

TOBACCO SMOKING

Tobacco smoking was considered by previous IARC Working Groups in 1986, 1987 and 2002 ([IARC, 1986, 1987, 2004a](#)). Since that time, new data have become available, these have been incorporated into the *Monograph*, and taken into consideration in the present evaluation.

1. Exposure Data

1.1 Smoked tobacco products

Smoked forms of tobacco include various kinds of cigarettes (manufactured, hand-rolled, filtered, un-filtered and flavoured), cigars and pipes. While cigarette smoking, particularly manufactured cigarettes, is by far the main form of tobacco smoked globally, in some countries other forms of smoked tobacco are dominant ([IARC, 2004a](#)). In India, for example, bidis (made of coarse and uncured tobacco) account for about 60% of smoked tobacco products whereas cigarettes account for 20% ([Ray & Gupta, 2009](#); [IIPS, 2010](#)). Water pipes, another form of smoked tobacco known by other various names such as gaza, hookah, narghile, shisha, hubble-bubble, are commonly smoked in the Eastern Mediterranean region, in some parts of Asia including India, and in North Africa ([Asma et al., 2009](#)).

1.2 Chemical composition of tobacco smoke

1.2.1 Smoke from cigarettes

One cubic cm of fresh, un-aged cigarette mainstream smoke [the smoke emerging from the mouth end of a cigarette during smoking] has about 4×10^9 particles with a mean diameter of about $0.2 \mu\text{m}$ ([Borgerding & Klus, 2005](#)). The size of the particles increases as the smoke ages. Temperatures in the burning cone of the cigarette are about 800°C during the smoulder period between puffs and increase to $910\text{--}920^\circ\text{C}$ at the periphery of the cone during puffing ([Borgerding & Klus, 2005](#)). Hydrogen is generated in the glowing cone, resulting in an oxygen deficient reducing atmosphere ([Borgerding & Klus, 2005](#)). The approximate composition of mainstream smoke of a plain cigarette is summarized in [Table 1.1](#) ([Borgerding & Klus, 2005](#)). The total particulate matter, after subtraction of the amounts of nicotine and water, is referred to as 'tar'.

Over 5300 compounds have been identified in tobacco smoke ([Rodgman & Perfetti, 2009](#)). Classes of compounds include but are not limited to neutral gases, carbon and nitrogen oxides, amides, imides, lactams, carboxylic acids, lactones, esters, aldehydes, ketones,

Table 1.1 Approximate chemical composition of mainstream smoke generated by a plain cigarette

Compound or class of components	Relative amount w/w (%)
Nitrogen	58
Oxygen	12
Carbon dioxide	13
Carbon monoxide	3.5
Hydrogen, argon	0.5
Water	1
Volatile organic substances	5
Particulate phase	8

From [Borgerding & Klus \(2005\)](#)

alcohols, phenols, amines, *N*-nitrosamines, *N*-heterocyclics, aliphatic hydrocarbons, monocyclic and polycyclic aromatic hydrocarbons (PAHs), nitriles, anhydrides, carbohydrates, ethers, nitro compounds and metals ([Rodgman & Perfetti, 2009](#)).

The addictive properties of tobacco smoke are attributed to nicotine, the principal tobacco alkaloid in smoke ([Hukkanen et al., 2005](#)). Minor tobacco alkaloids include nornicotine, anatabine and anabasine ([Hukkanen et al., 2005](#)). The tobacco alkaloids are not generally considered carcinogenic, but are accompanied by carcinogens in each puff of smoke.

There are over 70 carcinogens in tobacco smoke that have been evaluated by the *IARC Monographs* programme as having sufficient evidence for carcinogenicity in either laboratory animals or humans ([IARC, 2004a](#)). The different chemical classes of carcinogens and representatives of each are presented in [Table 1.2](#) ([IARC, 2004a](#)). Sixteen of these – benzo[*a*]pyrene (BaP), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and *N'*-nitrosonornicotine (NNN), 2-naphthylamine, 4-aminobiphenyl, formaldehyde, 1,3-butadiene, benzene, vinyl chloride, ethylene oxide, arsenic, beryllium, nickel compounds, chromium VI, cadmium, and polonium-210 – are classified as carcinogenic to

humans (Group 1). Structures of some representative carcinogens in cigarette smoke are shown in Fig. 1.1. There are other likely carcinogens in cigarette smoke that have not been evaluated by the *IARC Monographs* programme. These include, for example, PAHs with incompletely characterized occurrence levels and carcinogenic activities; over 500 PAHs have been identified ([Rodgman & Perfetti, 2006](#)).

PAHs, tobacco-specific *N*-nitrosamines, aromatic amines, aldehydes and certain volatile organics likely contribute significantly to the carcinogenic activity of tobacco smoke ([Hecht, 2003](#)).

In the early part of the 20th century, PAHs were identified as carcinogenic constituents of coal tar ([Phillips, 1983](#)). They are products of incomplete combustion of all organic matter and occur, always as complex mixtures, in tars, soots, broiled foods, vehicle engine exhaust and tobacco smoke. PAHs are generally locally acting carcinogens, and some, such as the prototypic compound BaP, have strong carcinogenic activity on mouse skin and in rodent lung. Heterocyclic analogues of PAHs also occur in cigarette smoke. Concentrations of individual PAHs in mainstream cigarette smoke are generally in the range of 1–50 ng per cigarette ([IARC, 2004a](#)).

Among the carcinogenic *N*-nitrosamines in tobacco smoke are tobacco-specific *N*-nitrosamines, which are derived from, and structurally related to, the tobacco alkaloids. Two of the most important of these are NNK and NNN ([Hecht & Hoffmann, 1988](#)). Levels of NNK and NNN in cigarette smoke vary depending on tobacco type and other factors, but are frequently in the range of 50–200 ng per cigarette ([IARC, 2004a](#)).

Aromatic amines were first identified as human carcinogens from industrial exposures in the dye industry in the early part of the 20th century. They include the well known human bladder carcinogens 2-naphthylamine and 4-aminobiphenyl which, along with other

Table 1.2 Tobacco smoke carcinogens evaluated in the IARC Monographs

Chemical Class	Number of Carcinogens	Representative Carcinogens
Polycyclic aromatic hydrocarbons (PAHs) and their heterocyclic analogues	15	Benzo[a]pyrene (BaP) Dibenz[a,h]anthracene
N-Nitrosamines	8	4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) N'-Nitrosoornicotine (NNN)
Aromatic amines	12	4-Aminobiphenyl 2-Naphthylamine
Aldehydes	2	Formaldehyde Acetaldehyde
Phenols	2	Catechol Caffeic acid
Volatile hydrocarbons	3	Benzene 1,3-Butadiene Isoprene
Other organics	12	Ethylene oxide Acrylonitrile
Inorganic compounds	8	Cadmium Polonium-210

There are many other carcinogens in cigarette smoke that have not been evaluated in an *IARC Monograph*.
From [IARC \(2004a\)](#)

isomers, are found in cigarette smoke, but their levels are generally quite low (1–20 ng per cigarette) ([IARC, 2004a](#)).

Aldehydes such as formaldehyde and acetaldehyde occur widely in the human environment and are also found in human blood. Concentrations of acetaldehyde and formaldehyde in cigarette smoke are far higher than those of PAHs, N-nitrosamines or aromatic amines but their carcinogenic activities are weak ([Hecht, 2003](#)). Cigarette mainstream smoke typically contains 10–30 µg formaldehyde/cigarette and 800–900 µg acetaldehyde/cigarette ([IARC, 2004a](#)).

Volatile hydrocarbons in cigarette smoke include 1,3-butadiene, a powerful multi-organ carcinogen in the mouse, and benzene, a known human leukaemogen. 1,3-Butadiene (20–40 µg/cigarette) and benzene (12–50 µg/cigarette) are two of the most prevalent strong carcinogens in cigarette smoke ([IARC, 2004a](#)).

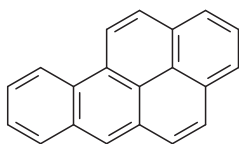
In summary, cigarette smoke is an exceedingly complex mixture which contains over 5300

compounds including multiple toxicants and carcinogens.

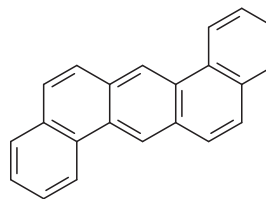
1.2.2 Smoke from other tobacco products

Some constituents have been measured in roll-your-own cigarettes, and their levels are comparable to or higher than those in commercial brands. Carcinogen and toxicant levels expressed per unit are higher in cigars than in cigarettes because of their larger size, and in some instances are also higher per litre of smoke. Levels of nicotine and tobacco-specific nitrosamines were comparable in bidis and commercial Indian cigarettes; bidis also contain high levels of eugenol, as do kreteks. Levels of NNK and NNN in chuttas were considerably higher than in standard cigarettes ([IARC, 2004a](#)).

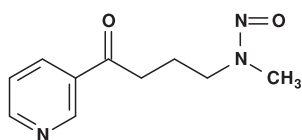
Fig. 1.1 Structures of some representative tobacco smoke carcinogens



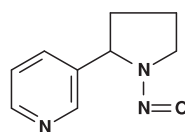
benzo[a]pyrene (BaP)



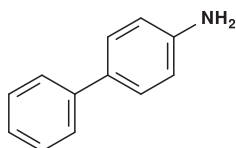
dibenz[a,h]anthracene



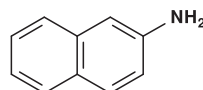
4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)



N'-nitrososornicotine (NNN)



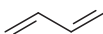
4-aminobiphenyl



2-naphtylamine



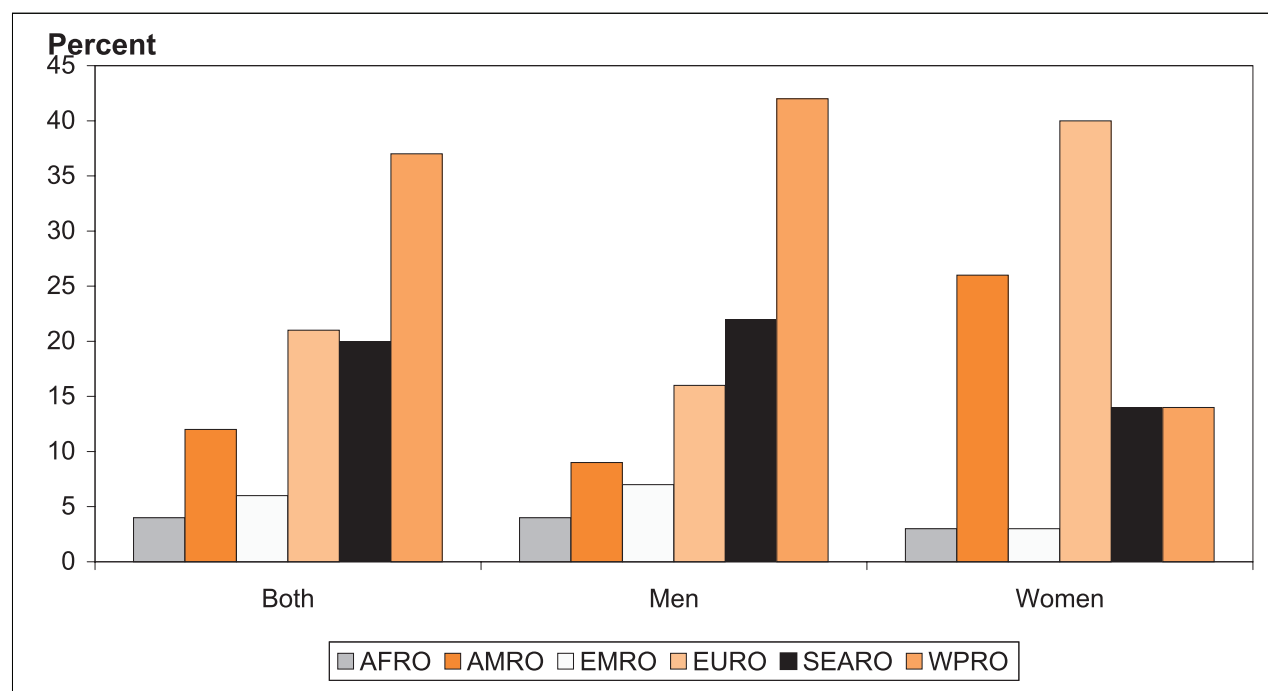
benzene



1,3-butadiene



ethylene oxide

Fig. 1.2 Proportion of adult smokers by WHO region in 2009

From [WHO \(2011\)](#)

1.3 Prevalence of tobacco smoking

1.3.1 Data collection and methods

Data on smoking tobacco are available from WHO's Global Infobase (www.who.int/infobase) and the WHO Global Health Observatory (www.who.int/gho/en) – repositories of information on tobacco use and other risk factors in young people (13–15 years old) and adults (aged 15 years and over). The data span several years and are acquired from government reports, journals and unpublished sources. WHO has in the recent past used and modelled these data to produce estimates of tobacco smoking prevalence, published in the WHO Reports on the Global Tobacco Epidemic. For a complete explanation of methods used, the reader is referred to the Technical Note on Prevalence in the 3rd WHO Report on the Global Tobacco Epidemic ([WHO, 2011](http://www.who.int/2011)). The six WHO regions are: EMRO, Eastern Mediterranean Region; EURO, European

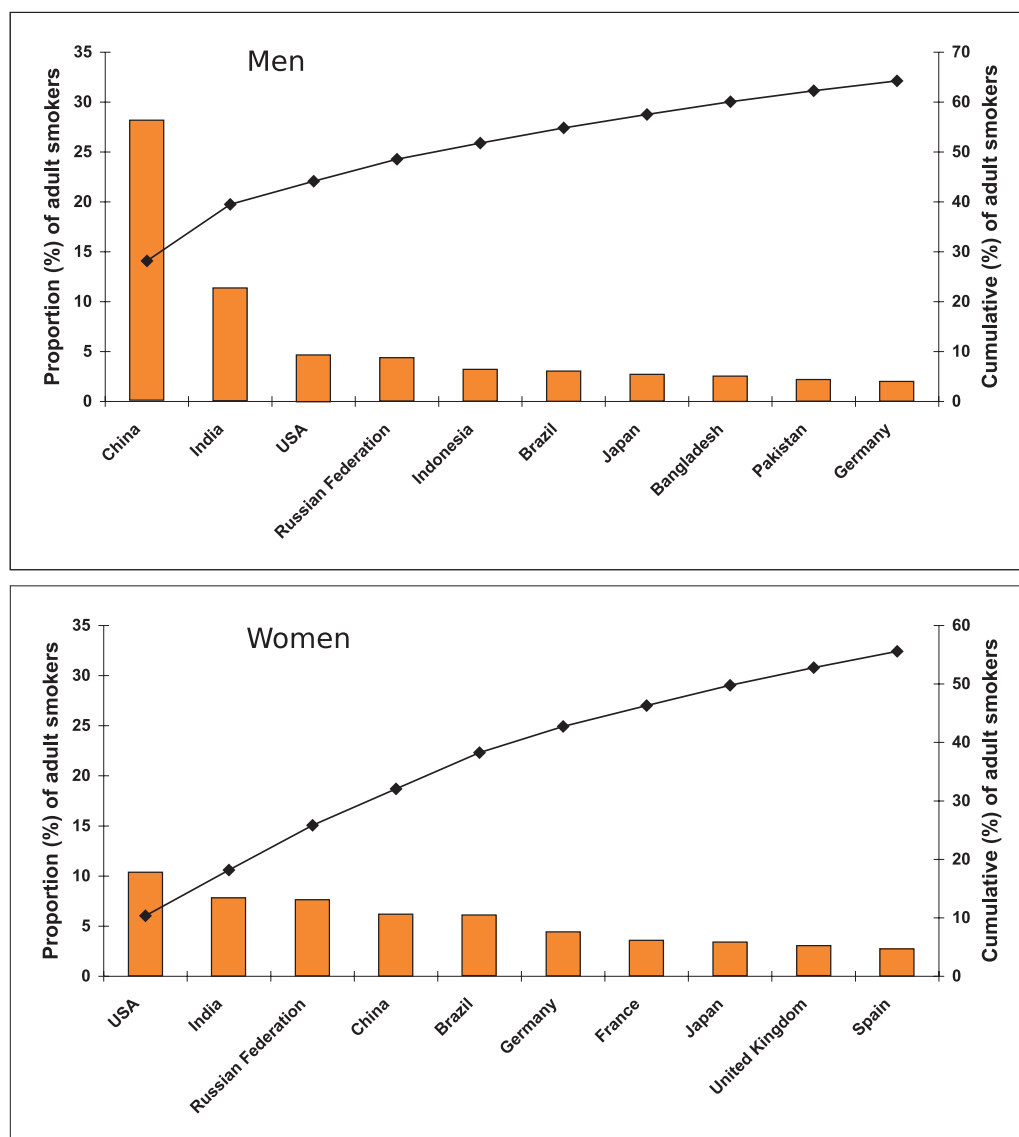
Region; AFRO, African Region; WPRO, Western Pacific Region; SEARO, South East Asian Region; AMRO, Region of the Americas. A listing of the countries in each region can be viewed at <http://www.who.int/about/structure/en/index.html>.

1.3.2 Distribution of smokers by WHO region and country

WHO estimates that in 2009, there was about 1.1 billion adult smokers worldwide, representing nearly a quarter (22%) of the global adult population ([WHO, 2011](#)). A disaggregation by the six WHO regions (Fig. 1.2) shows that over a third of smokers worldwide live in WPRO (highly influenced by the People's Republic of China), followed by SEARO, which has around a fifth of the world's smokers (influenced by India and Indonesia).

The number of smokers in any country is a function of both the prevalence of smoking and the size of the population. A further

Fig. 1.3 Proportion and cumulative percentage of smokers in high-burden countries, in men (A) and women (B) in 2009



From [WHO \(2011\)](#)

disaggregation of the regions by country shows that a few countries account for a large proportion of tobacco smokers. Ranked in descending order of the number of smokers, the five countries of China, India, United States of America (USA), Russian Federation and Indonesia account for about 52% of adult smokers in the world, with China and India alone accounting for 40% (Fig. 1.3). Furthermore, nearly two-thirds of the

world's smokers live in only ten countries of the world.

1.3.3 Distribution of smokers by sex

With a global average smoking prevalence of 36%, men account for just over 80% of all smokers. The male adult prevalence is 4–5 times that for women, at 8%. This difference varies across WHO

regions. Smoking among men, concentrated in the five countries of China, India, Indonesia, Russian Federation and USA (Fig. 1.3), accounts for about 56% of global smoking among men. Women smokers are mostly concentrated in EURO and AMRO. These two regions account for 40% and 26% of all women smokers globally, respectively. The prevalences for women in these two regions are about half of those in men, whereas the difference is substantially greater in the other regions. Just as men smoke more than women everywhere, so too among young people, boys generally smoke more than girls. There is an increasing concern, however, that the gap may diminish, not because of a reduction in boys prevalence but because of an increase in the proportion of girls who are taking up smoking ([Warren et al., 2006](#)).

1.3.4 The four stage smoking model

(a) The four stages of tobacco use

[Lopez et al. \(1994\)](#) used trend data on smoking prevalence and tobacco attributable mortality to show the evolution of tobacco use in a country. Four stages of smoking and attributable mortality have been identified to represent the growth and eventual decline of smoking among men and women (Fig. 1.4).

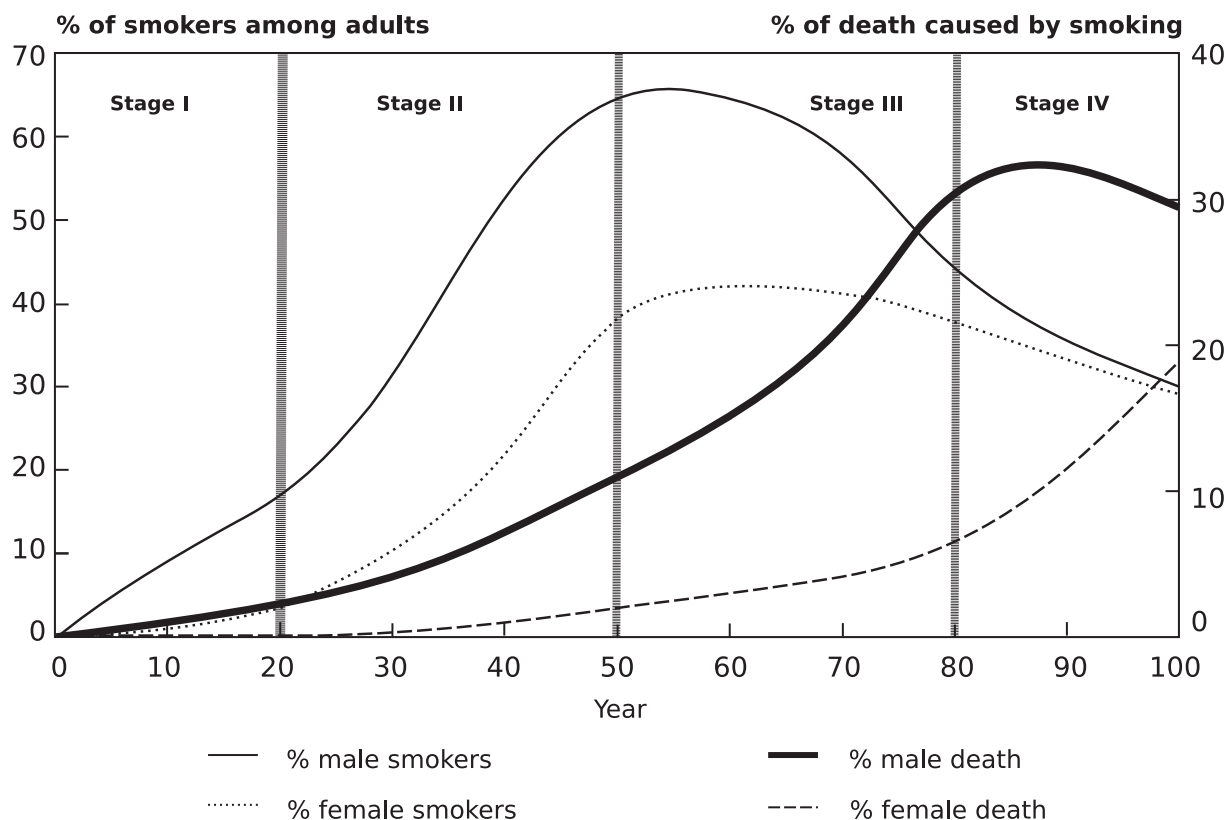
Stage 1 is characterized by low smoking prevalence in men (less than 15%) and very low in women (less than 10%). Death and disease from smoking are not apparent in this phase, as nearly all health effects from smoking are related to past smoking habits and their cumulative effects rather than current smoking. In Stage 2, smoking prevalence in men rapidly increases while it increases more slowly in women. Towards the end of this stage, smoking prevalence in men typically peaks to lie at 50–60%, with 10% of deaths in men attributable to smoking; deaths in women are comparatively fewer. After a protracted period of high smoking prevalence, Stage 3 shows a decline in smoking prevalence in men to around 40%.

Smoking prevalence in women peaks and then begins to decline; towards the end of this stage the gap between men's and women's prevalence starts to narrow. However, smoking attributable deaths in men increase from around 10% to 25–30% within a span of three decades; in women the deaths are increasing but are still quite low. In the final Stage 4, smoking prevalences in both men and women continue to decline albeit relatively slowly in comparison with Stage 3, with the gap substantially narrowing to lie at around five percentage points, and as little as one percentage point in some countries. In Stage 4, smoking mortality in men peaks to between 30–35% and then declines to below 30% at the end of this period. In women, the health effects from past smoking persist, with increasing mortality, but remain lower than in men, and recently have begun to decline in some countries.

(b) Smoking prevalence worldwide

Using prevalence data for men and women collected in 2006 for 140 countries, WHO determined at which stage of the tobacco epidemic countries are in the model of [Lopez et al. \(1994\)](#). In Fig. 1.5, countries have been ranked by smoking prevalence in men in ascending order for Stages 1 and 2, and then in descending order for Stages 3 and 4. (Smoking prevalence in men is almost always higher than in women, with a few exceptions observed in the fourth stage.) While most countries fit the classification, there are a few exceptions, most of which in the last stage. Prevalence between Stage 3 and Stage 4 is discontinuous in both sexes. This is due to the classification followed, which puts countries with a relatively narrow difference in prevalence between men and women in Stage 4 even though their prevalence is largely comparable with those in Stage 3.

Most African countries fall in the first stage of the smoking model, characterized by low smoking prevalence in men and very low prevalence in women. Three of the five high burden

Fig. 1.4 The four stages of the tobacco epidemic

From [Lopez et al. \(1994\)](#)

countries fall in stage 2 (India, Indonesia and China), with the rest comprising a combination of countries from Africa, South East Asia, eastern Europe and the Middle East. At this stage smoking prevalences in women continue to remain very low, most countries having a prevalence in adult women of less than 10%.

Stage 3 includes the fourth high burden country (Russian Federation), along with countries in eastern Europe, South America and western Europe, which fall at the end of Stage 3. Stage 4 is populated entirely by the developed countries of western Europe, North America and Oceania. The USA, the fifth high burden country, fall in the last stage as a result of the relatively small difference in the smoking prevalence between men and women compared to the

other intermediate stages. As mentioned before, Stage 4 includes countries where the smoking prevalence is higher in women than in men, with a small (< 8%) difference.

(c) Age-specific prevalence

Age-specific prevalence for men and women aged 15 years or older is presented for six representative countries for current smoking (Fig. 1.6). There are wide variations in age-specific prevalence between these countries. In men, prevalence varies from less than 10% to 75% in the 15–19 years age range to lie between 10% and 55% in the oldest age range. Prevalence among women varies from less than 1% to as high as 45% in young adults (15–19 years). Unlike men, prevalence in women tends to converge after age

Fig. 1.5 Prevalence of smoking in 140 countries in 2009, staged according to the model by Lopez

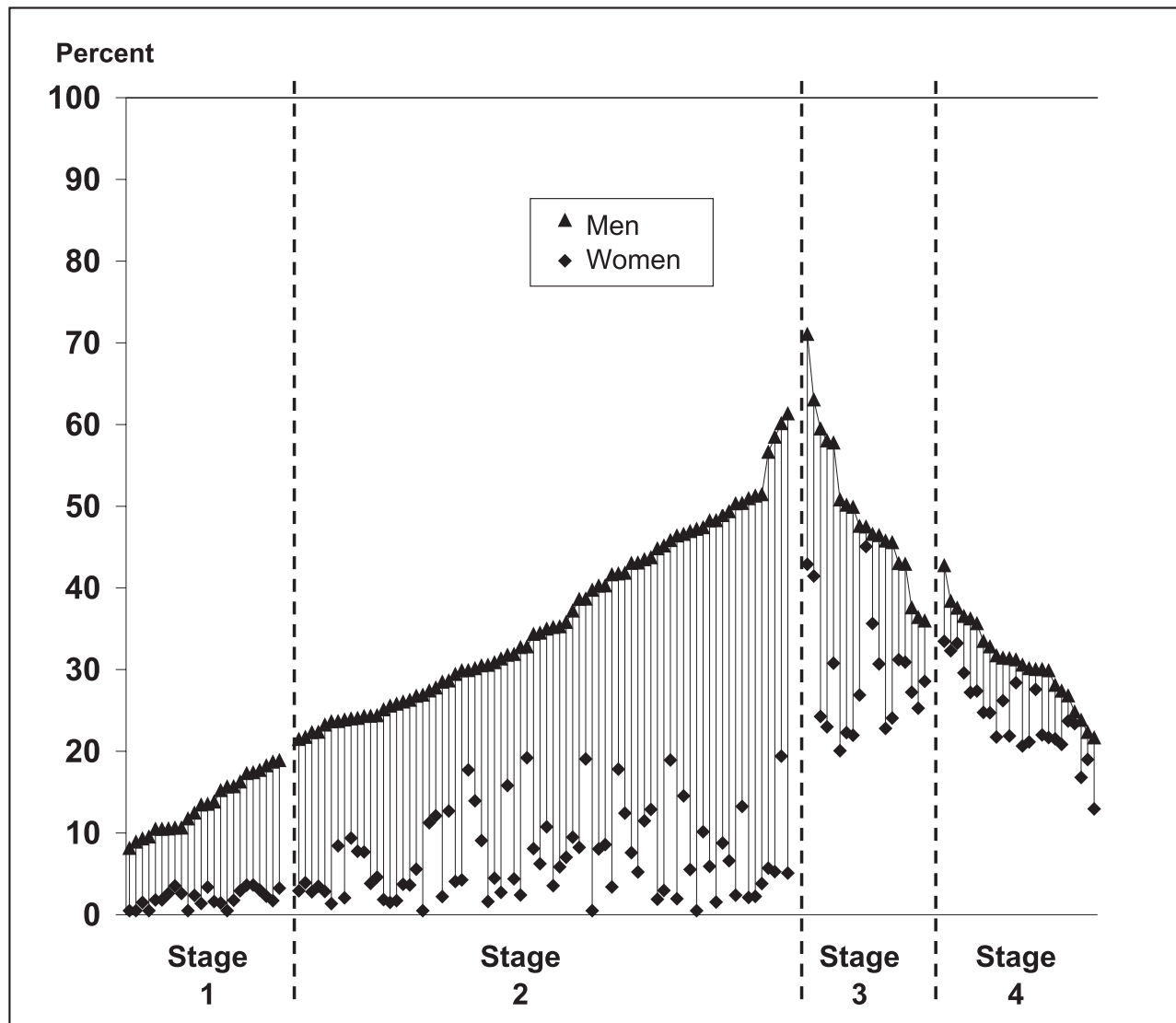
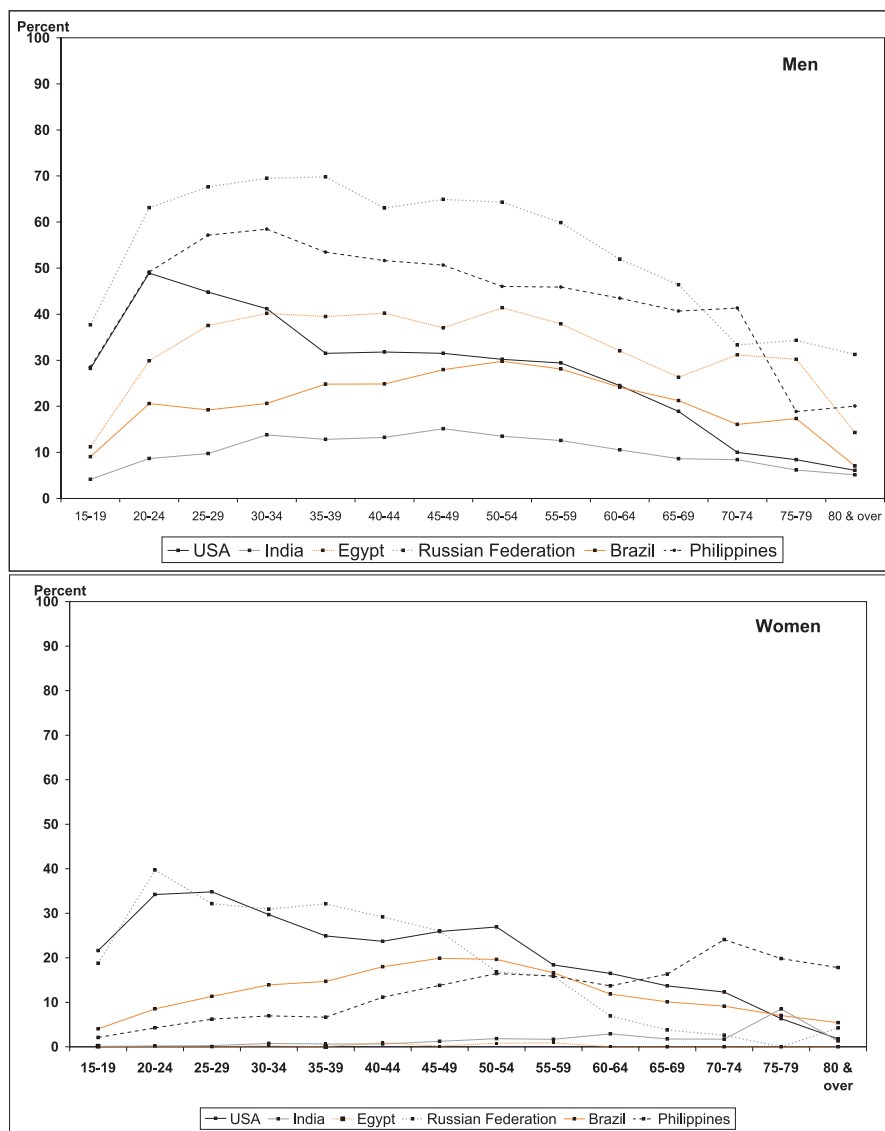


Fig. 1.6 Age-specific rates of smoking prevalence, in men and women in 2009

50, lying within 15 percentage points. Prevalence in women is almost always lower than in men in all age groups.

Initiation of smoking is shifting, and is taking place at earlier ages in both developed and developing countries. In developed countries, quitting smoking is also shifting to occur at a younger age, whereas in developing countries there is no such evidence.

(d) *Smoking in youth*

Information on smoking habits in youth are collected from a variety of youth surveys that include the Global Youth Tobacco Survey (GYTS), Global school-based Student Health Survey (GSHS) and Health Behaviour in School Aged Children (HBSC). Some countries have their own youth surveys, or have them as part of a general health or household survey, such as the Student Survey in Argentina, the Youth Smoking Survey in Canada, and New Zealand's Tobacco Survey.

The GYTS is a school-based survey designed to monitor tobacco use among youths aged 13 to 15 years. The GYTS uses a standard set of questions and sampling methods in over 160 countries. The survey has core questions that span seven thematic areas pertinent to tobacco. In addition to these, countries can include country-specific questions that allow assessment of tobacco use unique to the country. To assess prevalence of smoking, students are asked to report their smoking habits for both cigarettes and other tobacco products that they may have consumed over the past 30 days. Since its inception in 1999, the GYTS has covered over 2 million students. Although most GYTS are national surveys, in some countries they are limited to subnational locations. Further, countries conduct the GYTS in different years, rendering comparison for the same year difficult.

