terminally differentiated (Murrah *et al.*, 1993).

Hamster cheek-pouch epithelium showed histological changes, including hyperplasia, hyperkeratosis and, in one animal, moderate dysplasia, when treated with nicotine combined with NNN. These changes were more frequent than after treatment with NNN or nicotine alone (Chen *et al.*, 1994).

Exposure of Syrian hamster buccal mucosa to NNN, five times per week for 24 weeks, did not result in clinical or histological changes (Papageorge *et al.*, 1996).

(*c*) *NAB*

Using genetically engineered *S. typhimurium* strain YG7108 that overexpresses human CYP, NAB induced mutation in the strains that contain CYP3A4, CYP2A6, CYP1A1 or CYP3A5. CYP3A4-carrying constructs induced the greatest mutagenicity (0.071 revertants/nmol NAB/pmol CYP) (Fujita & Kamataki, 2001).

(*d*) *NAT*

Using genetically engineered *S. typhimurium* strain YG7108 that overexpresses human CYP, NAT induced mutation in the strain that contains CYP2A6 (0.164 revertants/nmol NAB/pmol CYP) (Fujita & Kamataki, 2001).

4.5 Mechanistic considerations

4.5.1 *4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)*

The formation of DNA adducts is pivotal in the carcinogenic process (Miller, 1994). If not repaired, DNA adducts cause permanent mutations due to miscoding events during the replication of adducted DNA (Singer & Essigmann, 1991; Seo *et al.*, 2000). When these mutations occur in critical regions of growth control genes, such as oncogenes and tumour-suppressor genes, cancer can result. The occurrence of multiple mutated genes in tumours that are caused by tobacco products is consistent with the extensive range of DNA damage that is caused by metabolically activated tobacco carcinogens (Hecht, 2003). However, NNK requires metabolic activation, generally through catalysis by CYP enzymes, before this genotoxic mechanism of cancer causation can occur. Extensive studies in laboratory animals have clearly demonstrated that the pathway of metabolic activation → persistent DNA adducts → mutations is critical to the carcinogenesis of NNK in the lung and nasal cavity. Fewer experimental studies have examined the genotoxic mechanism of the carcinogenicity of NNK in oral and pancreatic cells and tissues. Studies that used human cells and tissues have investigated potential parallels between experimental and human systems. Other mechanisms that contribute to the carcinogenesis of NNK have also emerged in recent years. This section examines the genotoxic and other mechanisms of the carcinogenesis of NNK, and focuses on comparisons between experimental and human systems.

(*a*) *Genotoxic mechanisms*

(i) *Metabolism*

A common metabolic event in virtually all systems (rodent and human) is the conversion of NNK to NNAL, during which (*S*)-NNAL predominates (Hecht, 1998, 2002). Studies in rats have demonstrated that (*S*)-NNAL is extensively distributed in the body and accumulates and persists in the lung, possibly at a receptor site (Wu *et al.*, 2002; Zimmerman *et al.*, 2004). (*S*)-NNAL is efficiently reconverted to NNK in rats (Zimmerman *et al.*, 2004). (*S*)-NNAL also persists in humans as shown by its slow excretion relative to that of (*R*)-NNAL

after cessation of tobacco use (Hecht *et al.*, 2002). Rat CYP2A3 and human CYP2A13 have similarly high catalytic efficiencies for the reconversion of (*S*)-NNAL to NNK (Tables 11 and 16; Jalas *et al.*, 2003a, 2005). The accumulation of (*S*)-NNAL in the lung may be a critical feature of the selectivity of NNK for induction of lung tumours in rodents. NNAL and its glucuronides are excreted in the urine of rodents, primates and humans (Hecht, 1998, 2002).

Rodent lung, oral mucosa, nasal mucosa and liver all metabolize NNK by α-hydroxylation at its methylene and methyl carbons to produce intermediates that bind to DNA (Hecht, 1998). These reactions are catalysed by CYP enzymes. Steady-state kinetic parameters for CYP-catalysed NNK metabolism have been reported for five rat enzymes, two mouse enzymes, two rabbit enzymes and eight human enzymes (Tables 11 and 14; Jalas *et al.*, 2005). Of these, rat CYP2A3, mouse CYP2A5, rabbit CYPs 2A10/11 and human CYPs 2A13 and 2B6 exhibit the lowest K_m values and may be the most important catalysts of NNK bioactivation in the respective species (Table 14). Members of the CYP2A subfamily appear to be the best catalysts of NNK α-hydroxylation across species, and CYP2A13, which has catalytic properties for NNK metabolism that are very similar to those of rat CYP2A3, may be particularly important in the bioactivation of NNK by the human lung (Jalas *et al.*, 2005). Rat pancreatic microsomes converted NNK to NNAL, (NNK)ADP$^+$ and (NNK)ADPH, and converted NNAL to (NNAL)ADP$^+$; products of α-hydroxylation were not observed (Peterson *et al.*, 1994).

NNK and/or NNAL are metabolically activated by a variety of human tissues and cells including those from the oral cavity, lung, oesophagus, cervix, urinary bladder and liver (Hecht, 1998; Prokopczyk *et al.*, 2001; Vondracek *et al.*, 2001). The extents of metabolism by α-hydroxylation are generally lesser than those observed in rodents. NNK and NNAL have been detected in human pancreatic juice (Prokopczyk *et al.*, 2002). Human pancreatic microsomes converted NNK to NNAL, but no products of α-hydroxylation of NNK or NNAL were observed (Anderson *et al.*, 1997).

(ii) *DNA adducts*

Methyl and pyridyloxobutyl DNA adducts of NNK have been characterized *in vitro* and *in vivo* (Hecht, 1998; Wang *et al.*, 2003; Hecht *et al.*, 2004c). Extensive studies have examined the mechanisms of lung tumour formation in A/J mice treated with a single dose of NNK. A consistent body of evidence including structure–activity studies, investigations of deuterated NNK analogues, analysis of the occurrence and persistence of DNA adducts and effects on the AGT-repair enzyme strongly implicates *O*^{6}-MeGua as the critical DNA adduct in lung tumour induction by NNK in this mouse strain (Peterson & Hecht, 1991; Hecht, 1998; Peterson *et al.*, 2001; Jalas & Hecht, 2003; Jalas *et al.*, 2003b; Thomson *et al.*, 2003). *O*^{6}-MeGua is known to have miscoding properties that cause G→A transitions (Loechler *et al.*, 1984). Mutations in the K-*ras* gene in A/J mouse lung tumours induced by NNK are predominantly G→A transitions, which is consistent with the important role of *O*^{6}-MeGua (Hecht, 1998). A different picture emerges from studies of mechanisms of NNK-induced lung and nasal cavity carcinogenesis in Fischer 344 rats. Strong

evidence, based on structure–activity considerations and extensive studies of the formation and persistence of DNA adducts in individual cell types of the lung and different regions of the nasal mucosa and the effects of inhibitors of NNK carcinogenesis, indicate that a combination of O^6-MeGua and HPB-releasing pyridyloxobutyl DNA adducts is important in lung carcinogenesis and that these latter adducts are critical in nasal cavity carcinogenesis by NNK (Hecht, 1998). Fewer studies have been carried out in oral tissue. However, it is known that rat oral tissue actively metabolizes NNK by all known pathways including α-hydroxylation, and 7-MeGua has been identified in these tissues (Murphy *et al.*, 1990a).

Methyl and pyridyloxobutyl DNA adducts have been identified in the lungs of smokers (Hecht, 1998). While the methyl adducts, 7-MeGua and O^6-MeGua, may have multiple sources and are also found in nonsmokers, only NNK and NNN are probable precursors to pyridyloxobutyl DNA adducts. Cellular binding has also been observed in human oral keratinocyte cell lines exposed to NNK (Vondracek *et al.*, 2001).

(iii) *Mutations*

Mutations in the K-*ras* gene are frequently found in A/J mouse and hamster lung tumours induced by NNK (Hecht, 1998). The most common mutation is a GGT→GAT transition in codon 12 of the K-*ras* gene. In-vitro studies have demonstrated that 7-MeGua, O^6-MeGua and O^6(POB-1-yl)Gua are preferentially formed at the second G of codon 12 of the K-*ras* gene (Ziegel *et al.*, 2003). Both O^6-MeGua and O^6(POB-1-yl)Gua are known to cause predominantly G→A transition mutations (Loechler *et al.*, 1984; Pauly *et al.*, 2002). The pyridyloxobutylating agent, NNKOAc, causes GGT→TGT and GGT→GTT mutations in codon 12 in addition to GGT→GAT mutations (Ronai *et al.*, 1993). Although mutations in K-*ras* in lung tumours from mice and hamsters are consistent with the properties of the DNA adducts formed by NNK, other factors are also involved. For example, only two of 22 lung tumours induced by NNK in relatively insensitive C57BL/6 mice had K-*ras* mutations (Devereux *et al.*, 1993). In another study, treatment of mice with NNK followed by butylated hydroxytoluene increased lung tumour induction compared with NNK alone, but decreased the frequency of GGT→GAT mutations in codon 12 of K-*ras* (Matzinger *et al.*, 1994). The frequency of activation of K-*ras* and GGT→GAT mutation in codon 12 is not affected by the time after NNK treatment, nor are the proliferative activity of the lung lesions and the presence of mutations correlated. Thus, K-*ras* gene mutations appear to play a minor role in the selective growth advantage of NNK-induced lung lesions in A/J mice (Kawano *et al.*, 1996). In the Muta Mouse treated with NNK, A:T→T:A and A:T→C:G transversions were the major mutations observed in the lung and liver *cII* genes; G:C→A:T transitions were also observed, but to a lesser extent (Hashimoto *et al.*, 2004). There is no evidence of K-*ras* or *p53* mutations in lung tumours induced by NNK in rats (Hecht, 1998). No mutations have been detected in the *p53* gene from NNK-induced mouse lung tumours and only one of 24 hamster lung tumours examined had a mutation in the *p53* gene (Oreffo *et al.*, 1993; Hecht, 1998).

Mutations in codon 12 of the K-*RAS* gene are present in 24–50% of human primary lung adenocarcinomas, but are rarely seen in other types of lung tumour (Rodenhuis & Slebos, 1992; Mills *et al.*, 1995; Westra *et al.*, 1996). These mutations are more common in smokers and former smokers than in nonsmokers, which suggests that they may be induced by a component of tobacco smoke (Westra *et al.*, 1993). The most frequently observed mutation is GGT→TGT, which typically represents about 60% of the mutations in codon 12, followed by GGT→GAT (20%) and GGT→GTT (15%). The prevalence of G→T mutations has led to speculation that they may be due to polycyclic aromatic hydrocarbons, which can induce such mutations through the diol epoxide metabolic activation pathway (You *et al.*, 1989; Westra *et al.*, 1993). However, G→T mutations are also induced by NNKOAc (Ronai *et al.*, 1993). In A/J mice, the O^6-MeGua pathway of NNK metabolic activation is clearly the major pathway involved in tumour induction, which is consistent with the high percentage of GGT→GAT mutations in the K-*ras* gene isolated from mouse lung tumours induced by NNK. However, in Fischer 344 rats, both the pyridyloxobutylation and methylation pathways are critical in the lung tumorigenesis of NNK. The relative importance of these pathways in human lung carcinogenesis is not known. If pyridyloxobutylation is critical, as in the rat, a higher percentage of G→T transversions than that observed in mice would be expected as a result of exposure to NNK. In the absence of additional information, it is difficult to assign mutations in human genes to particular carcinogen adducts for an exposure that is as complex as that to tobacco smoke. Numerous compounds that damage DNA are present in tobacco smoke and many of these cause G→T transversion mutations: examples, in addition to nitrosamines and polycyclic aromatic hydrocarbons, include aromatic amines, oxygen radicals and α,β-unsaturated aldehydes (Singer & Essigmann, 1991; Moriya, 1993; Moriya *et al.*, 1994; Nesnow *et al.*, 1995). With respect to the *p53* gene, which is commonly mutated in tobacco-related cancers, mutations at G are frequently observed, which is consistent with the multiple carcinogens in tobacco products that bind to G. The spectrum of mutations in the *p53* gene from lung tumours has been attributed in part to reactions with polycyclic aromatic hydrocarbon diol epoxides, subject to the limitations discussed above; at present, no evidence has been found that NNK produces a similar mutational spectrum (Pfeifer *et al.*, 2002; Ziegel *et al.*, 2004).

(*b*) *Other mechanisms*

NNK is a high-affinity agonist for both the β_1- and β_2-adrenergic receptors in human pulmonary adenocarcinoma cell lines and in Chinese hamster ovary cell lines that have been transfected with the human β_1- or β_2-adrenergic-receptor gene (Schuller, 2002). NNK and other β-adrenergic receptor agonists stimulate the release of arachidonic acid from cell membrane phospholipids, which results in stimulated DNA synthesis and proliferation of human pulmonary adenocarcinoma cells. The mitogenic response to NNK is reduced upon treatment with β-adrenergic receptor antagonists, such as propanolol, and with cyclooxygenase and lipoxygenase inhibitors. Similar results have been obtained in pancreatic cells. In cell lines derived from human pancreatic adenocarcinomas, NNK

stimulated the release of arachidonic acid, which led to DNA synthesis and cell proliferation. In a model of pancreatic carcinogenesis that was induced transplacentally by treatment of pregnant hamsters with NNK and ethanol, treatment of the offspring with ibuprofen and the 5-lipoxygenase-activating protein inhibitor MK886 began 4 weeks after the birth and inhibited pancreatic tumorigenesis (Schuller *et al.*, 2002). These results indicate that receptor binding of NNK could play a role in human lung and pancreatic carcinogenesis (Schuller, 2002).

NNK binds to the α_7 nicotinic acetylcholine receptor (α_7nAChR) in small-cell lung carcinoma and pulmonary neuroendocrine cells, which results in the influx of Ca^{2+}, release of 5-hydroxytryptamine (serotonin) and activation of a mitogenic pathway mediated by protein kinase C, Raf-1, mitogen-activated protein kinase and c-*Myc*. Unstimulated small-cell lung carcinoma cells from smokers demonstrated high base levels of 5-hydroxytryptamine release and individual downstream signalling components in comparison with pulmonary neuroendocrine cells. Subchronic exposure of the latter cells to NNK up-regulated the α_7nAChR and its associated mitogenic pathways (Schuller *et al.*, 2003). NNK at 0.1 nM simultaneously stimulates phosphorylation of the oncogenic proteins Bcl2 and c-Myc through the α_7nAChR in association with increased proliferation of human small-cell lung carcinoma cells, which suggests that NNK facilitates a functional cooperation between Bcl2 and c-Myc in a mechanism that involves phosphorylation of both regulators (Jin *et al.*, 2004).

The *p16*^{INK4a} (*p16*) tumour-suppressor gene can be inactivated by hypermethylation of its promoter region. In Fischer 344 rats, 94% of adenocarcinomas induced by NNK were hypermethylated at the *p16* gene promoter and this change was frequently detected in precursor lesions of the tumours such as adenomas and hyperplasias. This timing of *p16* hypermethylation was reproduced in human squamous-cell carcinomas in which the *p16* gene was coordinately methylated in 75% of carcinoma *in situ* lesions adjacent to squamous-cell carcinomas that harboured this change. The frequency of *p16* hypermethylation increased during disease progression from basal-cell hyperplasia to squamous metaplasia to carcinoma *in situ* (Belinsky *et al.*, 1998). Hypermethylation of *p16* was also detected in 45% of rat liver tumours induced by NNK, and is a common event in human hepatocellular carcinoma (Pulling *et al.*, 2001). The death-associated protein kinase (*DAPK*) gene is methylated in 23–44% of human non-small-cell lung cancers and in 52% of mouse lung tumours induced by NNK (Pulling *et al.*, 2004). *DAPK* methylation was observed at a similar prevalence in NNK-induced hyperplasias and adenocarcinomas, which suggests that inactivation of this gene is one pathway for tumour development in the mouse lung (Pulling *et al.*, 2004). The retinoic acid receptor gene β (*RAR-*β), which encodes one of the primary receptors for retinoic acid, is down-regulated by methylation in human lung cancer (Vuillemenot *et al.*, 2004). Methylated alleles of this gene were detected in virtually all primary lung tumours induced in mice by NNK and in 54% of preneoplastic hyperplasias induced by NNK (Vuillemenot *et al.*, 2004).