Prevalence of current tobacco use [including smokeless tobacco] in youth in 2004–09 for fourteen high burden low and middle income

countries is shown in Fig. 1.7. The Russian Federation has the highest prevalence of current tobacco use among the high burden countries for which national data are available. Further, in the Americas and Europe the difference in prevalence between boys and girls is smaller than in other regions. In contrast, in Egypt, India and Thailand, prevalences in boys are significantly higher than in girls.

Fig. 1.8 shows the range of current tobacco use by WHO region for boys and for girls and for both sexes combined. There are wide variations in current tobacco use within each region. The largest variations are observed in EMRO and SEARO irrespective of sex, reflecting potentially disparate initiation rates in countries within the region. In AFRO, the range of current tobacco use between boys and girls is virtually the same. In some countries (e.g. Argentina, Peru, Sierra Leone, Bulgaria, Croatia, Cook Islands, New Zealand), tobacco use in girls exceeds that in boys; but overall boys and girls show remarkably similar propensity to take up tobacco use.

[Warren *et al.* \(2006\)](#) present global estimates and regional averages for current tobacco smoking in youth using GYTS data spanning 1999–2005. Their estimates show that one in five boys and one in seven girls currently smoke tobacco. Prevalence of current smoking for both boys and girls combined was highest in AMRO (22.2%) and lowest in WPRO (11.4%). AMRO have the highest average for current tobacco smoking for boys (24%) and for girls (20.4%) whereas the lowest prevalence was in WPRO among boys (15%) and in SEARO among girls (7.1%).

1.4 Regulations and policies: the WHO Framework Convention on Tobacco Control

The WHO Framework Convention on Tobacco Control (WHO FCTC) – the first multi-lateral evidence-based treaty on tobacco control

Fig. 1.7 Prevalence of current tobacco use in youth for selected countries, 2005–2009

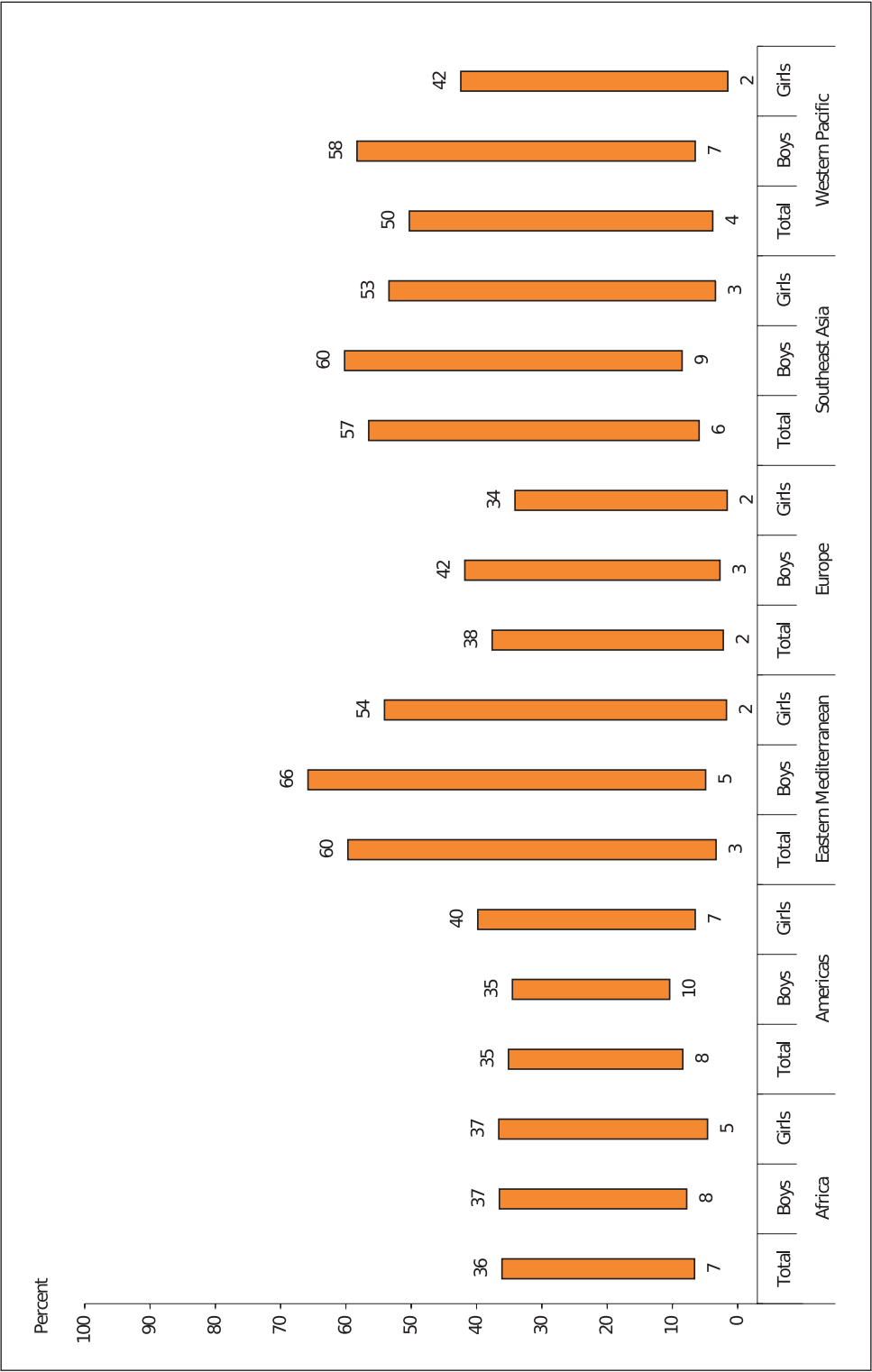
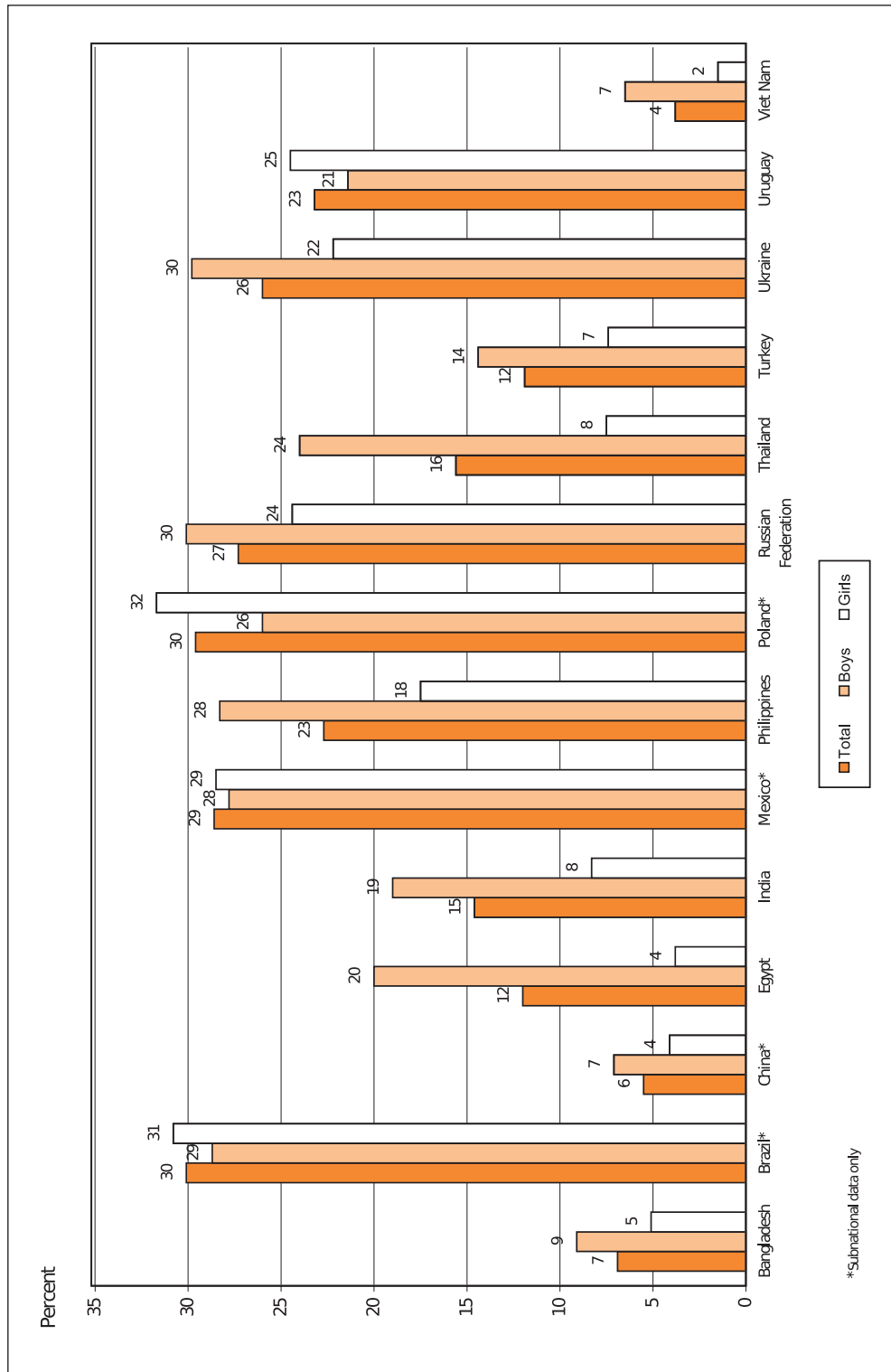


Fig. 1.8 Range of prevalence of current tobacco use in youth, 2005–2009, by WHO region



Figures have been rounded off and show prevalences in countries with national and subnational jurisdiction.

– articulates tobacco control measures available to countries to counter the growing tobacco epidemic. This treaty, which entered into force in 2005, represents one of the most universal treaties in the United Nations history. In 2008, the WHO launched MPOWER, a technical assistance package comprised of six strategies that reflects one or more of the WHO FCTC measures and helps countries meet their commitments to the WHO FCTC.

2. Cancer in Humans

2.1 Introduction

The available knowledge on the relationship between tobacco smoking and a variety of human cancers is based primarily on epidemiological evidence. An immense amount of such evidence has been obtained, and only a small proportion can be referred to here. The cancers considered to be causally related to tobacco smoking in the previous *IARC Monograph* on tobacco smoking ([IARC, 2004a](#)) included lung, oral cavity, nasal cavity and paranasal sinuses, nasopharynx, oropharynx, hypopharynx, larynx, oesophagus (adenocarcinoma and squamous cell carcinoma), upper aerodigestive tract combined, stomach, pancreas, liver, kidney (body and pelvis), ureter, urinary bladder, cervix and myeloid leukaemia. In addition, it was concluded that there was evidence suggesting lack of carcinogenicity for cancers of the breast and of the endometrium.

Since 2002, there have been additional cohort and case-control studies on the relationship of tobacco smoking in different forms to these and other cancers in many countries. A large body of evidence has been obtained from cohort studies with respect to different cancer sites and types of tobacco product. These cohort studies are described briefly in Table 2.1 (available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.1.pdf>), listed by country.

Case-control studies are described in the sections pertaining to cancer sites. More studies are now available from countries and populations that are still at an early stage of the tobacco epidemic. These studies are prone to underestimate the true strengths of the association between tobacco smoking and any specific cancer as the full effect of duration of smoking cannot be evaluated.

2.2 Cancer of the lung

2.2.1 Overview of studies

The main cause of lung cancer in humans is tobacco smoking and most information establishing this fact comes from epidemiological studies in which the assessment of exposure was based on self-reported information on personal smoking habits via self-administered questionnaire or in-person interviews. Since the previous *IARC Monograph* ([IARC, 2004a](#)), numerous studies have been published on the issues of tobacco smoking and sex and racial/ethnic susceptibility, ‘tar’ yields as measured by machine smoking, the relationship between histological changes and the design of cigarettes, dose-response association, genetic susceptibilities and interactions.

2.2.2 Factors affecting risk

Recent epidemiological studies incorporating measures of smoking metabolites in serum or urine are helping to refine our understanding of exposure-response relationships with tobacco smoke. Dose-response evidence has been obtained from three cohort studies ([Flanders et al., 2003](#); [Boffetta et al., 2006](#); [Yuan et al., 2009](#); Table 2.2 available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.2.pdf>) and four pooled analyses ([Lubin & Caporaso, 2006](#); [Lubin et al., 2007a, b, 2008](#); Table 2.3 available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.3.pdf>)

since the previous *IARC Monograph* ([IARC, 2004a](#)).

The US American Cancer Society Cancer Prevention Study-II (CPS-II) is the largest cohort study on smoking and lung cancer risk using questionnaire assessment of exposure ([Flanders et al., 2003](#)). In this study cigarette smoking duration is a much stronger predictor of lung cancer mortality than is cigarette smoking intensity, regardless of age in both men and women. These results are qualitatively similar to those reported by [Doll & Peto \(1978\)](#) and are consistent with [IARC \(2004a\)](#).

In a questionnaire-based assessment of the association of tobacco smoking with lung cancer risk, smokers at higher smoking intensities seem to experience a “reduced potency” per pack such that for equal total exposure, the excess odds ratio per pack-year decreases with intensity ([Lubin et al. 2008](#)). Below 15–20 cigarettes/day, the excess odds ratio/pack-year increases with intensity ([Lubin & Caporaso, 2006](#); [Lubin et al., 2007a](#)) while above 20 cigarettes/day, there is an ‘inverse-exposure-rate’ effect ([Lubin et al., 2007a](#)) suggesting a greater risk for total exposure delivered at lower intensity (or a longer duration) than the equivalent exposure delivered at a higher intensity. The intensity effects were also statistically homogeneous across diverse cancer types, indicating that after accounting for risk from total pack-years, intensity patterns were comparable for cancer of the lung, bladder, oral cavity, pancreas and oesophagus. These analyses suggest that the risk of lung cancer increases with increasing tobacco exposure at all dose levels, but there is some levelling-off effect at the highest intensity of tobacco smoking.

However, when serum cotinine was used as a measure of exposure to tobacco smoking, rather than questionnaire-based data, the odds ratio of lung cancer increased linearly over the full range of exposure from ≤ 5 ng/mL through ≥ 378 ng/mL, with an odds ratio of 55.1 (95% confidence interval (CI): 35.7–85.0) in the

highest exposure group. These results suggest that the decreased rate of lung cancer risk at high intensity of tobacco smoke previously described is a statistical artefact. Such an effect may be due to an inaccurate assessment of total tobacco smoke exposure from questionnaire-based studies at high exposure levels ([Boffetta et al., 2006](#)). Somewhat similar results were obtained when both 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) and total cotinine in urine were measured in subjects of two large cohort studies from Shanghai men and Singapore men and women ([Yuan et al., 2009](#)). Among smokers with comparable smoking histories (as noted in questionnaire data) there is a 9-fold variation in subsequent risk of lung cancer between those with high and those with low levels of total urinary NNAL and cotinine. Thus measurements of urinary cotinine and total NNAL at a single point in time in a smoker could substantially improve the predictive power of a lung cancer assessment model based solely on self-reported smoking history (number of cigarettes/day, number of years of regular smoking). A positive NNAL-lung cancer association of comparable magnitude was observed in both Shanghai and Singapore subjects despite differences in the NNK content of tobacco smoked. The independent association between total urinary cotinine and lung cancer risk, after adjustment for total urinary NNAL and smoking history, suggests that tobacco smoke compounds other than NNK play a role in lung cancer development in smokers. Further, a single measurement of urinary NNAL may closely predict the average level of NNAL measured over a much longer period of time.

2.2.3 Types of tobacco or of cigarette

(a) Tar levels

In a previous *IARC Monograph* ([IARC, 1986](#)), it was concluded on the basis of the case-control, cohort studies and ecological evaluations

available at the time that prolonged use of ‘high-tar’ and unfiltered cigarettes is associated with greater risks than prolonged use of filter-tipped and ‘low-tar’ cigarettes. More recently ([IARC, 2004a](#)), it has been recognized that the actual quantitative impact of reduced ‘tar’ and filter-tipped cigarettes is difficult to assess because of, respectively, the concomitant increase in tobacco-specific nitrosamines that accompanies the greater use of blend tobacco and the compensatory changes in smoking behaviour by smokers attempting to maintain their accustomed level of nicotine intake. Nevertheless, it was concluded that changes in cigarette types since the 1950s have probably tended to reduce the risk for lung cancer associated with tobacco smoking.

Additional refinement in assessing the health effects associated with smoking cigarettes of various tar content has been possible since the publication of the earlier reports. Compared with smokers of medium tar (15–21 mg) filtered cigarettes risk was higher among men and women who smoked high tar (≥ 22 mg) non-filtered brands but there was no difference in risk among men and women who smoked ‘very low tar’ or ‘low tar’ brands compared with those who smoked ‘medium tar’ brands ([Harris et al., 2004](#)). Regardless of tar content of their cigarettes, all current smokers had a far greater risk for lung cancer than people who had stopped smoking or had never smoked ([Harris et al., 2004](#)).

(b) *Mentholated cigarettes*

In the previous *IARC Monograph* ([IARC, 2004a](#)) the conclusion was drawn that there is no additional risk associated with smoking mentholated cigarettes when total consumption (pack-years) was controlled versus non-mentholated ones. Recent evidence supports that conclusion.

Mentholated cigarettes first appeared in the 1920s, but were not widely used until the mid-1950s ([Bogen, 1929](#); [Federal Trade Commission, 2001](#)). Since the early 1970s, menthol varieties have accounted for 25–60% of all cigarettes

sold in the USA ([Federal Trade Commission, 2001](#)). There are strong ethnic differences in the use of menthol cigarettes; more than 60% of Black smokers of both sexes use menthol brands compared to fewer than 25% of White smokers ([Royce et al., 1993](#); [Hymowitz et al., 1995](#)). Studies have generally not demonstrated an increased risk of lung cancer for mentholated cigarettes versus non-mentholated cigarettes ([Kabat & Hebert, 1994](#); [Carpenter et al., 1999](#); [Brooks et al., 2003](#); [Stellman et al., 2003](#)). Recent evidence also suggests that users of mentholated cigarettes smoke fewer pack-years than those of non-mentholated cigarettes.

The higher incidence of lung cancer among Blacks is an important public health concern but the causes remain unclear. Mentholated cigarette use does not appear to explain the racial disparity observed in lung cancer risk among those having the same total tobacco consumption.

2.2.4 *Histology*

Compiled databases from IARC and other sources indicated that squamous cell carcinoma rates [per 100000 person-years] among men declined by 30% or more in North America and some European countries between 1980–82 and 1995–97, while changing less dramatically in other areas; small cell carcinoma rates decreased less rapidly. In contrast, the proportion of adenocarcinoma cases rose among men and women in virtually all areas, with the increases among men exceeding 50% in many areas of Europe ([Devesa et al., 2005](#)).

Based on a comparison of two large cohort studies initiated by the American Cancer Society (ACS) (CPS-I and CPS-II) in 1960 and 1980, respectively, a stronger association between smoking and adenocarcinoma was observed in recent compared to earlier follow-up periods ([Thun & Heath, 1997](#)). Additionally, an association between cigarette smoking and bronchioloalveolar carcinoma was also found in several

studies ([Falk et al., 1992](#); [Morabia & Wynder, 1992](#)).

A meta-analysis of 8 cohort and 14 case-control studies conducted in Japan among active smokers indicated significant excess lung cancer risks among men for both squamous cell carcinoma (relative risk (RR), 11.7) and adenocarcinoma (RR, 2.30). Among women the risks were 11.3 for squamous cell carcinoma and 1.37 for adenocarcinoma ([Wakai et al., 2006](#)).

2.2.5 Population characteristics

(a) Sex

Meta-analyses on sex-specific susceptibility to lung cancer associated with tobacco smoking are presented in Table 2.4 (available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.4.pdf>) and cohort studies in Table 2.5 (available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.5.pdf>).

In the 1990s, two case-control studies indicated that relative risks for lung cancer associated with specific amounts and duration of cigarette smoking may be higher among women than among men ([Risch et al., 1993](#); [Zang & Wynder, 1996](#)).

In the large NIH-AARP [National Institutes of Health-American Association of Retired People] cohort ([Freedman et al., 2008](#)), smoking was associated with lung cancer risk in both men and women. Age-standardized incidence rates for lung cancer tended to be higher in men than in women with comparable smoking histories (for current smokers and for quitters of less than 10 years), and in cases with squamous cell tumours. However, lung cancer risk was generally similar between men and women.

In a joint analysis, results from the Nurses' Health Study of women and the Health Professionals Follow-up Study in men ([Bain et al., 2004](#)) suggest little difference in lung cancer susceptibility between men and women given equal smoking exposure. The hazard ratio

in women ever smokers compared with men was 1.11 (95%CI: 0.95–1.31).

Serum cotinine levels were analysed in lung cancer cases and controls ([Boffetta et al., 2006](#)). The lung cancer odds ratios (ORs) estimated for men and women were very similar for those with comparable serum cotinine levels. Other studies that have carefully quantified tobacco exposure via self-administered questionnaire or interview provide additional evidence of a comparable increase in lung cancer risk in the two sexes ([Kreuzer et al., 2000](#); [Flanders et al., 2003](#); [Bain et al., 2004](#)).

In a meta-analysis of observational studies on cigarette smoking and cancer from 1961–2003 (conducted on 177 case-control studies, 75 cohort studies and two nested case-control studies), dose-response estimates were available in 44 studies: 19 with estimates for men only, 11 with estimates for women only and 14 with separate estimates for men and women ([Gandini et al., 2008](#)). Overall, the risk of lung cancer for men and women increased by 7% for each additional cigarette smoked per day (RR, 1.07; 95%CI: 1.06–1.08). The increased risk appears to be slightly higher in women (RR, 1.08; 95%CI: 1.07–1.10) than in men (RR, 1.07; 95%CI: 1.05–1.08) ($P < 0.001$; adjusting for study type).

(b) Ethnicity

It has been postulated that susceptibility to lung cancer from tobacco smoking may differ by race and ethnicity ([Schwartz & Swanson, 1997](#); [Peto et al., 1999](#); [Stellman et al., 2001](#); [Kiyohara et al., 2004, 2005, 2006](#); [Pinsky, 2006](#); [Wakai et al., 2006](#); [Vineis et al., 2007](#); [Takahashi et al., 2008](#); Table 2.6 available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.6.pdf>). Lung cancer incidence rates vary considerable across racial/ethnic groups in the USA and elsewhere. Black men have higher rates than white men, while Hispanics, Asians and American Indians of both sexes have lower rates than whites ([Stellman et al., 2003](#); [SEER, 2004](#)).

Nutritional habits, smoking patterns, type of tobacco smoked and genetic factors may play a role in such differences between racial and ethnic groups.

The association of tobacco smoking and lung cancer does not appear to be as strong among Japanese as among populations of North America or Europe ([Wakai et al., 2006](#)). In a meta-analysis of 8 cohort studies and 14 case-control studies conducted in Japan, the excess lung cancer risks observed for both men (RR, 4.39; 95%CI: 3.92–4.92) and women (RR, 2.79; 95%CI: 2.44–3.20) in both case-control and cohort studies were lower than would have been expected from studies in North America and Europe. The lower lifetime consumption of cigarettes in Japanese, due in part to a later initiation of smoking and a lower consumption per day has been suggested to explain this. Other differences that may have etiological significance include tobacco ingredients, different filters on cigarettes, lifestyle factors including diet, and possibly differences in genetic susceptibility. [The Working Group noted that North American or European populations were not directly included in any of these studies.]

Data from the Asian Pacific Cohort Studies Collaboration, 31 studies involving 480125 persons, evaluated the risk of death from lung cancer associated with smoking habits in Australia, New Zealand and Asia ([Huxley et al., 2007b](#)). Among Asian men the hazard ratio was 2.48 versus 9.87 in men in Australia and New Zealand. Among women, the corresponding estimates were 2.35 and 19.33, respectively. [In these studies, Asian populations smoked fewer cigarettes for a shorter period of time compared to those in Australia and New Zealand.]

Based on data from the National Cancer Institute's Surveillance, Epidemiology, and End Results program (SEER), Chinese women residing in the USA have a fourfold increased risk of lung cancer, and Filipino women a twofold increased risk, compared to that expected based on rates in

non-Hispanic whites in the USA with a similar amount of cigarettes smoked ([Epplein et al., 2005](#)). Among Chinese women, the increased risk was largely restricted to adenocarcinoma and large cell undifferentiated carcinoma. Chinese females residents of the western US mainland have a much higher risk of lung cancer than would be expected from their tobacco use patterns, just as they do in Asia ([Peto et al., 1999](#); [Epplein et al., 2005](#)), the reason for these difference have not been identified. [Controlling for potential confounding factors was limited using aggregate data from SEER.]

Age, sex and race-specific risks of lung cancer mortality among lifetime non-smokers were compared in the two large ACS Cancer Prevention Study cohorts (CPS-I; CPS-II). The mortality rate was higher among African American women than among white women in CPS-II (hazard ratio (HR), 1.43; 95%CI: 1.11–1.36) ([Thun et al., 2006](#)). This suggests an inherent susceptibility difference between white and black women but it could also be explained by access to care, diet, or exposure to environmental carcinogens.

The risk for lung cancer associated with cigarette smoking in 183813 African-American, Japanese-American, Latino, native Hawaiian and white men and women was examined in the Multiethnic Cohort Study in the USA ([Haiman et al., 2006](#)). Information on demographic factors, smoking status, cigarettes/day smoked, years of smoking, years since quitting, diet, occupations, educational level and racial and ethnic group were collected for all subjects through a self-administered questionnaire at enrolment. Information about age of smoking initiation and cessation rates were collected on a subgroup of 5090 study subjects. Incident lung cancer cases were identified by linkage to the SEER cancer registries covering California and Hawaii. Among those who smoked no more than 10 cigarettes/day and those who smoked 11–20 cigarettes/day, relative risks ranged from 0.21 to 0.39 ($P < 0.001$) among Japanese Americans and Latinos and from 0.45

to 0.57 ($P < 0.001$) among whites as compared with African Americans. However, at levels exceeding 30 cigarettes/day, differences between racial/ethnic groups were no longer significant. The differences in lung cancer risk by racial group associated with smoking were observed for both men and women and for all histological types of lung cancer. These findings could not be explained by differences between populations in other known or suspected risk factors, including diet, occupation, and education level or by age at starting smoking or cessation of smoking.

Polymorphisms in glutathione-S-transferase (GST), GSTM1, GSTT1 and GSTP1 genes in humans are associated with reduction of enzymatic activity towards several substrates, including those found in tobacco smoke. In a population based case-control study involving early-onset lung cancer, African Americans carrying at least one G allele at the GSTP1 locus were more likely to have lung cancer compared with African Americans without a G allele after adjustment for age, sex, pack-years of smoking and a history of lung cancer in a first degree relative (OR, 2.9; 95%CI: 1.29–6.20). African Americans with either one or two risk genotypes at the GSTM1 (i.e. null genotype) and GSTP1 loci were at increased risk of having lung cancer compared with those having fully functional GSTM1 and GSTP1 genes (one risk genotype: OR, 2.8; 95%CI: 1.1–7.2 and two risk genotypes: OR, 4.0; 95%CI: 1.3–12.2). No significant single gene associations between GSTM1, GSTT1 and GSTP1 and early-onset lung cancer were observed in Caucasians, after adjusting for age, sex, pack-years and a family history of lung cancer ([Cote et al., 2005](#)).

The cytochrome P450 (CYP) superfamily of enzymes catalyses one of the first steps in the metabolism of carcinogens such as polycyclic aromatic hydrocarbons, nitroaromatics and arylamines. A population-based case-control study of lung cancer in the metropolitan Detroit area found that neither CYP1A1 MspI nor CYP1A1

Ile⁴⁶²Val was associated with lung cancer susceptibility among Caucasians or African Americans. Among Caucasians, however, CYP1B1 Leu⁴³² Val was significantly associated with lung cancer susceptibility (OR for at least one Val allele, 2.87; 95%CI: 1.63–5.07). Individuals with both this polymorphism and exposure to second-hand tobacco smoke were at particularly high risk for lung cancer. Combinations of particular CYP1B1 polymorphisms appeared to increase risk, although no combination differed significantly from the risk associated with CYP1B1 Leu⁴³² Val alone ([Cote et al., 2005](#); [Wenzlaff et al., 2005](#)).

The hypothesis that polymorphisms in TP53 may modulate the risk for lung cancer associated with tobacco smoke was evaluated in a case-control study of lung cancer in Baltimore, Maryland. African-Americans with Pro-T-A-G-G haplotype (combining the polymorphisms TP53_01 (rs1042522), TP53_65 (rs9895829), TP53_66 (rs2909430), TP53_16 (rs1625895), and TP_11 (rs12951053)) had both an increased risk for lung cancer (HR, 2.32; 95%CI: 1.38–4.10) and a worsened lung cancer prognosis (HR, 2.38; 95%CI: 0.38–4.10) compared with those having the Arg-T-A-G-T haplotype. No association of TP53 polymorphisms with lung cancer was observed in Caucasians ([Mechanic et al., 2007](#)). Common genetic variation in TP53 could modulate lung cancer pathways in African Americans. Differences in lung cancer susceptibility may exist based on race, tobacco exposure and selected genetic polymorphisms ([Mechanic et al., 2007](#)).

2.2.6 Interactions

(a) Diet and exercise

Antioxidant vitamins, carotenoids, isothiocyanates, total dietary vegetables and fruit, and physical exercise have been associated with a decreased risk for cancer in some studies but the overall protective effect of diet and exercise account for only a small fraction of the total risk associated with tobacco smoking.

The association of fruit and vegetable with lung cancer incidence among both smokers and non-smokers was evaluated in the European Prospective Investigation into Cancer and Nutrition (EPIC). In current smokers lung cancer risk was significantly decreased with higher vegetable consumption, the hazard ratio being 0.78 (95%CI: 0.62–0.98) per 100 g increase in daily vegetable consumption, and 0.90 (95%CI: 0.81–0.99) per 100 g fruit ([Linseisen et al., 2007](#)). While overall consumption of fruits and vegetables was not found to be protective of lung cancer in the NIH-AARP Diet and Health Study, higher consumption of several botanical subgroups (i.e. rosaceae, convolvulaceae, and umbelliferae) was significantly inversely associated with risk, but only in men ([Wright et al., 2008](#)).

Cruciferous vegetables (i.e. broccoli, cabbage, cauliflower, Brussels sprouts, kale) are rich in isothiocyanates and have been hypothesized to have anticancer properties that may contribute to reduced risk for lung cancer. Isothiocyanates may inhibit the bioactivation of procarcinogens found in tobacco smoke such as polycyclic aromatic hydrocarbons and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone ([Hecht, 2000](#)). Isothiocyanates may also enhance excretion of carcinogenic metabolites before they can damage DNA ([Gasper et al., 2005](#)). Furthermore, sulforaphane, a major isothiocyanate found in broccoli, can induce cell cycle arrest and apoptosis ([Seow et al., 2005](#)). GSTM1 and GSTT1 encode isoenzymes that play an important role in xenobiotic metabolism ([Hecht, 2000](#)). Individuals with homozygous deletion of GSTM1 and GSTT1, or both may metabolize isothiocyanates less efficiently and may be more intensely exposed to isothiocyanates after consumption of cruciferous vegetables. Epidemiological evidence from 30 studies on the association between lung cancer and either total cruciferous vegetable consumption (6 cohort and 12 case–control studies) or specific cruciferous vegetables (1 cohort and 11 case–control studies) was recently evaluated

([Lam et al., 2009](#)). The risk for lung cancer among those in the highest category of total cruciferous vegetable intake was 22% lower in case–control studies (pooled OR, 0.78; 95%CI: 0.70–0.88) and 17% lower in cohort studies (pooled RR, 0.83; 95%CI: 0.62–1.08). The strongest inverse association of total cruciferous vegetable intake with lung cancer was seen among individuals with GSTM1 and GSTT1 double null genotypes (OR, 0.41; 95%CI: 0.26–0.65; *p* for interaction = 0.01). The inverse association was observed in both smokers and non-smokers.

The potential role of vitamin A in the development of lung cancer attracted early research interest ([Bjelke, 1975](#)). Carotenoids were thought to have anti-cancer properties and early evidence from case–control studies tended to support an inverse association of lung cancer incidence with β -carotene intake and with serum concentrations of β -carotene. However, the case–control design is not ideal for assessing the effect of serum carotenoids as a risk factor for lung cancer risk since the disease is likely to effect serum levels. In a meta-analysis of six randomized clinical trials and 25 prospective observational studies, [Gallicchio et al. \(2008\)](#) computed a pooled relative risk for studies comparing β -carotene supplements with placebo of 1.10 (95%CI: 0.89–1.36). Among observational studies, the pooled relative risk for total carotenoid dietary intake from six studies was 0.86 (95%CI: 0.75–0.99) among current smokers. For dietary intake of β -cryptoxanthin, data from six studies gave a pooled relative risk among smokers of 0.75 (95%CI: 0.58–0.96). No other carotenoids significantly reduced the risk in current, former or never smokers.

Based on a review of the literature, antioxidant vitamins show no clear protective effect on lung cancer risk in smokers or non-smokers, although there was some, albeit inconsistent, evidence pointing to a protective role for vitamin C and E. No clear protective role was observed for vitamin A ([Ruano-Ravina et al., 2006](#)).

Increased physical activity has been associated with a reduction in the incidence and mortality from all-site cancer and some site-specific cancers in studies of non-smokers, but less is known about whether physical activity is associated with similar risk reduction in smokers. Several early studies suggested that physical activity is associated with decreased risk of lung cancer in men and women after adjusting for smoking, with risk reductions estimated from 18% (Peterson *et al.*, 2001) to 62% (Kubík *et al.*, 2001). The effect of physical activity on lung cancer risk was assessed in a sample drawn from participants in the Beta-Carotene and Retinol Efficacy Trial. The results suggested that physical activity may play a small role in reducing cancer risk and mortality among those with significant tobacco exposure. The incidence of lung cancer and of all cancer sites combined seemed to be more attenuated by exercise in men than in women, while the attenuation in lung cancer mortality was greater in women than in men. These effects may be more pronounced for younger people and may differ inconsistently by pack-years of smoking (Alfano *et al.*, 2004).

(b) Radon

In a pooled analysis of data from 13 case-control studies of residential radon and lung cancer from nine European countries (7148 cases of lung cancer and 14208 controls), the dose-response relation seemed to be linear with no threshold and remained significant in analyses limited to individuals from homes with measured radon < 200 Bq/m³. The absolute risks of lung cancer by age 75 years at radon concentrations of 0, 100, and 400 Bq/m³ would be about 0.4%, 0.5% and 0.7%, respectively, for lifelong non-smokers, and about 25 times greater (10%, 12% and 16%) for cigarette smokers. These studies show appreciable hazards from residential radon, particularly for smokers and recent ex-smokers (Darby *et al.*, 2005). Similar risks were identified in a

pooling project of North American case-control studies (Krewski *et al.*, 2005).

(c) Asbestos

Exposure to asbestos and tobacco smoking are both known causes of lung cancer in humans (Doll & Peto, 1978; de Klerk *et al.*, 1996). Some studies suggest a multiplicative effect [where the effect of asbestos exposure is a multiple of the effect of smoking] (Hammond *et al.*, 1979; Doll & Peto, 1985), and meta-analyses have suggested that the additive model [where asbestos exposure and smoking are independent of each other] is unsound (Lee, 2001; Liddell, 2001). In a recent study of 2935 asbestos miners, persons exposed to asbestos and tobacco who subsequently quit smoking remained at a 90% increased risk of lung cancer up to 20 years after smoking cessation, compared to never-smoker asbestos workers (Reid *et al.*, 2006a).

(d) Genetic polymorphisms

Lung cancer is plausibly caused by the interplay between environmental factors and several low-risk alleles. Attempts in identifying specific single nucleotide polymorphisms (SNPs) responsible for modulating lung cancer risk have yielded few conclusive results. Recent studies have focused on mechanistically plausible polymorphisms in genes coding for enzymes involved in the activation, detoxification and repair of chemical damage caused by tobacco smoke. Genetic association studies indicate that several inherited genetic polymorphisms may be associated with lung cancer risk, but the data from individual studies with low statistical power are conflicting. Evidence from pooled or meta-analyses, along with some individual studies, is briefly summarized below.

(i) Metabolic genes

Most of the 70 carcinogens in tobacco smoke are procarcinogens that must be activated by phase I enzymes and may then be deactivated by

phase II enzymes. Polymorphisms that alter the function of the genes involved in the activation or detoxification of tobacco smoke carcinogens can potentially influence an individual's risk of developing a tobacco-related cancer.

Meta and pooled analyses of 34 case-control, genotype-based studies were conducted to assess the effect of GSTT1 genotypes and smoking on lung cancer risk. No significant interaction was observed ([Raimondi et al., 2006](#)). A pooled analysis of 21 case-control studies from the International Collaborative study of Genetic Susceptibility to Environmental Carcinogens showed no evidence of increased risk for lung cancer among carriers of the GSTM1 null genotype and there was no evidence of interaction between GSTM1 genotype and either smoking status or cumulative tobacco consumption ([Benhamou et al., 2002](#)). Similarly, in another pooled analysis the summary OR indicated the slow acetylator genotype of N-acetyltransferase 2 (NAT2) detoxification enzyme was not associated with lung cancer risk among Caucasians ([Borlak & Reamon-Buettner, 2006](#)). In a pooled analysis to test the hypothesis of interaction among genetic variants in increasing the individual risk for cancer, the cumulative effect of variants in three metabolic genes, CYP1A1, GSTM1 and GSTT1 was assessed. The risk for lung cancer was increased with the combination of CYP1A1*2B or CYP1A1*4 alleles and the double deletion of both GSTM1 and GSTT1 up to an OR of 8.25 (95%CI: 2.29–29.77). The combination including CYP1A1*4 among never smokers was associated with an OR of 16.19 (95%CI: 1.90–137). These estimates did not change after adjustment by the number of cigarettes smoked and duration of smoking. The results were consistent across ethnicities and were approximately the same for adenocarcinoma and squamous cell carcinoma ([Vineis et al., 2007](#)).

Microsomal epoxide hydrolase 1 (EPHX1) plays an important role in both the activation and detoxification of tobacco-derived carcinogens.

Polymorphisms at exons 3 and 4 of the EPHX1 gene have been reported to be associated with variations in EPHX1 activity. In a meta-analysis of 13 case-control studies the low-activity (variant) genotype of EPHX1 polymorphism at exon 3 was associated with decreased risk for lung cancer (OR, 0.65; 95%CI: 0.44–0.96) among whites. In white-populations, the high activity (variant) genotype of EPHX1 polymorphism at exon 4 was associated with a modest increased risk of lung cancer (OR, 1.22; 95%CI: 0.79–1.90) and the predicted low activity was associated with a modest decrease in risk (OR, 0.72; 95%CI: 0.43–1.22) ([Kiyohara et al., 2006](#)).

(ii) DNA repair and cell cycle pathways

Data from 14 studies of lung cancer were used in a pooled analysis focusing on 18 sequence variants in 12 DNA repair genes, including APEX1, OGG1, XRCC1, XRCC2, XRCC3, ERCC1, XPD, XPF, XPG, XPA, MGMT and TP53 ([Hung et al., 2008a](#)). None of the variants appeared to have a large effect on lung cancer risk. In a recent meta-analysis the X-ray repair cross-complementing protein group 3 (XRCC3) and the xeroderma pigmentosum group D (XPD)/excision repair cross-complementing group 2 (ERCC2) genes were evaluated ([Manuguerra et al., 2006](#)). The authors found no association between these genes and the cancer sites investigated (skin, breast and lung). A significant association was identified for XPD/ERCC2 single nucleotide polymorphisms (codons 312 and 751) and lung cancer.

(iii) Nicotine acetylcholine receptor genes

A series of large genome-wide association studies for lung cancer have identified susceptibility loci for lung cancer in chromosome arms 5p, 6p and 15q ([Landi et al., 2009](#)). In particular, the susceptibility locus at chromosome region 15q25 includes several genes, including three that encode nicotinic acetylcholine receptor subunits (CHRNA5, CHRNA3 and CHRNB4). Nicotinic acetylcholine receptor subunit genes

code for proteins that form receptors present in neuronal and other tissue, in particular alveolar epithelial cells, pulmonary neuroendocrine cells, and lung cancer cell lines ([Wang et al., 2001](#); [Minna, 2003](#)) and bind to nicotine and nicotine derivatives including NNN. An association of CHRNA3 and CHRNA5 variants with nicotine dependence has been reported ([Saccone et al., 2007](#); [Berrettini et al., 2008](#)). These genes may act, at least partially, upon cigarette smoking. Current smokers with one or two copies of the susceptibility variant are likely to smoke between one and two cigarettes more a day ([Spitz et al., 2008](#)). Evidence for an effect of the 15q25 locus among never smokers is conflicting, with an association found in one study in Europe ([Hung et al., 2008b](#)) and one in Asia ([Wu et al., 2009a](#)), but not in others. Whether genes in the 15q25 locus have an effect on lung cancer beyond their propensity to increase numbers of cigarettes smoked is unclear.

Three genome-wide association studies identified genetic factors that modified disease risk. The first was a genome-wide association analysis to identify genetic polymorphisms associated with lung cancer risk in 1154 lung cancer patients of European ancestry who were current or former smokers and 1137 control subjects who were frequency matched to the lung cancer patients by age, sex, race and smoking status. Two SNPs, rs105173 and rs803419, which mapped to a region of strong linkage disequilibrium within 15q25.1, were strongly associated with risk of lung cancer, with an odds ratio for rs105173 of 1.31 ($P = 9.84 \times 10^{-6}$). This finding was replicated with an additional 711 case subjects and 632 control subjects from Texas ($P = 0.00042$) and in 2013 case subjects and 3062 control subjects in the United Kingdom ($P = 2.33 \times 10^{-10}$). The region of interest encompasses the nicotinic acetylcholine receptor subunit genes CHRNA3 and CHRNA5 (as well as CHRNB4) ([Spitz et al., 2008](#)). A second genome-wide association study conducted among 1989 lung cancer cases and

2625 controls from six central European countries confirm these results ([Hung et al., 2008a](#)). In a third genome-wide association study of 665 Icelandic, 269 Spanish and 90 Dutch lung cancer cases and 32244 controls a common variant in the nicotinic acetylcholine receptor gene cluster [chromosome region 15q24] was significantly associated with lung cancer risk (OR, 1.31; 95%CI: 0.1.19–1.44). The variant was observed to have a significant effect on the number of cigarettes smoked per day ([Thorgeirsson et al., 2008](#)). These studies have all shown a link between this variant and lung cancer risk either through a mechanism involving nicotine dependence or a direct role in downstream signalling pathways that promote carcinogens. Together these results provide compelling evidence of a locus at 15q25 and 15q24 predisposing to lung cancer.

(iv) *Alpha(1)-antitrypsin*

Alpha(1)-antitrypsin deficiency ($\alpha(1)$ ATD) is one of the most common genetic disorders, especially among European descendants. Recent results suggest that $\alpha(1)$ ATD carriers are at a 70–100% increased risk of lung cancer, accounting for 11% to 12% of patients with lung cancer ([Yang et al., 2008](#)). [The specific effect by smoking status was not evaluated.]

(v) *Other genes*

Mutations in the checkpoint CHEK2 gene have been associated with increased risk of breast, prostate and colon cancer and a decreased risk of lung cancer among those with the I157T missense variant of the CHEK2 gene. In a large Polish case–control study CHEK2 mutations were protective against lung cancer (OR, 0.3; 95%CI: 0.2–0.5) ([Cybulski et al., 2008](#)).

The Swedish Family-Cancer Database was used to compare the rate of lung cancers among persons without family history of lung cancer to those with a family history ([Li & Hemminki, 2004](#)). A high risk by family history in adenocarcinoma (standardized incidence ratio (SIR),

2.03) and large cell carcinoma (SIR, 2.14) was found, a slightly lower risk among patients with squamous cell carcinoma (SIR, 1.63) and small cell carcinoma (SIR, 1.55). Among siblings, an increased risk was shown for concordant adenocarcinoma and small cell carcinoma at all ages and for all histological types when cancer was diagnosed before age 50. At young age, risks between siblings were higher than those between offspring and parents. These data suggest that a large proportion of lung cancers before age 50 are heritable and due to a high-penetrant recessive gene or genes that predispose to tobacco carcinogen susceptibility.

(e) *Viral infection*

Data are limited regarding lung cancer risk in human immunodeficiency virus (HIV)-infected persons with modest immune suppression, before the onset of acquired immunodeficiency syndrome (AIDS). Among 57350 HIV-infected persons registered in the USA during 1991–2002 (median CD4 counts 491 cells/mm³), 871 cancers occurred. Risk was elevated for several non-AIDS defining malignancies, including cancer of the lung (SIR, 2.6 [*n* = 109]) (Engels *et al.*, 2008). [Specific evaluation with smoking status was not performed.]

2.3 Cancers of the upper aerodigestive tract

Evidence relating to cancers of the upper aerodigestive tract obtained from relevant cohort and case-control studies on specific sites is described in Sections 2.3.1 to 2.3.6; studies that looked at several subsites combined are described in Section 2.3.7. The major potential confounders for the relationship between smoking and cancers of the upper aerodigestive tract are alcohol consumption and use of any form of smokeless tobacco, and for some sites infection with human papillomavirus (HPV) (especially HPV16). In

general, the studies examined by the Working Group had adjusted for these two confounders when appropriate. Some studies also adjusted for dietary intake, especially of fruits and vegetables, although few reported stratified relative risks.

2.3.1 *Cancer of the oral cavity*

Tobacco smoking was found to be causally related to oral cancer (IARC, 1986, 2004a). New studies on the relationship between oral cancer and cigarette smoking published since the most recent IARC Monograph (IARC, 2004a) include four cohort studies (Table 2.7 available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.7.pdf>), and eight case-control studies (Tables 2.8–2.11 online; see below).

(a) *Intensity and duration of smoking*

Intensity of smoking was measured in almost all cohort (Table 2.7 online) and case-control studies (IARC 2004a; Table 2.8 available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.8.pdf> and Table 2.9 available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.9.pdf>). In addition to the number of cigarettes or amount of tobacco smoked daily, cumulative exposure to cigarette smoke was also measured in terms of pack-years, tobacco-years or lifetime tobacco consumption. The link between duration of cigarette consumption and oral cancer was examined in 15 case-control studies. Seven case-control studies also considered age at starting smoking.

One cohort study (McLaughlin *et al.*, 1995) and 14 case-control studies reported a dose-dependent increase in risk with increasing number of cigarettes smoked daily or increasing daily tobacco consumption (Franceschi *et al.*, 1990, 1992, 1999; Nandakumar *et al.*, 1990; Zheng *et al.*, 1990; Choi & Kahyo, 1991; Oreggia *et al.*, 1991; Bundgaard *et al.*, 1995; Zheng *et al.*, 1997; De Stefani *et al.*, 1998; Hayes *et al.*, 1999; De Stefani

et al., 2007; Subapriya *et al.*, 2007; Muwonge *et al.* 2008). Whenever analysed, the trend was always statistically significant (Franceschi *et al.*, 1990, 1992; Oreggia *et al.*, 1991; Bundgaard *et al.*, 1995; McLaughlin *et al.* 1995; Hayes *et al.*, 1999; Subapriya *et al.*, 2007), except in the study of Muwonge *et al.* (2008) which also included bidi smokers.

Bundgaard *et al.* (1995) used lifetime tobacco consumption divided into four categories and reported a positive, significant trend after adjustment for life-time consumption of alcohol and other risk factors. A positive trend was also found in all studies that have analysed consumption in pack-years or tobacco-years (Zheng *et al.*, 1990; Maier *et al.*, 1992a; Macfarlane *et al.*, 1995; Hung *et al.*, 1997; Zheng *et al.*, 1997; De Stefani *et al.*, 1998, 2007; Applebaum *et al.*, 2007; Muwonge *et al.*, 2008), except Muwonge *et al.* (2008).

Ten studies (Franceschi *et al.*, 1990, 1992; Nandakumar *et al.*, 1990; Zheng *et al.*, 1990; Choi & Kahyo, 1991; Oreggia *et al.*, 1991; Zheng *et al.*, 1997; De Stefani *et al.*, 1998, 2007; Znaor *et al.*, 2003; Subapriya *et al.*, 2007; Muwonge *et al.*, 2008) classified the duration of smoking in up to four categories, and all but one (Nandakumar *et al.*, 1990) reported increased relative risks and a positive trend.

Of six studies that considered age at start of smoking (Franceschi *et al.*, 1990, 1992; Choi & Kahyo, 1991; Oreggia *et al.*, 1991; Zheng *et al.*, 1997; Balaram *et al.*, 2002) two reported a statistically significant trend of increasing risk with decreasing age at starting (Franceschi *et al.*, 1990, 1992).

(b) Cessation of smoking

Three cohort studies (McLaughlin *et al.*, 1995; Freedman *et al.*, 2007a; Friborg *et al.* 2007) and nine case-control studies (Zheng *et al.*, 1990; Choi & Kahyo, 1991; Oreggia *et al.*, 1991; Franceschi *et al.*, 1992; Ko *et al.*, 1995; Zheng *et al.*, 1997; De Stefani *et al.*, 1998, 2007; Schildt *et al.*, 1998; Balaram *et al.*, 2002; Pacella-Norman

et al., 2002; Muwonge *et al.* 2008) estimated risks for former smokers which were always lower than those for current smokers and in five studies almost reached unity (Zheng *et al.*, 1990; Choi & Kahyo, 1991; Zheng *et al.*, 1997; Schildt *et al.*, 1998; Muwonge *et al.*, 2008). Twelve case-control studies examined the risk by years since quitting and all reported a negative trend, with relative risks compared with those in non-smokers decreasing to near unity after 10 or more years (Franceschi *et al.*, 1990, 1992; De Stefani *et al.*, 1998, 2007; Schlecht *et al.*, 1999a; Table 2.7 online; Table 2.10 available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.10.pdf>).

(c) Type of cigarette

The effect of the type of cigarette smoked was examined in several case-control studies (Table 2.11 available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.11.pdf>). The characteristics of the cigarettes included the presence of a filter, the type of tobacco, the tar content and whether the product was manufactured or hand-rolled. Two studies reported a statistically significantly higher risk for black than for blond tobacco (Oreggia *et al.*, 1991; De Stefani *et al.*, 1998, 2007). Similarly, a much higher risk was found for hand-rolled cigarettes than for manufactured cigarettes, and plain cigarettes had a much higher risk than filter-tipped cigarettes (De Stefani *et al.*, 1998, 2007). In one study the differences between black and blond tobacco and between hand-rolled and manufactured cigarettes persisted after stratification by duration of smoking (De Stefani *et al.*, 1998). Smoking cigarettes with a high-tar content led to higher risks than smoking cigarettes with a low-tar content (Franceschi *et al.*, 1992) and the same trend was observed for cigarettes without filter compared to cigarettes with filter (De Stefani *et al.*, 2007).

(d) Sex

Sex-specific effects were examined in two case-control studies ([Zheng et al., 1990](#); [Hayes et al., 1999](#)). In both studies, the relative risks for all categories of intensity, duration of smoking and pack-years were higher for women than for men. [The Working Group noted that the background risk of oral cancer is considerably lower in women than men. Thus, the higher relative risk estimates in women than men indicate a higher proportionate contribution from smoking in women than men, rather than higher absolute risk.]

2.3.2 Cancer of the pharynx

Tobacco smoking was considered to be an important cause of oropharyngeal and hypopharyngeal cancers in the previous *IARC Monographs* on tobacco smoking ([IARC, 1986, 2004a](#)). Since then, results available from three cohort (Table 2.12 available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.12.pdf>) and seven case-control studies (Table 2.13 available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.13.pdf> and Table 2.14 available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.14.pdf>) provide further support for the association. Many studies, however, combine cancers of the oral cavity and pharynx (see Section 2.3.7). This section summarizes the evidence from all eight cohort and 21 case-control studies that reported results specifically on oropharyngeal and hypopharyngeal cancer, or on pharyngeal cancer in general; the latter may include data on nasopharyngeal cancer.

The risk for pharyngeal cancer was significantly increased in smokers in four cohort studies ([Doll et al., 2005](#); [McLaughlin et al., 1995](#); [Freedman et al., 2007a](#); [Friborg et al., 2007](#)) and all but one of the case-control studies ([Rao et al., 1999](#)). The trend of increasing risk associated with increasing daily or cumulative consumption

of cigarettes was evident from all these studies, particularly those from Europe ([Brugere et al., 1986](#); [Tuyns et al., 1988](#); [Franceschi et al., 1990, 1999](#); [Maier et al., 1994](#); [Escribano Uzcudun et al., 2002](#); [Vlajinac et al., 2006](#)), India ([Znaor et al., 2003](#); [Sapkota et al., 2007](#)), Uruguay ([De Stefani et al., 1998, 2007](#)) and the USA ([McLaughlin et al., 1995](#); [Applebaum et al., 2007](#)), and less strongly so in studies from Canada ([Elwood et al., 1984](#)) and the Republic of Korea ([Choi & Kahyo, 1991](#)). The multicentre study in Europe, North and South America of [Hashibe et al. \(2007c\)](#) showed increased risks according to frequency (cigarettes/day) and duration (years) in never drinkers. [Applebaum et al. \(2007\)](#) found a relationship between increasing risk of pharyngeal cancer and increased pack-years of smoking in subjects with negative HPV16 serology but not in those with positive HPV16 serology (p value for interaction = 0.007).

In two case-control studies the risk increased with decreasing age at starting smoking ([Franceschi et al., 1990](#); [Choi & Kahyo, 1991](#)), but adjustment was not made for duration and intensity of smoking. In a case-control study from Spain ([Escribano Uzcudun et al., 2002](#)) the risk increased with the age of starting smoking.

Former smokers had consistently lower relative risks than did current smokers in both cohort ([McLaughlin et al., 1995](#); [Freedman et al., 2007a](#)) and case-control studies ([Choi & Kahyo, 1991](#); [De Stefani et al., 1998](#); [Vlajinac et al., 2006](#)). In comparison with non-smokers, the relative risks for former smokers who had quit smoking for more than 10 years were between 2 and 4 ([Franceschi et al., 1990](#); [De Stefani et al., 1998](#); [La Vecchia et al., 1999](#)), whereas the relative risks for current smokers in these studies were 10–14. In one study in Brazil ([Schlecht et al., 1999a](#)), relative risks for former smokers who had stopped smoking for more than 10 years approached 1, whereas that for current smokers was just below 6. Consumption of black tobacco, hand-rolled cigarettes or plain cigarettes resulted in a higher

risk for pharyngeal cancer than consumption of blond tobacco, manufactured cigarettes or filter-tipped cigarettes (De Stefani *et al.*, 1998; 2007).

2.3.3 Cancer of the nasal cavity and accessory sinuses

In the Life Span Study in Japan (Akiba, 1994) the association of tobacco use with sinonasal cancer was examined. A total of 26 cases of sino-nasal cancer were identified among 61505 adults during follow-up. Relative risk estimates, adjusted for sex, location, population group, atomic bomb exposure, year of birth and attained age, were 2.9 (95%CI: 0.5–) and 4.0 (95%CI: 1.2–) for former and current smokers, respectively, when compared with non-smokers [upper confidence limits were not reported]. The cohort of 34439 British doctors followed up to 50 years (Doll *et al.*, 2005) showed increased risk for current smokers and smokers of more than 25 cigarettes per day, but only six deaths from nasal cavity and sinuses cancers were observed (Table 2.15 available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.15.pdf>).

A total of nine case-control studies of nasal cavity and sinus cancers have been conducted. When histological types were combined, all studies found an increased risk associated with cigarette smoking, but only one was statistically significant (Caplan *et al.*, 2000). In seven studies, dose-response in terms of intensity of smoking (cigarettes/day), duration of smoking or pack-years was considered. A positive significant trend was found in five studies (Brinton *et al.*, 1984; Hayes *et al.*, 1987; Fukuda & Shibata, 1990; Zheng *et al.*, 1993; Caplan *et al.*, 2000) and suggested in the other two (Strader *et al.*, 1988; Zheng *et al.*, 1992c).

One study (Zheng *et al.*, 1993a) found a significant decrease in risk for sinonasal cancer associated with increasing number of years since cessation of smoking. In a previous study, the

same authors had found a negative, non-significant association (Zheng *et al.*, 1992c).

Five studies analysed squamous-cell carcinomas and adenocarcinomas separately (Brinton *et al.*, 1984; Hayes *et al.*, 1987; Strader *et al.*, 1988; Zheng *et al.*, 1992c; 't Mannetje *et al.*, 1999). In all studies, there was a significantly increased risk for squamous-cell carcinomas, whereas the risk was generally not increased for adenocarcinomas.

2.3.4 Cancer of the nasopharynx

(a) Cohort studies

The risk for nasopharyngeal carcinoma has been examined in relation to tobacco use in six cohort studies, three of them reported since the last evaluation (IARC 2004a; Table 2.16 available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.16.pdf>). In one study, conducted in a low-risk area (Chow *et al.*, 1993a), a significant increase in risk among smokers and suggestive positive dose-response relationships by duration of smoking and age at starting smoking were found. In another study, conducted in Province of Taiwan, China, an area in which nasopharyngeal cancer area is endemic, a similarly increased risk was found, but it was not statistically significant (Liaw & Chen, 1998). Doll *et al.* (2005) identified a risk only for smokers of more than 25 cigarettes per day, however, this result was based on only four deaths. Friberg *et al.* (2007) in Singapore found statistically significant increased risk of nasopharyngeal cancer only for those smoking for 40 years or more. Hsu *et al.* (2009) in Taiwan, China observed increased statistically significant risks only for those smoking for 30 years or more and those with cumulative exposure of 30 pack-years or more.

(b) Case-control studies

The study designs and the results of the case-control studies on the association of nasopharyngeal carcinoma with cigarette smoking

reported since the previous *IARC Monograph* (IARC, 2004a) are given in Table 2.17 (available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.17.pdf>) and Table 2.18 (available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.18.pdf>), one being a nested case–control analysis within a cohort study (Marsh *et al.*, 2007).

In total, 14 informative case–control studies were available. In almost all of these, the risk for nasopharyngeal carcinoma was higher in smokers than in non-smokers. In Taiwan, China (Cheng *et al.*, 1999) high risks were statistically significant only for duration of smoking of 20 years or more. In the five studies conducted in the USA (Mabuchi *et al.*, 1985; Nam *et al.*, 1992; Zhu *et al.*, 1995; Vaughan *et al.*, 1996; Marsh *et al.*, 2007), where the incidence of nasopharyngeal carcinoma is low, the relative risks for current smokers ranged between 2 and 4, but were not statistically significant in the two studies (Mabuchi *et al.*, 1985; Marsh *et al.*, 2007). In a study conducted in Shanghai, an area of China in which nasopharyngeal carcinoma is not endemic (Yuan *et al.*, 2000), the relative risk was just below 2. In one study from the Philippines there was a sevenfold increase in risk after more than 30 years of smoking (West *et al.*, 1993). The four studies (Lin *et al.*, 1973; Yu *et al.*, 1990; Ye *et al.*, 1995; Cao *et al.*, 2000) conducted in areas of China in which nasopharyngeal carcinoma is endemic (Taiwan, China, Guangzhou, and Sihui) found relative risks for ever smoking ranging between 2 and 5. In the study from the North of Africa (Feng *et al.*, 2009) the only statistically significant increased risk was found for differentiated nasopharyngeal cancer in those that had smoked more than 22 cigarettes/day. [The result, based only on three cases, is very unstable (RR, 313; 95%CI: 1.94–50336).]

A statistically significant dose–response relationship was detected in seven studies that evaluated the effects of daily or cumulative exposure to tobacco smoke (Yu *et al.*, 1990; Nam *et al.*, 1992;

Zhu *et al.*, 1995; Vaughan *et al.*, 1996; Cao *et al.*, 2000; Yuan *et al.*, 2000; Feng *et al.*, 2009) and was suggestive in two others (Lin *et al.*, 1973; West *et al.*, 1993). In two studies the risk of nasopharyngeal carcinoma decreased with increasing time since quitting smoking (Nam *et al.*, 1992; Vaughan *et al.*, 1996).

In the remaining studies, six from areas in which nasopharyngeal carcinoma is endemic (Ng, 1986; Yu *et al.*, 1986; Sriamporn *et al.*, 1992; Zheng *et al.*, 1994; Cheng *et al.*, 1999; Feng *et al.*, 2009; Guo *et al.*, 2009) and seven from areas in which it was not endemic (Henderson *et al.*, 1976; Lanier *et al.*, 1980; Mabuchi *et al.*, 1985; Ning *et al.*, 1990; Armstrong *et al.*, 2000; Marsh *et al.*, 2007), the relative risks for nasopharyngeal carcinoma for ever smoking were not significantly increased (Lanier *et al.*, 1980; Mabuchi *et al.*, 1985; Cheng *et al.*, 1999) or were close to 1.0 (Henderson *et al.*, 1976; Ng, 1986; Yu *et al.*, 1986; Ning *et al.*, 1990; Sriamporn *et al.*, 1992; Zheng *et al.*, 1994; Guo *et al.*, 2009).

In the two studies that distinguished between different histological types, relative risks were higher for keratinized (squamous-cell) carcinoma than for unkeratinized carcinoma (Zhu *et al.*, 1995; Vaughan *et al.*, 1996).

In the three studies in which men and women were analysed separately (Lin *et al.*, 1973; Nam *et al.*, 1992; Yuan *et al.*, 2000), the relative risks were found to increase similarly in both sexes in two studies (Nam *et al.*, 1992; Yuan *et al.*, 2000) and were higher among women in the study of Lin *et al.* (1973).

2.3.5 Cancer of the oesophagus

In the previous *IARC Monograph* (IARC, 2004a), both histological subtypes of oesophageal cancer (squamous-cell carcinoma and adenocarcinoma) were considered to be causally related to cigarette smoking. Many more epidemiological studies have since been conducted, and results of these studies further support this conclusion.

(a) *Squamous cell carcinoma and unspecified cancer of the oesophagus*

Since the previous *IARC Monograph* (IARC, 2004a), there have been reports on 9 cohort studies (Table 2.19 available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.19.pdf>) and 22 case-control studies (Tables 2.20–2.23; see below), making 30 cohort and 55 case-control studies in all. All showed that the risk of oesophageal squamous cell carcinoma was associated with cigarette smoking. In one study (Li *et al.*, 1989), the elevated risk was observed only in an area with a relatively low incidence of oesophageal cancer. However, two later studies in the same area, Lin County, China, found a twofold increase in risk for oesophageal cancer among smokers (Gao *et al.*, 1994; Lu *et al.*, 2000).

In most cohort studies and in most case-control studies with relatively large sample sizes (IARC, 2004a; Table 2.19 online; Table 2.20 available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.20.pdf>; Table 2.21 available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.21.pdf>), the risk for oesophageal cancer was shown to increase with increasing duration of smoking (11 cohort and 32 case-control studies) or number of cigarettes smoked daily (18 cohort and 31 case-control studies), and to decrease with increasing age at starting smoking (12 case-control studies). In comparison with pharyngeal and laryngeal cancers, relative risks for oesophageal cancer estimated by duration and by intensity of smoking were somewhat lower (see Sections 2.3.2 and 2.3.6, respectively).

Ten cohort and 20 case-control studies (IARC, 2004a; Table 2.19 online; Table 2.22 available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.22.pdf>) investigated the effect of smoking cessation on risk of oesophageal cancer. Although not all studies analysed the trend, all found a decreasing

relative risk with increasing number of years since quitting. In some studies, the risk first started to decrease after 10 years of cessation (Brown *et al.*, 1988; Rolón *et al.*, 1995; Gammon *et al.*, 1997; Castellsagué *et al.*, 1999; Freedman *et al.*, 2007b; Bosetti *et al.*, 2008) or after 30 years of cessation (Pandeya *et al.*, 2008).

When comparing the types of tobacco smoked (Table 2.23 available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.23.pdf>), consumption of black tobacco resulted in a higher risk for oesophageal cancer than did consumption of blond tobacco (De Stefani *et al.*, 1990; Rolón *et al.*, 1995; Castellsagué *et al.*, 1999; Launoy *et al.*, 2000; Vioque *et al.*, 2008). Similarly, smoking untipped cigarettes generally resulted in a higher risk than smoking filter-tipped cigarettes (Vaughan *et al.*, 1995; Gammon *et al.*, 1997; Castellsagué *et al.*, 1999).

Two studies from the USA reported risks separately for blacks and whites. After adjustment for alcohol consumption, age and income, risks were very similar for former and current smokers and for the number of cigarettes smoked per day and duration of smoking (Brown *et al.*, 1994a; Brown *et al.*, 2001).

(b) *Adenocarcinoma of the oesophagus*

Two decades ago it was noted that incidence rates for adenocarcinoma of the oesophagus and gastric cardia had increased steadily in the USA, whereas the incidence rate for squamous-cell carcinoma of the oesophagus had remained relatively stable (Blot *et al.*, 1991). An increase in the incidence of adenocarcinoma of the distal oesophagus and cardia was also noted in the United Kingdom (Powell & McConkey, 1990), and in several other countries. Since 1990, several studies have focused on the risk factors for adenocarcinoma of the oesophagus. Since the last evaluation (IARC, 2004a) one cohort study (Freedman *et al.*, 2007b) and three case-control studies (Table 2.24 available at <http://monographs.iarc.fr/ENG/Monographs/>

[vol100E/100E-01-Table2.24.pdf](#); Table 2.25 available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.25.pdf>) have been reported, totaling 13 case-control studies on the association of cigarette smoking and adenocarcinoma of the oesophagus.

(i) *Intensity and duration of smoking*

Ten studies, three that included only cases of adenocarcinoma of the oesophagus ([Menke-Pluymers et al., 1993](#); [Gammon et al., 1997](#); [Wu et al., 2001](#)), three that included cases of adenocarcinoma of the oesophagus, gastro-oesophageal junction and gastric cardia combined ([Kabat et al., 1993](#); [Brown et al., 1994b](#); [Vaughan et al., 1995](#)), and four that stratified by histology ([Lindblad et al., 2005](#); [Freedman et al., 2007b](#); [Hashibe et al., 2007a](#); [Pandeya et al., 2008](#)), showed a significant positive association of adenocarcinoma of the oesophagus with cigarette smoking. The relative risks were somewhat lower than those for squamous cell carcinoma of the oesophagus. Three studies, one in China ([Gao et al., 1994](#)), one in Sweden ([Lagergren et al., 2000](#)), and one in the USA ([Zhang et al., 1996](#)), reported similarly elevated relative risks, but some of these risks were not statistically significant, probably because of relatively small numbers of cases.

Of those studies that reported risks adjusted for alcohol consumption, a positive, significant dose-response relationship was found with intensity of smoking ([Kabat et al., 1993](#); [Brown et al., 1994b](#); [Gammon et al., 1997](#); [Hashibe et al., 2007a](#)), duration of smoking ([Gammon et al., 1997](#); [Pandeya et al., 2008](#)) and/or pack-years ([Vaughan et al., 1995](#); [Zhang et al., 1996](#); [Gammon et al., 1997](#); [Pandeya et al., 2008](#)).

(ii) *Cessation of smoking*

Ten studies provided point estimates for former smokers. In eight, relative risks were lower in former smokers than in current smokers, although they remained elevated ([Kabat et al.,](#)

[1993](#); [Gao et al., 1994](#); [Vaughan et al., 1995](#); [Gammon et al., 1997](#); [Wu et al., 2001](#); [Lindblad et al., 2005](#); [Freedman et al., 2007b](#); [Pandeya et al., 2008](#)), and were increased in the other studies ([Lagergren et al., 2000](#); [Hashibe et al., 2007a](#)). The decrease in relative risk associated with years since cessation was weak, but a significant trend was found in two out of six studies ([Gammon et al., 1997](#); [Wu et al., 2001](#)).

(iii) *Confounding*

With the exception of two studies ([Levi et al., 1990](#); [Wu et al., 2001](#)), all studies adjusted for alcohol intake as a potential confounder. Three more recent studies also adjusted for fruit and vegetables intake ([Freedman et al., 2007b](#); [Hashibe et al., 2007a](#); [Pandeya et al., 2008](#)). Ten of these studies were conducted in the USA ([Kabat et al., 1993](#); [Brown et al., 1994b](#); [Vaughan et al., 1995](#); [Zhang et al., 1996](#); [Gammon et al., 1997](#); [Freedman et al., 2007b](#)) the Netherlands ([Menke-Pluymers et al., 1993](#)), the United Kingdom ([Lindblad et al., 2005](#)), central and eastern Europe ([Hashibe et al., 2007a](#)) and Australia ([Pandeya et al., 2008](#)), where chewing of betel quid with tobacco or use of other forms of smokeless tobacco are not likely confounders. One study conducted in Sweden was adjusted for snuff use ([Lagergren et al., 2000](#)).