The serine/threonine kinase Akt (or protein kinase B) is activated in non-small-cell lung cancer cells and promotes cellular survival and resistance to chemotherapy or radiation

(Brognard *et al.*, 2001). NNK activates Akt in non-immortalized human airway epithelial cells. Activation of Akt by NNK occurred dose dependently within minutes and depended upon α_7nAChR. Activated Akt increased phosphorylation of certain downstream substrates that control the cellular cell cycle and protein translation, and partially induced a transformed phenotype. Active Akt was detected in airway epithelial cells and lung tumours from NNK-treated A/J mice and in human lung cancers from smokers (West *et al.*, 2003). These studies were extended by examining Akt activation at intermediate steps in carcinogenesis. The phosphatidylinositol 3′-kinase/Akt pathway was analysed in isogenic, immortalized or tumorigenic human lung bronchial epithelial cells *in vitro* and in a spectrum of NNK-induced mouse lung lesions *in vivo*. Progressive activation of the phosphatidylinositol 3′-kinase/Akt pathway correlated with phenotypic progression of lung epithelial cells, which strengthens the hypothesis that Akt activity plays a role in lung tumorigenesis (West *et al.*, 2004).

4.5.2 N′-*Nitrosonornicotine (NNN)*

A convincing body of data demonstrates that NNN requires metabolic activation to form DNA adducts that drive the mutagenic and carcinogenic processes. Metabolic activation of NNN occurs by 2′-hydroxylation and 5′-hydroxylation and leads to the formation of diazohydroxides that bind to DNA and are mutagenic. This process is observed in both rodent and human tissues. Metabolic activation of NNN is catalysed by CYP enzymes and, among these, CYP2A enzymes are outstanding catalysts in both rodents and humans. The formation of HPB-releasing DNA adducts of NNN has been clearly demonstrated in the rat nasal mucosa and oesophagus, which are target tissues of NNN carcinogenesis. HPB-releasing haemoglobin adducts of NNN are also produced in rats. As discussed above, HPB-releasing DNA and haemoglobin adducts have been detected in humans exposed to tobacco products, and their sources could be either NNK or NNN or both. Thus, there are clear parallels between the mechanisms of metabolic activation of NNN in rodents and humans.

5. Summary of Data Reported and Evaluation

5.1 Exposure data

Tobacco-specific *N*-nitrosamines, including 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), *N*′-nitrosonornicotine (NNN), *N*′-nitrosoanabasine (NAB) and *N*′-nitrosoanatabine (NAT), occur widely in tobacco and tobacco smoke. They are formed by the nitrosation of nicotine and other tobacco alkaloids and have been detected in green tobacco leaves from *Nicotiana tabacum* and *N. rustica* species; however, the largest quantities of tobacco-specific *N*-nitrosamines are formed during tobacco curing and processing and additional amounts are formed during smoking. Tobacco-specific *N*-nitrosamines occur in all

commercially and non-commercially prepared tobacco products including cigarettes, cigars, *bidis*, pipe tobacco and smokeless tobacco products. *N*-Nitrosamines occur in a wide variety of both food and non-food products, but the amounts of tobacco-specific *N*-nitrosamines in all tobacco products exceed the levels of other *N*-nitrosamines in other commercial products by several orders of magnitude. The highest levels of tobacco-specific *N*-nitrosamines are measured in smokeless tobacco products. For example, levels of NNK up to 17.8 μg/g have been measured in North American and European smokeless tobacco products; up to 245 μg/g have been measured in products used in India; and up to 7870 μg/g have been measured in Sudanese *toombak*. Levels of NNN up to 135 μg/g have been measured in North American and European smokeless tobacco products; up to 1356 μg/g have been measured in products used in India; and up to 3085 μg/g have been measured in Sudanese *toombak*. These compounds are also present in secondhand tobacco smoke. The degree of exposure to tobacco-specific *N*-nitrosamines depends not only on the levels of these compounds in tobacco products or smoke, but also on the manner in which the products are used.

5.2 Human carcinogenicity data

No data were available to the Working Group.

5.3 Animal carcinogenicity data

NNK

In numerous studies in mice, NNK induced lung adenomas independent of the route of administration.

In studies by subcutaneous injection, benign and malignant tumours of the lung, nasal cavity and liver were induced in rats. In two of four experiments in hamsters, lung adenomas and adenocarcinomas or adenosquamous carcinomas were induced in males and females. In the two other experiments, adenomas were observed. Nasal cavity tumours involving the forebrain were observed in a limited study in mink.

In a study by administration in the drinking-water and another by oral swabbing, combined benign and malignant lung tumours (adenoma, adenosquamous carcinoma and carcinoma) were induced in male rats. In the drinking-water study, NNK produced benign and malignant pancreatic tumours. In the oral swabbing study, combined benign and malignant tumours of the liver and nasal cavity were observed. A significant increase in the incidence of liver and lung tumours was reported in female rats when NNK was instilled into the urinary bladder.

In two studies, the offspring of mice were exposed transplacentally by intraperitoneal injection of the dams. Liver tumours were observed in male offspring in both studies and in female offspring in one study. In one of these studies, lung tumours were also observed in male offspring.

In studies of the offspring of hamsters given NNK during pregnancy, intratracheal instillation of the dams resulted in adenocarcinomas of the nasal cavity in male offspring and adrenal pheochromocytomas in male and female offspring in one study. In a second study, subcutaneous injection of NNK into dams induced respiratory tract (nasal cavity, larynx and trachea) tumours in male and female offspring. When dams were injected subcutanously or treated by intratracheal instillation, nasal cavity and adrenal gland tumours developed in male and female offspring in a third study.

Intraperitoneal administration of NNK-*N*-oxide induced lung adenomas in female mice.

In an oral swabbing study, NNK in combination with NNN increased the incidence of oral tumours in rats.

4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL)

In one study in male rats in which NNAL, a principal metabolite of NNK, was administered in the drinking-water, adenomas, adenocarcinomas and adenosquamous carcinomas of the lung and benign and malignant pancreatic tumours were induced.

In three studies in female mice, intraperitoneal injection of NNAL induced lung adenomas. In one of these studies, adenocarcinomas were also observed.

NNN

In four studies in female mice, intraperitoneal injection of NNN produced lung adenomas. In another study in mice, lung adenomas were induced by intraperitoneal injection of NNN in males and females.

In two studies in rats in which NNN was given in the drinking-water and one study in which it was added to a liquid diet, benign and malignant oesophageal tumours were observed in males and females. Benign nasal cavity tumours were also observed in rats treated through the liquid diet.

In rats, subcutaneous injection of NNN induced malignant or benign (combined) and malignant nasal cavity tumours in males and females in two studies. In one study in hamsters, subcutaneous injection of NNN induced tracheal tumours in males and females and benign and malignant tumours of the nasal cavity in males. In two limited studies in mink treated with NNN by subcutaneous injection, nasal cavity tumours that invaded the forebrain were observed in females.

Skin application of NNN in female mice induced a non-significant increase in the incidence of skin papillomas and carcinomas.

Metabolites of NNN (3′-hydroxy-NNN, 4′-hydroxy-NNN or NNN-1-*N*-oxide) were tested by intraperitoneal injection into female mice and resulted in the induction of lung adenomas in mice exposed to 4′-hydroxy-NNN. Administration of NNN-1-*N*-oxide in the drinking-water increased the incidence of benign and malignant oesophageal tumours in male and female rats in one study and that of colon tumours in female hamsters in another study.

In one oral swabbing study, NNN in combination with NNK increased the incidence of oral tumours in rats.

NAB

In one study in female mice, intraperitoneal injection of NAB induced lung adenomas.

In one study in rats in which NAB was administered in the drinking-water, oesophageal carcinomas and/or papillomas were induced in males and females. Another study in male rats gave negative results.

Subcutaneous injection of NAB into hamsters gave negative results in one study.

NAT

Subcutaneous injection of NAT into male and female rats did not induce tumours at any site.

5.4 Other relevant data

NNK and its metabolite NNAL

Extensive studies have examined the metabolism of NNK and the formation of DNA adducts by NNK and its metabolite NNAL in humans and laboratory animals; the metabolic pathways and structures of DNA adducts have been characterized comprehensively. NNK and NNAL have been detected in the saliva of smokeless tobacco users, and NNAL and another metabolite of NNK, NNAL-glucuronide, have been quantified in human urine. The presence of these metabolites, which are specific to exposure to tobacco products (e.g. in smokers, users of smokeless tobacco and nonsmokers exposed to secondhand tobacco smoke), signals human uptake and metabolism of NNK, and their quantification allows an estimation of the dose of NNK absorbed. Dose calculations show that the total amounts of NNK taken up by people who used tobacco products for a period of 30 years or more approximate the total amounts that induce tumours in rats.

The metabolic activation of NNK and NNAL to DNA adducts is critical for the expression of their carcinogenic activities. The metabolic activation process has been documented extensively in laboratory animals. Cytochrome P450 enzymes are the principal catalysts of this process, and those in the 2A family appear to be the most efficient in both humans and laboratory animals. Macromolecular adducts formed after the metabolic activation of NNK and/or NNN have been detected in smokers, in smokeless tobacco users and in laboratory animals treated with these carcinogens. In laboratory animals, persistence of these adducts is associated with tumour formation.

NNK is a genotoxic compound. It was shown to be mutagenic in bacteria, in rodent fibroblasts and in human lymphoblastoid cells *in vitro*. It caused cytogenic effects in a variety of mammalian cells *in vitro* and induced transformation of the pancreatic duct cells of hamsters. *In vivo,* NNK induced micronucleus formation in the bone marrow of

mice and DNA strand breaks in the hepatocytes of rats and hamsters. NNAL was reported to be mutagenic in *Salmonella* in a single study.

In addition to the classical mechanisms of carcinogenesis that proceed through the formation of DNA adducts, NNK also binds to nicotinic and other receptors, which leads to downstream effects that contribute to the development of cancer. These effects have been observed in experimental systems including pancreatic and lung cells from humans and laboratory animals.

NNN

The major route of metabolic activation to DNA adducts is α-hydroxylation adjacent to the nitroso group, which is mediated principally by cytochrome P450 enzymes and, in particular, by those of the 2A family. The human oesophagus catalyses α-hydroxylation of NNN, and this process is especially efficient in the rat oesophagus and nasal mucosa, which are target tissues for the carcinogenicity of NNN. NNN has been detected in the saliva of smokeless tobacco users. The uptake and metabolism of NNN by smokers and smokeless tobacco users has been demonstrated by its quantitation and that of its glucuronide in human urine. The metabolic pathways of NNN have been characterized extensively in laboratory animals and there are distinct parallels can be seen between the metabolism of NNN in humans and laboratory animals.

NNN is a genotoxic compound. It was shown to be mutagenic in bacteria, but not in mammalian cells *in vitro*. NNN induced DNA strand breaks in human fetal lung cells and in primary rat hepatocytes *in vitro*. It did not show cytogenetic activity *in vitro*, but induced micronuclei in the bone marrow of mice *in vivo* in a single study.

NAB

NAB has been detected in the saliva of *toombak* users and has been quantified together with its glucuronide in the urine of smokers and smokeless tobacco users. The metabolism of NAB by α-hydroxylation and other pathways has been characterized in rats.

The genotoxicity of NAB has not been tested extensively. It was shown to be mutagenic in various strains of *Salmonella typhimurium*, each of which co-expressed a different form of human cytochrome P450 enzyme.

NAT

NAT has been detected in the saliva of smokeless tobacco users, and has been quantified together with its glucuronide in the urine of smokers and smokeless tobacco users.

The genotoxicity of NAT has not been tested extensively. It was shown to be mutagenic in a strain of *S. typhimurium* that expressed human cytochrome P450 2A6.

5.5 Evaluation

There is *inadequate evidence* in humans for the carcinogenicity of tobacco-specific *N*-nitrosamines.

There is *sufficient evidence* in experimental animals for the carcinogenicity of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and its metabolite 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL).

There is *sufficient evidence* in experimental animals for the carcinogenicity of *N*′-nitrosonornicotine (NNN).

There is *limited evidence* in experimental animals for the carcinogenicity of *N*′-nitrosoanabasine (NAB).

There is *inadequate evidence* in experimental animals for the carcinogenicity of *N*′-nitrosoanatabine (NAT).

Overall evaluation

4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and *N*′-nitrosonornicotine (NNN) are *carcinogenic to humans (Group 1).*

N′-Nitrosoanabasine (NAB) is *not classifiable as to its carcinogenicity to humans (Group 3).*

N′-Nitrosoanatabine (NAT) is *not classifiable as to its carcinogenicity to humans (Group 3).*

In making the overall evaluation of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and *N*′-nitrosonornicotine, the Working Group took into consideration the following mechanistic evidence (detailed in Section 5.4).

4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone and *N*′-nitrosonornicotine are the most abundant strong carcinogens in smokeless tobacco; uptake and metabolic activation in smokeless tobacco users have been clearly observed. In rats, combined application of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and *N*′-nitrosonornicotine induced oral tumours consistent with their induction by smokeless tobacco. One of the mechanisms of carcinogenicity is cytochrome P450-mediated α-hydroxylation, which leads to the formation of DNA and haemoglobin adducts that are commonly detected in users of tobacco.

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GLOSSARY

Bajjar: Dry snuff applied on the teeth and gums; also known as *tapkir*/*tapkeer*

Betel quid with tobacco: Mixture of betel leaf, areca nut, catechu, lime and tobacco; the tobacco may be used raw, sun-dried or roasted, then finely chopped, powdered and scented. Alternatively, tobacco may be boiled, made into a paste and scented with rose water or perfume. Also known as *paan* or *pan*

Bidi: Hand-rolled Indian cigarette consisting of flaked tobacco rolled in temburni leaf

Catechu: Astringent reddish-brown substance often smeared on the betel leaf used to wrap the betel quid ingredients. Also known as cutch

Chimó: Tobacco paste made from tobacco leaves, sodium bicarbonate, brown sugar, ashes from the Mamón tree (*Melicocca bijuga*), vanilla and anisette flavours. *Chimó* is specific to Venezuela.

Chutta: Reverse smoking

Creamy snuff: Tobacco toothpaste made from finely ground tobacco mixed with aromatic substances such as clove oil, glycerine, spearmint and menthol

Daqqa: Marijuana

Dry snuff: Fire-cured, fermented tobacco powder that may contain aroma and flavour additives. See also ***khaini***, ***neffa***

Gambir: Woody, climbing shrub native to China and other parts of Southeast Asia; the main ingredients of the extract are tannins and catechins.

Gudhaku: Tobacco paste consisting of powdered tobacco and molasses. Also spelled *gudaku*, *gudakhu*

Gul: Mixture of tobacco powder, molasses and other flavouring ingredients; sold as a powder and used as a dentrifice

Gutka: Commercially prepared betel quid which consists of sun-dried or roasted, finely chopped tobacco mixed with areca nut, slaked lime, catechu and flavouring ingredients. Also spelled *gutkha*

Hookah: Waterpipe used for smoking; also called *nargile*, *arghileh*, *sheesha*/*shisha*

Iq'mik: Fire-cured tobacco leaves mixed with punk ash derived from the burnt fungus that grows on birch tree bark

Khaini: Mixture of sun-dried, coarsely cut tobacco leaves crushed into smaller pieces and mixed with slaked lime. Also known as *chada*, *chadha* or *sada*, or as *surti* in Nepal and neighbouring parts of India

Khiwam: Thick paste prepared from tobacco leaf extract, rose extract water and powdered spices. Also spelled *qiwam*, *qimam*, *khimam*, *kiwam*

Liquid snuff: Substance used nasally in East Africa by the Nandi tribe

Loose-leaf: Made from cigar leaf tobacco that is air-cured, stemmed, cut or granulated and loosely packed; generally sweetened and flavoured with liquorice

Maras: Sun-dried tobacco leaf powder mixed with ash of oak, walnut or grapevine wood. Water is sprinkled on for humidification. *Maras* is used in Turkey.