(iv) *Sex*

[Kabat et al. \(1993\)](#) examined risks for men and women separately and observed similar patterns in both sexes, although risks among current smokers and heavy smokers were somewhat higher for women than for men. [Lindblad et al. \(2005\)](#) also found higher risks in women than in men, but they were not statistically significant.

2.3.6 Cancer of the larynx

Laryngeal cancer is one of the cancers most strongly associated with cigarette smoking ([IARC, 1986, 2004a](#)). Since the previous IARC

Monograph, more epidemiological evidence has become available to strengthen this conclusion.

(a) Potential confounders

Other causes of laryngeal cancer include alcohol consumption, some occupational exposures (e.g. sulphuric acid; [IARC, 2012a](#)) and possibly some dietary habits. In investigating associations between smoking and laryngeal cancer, potential confounding by alcohol consumption has been considered in most of the studies.

(b) Intensity and duration of smoking

Cohort and case-control studies have been carried out in Asia, Europe, North and South America, and South Africa. In all, the risk for laryngeal cancer was consistently higher in smokers, and a positive significant trend was observed with increasing duration and intensity of smoking ([IARC, 2004a](#); Table 2.26 available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.26.pdf>; Table 2.27 available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.27.pdf>; Table 2.28 available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.28.pdf>).

In most case-control studies, the relative risks for laryngeal cancer were near to or greater than 10 for smokers who had smoked for longer than 40 years ([Falk et al., 1989](#); [Zheng et al., 1992b](#)) or had smoked more than 20 cigarettes per day ([Tuyns et al., 1988](#); [Falk et al., 1989](#); [Choi & Kahyo, 1991](#); [Zatonski et al., 1991](#); [Muscat & Wynder, 1992](#); [Zheng et al., 1992b](#); [Hedberg et al., 1994](#); [Sokić et al., 1994](#); [Talamini et al., 2002](#)). Cancer of the larynx in non-smokers is so rare that several studies used as the reference category light smokers ([Herity et al., 1982](#); [Olsen et al., 1985a](#); [De Stefani et al., 1987](#); [Zatonski et al., 1991](#); [López-Abente et al., 1992](#); [Maier & Tisch, 1997](#)), or former smokers ([Hashibe et al., 2007b](#)). Consequently, relative risks were lower

in these studies, although the increases were still statistically significant.

Three case-control studies reported odds ratios for cancer of the larynx that increased with decreasing age of starting smoking ([Franceschi et al., 1990](#); [Zatonski et al., 1991](#); [Talamini et al., 2002](#)).

(c) Cessation of smoking

The risk for cancer of the larynx declines rather rapidly after cessation of smoking ([IARC, 2004a](#); Table 2.29 available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.29.pdf>). No detectable higher risk compared with never-smokers was seen among subjects who had quit smoking for at least 10 years ([Franceschi et al., 1990](#); [Ahrens et al., 1991](#); [Schlecht et al., 1999a, b](#); [Bosetti et al., 2006](#); [Hashibe et al., 2007b](#)).

(d) Types of tobacco or of cigarette

Some investigators considered the role of type of tobacco ([IARC, 2004a](#); Table 2.30 available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.30.pdf>). An average 2.5-fold higher risk was observed in smokers of black tobacco compared to smokers of blond tobacco ([De Stefani et al., 1987](#); [Tuyns et al., 1988](#); [López-Abente et al., 1992](#)). Smoking untipped cigarettes also led to a higher risk than smoking filter-tipped cigarettes ([Wynder & Stellman, 1979](#); [Tuyns et al., 1988](#); [Falk et al., 1989](#)). Those that smoke cigarettes only had higher risks of larynx cancer than those that smoke cigars only ([Hashibe et al., 2007b](#)).

(e) Subsites

Six studies investigated the risk for glottic and supraglottic cancer separately ([Olsen et al., 1985a](#); [Tuyns et al., 1988](#); [López-Abente et al., 1992](#); [Maier et al., 1992b](#); [Muscat & Wynder, 1992](#); [Sapkota et al., 2007](#)). The cancer risk increased with increasing amount smoked per

day and with cumulative exposure for both subsites (IARC, 2004a; Table 2.28 online). In addition, the observed relative risks were higher for supraglottic cancer than for glottic cancer (Maier *et al.*, 1992b; Sapkota *et al.*, 2007).

(f) Sex

Few studies investigated sex-specific effects. In one cohort study (Raitiola & Pukander, 1997) similar risks were found for men and women, whereas in two case-control studies (Zheng *et al.*, 1992b; Tavani *et al.*, 1994), the relative risks for women were up to 10-fold higher than for the corresponding categories in men, though a small number of cases were involved. However, Freedman *et al.* (2007a) observed higher relative risks in men than women (Table 2.26 online). One study looked at women only and found higher risks of laryngeal cancer in former and current smokers relative to non-smokers, and also according to the number of cigarettes per day with a clear dose-response effect ($P < 0.001$) (Gallus *et al.*, 2003b).

2.3.7 Cancer of the upper aerodigestive tract combined

In epidemiological studies, especially in cohort studies in which there are few cases at some sites, investigators often combine cancers of the oral cavity, pharynx, larynx and oesophagus and term these ‘cancer of the upper aerodigestive tract’. This section summarizes the data from 19 cohort studies (IARC, 2004a; Table 2.31 available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.31.pdf>), and 40 case-control studies (IARC, 2004a; Tables 2.32–2.35; see below).

(a) Intensity and duration of smoking

In all but two cohort studies from Japan (Kono *et al.*, 1987; Akiba, 1994), the risk for cancer of the upper aerodigestive tract was strongly associated with cigarette smoking. Relative risks increased

with increasing daily cigarette consumption (Hammond & Horn, 1958; Doll *et al.*, 1980, 1994; Akiba & Hirayama, 1990; Kuller *et al.*, 1991; Chyou *et al.*, 1995; Engeland *et al.*, 1996; Murata *et al.*, 1996; Yuan *et al.*, 1996; Kjaerheim *et al.*, 1998; Liaw & Chen, 1998; Yun *et al.*, 2005; Freedman *et al.*, 2007a), duration of smoking (Chyou *et al.*, 1995; Yun *et al.*, 2005; Friberg *et al.*, 2007) or pack-years (Liaw & Chen, 1998; Freedman *et al.*, 2007a).

The main characteristics and results of the case-control studies are presented in IARC (2004a), and in Table 2.32 (available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.32.pdf>) and Table 2.33 (available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.33.pdf>), respectively. Intensity of smoking was measured in most of these studies. The link between duration of smoking and cancer of the upper aerodigestive tract was examined in 20 case-control studies (Blot *et al.*, 1988; Merletti *et al.*, 1989; Barra *et al.*, 1991; De Stefani *et al.*, 1992, 2007; Franceschi *et al.*, 1992; Day *et al.*, 1993; Mashberg *et al.*, 1993; Kabat *et al.*, 1994; Lewin *et al.*, 1998; Bosetti *et al.*, 2000a; Garrote *et al.*, 2001; Gallus *et al.*, 2003a; Lissowska *et al.*, 2003; Znaor *et al.*, 2003; Castellsagué *et al.*, 2004; Menvielle *et al.*, 2004a, b; Rodriguez *et al.*, 2004; Hashibe *et al.*, 2007c; Sapkota *et al.*, 2007). Nine also considered age at starting smoking (Blot *et al.*, 1988; Merletti *et al.*, 1989; Barra *et al.*, 1991; Franceschi *et al.*, 1992; Day *et al.*, 1993; Lewin *et al.*, 1998; Garrote *et al.*, 2001; Lissowska *et al.*, 2003; Menvielle *et al.*, 2004a).

In all but one study (Rao *et al.*, 1999) there was an increased risk for cancer of the upper aerodigestive tract associated with cigarette smoking. A clear dose-response relationship was seen with increasing daily tobacco consumption and duration of smoking as well as with decreasing age at starting smoking in most of the studies examined.

(b) Cessation of smoking

Twelve cohort studies ([Doll et al., 1980, 1994](#); [Tomita et al., 1991](#); [Akiba, 1994](#); [Chyou et al., 1995](#); [Engeland et al., 1996](#); [Nordlund et al., 1997](#); [Kjaerheim et al., 1998](#); [Yun et al., 2005](#); [Freedman et al., 2007a](#); [Friborg et al., 2007](#); [Ide et al., 2008](#)) provided point estimates for former smokers ([IARC 2004a](#); Table 2.31 online). The relative risks for former smokers were always lower than those for current smokers.

In 16 case-control studies the relative risk by years since quitting was examined and generally a statistically significant negative trend was found (Table 2.34 available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.34.pdf>).

(c) Types of cigarette

The characteristics studied in several case-control studies included the use of a filter, the type of tobacco, the tar content and whether the product was manufactured or hand-rolled ([IARC, 2004a](#); Table 2.35 available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.35.pdf>). Consumption of black tobacco, cigars, untipped cigarettes, hand-rolled cigarettes, or cigarettes with a high-tar yield generally resulted in a higher risk than consumption of blond tobacco ([Merletti et al., 1989](#); [Castellsagué et al., 2004](#); [De Stefani et al., 2007](#)), filter-tipped cigarettes ([Merletti et al., 1989](#); [Mashberg et al., 1993](#); [Kabat et al., 1994](#); [Lissowska et al., 2003](#); [De Stefani et al., 2007](#)), manufactured cigarettes ([De Stefani et al., 1992, 2007](#)) or low-tar cigarettes ([Franceschi et al., 1992](#)). Two studies from India ([Znaor et al., 2003](#); [Sapkota et al., 2007](#)) revealed higher risks of *bidi* smoking related to cigarettes smoking.

(d) Sex

Sex-specific effects were analysed in four cohort studies ([IARC 2004a](#); Table 2.31 online). In three cohort studies ([Hammond & Seidman,](#)

[1980](#); [Akiba & Hirayama, 1990](#); [Freedman et al., 2007a](#)) a higher relative risk was found for male smokers than for female smokers; however, [Ide et al. \(2008\)](#) detected a higher risk among women in a study with a small number of cases.

In three case-control studies ([Blot et al., 1988](#); [Kabat et al., 1994](#); [Muscat et al., 1996](#)) the relative risks were higher for women than for men in all categories of intensity of smoking (number of cigarettes per day), cumulative exposure (cumulative tar consumption, pack-years, duration of smoking) and age at starting smoking, as well as for former smokers. However, the trends in men were always in the same direction and of the same order of magnitude. An exception to the pattern was that in one study ([Merletti et al., 1989](#)) the relative risk for smoking filter-tipped cigarettes was higher than that for smoking untipped cigarettes for women.

Overall, the strength of association by sex was generally similar, especially when taking into account the fact that women generally under-report levels of smoking and that most studies included many fewer women than men.

(e) Ethnicity

Relative risks were reported separately for blacks and whites in a large case-control study from the USA ([Day et al., 1993](#)). Relative risks adjusted for alcohol consumption, sex and other relevant variables were very similar for the number of cigarettes smoked per day, years of cigarette smoking, age at starting smoking and number of years since stopping smoking.

2.4 Cancer of the stomach

2.4.1 Overview of studies

In the previous *IARC Monograph* ([IARC, 2004a](#)) it was concluded that there was *sufficient evidence* that tobacco smoking causes cancer of the stomach. Three meta-analyses have since examined the evidence for gastric cancer in 42

independent cohort studies published between 1958 and July 2007 ([Ladeiras-Lopes et al., 2008](#)), in 46 case–control studies published between 1997 and June 2006 ([La Torre et al., 2009](#)), and in 10 cohort and 16 case–control studies conducted in Japanese populations published between 1966 and March 2005 ([Nishino et al., 2006](#); Table 2.36 available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.36.pdf>). For current smokers compared to never smokers, the risk for stomach cancer was found to be statistically significantly increased by 53% ([Ladeiras-Lopes et al., 2008](#)), 56% ([Nishino et al., 2006](#)), and 57% when considering high quality case–control studies ([La Torre et al., 2009](#)), with moderate to high heterogeneity.

Since the previous *IARC Monograph* ([IARC, 2004a](#)), the association between cigarette smoking and stomach cancer risk (15 studies) and mortality (4 studies) has been examined in 19 cohort studies (Table 2.37 available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.37.pdf>). Eleven of these were conducted in Asia ([Sasazuki et al., 2002](#); [Jee et al., 2004](#); [Koizumi et al., 2004](#); [Wen et al., 2004](#); [Fujino et al., 2005](#); [Sauvaget et al., 2005](#); [Tran et al., 2005](#); [Kurosawa et al., 2006](#); [Kim et al., 2007](#); [Sung et al., 2007](#); [Shikata et al., 2008](#)), seven in Europe ([Simán et al., 2001](#); [González et al., 2003](#); [Doll et al., 2005](#); [Lindblad et al., 2005](#); [Sjödahl et al., 2007](#); [Batty et al., 2008](#); [Zendehdel et al., 2008](#)) and one in the USA ([Freedman et al., 2007a](#)). Only the updated British Doctors' study ([Doll et al., 2005](#)) and the most recent studies ([Shikata et al., 2008](#); [Zendehdel et al., 2008](#)) were not included in the meta-analysis of cohort studies ([Ladeiras-Lopes et al., 2008](#)). Elevated risks in current smokers were found in all studies. The reported association of current smoking with mortality in the four cohort studies conducted in Taiwan, China ([Wen et al., 2004](#)), Japan ([Kurosawa et al., 2006](#)) and the United Kingdom ([Doll et al., 2005](#); [Batty et al., 2008](#)) was comparable to that with incidence.

In addition, the association between smoking and stomach cancer risk has been reported in 37 case–control studies since the previous *IARC Monograph*, of which 22 are hospital-based and 15 population-based. With the exception of three studies ([Campos et al., 2006](#); [García-González et al., 2007](#); [Suwanrungruang et al., 2008](#); Table 2.38 available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.38.pdf>), all these studies were included in the meta-analysis conducted by ([La Torre et al., 2009](#)).

2.4.2 Factors affecting risk

(a) Intensity and duration

Clear evidence has been provided by the meta-analyses as well as by the additional cohort studies that the risk for stomach cancer increases significantly with increasing daily cigarette consumption, duration or pack-years of smoking, although individual studies did not always find statistically significant dose–response relationships. In one meta-analysis based on 21 cohort studies, the risk for stomach cancer increased statistically significantly by 53% with consumption of approximately 20 cigarettes per day ([Ladeiras-Lopes et al., 2008](#)). Using trend estimation analysis as proposed by [Greenland & Longnecker \(1992\)](#), the authors found an increase in relative risk from 1.3 for the lowest consumption to 1.7 for smoking 30 cigarettes per day.

(b) Cessation of smoking

Risk for stomach cancer has been generally found to be lower in former smokers than in current smokers. In six of the cohort studies decreasing risk with increasing years since stopping smoking was found although none found statistically significant dose–response relationships ([González et al., 2003](#); [Koizumi et al., 2004](#); [Sauvaget et al., 2005](#); [Freedman et al., 2007a](#); [Kim et al., 2007](#); [Zendehdel et al., 2008](#)). Risk in former smokers was comparable to never smokers after quitting for 5 years ([Kim et al.,](#)

2007), 10 years ([González et al., 2003](#); [Sauvaget et al., 2005](#); [Freedman et al., 2007a](#)) or 15 years ([Koizumi et al., 2004](#)).

2.4.3 Subsites

The effect of current smoking on the risk for stomach cancer by subsite was assessed in ten cohort studies. Elevated risks were found for both cardia and non-cardia cancers. In six studies higher risks were found for cancer of the gastric cardia than for cancer of the distal stomach ([Simán et al. 2001](#); [González et al., 2003](#); [Freedman et al., 2007a](#); [Sung et al., 2007](#); [Shikata et al., 2008](#); [Zendehdel et al., 2008](#)), three studies found no difference ([Sasazuki et al., 2002](#); [Lindblad et al., 2005](#); [Tran et al., 2005](#)), and in one study higher risk for cancer in the antrum rather than the body or the cardia was found ([Koizumi et al., 2004](#)). A meta-analysis yielded statistically significant summary relative risks of 1.87 for cardia cancers and 1.60 for non-cardia cancers based on nine cohort studies ([Ladeiras-Lopes et al., 2008](#)). However, there was substantial heterogeneity across studies for cardia cancers. For case–controls studies, the corresponding odds ratios were 2.05 (95%CI: 1.50–2.81) and 2.04 (95%CI: 1.66–2.50), respectively, with greater heterogeneity for non-cardia cancers. Criteria for the classification by subsite were not always described ([Simán et al., 2001](#); [Koizumi et al., 2004](#); [Lindblad et al., 2005](#); [Tran et al., 2005](#)) and some studies included tumours located in the upper third of the stomach in the group of cardia cancer ([Sasazuki et al., 2002](#); [Sung et al., 2007](#); [Shikata et al. 2008](#)).

In three studies risk estimates for smoking associated stomach cancer were estimated by histological type ([Sasazuki et al., 2002](#); [Koizumi et al., 2004](#); [Shikata et al., 2008](#)). The relative risks were 2.1 (95%CI: 1.2–3.6), 1.6 (95%CI: 1.1–2.3) and 2.3 (95%CI: 1.3–4.1) for the differentiated type, respectively, and 0.6 (95%CI: 0.3–1.1), 2.1

(95%CI: 1.1–4.1), and 1.3 (95%CI: 0.5–3.5) for the non-differentiated type, respectively.

2.4.4 Population characteristics

In four of the additional cohort studies risk was reported separately for men and women ([González et al., 2003](#); [Jee et al., 2004](#); [Fujino et al., 2005](#); [Kim et al., 2007](#)), in three studies only for men ([Koizumi et al., 2004](#); [Tran et al., 2005](#); [Sung et al., 2007](#)) and in one mortality study for men as well as for women ([Wen et al., 2004](#)). Generally, the relative risks were smaller in women than in men. For all stomach cancers, risk in current smokers compared to never smokers was found to be significantly increased by 62% in men (based on 18 studies) and by 20% in women (based on nine studies) in the meta-analysis of cohort studies ([Ladeiras-Lopes et al., 2008](#)). The men–women differences were independent of exposure level but could be explained by the sex difference in the distribution by histological type and other factors associated with socioeconomic status.

Ethnicity does not appear to modify the effect of smoking on stomach cancer risk. In the meta-analysis of case–control studies risk in current smokers was increased by 78% in Caucasians and by 48% in Asians ([La Torre et al., 2009](#)). The summary risk based on the cohort studies increased by 46% and 47% in Caucasian and Asian studies, respectively. In a meta-regression analysis including the variables sex, population, and fruit and vegetable consumption, sex but not origin of the population showed significant differences in risk estimates ([Ladeiras-Lopes et al., 2008](#)).

2.4.5 Bias and confounding

Generally, most cohort studies have relied on baseline information and did not update the exposure information, possibly leading to misclassification of smoking status. Most of

the recent cohort studies have accounted for confounding by alcohol consumption ([Fujino et al., 2005](#); [Lindblad et al., 2005](#); [Sjödahl et al., 2007](#); [Sung et al., 2007](#)) as well as fruit and vegetable consumption ([González et al., 2003](#); [Koizumi et al., 2004](#); [Freedman et al., 2007a](#)) and still observed significantly increased risk of stomach cancer in current smokers.

2.4.6 *Helicobacter pylori* infection

The association between tobacco smoking and stomach cancer could be confounded or modified by the effect of *H. pylori* infection, an established risk factor for stomach cancer. In three case-control studies ([Zaridze et al., 2000](#); [Brenner et al., 2002](#); [Wu et al., 2003](#)), and two cohort studies ([Simán et al., 2001](#); [Shikata et al., 2008](#)) the joint effects and possible interaction between *H. pylori* status and smoking in relation to risk for stomach cancer was investigated. Among subjects who had *H. pylori* infection, the risk for stomach cancer was higher in current smokers than in non-smokers by 1.6 to 2.7 fold, providing evidence for a causal effect of tobacco smoking independently of *H. pylori* infection. Smoking was associated with risk elevations of the same order of magnitude among subjects without *H. pylori* infection. Smoking and *H. pylori* therefore may act synergistically, leading to very high risks in current smokers with *H. pylori* infection compared to non-smokers without *H. pylori* infection. In one study that examined risk by subsite an effect of smoking independent of *H. pylori* infection for gastric cardia as well as distal gastric cancer was found ([Wu et al., 2003](#)). In none of the studies was there statistically significant evidence for interaction.

2.5 Cancer of the pancreas

2.5.1 Overview of studies

Previous *IARC Monographs* ([IARC, 1986, 2004a](#)) concluded that exposure to tobacco smoke caused cancer of the pancreas. Additional evidence has come from a pooled analysis of eight cohort studies with almost 1500 incident cases of pancreatic cancer and an equal number of controls ([Lynch et al., 2009](#)) as well as a meta-analysis of 82 independent studies (42 case-control studies, 40 cohort studies) published between 1950 and 2007 ([Iodice et al., 2008](#); Table 2.39 available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.39.pdf>). In the meta-analysis 74% and 20% significant increased risks for current and former smokers, respectively, were found with significant heterogeneity of effect regarding current smoking across studies. Adjustment for confounders explained some of the heterogeneity ([Iodice et al., 2008](#)). A similar significant risk elevation of 77% for current smokers was found in the pooled analysis, without study heterogeneity ([Lynch et al., 2009](#)). For former smokers, risk was increased non-significantly by 9%.

Since the previous *IARC Monograph* ([IARC, 2004a](#)), a total of 15 cohort studies have reported on the association between cigarette smoking and pancreatic cancer incidence (8 studies) and mortality (5 studies) or both (one study) (Table 2.40 available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.40.pdf>), two of which were included in the pooled analysis ([Coughlin et al., 2000](#); [Vrieling et al., 2009](#)). Excluding case-control studies that did not report odds ratios for current smokers, there were three additional case-control studies ([Duell et al., 2002](#); [Inoue et al., 2003](#); [Alguacil & Silverman, 2004](#); Table 2.41 available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.41.pdf>). The effect of cigar and pipe smoking on pancreatic cancer was

also examined in the ACS Cancer Prevention Study II regarding mortality ([Shapiro et al., 2000](#); [Henley et al., 2004](#)) and in the Kaiser Permanente Medical Care Program regarding incidence ([Iribarren et al., 1999](#)). All the additional studies showed an increased risk for pancreatic cancer associated with tobacco smoking, generally higher in current than in former smokers. The reported risk estimates were not always statistically significant, predominantly due to the small size of some studies and therefore lack of statistical precision.

2.5.2 Factors affecting risks

(a) Intensity and duration

Clear evidence has been provided by the meta-analysis, the pooled analysis as well as the additional studies that the risk for cancer of the pancreas increases significantly with increasing daily cigarette consumption, duration and pack-years of smoking ([Coughlin et al., 2000](#); [Gapstur et al., 2000](#); [Nilsen & Vatten, 2000](#); [Nilsson et al., 2001](#); [Isaksson et al., 2002](#); [Doll et al., 2005](#); [Yun et al., 2005](#); [Ansary-Moghaddam et al., 2006](#); [Gallicchio et al., 2006](#); [Vrieling et al., 2009](#)). In the meta-analysis risk of pancreatic cancer increased significantly by 62% with an increase of 20 cigarettes per day (based on 45 studies) and by 16% with a 10-year increase in smoking duration (based on 16 studies), but with significant study heterogeneity. In the pooled analysis, the excess odds ratio per pack-years generally declined with increasing smoking intensity ([Lynch et al., 2009](#)).

(b) Cessation of smoking

A reduction in risk in former smokers who had stopped smoking for at least 10 years was found in the meta-analysis ([Iodice et al., 2008](#)) and the pooled study ([Lynch et al., 2009](#)). In some cohort studies risk was already comparable to never smokers five years after quitting ([Boyle et al., 1996](#); [Fuchs et al., 1996](#); [Nilsen & Vatten, 2000](#); [Vrieling et al., 2009](#)).

(c) Types of tobacco

In non-cigarette smokers, mortality from pancreatic cancer was increased although not statistically significantly so in cigar smokers in the CPS-II cohort study ([Shapiro et al., 2000](#)) as well as a large case-control study ([Alguacil & Silverman, 2004](#)) but was less clearly elevated in the smaller Kaiser Permanente cohort study ([Iribarren et al., 1999](#)). There was a significantly increased mortality for current cigar smokers who reported inhaling cigar smoke ([Shapiro et al., 2000](#)). Pipe smoking was also found to be associated with an increased risk of cancer of the pancreas, which was stronger in those who reported that they inhaled the smoke ([Henley et al., 2004](#)). A limitation of the cohort studies is that smoking habits were reported only at baseline, misclassification of smoking exposure is likely to underestimate the associated risks. In the meta-analysis there was a significant increase in risk of 47% associated with current cigar and/or pipe smoking (18 studies) and a non-significant risk elevation of 29% with former cigar and/or pipe smoking (5 studies) ([Iodice et al., 2008](#)).

2.5.3 Population characteristics

The effect of sex on pancreatic cancer risk was investigated in two cohort studies ([Nilsen & Vatten, 2000](#); [Larsson et al., 2005](#)) and on pancreatic cancer mortality in four cohort studies ([Coughlin et al., 2000](#); [Gapstur et al., 2000](#); [Nilsson et al., 2001](#); [Lin et al., 2002a](#)). The relative risks were comparable between men and women and no consistent evidence for an effect modification by sex was observed.

Ethnicity does not appear to modify the association of smoking with pancreatic cancer risk. The roughly twofold elevated risk in current smokers compared to never smokers was observed both in studies of Caucasians ([Lynch et al., 2009](#)) and of Asians ([Lin et al., 2002a](#); [Jee et al., 2004](#); [Yun et al., 2005](#); [Li et al., 2006](#)). In populations of the Asia-Pacific Region, there

was also no difference in the strength of association between Asia and Australia/New Zealand ([Ansary-Moghaddam et al., 2006](#)).

2.5.4 Confounding factors

In two large cohort studies the risk estimates for pancreatic cancer associated with cigarette smoking were not substantially influenced by adjustment for further potential confounding factors, including diabetes, body mass index (BMI), alcohol and dietary intake ([Coughlin et al., 2000](#); [Vrieling et al., 2009](#)).

2.6 Cancer of the colorectum

2.6.1 Overview of studies

In the previous *IARC Monograph* ([IARC, 2004a](#)) it was not possible to conclude that the association between tobacco smoking and colorectal cancer is casual, principally because of concern about confounding by other risk factors. That evaluation was based on a total of 60 epidemiologic studies, although only few were specifically designed to study the effects of smoking. Studies have however shown consistently that cigarette smoking is a risk factor for colorectal adenomatous polyps, which are recognized precursor lesions of colorectal cancer ([Hill, 1978](#)). To explain this discrepancy, [Giovannucci et al. \(1994\)](#) hypothesized that a long induction period is required for tobacco to play a role in colorectal carcinogenesis, which would not be captured by studies with shorter follow-up time.

Four recent meta-analyses consistently showed a strong association between cigarette smoking and colorectal cancer ([Botteri et al., 2008a](#); [Liang et al., 2009](#); [Huxley et al., 2009](#); [Tsoi et al., 2009](#)).

2.6.2 Cohort studies

Since the previous *IARC Monograph* ([IARC, 2004a](#)), 22 additional cohort studies have investigated the association between tobacco smoke and colorectal cancer (Table 2.42 available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.42.pdf>). [Studies that did not provide point estimates of risk ([Andersen et al., 2009](#); [Hansen et al., 2009](#); [Murphy et al., 2009](#)) and included prevalent colorectal cancer in patients with other diagnosis ([Chan et al., 2007](#)) are excluded from this review]. Seven of the studies were conducted in Europe, nine in Asia and five in the USA. In eleven studies, risk estimates were reported solely for colorectal cancer ([Tiemersma et al., 2002a](#); [Limburg et al., 2003](#); [Otani et al., 2003](#); [Colangelo et al., 2004](#); [Sanjoaquin et al., 2004](#); [Lüchtenborg et al., 2005a](#); [Kim et al., 2006](#); [Akhter et al., 2007](#); [Huxley, 2007a](#); [Kenfield et al., 2008](#); [Hannan et al., 2009](#)), five studies separately for colon cancer and rectal cancer ([Shimizu et al., 2003](#); [Wakai et al., 2003](#); [Jee et al., 2004](#); [Yun et al., 2005](#); [Batty et al., 2008](#)) and five studies both for colorectal cancer as well as for colon and rectal cancers ([Terry et al., 2002a](#); [van der Hel et al., 2003a](#); [Doll et al., 2005](#); [Paskett et al., 2007](#); [Tsong et al., 2007](#); [Gram et al., 2009](#)). Six studies were restricted to women ([Terry et al., 2002a](#); [Limburg et al., 2003](#); [van der Hel et al., 2003a](#); [Paskett et al., 2007](#); [Kenfield et al., 2008](#); [Gram et al., 2009](#)), and two studies to men ([Doll et al., 2005](#); [Yun et al., 2005](#); [Akhter et al., 2007](#)). One study reported both colorectal incidence and mortality ([Limburg et al., 2003](#)) and three studies only reported colorectal cancer mortality ([Doll et al., 2005](#); [Huxley, 2007a](#); [Batty et al., 2008](#); [Kenfield et al., 2008](#)).

(a) Smoking status

Virtually all studies reported elevated risk associated with smoking, although results were not always statistically significant. The largest meta-analysis based on 36 prospective studies

with data from a total of 3007002 subjects found that compared to never smokers, current smokers had a 15% significantly higher risk of developing colorectal cancer and 27% significantly higher risk of colorectal cancer mortality (Liang *et al.*, 2009; Table 2.43 available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.43.pdf>). In former smokers, colorectal cancer risk was also significantly elevated by 20% whereas colorectal cancer mortality was non-significantly increased by 20%. The risk estimates were not significantly different between colon and rectal cancer for current smokers (RR, 1.10 versus 1.19) and for former smokers (RR, 1.10 versus 1.20). There was no heterogeneity among colorectal cancer studies and no evidence for publication bias. Comparable risk elevations in current and former smokers were found in the other meta-analyses. For current smokers, the risk for colorectal cancer increased significantly by 16% when using data from 22 cohort studies (Huxley *et al.*, 2009), by 20% based on 28 cohort studies (Tsoi *et al.*, 2009), and by 7% based on data from 45 cohort and case-control studies (Botteri *et al.*, 2008a). In the latter meta-analysis a 17% significantly higher risk of colorectal cancer in former smokers was found.

(b) Intensity of smoking

All but three of the recent 21 cohort studies (van der Hel *et al.*, 2003a; Jee *et al.*, 2004; Sanjoaquin *et al.*, 2004) investigated dose-response relationships, using at least one of number of cigarettes smoked, duration of smoking, pack-years of smoking, age at smoking initiation, time since smoking cessation. In two further studies (Tiemersma *et al.*, 2002a; Batty *et al.*, 2008) these parameters were examined separately in current and former smokers, as by Chao *et al.* (2000). Statistically significant dose-response trends with amount smoked daily were reported for colorectal cancer (Lüchtenborg *et al.*, 2005a; Akhter *et al.*, 2007; Paskett *et al.*, 2007; Kenfield *et al.*, 2008), for colon cancer (Paskett

et al., 2007), and for rectal cancer (Paskett *et al.*, 2007; Tsong *et al.*, 2007). The dose-response of daily cigarette consumption and colorectal cancer was assessed in two meta-analyses (Liang *et al.*, 2009; Tsoi *et al.*, 2009) and both found statistically significant relationships. Based on eleven studies, Liang *et al.* (2009) found that risk for colorectal cancer increased significantly by 17% with an increase of 20 cigarettes/day and by 38% with an increase of 40 cigarettes/day, while colorectal cancer mortality increased by 41% and 98%, respectively (Table 2.43 online). The risk elevation associated with an increase of 20 cigarettes/day was greater for rectal than for colon cancer (13% versus 3%) but this difference was not statistically significant.

(c) Duration of smoking

In addition to two previously reported studies (Hsing *et al.*, 1998; Chao *et al.*, 2000), thirteen studies have examined duration of smoking and colorectal cancer risk. A statistically significant trend of increasing risk with increasing duration was found for colorectal (Limburg *et al.*, 2003; Kim *et al.*, 2006; Paskett *et al.*, 2007; Gram *et al.*, 2009), for colon cancer (Paskett *et al.*, 2007) and for rectal cancer (Terry *et al.*, 2002a; Paskett *et al.*, 2007; Tsong *et al.*, 2007). In one study, increasing duration of smoking was significantly associated with risk for colorectal cancer solely in former smokers (Tiemersma *et al.*, 2002a). Based on eight studies (Terry *et al.*, 2002a; Tiemersma *et al.*, 2002a; Limburg *et al.*, 2003; Lüchtenborg *et al.*, 2005a; Kim *et al.*, 2006; Akhter *et al.*, 2007; Paskett *et al.*, 2007; Tsong *et al.*, 2007), a meta-analysis for duration of smoking and colorectal cancer incidence yielded highly significant results (Liang *et al.*, 2009). Risk was increased by 9.4% with a 20-year increase in smoking duration and 19.7% with a 40-year increase. Smoking duration was also significantly associated with risk for rectal cancer but not for colon cancer. In another meta-analysis where dose-response relationship was modelled, a nonlinear increase in risk with

increasing duration was observed ([Botteri et al., 2008a](#)). The risk started to increase after approximately 10 years of smoking and reached statistical significance after 30 years.

(d) *Pack-years*

Since the previous *IARC Monograph*, the association of colorectal cancer with pack-years of cigarette smoking has been evaluated in six studies ([Limburg et al., 2003](#); [Otani et al., 2003](#); [Shimizu et al., 2003](#); [Wakai et al., 2003](#); [Kim et al., 2006](#); [Gram et al., 2009](#)). In addition to the previously reported significant results ([Giovannucci et al., 1994](#); [Heineman et al., 1994](#); [Chao et al., 2000](#); [Stürmer et al., 2000](#)), a statistically significant trend of increasing risk with increasing pack-years was found for colorectal cancer in two studies ([Limburg et al., 2003](#); [Gram et al., 2009](#)), and for colon cancer in one study ([Gram et al., 2009](#)). In their dose-response analysis of pack-years and colorectal incidence, [Liang et al. \(2009\)](#) included five studies ([Giovannucci et al., 1994](#); [Stürmer et al., 2000](#); [Limburg et al., 2003](#); [Otani et al., 2003](#); [Kim et al., 2006](#)) and found a statistically significant trend of increasing risk with increasing pack-years of smoking for colorectal cancer but not specifically for colon or rectal cancer. Risk for colorectal cancer increased by 27% for an increase of 35 pack-years and by 50% for an increase of 60 pack-years.