Mawa: Sun-dried powdered tobacco flakes mixed with slaked lime and areca nut, and rubbed together

Mishri: Roasted tobacco powder used as a dentifrice. Also known as *masheri*, *misheri*

Moist snuff: Air- and fire-cured tobacco, including stems and leaves, that is powdered into fine particles or strips containing 20–55% moisture by weight. Also includes flavouring agents and chemical buffering agents

Naffa*/*Neffa: Dry snuff product used in Tunisia, Lybia and Algeria; known as *tenfeha* in Morocco and *nufha* in Algeria

Naswar: Mixture of powdered tobacco, ash, flavouring and colouring agents, oil and sometimes lime. Also known as *niswar*, *nass*, *nasswar*

Pattiwala: Sun-cured tobacco leaf used with or without lime

Plug: Chewing tobacco made from heavier grades of tobacco leaves harvested from the top of the plant. The stems of the leaves are removed, immersed in a mixture of liquorice and sugar, pressed into a plug, and reshaped into flat bars and rolls. Also known as 'pressed leaf'

Red tooth powder: Fine red tobacco powder mixed with herbs and flavouring agents. Also known as *lal dant manjan*

Shammah: Mixture of powdered tobacco, lime, ash, black pepper, oils and flavouring. Also known as *al-shammah*, *alqat* or Yemeni snuff

Slaked lime: Prepared from coral, sea shells (shell lime) or quarried limestone mixed with water. Red and white varieties are available in Taiwan (China), Thailand and Myanmar. Also known as *chuna* or *chunam*

Snuff: General term for finely cut or powdered, flavoured tobacco; snuff can be prepared as three types: moist snuff, consisting of fine-cut or long-cut tobacco particles, and dry snuff.

Snus: Swedish-type moist snuff consisting of finely ground dry tobacco mixed with aromatic substances, salt, water, humidifying agents and chemical buffering agents

Supari: Areca nut

Tamol: Fermented form of areca nut

Tobacco chewing gum: Chewing gum that contains tobacco, currently marketed in Japan.

Tobacco tablet: Commercially manufactured compressed tobacco product that contains approximately 1.3 mg nicotine. Also known as Cigalett® or Ariva®

Toombak: Fermented tobacco and sodium bicarbonate rolled into a ball; used in Sudan. Also known as *saffa*

Tuibur: Water through which tobacco smoke is passed, used for gargling; also known as *hidakphu*

Twist or roll: Made from either air-cured or fire-cured Burley tobacco leaves that are flavoured and twisted in form of a rope

Zarda: Flaked tobacco leaves boiled in water with lime and spices until evaporation, then dried and coloured with vegetable dyes, generally chewed mixed with finely cut areca nut and spices. Also known as *dokta*

LIST OF ABBREVIATIONS

α_7nAChR	α_7 nicotine acetylcholine receptor
α-HOMeNNAL	4-(hydroxymethylnitrosamino)-1-(3-pyridyl)-1-butanone
α-HOMeNNK	4-(hydroxymethylnitrosamino)-1-(3-pyridyl)-1-butanone
α-HOMethyleneNNAL	4-(methylnitrosamino)-1-(3-pyridyl)-1-(4-hydroxy)butanol
α-HOMethyleneNNK	4-(methylnitrosamino)-1-(3-pyridyl)-1-(4-hydroxy)butanone
4-HPO	4-hydroxy-1-phenyl-1-octanone
4-NQO	4-nitroquinoline *N*-oxide
6-HONNK	4-(methylnitrosamino)-1-[3-(6-hydroxypyridyl)]-1-butanone
8-OHdGuo	8-hydroxydeoxyguanosine
AFRO	WHO Regional Office for Africa
AGT	O^6-alkylguanine–DNA alkyltransferase
Akt	serine/threonine kinase
ALDH	aldehyde dehydrogenase
AMMN	acetoxymethylmethylnitrosamine
APC	adenomatous polyposis coli
Apo	apolipoprotein
AUC	area under the curve
BHA	butylated hydroxyanisole
BOP	*N*-nitrosobis(2-oxypropyl)amine
BrdU	bromodeoxyuridine
CDC	Centers for Disease Control and Prevention
CI	confidence interval
$_{cot}$	cotinine
COX	cyclooxygenase
CPS	American Cancer Society cohort
CS	cigarette smoke
Cyd	cytidine
CYP	cytochrome-P450
DAPK	death-associated protein kinase
DBP	diastolic blood pressure
dCyd	deoxycytidine

dGuo	deoxyguanosine
DMSO	dimethyl sulfoxide
dR	deoxyribose
dThd	deoxythymidine
EFTA	European Free Trade Association
EMRO	WHO Regional Office for the Eastern Mediterranean
EURO	WHO Regional Office for Europe
FA	filtered air
FCTC	Framework Convention on Tobacco Control
FMD	flow-mediated dilation
FTC	Federal Trade Commission
GED	General Educational Development
GSH	glutathione
GST	glutathione *S*-transferase
Guo	guanosine
GYTS	Global Youth Tobacco Survey
Hb	haemoglobin
HCFA	Health Care Financing Administration
HCPC	hamster cheek pouch carcinoma
HDL	high-density lipoprotein
HF	high fat
HONNN	hydroxy-NNN
HPB	4-hydroxy-1-(3-pyridyl)-1-butanone
HR	heart rate
HSV	herpes simplex virus
ICP	infected cell protein
IL	interleukin
ISO	International Standardization Organization
iso-NNAC	4-(methylnitrosamino)-4-(3-pyridyl)butyric acid
iso-NNAL	4-(methylnitrosamino)-4-(3-pyridyl)-1-butanol
K_m	Michaelis constant
LD_{50}	dose that causes 50% lethality
LDL	low-density lipoprotein
LF	low fat
MCC	mutated colon cancer
MDPH	Massachusetts Department of Public Health
MedGuo	methyldeoxyguanosine
MedThd	methyldeoxythymidine
Me-DZH	methanediazohydroxide
MeGua	methylguanine
MGMT	O^6-methylguanine–DNA methyltransferase
MNBA	4-(methylnitrosamino)butyric acid

MNPA	3-(methylnitrosamino)propionic acid
MNPhPA	2-(methylnitrosamino)-3-phenyl propionic acid
MNTCA	*N*-nitroso-2-methylthiazolidine-4-carboxylic acid
MONICA	Monitoring of Trends and Determinants in Cardiovascular Disease Project
NA	not available
NAB	*N*′-nitrosoanabasine
NAB-*N*-Gluc	NAB-*N*-glucuronide
NADPH	nicotinamide adenine dinucleotide phosphate
NAT	*N*′-nitrosoanatabine
NAT-*N*-Gluc	NAT-*N*-glucuronide
NAzCA	*N*-nitrosoazatidine-4-carboxylic acid
ND	not detected
NDEA	*N*-nitrosodiethylamine
NDELA	*N*-nitrosodiethanolamine
NDMA	*N*-nitrosodimethylamine
NE	not evaluted
NHANES	National Health and Nutrition Examination Survey
NHEFS	NHANES I Epidemiological Follow-up Study
NHIS	National Health Interview Study
NHPRO	*N*-nitrosohydroxyproline
$_{\text{nic}}$	nicotine
NMOR	*N*-nitrosomorpholine
NNA	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanal
NNAL	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol
NNAL-Gluc	NNAL-glucuronides
NNAL-*N*-Gluc	4-(methylnitrosamino)-1-(3-pyridyl-*N*-β-D-glucopyranurono-syl)-1-butalonium inner salt
NNAL-*O*-Gluc	4-(methylnitrosamino)-1-(3-pyridyl)-1-(*O*-β-D-glucopyranu-ronosyl)butane
NNK	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
(NNK)ADP$^+$	(NNK)adenosine dinucleotide phosphate
(NNK)ADPH	(NNK)adenosine dinucleotide phosphate (reduced form)
NNKOAc	4-(acetoxymethylnitrosamino)-1-(3-pyridyl)-1-butanone
NNN	*N*′-nitrosonornicotine
NNN-*N*-Gluc	NNN-*N*-glucuronide
NPIC	*N*-nitrosopipicolic acid
NPIP	*N*-nitrosopiperidine
NPRO	*N*-nitrosoproline
NPYR	*N*-nitrosopyrrolidine
NR	not reported
NSAR	*N*-nitrososarcosine

NTCA	*N*-nitrosothiazolidine-4-carboxylic acid
OGTT	oral glucose tolerance test
OSMF	oral submucosal fibrosis
PAH	polycyclic aromatic hydrocarbon
PEITC	phenylisothiocyanate
PG	prostaglandin
PHB-DZH	4-hydroxy-4-(3-pyridyl)-1-butanediazohydroxide
pKa	dissociation constant
POB-DZH	4-oxo-4-(3-pyridyl)-1-butanediazohydroxide
PREP	potential reduced exposure product
RDD	random-digit dialling
ROS	reactive oxygen species
SAR	Special Administrative Region
SBP	systolic blood pressure
SD	standard deviation
SE	standard error
SEARO	WHO Regional Office for South-East Asia
Sf9	*Spodoptera frugiperda* cells
Thd	thymidine
THF	tetrahydrofuran
TPA	12-*O*-tetradecanoylphorbol-13-acetate
TSNA	tobacco-specific *N*-nitrosamines
UAE	United Arab Emirates
UDP	Uridine diphosphate
UGT	UDP-glucuronyltransferase
VLDL	very low-density lipoprotein
V_{max}	Maximum velocity of an enzymatic reaction

CUMULATIVE CROSS INDEX TO *IARC MONOGRAPHS ON THE EVALUATION OF CARCINOGENIC RISKS TO HUMANS*

The volume, page and year of publication are given. References to corrigenda are given in parentheses.

A

A-α-C — *40*, 245 (1986); *Suppl. 7*, 56 (1987)
Acetaldehyde — *36*, 101 (1985) (*corr. 42*, 263); *Suppl. 7*, 77 (1987); *71*, 319 (1999)
Acetaldehyde formylmethylhydrazone (*see* Gyromitrin)
Acetamide — *7*, 197 (1974); *Suppl. 7*, 56, 389 (1987); *71*, 1211 (1999)
Acetaminophen (*see* Paracetamol)
Aciclovir — *76*, 47 (2000)
Acid mists (*see* Sulfuric acid and other strong inorganic acids, occupational exposures to mists and vapours from)
Acridine orange — *16*, 145 (1978); *Suppl. 7*, 56 (1987)
Acriflavinium chloride — *13*, 31 (1977); *Suppl. 7*, 56 (1987)
Acrolein — *19*, 479 (1979); *36*, 133 (1985); *Suppl. 7*, 78 (1987); *63*, 337 (1995) (*corr. 65*, 549)
Acrylamide — *39*, 41 (1986); *Suppl. 7*, 56 (1987); *60*, 389 (1994)
Acrylic acid — *19*, 47 (1979); *Suppl. 7*, 56 (1987); *71*, 1223 (1999)
Acrylic fibres — *19*, 86 (1979); *Suppl. 7*, 56 (1987)
Acrylonitrile — *19*, 73 (1979); *Suppl. 7*, 79 (1987); *71*, 43 (1999)
Acrylonitrile-butadiene-styrene copolymers — *19*, 91 (1979); *Suppl. 7*, 56 (1987)
Actinolite (*see* Asbestos)
Actinomycin D (*see also* Actinomycins) — *Suppl. 7*, 80 (1987)
Actinomycins — *10*, 29 (1976) (*corr. 42*, 255)
Adriamycin — *10*, 43 (1976); *Suppl. 7*, 82 (1987)
AF-2 — *31*, 47 (1983); *Suppl. 7*, 56 (1987)
Aflatoxins — *1*, 145 (1972) (*corr. 42*, 251); *10*, 51 (1976); *Suppl. 7*, 83 (1987); *56*, 245 (1993); *82*, 171 (2002)
Aflatoxin B_1 (*see* Aflatoxins)
Aflatoxin B_2 (*see* Aflatoxins)
Aflatoxin G_1 (*see* Aflatoxins)
Aflatoxin G_2 (*see* Aflatoxins)
Aflatoxin M_1 (*see* Aflatoxins)
Agaritine — *31*, 63 (1983); *Suppl. 7*, 56 (1987)
Alcohol drinking — *44* (1988)
Aldicarb — *53*, 93 (1991)

Aldrin	*5*, 25 (1974); *Suppl. 7*, 88 (1987)
Allyl chloride	*36*, 39 (1985); *Suppl. 7*, 56 (1987); *71*, 1231 (1999)
Allyl isothiocyanate	*36*, 55 (1985); *Suppl. 7*, 56 (1987); *73*, 37 (1999)
Allyl isovalerate	*36*, 69 (1985); *Suppl. 7*, 56 (1987); *71*, 1241 (1999)
Aluminium production	*34*, 37 (1984); *Suppl. 7*, 89 (1987)
Amaranth	*8*, 41 (1975); *Suppl. 7*, 56 (1987)
5-Aminoacenaphthene	*16*, 243 (1978); *Suppl. 7*, 56 (1987)
2-Aminoanthraquinone	*27*, 191 (1982); *Suppl. 7*, 56 (1987)
para-Aminoazobenzene	*8*, 53 (1975); *Suppl. 7*, 56, 390 (1987)
ortho-Aminoazotoluene	*8*, 61 (1975) (*corr. 42*, 254); *Suppl. 7*, 56 (1987)
para-Aminobenzoic acid	*16*, 249 (1978); *Suppl. 7*, 56 (1987)
4-Aminobiphenyl	*1*, 74 (1972) (*corr. 42*, 251); *Suppl. 7*, 91 (1987)
2-Amino-3,4-dimethylimidazo[4,5-*f*]quinoline (*see* MeIQ)	
2-Amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (*see* MeIQx)	
3-Amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (*see* Trp-P-1)	
2-Aminodipyrido[1,2-*a*:3′,2′-*d*]imidazole (*see* Glu-P-2)	
1-Amino-2-methylanthraquinone	*27*, 199 (1982); *Suppl. 7*, 57 (1987)
2-Amino-3-methylimidazo[4,5-*f*]quinoline (*see* IQ)	
2-Amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole (*see* Glu-P-1)	
2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (*see* PhIP)	
2-Amino-3-methyl-9*H*-pyrido[2,3-*b*]indole (*see* MeA-α-C)	
3-Amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (*see* Trp-P-2)	
2-Amino-5-(5-nitro-2-furyl)-1,3,4-thiadiazole	*7*, 143 (1974); *Suppl. 7*, 57 (1987)
2-Amino-4-nitrophenol	*57*, 167 (1993)
2-Amino-5-nitrophenol	*57*, 177 (1993)
4-Amino-2-nitrophenol	*16*, 43 (1978); *Suppl. 7*, 57 (1987)
2-Amino-5-nitrothiazole	*31*, 71 (1983); *Suppl. 7*, 57 (1987)
2-Amino-9*H*-pyrido[2,3-*b*]indole (*see* A-α-C)	
11-Aminoundecanoic acid	*39*, 239 (1986); *Suppl. 7*, 57 (1987)
Amitrole	*7*, 31 (1974); *41*, 293 (1986) (*corr. 52*, 513; *Suppl. 7*, 92 (1987); *79*, 381 (2001)
Ammonium potassium selenide (*see* Selenium and selenium compounds)	
Amorphous silica (*see also* Silica)	*42*, 39 (1987); *Suppl. 7*, 341 (1987); *68*, 41 (1997) (*corr. 81*, 383)
Amosite (*see* Asbestos)	
Ampicillin	*50*, 153 (1990)
Amsacrine	*76*, 317 (2000)
Anabolic steroids (*see* Androgenic (anabolic) steroids)	
Anaesthetics, volatile	*11*, 285 (1976); *Suppl. 7*, 93 (1987)
Analgesic mixtures containing phenacetin (*see also* Phenacetin)	*Suppl. 7*, 310 (1987)
Androgenic (anabolic) steroids	*Suppl. 7*, 96 (1987)
Angelicin and some synthetic derivatives (*see also* Angelicins)	*40*, 291 (1986)
Angelicin plus ultraviolet radiation (*see also* Angelicin and some synthetic derivatives)	*Suppl. 7*, 57 (1987)
Angelicins	*Suppl. 7*, 57 (1987)
Aniline	*4*, 27 (1974) (*corr. 42*, 252); *27*, 39 (1982); *Suppl. 7*, 99 (1987)

ortho-Anisidine *27*, 63 (1982); *Suppl. 7*, 57 (1987); *73*, 49 (1999)
para-Anisidine *27*, 65 (1982); *Suppl. 7*, 57 (1987)
Anthanthrene *32*, 95 (1983); *Suppl. 7*, 57 (1987)
Anthophyllite (*see* Asbestos)
Anthracene *32*, 105 (1983); *Suppl. 7*, 57 (1987)
Anthranilic acid *16*, 265 (1978); *Suppl. 7*, 57 (1987)
Anthraquinones *82*, 129 (2002)
Antimony trioxide *47*, 291 (1989)
Antimony trisulfide *47*, 291 (1989)
ANTU (*see* 1-Naphthylthiourea)
Apholate *9*, 31 (1975); *Suppl. 7*, 57 (1987)
para-Aramid fibrils *68*, 409 (1997)
Aramite® *5*, 39 (1974); *Suppl. 7*, 57 (1987)
Areca nut (*see also* Betel quid) *85*, 39 (2004)
Aristolochia species (*see also* Traditional herbal medicines) *82*, 69 (2002)
Aristolochic acids *82*, 69 (2002)
Arsanilic acid (*see* Arsenic and arsenic compounds)
Arsenic and arsenic compounds *1*, 41 (1972); *2*, 48 (1973); *23*, 39 (1980); *Suppl. 7*, 100 (1987)
Arsenic in drinking-water *84*, 39 (2004)
Arsenic pentoxide (*see* Arsenic and arsenic compounds)
Arsenic trioxide (*see* Arsenic in drinking-water)
Arsenic trisulfide (*see* Arsenic in drinking-water)
Arsine (*see* Arsenic and arsenic compounds)
Asbestos *2*, 17 (1973) (*corr. 42*, 252); *14* (1977) (*corr. 42*, 256); *Suppl. 7*, 106 (1987) (*corr. 45*, 283)
Atrazine *53*, 441 (1991); *73*, 59 (1999)
Attapulgite (*see* Palygorskite)
Auramine (technical-grade) *1*, 69 (1972) (*corr. 42*, 251); *Suppl. 7*, 118 (1987)
Auramine, manufacture of (*see also* Auramine, technical-grade) *Suppl. 7*, 118 (1987)
Aurothioglucose *13*, 39 (1977); *Suppl. 7*, 57 (1987)
Azacitidine *26*, 37 (1981); *Suppl. 7*, 57 (1987); *50*, 47 (1990)
5-Azacytidine (*see* Azacitidine)
Azaserine *10*, 73 (1976) (*corr. 42*, 255); *Suppl. 7*, 57 (1987)
Azathioprine *26*, 47 (1981); *Suppl. 7*, 119 (1987)
Aziridine *9*, 37 (1975); *Suppl. 7*, 58 (1987); *71*, 337 (1999)
2-(1-Aziridinyl)ethanol *9*, 47 (1975); *Suppl. 7*, 58 (1987)
Aziridyl benzoquinone *9*, 51 (1975); *Suppl. 7*, 58 (1987)
Azobenzene *8*, 75 (1975); *Suppl. 7*, 58 (1987)
AZT (*see* Zidovudine)