(e) *Age at initiation*

In nine of the cohort studies the age at smoking initiation in relation to colorectal cancer (eight studies) or colon and rectal cancer (four studies) was investigated. In four studies a statistically significant trend of increasing risk with decreasing age at initiation of smoking for colorectal cancer was found ([Limburg et al., 2003](#); [Kim et al., 2006](#); [Akhter et al., 2007](#); [Gram et al., 2009](#)) and for colon cancer ([Gram et al., 2009](#)) and rectal cancer ([Tsong et al., 2007](#)). In one meta-analysis ([Liang et al., 2009](#)), a highly significant association was found for age at

smoking initiation and colorectal cancer incidence based on six studies ([Limburg et al., 2003](#); [Kim et al., 2006](#); [Akhter et al., 2007](#); [Paskett et al., 2007](#); [Tsong et al., 2007](#); [Gram et al., 2009](#)). Risk for colorectal cancer was reduced by 2.2% for a 5-year delay in smoking initiation and by 4.4% for a 10-year delay.

(f) *Smoking cessation*

The effect of smoking cessation by years since stopping was assessed in seven studies, six for colorectal cancer ([Tiemersma et al., 2002a](#); [Lüchtenborg et al., 2005a, 2007](#); [Paskett et al., 2007](#); [Kenfield et al., 2008](#); [Gram et al., 2009](#); [Hannan et al., 2009](#)) and three for colon and/or rectal cancer ([Wakai et al., 2003](#); [Paskett et al., 2007](#); [Gram et al., 2009](#)). In one study a statistically significant trend in risk reduction with years since quitting was found both overall as well as separately for men and for women ([Hannan et al., 2009](#)).

(g) *Population characteristics*

It has been suggested that the association between smoking and colorectal cancer may be stronger in men than in women. In the three recent cohort studies reporting sex-specific results ([Shimizu et al., 2003](#); [Wakai et al., 2003](#); [Colangelo et al., 2004](#)), this was only observed in studies in Japan ([Shimizu et al., 2003](#); [Wakai et al., 2003](#)), but could be attributed to the very low prevalence of smoking in women. The studies restricted to women have generally shown associations with cigarette smoking that were of comparable magnitude to those observed in men ([Terry et al., 2002a](#); [Limburg et al., 2003](#); [van der Hel et al., 2003a](#); [Paskett et al., 2007](#); [Kenfield et al., 2008](#); [Gram et al., 2009](#)).

Recent studies have been carried out either in Europe and in USA, with predominantly Caucasian study subjects, or in Asia, mostly in Japan and in the Republic of Korea. The results from these studies suggest no differences in the association between tobacco smoking and

colorectal cancer between different ethnic groups.

(h) *Subsites*

Smoking and risks for colon cancer and for rectal cancer were investigated in eleven of the 21 additional studies. Risk patterns are generally consistent between colon and rectal cancer ([Otani et al., 2003](#); [van der Hel et al., 2003a](#); [Wakai et al., 2003](#); [Jee et al., 2004](#); [Yun et al., 2005](#); [Batty et al., 2008](#)). In some studies, dose-response relationships were stronger for rectal cancer than for colon cancer ([Terry et al., 2002a](#); [Paskett et al., 2007](#)) or were statistically significant only for rectal cancer ([Shimizu et al., 2003](#); [Doll et al., 2005](#); [Tsong et al., 2007](#)). In a meta-analysis ([Liang et al., 2009](#)) the association was stronger for rectal cancer than for colon cancer in the subset of cohort studies that differentiated cancer by site. Most dose-response variables were not associated with colon cancer incidence whereas associations were stronger for rectal cancer incidence and statistically significant with longer duration of smoking, albeit based only on a small number of studies. In one cohort study the increased risk associated with smoking was more apparent for proximal than for distal colon cancer ([Lüchtenborg et al., 2005a](#)), which was not found in an earlier study ([Heineman et al., 1994](#)).

(i) *Confounding and effect modification*

Smokers have been shown to be more likely than non-smokers to be physically inactive, to use alcohol, to have lower consumption of fruits and vegetables and higher consumption of fat and meat, and they are less likely to be screened for colorectal cancer ([Le Marchand et al., 1997](#); [Ghadirian et al., 1998](#); [Nkondjock & Ghadirian, 2004](#); [Reid et al., 2006b](#); [Mutch et al., 2009](#)).

Few potential confounders were considered in the cohort studies evaluated in the previous IARC Monograph ([IARC, 2004a](#)). Of the cohort studies published since, all except three ([van der Hel et al., 2003a](#); [Jee et al., 2004](#); [Doll et al., 2005](#))

considered two or more potential confounders. In eleven of the recent studies adjustments were made for physical activity, alcohol consumption, overweight/obesity ([Terry et al., 2002a](#); [Limburg et al., 2003](#); [Otani et al., 2003](#); [Wakai et al., 2003](#); [Yun et al., 2005](#); [Akhter et al., 2007](#); [Ashktorab et al., 2007](#); [Paskett et al., 2007](#); [Tsong et al., 2007](#); [Kenfield et al., 2008](#); [Hannan et al., 2009](#)), and seven also adjusted for dietary habits (e.g. intake of fruits and vegetables, dietary fibres, fat, red meat). Among the studies with the latter adjustments, eight ([Giovannucci et al., 1994](#); [Chao et al., 2000](#); [Stürmer et al., 2000](#); [Limburg et al., 2003](#); [Yun et al., 2005](#); [Akhter et al., 2007](#); [Paskett et al., 2007](#); [Hannan et al., 2009](#)) found significant dose-response relationships with at least one of the smoking variables. In two studies a significant association of smoking with colorectal cancer risk was observed after accounting for history of colonoscopy ([Paskett et al., 2007](#); [Hannan et al., 2009](#)). Risk factors in multivariable analyses in several studies were level of education, use of menopausal hormone therapy, family history and regular aspirin use. The association between smoking and colorectal cancer was not modified by these other characteristics, or by alcohol consumption in two studies ([Otani et al., 2003](#); [Tsong et al., 2007](#)). Therefore, confounding factors do not seem to affect the observed significant increase in risk for colorectal cancer associated with tobacco smoking and the dose-response relationships with smoking variables.

When considering other types of smoking, it is generally found that cigar and pipe smoking are less associated with socioeconomic class and other life-style habits than cigarette smoking. Therefore, it is logical to assume that, for these types of smoking, risk associations derived from epidemiologic studies may be less prone to potential confounding. In all the cohort studies reviewed in the previous IARC Monograph ([IARC, 2004a](#)) an elevated, though not always statistically significant, risk was consistently reported for cancers of the colon and the rectum

associated with exclusive pipe and/or cigar smoking.

Infection with JC virus has been proposed as a potential risk factor for colon cancer ([Rollison et al., 2009](#)) but results still need further validation.

Three cohort studies assessed possible modifying effects by genetic susceptibility. Rapid acetylator phenotype (as determined by polymorphisms of the *NAT2* gene involved in metabolism of heterocyclic aromatic amines) was found to increase the risk for colorectal cancer in smokers, in one ([van der Hel et al., 2003a](#)) but not in another study ([Tiemersma et al., 2002a](#)). For genes involved in the metabolism of polycyclic aromatic hydrocarbons such as *GSTM1* or *GSTT1*, no statistical contribution to the risk of colorectal cancer associated with smoking was observed ([Tiemersma et al., 2002a](#); [Lüchtenborg et al., 2005a](#)).

2.6.3 Case-control studies

Thirty-one case-control studies were included in the previous *IARC Monograph* ([IARC, 2004a](#)). Although results were inconsistent with respect to risk association in ever versus former and current smokers, a dose-response relationship with smoking variables was found in some studies. Since then, seventeen case-control studies investigating the association between tobacco smoke and colorectal cancer risk have been published, seven carried out in Asia, four in Europe, five in North America and one in Hawaii (Table 2.44 available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.44.pdf>). Six studies reported solely for colorectal cancer ([Ateş et al., 2005](#); [Chia et al., 2006](#); [Verla-Tebit et al., 2006](#); [Lüchtenborg et al., 2007](#); [Steinmetz et al., 2007](#); [Wu et al., 2009b](#)), four separately for colon and rectal cancer ([Ji et al., 2002](#); [Sharpe et al., 2002](#); [Minami & Tateno, 2003](#); [Goy et al., 2008](#)), two for colorectal cancer as well as for colon and rectal cancer ([Ho et al., 2004](#); [Gao](#)

[et al., 2007](#); [Wei et al., 2009](#)), three for colon cancer only ([Diergaarde et al., 2003](#); [Kim et al., 2003](#); [Hu et al., 2007](#)) and one for rectal cancer only ([Slattery et al., 2003](#)). Nine of the studies reported risk estimates separately for men and for women.

(a) Smoking status

Most case-control studies considered the effects of current and former smoking separately. A positive association between smoking and colorectal cancer was found in virtually all the studies, although the results were generally not statistically significant. Statistically significant increased risk was reported in current smokers for colorectal cancer ([Chia et al., 2006](#); [Wu et al., 2009b](#)), for rectal cancer ([Slattery et al., 2003](#); [Ho et al., 2004](#)), and in former smokers for colorectal cancer both in men and women combined ([Chia et al., 2006](#)) and in women only ([Lüchtenborg et al., 2007](#)). Five studies, which did not focus on the main effects of smoking, only evaluated risks for ever smoking ([Diergaarde et al., 2003](#); [Kim et al., 2003](#); [Ateş et al., 2005](#); [Gao et al., 2007](#); [Hu et al., 2007](#)); none of these reported significant risk estimates.

(b) Intensity of smoking

Nine case-control studies investigated dose-response relationships considering at least one smoking variable. Number of cigarettes smoked daily was evaluated in seven studies, three for colorectal cancer ([Verla-Tebit et al., 2006](#); [Lüchtenborg et al., 2007](#); [Wu et al., 2009b](#)), two for colon and rectal cancer ([Ji et al., 2002](#); [Minami & Tateno, 2003](#)), one for rectal cancer ([Slattery et al., 2003](#)) and one for colorectal cancer and both subsites ([Ho et al., 2004](#)). Statistically significant positive trends of increasing risk with increasing number of cigarettes smoked daily were found for colorectal cancer in only one study ([Wu et al., 2009b](#)).

(c) *Duration of smoking, pack-years, age at initiation, smoking cessation*

Duration of smoking was examined in several studies in relation to colorectal cancer ([Ho et al., 2004](#); [Chia et al., 2006](#); [Verla-Tebit et al., 2006](#); [Lüchtenborg et al., 2007](#); [Wu et al., 2009b](#)) and/or to colorectal cancer by subsite ([Ji et al., 2002](#); [Minami & Tateno, 2003](#); [Ho et al., 2004](#)). A statistically significant trend with increasing number of years smoked was found in two of the five studies of colorectal cancer ([Chia et al., 2006](#); [Wu et al., 2009b](#)). In one study, increasing duration of smoking was significantly associated with risk for rectal cancer in ever smokers but not in current smokers ([Ho et al., 2004](#)). In only one earlier case-control study was a significant association in ever smokers with increasing number of years of smoking for colon as well as rectal cancer found ([Newcomb et al., 1995](#)).

Duration of smoking exposure was assessed by pack-years of smoking in seven studies ([Ji et al., 2002](#); [Slattery et al., 2003](#); [Chia et al., 2006](#); [Verla-Tebit et al., 2006](#); [Lüchtenborg et al., 2007](#); [Goy et al., 2008](#); [Wu et al., 2009b](#)) and by age at smoking initiation in three studies ([Ji et al., 2002](#); [Slattery et al., 2003](#); [Wu et al., 2009b](#)). All four studies that evaluated pack-years of smoking with respect to colorectal cancer risk found statistically significant associations. Two studies found a significant association with increasing pack-years in men and women combined; when investigated separately, the increasing trend was statistically significant only in women ([Verla-Tebit et al., 2006](#)) or only in men ([Wu et al., 2009b](#)). In one study a statistically significant trend with pack-years of smoking in both men and women was found only with non-filtered cigarettes ([Lüchtenborg et al., 2007](#)); the relative risk was significant for colon as well as rectal cancer and was greater for rectal cancer.

In two studies a non-significant trend of decreasing risk with increasing time since

stopped smoking was found ([Verla-Tebit et al., 2006](#); [Lüchtenborg et al., 2007](#)).

(d) *Subsites and molecular subtypes*

A stronger association between tobacco smoking and rectal cancer compared with colon cancer has generally been observed in the studies that reported risk estimates by cancer site. In a recent meta-analysis including both cohort and case-control studies, higher smoking-related risk estimates for rectal cancer were found than for proximal and distal colon cancer ([Botteri et al., 2008a](#)). Stronger relative risk in ever smokers, but not in current smokers, was found for proximal compared to distal tumours in one recent study ([Hu et al., 2007](#)).

Colorectal cancer is a multipathway disease. A molecular approach to its classification utilizes: (1) the type of genetic instability, specifically microsatellite instability, and (2) the presence of DNA methylation or the CpG island methylator phenotype (CIMP) ([Jass, 2007](#)). Smoking has been associated with microsatellite instability in sporadic colon cancer. Higher risk for microsatellite-unstable than for microsatellite-stable tumours was found in four studies ([Slattery et al., 2000](#); [Yang et al., 2000](#); [Chia et al., 2006](#); [Campbell et al., 2009](#)). The observed twofold risk elevation for colorectal cancer showing microsatellite instability is similar in order of magnitude to that found for colorectal polyps. In only one small study similar risk estimates for stable and unstable tumours were found ([Diergaarde et al., 2003](#)). Microsatellite instability is characteristic of hereditary nonpolyposis colorectal cancer syndrome and smoking has been associated with colorectal cancer in patients with this syndrome ([Watson et al., 2004](#); [Diergaarde et al., 2007](#)). Among sporadic colorectal tumours with microsatellite instability, about 11–28% carry somatic genetic mutations. In addition, the association of colon cancer with smoking was increased two to threefold when widespread CIMP and/or *BRAF* mutation, irrespective of microsatellite instability

status, was present ([Samowitz et al., 2006](#)). These data indicate that the association with MSI-high tumours may be attributed to the association of smoking with CIMP and *BRAF* mutation.

(e) Effect modification

Effect modification by genetic polymorphisms in enzymes metabolizing tobacco smoke constituents could provide further evidence for a causal association between smoking and colorectal cancer. Most studies that have investigated modification of colorectal cancer risk associated with smoking by genetic polymorphisms of xenobiotic enzymes were too small to be informative ([Inoue et al., 2000](#); [Smits et al., 2003](#); [Jin et al., 2005](#); [Tranah et al., 2005](#); [van den Donk et al., 2005](#); [Tijhuis et al., 2008](#)). Studies on the possible differential effect by acetylation status have reported stronger association of tobacco smoking (in terms of pack-years) with colorectal cancer risk in slow acetylators phenotypes ([Lilla et al., 2006](#)), and with rectal cancer in rapid acetylators phenotypes ([Curtin et al., 2009](#)). Furthermore, *CYP1A1* and *GSTM1* variant alleles were found to greatly affect colon cancer or rectal cancer risk in smokers ([Slattery et al., 2004](#)).

2.6.4 Colorectal polyps

Colorectal adenomas and possibly some hyperplastic polyps are considered precursors of colorectal cancer. The epidemiologic evidence on the relationship between cigarette smoking and colorectal polyps has been generally consistent. Since the previous *IARC Monograph* ([IARC, 2004a](#)), twelve further independent studies have investigated this association (Table 2.45 available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.45.pdf>). All studies found a significantly increased risk for polyps in association with one or more smoking variables. A recent meta-analysis including 42 studies reported a statistically significant positive association between smoking and colorectal adenomas

([Botteri et al., 2008b](#)). The meta-analysis, which included several studies that did not explicitly report relative risks for tobacco smoking ([Cardoso et al., 2002](#); [Voskuil et al., 2002](#); [Sparks et al., 2004](#); [Gong et al., 2005](#); [Jiang et al., 2005](#); [Kim et al., 2005](#); [Mitrou et al., 2006](#); [Otani et al., 2006](#); [Skjelbred et al., 2006](#)), found a twofold risk elevation for colorectal adenomas in current smokers and a 50% increase in former smokers. The association had been previously found to be equally strong in men and women. In one of two recent studies, there was no difference in the results for men and women separately ([Tranah et al., 2004](#)) but significantly greater effects in women were found in the other ([Hermann et al., 2009](#)).

Significant positive trends with number of cigarettes per day were found in four ([Ji et al., 2006](#); [Larsen et al., 2006](#); [Stern et al., 2006](#); [Shrubsole et al., 2008](#)) of five studies ([Tiemersma et al., 2004](#)). Dose-response with duration of smoking was assessed in four studies ([Ji et al., 2002](#); [Tiemersma et al., 2004](#); [Stern et al., 2006](#); [Shrubsole et al., 2008](#)) and with pack-years of smoking in five studies ([Hoshiyama et al., 2000](#); [Ulrich et al., 2001](#); [Tranah et al., 2004](#); [Ji et al., 2006](#); [Shrubsole et al., 2008](#); [Omata et al., 2009](#)). All nine studies found statistically significant trends, which were consistent with those for adenomas and hyperplastic polyps when reported separately ([Ulrich et al., 2001](#); [Ji et al., 2006](#); [Shrubsole et al., 2008](#)). Ever smokers were estimated to have a 13% (95%CI: 9–18%) increasing risk of presenting with adenomatous polyps for every additional 10 pack-years smoked in comparison to never smokers, based on data from 19 studies ([Botteri et al., 2008b](#)).

Decreasing risks with years since quitting smoking were found in four studies ([Ulrich et al., 2001](#); [Tiemersma et al., 2004](#); [Ji et al., 2006](#); [Shrubsole et al., 2008](#)), statistically significant so in the latter three studies. In comparison to never smokers, former smokers retained moderately elevated risk for colorectal polyps even 20

years after quitting smoking. One study examined both dose metrics (cigarettes per day, duration, and pack-years) and recency of tobacco use: in subjects who had quit smoking for at least 20 years, only the heaviest users of tobacco still had modest excess risks ([Ji et al., 2006](#)).

It has been proposed that the association between cigarette smoking and polyps may be stronger with non-progressing adenomas, such as those that are smaller and less villous but the hypothesis is not supported in most studies ([Anderson et al., 2003](#); [Toyomura et al., 2004](#); [Ji et al., 2006](#); [Skjelbred et al., 2006](#)). In one study a clearly higher risk for large and multiple adenomas in every anatomic site of the colon was found in a dose-response manner ([Toyomura et al., 2004](#)). A meta-analysis found that the combined risk estimate for high-risk adenomas associated with smoking was greater than that for low-risk adenomas and that the difference was statistically significant for current smokers but not former smokers ([Botteri et al., 2008b](#)). In addition, a stronger association of smoking with hyperplastic polyps than with adenomas was found in some studies ([Ulrich et al., 2001](#); [Ji et al., 2006](#); [Shrubsole et al., 2008](#)) but not in another ([Erhardt et al., 2002](#)). The risk associated with smoking may be even higher in subjects presenting with concurrent benign hyperplastic and adenomatous polyps ([Ji et al., 2006](#); [Shrubsole et al., 2008](#)).

Relative risk estimates for tobacco smoking and polyps generally range between 2 and 3 whereas those for colorectal cancer range between 1.2 and 1.4. One possible explanation is the effect dilution due to the inclusion of a high proportion of individuals with precursor lesions in the unscreened control groups in most colorectal cancer studies ([Terry & Neugut, 1998](#)). Some indirect evidence for this hypothesis is provided by the meta-analysis of colorectal adenomas, which showed that the smoking-associated risk for adenomas was significantly higher in studies including subjects who had undergone complete

colonoscopy in comparison to those in which some or all controls had undergone incomplete examination (i.e. only sigmoidoscopy) ([Abrams et al., 2008](#); [Botteri et al., 2008b](#)).

It is also possible that smoking is associated with a subset of colorectal cancers so that relative risk estimates for colorectal cancer as a whole are diluted. The pattern of risk observed for colorectal cancer by microsatellite instability status and for type of colorectal polyps suggests that the traditional (non-serrated) adenoma-carcinoma sequence may proceed through a hyperplastic polyps-mixed polyps-serrated adenoma progression and that smoking may be more strongly related to the development of these subtypes ([Jass et al., 2000](#); [Hawkins & Ward, 2001](#)). More recently, a *BRAF* mutation was shown to be a specific marker for the serrated polyp neoplasia pathway originating from a hyperplastic polyp, in which the CIMP-high develops early and the microsatellite instability carcinoma develops late ([O'Brien et al., 2006](#)). The findings of strong associations between smoking and colon cancer with CIMP and/or *BRAF* mutation, irrespective of microsatellite status, are compatible with this observation ([Samowitz et al., 2006](#)).

2.7 Hepatocellular carcinoma

2.7.1 Overview of studies

In the previous *IARC Monograph* ([IARC, 2004a](#)), a causal relationship between liver cancer (hepatocellular carcinoma) and smoking was established. Two case-control and one cohort studies have been published since (Table 2.46 available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.46.pdf>). Overall, most cohort studies and the largest case-control studies, most notably those that included community controls, showed a moderate association between tobacco smoking and risk for hepatocellular carcinoma.

Confounding from alcohol has been addressed in the best studies. The association between alcohol drinking and hepatocellular carcinoma is strong, and alcohol intake is frequently misclassified, leading to potential residual confounding. However an association with smoking has been demonstrated also among non-drinkers.

A meta-analysis was based on 38 cohort studies and 58 case-control studies ([Lee et al., 2009](#)). Compared to never smokers, the meta-relative risks adjusted for appropriate confounders were 1.51 (95%CI: 1.37–1.67) for current smokers and 1.12 (0.78–1.60) for former smokers. The increased liver cancer risk among current smokers appeared to be consistent in strata of different regions, study designs, study sample sizes, and publication periods. The association with smoking was observed in non-alcohol-drinkers (RR, 1.34; 95%CI: 0.92–1.94 in men and 1.31; 95%CI: 0.70–2.44 in women). Further supportive evidence is provided by the association between smoking and liver cancer observed among Chinese women and Japanese women, in whom alcohol drinking is extremely rare ([Li et al., 2011](#)). One difficulty is that sometimes studies do not specify the histology of liver cancer (hepatocellular versus intra-hepatic biliary tract).

In the update of the Whitehall study ([Batty et al., 2008](#)) (a cohort of 17363 government employees in London, followed-up for 38 years), the hazard ratio for death from liver cancer was 1.03 (0.49–2.16) in former smokers and 1.43 (0.69–2.95) in current smokers (based on 57 deaths). In the 50-year follow-up of the British doctors cohort ([Doll et al., 2005](#)), there were 74 deaths from liver cancer. Death rates per 100000 per year were 4.4 in never smokers, 10.7 in smokers of 1–14 cigarettes/day, 2.6 in smokers of 15–24 cigarettes/day, and 31.3 in smokers of ≥ 25 cigarettes/day.

2.7.2 Factors affecting risks

(a) Dose-response relationship

Most studies, including the recent ones (Table 2.46 online), show a dose-response relationship with the number of cigarettes smoked and with smoking duration, with exceptions such as [Franceschi et al. \(2006\)](#) and some older studies from Asia. Relative risk estimates increased to 2.0 after 20 years of smoking.

(b) Cessation

Though former smokers tend to have lower relative risks than current smokers, there were no consistent patterns of risks after cessation, including in the recent studies (Table 2.46 online).

2.7.3 Interaction with hepatitis B or C

Infection with hepatitis B virus (HBV) is one of the major causes of liver cancer worldwide, whereas hepatitis C virus (HCV) infection causes a large fraction of liver cancer in Japan, Northern Africa and southern Europe. While many studies, most notably from Asia, have found no attenuation of the association between smoking and liver cancer after adjustment/stratification for markers of HBV or HCV infection, an apparent interaction between smoking and HBV or HCV infection has been reported. The increase in risk for liver cancer associated with cigarette smoking appears to be greater among HBV carriers than among uninfected persons in some studies ([Tu et al., 1985](#)), but not in others ([Kuper et al., 2000a](#)). Two recent reports ([Franceschi et al., 2006](#); [Hassan et al., 2008a](#)) studied possible interactions between smoking and hepatitis virus infection and both reported an apparent interaction between smoking and hepatitis C infection. Interactions between smoking and hepatitis B infection were not found among men in one study ([Hassan et al., 2008a](#)) and the rarity of HBsAg prevented the evaluation of HBV and smoking in the other ([Franceschi et al., 2006](#);

Table 2.46 online). In the meta-analysis by [Lee et al. \(2009\)](#) adjustment for HBV reduced the relative risks in both men and women, while adjustment for HCV did not change the risk in women and increased it in men.

2.8 Renal cell carcinoma

2.8.1 Overview of studies

The previous *IARC Monograph* ([IARC, 2004a](#)) concluded that renal-cell carcinoma is associated with tobacco smoking in both men and women. Four case-control studies and no cohort studies have become available since then (Table 2.47 available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.47.pdf>). Overall these confirm the previous evidence, though with some conflicting results. In particular, both the study by [Hu et al. \(2005\)](#) in Canada and the multicentre European study by [Brennan et al. \(2008\)](#) do not show a clear effect of smoking. In contrast, the study by [Theis et al. \(2008\)](#) shows an increased risk with smoking duration (irregular, levelling-off after 40 years) and a statistically significant dose-response relationship with pack-years.

In the update of the Whitehall study ([Batty et al., 2008](#)) (a cohort of 17363 government employees in London, followed for 38 years), the hazard ratio for deaths from kidney cancer was 0.64 (0.32–1.26) for former smokers, and 1.29 (0.69–2.41) for current smokers (based on 68 deaths). In the 50-year follow-up of the British doctor cohort ([Doll et al., 2005](#)) there were 140 deaths from kidney cancer. Mortality rates per 100000 per year were 9.3 in never smokers, 16.4 in smokers of 1–14 cigarettes/day, 16.6 in smokers of 15–24 cigarettes/day, and 15.5 in smokers of ≥ 25 cigarettes/day (age-adjusted).

[Hunt et al. \(2005\)](#) performed a meta-analysis based on 19 case-control studies and 5 cohort studies (total 8032 cases in case-control and 1326 in cohort studies). The relative risk for smoking

men was 1.54 (1.42–1.68), and for smoking women was 1.22 (1.09–1.36). A dose-response relationship was found in both men and women. The association observed was more convincing in population-based compared to hospital-based studies.

2.8.2 Confounding

Hypertension is a well established risk factor for kidney cancer but the association with smoking is only indirect. Potential confounding from hypertension was considered only by [Brennan et al. \(2008\)](#).

Other potential confounders such as BMI have been appropriately addressed in most studies.

2.8.3 Cessation

Most studies reviewed in the previous *Monograph* showed a lower risk for former smokers compared to current smokers, with a significant negative trend with increasing number of years since quitting ([IARC, 2004a](#)). In case-control study on smoking cessation and renal-cell carcinoma, the decrease in risk became significant only after 30 years of quitting ([Parker et al., 2003](#)). In the meta-analysis ([Hunt et al., 2005](#)), former smokers were at reduced risk after 10 years or more of quitting. A clear decline in risk after cessation was also reported by [Theis et al. \(2008\)](#). [The Working Group noted the poor quality of the study, considering the low response rate among controls.]

2.9 Cancer of the lower urinary tract (including cancer of the bladder, ureter, and renal pelvis)

2.9.1 Overview of studies

The previous *IARC Monograph* ([IARC, 2004a](#)) clearly identified a causal relationship of smoking with transitional-cell carcinomas and squamous-cell carcinomas of the bladder, ureter and renal pelvis both in men and women. Two new case-control studies ([Cao et al., 2005](#); [Samanic et al., 2006](#); Table 2.48 available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.48.pdf>) and two cohort studies ([Bjerregaard et al., 2006](#); [Alberg et al., 2007](#); Table 2.49 available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.49.pdf>) have been reported since then in addition to updates of cohort studies with longer follow-up.

In the update of the Whitehall study ([Batty et al., 2008](#)) (a cohort of 17363 government employees in London, followed-up for 38 years), the hazard ratio for death from bladder cancer was 0.98 (0.62–1.54) in former smokers and 1.66 (1.06–2.59) in current smokers (based on 164 deaths). In the 50-year follow-up of the British doctors cohort ([Doll et al., 2005](#)), there were 220 deaths from bladder cancer. Death rates per 100000 per year were 13.7 in never smokers, 37.7 in smokers of 1–14 cigarettes/day, 31.8 in smokers of 15–24 cigarettes/day, and 51.4 in smokers of ≥ 25 cigarettes/day. All the new studies confirm the existence of a dose-response relationship with the number of cigarettes smoked and with duration, and a decline in relative risk with time since quitting smoking, compared to non-quitters.

2.9.2 Types of tobacco

The risk of lower urinary tract cancer was more strongly associated with smoking air-cured (black) tobacco than smoking flue-cured

(blond) tobacco in several studies ([IARC, 2004a](#)). The stronger association with air-cured (black) than blond tobacco among current smokers has not been clearly confirmed in a re-analysis of the Spanish multicentre case-control study ([Samanic et al., 2006](#)). Relative risks in current smokers were 7.3 (4.9–10.9) in black tobacco smokers and 5.8 (3.4–10.0) in blond tobacco smokers; in former smokers, 4.2 (2.9–6.0) for black tobacco and 1.8 (1.0–3.2) for blond tobacco (Table 2.48 online). The effect of cessation was more pronounced in blond tobacco smokers than in black tobacco smokers, suggesting potentially different mechanisms of action of the two types of tobacco. Air-cured (black) tobacco is richer in arylamines.

2.9.3 Gene-environment interactions

A large number of studies have considered gene-environment interactions between tobacco smoking and genetic polymorphisms, including DNA repair genes ([Vineis et al., 2009](#)) and genes involved in carcinogen metabolism ([Malats, 2008](#); [Dong et al., 2008](#)). Overall, there is evidence that the slow acetylator variant of the *NAT2* gene is involved in bladder carcinogenesis and may interact with smoking. The meta-relative risk for *NAT2* slow acetylator and bladder cancer was 1.46 (95%CI: 1.26–1.68; $P = 2.5 \times 10^{-7}$), based on 36 studies and 5747 cases ([Dong et al., 2008](#)). Similar but weaker evidence has been provided for *GSTM1* ([Malats, 2008](#)).

The extent of interaction between *NAT2* variants and smoking is still unclear. In one study the *NAT2* acetylation status was found to modulate the association of bladder cancer and cigarette smoking through smoking intensity and not smoking duration ([Lubin et al., 2007](#)). Studies are not consistent concerning the three-way association between smoking intensity, *NAT2* and bladder cancer. Some studies found greater effects at a lower level of exposure and others the opposite ([Malats, 2008](#)). Genome-wide

association studies have indicated 8q24 as a region that may confer high risk for bladder cancer ([Kiemenev et al., 2008](#)).

2.10 Myeloid leukaemia (acute and chronic)

Myeloid leukaemia in adults was observed to be causally related to cigarette smoking in the previous *IARC Monograph* ([IARC, 2004a](#)). Risk increased with amount of tobacco smoked in a substantial number of adequate studies, with evidence of a dose–response relationship. Biological plausibility for a causal relationship of smoking with myeloid leukaemia is provided by the finding of known leukaemogens in tobacco smoke, one of which (benzene) is present in relatively large amounts. No evidence was found for an association with acute lymphocytic leukaemia.

One recently published cohort study included information on acute and chronic myeloid leukaemias ([Fernberg et al., 2007](#)), based on 372 incident cases. A weak association was found between acute myeloid leukaemia and intensity of smoking, and a statistically significant association with current smoking (RR, 1.5; 95%CI: 1.06–2.11). No association was found with chronic myeloid leukaemia.

In the update of the Whitehall study ([Batty et al., 2008](#)) (a cohort of 17363 government employees in London, followed-up for 38 years), the hazard ratio for mortality from myeloid leukaemias (acute plus chronic) was 5.08 (95%CI: 1.78–14.5) for current smokers, and 3.84 (95%CI: 1.35–11.0) for former smokers (based on 66 deaths). In the 50-year follow-up of the British doctors cohort ([Doll et al., 2005](#)), there were 100 deaths from myeloid leukaemias. The mortality rates per 100000 per year were 6.3 in never smokers, 2.8 in smokers of 1–14 cigarettes/day, 14.0 in smokers of 15–24, and 18.3 in smokers of ≥ 25 cigarettes/day (age-adjusted).

2.11 Other leukaemias and lymphomas

2.11.1 Non-Hodgkin lymphoma

Six cohort studies have been published on the association between non-Hodgkin lymphoma and smoking, all reviewed in the previous *IARC Monograph* ([IARC, 2004a](#)). In five of these, no increased risk among smokers was evident ([Doll et al., 1994](#); [McLaughlin et al., 1995](#); [Adami et al., 1998](#); [Herrinton & Friedman, 1998](#); [Parker et al., 2000](#)). However, in one study, men who had ever smoked cigarettes had a twofold increase in risk for non-Hodgkin lymphoma, and the risk was still higher among the heaviest smokers ([Linnet et al., 1992](#)). Data from case–control studies generally also fail to support an effect of smoking on the incidence of non-Hodgkin lymphoma ([Peach & Barnett, 2001](#); [Stagnaro et al., 2001](#); [Schöllkopf et al., 2005](#); [Bracci & Holly, 2005](#); Table 2.50 available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.50.pdf>). Reanalysis of data of an Italian study ([Stagnaro et al., 2004](#)) found a statistically significant association (OR, 1.4; 95%CI: 1.1–1.7) for blond tobacco exposure and non-Hodgkin lymphoma risk.