B

Barium chromate (*see* Chromium and chromium compounds)
Basic chromic sulfate (*see* Chromium and chromium compounds)
BCNU (*see* Bischloroethyl nitrosourea)
Benz[*a*]acridine *32*, 123 (1983); *Suppl. 7*, 58 (1987)

Benz[*c*]acridine *3*, 241 (1973); *32*, 129 (1983); *Suppl. 7*, 58 (1987)

Benzal chloride (*see also* α-Chlorinated toluenes and benzoyl chloride) *29*, 65 (1982); *Suppl. 7*, 148 (1987); *71*, 453 (1999)

Benz[*a*]anthracene *3*, 45 (1973); *32*, 135 (1983); *Suppl. 7*, 58 (1987)

Benzene *7*, 203 (1974) (*corr. 42*, 254); *29*, 93, 391 (1982); *Suppl. 7*, 120 (1987)

Benzidine *1*, 80 (1972); *29*, 149, 391 (1982); *Suppl. 7*, 123 (1987)

Benzidine-based dyes *Suppl. 7*, 125 (1987)

Benzo[*b*]fluoranthene *3*, 69 (1973); *32*, 147 (1983); *Suppl. 7*, 58 (1987)

Benzo[*j*]fluoranthene *3*, 82 (1973); *32*, 155 (1983); *Suppl. 7*, 58 (1987)

Benzo[*k*]fluoranthene *32*, 163 (1983); *Suppl. 7*, 58 (1987)

Benzo[*ghi*]fluoranthene *32*, 171 (1983); *Suppl. 7*, 58 (1987)

Benzo[*a*]fluorene *32*, 177 (1983); *Suppl. 7*, 58 (1987)

Benzo[*b*]fluorene *32*, 183 (1983); *Suppl. 7*, 58 (1987)

Benzo[*c*]fluorene *32*, 189 (1983); *Suppl. 7*, 58 (1987)

Benzofuran *63*, 431 (1995)

Benzo[*ghi*]perylene *32*, 195 (1983); *Suppl. 7*, 58 (1987)

Benzo[*c*]phenanthrene *32*, 205 (1983); *Suppl. 7*, 58 (1987)

Benzo[*a*]pyrene *3*, 91 (1973); *32*, 211 (1983) (*corr. 68*, 477); *Suppl. 7*, 58 (1987)

Benzo[*e*]pyrene *3*, 137 (1973); *32*, 225 (1983); *Suppl. 7*, 58 (1987)

1,4-Benzoquinone (see *para*-Quinone)

1,4-Benzoquinone dioxime *29*, 185 (1982); *Suppl. 7*, 58 (1987); *71*, 1251 (1999)

Benzotrichloride (*see also* α-Chlorinated toluenes and benzoyl chloride) *29*, 73 (1982); *Suppl. 7*, 148 (1987); *71*, 453 (1999)

Benzoyl chloride (*see also* α-Chlorinated toluenes and benzoyl chloride) *29*, 83 (1982) (*corr. 42*, 261); *Suppl. 7*, 126 (1987); *71*, 453 (1999)

Benzoyl peroxide *36*, 267 (1985); *Suppl. 7*, 58 (1987); *71*, 345 (1999)

Benzyl acetate *40*, 109 (1986); *Suppl. 7*, 58 (1987); *71*, 1255 (1999)

Benzyl chloride (*see also* α-Chlorinated toluenes and benzoyl chloride) *11*, 217 (1976) (*corr. 42*, 256); *29*, 49 (1982); *Suppl. 7*, 148 (1987); *71*, 453 (1999)

Benzyl violet 4B *16*, 153 (1978); *Suppl. 7*, 58 (1987)

Bertrandite (*see* Beryllium and beryllium compounds)

Beryllium and beryllium compounds *1*, 17 (1972); *23*, 143 (1980) (*corr. 42*, 260); *Suppl. 7*, 127 (1987); *58*, 41 (1993)

Beryllium acetate (*see* Beryllium and beryllium compounds)

Beryllium acetate, basic (*see* Beryllium and beryllium compounds)

Beryllium-aluminium alloy (*see* Beryllium and beryllium compounds)

Beryllium carbonate (*see* Beryllium and beryllium compounds)

Beryllium chloride (*see* Beryllium and beryllium compounds)

Beryllium-copper alloy (*see* Beryllium and beryllium compounds)

Beryllium-copper-cobalt alloy (*see* Beryllium and beryllium compounds)

Beryllium fluoride (*see* Beryllium and beryllium compounds)
Beryllium hydroxide (*see* Beryllium and beryllium compounds)
Beryllium-nickel alloy (*see* Beryllium and beryllium compounds)
Beryllium oxide (*see* Beryllium and beryllium compounds)
Beryllium phosphate (*see* Beryllium and beryllium compounds)
Beryllium silicate (*see* Beryllium and beryllium compounds)
Beryllium sulfate (*see* Beryllium and beryllium compounds)
Beryl ore (*see* Beryllium and beryllium compounds)
Betel quid with tobacco — *37*, 141 (1985); *Suppl. 7*, 128 (1987); *85*, 39 (2004)
Betel quid without tobacco — *37*, 141 (1985); *Suppl. 7*, 128 (1987); *85*, 39 (2004)
BHA (*see* Butylated hydroxyanisole)
BHT (*see* Butylated hydroxytoluene)
Bis(1-aziridinyl)morpholinophosphine sulfide — *9*, 55 (1975); *Suppl. 7*, 58 (1987)
2,2-Bis(bromomethyl)propane-1,3-diol — *77*, 455 (2000)
Bis(2-chloroethyl)ether — *9*, 117 (1975); *Suppl. 7*, 58 (1987); *71*, 1265 (1999)
N,*N*-Bis(2-chloroethyl)-2-naphthylamine — *4*, 119 (1974) (*corr. 42*, 253); *Suppl. 7*, 130 (1987)
Bischloroethyl nitrosourea (*see also* Chloroethyl nitrosoureas) — *26*, 79 (1981); *Suppl. 7*, 150 (1987)
1,2-Bis(chloromethoxy)ethane — *15*, 31 (1977); *Suppl. 7*, 58 (1987); *71*, 1271 (1999)
1,4-Bis(chloromethoxymethyl)benzene — *15*, 37 (1977); *Suppl. 7*, 58 (1987); *71*, 1273 (1999)
Bis(chloromethyl)ether — *4*, 231 (1974) (*corr. 42*, 253); *Suppl. 7*, 131 (1987)
Bis(2-chloro-1-methylethyl)ether — *41*, 149 (1986); *Suppl. 7*, 59 (1987); *71*, 1275 (1999)
Bis(2,3-epoxycyclopentyl)ether — *47*, 231 (1989); *71*, 1281 (1999)
Bisphenol A diglycidyl ether (*see also* Glycidyl ethers) — *71*, 1285 (1999)
Bisulfites (see Sulfur dioxide and some sulfites, bisulfites and metabisulfites)
Bitumens — *35*, 39 (1985); *Suppl. 7*, 133 (1987)
Bleomycins (*see also* Etoposide) — *26*, 97 (1981); *Suppl. 7*, 134 (1987)
Blue VRS — *16*, 163 (1978); *Suppl. 7*, 59 (1987)
Boot and shoe manufacture and repair — *25*, 249 (1981); *Suppl. 7*, 232 (1987)
Bracken fern — *40*, 47 (1986); *Suppl. 7*, 135 (1987)
Brilliant Blue FCF, disodium salt — *16*, 171 (1978) (*corr. 42*, 257); *Suppl. 7*, 59 (1987)
Bromochloroacetonitrile (*see also* Halogenated acetonitriles) — *71*, 1291 (1999)
Bromodichloromethane — *52*, 179 (1991); *71*, 1295 (1999)
Bromoethane — *52*, 299 (1991); *71*, 1305 (1999)
Bromoform — *52*, 213 (1991); *71*, 1309 (1999)
1,3-Butadiene — *39*, 155 (1986) (*corr. 42*, 264); *Suppl. 7*, 136 (1987); *54*, 237 (1992); *71*, 109 (1999)
1,4-Butanediol dimethanesulfonate — *4*, 247 (1974); *Suppl. 7*, 137 (1987)
2-Butoxyethanol — *88*, 329
1-*tert*-Butoxypropan-2-ol — *88*, 415
n-Butyl acrylate — *39*, 67 (1986); *Suppl. 7*, 59 (1987); *71*, 359 (1999)
Butylated hydroxyanisole — *40*, 123 (1986); *Suppl. 7*, 59 (1987)

Butylated hydroxytoluene — *40*, 161 (1986); *Suppl. 7*, 59 (1987)
Butyl benzyl phthalate — *29*, 193 (1982) (*corr. 42*, 261); *Suppl. 7*, 59 (1987); *73*, 115 (1999)
β-Butyrolactone — *11*, 225 (1976); *Suppl. 7*, 59 (1987); *71*, 1317 (1999)
γ-Butyrolactone — *11*, 231 (1976); *Suppl. 7*, 59 (1987); *71*, 367 (1999)

C

Cabinet-making (*see* Furniture and cabinet-making)
Cadmium acetate (*see* Cadmium and cadmium compounds)
Cadmium and cadmium compounds — *2*, 74 (1973); *11*, 39 (1976) (*corr. 42*, 255); *Suppl. 7*, 139 (1987); *58*, 119 (1993)
Cadmium chloride (*see* Cadmium and cadmium compounds)
Cadmium oxide (*see* Cadmium and cadmium compounds)
Cadmium sulfate (*see* Cadmium and cadmium compounds)
Cadmium sulfide (*see* Cadmium and cadmium compounds)
Caffeic acid — *56*, 115 (1993)
Caffeine — *51*, 291 (1991)
Calcium arsenate (*see* Arsenic in drinking-water)
Calcium chromate (*see* Chromium and chromium compounds)
Calcium cyclamate (*see* Cyclamates)
Calcium saccharin (*see* Saccharin)
Cantharidin — *10*, 79 (1976); *Suppl. 7*, 59 (1987)
Caprolactam — *19*, 115 (1979) (*corr. 42*, 258); *39*, 247 (1986) (*corr. 42*, 264); *Suppl. 7*, 59, 390 (1987); *71*, 383 (1999)
Captafol — *53*, 353 (1991)
Captan — *30*, 295 (1983); *Suppl. 7*, 59 (1987)
Carbaryl — *12*, 37 (1976); *Suppl. 7*, 59 (1987)
Carbazole — *32*, 239 (1983); *Suppl. 7*, 59 (1987); *71*, 1319 (1999)
3-Carbethoxypsoralen — *40*, 317 (1986); *Suppl. 7*, 59 (1987)
Carbon black — *3*, 22 (1973); *33*, 35 (1984); *Suppl. 7*, 142 (1987); *65*, 149 (1996)
Carbon tetrachloride — *1*, 53 (1972); *20*, 371 (1979); *Suppl. 7*, 143 (1987); *71*, 401 (1999)
Carmoisine — *8*, 83 (1975); *Suppl. 7*, 59 (1987)
Carpentry and joinery — *25*, 139 (1981); *Suppl. 7*, 378 (1987)
Carrageenan — *10*, 181 (1976) (*corr. 42*, 255); *31*, 79 (1983); *Suppl. 7*, 59 (1987)
Cassia occidentalis (*see* Traditional herbal medicines)
Catechol — *15*, 155 (1977); *Suppl. 7*, 59 (1987); *71*, 433 (1999)
CCNU (*see* 1-(2-Chloroethyl)-3-cyclohexyl-1-nitrosourea)
Ceramic fibres (*see* Man-made vitreous fibres)

Chemotherapy, combined, including alkylating agents (*see* MOPP and other combined chemotherapy including alkylating agents)
Chloral (*see also* Chloral hydrate) *63*, 245 (1995); *84*, 317 (2004)
Chloral hydrate *63*, 245 (1995); *84*, 317 (2004)
Chlorambucil *9*, 125 (1975); *26*, 115 (1981); *Suppl. 7*, 144 (1987)
Chloramine *84*, 295 (2004)
Chloramphenicol *10*, 85 (1976); *Suppl. 7*, 145 (1987); *50*, 169 (1990)
Chlordane (*see also* Chlordane/Heptachlor) *20*, 45 (1979) (*corr. 42*, 258)
Chlordane and Heptachlor *Suppl. 7*, 146 (1987); *53*, 115 (1991); *79*, 411 (2001)
Chlordecone *20*, 67 (1979); *Suppl. 7*, 59 (1987)
Chlordimeform *30*, 61 (1983); *Suppl. 7*, 59 (1987)
Chlorendic acid *48*, 45 (1990)
Chlorinated dibenzodioxins (other than TCDD) (*see also* Polychlorinated dibenzo-*para*-dioxins) *15*, 41 (1977); *Suppl. 7*, 59 (1987)
Chlorinated drinking-water *52*, 45 (1991)
Chlorinated paraffins *48*, 55 (1990)
α-Chlorinated toluenes and benzoyl chloride *Suppl. 7*, 148 (1987); *71*, 453 (1999)
Chlormadinone acetate *6*, 149 (1974); *21*, 365 (1979); *Suppl. 7*, 291, 301 (1987); *72*, 49 (1999)
Chlornaphazine (*see N,N*-Bis(2-chloroethyl)-2-naphthylamine)
Chloroacetonitrile (*see also* Halogenated acetonitriles) *71*, 1325 (1999)
para-Chloroaniline *57*, 305 (1993)
Chlorobenzilate *5*, 75 (1974); *30*, 73 (1983); *Suppl. 7*, 60 (1987)
Chlorodibromomethane *52*, 243 (1991); *71*, 1331 (1999)
3-Chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone *84*, 441 (2004)
Chlorodifluoromethane *41*, 237 (1986) (*corr. 51*, 483); *Suppl. 7*, 149 (1987); *71*, 1339 (1999)
Chloroethane *52*, 315 (1991); *71*, 1345 (1999)
1-(2-Chloroethyl)-3-cyclohexyl-1-nitrosourea (*see also* Chloroethyl nitrosoureas) *26*, 137 (1981) (*corr. 42*, 260); *Suppl. 7*, 150 (1987)
1-(2-Chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea (*see also* Chloroethyl nitrosoureas) *Suppl. 7*, 150 (1987)
Chloroethyl nitrosoureas *Suppl. 7*, 150 (1987)
Chlorofluoromethane *41*, 229 (1986); *Suppl. 7*, 60 (1987); *71*, 1351 (1999)
Chloroform *1*, 61 (1972); *20*, 401 (1979); *Suppl. 7*, 152 (1987); *73*, 131 (1999)
Chloromethyl methyl ether (technical-grade) (*see also* Bis(chloromethyl)ether) *4*, 239 (1974); *Suppl. 7*, 131 (1987)
(4-Chloro-2-methylphenoxy)acetic acid (*see* MCPA)
1-Chloro-2-methylpropene *63*, 315 (1995)
3-Chloro-2-methylpropene *63*, 325 (1995)
2-Chloronitrobenzene *65*, 263 (1996)
3-Chloronitrobenzene *65*, 263 (1996)
4-Chloronitrobenzene *65*, 263 (1996)
Chlorophenols (*see also* Polychlorophenols and their sodium salts) *Suppl. 7*, 154 (1987)

Chlorophenols (occupational exposures to) *41*, 319 (1986)
Chlorophenoxy herbicides *Suppl. 7*, 156 (1987)
Chlorophenoxy herbicides (occupational exposures to) *41*, 357 (1986)
4-Chloro-*ortho*-phenylenediamine *27*, 81 (1982); *Suppl. 7*, 60 (1987)
4-Chloro-*meta*-phenylenediamine *27*, 82 (1982); *Suppl. 7*, 60 (1987)
Chloroprene *19*, 131 (1979); *Suppl. 7*, 160 (1987); *71*, 227 (1999)
Chloropropham *12*, 55 (1976); *Suppl. 7*, 60 (1987)
Chloroquine *13*, 47 (1977); *Suppl. 7*, 60 (1987)
Chlorothalonil *30*, 319 (1983); *Suppl. 7*, 60 (1987); *73*, 183 (1999)
para-Chloro-*ortho*-toluidine and its strong acid salts (*see also* Chlordimeform) *16*, 277 (1978); *30*, 65 (1983); *Suppl. 7*, 60 (1987); *48*, 123 (1990); *77*, 323 (2000)
4-Chloro-*ortho*-toluidine (see *para*-chloro-*ortho*-toluidine)
5-Chloro-*ortho*-toluidine *77*, 341 (2000)
Chlorotrianisene (*see also* Nonsteroidal oestrogens) *21*, 139 (1979); *Suppl. 7*, 280 (1987)
2-Chloro-1,1,1-trifluoroethane *41*, 253 (1986); *Suppl. 7*, 60 (1987); *71*, 1355 (1999)
Chlorozotocin *50*, 65 (1990)
Cholesterol *10*, 99 (1976); *31*, 95 (1983); *Suppl. 7*, 161 (1987)
Chromic acetate (*see* Chromium and chromium compounds)
Chromic chloride (*see* Chromium and chromium compounds)
Chromic oxide (*see* Chromium and chromium compounds)
Chromic phosphate (*see* Chromium and chromium compounds)
Chromite ore (*see* Chromium and chromium compounds)
Chromium and chromium compounds (*see also* Implants, surgical) *2*, 100 (1973); *23*, 205 (1980); *Suppl. 7*, 165 (1987); *49*, 49 (1990) (*corr. 51*, 483)
Chromium carbonyl (*see* Chromium and chromium compounds)
Chromium potassium sulfate (*see* Chromium and chromium compounds)
Chromium sulfate (*see* Chromium and chromium compounds)
Chromium trioxide (*see* Chromium and chromium compounds)
Chrysazin (*see* Dantron)
Chrysene *3*, 159 (1973); *32*, 247 (1983); *Suppl. 7*, 60 (1987)
Chrysoidine *8*, 91 (1975); *Suppl. 7*, 169 (1987)
Chrysotile (*see* Asbestos)
CI Acid Orange 3 *57*, 121 (1993)
CI Acid Red 114 *57*, 247 (1993)
CI Basic Red 9 (*see also* Magenta) *57*, 215 (1993)
Ciclosporin *50*, 77 (1990)
CI Direct Blue 15 *57*, 235 (1993)
CI Disperse Yellow 3 (see Disperse Yellow 3)
Cimetidine *50*, 235 (1990)
Cinnamyl anthranilate *16*, 287 (1978); *31*, 133 (1983); *Suppl. 7*, 60 (1987); *77*, 177 (2000)
CI Pigment Red 3 *57*, 259 (1993)
CI Pigment Red 53:1 (*see* D&C Red No. 9)
Cisplatin (*see also* Etoposide) *26*, 151 (1981); *Suppl. 7*, 170 (1987)
Citrinin *40*, 67 (1986); *Suppl. 7*, 60 (1987)

Citrus Red No. 2	*8*, 101 (1975) (*corr. 42*, 254); *Suppl. 7*, 60 (1987)
Clinoptilolite (*see* Zeolites)	
Clofibrate	*24*, 39 (1980); *Suppl. 7*, 171 (1987); *66*, 391 (1996)
Clomiphene citrate	*21*, 551 (1979); *Suppl. 7*, 172 (1987)
Clonorchis sinensis (infection with)	*61*, 121 (1994)
Coal dust	*68*, 337 (1997)
Coal gasification	*34*, 65 (1984); *Suppl. 7*, 173 (1987)
Coal-tar pitches (*see also* Coal-tars)	*35*, 83 (1985); *Suppl. 7*, 174 (1987)
Coal-tars	*35*, 83 (1985); *Suppl. 7*, 175 (1987)
Cobalt[III] acetate (*see* Cobalt and cobalt compounds)	
Cobalt-aluminium-chromium spinel (*see* Cobalt and cobalt compounds)	
Cobalt and cobalt compounds (*see also* Implants, surgical)	*52*, 363 (1991)
Cobalt[II] chloride (*see* Cobalt and cobalt compounds)	
Cobalt-chromium alloy (*see* Chromium and chromium compounds)	
Cobalt-chromium-molybdenum alloys (*see* Cobalt and cobalt compounds)	
Cobalt metal powder (*see* Cobalt and cobalt compounds)	
Cobalt metal with tungsten carbide	*86*, 37 (2006)
Cobalt metal without tungsten carbide	*86*, 37 (2006)
Cobalt naphthenate (*see* Cobalt and cobalt compounds)	
Cobalt[II] oxide (*see* Cobalt and cobalt compounds)	
Cobalt[II,III] oxide (*see* Cobalt and cobalt compounds)	
Cobalt sulfate and other soluble cobalt(II) salts	*86*, 37 (2006)
Cobalt[II] sulfide (*see* Cobalt and cobalt compounds)	
Coffee	*51*, 41 (1991) (*corr. 52*, 513)
Coke production	*34*, 101 (1984); *Suppl. 7*, 176 (1987)
Combined oral contraceptives (*see* Oral contraceptives, combined)	
Conjugated equine oestrogens	*72*, 399 (1999)
Conjugated oestrogens (*see also* Steroidal oestrogens)	*21*, 147 (1979); *Suppl. 7*, 283 (1987)
Continuous glass filament (*see* Man-made vitreous fibres)	
Contraceptives, oral (*see* Oral contraceptives, combined; Sequential oral contraceptives)	
Copper 8-hydroxyquinoline	*15*, 103 (1977); *Suppl. 7*, 61 (1987)
Coronene	*32*, 263 (1983); *Suppl. 7*, 61 (1987)
Coumarin	*10*, 113 (1976); *Suppl. 7*, 61 (1987); 77, 193 (2000)
Creosotes (*see also* Coal-tars)	*35*, 83 (1985); *Suppl. 7*, 177 (1987)
meta-Cresidine	*27*, 91 (1982); *Suppl. 7*, 61 (1987)
para-Cresidine	*27*, 92 (1982); *Suppl. 7*, 61 (1987)
Cristobalite (*see* Crystalline silica)	
Crocidolite (*see* Asbestos)	
Crotonaldehyde	*63*, 373 (1995) (*corr. 65*, 549)
Crude oil	*45*, 119 (1989)
Crystalline silica (*see* also Silica)	*42*, 39 (1987); *Suppl. 7*, 341 (1987); *68*, 41 (1997) (*corr. 81*, 383)
Cycasin (*see also* Methylazoxymethanol)	*1*, 157 (1972) (*corr. 42*, 251); *10*, 121 (1976); *Suppl. 7*, 61 (1987)
Cyclamates	*22*, 55 (1980); *Suppl. 7*, 178 (1987); *73*, 195 (1999)

Cyclamic acid (*see* Cyclamates)	
Cyclochlorotine	*10*, 139 (1976); *Suppl. 7*, 61 (1987)
Cyclohexanone	*47*, 157 (1989); *71*, 1359 (1999)
Cyclohexylamine (*see* Cyclamates)	
Cyclopenta[*cd*]pyrene	*32*, 269 (1983); *Suppl. 7*, 61 (1987)
Cyclopropane (*see* Anaesthetics, volatile)	
Cyclophosphamide	*9*, 135 (1975); *26*, 165 (1981); *Suppl. 7*, 182 (1987)
Cyproterone acetate	*72*, 49 (1999)

D

2,4-D (*see also* Chlorophenoxy herbicides; Chlorophenoxy herbicides, occupational exposures to)	*15*, 111 (1977)
Dacarbazine	*26*, 203 (1981); *Suppl. 7*, 184 (1987)
Dantron	*50*, 265 (1990) (*corr. 59*, 257)
D&C Red No. 9	*8*, 107 (1975); *Suppl. 7*, 61 (1987); *57*, 203 (1993)
Dapsone	*24*, 59 (1980); *Suppl. 7*, 185 (1987)
Daunomycin	*10*, 145 (1976); *Suppl. 7*, 61 (1987)
DDD (*see* DDT)	
DDE (*see* DDT)	
DDT	*5*, 83 (1974) (*corr. 42*, 253); *Suppl. 7*, 186 (1987); *53*, 179 (1991)
Decabromodiphenyl oxide	*48*, 73 (1990); *71*, 1365 (1999)
Deltamethrin	*53*, 251 (1991)
Deoxynivalenol (*see* Toxins derived from *Fusarium graminearum*, *F. culmorum* and *F. crookwellense*)	
Diacetylaminoazotoluene	*8*, 113 (1975); *Suppl. 7*, 61 (1987)
N,*N*′-Diacetylbenzidine	*16*, 293 (1978); *Suppl. 7*, 61 (1987)
Diallate	*12*, 69 (1976); *30*, 235 (1983); *Suppl. 7*, 61 (1987)
2,4-Diaminoanisole and its salts	*16*, 51 (1978); *27*, 103 (1982); *Suppl. 7*, 61 (1987); *79*, 619 (2001)
4,4′-Diaminodiphenyl ether	*16*, 301 (1978); *29*, 203 (1982); *Suppl. 7*, 61 (1987)
1,2-Diamino-4-nitrobenzene	*16*, 63 (1978); *Suppl. 7*, 61 (1987)
1,4-Diamino-2-nitrobenzene	*16*, 73 (1978); *Suppl. 7*, 61 (1987); *57*, 185 (1993)
2,6-Diamino-3-(phenylazo)pyridine (*see* Phenazopyridine hydrochloride)	
2,4-Diaminotoluene (*see also* Toluene diisocyanates)	*16*, 83 (1978); *Suppl. 7*, 61 (1987)
2,5-Diaminotoluene (*see also* Toluene diisocyanates)	*16*, 97 (1978); *Suppl. 7*, 61 (1987)
ortho-Dianisidine (*see* 3,3′-Dimethoxybenzidine)	
Diatomaceous earth, uncalcined (*see* Amorphous silica)	
Diazepam	*13*, 57 (1977); *Suppl. 7*, 189 (1987); *66*, 37 (1996)
Diazomethane	*7*, 223 (1974); *Suppl. 7*, 61 (1987)
Dibenz[*a*,*h*]acridine	*3*, 247 (1973); *32*, 277 (1983); *Suppl. 7*, 61 (1987)
Dibenz[*a*,*j*]acridine	*3*, 254 (1973); *32*, 283 (1983); *Suppl. 7*, 61 (1987)

Dibenz[*a*,*c*]anthracene — *32*, 289 (1983) (*corr. 42*, 262); *Suppl. 7*, 61 (1987)
Dibenz[*a*,*h*]anthracene — *3*, 178 (1973) (*corr. 43*, 261); *32*, 299 (1983); *Suppl. 7*, 61 (1987)
Dibenz[*a*,*j*]anthracene — *32*, 309 (1983); *Suppl. 7*, 61 (1987)
7*H*-Dibenzo[*c*,*g*]carbazole — *3*, 260 (1973); *32*, 315 (1983); *Suppl. 7*, 61 (1987)
Dibenzodioxins, chlorinated (other than TCDD) (*see* Chlorinated dibenzodioxins (other than TCDD))
Dibenzo[*a*,*e*]fluoranthene — *32*, 321 (1983); *Suppl. 7*, 61 (1987)
Dibenzo[*h*,*rst*]pentaphene — *3*, 197 (1973); *Suppl. 7*, 62 (1987)
Dibenzo[*a*,*e*]pyrene — *3*, 201 (1973); *32*, 327 (1983); *Suppl. 7*, 62 (1987)
Dibenzo[*a*,*h*]pyrene — *3*, 207 (1973); *32*, 331 (1983); *Suppl. 7*, 62 (1987)
Dibenzo[*a*,*i*]pyrene — *3*, 215 (1973); *32*, 337 (1983); *Suppl. 7*, 62 (1987)
Dibenzo[*a*,*l*]pyrene — *3*, 224 (1973); *32*, 343 (1983); *Suppl. 7*, 62 (1987)
Dibenzo-*para*-dioxin — *69*, 33 (1997)
Dibromoacetonitrile (*see also* Halogenated acetonitriles) — *71*, 1369 (1999)
1,2-Dibromo-3-chloropropane — *15*, 139 (1977); *20*, 83 (1979); *Suppl. 7*, 191 (1987); *71*, 479 (1999)
1,2-Dibromoethane (*see* Ethylene dibromide)
2,3-Dibromopropan-1-ol — *77*, 439 (2000)
Dichloroacetic acid — *63*, 271 (1995); *84*, 359 (2004)
Dichloroacetonitrile (*see also* Halogenated acetonitriles) — *71*, 1375 (1999)
Dichloroacetylene — *39*, 369 (1986); *Suppl. 7*, 62 (1987); *71*, 1381 (1999)
ortho-Dichlorobenzene — *7*, 231 (1974); *29*, 213 (1982); *Suppl. 7*, 192 (1987); *73*, 223 (1999)
meta-Dichlorobenzene — *73*, 223 (1999)
para-Dichlorobenzene — *7*, 231 (1974); *29*, 215 (1982); *Suppl. 7*, 192 (1987); *73*, 223 (1999)
3,3′-Dichlorobenzidine — *4*, 49 (1974); *29*, 239 (1982); *Suppl. 7*, 193 (1987)
trans-1,4-Dichlorobutene — *15*, 149 (1977); *Suppl. 7*, 62 (1987); *71*, 1389 (1999)
3,3′-Dichloro-4,4′-diaminodiphenyl ether — *16*, 309 (1978); *Suppl. 7*, 62 (1987)
1,2-Dichloroethane — *20*, 429 (1979); *Suppl. 7*, 62 (1987); *71*, 501 (1999)
Dichloromethane — *20*, 449 (1979); *41*, 43 (1986); *Suppl. 7*, 194 (1987); *71*, 251 (1999)
2,4-Dichlorophenol (*see* Chlorophenols; Chlorophenols, occupational exposures to; Polychlorophenols and their sodium salts)
(2,4-Dichlorophenoxy)acetic acid (*see* 2,4-D)
2,6-Dichloro-*para*-phenylenediamine — *39*, 325 (1986); *Suppl. 7*, 62 (1987)
1,2-Dichloropropane — *41*, 131 (1986); *Suppl. 7*, 62 (1987); *71*, 1393 (1999)
1,3-Dichloropropene (technical-grade) — *41*, 113 (1986); *Suppl. 7*, 195 (1987); *71*, 933 (1999)