Three studies and a pooled analysis have examined histological subtypes of non-Hodgkin lymphoma. In one cohort study in women, smoking was associated with increased risk for follicular non-Hodgkin lymphoma ([Parker et al., 2000](#)). Similarly, two other studies reported a weak positive association between smoking and risk for follicular lymphoma, but no effect for other histological types ([Herrinton & Friedman, 1998](#); [Stagnaro et al., 2001](#)). A large pooled analysis based on nine North-American and European case–control studies found an overall odds ratio of 1.07 (95%CI: 1.0–1.15) for smokers; the association was particularly strong for follicular lymphoma (OR, 1.31; 95%CI: 1.12–1.52) ([Morton et al., 2005](#)).

2.11.2 Hodgkin lymphoma

In the previous *IARC Monograph* ([IARC, 2004a](#)) seven studies on the association between Hodgkin lymphoma and smoking were examined and null or weakly positive associations were noted. Among studies published since, a positive association was observed in two case-control ([Willett et al., 2007](#); [Kanda et al., 2009](#)) and three cohort studies ([Nieters et al., 2006](#); [Lim et al., 2007](#); [Nieters et al., 2008](#)), while one study found no clear association ([Monnereau et al., 2008](#)). Several other recent studies also reported a positive association, but with some internal inconsistencies. In a European multicentre case-control study, no association was observed between tobacco and Hodgkin lymphoma for subjects below age 35 years, whereas for older subjects, ever-smokers experienced a doubled risk of Hodgkin lymphoma as compared to never smokers ([Besson et al., 2006](#)). In contrast, a positive association was observed in young adults participating in the International Twin Study ([Cozen et al., 2009](#)). A positive association was observed in a Scandinavian case-control study, but without a clear dose-response ([Hjalgrim et al., 2007](#)). In a case-control study addressing infectious precursors, particularly Epstein-Barr virus (EBV), an increased risk for EBV-positive Hodgkin lymphoma was found among current smokers ([Glaser et al., 2004](#); Table 2.50 online).

Several of the above studies found positive associations for Hodgkin lymphoma while also demonstrating null or inverse associations with non-Hodgkin lymphoma ([Nieters et al., 2006](#); [Lim et al., 2007](#); [Nieters et al., 2008](#); [Kanda et al., 2009](#)).

2.11.3 Multiple myeloma

In the previous *IARC Monograph* ([IARC, 2004a](#)), the large majority of studies on tobacco smoking and risk for multiple myeloma evaluated showed no clear association. More recently,

two case-control studies found a positive association ([Vlajinac et al., 2003](#); [Nieters et al., 2006](#)), whereas no clear association was observed in another case-control study ([Monnereau et al., 2008](#)) or in a cohort study in Sweden ([Fernberg et al., 2007](#); Table 2.51 available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.51.pdf>).

2.12 Cancer of the breast

Approximately 150 epidemiological studies have been published on the relationship between breast cancer and active and passive smoking. The results from these studies have been comprehensively examined in peer-reviewed literature ([Palmer & Rosenberg, 1993](#); [Terry et al., 2002a](#); [Johnson et al., 2002](#); [Johnson, 2005](#); [Terry & Goodman, 2006](#); [Miller et al., 2007](#)). The previous *IARC Monograph* ([IARC, 2004a](#)) considered studies conducted through June 2002 and concluded that there is evidence suggesting lack of carcinogenicity of tobacco smoking in humans for cancers of the female breast.

Other consensus reviews have drawn different conclusions, based partly on the availability of new data, and partly on differences in interpretation:

- The 2001 US Surgeon General Report on Women and Smoking (Department of Health & Human Services, 2001) concluded that tobacco smoking does not appear to appreciably affect breast cancer risk overall. However, several issues were not entirely resolved, including whether starting to smoke at an early age increases risk, whether certain subgroups defined by genetic polymorphisms are differentially affected by smoking, and whether exposure to second-hand smoke affects risk.
- The 2004 US Surgeon General report on “The Health Consequences of Smoking” (Department of Health & Human

Services, 2004) concluded the evidence is suggestive of no causal relationship between tobacco smoking and breast cancer.

- The 2009 Canadian Expert Panel on Tobacco Smoke and Breast Cancer Risk ([Collishaw et al., 2009](#)) concludes that based on the weight of evidence from epidemiological and toxicological studies and understanding of biological mechanisms, the associations between tobacco smoking and both pre- and post-menopausal breast cancer are consistent with causality.

The lack of agreement in the conclusions from these groups is not surprising, given that the observed associations are weaker and less consistent for breast cancer than for other tobacco-related cancers. Furthermore, several methodological considerations could either obscure a small increase in risk caused by tobacco smoking, or alternatively introduce a spurious association where no causal relationship exists.

2.12.1 Methodological and related issues

The principal concerns about studies of tobacco smoking and breast cancer are the following: timing of exposure, the relevant disease endpoint, the potential for confounding by factors associated with both smoking and the occurrence/detection of breast cancer, the hypothesis that tobacco smoking may have opposing effects on breast cancer risk (protective and detrimental), and the hypothesis that some women may be genetically more susceptible to develop breast cancer from smoking, and that increased risk in these subgroups may be obscured in analyses of average risk in the population.

(a) Misclassification of exposure

Self-reported information on tobacco smoking is generally considered more reliable than questionnaire information on exposure to second-hand tobacco smoke. However, studies of tobacco smoking have not uniformly considered the duration of smoking (years), the average amount smoked (cigarettes/day), or the timing of initiation in relation to first full-term pregnancy. Only one ([Al-Delaimy et al., 2004](#)) of the seven available cohort studies updated the information on smoking behaviour during follow-up. Whereas some exposure variables, such as age at initiation and age at first full-term pregnancy remain constant over time, others, such as smoking status, duration and age at cessation do not. Furthermore, the average age at initiation and duration of smoking are highly correlated with birth cohort and attained age. While the number of years of smoking before first full term pregnancy has been proposed as a potentially relevant measure of exposure, the range of this variable is constrained except among women whose first pregnancy occurs at an older age, which is itself an independent risk factor for breast cancer.

(b) Specificity of disease endpoints

Breast cancer is not a single disease. Accordingly, some researchers have postulated that exposure to tobacco smoke (from tobacco smoking or second-hand tobacco smoke) could differentially affect certain clinical subtypes of breast such as pre- or post-menopausal cancers or tumours with or without hormonal receptors. It is also possible that smoking might affect the survival of women with breast cancer, whether or not it affects incidence rates. Most published studies have measured incidence rates as the endpoint, although some have measured mortality rates or effects on survival.

(c) Confounding

Alcohol consumption is positively correlated with tobacco smoking ([Marshall et al., 1999](#)) and is an established cause of breast cancer ([IARC, 2010a](#); *Monograph on Consumption of Alcoholic Beverages in this Volume*). Most epidemiologic studies attempt to control for alcohol consumption using questionnaire information on usual drinking patterns. This approach is vulnerable to residual confounding, because self-reported data on lifetime alcohol consumption leave room for misclassification. Potential confounding by alcohol consumption is of greater concern for current than for former smokers, since, on average, current smokers drink more than former smokers ([Reynolds et al., 2004a, b](#)). One study by the Collaborative Group on Hormonal Factors and Breast Cancer ([Hamajima et al., 2002](#)) controlled rigorously for alcohol consumption by restricting the analysis of smoking and breast cancer to women who reported drinking no alcohol.

Conversely, mammography screening can be a negative confounder in studies of tobacco smoking and breast cancer incidence. Few studies of tobacco smoking in relation to breast cancer have controlled for mammography screening. Current smokers report a lower frequency of mammographic screening than never-smokers, whereas health conscious former smokers report higher screening rates ([Gross et al., 2006](#)). Mammography screening affects the detection rather than the occurrence of breast cancer; it detects some tumours that might otherwise never have been recognized and allows earlier diagnosis of others, thereby increasing breast cancer incidence in the short-term. The consequence of uncontrolled confounding by mammography screening would be to underestimate an association between current smoking and breast cancer incidence, and to overestimate the association in former smokers. Confounding by screening

would be expected to have the opposite effect in studies of breast cancer mortality.

Other correlates of tobacco smoking might also confound a potential association between tobacco smoking and breast cancer, although their net effect is likely to be smaller and harder to predict than confounding by alcohol and mammography screening. Women who smoke undergo menopause about two to three years earlier than never-smokers ([Baron et al., 1990](#)). The effect of this may be partly or wholly offset by the greater likelihood of girls who experience early menarche to initiate smoking in early adolescence ([Jean et al., 2011](#)). There is no documentation that smokers and never-smokers differ with respect to average years of ovulation. Tobacco smoking also has a complex relationship to body mass index. Post-menopausal women who smoke are less likely to be overweight or obese than former or never smokers, but overweight adolescent girls are more likely to begin smoking for weight control ([Fine et al., 2004](#)). Similarly complex relationships exist between smoking and physical activity. Current smokers report less physical activity than either former or never smokers ([Kaczynski et al., 2008](#); [Trost et al., 2002](#)), but only a small proportion of the population engages in the vigorous physical activity that is needed to protect against breast cancer. The socioeconomic correlates of smoking have changed over time. Women who attended college during the 1960s and 1970s were more likely to initiate smoking than less educated women, but subsequently college-educated women have been more likely to quit. Thus, the potential for confounding by reproductive patterns and use of post-menopausal hormone treatment varies by birth cohort and differs for current and former smokers.

Most epidemiological studies have attempted to control for factors that might confound the relationship between breast cancer and tobacco smoking using questionnaire information collected on these factors. None of the published

studies have been able to control for all of the potential confounders, however. Most studies lack data on screening behaviour and have limited information on alcohol consumption, use of post-menopausal hormones, and physical activity.

(d) *Potential anti-estrogenic effects of tobacco smoking*

Indirect evidence suggests that tobacco smoking may have anti-estrogenic effects that might offset the adverse effects of tobacco smoke carcinogens on breast cancer risk. [Baron et al. \(1990\)](#) pointed to observations suggesting lower estrogen activity levels in women who smoke compared to those who do not. Smokers have lower risk of endometrial cancer (Department of Health & Human Services, 2004), higher risk of osteoporosis ([Jensen et al., 1985](#); [Jensen & Christiansen, 1988](#)), earlier age at natural menopause ([Baron et al., 1990](#)) and lower mammography density ([Roubidoux et al., 2003](#)) than women who do not smoke. Smoking also attenuates the effects of hormone replacement therapy (HRT) on lipid profiles ([Jensen & Christiansen, 1988](#)) and serum estrone ([McDivitt et al., 2008](#)). No difference in serum concentrations of estradiol and estrone between post-menopausal smokers and non-smokers have been reported in several studies ([Cassidenti et al., 1992](#); [Khaw et al., 1988](#); [Berta et al., 1991](#); [Longcope et al., 1986](#); [Berta et al., 1992](#); [Cauley et al., 1989](#); [Friedman et al., 1987](#); [Key et al., 1991](#)). However, smokers have been observed to have higher levels of androgens ([Cassidenti et al., 1992](#)) (specifically androstenedione) ([Khaw et al., 1988](#); [Cauley et al., 1989](#); [Friedman et al., 1987](#); [Key et al., 1991](#)), prolactin ([Berta et al., 1991](#)), and unbound serum estradiol ([Cassidenti et al., 1992](#)).

(e) *Genetically susceptible subgroups*

Certain subgroups of women may have greater risk of breast cancer when exposed to tobacco smoke because of genetic or other factors

affecting cancer susceptibility. Potential interactions between inherited polymorphisms and tobacco smoking have been studied for selected candidate genes that affect carcinogen metabolism, modulation of oxidative damage, immune responses, and DNA repair (see Sections 2.12.4b and 4.2).

2.12.2 Analytical studies

Over 130 epidemiological studies on tobacco smoking and breast cancer were reviewed.

(a) *Incidence in current and former smokers*

Since the previous IARC Monograph ([IARC, 2004a](#)), seven reports on cohort studies ([Al-Delaimy et al., 2004](#); [Reynolds et al., 2004a](#); [Gram et al., 2005](#); [Hanaoka et al., 2005](#); [Olson et al., 2005](#); [Cui et al., 2006](#); [Ha et al., 2007](#)) have been published on breast cancer incidence in relation to tobacco smoking (Table 2.52 available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.52.pdf>). Breast cancer incidence was significantly associated with current tobacco smoking in three studies ([Reynolds et al., 2004a](#); [Olson et al., 2005](#); [Cui et al., 2006](#)), with relative risk estimates among the larger studies ranging from 1.12 (95%CI: 0.92–1.37) ([Al-Delaimy et al., 2004](#)) to 1.32 (95%CI: 1.10–1.57) ([Reynolds et al., 2004a](#)). Former smoking was significantly associated with risk in only one cohort ([Al-Delaimy et al., 2004](#)), with relative risk estimates across all of the cohorts ranging from 1.00 (95%CI: 0.93–1.08) ([Cui et al., 2006](#)) to 1.18 (95%CI: 1.02–1.36) ([Al-Delaimy et al., 2004](#)). The association with breast cancer is stronger in current than in former smokers in four of the seven cohort studies ([Reynolds et al., 2004a](#); [Hanaoka et al., 2005](#); [Olson et al., 2005](#); [Cui et al., 2006](#)), although the confidence intervals overlap widely in all but one ([Cui et al., 2006](#)). [The Working group noted that three cohort studies ([Gram et al., 2005](#); [Hanaoka et al., 2005](#); [Olson et al., 2005](#)) provided data on both

the age-adjusted and the multivariate-adjusted risk estimates for current and former smoking. None of these showed attenuation of the estimate associated with current smoking, and two (Hanaoka *et al.*, 2005; Olson *et al.*, 2005) reported somewhat stronger estimates when adjusted for established risk factors besides age. None of the studies adjusted for the frequency of mammography screening. Residual confounding by screening and incomplete control for other risk factors would be expected to cause underestimation of the association with current smoking, and overestimation of the association with former smoking.]

Since the previous *IARC Monograph* (IARC, 2004a), a total of 12 case-control studies on tobacco smoking and breast cancer incidence have been published (Table 2.53 available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.53.pdf>). Results from the case-control studies are less consistent than those from the cohort studies. Six studies (Li *et al.*, 2004; Mechanic *et al.*, 2006; Magnusson *et al.*, 2007; Prescott *et al.*, 2007; Roddam *et al.*, 2007; Slattery *et al.*, 2008) differentiated between current and former smokers, while the six other reports (Band *et al.*, 2002; Lash & Aschengrau, 2002; Gammon *et al.*, 2004; Rollison *et al.*, 2008; Ahern *et al.*, 2009; Young *et al.*, 2009) specify only ever or never smokers. Only one study (Li *et al.*, 2004) reported a borderline significant increase in risk associated with current smoking, and two studies (Band *et al.*, 2002; Rollison *et al.*, 2008) with ever smoking.

None of the six case-control studies that presented data on breast cancer incidence separately for current and former smokers found a significant difference in risk between the two smoking categories; the relative risk estimates were higher for former than for current smokers in four of the studies (Mechanic *et al.*, 2006; Prescott *et al.*, 2007; Roddam *et al.*, 2007; Slattery *et al.*, 2008) and identical in the fifth (Magnusson *et al.*, 2007).

(b) *Years of cessation*

When the relative risk for breast cancer incidence in former smokers is examined by years since cessation in cohort studies (Table 2.54 available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.54.pdf>), the point estimates do not consistently decrease with longer time since cessation. In none of the four cohort studies (London *et al.*, 1989b; Egan *et al.*, 2002; Reynolds *et al.*, 2004a; Cui *et al.*, 2006) and in only one (Li *et al.*, 2005) of the five case-control studies (Chu *et al.*, 1990; Gammon *et al.*, 1998; Johnson *et al.*, 2000; Kropp & Chang-Claude, 2002; Li *et al.*, 2005) that formally tested for trend (Table 2.55 available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.55.pdf>) was there a statistically significant decrease in relative risk observed with longer time since cessation. Only one study has reported data on breast cancer mortality in relation to years since quitting or age at cessation (Calle *et al.*, 1994). A statistically significant inverse trend in the relative risk estimates was reported with both years since quitting (p trend = 0.04) and younger age at cessation (p trend = 0.02). [The Working Group noted that the inverse trends in the relative risk of dying from breast cancer observed in this study are weaker than those observed with most other cancers designated as causally associated with smoking.]

(c) *Duration of smoking and age at initiation*

Tables 2.56–2.61 (see below for links) list the published epidemiologic studies that relate breast cancer incidence to duration of tobacco smoking, age at initiation and/or timing relative to first full term pregnancy.

Longer duration of smoking is associated with higher breast cancer incidence in five of seven cohort studies (Table 2.56 available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.56.pdf>). A similar trend is seen inconsistently among the 33 case-control

studies that report relative risk estimates by duration of smoking (Table 2.57 available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.57.pdf>). Among the 18 studies that reported a formal test of trend, eight studies (Gammon *et al.*, 1998; Johnson *et al.*, 2000; Reynolds *et al.*, 2004a; Gram *et al.*, 2005; Li *et al.*, 2005; van der Hel *et al.*, 2005; Cui *et al.*, 2006; Mechanic *et al.*, 2006) reported a statistically significant or borderline increase in the relative risk of incident breast cancer with the duration of smoking; seven studies (Ewertz, 1990; Palmer *et al.*, 1991; Egan *et al.*, 2002; Al-Delaimy *et al.*, 2004; Lissowska *et al.*, 2006; Magnusson *et al.*, 2007; Prescott *et al.*, 2007) reported no trend, and one study (Brinton *et al.*, 1986) reported an inverse relationship.

Thirty studies, including cohort (Tables 2.58 available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.58.pdf>) and case-control studies (Table 2.59 available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.59.pdf>) related breast cancer incidence to age at smoking initiation. Fifteen of these (Chu *et al.*, 1990; Ewertz, 1990; Palmer *et al.*, 1991; Nordlund *et al.*, 1997; Gammon *et al.*, 1998; Johnson *et al.*, 2000; Egan *et al.*, 2002; Kropp & Chang-Claude, 2002; Gram *et al.*, 2005; Cui *et al.*, 2006; Lissowska *et al.*, 2006; Ha *et al.*, 2007; Lissowska *et al.*, 2007; Magnusson *et al.*, 2007; Prescott *et al.*, 2007; Slattery *et al.*, 2008) reported a formal test of trend. Among these, only two (Gram *et al.*, 2005; Ha *et al.*, 2007) found a statistically significant or borderline significantly higher risk in women who began smoking at a younger ages; twelve studies (Chu *et al.*, 1990; Ewertz, 1990; Palmer *et al.*, 1991; Nordlund *et al.*, 1997; Gammon *et al.*, 1998; Johnson *et al.*, 2000; Egan *et al.*, 2002; Cui *et al.*, 2006; Lissowska *et al.*, 2006; Magnusson *et al.*, 2007; Prescott *et al.*, 2007; Slattery *et al.*, 2008) found no relationship with age at initiation, and one (Kropp & Chang-Claude, 2002) reported higher risk among women who began

smoking later. [The Working Group noted that at least two studies (Cui *et al.*, 2006; Slattery *et al.*, 2008) appear to have included never-smokers in the tests of trend and that the categories that define age at initiation differ across studies.]

The relative risk of incident breast cancer according to the timing of smoking initiation relative to first full-term pregnancy was reported in 21 studies, of cohort (Table 2.60 available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.60.pdf>) and case-control (Table 2.61 available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.61.pdf>) design. For nine studies (Hunter *et al.*, 1997; Egan *et al.*, 2002; Al-Delaimy *et al.*, 2004; Reynolds *et al.*, 2004a; Li *et al.*, 2005; Cui *et al.*, 2006; Prescott *et al.*, 2007; Rollison *et al.*, 2008; Young *et al.*, 2009) categorical data on years of smoking before first pregnancy are presented, whereas for 12 (Lash & Aschengrau, 1999; Innes & Byers, 2001; Band *et al.*, 2002; Kropp & Chang-Claude, 2002; Lash & Aschengrau, 2002; Fink & Lash, 2003; Lawlor *et al.*, 2004; Gram *et al.*, 2005; Olson *et al.*, 2005; Lissowska *et al.*, 2006; Magnusson *et al.*, 2007; Slattery *et al.*, 2008) whether smoking was initiated before or after the initial pregnancy was considered. Breast cancer incidence is consistently higher when smoking began before or during first pregnancy in most (Hunter *et al.*, 1997; Lash & Aschengrau, 1999; Innes & Byers, 2001; Band *et al.*, 2002; Egan *et al.*, 2002; Al-Delaimy *et al.*, 2004; Reynolds *et al.*, 2004a; Gram *et al.*, 2005; Li *et al.*, 2005; Olson *et al.*, 2005; Cui *et al.*, 2006; Slattery *et al.*, 2008; Young *et al.*, 2009) but not all (Kropp & Chang-Claude, 2002; Lash & Aschengrau, 2002; Fink & Lash, 2003; Prescott *et al.*, 2007) studies that tested this. [The Working Group noted that the number of years of smoking before first pregnancy is highly correlated with age at first full-term pregnancy, which is itself an independent risk factor for breast cancer.]

It has been argued that some studies, and especially cohort studies, may underestimate

the true association between tobacco smoking and breast cancer risk by ignoring or underestimating lifetime exposure to second-hand tobacco smoke of those in the referent group ([California Environmental Protection Agency, 2005](#); [Johnson, 2005](#); [Collishaw *et al.*, 2009](#)). This criticism is based on the hypothesis that exposure to second-hand smoke may confer almost the same degree of breast cancer risk as tobacco smoking. Under this hypothesis, the inclusion of women exposed to second-hand smoke in the referent group dilutes the contrast between exposed and unexposed women in studies of tobacco smoking, and causes underestimation of the association between tobacco smoking and breast cancer. In several case-control studies the association between breast cancer and tobacco smoking strengthened when the referent group was defined as women with “never active, never-passive” exposure to tobacco smoke ([Morabia *et al.*, 1996](#); [Lash & Aschengrau, 1999](#); [Johnson *et al.*, 2000](#); [Kropp & Chang-Claude, 2002](#)). In contrast, a stronger association between tobacco smoking and breast cancer risk, when women exposed only to second-hand smoke are excluded from the referent group, has not been observed in cohort studies ([Egan *et al.*, 2002](#); [Reynolds *et al.*, 2004a](#)). Debate continues over whether the case-control studies should be considered “of highest quality” because they provide “lifetime exposure assessment” ([Collishaw *et al.*, 2009](#)) or whether the cohort studies are more credible, because prospectively-collected exposure data are not susceptible to the recall bias that can affect retrospective studies.

(d) *Survival and mortality from breast cancer*

The relationship between smoking and the natural history of breast cancer has been examined in several studies ([Daniell, 1988](#); [Ewertz *et al.*, 1991](#); [Daniell *et al.*, 1993](#); [Scanlon *et al.*, 1995](#); [Yu *et al.*, 1997](#); [Manjer *et al.*, 2000](#); [Murin & Inciardi, 2001](#); [Holmes *et al.*, 2007](#)). In cross-sectional analyses, [Daniell *et al.* \(1993\)](#) found that

smokers with breast cancer had more and larger lymph node metastases than non-smokers, after controlling for primary tumour size and other variables. Further, a case-control study ([Murin & Inciardi, 2001](#)) and a retrospective cohort study ([Scanlon *et al.*, 1995](#)) found smoking to be associated with an increased risk of developing pulmonary metastases from breast cancer. However, these studies could not definitively distinguish lung metastases from primary lung cancers.

Five cohort studies have focused specifically upon the association of tobacco smoking with either breast cancer survival ([Ewertz *et al.*, 1991](#); [Yu *et al.*, 1997](#); [Manjer *et al.*, 2000](#); [Holmes *et al.*, 2007](#)) or breast cancer death rates ([Calle *et al.*, 1994](#)). A study of 1774 Danish women showed no association between smoking and breast cancer survival ([Ewertz *et al.*, 1991](#)), as did a study of 5056 women with breast cancer in the Nurse’s Health Study ([Holmes *et al.*, 2007](#)). In contrast, follow-up of 792 women with in situ or invasive breast cancer detected in a screening study in Malmö, Sweden found a crude relative risk for smokers and ex-smokers, compared to never smokers, of 1.44 (95%CI: 1.01–2.06) and of 1.13 (95%CI: 0.66–1.94), respectively ([Manjer *et al.*, 2000](#)). The relative risk associated with smoking remained significant after adjustment for age and stage at diagnosis (RR, 2.14; 95%CI: 1.47–3.10). A study based on the ACS Cancer Prevention Study II reported an association between current smoking and increased breast cancer death rates after six years of follow-up (Table 2.56 online; [Calle *et al.*, 1994](#)). Risk of death attributed to breast cancer was positively and significantly related to the duration of current smoking reported at the time of enrolment. However, the authors acknowledge that mortality studies cannot exclude biases arising from the effect of smoking on overall death rates, which could increase the potential for prevalent breast cancer to be coded as the underlying cause of death on the death certificate ([Calle *et al.*, 1994](#)).

2.12.3 Subtypes

(a) Pre- versus post-menopausal

Since the previous *IARC Monograph* ([IARC, 2004a](#)), 19 case-control studies have published data on tobacco smoking in relation to pre- and post-menopausal breast cancer (Table 2.62 available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.62.pdf>). The results are inconsistent. Of the 12 studies that provide information separately for current smokers ([Schechter et al., 1985](#); [Brinton et al., 1986](#); [Rohan & Baron, 1989](#); [Ewertz, 1990](#); [Baron et al., 1996](#); [Gammon et al., 1998](#); [Millikan et al., 1998](#); [Johnson et al., 2000](#); [Zheng et al., 2002](#); [Magnusson et al., 2007](#); [Slattery et al., 2008](#)), only five ([Schechter et al., 1985](#); [Johnson et al., 2000](#); [Magnusson et al., 2007](#); [Slattery et al., 2008](#)) found a stronger association with pre- than with post-menopausal breast cancer. The other analyses show either similar associations ([Brinton et al., 1986](#); [Ewertz, 1990](#); [Baron et al., 1996](#); [Gammon et al., 1998](#); [Millikan et al., 1998](#); [Zheng et al., 2002](#)) or a stronger association with post-menopausal breast cancer ([Rohan & Baron, 1989](#); [Millikan et al., 1998](#); [Johnson et al., 2000](#); [Zheng et al., 2002](#)).

(b) Hormone receptor status

Two cohort studies ([London et al., 1989a](#); [Manjer et al., 2001](#)), one case-control study ([Morabia et al., 1998](#)) and a case series ([Yoo et al., 1997](#)) have examined the association between quantitative measures of cigarette smoking and breast cancer risk according to estrogen receptor (ER) status. In one of the cohort studies ([Manjer et al., 2001](#)), a statistically significant increased risk (RR, 1.6) of ER negative tumours associated with current smoking was found but no clear association between smoking and ER positive tumours, and no difference in the association with progesterone receptor (PR)-positive and PR-negative tumours. In the other three studies

there was no clear difference in the association related to ER or PR receptor status.

2.12.4 Susceptible populations

More than 30 studies and meta-analyses ([Alberg et al., 2004](#); [Terry & Goodman, 2006](#); [Ambrosone et al., 2008](#); [Collishaw et al., 2009](#)) have evaluated whether a family history of breast cancer and/or inherited polymorphisms in various genes may confer greater susceptibility to develop breast cancer from exposure to tobacco smoke. These are described below in relation to the measure indicating potential susceptibility.

(a) Family history

In two studies, whether a family history of breast cancer modifies susceptibility to develop breast cancer from tobacco smoking has been examined. [Couch et al. \(2001\)](#) measured breast cancer incidence among female family members in a cohort of breast cancer cases diagnosed between 1944 and 1952 at the University of Minnesota. Sisters and daughters in families with at least three breast and/or ovarian cancers were at 2.4 fold higher risk for breast cancer (95%CI: 1.2–5.1) if they smoked compared to never-smokers. No dose-response was observed in relation to pack-years of smoking.

[Suzuki et al. \(2007\)](#) reported a statistically significant interaction between family history of breast cancer and smoking history in a hospital-based case-control study of 3861 breast cancer cases treated at a large cancer centre in Japan between 1988 and 2000. A family history of breast cancer in the absence of smoking was associated with a relative risk of 1.44 (95%CI: 1.21–1.71); the relative risk estimate was 1.95 (95%CI: 1.36–2.81) in women who reported < 30 pack-years of tobacco smoking, and 4.33 (95%CI: 1.65–11.40) in women who reported > 30 pack-years of smoking.

[The Working group noted that Japanese women who smoked during this time period

may have differed from never-smokers in other characteristics related to breast cancer. Besides its strong correlation with female smoking, “Westernization” might be associated with delayed childbearing, smaller families, higher body mass index, and greater use of post-menopausal hormones.]

(b) Genetic polymorphisms

Studies of breast cancer, smoking and low penetrance genetic polymorphisms are summarized in Table 2.63 (available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.63.pdf>). The candidate genes in these studies are involved in carcinogen metabolism [*N*-acetyltransferases (*NAT1*, *NAT2*), cytochrome P450s (*CYP1A1*, *CYP1B1*, *CYP2E2*), GSTs], host responses to oxidative stress (superoxide dismutase) or to infectious organisms (myeloperoxidase and immunoglobulin binding protein) and DNA repair (*O*⁶-methylguanine DNA methyltransferase, nucleotide excision repair).

The most consistent associations with breast cancer risk have been observed among long-term smokers with the *NAT2* slow acetylation genotype (Terry & Goodman, 2006). *NAT2* slow acetylation genotype is thought to confer less capability to detoxify tobacco smoke carcinogens and is associated with an increase in breast cancer risk (Ambrosone *et al.*, 1996, 2008). Approximately 50–60% of Caucasian women are reported to be slow acetylators.

Table 2.63 (online) lists 15 studies of polymorphisms in *NAT2*, of which 9 were included in a pooled analysis and 13 in a meta-analysis (Ambrosone *et al.*, 2008). [The study by Delfino *et al.* (2000) was excluded from these analyses because cases included women with benign breast disease; the study by Lilla *et al.* (2005) was not considered because it is based on the same population as that by Chang-Claude *et al.* (2002).] The meta-analysis found a statistically significant association between ever tobacco

smoking and breast cancer risk among women with the *NAT2* slow acetylator genotype (meta-RR, 1.27; 95%CI: 1.16–1.40) but not in those with rapid acetylator genotype (meta-RR, 1.05; 95%CI: 0.95–1.17). Pack-years of tobacco smoking was significantly associated with increasing breast cancer risk among women with *NAT2* slow acetylator genotype (meta-RR for ever smokers, 1.44; 95%CI: 1.23–1.68, for > 20 pack-years versus never smokers), but not among rapid acetylators (Ambrosone *et al.*, 2008). No main effect was seen between *NAT2* status and breast cancer risk (meta-RR, 1.0; 95%CI: 0.93–1.07). In contrast to an earlier meta-analysis (Alberg *et al.*, 2004), this study observed no difference in risk for pre- or post-menopausal breast cancer. The pooled analysis of nine studies (Ambrosone *et al.*, 2008) reported pooled risk estimates for pre- and post-menopausal women of 1.49 (95%CI: 1.08–2.04) and 1.42 (95%CI: 1.16–1.74), respectively, among women with slow *NAT2* genotype and at least 20 pack-years of smoking compared to never-smokers. The corresponding values for women with rapid acetylator genotype were 1.29 (95%CI: 0.89–1.86) and 0.88 (95%CI: 0.69–1.13). A statistically significant interaction was observed between pack-years of smoking as a continuous variable and *NAT2* genotype (*p* interaction = 0.03).

A population-based case-control study published after the meta-analysis by Ambrosone *et al.* compared the prevalence of the *NAT2* genotypes and their joint effect with smoking on breast cancer risk in Hispanic and non-Hispanic white women (Baumgartner *et al.*, 2009). Non-Hispanic white women were more likely (*P* < 0.001) than Hispanics to have a slow (41.7% versus 33.5%) or very slow (19.0% versus 11.1%) *NAT2* acetylator status. Breast cancer risk was significantly increased in non-Hispanic smoking white women with a very slow acetylator genotype (RR, 2.46; 95%CI: 1.07–5.65 for current versus never).

[The Working Group noted that publication bias remains a concern in the studies of *NAT2* published to date. All of the studies included in the meta-analysis by Ambrosone *et al.* were published between 1996 and 2006; some among them (Morabia *et al.*, 2000; Sillanpää *et al.*, 2007) reported very strong associations that seem inconsistent with the rest of the data. Because genetic studies often examine multiple genes, it is plausible that studies that find no main effect with *NAT2* have not examined this association or that null results for smoking have not been published.]

Fewer studies with less consistent findings have been published on polymorphisms in other genes such as *NAT1*, *CYP1A1*, *GST*, *NOS3*, *MPO*, *MnSOD2* and various DNA repair genes (Table 2.63 online).

2.12.5 High penetrance genes & prognosis

At least seven studies have examined the hypothesis that tobacco smoking may modify breast cancer risk among women who carry *BRCA1* and *BRCA2* mutations (Brunet *et al.*, 1998; Ghadirian *et al.*, 2004; Colilla *et al.*, 2006; Gronwald *et al.*, 2006; Nkondjock *et al.*, 2006; Breast Cancer Family Registry, 2008; Ginsburg *et al.*, 2009). The results have been inconsistent. A recent case-control study of women under age 50 years who were carriers of mutations in *BRCA1* or *BRCA2* reported increased risk for breast cancer associated with as little as five pack-years of smoking. Compared to non-smokers, the risk associated with five or more pack-years of smoking was 2.3 (95%CI: 1.6–3.5) for *BRCA1* mutation carriers and 2.6 (95%CI: 1.8–3.9) for *BRCA2* mutation carriers (Breast Cancer Family Registry, 2008). In contrast, six other studies reported no increased risk among *BRCA1* or *BRCA2* carriers who smoke. The Canadian Panel review (Collishaw *et al.*, 2009) postulated that the five previous studies (Brunet *et al.*, 1998; Ghadirian *et al.*, 2004; Colilla *et al.*,

2006; Gronwald *et al.*, 2006; Nkondjock *et al.*, 2006) may have failed to observe a relationship because they included prevalent cases. However, a sixth study published since the Canadian panel review is also negative (Ginsburg *et al.*, 2009).

2.13 Cancer of the cervix

The association between smoking and cervical cancer has been examined in many epidemiological studies over the past few decades.