Dichlorvos	*20*, 97 (1979); *Suppl. 7*, 62 (1987); *53*, 267 (1991)
Dicofol	*30*, 87 (1983); *Suppl. 7*, 62 (1987)
Dicyclohexylamine (*see* Cyclamates)	
Didanosine	*76*, 153 (2000)
Dieldrin	*5*, 125 (1974); *Suppl. 7*, 196 (1987)
Dienoestrol (*see also* Nonsteroidal oestrogens)	*21*, 161 (1979); *Suppl. 7*, 278 (1987)
Diepoxybutane (*see also* 1,3-Butadiene)	*11*, 115 (1976) (*corr. 42*, 255); *Suppl. 7*, 62 (1987); *71*, 109 (1999)
Diesel and gasoline engine exhausts	*46*, 41 (1989)
Diesel fuels	*45*, 219 (1989) (*corr. 47*, 505)
Diethanolamine	*77*, 349 (2000)
Diethyl ether (*see* Anaesthetics, volatile)	
Di(2-ethylhexyl) adipate	*29*, 257 (1982); *Suppl. 7*, 62 (1987); *77*, 149 (2000)
Di(2-ethylhexyl) phthalate	*29*, 269 (1982) (*corr. 42*, 261); *Suppl. 7*, 62 (1987); *77*, 41 (2000)
1,2-Diethylhydrazine	*4*, 153 (1974); *Suppl. 7*, 62 (1987); *71*, 1401 (1999)
Diethylstilboestrol	*6*, 55 (1974); *21*, 173 (1979) (*corr. 42*, 259); *Suppl. 7*, 273 (1987)
Diethylstilboestrol dipropionate (*see* Diethylstilboestrol)	
Diethyl sulfate	*4*, 277 (1974); *Suppl. 7*, 198 (1987); *54*, 213 (1992); *71*, 1405 (1999)
N,*N*′-Diethylthiourea	*79*, 649 (2001)
Diglycidyl resorcinol ether	*11*, 125 (1976); *36*, 181 (1985); *Suppl. 7*, 62 (1987); *71*, 1417 (1999)
Dihydrosafrole	*1*, 170 (1972); *10*, 233 (1976) *Suppl. 7*, 62 (1987)
1,8-Dihydroxyanthraquinone (*see* Dantron)	
Dihydroxybenzenes (*see* Catechol; Hydroquinone; Resorcinol)	
1,3-Dihydroxy-2-hydroxymethylanthraquinone	*82*, 129 (2002)
Dihydroxymethylfuratrizine	*24*, 77 (1980); *Suppl. 7*, 62 (1987)
Diisopropyl sulfate	*54*, 229 (1992); *71*, 1421 (1999)
Dimethisterone (*see also* Progestins; Sequential oral contraceptives)	*6*, 167 (1974); *21*, 377 (1979))
Dimethoxane	*15*, 177 (1977); *Suppl. 7*, 62 (1987)
3,3′-Dimethoxybenzidine	*4*, 41 (1974); *Suppl. 7*, 198 (1987)
3,3′-Dimethoxybenzidine-4,4′-diisocyanate	*39*, 279 (1986); *Suppl. 7*, 62 (1987)
para-Dimethylaminoazobenzene	*8*, 125 (1975); *Suppl. 7*, 62 (1987)
para-Dimethylaminoazobenzenediazo sodium sulfonate	*8*, 147 (1975); *Suppl. 7*, 62 (1987)
trans-2-[(Dimethylamino)methylimino]-5-[2-(5-nitro-2-furyl)-vinyl]-1,3,4-oxadiazole	*7*, 147 (1974) (*corr. 42*, 253); *Suppl. 7*, 62 (1987)
4,4′-Dimethylangelicin plus ultraviolet radiation (*see also* Angelicin and some synthetic derivatives)	*Suppl. 7*, 57 (1987)
4,5′-Dimethylangelicin plus ultraviolet radiation (*see also* Angelicin and some synthetic derivatives)	*Suppl. 7*, 57 (1987)
2,6-Dimethylaniline	*57*, 323 (1993)
N,*N*-Dimethylaniline	*57*, 337 (1993)
Dimethylarsinic acid (*see* Arsenic and arsenic compounds)	
3,3′-Dimethylbenzidine	*1*, 87 (1972); *Suppl. 7*, 62 (1987)

Dimethylcarbamoyl chloride — *12*, 77 (1976); *Suppl. 7*, 199 (1987); *71*, 531 (1999)
Dimethylformamide — *47*, 171 (1989); *71*, 545 (1999)
1,1-Dimethylhydrazine — *4*, 137 (1974); *Suppl. 7*, 62 (1987); *71*, 1425 (1999)
1,2-Dimethylhydrazine — *4*, 145 (1974) (*corr. 42*, 253); *Suppl. 7*, 62 (1987); *71*, 947 (1999)
Dimethyl hydrogen phosphite — *48*, 85 (1990); *71*, 1437 (1999)
1,4-Dimethylphenanthrene — *32*, 349 (1983); *Suppl. 7*, 62 (1987)
Dimethyl sulfate — *4*, 271 (1974); *Suppl. 7*, 200 (1987); *71*, 575 (1999)
3,7-Dinitrofluoranthene — *46*, 189 (1989); *65*, 297 (1996)
3,9-Dinitrofluoranthene — *46*, 195 (1989); *65*, 297 (1996)
1,3-Dinitropyrene — *46*, 201 (1989)
1,6-Dinitropyrene — *46*, 215 (1989)
1,8-Dinitropyrene — *33*, 171 (1984); *Suppl. 7*, 63 (1987); *46*, 231 (1989)
Dinitrosopentamethylenetetramine — *11*, 241 (1976); *Suppl. 7*, 63 (1987)
2,4-Dinitrotoluene — *65*, 309 (1996) (*corr. 66*, 485)
2,6-Dinitrotoluene — *65*, 309 (1996) (*corr. 66*, 485)
3,5-Dinitrotoluene — *65*, 309 (1996)
1,4-Dioxane — *11*, 247 (1976); *Suppl. 7*, 201 (1987); *71*, 589 (1999)
2,4′-Diphenyldiamine — *16*, 313 (1978); *Suppl. 7*, 63 (1987)
Direct Black 38 (*see also* Benzidine-based dyes) — *29*, 295 (1982) (*corr. 42*, 261)
Direct Blue 6 (*see also* Benzidine-based dyes) — *29*, 311 (1982)
Direct Brown 95 (*see also* Benzidine-based dyes) — *29*, 321 (1982)
Disperse Blue 1 — *48*, 139 (1990)
Disperse Yellow 3 — *8*, 97 (1975); *Suppl. 7*, 60 (1987); *48*, 149 (1990)
Disulfiram — *12*, 85 (1976); *Suppl. 7*, 63 (1987)
Dithranol — *13*, 75 (1977); *Suppl. 7*, 63 (1987)
Divinyl ether (*see* Anaesthetics, volatile)
Doxefazepam — *66*, 97 (1996)
Doxylamine succinate — *79*, 145 (2001)
Droloxifene — *66*, 241 (1996)
Dry cleaning — *63*, 33 (1995)
Dulcin — *12*, 97 (1976); *Suppl. 7*, 63 (1987)

E

Endrin — *5*, 157 (1974); *Suppl. 7*, 63 (1987)
Enflurane (*see* Anaesthetics, volatile)
Eosin — *15*, 183 (1977); *Suppl. 7*, 63 (1987)
Epichlorohydrin — *11*, 131 (1976) (*corr. 42*, 256); *Suppl. 7*, 202 (1987); *71*, 603 (1999)
1,2-Epoxybutane — *47*, 217 (1989); *71*, 629 (1999)
1-Epoxyethyl-3,4-epoxycyclohexane (*see* 4-Vinylcyclohexene diepoxide)
3,4-Epoxy-6-methylcyclohexylmethyl 3,4-epoxy-6-methylcyclohexane carboxylate — *11*, 147 (1976); *Suppl. 7*, 63 (1987); *71*, 1441 (1999)
cis-9,10-Epoxystearic acid — *11*, 153 (1976); *Suppl. 7*, 63 (1987); *71*, 1443 (1999)

Epstein-Barr virus *70*, 47 (1997)
d-Equilenin *72*, 399 (1999)
Equilin *72*, 399 (1999)
Erionite *42*, 225 (1987); *Suppl. 7*, 203 (1987)
Estazolam *66*, 105 (1996)
Ethinyloestradiol *6*, 77 (1974); *21*, 233 (1979); *Suppl. 7*, 286 (1987); *72*, 49 (1999)
Ethionamide *13*, 83 (1977); *Suppl. 7*, 63 (1987)
Ethyl acrylate *19*, 57 (1979); *39*, 81 (1986); *Suppl. 7*, 63 (1987); *71*, 1447 (1999)
Ethylbenzene *77*, 227 (2000)
Ethylene *19*, 157 (1979); *Suppl. 7*, 63 (1987); *60*, 45 (1994); *71*, 1447 (1999)
Ethylene dibromide *15*, 195 (1977); *Suppl. 7*, 204 (1987); *71*, 641 (1999)
Ethylene oxide *11*, 157 (1976); *36*, 189 (1985) (*corr. 42*, 263); *Suppl. 7*, 205 (1987); *60*, 73 (1994)
Ethylene sulfide *11*, 257 (1976); *Suppl. 7*, 63 (1987)
Ethylenethiourea *7*, 45 (1974); *Suppl. 7*, 207 (1987); *79*, 659 (2001)
2-Ethylhexyl acrylate *60*, 475 (1994)
Ethyl methanesulfonate *7*, 245 (1974); *Suppl. 7*, 63 (1987)
N-Ethyl-*N*-nitrosourea *1*, 135 (1972); *17*, 191 (1978); *Suppl. 7*, 63 (1987)
Ethyl selenac (*see also* Selenium and selenium compounds) *12*, 107 (1976); *Suppl. 7*, 63 (1987)
Ethyl tellurac *12*, 115 (1976); *Suppl. 7*, 63 (1987)
Ethynodiol diacetate *6*, 173 (1974); *21*, 387 (1979); *Suppl. 7*, 292 (1987); *72*, 49 (1999)
Etoposide *76*, 177 (2000)
Eugenol *36*, 75 (1985); *Suppl. 7*, 63 (1987)
Evans blue *8*, 151 (1975); *Suppl. 7*, 63 (1987)
Extremely low-frequency electric fields *80* (2002)
Extremely low-frequency magnetic fields *80* (2002)

F

Fast Green FCF *16*, 187 (1978); *Suppl. 7*, 63 (1987)
Fenvalerate *53*, 309 (1991)
Ferbam *12*, 121 (1976) (*corr. 42*, 256); *Suppl. 7*, 63 (1987)
Ferric oxide *1*, 29 (1972); *Suppl. 7*, 216 (1987)
Ferrochromium (*see* Chromium and chromium compounds)
Fluometuron *30*, 245 (1983); *Suppl. 7*, 63 (1987)
Fluoranthene *32*, 355 (1983); *Suppl. 7*, 63 (1987)
Fluorene *32*, 365 (1983); *Suppl. 7*, 63 (1987)
Fluorescent lighting (exposure to) (*see* Ultraviolet radiation)
Fluorides (inorganic, used in drinking-water) *27*, 237 (1982); *Suppl. 7*, 208 (1987)

5-Fluorouracil *26*, 217 (1981); *Suppl. 7*, 210 (1987)
Fluorspar (*see* Fluorides)
Fluosilicic acid (*see* Fluorides)
Fluroxene (*see* Anaesthetics, volatile)
Foreign bodies *74* (1999)
Formaldehyde *29*, 345 (1982); *Suppl. 7*, 211 (1987); *62*, 217 (1995) (*corr. 65*, 549; *corr. 66*, 485); *88*, 39
2-(2-Formylhydrazino)-4-(5-nitro-2-furyl)thiazole *7*, 151 (1974) (*corr. 42*, 253); *Suppl. 7*, 63 (1987)
Frusemide (*see* Furosemide)
Fuel oils (heating oils) *45*, 239 (1989) (*corr. 47*, 505)
Fumonisin B_1 (*see* also Toxins derived from *Fusarium moniliforme*) *82*, 301 (2002)
Fumonisin B_2 (*see* Toxins derived from *Fusarium moniliforme*)
Furan *63*, 393 (1995)
Furazolidone *31*, 141 (1983); *Suppl. 7*, 63 (1987)
Furfural *63*, 409 (1995)
Furniture and cabinet-making *25*, 99 (1981); *Suppl. 7*, 380 (1987)
Furosemide *50*, 277 (1990)
2-(2-Furyl)-3-(5-nitro-2-furyl)acrylamide (*see* AF-2)
Fusarenon-X (*see* Toxins derived from *Fusarium graminearum*, *F. culmorum* and *F. crookwellense*)
Fusarenone-X (*see* Toxins derived from *Fusarium graminearum*, *F. culmorum* and *F. crookwellense*)
Fusarin C (*see* Toxins derived from *Fusarium moniliforme*)

G

Gallium arsenide *86*, 163 (2006)
Gamma (γ)-radiation *75*, 121 (2000)
Gasoline *45*, 159 (1989) (*corr. 47*, 505)
Gasoline engine exhaust (*see* Diesel and gasoline engine exhausts)
Gemfibrozil *66*, 427 (1996)
Glass fibres (*see* Man-made mineral fibres)
Glass manufacturing industry, occupational exposures in *58*, 347 (1993)
Glass wool (*see* Man-made vitreous fibres)
Glass filaments (*see* Man-made mineral fibres)
Glu-P-1 *40*, 223 (1986); *Suppl. 7*, 64 (1987)
Glu-P-2 *40*, 235 (1986); *Suppl. 7*, 64 (1987)
L-Glutamic acid, 5-[2-(4-hydroxymethyl)phenylhydrazide] (*see* Agaritine)
Glycidaldehyde *11*, 175 (1976); *Suppl. 7*, 64 (1987); *71*, 1459 (1999)
Glycidol *77*, 469 (2000)
Glycidyl ethers *47*, 237 (1989); *71*, 1285, 1417, 1525, 1539 (1999)
Glycidyl oleate *11*, 183 (1976); *Suppl. 7*, 64 (1987)
Glycidyl stearate *11*, 187 (1976); *Suppl. 7*, 64 (1987)
Griseofulvin *10*, 153 (1976); *Suppl. 7*, 64, 391 (1987); *79*, 289 (2001)
Guinea Green B *16*, 199 (1978); *Suppl. 7*, 64 (1987)

Gyromitrin	*31*, 163 (1983); *Suppl. 7*, 64, 391 (1987)

H

Haematite	*1*, 29 (1972); *Suppl. 7*, 216 (1987)
Haematite and ferric oxide	*Suppl. 7*, 216 (1987)
Haematite mining, underground, with exposure to radon	*1*, 29 (1972); *Suppl. 7*, 216 (1987)
Hairdressers and barbers (occupational exposure as)	*57*, 43 (1993)
Hair dyes, epidemiology of	*16*, 29 (1978); *27*, 307 (1982);
Halogenated acetonitriles	*52*, 269 (1991); *71*, 1325, 1369, 1375, 1533 (1999)
Halothane (*see* Anaesthetics, volatile)	
HC Blue No. 1	*57*, 129 (1993)
HC Blue No. 2	*57*, 143 (1993)
α-HCH (*see* Hexachlorocyclohexanes)	
β-HCH (*see* Hexachlorocyclohexanes)	
γ-HCH (*see* Hexachlorocyclohexanes)	
HC Red No. 3	*57*, 153 (1993)
HC Yellow No. 4	*57*, 159 (1993)
Heating oils (*see* Fuel oils)	
Helicobacter pylori (infection with)	*61*, 177 (1994)
Hepatitis B virus	*59*, 45 (1994)
Hepatitis C virus	*59*, 165 (1994)
Hepatitis D virus	*59*, 223 (1994)
Heptachlor (*see also* Chlordane/Heptachlor)	*5*, 173 (1974); *20*, 129 (1979)
Hexachlorobenzene	*20*, 155 (1979); *Suppl. 7*, 219 (1987); *79*, 493 (2001)
Hexachlorobutadiene	*20*, 179 (1979); *Suppl. 7*, 64 (1987); *73*, 277 (1999)
Hexachlorocyclohexanes	*5*, 47 (1974); *20*, 195 (1979) (*corr. 42*, 258); *Suppl. 7*, 220 (1987)
Hexachlorocyclohexane, technical-grade (*see* Hexachlorocyclohexanes)	
Hexachloroethane	*20*, 467 (1979); *Suppl. 7*, 64 (1987); *73*, 295 (1999)
Hexachlorophene	*20*, 241 (1979); *Suppl. 7*, 64 (1987)
Hexamethylphosphoramide	*15*, 211 (1977); *Suppl. 7*, 64 (1987); *71*, 1465 (1999)
Hexoestrol (*see also* Nonsteroidal oestrogens)	*Suppl. 7*, 279 (1987)
Hormonal contraceptives, progestogens only	*72*, 339 (1999)
Human herpesvirus 8	*70*, 375 (1997)
Human immunodeficiency viruses	*67*, 31 (1996)
Human papillomaviruses	*64* (1995) (*corr. 66*, 485)
Human T-cell lymphotropic viruses	*67*, 261 (1996)
Hycanthone mesylate	*13*, 91 (1977); *Suppl. 7*, 64 (1987)
Hydralazine	*24*, 85 (1980); *Suppl. 7*, 222 (1987)
Hydrazine	*4*, 127 (1974); *Suppl. 7*, 223 (1987); *71*, 991 (1999)
Hydrochloric acid	*54*, 189 (1992)
Hydrochlorothiazide	*50*, 293 (1990)
Hydrogen peroxide	*36*, 285 (1985); *Suppl. 7*, 64 (1987); *71*, 671 (1999)