Since the previous *IARC Monograph* (IARC, 2004a), additional epidemiological studies have been published. Study design and results of the case-control studies restricted to HPV positive women or that adjusted for HPV status are presented in Table 2.64 (available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.64.pdf>) and Table 2.65 (available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.65.pdf>). Cohort studies and pooled analyses are presented in Table 2.66 (available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.66.pdf>) and Table 2.67 (available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.67.pdf>), respectively. Table 2.68 (available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.68.pdf>) and Table 2.69 (available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.69.pdf>) present additional cohort studies and pooled analyses on tobacco smoking and cervical, cervical intraepithelia neoplasia and carcinoma in situ, with or without controlling for HPV status, respectively.

2.13.1 Dose-response relationship

A positive association between smoking and incidence of cervical squamous-cell carcinoma, which account for approximately 90% of all cervical cancers, has been shown consistently over several decades in many epidemiological studies

of various designs conducted across different geographic regions. Dose–response associations with smoking intensity and duration were noted in many of the studies where such associations were examined ([Berrington de González et al., 2004](#); [Appleby et al., 2006](#)). Conversely, no clear association was found among former smokers. For adenocarcinoma of the cervix, which usually account for less than 10% of the total of all types of cervical cancer, there appears to be no clear association with smoking ([Berrington de González et al., 2004](#)).

2.13.2 Interaction with HPV positivity

Epidemiological studies of smoking and cervical cancer increasingly have considered the effects of HPV infection, which is recognized as the main etiological factor for invasive and pre-invasive cervical neoplasia worldwide ([IARC, 1995, 2012b](#)). HPV infection has been considered not only with respect to possible effect modification ([Hellberg & Stendahl, 2005](#); [Gunnell et al., 2006](#)), but also to confounding, as both HPV infection and smoking habits are directly associated with number of sexual partners and other indications of high-risk sexual behaviours ([Sikström et al., 1995](#); [Wang et al., 2004](#); [Hellberg & Stendahl, 2005](#); [McIntyre-Seltman et al., 2005](#); [Syrjänen et al., 2007](#)). Although there have been exceptions ([Syrjänen et al., 2007](#)), recent studies have generally continued to show that statistical adjustment for the potential confounding effects of HPV infection, or restricting studies to women with high risk HPV infection ([Plummer et al., 2003](#)), does not appreciably alter the finding of a positive association or its magnitude ([McIntyre-Seltman et al., 2005](#); [Appleby et al., 2006](#); [Tolstrup et al., 2006](#); [Tsai et al., 2007](#); [Nishino et al., 2008](#); [Kapeu et al., 2009](#)).

Statistical adjustment for the potentially confounding effect of HPV infection was usually based on the measured presence of HPV DNA in cervical cells or anti-HPV serum antibodies

in multivariate analytical models; as noted above, studies have also restricted their analyses to HPV-positive cases and controls. As there is currently no reliable marker of persistent HPV infection, case–control studies based on a cross-sectional measurement of HPV cannot distinguish between transient and persistent infections ([Franco et al., 1999](#)). Tobacco smoking is suspected to facilitate acquisition or persistence of an HPV infection through a reduced number of Langerhans cells and CD4 lymphocytes, which are markers of local immune response in the cervix ([Vaccarella et al., 2008](#)). In addition, smoking may affect innate immunity ([Ferson et al., 1979](#)). Current smokers have been shown to have a slightly higher HPV prevalence than non-smokers in a broad range of world populations after adjustment for life-time number of sexual partners (OR, 1.18; 95%CI: 1.01–1.39) ([Vaccarella et al., 2008](#)). Studies have evaluated the effect of smoking on HPV persistence. One study shows lower probability of HPV clearance among ever smokers ([Giuliano et al., 2002](#)) but a few others found no relationship ([Molano et al., 2003](#); [Richardson et al., 2005](#)).

2.14 Cancer of the endometrium

2.14.1 Overview of studies

To date, at least 42 epidemiological studies have examined the association between smoking and endometrial cancer, 25 reviewed in the previous *IARC Monograph* ([IARC, 2004a](#)) and 17 published since then ([Petridou et al., 2002](#); [Folsom et al., 2003](#); [Furberg & Thune, 2003](#); [Newcomb & Trentham-Dietz, 2003](#); [Beral et al., 2005](#); [Matthews et al., 2005](#); [Viswanathan et al., 2005](#); [Okamura et al., 2006](#); [Strom et al., 2006](#); [Trentham-Dietz et al., 2006](#); [Weiss et al., 2006a](#); [Al-Zoughool et al., 2007](#); [Bjørge et al., 2007](#); [Lacey et al., 2007](#); [Loerbroks et al., 2007](#); [Setiawan et al., 2007](#); [Lindemann et al., 2008](#)). Study design and results of the additional studies

are presented separately for the case-control studies (Table 2.70 available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.70.pdf> and Table 2.71 available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.71.pdf>, respectively) and for the cohort studies (Table 2.72 available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.72.pdf> and Table 2.73 available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.73.pdf>, respectively).

(a) Cohort studies

The majority of the 13 cohort studies (Engeland *et al.*, 1996; Terry *et al.*, 1999, 2002b; Folsom *et al.*, 2003; Furberg & Thune 2003; Beral *et al.*, 2005; Viswanathan *et al.*, 2005; Al-Zoughool *et al.*, 2007; Bjørge *et al.*, 2007; Lacey *et al.*, 2007; Loerbroks *et al.*, 2007; Setiawan *et al.*, 2007; Lindemann *et al.*, 2008) suggest a decreased risk among current smokers, including the largest study with over 9000 cases (Bjørge *et al.*, 2007). In five of these studies quantitative smoking measures have been examined in relation to endometrial cancer risk (Terry *et al.*, 1999, 2002b; Viswanathan *et al.*, 2005; Al-Zoughool *et al.*, 2007; Loerbroks *et al.*, 2007). Of these, one (Terry *et al.*, 1999) found a 50% reduced risk among current smokers in the highest level of intensity (11 cigarettes per day or more) compared with non-smokers, but the number of cases was low and the confidence intervals correspondingly wide. A more recent and larger cohort study (Terry *et al.*, 2002b) found a statistically significant 40% reduced risk among current smokers of more than 20 cigarettes per day, but showed somewhat weaker and statistically non-significant reductions in risk with smoking of long duration or high cumulative consumption (i.e. pack-years). In contrast, the risk among former smokers was similar to that among never smokers. The largest of these studies generally showed decreasing risk of endometrial cancer with increasing

smoking intensity, duration, and pack-years of consumption (Viswanathan *et al.*, 2005). Three studies examined the association between time since smoking cessation and endometrial cancer risk. Two of these studies suggested a positive association with time since quitting (compared with non-smokers) (Viswanathan *et al.*, 2005; Loerbroks *et al.*, 2007), whereas one found no association (Terry *et al.*, 2002b).

(b) Case-control studies

The results of 17 population-based case-control studies (Smith *et al.*, 1984; Tyler *et al.*, 1985; Franks *et al.*, 1987; Elliott *et al.*, 1990; Rubin *et al.*, 1990; Brinton *et al.*, 1993; Goodman *et al.*, 1997; Shields *et al.*, 1999; Jain *et al.*, 2000; McCann *et al.*, 2000; Newcomer *et al.*, 2001; Weiderpass & Baron, 2001; Newcomb & Trentham-Dietz, 2003; Matthews *et al.*, 2005; Strom *et al.*, 2006; Trentham-Dietz *et al.*, 2006; Weiss *et al.*, 2006a), that have included between 46 and 1304 endometrial cancer cases, generally have shown reductions in risk among current smokers compared with never smokers (although the magnitude of the reduction in risk has varied somewhat); results among former smokers compared with never smokers were equally variable, albeit somewhat weaker overall. The results of eight hospital-based case-control studies (Kelsey *et al.*, 1982; Lesko *et al.*, 1985; Levi *et al.*, 1987; Stockwell & Lyman, 1987; Koumantaki *et al.*, 1989; Austin *et al.*, 1993; Petridou *et al.*, 2002; Okamura *et al.*, 2006), which included between 83 and 1374 endometrial cancer cases, are somewhat consistent with those of population-based studies. They showed moderate (e.g. 30–40%) reduction in risks among current compared with never smokers, and unaltered risks (or perhaps a small 10–20% reduction in risk) in former compared with never smokers. The largest of the hospital-based studies (Stockwell & Lyman, 1987), with 1374 cases and 3921 controls, found both former and current smokers to be at moderately (approximately 30%) reduced risk

of endometrial cancer. To date, six population-based case-control studies ([Tyler et al., 1985](#); [Lawrence et al., 1987, 1989](#); [Brinton et al., 1993](#); [Newcomer et al., 2001](#); [Weiderpass & Baron, 2001](#)) have examined quantitative measures of smoking in relation to endometrial cancer risk, generally showing inverse associations to be strongest among current smokers of high intensity or long duration.

2.14.2 Confounders

Whereas the majority of these studies adjusted their relative risk estimates for potentially confounding variables, such as BMI, HRT, parity, diabetes, and age at menopause, studies that did not adjust for these variables tended to show similar inverse associations. Within individual studies, statistical adjustment for the effects of BMI and other covariates often made little difference, although some attenuation of relative risk estimates has been noted ([Weiderpass & Baron, 2001](#); [Terry et al., 2002c](#)).

2.14.3 Effect modification

The association between smoking and endometrial cancer risk according to factors that are known determinants of endogenous hormone concentrations, and which may counteract or augment possible tobacco-related hormonal changes, have been examined in several studies. These factors include menopausal status, HRT and BMI. Effect modification can reflect true underlying differences in the association across strata (for example, if cigarette smoking acts to reduce or modify estrogen concentrations differently in one group compared with another), but can also reflect methodological factors, such as differences that occur by chance or through the varying prevalence of confounding variables.

(a) Menopausal status

Although endometrial cancer is rare among pre-menopausal women, several studies have examined the association between cigarette smoking and endometrial cancer risk according to menopausal status, because the effect of smoking (if any) might vary according to the underlying hormonal milieu. The studies have included two cohort studies ([Terry et al., 2002b](#); [Al-Zoughool et al., 2007](#)), five population-based case-control studies ([Smith et al., 1984](#); [Franks et al., 1987](#); [Lawrence et al., 1987](#); [Brinton et al., 1993](#); [Weiderpass & Baron, 2001](#)), and four hospital-based case-control studies ([Lesko et al., 1985](#); [Levi et al., 1987](#); [Stockwell & Lyman, 1987](#); [Koumantaki et al., 1989](#)). In all but one of these studies, a study of early stage endometrial cancer ([Lawrence et al., 1987](#)), the inverse association was (to varying degrees) stronger among post-menopausal than pre-menopausal women. Among pre-menopausal women, the relative risk estimates for cigarette smoking have been inconsistent, sometimes showing increased risks with certain measures of cigarette smoking ([Smith et al., 1984](#); [Stockwell & Lyman, 1987](#); [Koumantaki et al., 1989](#); [Brinton et al., 1993](#); [Al-Zoughool et al., 2007](#)), sometimes showing decreased risks ([Lawrence et al., 1987](#); [Levi et al., 1987](#); [Brinton et al., 1993](#); [Terry et al., 2002b](#)), and sometimes showing practically no association ([Lesko et al., 1985](#); [Weiderpass & Baron, 2001](#); [Al-Zoughool et al., 2007](#)). In analyses limited to post-menopausal women, on the other hand, all showed between 10% and 80% reduced risks of endometrial cancer with the various smoking measures.

(b) Hormone replacement therapy

Given the possibility that cigarette smoking affects hormone concentrations mostly among women who are taking HRT ([Jensen et al., 1985](#); [Jensen & Christiansen, 1988](#); [Cassidenti et al., 1990](#)), the inverse association between tobacco

smoking and endometrial cancer risk might be stronger among HRT users than among non-users. However, the results of studies that have examined the association between smoking and endometrial cancer risk according to HRT use have been equivocal ([Weiss *et al.*, 1980](#); [Franks *et al.*, 1987](#); [Lawrence *et al.*, 1987](#); [Levi *et al.*, 1987](#); [Terry *et al.*, 2002b](#); [Beral *et al.*, 2005](#)). Whereas in two studies ([Franks *et al.*, 1987](#); [Levi *et al.*, 1987](#)) a larger reduction in risk among smokers taking HRT than among smokers not taking HRT was observed, in two other studies ([Lawrence *et al.*, 1987](#); [Terry *et al.*, 2002b](#)) there was no difference in the association according to HRT status. A cohort study that examined associations only among women using HRT showed no clear association among users of continuous combined HRT and cyclic combined HRT, but some suggestion of increased risk among smokers who used tibolone (perhaps more clearly among former smokers) ([Beral *et al.*, 2005](#)). Thus, although effect modification by HRT status is biologically plausible, the available epidemiological evidence is equivocal.

(c) *Relative body weight*

Obesity is an established risk factor for endometrial cancer ([IARC, 2002](#)). Smokers tend to have a lower BMI than non-smokers, although former smokers tend to have a higher BMI than current or never smokers ([Baron *et al.*, 1990](#)). Two case-control studies have examined the association between cigarette smoking and endometrial cancer risk according to BMI, one population-based ([Elliott *et al.*, 1990](#)) and one hospital-based ([Levi *et al.*, 1987](#)). Neither of these studies found clear differences in the association between smoking and endometrial cancer risk according to BMI. In a population-based case-control study of early stage endometrial cancer ([Lawrence *et al.*, 1987](#)), the inverse association with cigarette smoking tended to become stronger with increasing absolute rather than relative body weight.

2.14.4 *Gene polymorphisms*

Cigarette smoking and estrogen are both thought to influence cancer risk through pathways that are under the control of specific genes, such as those involved in the formation of bulky DNA adducts by estrogen metabolites ([Cavalieri *et al.*, 2000](#)) and both bulky and non-bulky adducts formed by carcinogens in tobacco smoke ([Terry & Rohan, 2002](#)). Therefore, studies have been conducted to examine the association between smoking and endometrial cancer risk according to genes that repair these types of DNA damage. In a moderately-sized population-based case-control study no clear effect modification according to certain polymorphisms in the *XPA* and *XPC* genes, both of which are involved in the nucleotide excision repair of bulky DNA adducts and may influence endometrial cancer risk, were found ([Weiss *et al.*, 2005, 2006b](#)). A nested case-control study also showed no clear effect modification according to three polymorphisms in *CYP1A1* ([McGrath *et al.*, 2007](#)), a gene that encodes microsomal *CYP1A1*, which contributes to aryl hydrocarbon hydroxylase activity, catalysing the metabolism of PAHs and other carcinogens found in tobacco smoke ([Masson *et al.*, 2005](#)). In another nested case-control study some evidence was found that the association between smoking and endometrial cancer may vary according to a polymorphism (Ile¹⁴³Val) in *O*⁶-methylguanine DNA methyltransferase (*MGMT*). Overall, studies that address the association between smoking and endometrial cancer risk according to genotype are scarce.

2.15 Cancer of the prostate

Many epidemiological studies have examined the association between cigarette smoking and prostate cancer risk, and most have shown no consistent association ([Hickey *et al.*, 2001](#); [Levi & La Vecchia, 2001](#); [Batty *et al.*, 2008](#); [Butler](#)

et al., 2009; *Huncharek et al.*, 2010; Table 2.74 available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.74.pdf>; Table 2.75 available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.75.pdf>). However, questions remain regarding whether smoking may alter risk in various population subgroups, for example, those defined by certain genotypes, and whether any association with smoking may be stronger for, or limited to, advanced tumours or prostate cancer mortality. Regarding this latter issue, the majority of epidemiological studies, including several large, long-term cohort studies, have reported a positive association between smoking and prostate cancer mortality (*Rohrmann et al.*, 2007; *Zu & Giovannucci*, 2009). Several studies that examined smoking in relation to both prostate cancer incidence and mortality tend to show positive results only for the latter (*Rohrmann et al.*, 2007; *Zu & Giovannucci*, 2009). Given the largely null results with respect to prostate cancer incidence, the latter findings suggest that smoking is less likely to be a causal agent in prostate cancer initiation than an agent that acts on existing tumours to promote their progression (*Zu & Giovannucci*, 2009).

A recent review of smoking and prostate cancer that focused specifically on aggressive and fatal tumours, considered the findings from 14 cohort studies (*Zu & Giovannucci*, 2009). Nine of these studies showed statistically significant increased risk with at least one smoking measure, and five showed increased risks that were not statistically significant for any measure. Only one study showed no association with any measure of tobacco consumption. Seven studies of various designs examined smoking with respect to indicators of cancer aggressive behaviour at the time of diagnosis. In these studies smoking was associated positively with tumour grade, risk of regional, distant, extraprostatic or metastatic disease, Gleason score, and biochemical outcome (failure) after prostate brachytherapy

and in several dose–response associations with the respective endpoint were demonstrated. In one study smoking cessation was associated with a decline in risk compared with that among current smokers.

The association between smoking and prostate cancer risk according to genotype and other potentially effect-modifying factors have been examined in several studies. For example, in a population-based case–control study tobacco use was a risk factor for prostate cancer primarily among men with high BMI (*Sharpe & Siemiatycki*, 2001). The results of a cohort study in Switzerland suggest that risk of prostate cancer mortality is increased in smokers, particularly those with low plasma vitamin E levels (*Eichholzer et al.*, 1999). These latter associations, as well as those regarding several genotypes that may modify the association (*Mao et al.*, 2004; *Nock et al.*, 2006; *Quiñones et al.*, 2006; *Yang et al.*, 2006; *Iguchi et al.*, 2009; *Kesarwani et al.*, 2009), have yet to be fully clarified.

[The Working Group noted that several of the studies of smoking and prostate cancer mortality did not demonstrate clear dose–response associations with risk, and noted the possibility of bias due to confounding by screening behaviour. However, in the Health Professionals Follow-up Study, screening behaviour was not found to differ appreciably between smokers and non-smokers. In an analysis limited to men with a negative digital rectal examination in the prior two years, stronger associations were found between smoking and metastatic prostate cancer risk among high intensity smokers (RR, 4.2; 95%CI: 1.6–10.9) (*Zu & Giovannucci*, 2009). This finding was evidence against bias from screening behaviour.]

2.16 Cancer of the ovary

2.16.1 Overview of studies

A total of over 30 epidemiological studies have investigated the association between tobacco smoking and ovarian cancer risk. Of these, 24 were case-control studies (IARC, 2004a; Table 2.76 available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.76.pdf>; Table 2.77 available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.77.pdf>) and six were cohort studies (IARC, 2004a; Table 2.78 available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.78.pdf>; Table 2.79 available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.79.pdf>). Most studies showed no statistically significant association between a measure of smoking and risk for ovarian cancer overall (Newhouse *et al.*, 1977; Smith *et al.*, 1984; Tzonou *et al.*, 1984; Baron *et al.*, 1986; Stockwell & Lyman, 1987; Whittemore *et al.*, 1988; Hartge *et al.*, 1989; Polychronopoulou *et al.*, 1993; Engeland *et al.*, 1996; Goodman *et al.*, 2001; Goodman & Tung, 2003; Pan *et al.*, 2004; Zhang *et al.*, 2004; Kurian *et al.*, 2005; Niwa *et al.*, 2005; Baker *et al.*, 2006; Huusom *et al.*, 2006; Fujita *et al.*, 2008; Lurie *et al.*, 2008; Nagle *et al.*, 2008; Tworoger *et al.*, 2008); some showed positive associations (Doll *et al.*, 1980; Tverdal *et al.*, 1993; Kuper *et al.*, 2000b; Marchbanks *et al.*, 2000; Green *et al.*, 2001; Modugno *et al.*, 2002; Gram *et al.*, 2008; Rossing *et al.*, 2008) and one (Riman *et al.*, 2004) showed an inverse association.

2.16.2 Histological subtypes

Differences in ovarian cancer risk factor profiles have been observed according to histological type, on the basis of which it has been suggested that mucinous and non-mucinous tumours are etiologically distinct diseases (Risch *et al.*, 1996). Epidemiological studies that have considered histological type tend to support a

positive association primarily between cigarette smoking and mucinous ovarian tumours (Kuper *et al.*, 2000b; Marchbanks *et al.*, 2000; Green *et al.*, 2001; Modugno *et al.*, 2002; Pan *et al.*, 2004; Zhang *et al.*, 2004; Kurian *et al.*, 2005; Tworoger *et al.*, 2008). In contrast, two studies showed no clear association between smoking and risk of mucinous or non-mucinous ovarian tumours (Riman *et al.*, 2004; Baker *et al.*, 2006). In addition, one early case-control study (Newhouse *et al.*, 1977), with 300 ovarian cancer cases and with both population and hospital controls, found no clear association with “ever” compared with “never” smoking, and reported no differences according to histological type.

A pooled analysis of 10 case-control studies (Kurian *et al.*, 2005) with 254 cases of mucinous and 1580 non-mucinous tumours found an increased risk of mucinous tumours among current smokers (RR, 2.4; 95%CI: 1.5–3.8), a positive association that was not observed with other histological types. Former smokers in that analysis did not have an increased risk of any histological type of ovarian cancer. This type of dose-response, whereby current smokers have a higher risk than former smokers, was observed in most, but not all, studies of mucinous ovarian cancer (Tables 2.77 and 2.79 online). Overall, the positive association between cigarette smoking and risk of mucinous ovarian tumours is generally consistent across both case-control and cohort studies conducted among various populations. In contrast, associations with smoking have been mostly null with respect to non-mucinous ovarian tumours, suggesting that recall bias is unlikely to explain the association with mucinous tumours.

[The Working Group considered the possibility that women who smoke may come to medical attention more frequently. This raises the possibility of detection bias, because mucinous tumours, benign or malignant, tend to be quite large and could be more easily detected on routine physical exam or testing. However, the

Working Group felt that detection bias would not account for the association entirely.

2.17 Cancer of the thyroid

The previous *IARC Monograph* ([IARC, 2004a](#)) noted inconsistent associations between smoking and thyroid cancer risk. In 2003, a pooled analysis of 14 case–control studies showed that smoking was inversely associated with thyroid cancer risk ([Mack et al., 2003](#); Table 2.80 available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.80.pdf>; Table 2.81 available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.81.pdf>). The sample consisted of 2725 thyroid cancer cases (2247 women, 478 men) and 4776 controls (3699 women, 1077 men). The inverse association was stronger among current smokers (RR, 0.6; 9% CI: 0.6–0.7) than former smokers (RR, 0.9; 9% CI: 0.8–1.1) and were similar in both men and women, for both papillary and follicular thyroid cancers, as well as by age and region. An inverse association between smoking and thyroid cancer risk was also found in a subsequent case–control study ([Nagano et al., 2007](#)). In contrast, two case–control studies ([Zivaljevic et al., 2004](#); [Bufalo et al., 2006](#)) reported no clear association between smoking and thyroid cancer risk (no risk ratio estimates were reported; hence, data are not shown in the tables) and a cohort study with 169 incident cases of thyroid cancer, also found no clear association with any qualitative or quantitative smoking measure ([Navarro Silvera et al., 2005](#); Table 2.82 available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.82.pdf>; Table 2.83 available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.83.pdf>).

2.18 Other cancers

The cancers reviewed in this section generally have low incidence and mortality rates and are not considered to be strongly associated with cigarette smoking. This raises the possibility of preferential reporting of positive associations in epidemiological studies.

2.18.1 Cancer of the salivary gland

Studies of smoking and cancers of the salivary gland reviewed in the previous *IARC Monograph* ([IARC, 2004a](#)) were sparse and their results were inconsistent ([Spitz et al., 1990](#); [Swanson & Burns, 1997](#); [Hayes et al., 1999](#)). A few additional studies also show inconsistent results ([Kotwall, 1992](#); [Pinkston & Cole, 1996](#); [Horn-Ross et al., 1997](#); [Vories & Ramirez, 1997](#); [Muscat & Wynder, 1998](#)). Studies that focused specifically on Warthin's tumour [papillary cystadenoma lymphomatosum or adenolymphoma, a benign tumour of the parotid gland] tend to show strong positive associations with smoking ([Kotwall, 1992](#); [Pinkston & Cole, 1996](#); [Vories & Ramirez, 1997](#)). One study ([Pinkston & Cole, 1996](#)) compared the risk for Warthin's tumour with that for other salivary gland tumours and found that smoking increased risk significantly only for Warthin's tumour.

2.18.2 Cancer of the small intestine

Epidemiological studies (all of case–control design) reviewed in the previous *IARC Monograph* ([IARC, 2004a](#)) have been inconsistent in showing a positive association between smoking and cancers of the small intestine ([Chow et al., 1993b](#); [Chen et al., 1994](#); [Wu et al., 1997](#); [Negri et al., 1999](#); [Kaerlev et al., 2002](#)). A more recent study showed no clear association ([Hassan et al., 2008b](#)).

2.18.3 Cancers of the gallbladder and extra-hepatic bile ducts

Epidemiological studies of smoking and risk of cancers of the gallbladder and extra-hepatic bile ducts reviewed in the previous *IARC Monograph* ([IARC, 2004a](#)) tended to show null, weak, or moderately strong positive associations. More recent studies also tend to show either no clear association with biliary tract carcinoma/extra-hepatic cholangiocarcinoma ([Shaib et al., 2007](#); [Welzel et al., 2007](#)) or suggest positive associations with gallbladder/biliary cancers ([Pandey & Shukla, 2003](#); [Yagyu et al., 2008](#); [Grainge et al., 2009](#)). Attention should be paid to potential confounders, especially BMI, when considering the results of epidemiological studies of risk of cancers of the gallbladder and extra-hepatic bile ducts. Recent studies that statistically adjusted for BMI, on gallbladder disease risk ([Grainge et al., 2009](#)) or on extrahepatic biliary tract carcinoma risk ([Ahrens et al., 2007](#)), showed a positive and null association with smoking, respectively. To date, there are too few studies with adequate control for potentially confounding factors to determine any clear pattern.

2.18.4 Soft-tissue sarcoma

As reported in the previous *IARC Monograph* ([IARC, 2004a](#)), one cohort study found an association between cigarette smoking and mortality from soft-tissue sarcoma after 26 years of follow-up but no dose-response relationship with the number of cigarettes/day, duration of smoking or pack-years ([Zahm et al., 1992](#)). No effect of cigarette smoking was detected in an Italian hospital-based case-control study ([Franceschi & Serraino, 1992](#)).

2.18.5 Cancer of the skin

(a) Melanoma

Several case-control studies found no difference in the prevalence of tobacco smoking between patients with malignant melanoma and controls, and one study found an inverse association ([IARC, 2004a](#)). An inverse association with smoking was also found in the US Radiologic Technologists cohort Study ([Freedman et al., 2003a](#)). In that study, smoking for at least 30 years compared with never smoking was inversely related to melanoma risk (RR, 0.6; 95%CI: 0.3–1.3), though risk was not associated with number of cigarettes/day. An inverse association was also observed in a cohort of Swedish construction workers ([Odenbro et al., 2007](#)). In this study, the risk for malignant melanoma was reduced in a dose-dependant manner for both cigarette and pipe smokers. The possibility that smoking may reduce the risk for melanoma should, therefore, be considered.

(b) Non-melanoma skin cancer

Four studies showed a positive association between smoking and non-melanoma skin cancer risk ([De Stefani et al., 1995](#); [Wojno, 1999](#); [Smith & Randle, 2001](#); [Boyd et al., 2002](#)), and two found no clear association ([van Dam et al., 1999](#); [Corona et al., 2001](#)). When distinguishing between histological subtypes, tobacco smoking was linked to the incidence of squamous-cell carcinoma of the skin in most studies, whereas the results for basal cell carcinoma remain inconsistent ([Zak-Prelich et al., 2004](#)). No clear association between smoking and risk for basal cell carcinoma was found in a cohort study ([Freedman et al., 2003b](#)).

2.18.6 Cancer of the penis

Case-control studies of smoking and penile cancer ([Hellberg et al., 1987](#); [Daling et al., 1992, 2005](#); [Maden et al., 1993](#); [Harish & Ravi, 1995](#);

Table 2.84 available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.84.pdf>; Table 2.85 available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.85.pdf>) and reviews of studies of smoking and penile cancer and population surveys (Dillner *et al.*, 2000; Favorito *et al.*, 2008; Bleeker *et al.*, 2009; Table 2.86 available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.86.pdf>; Table 2.87 available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.87.pdf>) consistently showed a positive association. In most studies there was a dose–response relationship, with higher risks among those with increased smoking intensity and/or duration. A study in Brazil showed a positive correlation with penile tumour grade (Favorito *et al.*, 2008). Based on the two reviews (Dillner *et al.*, 2000; Bleeker *et al.*, 2009), relative risks were generally increased twofold to fivefold among smokers.

Most studies did not adjust for HPV infection. In one case–control study (Daling *et al.*, 2005), current smoking was associated with a 160% increased risk of HPV-positive penile cancer ($n = 75$), and a 180% increased risk of HPV-negative penile cancer ($n = 19$), suggesting no important effect modification.

2.18.7 Cancer of the testis

Studies reviewed in the previous IARC Monograph (IARC, 2004a) showed no association between cigarette smoking and risk for testicular cancer. More recently, two case–control studies showed positive associations with smoking, one in Canada (Srivastava & Kreiger, 2004) and one in the Czech Republic (Dusek *et al.*, 2008).

2.18.8 Cancer of the central nervous system

A recent meta-analysis was conducted on smoking in relation to glioma risk (Mandelzweig *et al.*, 2009), which included 17 epidemiological

studies (6 cohort and 11 case–control). It was concluded that smoking is not associated with risk of glioma, despite a small significant increased risk seen in cohort studies. A recent cohort study found no association between smoking and carcinoma of the brain (Batty *et al.*, 2008). There have been no consistent associations of smoking with other CNS tumours (IARC, 2004a). In a population-based case–control study in the USA, smoking was associated with increased risk of intracranial meningioma in men (OR, 2.1; 95%CI: 1.1–4.2) but not in women (Phillips *et al.*, 2005).

2.18.9 Cancer of the adrenal gland

Data on risk factors for adrenal carcinoma are sparse. In the US Veterans' Study there was a fivefold increase in risk among current cigarette smokers during 26 years of follow-up, with risk being particularly high among those who smoked most intensely (Chow *et al.*, 1996). Other forms of tobacco use were associated with a statistically non-significant increase in risk. A case–control study in the USA found a twofold increase in risk for adrenal cancer among heavy smokers in men, but not in women (Hsing *et al.*, 1996).

2.19 Bidi smoking

2.19.1 Cancer of the oral cavity

(a) Overview of studies

The association between cancers of oral cavity and bidi smoking has been examined in 10 case–control studies conducted in India (Sankaranarayanan *et al.*, 1989a, b, 1990a; Rao *et al.*, 1994; Rao & Desai, 1998; Dikshit & Kanhere, 2000; Balaram *et al.*, 2002; Znaor *et al.*, 2003; Subapriya *et al.*, 2007; Muwonge *et al.*, 2008; Table 2.88 available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.88.pdf>). In these studies both cases and controls were interviewed and analyses were restricted

to men, except for the studies by [Balaram *et al.* \(2002\)](#) and [Subapriya *et al.* \(2007\)](#), because very few women smoked among study subjects.

Three hospital-based case-control studies considered cancers of subsites of the oral cavity (gingiva, tongue and floor of the mouth, buccal and labial mucosa) ([Sankaranarayanan *et al.*, 1989a, b, 1990a](#)). All three studies showed a higher oral cancer risk for bidi smoking. In one early study an unadjusted relative risk of 1.6 (95%CI: 1.3–2.0) for oral cancer in bidi smokers was reported ([Rao *et al.*, 1994](#)). [The Working Group noted that the study had several deficiencies, particularly in the selection of controls that resulted in cigarette smoking apparently being protective for oral cancer.] In another early study ([Rao & Desai, 1998](#)) relative risks were estimated after stratification by age and place of residence. Bidi smoking was a significant risk factor for cancer of the base of the tongue (RR, 5.9; 95%CI: 4.2–8.2) but not significant for cancer of the anterior tongue. Relative risk for bidi smoking adjusted for alcohol drinking, illiteracy, non-vegetarian diet and tobacco chewing showed significant risk for cancer of the base of the tongue (RR, 4.7; 95%CI: 3.5–6.3) but not for cancer of the anterior tongue. In a population-based case-control study a relative risk of 1.5 (95%CI: 0.9–2.4), adjusted for age and tobacco quid chewing for smokers (bidis and/or cigarettes), was found ([Dikshit & Kanhere, 2000](#)).

Two hospital-based multi centre case-control studies on cancer of the oral cavity were conducted in southern India. One included 309 cases and 292 controls ([Balaram *et al.*, 2002](#)). The risk for oral cavity cancer among those who smoked < 20 bidis per day was 2.0 (95%CI: 1.1–3.8) and 2.5 (95%CI: 1.4–4.4) for ≥ 20 per day. The second study included 1563 cases and 3638 controls and found a risk for bidi smoking only of 2.2 (95%CI: 1.75–2.63) compared to never smokers, adjusted for age, centre, level of education, alcohol consumption and chewing ([Znaor *et al.*, 2003](#)).

In a hospital-based case-control study with 388 oral squamous cell carcinoma cases (202 men and 186 women) and an equal number of age and sex-matched controls the effect of life-style factors (tobacco chewing, smoking and alcohol drinking, diet and dental care) on the risk of oral cancer was evaluated ([Subapriya *et al.*, 2007](#)). Both cases and controls were interviewed using a structured questionnaire. The risk estimate for bidi smoking based on 22 cases (84 cases included in the model) and 22 controls was 4.6 (95%CI not given).

Data from a randomized control trial conducted between 1996 and 2004 in Trivandrum, southern India were used in a nested case-control analysis with 282 (163 men and 119 women) incident oral cancer cases and 1410 matched population controls aged 35 years and over ([Muwonge *et al.*, 2008](#)). Oral cancer risk among men, adjusted for education and religion, was 1.9 (95%CI: 1.1–3.2) for bidi smokers only compared to never smokers. No association was found between mixed smoking of bidi and cigarette and risk of oral cancer.

[Rahman *et al.* \(2003\)](#) performed a meta-analysis to investigate the relationship between bidi smoking and oral cancer. They identified 12 case-control studies published in English during 1996–2002 with quantitative information on bidi smoking and oral cancer. Of these, ten studies were conducted in India, one in Sri Lanka and one in Pakistan. All cases were confirmed histologically and exposure data were collected by direct interview. In these studies ORs were not adjusted for tobacco chewing or alcohol drinking. The OR for bidi smokers compared to never smokers based on random effects model was 3.1 (95%CI: 2.0–5.0). The ORs ranged from 2.0 to 3.6 in different regions of India: studies conducted in Mumbai had an OR of 3.6 (95%CI: 1.6–7.9), in central India 2.7 (95%CI: 1.6–4.6), in Kerala 2.0 (95%CI: 1.5–2.9) and in Bangalore 2.0 (95%CI: 1.1–3.7).