Hydroquinone — *15*, 155 (1977); *Suppl. 7*, 64 (1987); *71*, 691 (1999)
1-Hydroxyanthraquinone — *82*, 129 (2002)
4-Hydroxyazobenzene — *8*, 157 (1975); *Suppl. 7*, 64 (1987)
17α-Hydroxyprogesterone caproate (*see also* Progestins) — *21*, 399 (1979) (*corr. 42*, 259)
8-Hydroxyquinoline — *13*, 101 (1977); *Suppl. 7*, 64 (1987)
8-Hydroxysenkirkine — *10*, 265 (1976); *Suppl. 7*, 64 (1987)
Hydroxyurea — *76*, 347 (2000)
Hypochlorite salts — *52*, 159 (1991)

I

Implants, surgical — *74*, 1999
Indeno[1,2,3-*cd*]pyrene — *3*, 229 (1973); *32*, 373 (1983); *Suppl. 7*, 64 (1987)
Indium phosphide — *86*, 197 (2006)
Inorganic acids (*see* Sulfuric acid and other strong inorganic acids, occupational exposures to mists and vapours from)
Inorganic lead compounds — *Suppl. 7*, 230 (1987); *87* (2006)
Insecticides, occupational exposures in spraying and application of — *53*, 45 (1991)
Insulation glass wool (*see* Man-made vitreous fibres)
Involuntary smoking — *83*, 1189 (2004)
Ionizing radiation (*see* Neutrons, γ- and X-radiation)
IQ — *40*, 261 (1986); *Suppl. 7*, 64 (1987); *56*, 165 (1993)
Iron and steel founding — *34*, 133 (1984); *Suppl. 7*, 224 (1987)
Iron-dextran complex — *2*, 161 (1973); *Suppl. 7*, 226 (1987)
Iron-dextrin complex — *2*, 161 (1973) (*corr. 42*, 252); *Suppl. 7*, 64 (1987)
Iron oxide (*see* Ferric oxide)
Iron oxide, saccharated (*see* Saccharated iron oxide)
Iron sorbitol-citric acid complex — *2*, 161 (1973); *Suppl. 7*, 64 (1987)
Isatidine — *10*, 269 (1976); *Suppl. 7*, 65 (1987)
Isoflurane (*see* Anaesthetics, volatile)
Isoniazid (*see* Isonicotinic acid hydrazide)
Isonicotinic acid hydrazide — *4*, 159 (1974); *Suppl. 7*, 227 (1987)
Isophosphamide — *26*, 237 (1981); *Suppl. 7*, 65 (1987)
Isoprene — *60*, 215 (1994); *71*, 1015 (1999)
Isopropanol — *15*, 223 (1977); *Suppl. 7*, 229 (1987); *71*, 1027 (1999)
Isopropanol manufacture (strong-acid process) (*see also* Isopropanol; Sulfuric acid and other strong inorganic acids, occupational exposures to mists and vapours from) — *Suppl. 7*, 229 (1987)
Isopropyl oils — *15*, 223 (1977); *Suppl. 7*, 229 (1987); *71*, 1483 (1999)
Isosafrole — *1*, 169 (1972); *10*, 232 (1976); *Suppl. 7*, 65 (1987)

J

Jacobine — *10*, 275 (1976); *Suppl. 7*, 65 (1987)

Jet fuel — *45*, 203 (1989)
Joinery (*see* Carpentry and joinery)

K

Kaempferol — *31*, 171 (1983); *Suppl. 7*, 65 (1987)
Kaposi's sarcoma herpesvirus — *70*, 375 (1997)
Kepone (*see* Chlordecone)
Kojic acid — *79*, 605 (2001)

L

Lasiocarpine — *10*, 281 (1976); *Suppl. 7*, 65 (1987)
Lauroyl peroxide — *36*, 315 (1985); *Suppl. 7*, 65 (1987); *71*, 1485 (1999)
Lead acetate (*see* Lead and lead compounds)
Lead and lead compounds (*see also* Foreign bodies) — *1*, 40 (1972) (*corr. 42*, 251); *2*, 52, 150 (1973); *12*, 131 (1976); *23*, 40, 208, 209, 325 (1980); *Suppl. 7*, 230 (1987); *87* (2006)
Lead arsenate (*see* Arsenic and arsenic compounds)
Lead carbonate (*see* Lead and lead compounds)
Lead chloride (*see* Lead and lead compounds)
Lead chromate (*see* Chromium and chromium compounds)
Lead chromate oxide (*see* Chromium and chromium compounds)
Lead compounds, inorganic and organic — *Suppl. 7*, 230 (1987); *87* (2006)
Lead naphthenate (*see* Lead and lead compounds)
Lead nitrate (*see* Lead and lead compounds)
Lead oxide (*see* Lead and lead compounds)
Lead phosphate (*see* Lead and lead compounds)
Lead subacetate (*see* Lead and lead compounds)
Lead tetroxide (*see* Lead and lead compounds)
Leather goods manufacture — *25*, 279 (1981); *Suppl. 7*, 235 (1987)
Leather industries — *25*, 199 (1981); *Suppl. 7*, 232 (1987)
Leather tanning and processing — *25*, 201 (1981); *Suppl. 7*, 236 (1987)
Ledate (*see also* Lead and lead compounds) — *12*, 131 (1976)
Levonorgestrel — *72*, 49 (1999)
Light Green SF — *16*, 209 (1978); *Suppl. 7*, 65 (1987)
d-Limonene — *56*, 135 (1993); *73*, 307 (1999)
Lindane (*see* Hexachlorocyclohexanes)
Liver flukes (*see Clonorchis sinensis*, *Opisthorchis felineus* and *Opisthorchis viverrini*)
Lucidin (*see* 1,3-Dihydro-2-hydroxymethylanthraquinone)
Lumber and sawmill industries (including logging) — *25*, 49 (1981); *Suppl. 7*, 383 (1987)
Luteoskyrin — *10*, 163 (1976); *Suppl. 7*, 65 (1987)
Lynoestrenol — *21*, 407 (1979); *Suppl. 7*, 293 (1987); *72*, 49 (1999)

M

Madder root (*see also Rubia tinctorum*) *82*, 129 (2002)
Magenta *4*, 57 (1974) (*corr. 42*, 252); *Suppl. 7*, 238 (1987); *57*, 215 (1993)
Magenta, manufacture of (*see also* Magenta) *Suppl. 7*, 238 (1987); *57*, 215 (1993)
Malathion *30*, 103 (1983); *Suppl. 7*, 65 (1987)
Maleic hydrazide *4*, 173 (1974) (*corr. 42*, 253); *Suppl. 7*, 65 (1987)
Malonaldehyde *36*, 163 (1985); *Suppl. 7*, 65 (1987); *71*, 1037 (1999)
Malondialdehyde (*see* Malonaldehyde)
Maneb *12*, 137 (1976); *Suppl. 7*, 65 (1987)
Man-made mineral fibres (*see* Man-made vitreous fibres)
Man-made vitreous fibres *43*, 39 (1988); *81* (2002)
Mannomustine *9*, 157 (1975); *Suppl. 7*, 65 (1987)
Mate *51*, 273 (1991)
MCPA (*see also* Chlorophenoxy herbicides; Chlorophenoxy herbicides, occupational exposures to) *30*, 255 (1983)
MeA-α-C *40*, 253 (1986); *Suppl. 7*, 65 (1987)
Medphalan *9*, 168 (1975); *Suppl. 7*, 65 (1987)
Medroxyprogesterone acetate *6*, 157 (1974); *21*, 417 (1979) (*corr. 42*, 259); *Suppl. 7*, 289 (1987); *72*, 339 (1999)
Megestrol acetate *Suppl. 7*, 293 (1987); *72*, 49 (1999)
MeIQ *40*, 275 (1986); *Suppl. 7*, 65 (1987); *56*, 197 (1993)
MeIQx *40*, 283 (1986); *Suppl. 7*, 65 (1987) *56*, 211 (1993)
Melamine *39*, 333 (1986); *Suppl. 7*, 65 (1987); *73*, 329 (1999)
Melphalan *9*, 167 (1975); *Suppl. 7*, 239 (1987)
6-Mercaptopurine *26*, 249 (1981); *Suppl. 7*, 240 (1987)
Mercuric chloride (*see* Mercury and mercury compounds)
Mercury and mercury compounds *58*, 239 (1993)
Merphalan *9*, 169 (1975); *Suppl. 7*, 65 (1987)
Mestranol *6*, 87 (1974); *21*, 257 (1979) (*corr. 42*, 259); *Suppl. 7*, 288 (1987); *72*, 49 (1999)
Metabisulfites (*see* Sulfur dioxide and some sulfites, bisulfites and metabisulfites)
Metallic mercury (*see* Mercury and mercury compounds)
Methanearsonic acid, disodium salt (*see* Arsenic and arsenic compounds)
Methanearsonic acid, monosodium salt (*see* Arsenic and arsenic compounds)
Methimazole *79*, 53 (2001)
Methotrexate *26*, 267 (1981); *Suppl. 7*, 241 (1987)
Methoxsalen (*see* 8-Methoxypsoralen)

Methoxychlor — *5*, 193 (1974); *20*, 259 (1979); *Suppl. 7*, 66 (1987)

Methoxyflurane (*see* Anaesthetics, volatile)

5-Methoxypsoralen — *40*, 327 (1986); *Suppl. 7*, 242 (1987)

8-Methoxypsoralen (*see also* 8-Methoxypsoralen plus ultraviolet radiation) — *24*, 101 (1980)

8-Methoxypsoralen plus ultraviolet radiation — *Suppl. 7*, 243 (1987)

Methyl acrylate — *19*, 52 (1979); *39*, 99 (1986); *Suppl. 7*, 66 (1987); *71*, 1489 (1999)

5-Methylangelicin plus ultraviolet radiation (*see also* Angelicin and some synthetic derivatives) — *Suppl. 7*, 57 (1987)

2-Methylaziridine — *9*, 61 (1975); *Suppl. 7*, 66 (1987); *71*, 1497 (1999)

Methylazoxymethanol acetate (*see also* Cycasin) — *1*, 164 (1972); *10*, 131 (1976); *Suppl. 7*, 66 (1987)

Methyl bromide — *41*, 187 (1986) (*corr. 45*, 283); *Suppl. 7*, 245 (1987); *71*, 721 (1999)

Methyl *tert*-butyl ether — *73*, 339 (1999)

Methyl carbamate — *12*, 151 (1976); *Suppl. 7*, 66 (1987)

Methyl-CCNU (*see* 1-(2-Chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea)

Methyl chloride — *41*, 161 (1986); *Suppl. 7*, 246 (1987); *71*, 737 (1999)

1-, 2-, 3-, 4-, 5- and 6-Methylchrysenes — *32*, 379 (1983); *Suppl. 7*, 66 (1987)

N-Methyl-*N*,4-dinitrosoaniline — *1*, 141 (1972); *Suppl. 7*, 66 (1987)

4,4′-Methylene bis(2-chloroaniline) — *4*, 65 (1974) (*corr. 42*, 252); *Suppl. 7*, 246 (1987); *57*, 271 (1993)

4,4′-Methylene bis(*N*,*N*-dimethyl)benzenamine — *27*, 119 (1982); *Suppl. 7*, 66 (1987)

4,4′-Methylene bis(2-methylaniline) — *4*, 73 (1974); *Suppl. 7*, 248 (1987)

4,4′-Methylenedianiline — *4*, 79 (1974) (*corr. 42*, 252); *39*, 347 (1986); *Suppl. 7*, 66 (1987)

4,4′-Methylenediphenyl diisocyanate — *19*, 314 (1979); *Suppl. 7*, 66 (1987); *71*, 1049 (1999)

2-Methylfluoranthene — *32*, 399 (1983); *Suppl. 7*, 66 (1987)

3-Methylfluoranthene — *32*, 399 (1983); *Suppl. 7*, 66 (1987)

Methylglyoxal — *51*, 443 (1991)

Methyl iodide — *15*, 245 (1977); *41*, 213 (1986); *Suppl. 7*, 66 (1987); *71*, 1503 (1999)

Methylmercury chloride (*see* Mercury and mercury compounds)

Methylmercury compounds (*see* Mercury and mercury compounds)

Methyl methacrylate — *19*, 187 (1979); *Suppl. 7*, 66 (1987); *60*, 445 (1994)

Methyl methanesulfonate — *7*, 253 (1974); *Suppl. 7*, 66 (1987); *71*, 1059 (1999)

2-Methyl-1-nitroanthraquinone — *27*, 205 (1982); *Suppl. 7*, 66 (1987)

N-Methyl-*N*′-nitro-*N*-nitrosoguanidine — *4*, 183 (1974); *Suppl. 7*, 248 (1987)

3-Methylnitrosaminopropionaldehyde [*see* 3-(*N*-Nitrosomethylamino)-propionaldehyde]

3-Methylnitrosaminopropionitrile [*see* 3-(*N*-Nitrosomethylamino)-propionitrile]
4-(Methylnitrosamino)-4-(3-pyridyl)-1-butanal [*see* 4-(*N*-Nitrosomethylamino)-4-(3-pyridyl)-1-butanal]
4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone [*see* 4-(*N*-Nitrosomethylamino)-1-(3-pyridyl)-1-butanone]
N-Methyl-*N*-nitrosourea — *1*, 125 (1972); *17*, 227 (1978); *Suppl. 7*, 66 (1987)
N-Methyl-*N*-nitrosourethane — *4*, 211 (1974); *Suppl. 7*, 66 (1987)
N-Methylolacrylamide — *60*, 435 (1994)
Methyl parathion — *30*, 131 (1983); *Suppl. 7*, 66, 392 (1987)
1-Methylphenanthrene — *32*, 405 (1983); *Suppl. 7*, 66 (1987)
7-Methylpyrido[3,4-*c*]psoralen — *40*, 349 (1986); *Suppl. 7*, 71 (1987)
Methyl red — *8*, 161 (1975); *Suppl. 7*, 66 (1987)
Methyl selenac (*see also* Selenium and selenium compounds) — *12*, 161 (1976); *Suppl. 7*, 66 (1987)
Methylthiouracil — *7*, 53 (1974); *Suppl. 7*, 66 (1987); *79*, 75 (2001)
Metronidazole — *13*, 113 (1977); *Suppl. 7*, 250 (1987)
Mineral oils — *3*, 30 (1973); *33*, 87 (1984) (*corr. 42*, 262); *Suppl. 7*, 252 (1987)
Mirex — *5*, 203 (1974); *20*, 283 (1979) (*corr. 42*, 258); *Suppl. 7*, 66 (1987)
Mists and vapours from sulfuric acid and other strong inorganic acids — *54*, 41 (1992)
Mitomycin C — *10*, 171 (1976); *Suppl. 7*, 67 (1987)
Mitoxantrone — *76*, 289 (2000)
MNNG (*see N*-Methyl-*N*′-nitro-*N*-nitrosoguanidine)
MOCA (*see* 4,4′-Methylene bis(2-chloroaniline))
Modacrylic fibres — *19*, 86 (1979); *Suppl. 7*, 67 (1987)
Monochloramine (*see* Chloramine)
Monocrotaline — *10*, 291 (1976); *Suppl. 7*, 67 (1987)
Monuron — *12*, 167 (1976); *Suppl. 7*, 67 (1987); *53*, 467 (1991)
MOPP and other combined chemotherapy including alkylating agents — *Suppl. 7*, 254 (1987)
Mordanite (*see* Zeolites)
Morinda officinalis (see also Traditional herbal medicines) — *82*, 129 (2002)
Morpholine — *47*, 199 (1989); *71*, 1511 (1999)
5-(Morpholinomethyl)-3-[(5-nitrofurfurylidene)amino]-2-oxazolidinone — *7*, 161 (1974); *Suppl. 7*, 67 (1987)
Musk ambrette — *65*, 477 (1996)
Musk xylene — *65*, 477 (1996)
Mustard gas — *9*, 181 (1975) (*corr. 42*, 254); *Suppl. 7*, 259 (1987)
Myleran (*see* 1,4-Butanediol dimethanesulfonate)

N

Nafenopin — *24*, 125 (1980); *Suppl. 7*, 67 (1987)
Naphthalene — *82*, 367 (2002)
1,5-Naphthalenediamine — *27*, 127 (1982); *Suppl. 7*, 67 (1987)
1,5-Naphthalene diisocyanate — *19*, 311 (1979); *Suppl. 7*, 67 (1987); *71*, 1515 (1999)
1-Naphthylamine — *4*, 87 (1974) (*corr. 42*, 253); *Suppl. 7*, 260 (1987)
2-Naphthylamine — *4*, 97 (1974); *Suppl. 7*, 261 (1987)
1-Naphthylthiourea — *30*, 347 (1983); *Suppl. 7*, 263 (1987)
Neutrons — *75*, 361 (2000)
Nickel acetate (*see* Nickel and nickel compounds)
Nickel ammonium sulfate (*see* Nickel and nickel compounds)
Nickel and nickel compounds (*see also* Implants, surgical) — *2*, 126 (1973) (*corr. 42*, 252); *11*, 75 (1976); *Suppl. 7*, 264 (1987) (*corr. 45*, 283); *49*, 257 (1990) (*corr. 67*, 395)
Nickel carbonate (*see* Nickel and nickel compounds)
Nickel carbonyl (*see* Nickel and nickel compounds)
Nickel chloride (*see* Nickel and nickel compounds)
Nickel-gallium alloy (*see* Nickel and nickel compounds)
Nickel hydroxide (*see* Nickel and nickel compounds)
Nickelocene (*see* Nickel and nickel compounds)
Nickel oxide (*see* Nickel and nickel compounds)
Nickel subsulfide (*see* Nickel and nickel compounds)
Nickel sulfate (*see* Nickel and nickel compounds)
Niridazole — *13*, 123 (1977); *Suppl. 7*, 67 (1987)
Nithiazide — *31*, 179 (1983); *Suppl. 7*, 67 (1987)
Nitrilotriacetic acid and its salts — *48*, 181 (1990); *73*, 385 (1999)
5-Nitroacenaphthene — *16*, 319 (1978); *Suppl. 7*, 67 (1987)
5-Nitro-*ortho*-anisidine — *27*, 133 (1982); *Suppl. 7*, 67 (1987)
2-Nitroanisole — *65*, 369 (1996)
9-Nitroanthracene — *33*, 179 (1984); *Suppl. 7*, 67 (1987)
7-Nitrobenz[*a*]anthracene — *46*, 247 (1989)
Nitrobenzene — *65*, 381 (1996)
6-Nitrobenzo[*a*]pyrene — *33*, 187 (1984); *Suppl. 7*, 67 (1987); *46*, 255 (1989)
4-Nitrobiphenyl — *4*, 113 (1974); *Suppl. 7*, 67 (1987)
6-Nitrochrysene — *33*, 195 (1984); *Suppl. 7*, 67 (1987); *46*, 267 (1989)
Nitrofen (technical-grade) — *30*, 271 (1983); *Suppl. 7*, 67 (1987)
3-Nitrofluoranthene — *33*, 201 (1984); *Suppl. 7*, 67 (1987)
2-Nitrofluorene — *46*, 277 (1989)
Nitrofural — *7*, 171 (1974); *Suppl. 7*, 67 (1987); *50*, 195 (1990)
5-Nitro-2-furaldehyde semicarbazone (*see* Nitrofural)
Nitrofurantoin — *50*, 211 (1990)
Nitrofurazone (*see* Nitrofural)
1-[(5-Nitrofurfurylidene)amino]-2-imidazolidinone — *7*, 181 (1974); *Suppl. 7*, 67 (1987)
N-[4-(5-Nitro-2-furyl)-2-thiazolyl]acetamide — *1*, 181 (1972); *7*, 185 (1974); *Suppl. 7*, 67 (1987)

Nitrogen mustard *9*, 193 (1975); *Suppl. 7*, 269 (1987)
Nitrogen mustard *N*-oxide *9*, 209 (1975); *Suppl. 7*, 67 (1987)
Nitromethane *77*, 487 (2000)
1-Nitronaphthalene *46*, 291 (1989)
2-Nitronaphthalene *46*, 303 (1989)
3-Nitroperylene *46*, 313 (1989)
2-Nitro-*para*-phenylenediamine (*see* 1,4-Diamino-2-nitrobenzene)
2-Nitropropane *29*, 331 (1982); *Suppl. 7*, 67 (1987); *71*, 1079 (1999)
1-Nitropyrene *33*, 209 (1984); *Suppl. 7*, 67 (1987); *46*, 321 (1989)
2-Nitropyrene *46*, 359 (1989)
4-Nitropyrene *46*, 367 (1989)
N-Nitrosatable drugs *24*, 297 (1980) (*corr. 42*, 260)
N-Nitrosatable pesticides *30*, 359 (1983)
N′-Nitrosoanabasine (NAB) *37*, 225 (1985); *Suppl. 7*, 67 (1987); *89*, 419 (2007)
N′-Nitrosoanatabine (NAT) *37*, 233 (1985); *Suppl. 7*, 67 (1987); *89*, 419 (2007)
N-Nitrosodi-*n*-butylamine *4*, 197 (1974); *17*, 51 (1978); *Suppl. 7*, 67 (1987)
N-Nitrosodiethanolamine *17*, 77 (1978); *Suppl. 7*, 67 (1987); *77*, 403 (2000)
N-Nitrosodiethylamine *1*, 107 (1972) (*corr. 42*, 251); *17*, 83 (1978) (*corr. 42*, 257); *Suppl. 7*, 67 (1987)
N-Nitrosodimethylamine *1*, 95 (1972); *17*, 125 (1978) (*corr. 42*, 257); *Suppl. 7*, 67 (1987)
N-Nitrosodiphenylamine *27*, 213 (1982); *Suppl. 7*, 67 (1987)
para-Nitrosodiphenylamine *27*, 227 (1982) (*corr. 42*, 261); *Suppl. 7*, 68 (1987)
N-Nitrosodi-*n*-propylamine *17*, 177 (1978); *Suppl. 7*, 68 (1987)
N-Nitroso-*N*-ethylurea (*see N*-Ethyl-*N*-nitrosourea)
N-Nitrosofolic acid *17*, 217 (1978); *Suppl. 7*, 68 (1987)
N-Nitrosoguvacine *37*, 263 (1985); *Suppl. 7*, 68 (1987); *85*, 281 (2004)
N-Nitrosoguvacoline *37*, 263 (1985); *Suppl. 7*, 68 (1987); *85*, 281 (2004)
N-Nitrosohydroxyproline *17*, 304 (1978); *Suppl. 7*, 68 (1987)
3-(*N*-Nitrosomethylamino)propionaldehyde *37*, 263 (1985); *Suppl. 7*, 68 (1987); *85*, 281 (2004)
3-(*N*-Nitrosomethylamino)propionitrile *37*, 263 (1985); *Suppl. 7*, 68 (1987); *85*, 281 (2004)
4-(*N*-Nitrosomethylamino)-4-(3-pyridyl)-1-butanal *37*, 205 (1985); *Suppl. 7*, 68 (1987)
4-(*N*-Nitrosomethylamino)-1-(3-pyridyl)-1-butanone (NNK) *37*, 209 (1985); *Suppl. 7*, 68 (1987); *89*, 419 (2007)
N-Nitrosomethylethylamine *17*, 221 (1978); *Suppl. 7*, 68 (1987)
N-Nitroso-*N*-methylurea (*see N*-Methyl-*N*-nitrosourea)
N-Nitroso-*N*-methylurethane (*see N*-Methyl-*N*-nitrosourethane)
N-Nitrosomethylvinylamine *17*, 257 (1978); *Suppl. 7*, 68 (1987)
N-Nitrosomorpholine *17*, 263 (1978); *Suppl. 7*, 68 (1987)
N′-Nitrosonornicotine (NNN) *17*, 281 (1978); *37*, 241 (1985); *Suppl. 7*, 68 (1987); *89*, 419 (2007)
N-Nitrosopiperidine *17*, 287 (1978); *Suppl. 7*, 68 (1987)

N-Nitrosoproline *17*, 303 (1978); *Suppl. 7*, 68 (1987)
N-Nitrosopyrrolidine *17*, 313 (1978); *Suppl. 7*, 68 (1987)
N-Nitrososarcosine *17*, 327 (1978); *Suppl. 7*, 68 (1987)
Nitrosoureas, chloroethyl (*see* Chloroethyl nitrosoureas)
5-Nitro-*ortho*-toluidine *48*, 169 (1990)
2-Nitrotoluene *65*, 409 (1996)
3-Nitrotoluene *65*, 409 (1996)
4-Nitrotoluene *65*, 409 (1996)
Nitrous oxide (*see* Anaesthetics, volatile)
Nitrovin *31*, 185 (1983); *Suppl. 7*, 68 (1987)
Nivalenol (*see* Toxins derived from *Fusarium graminearum*, *F. culmorum* and *F. crookwellense*)
NNK (*see* 4-(*N*-Nitrosomethylamino)-1-(3-pyridyl)-1-butanone)
NNN (*see N′*-Nitrosonornicotine)
Nonsteroidal oestrogens *Suppl. 7*, 273 (1987)
Norethisterone *6*, 179 (1974); *21*, 461 (1979); *Suppl. 7*, 294 (1987); *72*, 49 (1999)
Norethisterone acetate *72*, 49 (1999)
Norethynodrel *6*, 191 (1974); *21*, 461 (1979) (*corr. 42*, 259); *Suppl. 7*, 295 (1987); *72*, 49 (1999)
Norgestrel *6*, 201 (1974); *21*, 479 (1979); *Suppl. 7*, 295 (1987); *72*, 49 (1999)
Nylon 6 *19*, 120 (1979); *Suppl. 7*, 68 (1987)

O

Ochratoxin A *10*, 191 (1976); *31*, 191 (1983) (*corr. 42*, 262); *Suppl. 7*, 271 (1987); *56*, 489 (1993)
Oestradiol *6*, 99 (1974); *21*, 279 (1979); *Suppl. 7*, 284 (1987); *72*, 399 (1999)
Oestradiol-17β (*see* Oestradiol)
Oestradiol 3-benzoate (*see* Oestradiol)
Oestradiol dipropionate (*see* Oestradiol)
Oestradiol mustard *9*, 217 (1975); *Suppl. 7*, 68 (1987)
Oestradiol valerate (*see* Oestradiol)
Oestriol *6*, 117 (1974); *21*, 327 (1979); *Suppl. 7*, 285 (1987); *72*, 399 (1999)
Oestrogen-progestin combinations (*see* Oestrogens, progestins (progestogens) and combinations)
Oestrogen-progestin replacement therapy (*see* Post-menopausal oestrogen-progestogen therapy)
Oestrogen replacement therapy (*see* Post-menopausal oestrogen therapy)
Oestrogens (*see* Oestrogens, progestins and combinations)
Oestrogens, conjugated (*see* Conjugated oestrogens)
Oestrogens, nonsteroidal (*see* Nonsteroidal oestrogens)

Oestrogens, progestins (progestogens) and combinations — *6* (1974); *21* (1979); *Suppl. 7*, 272 (1987); *72*, 49, 339, 399, 531 (1999)
Oestrogens, steroidal (*see* Steroidal oestrogens)
Oestrone — *6*, 123 (1974); *21*, 343 (1979) (*corr. 42*, 259); *Suppl. 7*, 286 (1987); *72*, 399 (1999)
Oestrone benzoate (*see* Oestrone)
Oil Orange SS — *8*, 165 (1975); *Suppl. 7*, 69 (1987)
Opisthorchis felineus (infection with) — *61*, 121 (1994)
Opisthorchis viverrini (infection with) — *61*, 121 (1994)
Oral contraceptives, combined — *Suppl. 7*, 297 (1987); *72*, 49 (1999)
Oral contraceptives, sequential (*see* Sequential oral contraceptives)
Orange I — *8*, 173 (1975); *Suppl. 7*, 69 (1987)
Orange G — *8*, 181 (1975); *Suppl. 7*, 69 (1987)
Organic lead compounds — *Suppl. 7*, 230 (1987); *87* (2006)
Organolead compounds (*see* Organic lead compounds)
Oxazepam — *13*, 58 (1977); *Suppl. 7*, 69 (1987); *66*, 115 (1996)
Oxymetholone (*see also* Androgenic (anabolic) steroids) — *13*, 131 (1977)
Oxyphenbutazone — *13*, 185 (1977); *Suppl. 7*, 69 (1987)

P

Paint manufacture and painting (occupational exposures in) — *47*, 329 (1989)
Palygorskite — *42*, 159 (1987); *Suppl. 7*, 117 (1987); *68*, 245 (1997)
Panfuran S (*see also* Dihydroxymethylfuratrizine) — *24*, 77 (1980); *Suppl. 7*, 69 (1987)
Paper manufacture (*see* Pulp and paper manufacture)
Paracetamol — *50*, 307 (1990); *73*, 401 (1999)
Parasorbic acid — *10*, 199 (1976) (*corr. 42*, 255); *Suppl. 7*, 69 (1987)
Parathion — *30*, 153 (1983); *Suppl. 7*, 69 (1987)
Patulin — *10*, 205 (1976); *40*, 83 (1986); *Suppl. 7*, 69 (1987)
Penicillic acid — *10*, 211 (1976); *Suppl. 7*, 69 (1987)
Pentachloroethane — *41*, 99 (1986); *Suppl. 7*, 69 (1987); *71*, 1519 (1999)
Pentachloronitrobenzene (see Quintozene)
Pentachlorophenol (*see also* Chlorophenols; Chlorophenols, occupational exposures to; Polychlorophenols and their sodium salts) — *20*, 303 (1979); *53*, 371 (1991)
Permethrin — *53*, 329 (1991)
Perylene — *32*, 411 (1983); *Suppl. 7*, 69 (1987)
Petasitenine — *31*, 207 (1983); *Suppl. 7*, 69 (1987)
Petasites japonicus (*see also* Pyrrolizidine alkaloids) — *10*, 333 (1976)
Petroleum refining (occupational exposures in) — *45*, 39 (1989)
Petroleum solvents — *47*, 43 (1989)
Phenacetin — *13*, 141 (1977); *24*, 135 (1980); *Suppl. 7*, 310 (1987)
Phenanthrene — *32*, 419 (1983); *Suppl. 7*, 69 (1987)
Phenazopyridine hydrochloride — *8*, 117 (1975); *24*, 163 (1980) (*corr. 42*, 260); *Suppl. 7*, 312 (1987)

Phenelzine sulfate	*24*, 175 (1980); *Suppl. 7*, 312 (1987)
Phenicarbazide	*12*, 177 (1976); *Suppl. 7*, 70 (1987)
Phenobarbital and its sodium salt	*13*, 157 (1977); *Suppl. 7*, 313 (1987); *79*, 161 (2001)
Phenol	*47*, 263 (1989) (*corr. 50*, 385); *71*, 749 (1999)
Phenolphthalein	*76*, 387 (2000)
Phenoxyacetic acid herbicides (*see* Chlorophenoxy herbicides)	
Phenoxybenzamine hydrochloride	*9*, 223 (1975); *24*, 185 (1980); *Suppl. 7*, 70 (1987)
Phenylbutazone	*13*, 183 (1977); *Suppl. 7*, 316 (1987)
meta-Phenylenediamine	*16*, 111 (1978); *Suppl. 7*, 70 (1987)
para-Phenylenediamine	*16*, 125 (1978); *Suppl. 7*, 70 (1987)
Phenyl glycidyl ether (*see also* Glycidyl ethers)	*71*, 1525 (1999)
N-Phenyl-2-naphthylamine	*16*, 325 (1978) (*corr. 42*, 257); *Suppl. 7*, 318 (1987)
ortho-Phenylphenol	*30*, 329 (1983); *Suppl. 7*, 70 (1987); *73*, 451 (1999)
Phenytoin	*13*, 201 (1977); *Suppl. 7*, 319 (1987); *66*, 175 (1996)
Phillipsite (*see* Zeolites)	
PhIP	*56*, 229 (1993)
Pickled vegetables	*56*, 83 (1993)
Picloram	*53*, 481 (1991)
Piperazine oestrone sulfate (*see* Conjugated oestrogens)	
Piperonyl butoxide	*30*, 183 (1983); *Suppl. 7*, 70 (1987)
Pitches, coal-tar (*see* Coal-tar pitches)	
Polyacrylic acid	*19*, 62 (1979); *Suppl. 7*, 70 (1987)
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