(b) Dose–response evidence

The trends in relative risks by intensity and duration of bidi smoking were both statistically significant in two studies ([Rao et al., 1994](#); [Rao & Desai, 1998](#)). A meta-analysis based on three studies on duration of bidi smoking and on five studies on number of bidi sticks per day, showed a dose–response relationship for duration of bidi smoking but not for number of sticks used per day ([Rahman et al., 2003](#)).

In a nested case–control analysis ([Muwonge et al., 2008](#)) a dose–response relationship was observed for duration of bidi smoking ($P = 0.045$). [It is not clear if the analysis was restricted to bidi smokers only ($n = 40$ men) and if smokers with combined smoking habits (bidi and cigarette) were excluded. Moreover, ORs for the dose–response analysis were not reported.]

2.19.2 Cancer of the pharynx

Five case–control studies, two hospital-based ([Wasnik et al., 1998](#); [Rao et al., 1999](#)), one population-based ([Dikshit & Kanhere, 2000](#)) and two multicentric studies ([Znaor et al., 2003](#); [Sapkota et al., 2007](#)) were conducted on cancers of oropharynx and hypopharynx in India (Table 2.88 online). In all these studies, analyses were restricted to men because very few women smoked among study subjects.

[Wasnik et al. \(1998\)](#) conducted a case–control study on oropharyngeal cancers with cases and controls were matched on age and sex. Odds ratios for tobacco smoking, predominantly in the form of bidi and/or chillum, were 2.3 (95%CI: 1.2–3.7) after adjustment for tobacco chewing and outdoor occupation. [The Working Group noted some problems with the data analysis.]

[Rao et al. \(1999\)](#) reported a relative risk for bidi smoking adjusted for alcohol, illiteracy, diet and tobacco chewing of 4.7 (3.6–6.3) for oropharyngeal cancer and of 2.8 (2.1–3.7) for cancer of the hypopharynx. [Dikshit & Kanhere \(2000\)](#) found

an odds ratio for oropharyngeal cancer among bidi smokers only of 7.9 (95%CI: 5.1–12.4).

[Znaor et al. \(2003\)](#) reported a risk for bidi smoking only for pharyngeal cancer of 4.7 (95%CI: 3.5–6.3) and for combined bidi and cigarette smoking of 3.6 (95%CI: 2.55–4.98). [Sapkota et al. \(2007\)](#) reported an odds ratio for hypopharyngeal cancer of 6.8 (95%CI: 4.6–10.0) for bidi smokers compared to never smokers.

A dose–response relationship was observed for intensity and duration of bidi smoking for both cancers of oropharynx and hypopharynx ([Rao et al., 1999](#); [Dikshit & Kanhere, 2000](#); [Sapkota et al., 2007](#)).

2.19.3 Cancer of the lung

One cohort study ([Jayalekshmy et al., 2008](#)), one population-based case–control study ([Dikshit & Kanhere, 2000](#)) and two hospital-based case–control studies ([Gupta et al., 2001](#); [Gajalakshmi et al., 2003](#)) in India (Table 2.88 online) have investigated the relationship between bidi smoking and lung cancer. In all these studies both cases and controls were interviewed and analyses were restricted to men because very few women smoked among study subjects. One hospital-based case–control study in Chiang Mai, Thailand, looked at the association between lung cancer and *khii yoo*, hand-rolled cigars. The risk for lung cancer for *khii yoo* smoking was 1.2 in men and 1.5 in women, $P > 0.05$ ([Simarak et al., 1977](#)).

In the population based case–control study by [Dikshit & Kanhere \(2000\)](#) the age-adjusted relative risk for lung cancer among bidi smokers only was 11.6 (95%CI: 6.4–21.3).

[Gupta et al. \(2001\)](#) reported an odds ratio for bidi smoking of 5.8 (95%CI: 3.4–9.7) from a hospital-based case–control study of lung cancer conducted in Chandigarh. [Gajalakshmi et al. \(2003\)](#) conducted a case–control study in two centres in which all subjects were interviewed by trained social investigators with standard

questionnaires. Odds ratios were adjusted for age, educational level, centre, chewing and alcohol habit. The odds ratios of lung cancer for former and current bidi smokers were 3.4 (95%CI: 2.1–5.4) and 5.3 (95%CI: 3.8–7.3), respectively. Odds ratios for former and current smokers of cigarette and bidi combined were 4.0 (95%CI: 2.5–6.6) and 9.1 (95%CI: 6.2–13.2), respectively.

Baseline data of a cohort of 359 619 residents in Kerala, India was collected by direct interview using standardized questionnaires during 1990–97 ([Jayalekshmy et al., 2008](#)). After excluding rare earth workers, those who died, were diagnosed with cancer before 1997 or died within three years of interview, there were 65 829 bidi-smoking men aged 30–84 years old. Two hundred and twelve lung cancer cases were identified by the Karunagappally Cancer Registry between 1997 and 2004. The relative risk for lung cancer for current compared to never bidi smokers calculated by Poisson regression analysis and adjusted for age, religion and education was 3.9 (95%CI: 2.6–6.0; $P < 0.001$). The risk was lower among former than among current smokers.

(a) Dose–response evidence

Lung cancer risks increased with increasing bidi smoking intensities. The highest odds ratio was found for 9 pack-years (3.9; 95%CI: 2.1–7.1) ([Gupta et al., 2001](#)). In a cohort study [Jayalekshmy et al. \(2008\)](#) found increased lung cancer incidence with increasing number of bidi sticks smoked per day ($P < 0.001$) and with increasing duration of bidi smoking ($P < 0.001$). [The number of lung cancer cases was small in each category, resulting in wide confidence intervals.] [Gajalakshmi et al. \(2003\)](#) also reported increased risk with duration and intensity of bidi smoking.

(b) Cessation of smoking

In two case–control studies ([Gupta et al., 2001](#); [Gajalakshmi et al., 2003](#)) there was a clear decreasing trend in risk for years since quitting.

[Gajalakshmi et al. \(2003\)](#) reported that lung cancer risk of former bidi smokers fell to 0.4 (0.1–1.2) after quitting for more than 15 years. The cohort study conducted in Kerala did not have the power to assess the risk associated with stopping bidi smoking ([Jayalekshmy et al., 2008](#)).

2.19.4 Cancer of the larynx

Two hospital based case–control studies ([Sankaranarayanan et al., 1990b](#); [Rao et al., 1999](#)) showed a higher risk for bidi smokers (Table 2.88 online). The relative risk was adjusted for age and religion in [Sankaranarayanan et al. \(1990b\)](#) study and for alcohol use, illiteracy, vegetarian/non-vegetarian diet and tobacco chewing in [Rao et al. \(1999\)](#) study. A multicentre case–control study on laryngeal cancer was conducted in four Indian centres using standardized questionnaires adjusting risks for centre, age, socio-economic status, alcohol consumption, tobacco snuffing and tobacco chewing ([Sapkota et al., 2007](#)). Compared to never smokers bidi smokers had a higher risk for cancers of the supraglottis (OR, 7.5; 95%CI: 3.8–14.7), glottis (OR, 5.3; 95%CI: 3.2–8.9) and rest of larynx (OR, 9.6; 95%CI: 5.6–16.4).

All levels of intensity and duration of bidi smoking were associated with significant relative risk estimates and dose–response for laryngeal cancer ([Sankaranarayanan et al., 1990b](#); [Rao et al., 1999](#)). A strong dose–response relationship was observed for duration and frequency of bidi smoking for cancers of supraglottis, glottis and rest of larynx ([Sapkota et al., 2007](#)).

2.19.5 Cancer of the oesophagus

Three hospital-based case–control studies and one multicentre study ([Sankaranarayanan et al., 1991](#); [Nandakumar et al., 1996](#); [Nayar et al., 2000](#); [Znaor et al., 2003](#)) showed increased risk for oesophageal cancer among bidi smokers in India (Table 2.88 online). A significantly elevated

risk for all three segments of the oesophagus was reported (Nandakumar *et al.*, 1996). One study (Nayar *et al.*, 2000) adjusted for chewing of betel leaf with tobacco and low consumption of vegetables other than leafy vegetables. The multicentre case–control study conducted in two centres in South India found an increased risk for oesophageal cancer for bidi smoking only (OR, 3.3; 95%CI: 2.45–4.39) (Znaor *et al.*, 2003). Odds ratios were adjusted for age, centre, level of education, alcohol consumption and chewing. Only men were analysed in all the above studies.

Significant effects were noted in men for all levels of intensity and for duration of more than 20 years of bidi smoking (Sankaranarayanan *et al.*, 1991).

2.19.6 Cancer of the stomach

In a hospital-based case–control study the association between stomach cancer and bidi smoking was analysed as part of a multicentre study (Gajalakshmi & Shanta, 1996). Cases and controls were matched on age, sex, religion and mother tongue. The odds ratio for stomach cancer for current bidi smokers only was 3.2 (95%CI: 1.8–5.7) and for current smokers of any type of tobacco was 2.7 (95%CI: 1.8–4.1).

Table 2.88 (online) summarizes the studies published since the last IARC Monograph (IARC, 2004a). A hospital-based case–control study of stomach cancer included 170 stomach cancer cases (121 men and 49 women) and 2184 controls (1309 men and 875 women) aged 30–75 years (Rao *et al.*, 2002). The association between bidi smoking and stomach cancer was not significant (RR, 0.8; 95%CI: 0.5–1.2) in a univariate analysis. The risk increased with increase in lifetime exposure to bidi smoking and was highly significant ($P < 0.001$).

One study investigated stomach cancer risk in association with smoking of *meiziol*, a local cigarette in Mizoram, India (Phukan *et al.*, 2005). Statistically significant higher risks were seen for

smokers of combined users of tobacco (cigarette and *meiziol*), with an odds ratio of 3.1 (95%CI: 2.0–11.1). Among users of a single type of tobacco, higher risks were seen for *meiziol* smokers (OR, 2.2; 95%CI: 1.3–9.3) in the multivariate model in comparison to cigarette smokers. Overall, the excess risk was limited to smokers of > 10 *meiziols* per day.

2.20 Synergistic effects of tobacco smoking and alcohol drinking

This section addresses the combined effects of smoking and alcohol consumption on cancers of oral cavity, pharynx, larynx and oesophagus, which have been examined extensively. For the purposes of this report interdependence of effects is termed *effect modification*, and *synergism* and *antagonism* are used to describe the consequences of the interdependence of disease risk when both risk factors are present (Rothman & Greenland, 1998). The studies varied in their methods and in the approaches used to assess effect modification, which ranged from descriptive to formal estimation of interaction terms in multivariate models. Study designs of the case–control and cohort studies are presented in Table 2.89 (available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.89.pdf>) and Table 2.90 (available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.90.pdf>), respectively; and the results for both study designs are presented in Table 2.91 (available at <http://monographs.iarc.fr/ENG/Monographs/vol100E/100E-01-Table2.91.pdf>).

2.20.1 Cancers of the upper aerodigestive tract

It was noted in the previous IARC Monograph (IARC, 2004a) with relatively large numbers of cases and controls that the pattern of increasing cancer risk with increasing alcohol consumption is strong (Mashberg *et al.*, 1993; Kabat *et al.*, 1994).

For cancers of the oral cavity, recent evidence comes from seven case-control studies and one cohort study. The pattern of odds ratios for smoking, across categories of alcohol consumption, is consistent with synergism. In four case-control studies with relatively large numbers of cases and controls (more than 200 cases and equivalent number of controls), the pattern of increasing cancer risks with increasing alcohol consumption was strong ([Schlecht *et al.*, 1999b](#); [Znaor *et al.*, 2003](#); [Castellsagué *et al.*, 2004](#); [Hashibe *et al.*, 2009](#)). In the cohort study from Taiwan, China ([Yen *et al.*, 2008](#)) similar strong risks were also observed. In all four case-control studies in which the estimate of formal statistical interaction was examined, the tests were statistically significant ([Schlecht *et al.*, 1999b](#); [Znaor *et al.*, 2003](#); [Castellsagué *et al.*, 2004](#); [Hashibe *et al.*, 2009](#)). In two case-control studies from India ([Znaor *et al.*, 2003](#); [Muwonge *et al.*, 2008](#)) and in the cohort study from Taiwan, China ([Yen *et al.*, 2008](#)) the interaction of tobacco smoking, alcohol and betel quid chewing was examined. In general, the results suggested increasing risks when betel quid chewing was included in the model.

Five case-control studies and one cohort study examined the effect of interaction between tobacco and alcohol in pharyngeal cancer. The results from case-control studies were similar to those observed for oral cancer ([Olsen *et al.*, 1985b](#); [Choi & Kahyo, 1991](#); [Schlecht *et al.*, 1999b](#); [Znaor *et al.*, 2003](#); [Hashibe *et al.*, 2009](#)). In a Singapore cohort study ([Friborg *et al.*, 2007](#)) the pattern of odds ratios for smoking across categories of alcohol consumption was consistent with synergism for oropharyngeal but not for nasopharyngeal cancer.

Two cohort and fourteen case-control studies reported on joint effects of tobacco smoking and alcohol drinking on the risk for oesophageal cancer. Since multiple logistic regression models were used for analysing most of these studies, some of them tested likelihood ratio test

for departure from multiplicativity of the individual effects of tobacco and alcohol. Generally, the positive results were stronger for squamous cell carcinoma. However, these tests for interaction are inadequate to assess synergy. Four studies from India and Taiwan, China, included betel quid chewing to the joint effect analysis of tobacco smoking and alcohol consumption and the results suggested increasing risks of oesophageal cancer.

Most of the twenty case-control studies of laryngeal cancer provided strong evidence for synergism of tobacco smoking and alcohol consumption. Only [Zheng *et al.* \(1992\)](#) did not find consistent evidence with synergism. In several studies, tests for interaction were carried out and reported to be 'non significant.' These were tests for departure from the multiplicative models, typically multiple logistic regression models, used to analyse the case-control data, and not tests for departure from additive model.

Several studies (14 case-control, 3 cohort) reported on cancer of the 'mixed upper aerodigestive tract', comprising studies on squamous cell carcinomas, regardless of specific sites. These studies also provided strong evidence for synergism.

The Working Group considers that there is strong evidence of tobacco smoking and alcohol consumption interaction on the incidence of upper aerodigestive tract cancers, as well as with regard to cancer of specific subsites of this anatomical region.

2.21 Synthesis

2.21.1 Lung

Tobacco smoking is the major cause of lung cancer, primarily from cigarettes. Duration of smoking is the strongest determinant of lung cancer in smokers. Risk also increases in proportion to the number of cigarettes smoked. The strong dose- and duration-response

relationships between lung cancer and tobacco smoking have been confirmed more recently in both questionnaire-based and biomarker-based studies. Tobacco smoking increases the risk of all histological types of lung cancer.

Differences in the intensity and/or duration of tobacco smoking may explain, in part, the lower lung cancer risks in Asian populations relative to whites. However, several studies of genetic polymorphisms among African-American and Caucasian populations provide some preliminary evidence supporting the hypothesis of a racial/ethnic disparity in susceptibility.

The results from observational studies do not provide strong support that a higher intake or a greater circulating concentration of carotenoids reduce lung cancer risk, particular in light of the elevated risk of lung cancer observed in the randomized trials of β -carotene supplementation. Residual confounding from smoking and the possibility that carotenoid measurements are serving as markers for a diet rich in total fruit and vegetables mitigate the likelihood of any protective role for total carotenoids or β -cryptoxanthins.

The specific genes that are responsible for enhanced lung cancer risk remain poorly understood, in spite of hundreds of candidate gene studies. Single-gene studies conducted to date have several limitations which contribute to inconclusive results, including small sample size and associated low power to detect moderate risks when allele frequencies are low.

2.21.2 Upper aerodigestive tract

(a) Oral cavity

Tobacco smoking is causally associated with cancer of the oral cavity in both men and women. Since the previous *Monograph*, additional evidence has accumulated that further confirms the association. Risk increases with duration and intensity of smoking, and decreases after quitting.

(b) Pharynx

Tobacco smoking is an important cause of oropharyngeal and hypopharyngeal cancers. The risk increases with increasing duration and intensity of smoking and decreases with increasing time since quitting.

(c) Nasal cavity and accessory sinuses

The evidence of an association between tobacco smoking and sinonasal cancer is based on the results from case-control studies, each of which may be subject to different sources of bias. However, presence of a dose-response relationship in most studies, the decrease in risk associated with time since quitting, the consistently higher risks for squamous-cell carcinoma than for adenocarcinoma and the lack of potential confounders support the existence of a causal association.

(d) Nasopharynx

Although the interpretation of the results is complicated by small sample sizes in several studies, by different criteria used for the selection of controls and by the control groups in some studies including smoking-related diseases, the combined evidence shows an association between tobacco smoking and nasopharyngeal carcinoma in both endemic and non-endemic areas. Most studies that adjusted for known and suspected causes of nasopharyngeal carcinoma such as intake of Chinese-style salted fish, other dietary factors, alcohol drinking and family history of nasopharyngeal carcinoma, suggested only a limited confounding effect of these factors. Adjustment for infection with Epstein-Barr virus (human herpes virus 4), a major cause of nasopharyngeal carcinoma worldwide, was possible in just one of the available studies. However, it is unlikely that confounding by infection with Epstein-Barr virus would explain the observed association between tobacco smoking and risk for nasopharyngeal carcinoma.

(e) Oesophagus

Several well conducted case-control studies found a statistically significant higher risk for adenocarcinoma of the oesophagus in smokers than in nonsmokers. Positive dose-response relationships obtained using various indicators of amount smoked support a causal association, which is further corroborated by the findings of decreasing risks after smoking cessation. Several of these studies reported relative risks adjusted for alcohol consumption and other potential confounders. Further risk factors, such as chewing betel quid with tobacco or use of other forms of smokeless tobacco, have not been considered in these populations, but are not likely to be strong confounders. Studies from Australia, China and Europe also found increased risks for smokers.

(f) Larynx

Laryngeal cancer is one of the cancers most strongly associated with cigarette smoking. Recent epidemiological evidence strengthens this conclusion.

2.21.3 Stomach

The additional epidemiologic data showing a consistent association of stomach cancer with tobacco smoking in both men and women greatly strengthens the previous conclusion of a causal association. There was insufficient evidence for differential risks between cardia and non-cardia stomach cancer. Confounding and effect modification by *H. pylori* has not been found.

2.21.4 Pancreas

The additional data supports the previous evaluation that cancer of the pancreas is causally associated with tobacco smoking. The risk increases with increasing daily consumption levels and duration of smoking and decreases with increasing time since cessation of smoking.

The risk remains elevated after accounting for potential confounding factors.

2.21.5 Colorectum

At the previous evaluation, there was already some evidence from prospective cohort and case-control studies that the risk of colorectal cancer is increased among tobacco smokers. However, inadequate adjustment for various potential confounders was considered to possibly account for some of the small increase in risk that appears to be associated with smoking. Since then, an appreciable amount of data has accumulated to support a causal association with smoking. In virtually all the cohort studies published since elevated risk associated with smoking was found, although not always statistically significant. More than half of the cohort studies that assessed dose-response relationships found statistically significant increasing risks with increasing daily cigarette consumption, duration of smoking and/or pack-years of smoking. Risk of colorectal cancer decreased with increasing delay in smoking initiation and years since cessation of smoking. A meta-analysis based on 36 cohort studies with data from a total of 3 million subjects found a significantly 15% increased risk of colorectal cancer and 27% higher risk of colorectal cancer mortality in current smokers compared to never smokers. A stronger association with smoking for rectal cancer than for colon cancer was found in the meta-analysis of the subset of cohort studies that differentiated colorectal cancer by site. Risk for colorectal cancer increased significantly by 17% and by 38% with 20 cigarettes and 40 cigarettes/day, respectively, and was elevated by 9.4% and by 19.7% with a 20-year and a 40-year duration of smoking, respectively. While these results are persuasive, this meta-analysis could not correct for the potential confounders in the individual studies. Convincing evidence has been provided by three large cohort studies that adjusted for at

least four important potential confounders (i.e. physical activity, alcohol consumption, body mass index and dietary intake of fruits and vegetables and/or meat); two studies also adjusted for history of colonoscopy. Significant dose–response relationships were found with one or more of the smoking variables, for risk of colorectal cancer and/or colon cancer and/or rectal cancer. Earlier cohort studies may not have been able to establish the association because of insufficient follow-up time and a limited number of cases. Updated results of several large cohort studies, which now show clearly significant increased risk of colorectal cancer associated with smoking, provide support for the lag-time hypothesis for smoking and colorectal risk.

Recent evidence suggests that smoking may be associated with the subtype of colorectal cancer characterized by microsatellite instability, and by CIMP status and BRAF mutation. For this subtype, the magnitude of risk associated with smoking reaches the twofold risk elevation consistently observed for colorectal adenomas and supported by a recent meta-analysis. Smoking has been associated with a stronger risk for hyperplastic polyps than for adenomas. Also, CIMP positivity and BRAF mutations have been associated with hyperplastic polyps, particularly serrated polyps. These data suggest that smoking may be associated primarily with a subtype of colorectal cancer that develops through a hyperplastic (serrated) polyp progression. The association with smoking may therefore be diluted when considering colon cancers overall.

2.21.6 Liver

Recent studies on smoking and hepatocellular carcinoma supports the established causal relationship. Supporting evidence comes from the consistency of the findings across regions (with the best evidence coming from Asian studies), and the observations of an association among non-drinkers and after controlling for hepatitis B or C virus infection.

2.21.7 Kidney

Recent evidence supports a causal association between kidney cancer and smoking. After adjustment for body mass index and hypertension, current and former smokers still had a greater risk for renal-cell cancer. A dose–response relationship with the number of cigarettes smoked has been noted in most studies, and a few also noted a reduction in risk after cessation.

2.21.8 Urinary bladder

Tobacco smoking is causally associated to bladder cancer, based on a large number of case–control and cohort studies that showed statistically significant associations not explained by confounding or bias. Risk increased with the duration of smoking and the number of cigarettes smoked. Also, stopping smoking at any age avoids the further increase in risk incurred by continued smoking. The evidence supporting a modulating role by *NAT2* polymorphisms is convincing.

2.21.9 Myeloid leukaemia

There is evidence for a causal association of tobacco smoking with myeloid leukaemia.

2.21.10 Breast

New evidence from cohort and case–control studies and from meta-analyses of genetic polymorphisms has become available since the previous *IARC Monograph* ([IARC, 2004a](#)). Results from seven new cohort studies consistently show a small overall association between current smoking and breast cancer incidence, with relative risk estimates ranging from 1.1–1.3 in studies with at least 100 exposed cases. The overall association is weaker than that observed with other cancers that have been designated as causally related to smoking, and the dose–response relationships (with years of smoking,

cigarettes smoked per day, age at initiation) are correspondingly small.

Emerging evidence from case-control studies suggests that inherited polymorphisms in the *NAT2* gene, which encode the slow acetylator phenotype, may modify (increase) the association between smoking and breast cancer. The p-value for interaction with pack-years of smoking as a continuous variable is statistically significant ($P = 0.03$) and another small study published since this meta-analysis supports the conclusion. The potential for publication bias remains of concern.

It is biologically plausible that tobacco smoke could be causally related to breast cancer risk. There are multiple chemicals in tobacco smoke that are known to cause mammary cancer in rodents. These substances reach the breast in humans; some are stored in adipose tissue, and some can be detected in nipple aspirate and DNA adducts.

Hypotheses have been proposed to explain why numerous well conducted epidemiological studies have generally not observed strong or consistent associations between tobacco smoking and breast cancer. Underlying all of these is the theory that tobacco smoking may have both protective and detrimental effects on breast cancer risk, which cancel each other out and which could explain the atypical dose-response relationship that has been reported between tobacco smoke and breast cancer from some studies.

2.21.11 Cervix

The largely positive findings observed in studies of cohort design, the relatively high consistency of positive associations found for squamous-cell carcinoma of the cervix (but not adenocarcinomas) across all epidemiological studies, including those with adjustment for a wide range of potentially confounding variables, and the positive associations observed in studies

restricted to HPV-positive individuals, all argue against the observed positive association being due to recall or selection bias or confounding.

2.21.12 Endometrium

The results of epidemiological studies to date, including recent studies, largely show inverse associations of smoking with risk of postmenopausal endometrial cancer. However, the Working Group noted the few studies of premenopausal cancer that were less consistent, as well as indications of an increased risk among smokers in a recent multicentre European study.

2.21.13 Prostate

Many epidemiological studies have examined the association between cigarette smoking and prostate cancer risk, and most have shown no consistent association. The question remains whether smoking may alter risk in various population subgroups.

2.21.14 Ovary

A causal association between cigarette smoking and risk for mucinous ovarian tumours is indicated by 1) the consistency of the positive association across the large majority of ten pooled case-control studies and ten additional independent epidemiological studies of both case-control and cohort design, 2) the relatively strong magnitude of the association (typically greater than a doubling of risk among current smokers), 3) the tendency to show dose-response associations with risk, such that current smokers generally have higher risk than former smokers and the dose-response observed with measures of smoking intensity in some (but not all) studies, and 4) the specificity of the positive association with the mucinous histological type, which argues against recall bias as an explanation of the findings.

2.21.15 Thyroid

A pooled analysis of 14 case–control studies showed that smoking was inversely associated with thyroid cancer risk. Similar inverse associations were also observed in two subsequent case–control studies.

2.21.16 Other sites

There is inconsistent or sparse evidence for an association between tobacco smoking and other cancer sites that were considered by the Working Group.

2.21.17 Bidi smoking

Overall, bidi smoking increases the risk for cancers of the oral cavity, oropharynx, hypopharynx, larynx, lung, oesophagus and stomach.

3. Cancer in Experimental Animals

3.1 Mainstream tobacco smoke

3.1.1 Mouse

There have been multiple studies of the carcinogenic potential of tobacco smoke in mice ([Table 3.1](#)). Lifetime exposure of several mouse strains to cigarette smoke failed to result in the production of lung tumours ([Harris & Negroni, 1967](#); [Otto & Elmenhorst, 1967](#); [Henry & Kouri, 1986](#)). However, studies involving lifetime exposure of C57BL mice to a mixture of flue-cured or air-cured cigarette smoke or to the gas phase of flue-cured cigarette smoke led to significant increases in the number of lung tumours (adenomas) ([Harris *et al.*, 1974](#)). Similarly, lifetime exposure of Snell's mice to the gas phase of cigarette smoke led to an increased incidence of lung adenocarcinomas ([Leuchtenberger & Leuchtenberger, 1970](#)). Exposure of B6C3F₁

female mice to smoke for lifetime led to increased incidence of lung adenomas, bronchiolar papillomas and lung adenocarcinomas in smoke-exposed mice. In addition, the occurrence of squamous cell carcinomas of the nasal cavity in smoke-exposed mice was increased ([Hutt *et al.*, 2005](#)). In a recent study, Swiss mice were exposed whole-body to cigarette smoke for 120 days, starting within 12 hours of the birth. Smoke-exposed mice developed microscopic lung tumours beginning only 75 days after birth and reaching an overall incidence of 78.3% after 181–230 days. The mean lung tumour multiplicity was 6.1 and 13.6 tumours per mouse in males and females, respectively. In addition, malignant tumours, some of which may have had a metastatic origin, were detected in the urinary tract of smoke-exposed mice ([Balansky *et al.*, 2007](#)).

3.1.2 Rat

Several studies have evaluated the carcinogenic potential of mainstream tobacco smoke in rats ([Table 3.1](#)). Exposure of Wistar rats to cigarette smoke for lifetime did not increase the lung tumour incidence ([Davis *et al.*, 1975](#)). In contrast, exposure of Fischer 344 rats to a mixture of non-filter cigarette smoke for 128 weeks resulted in an increased incidence of nasal and lung tumours. There was also an increase in subcutaneous sarcomas at forelimb ulceration sites ([Dalbey *et al.*, 1980](#)). CDF rats were exposed to low-dose cigarette smoke (LCS) or high-dose cigarette smoke (HCS) for 126 weeks. The incidence of lung tumours was significantly higher only in female rats that received HCS ([Finch *et al.*, 1995](#)). In a recent study, Fischer 344 rats received whole body exposure to smoke containing either 100 mg (LCS) or 250 mg (HCS) total particulate matter/m³ for 30 months. This led to significant increases in the incidence of lung and nasal cavity tumours in male rats treated with HCS but not with LCS. In female rats, there were significant increases in the incidence of lung adenomas

in animals treated with HCS and of all lung tumours in animals treated with both LCS and HCS. There was also a significant increase in the occurrence of nasal cavity tumours in female rats treated with HCS ([Mauderly et al., 2004](#)).

3.1.3 Hamster

Four studies have evaluated the ability of mainstream tobacco smoke to induce tumours in hamsters ([Table 3.1](#)). Syrian golden hamsters were exposed to either a mixture of German reference cigarette smoke or of dark air-cured cigarette smoke for lifetime. There were increases in the incidence of laryngeal carcinomas in hamsters exposed to both smoke preparations ([Dontenwill et al., 1973](#)). In a subsequent study, hamsters were exposed to a mixture of German reference cigarette smoke containing 1.5 mg nicotine, 0.173 mg phenol and 12.7 mL carbon monoxide/cigarette for lifetime. The incidence of laryngeal tumours in smoke-exposed hamsters was higher than in controls ([Dontenwill et al., 1977](#)). BIO male hamsters exposed to a mixture of US reference smoke for 100 weeks developed laryngeal and nasopharyngeal tumours ([Bernfeld et al., 1974](#)). In a subsequent study, male BIO hamsters exposed to smoke from commercial British filter cigarettes developed higher incidence of laryngeal tumours than controls ([Bernfeld et al., 1979](#)).

3.2 Co-administration of tobacco smoke with known carcinogens and other agents

Study design and results of the studies on co-administration of tobacco smoke with known carcinogens and other agents are summarized in [Table 3.2](#).

3.2.1 Rat

(a) Benzo[a]pyrene

Wistar rats received a single intratracheal instillation of 2 mg benzo[a]pyrene followed by lifetime exposure to cigarette smoke. This treatment led to a low incidence of lung tumours that was not significantly higher than in controls ([Davis et al., 1975](#)). In another study Wistar rats were given intratracheal instillations of benzo[a]pyrene mixed with ferric oxide and exposed to cigarette smoke either during initiation and post-initiation or only after treatment with benzo[a]pyrene/ferric oxide (post-initiation). Inhalation of cigarette smoke during the initiation and post-initiation phases of carcinogenesis resulted in a higher lung tumour (squamous-cell carcinoma) multiplicity than that seen in rats exposed during the post-initiation phase only ([Gupta et al., 1990](#)).

(b) Radon progeny

Sprague-Dawley rats were exposed to radon progeny at cumulative doses of 4000, 500 or 100 work-level-months (WLM), with or without concurrent exposure to cigarette smoke by inhalation for one year. Rats exposed to 4000 WLM radon progeny, without exposure to smoke, developed lung carcinomas (17/50). Thirty four carcinomas were seen in 50 rats exposed to radon and cigarette smoke. The 500 WLM radon progeny group exposed to radon only had 2/28 lung carcinomas as compared with 8/30 rats exposed to radon and cigarette smoke. No tumours were observed in rats treated with 100 WLM radon and one carcinoma was seen among 30 rats exposed to 100 WLM radon and cigarette smoke. Seventy five percent of the lung tumours were squamous-cell carcinomas, 20% were adenocarcinomas, and the remainder were undifferentiated carcinomas ([Chameaud et al., 1982](#)).

Table 3.1 Carcinogenicity in response to mainstream tobacco smoke in animals

Species, strain (sex) Reference	Animals/group at start Dosing regimen Duration	Lung burden	Results Target organ Incidence and/or multiplicity of tumours (%)	Significance	Comments
Mice, C57BL (M, F) Harris & Negroni (1967)	100 animals/sex/group Nose-only, mixture of fresh non-filter cigarette smoke/ air (1/39, v/v), nicotine, 0.1 mg/mL; CO, 0.064% (v/v), 12 min/every other d; lifetime	Nicotine, 14–17 µg	Alveolar adenocarcinomas: M: 4/100 (alveolar adenocarcinoma) Controls–0/100 F: 4/100 (alveolar adenocarcinoma) Controls–0/100	$P = 0.06$ $P = 0.06$	
Mice, C57BL and BLH (sex, NR) Otto & Elmenhorst (1967)	126 animals/group Whole-body, gas phase of 12 cigarettes puffed 2 sec/ min, concentration (NR), 90 min/d; lifetime (~27 mo)	NR	Lung (adenomas): C57BL–7/126 (5.5%) Controls–3/90 (3%) BLH–40/126 (32%) Controls–19/60 (32%)	NS NS	
Mice, (C57BL/Cum × C3H/ Anf Cum) _{F₁} (F) Henry & Kouri (1986)	2053, 1 014 sham Nose-only, 10% smoke from US reference cigarettes, concentration (NR), smoke 20 sec/min, 6–8 min/d, 5 d/wk for 110 wk; 116 wk	Particulate deposition, 125–200 µg	Alveolar adenocarcinomas: 19/978 (2%) Sham-exposed controls–7/651 (1%)	$P = 0.10$	Shorter latency of tumour occurrence in smoke-exposed group suggested
Mice, C57BL (M, F) Harris et al. (1974)	100 animals/sex/group Nose-only, mixture of fresh flue-cured or air-cured cigarette smoke/air (1/39, v/v), concentration (NR), 12 min/d on alternate d; lifetime	NR	M: 9/162 ^a (5%, flue-cured), 7/189 ^a (4%, air-cured) Controls–3/160 ^a (2%) F: 7/164 ^a (4%, flue-cured), 0/173 (air-cured) Controls–1/159 ^a (1%)	$P = 0.07$, flue-cured $P > 0.05$, air-cured $P = 0.04$, flue-cured	
	Nose-only, gas phase of flue-cured cigarette smoke, concentration (NR), 12 min/d on alternate d; lifetime	NR	M: 3/8 ^a (37%) Controls–3/160 ^a (2%) F: 2/88 ^a (2%) Controls–1/159 ^a (1%)	$P > 0.05$ $P > 0.05$	

Table 3.1 (continued)

Species, strain (sex) Reference	Animals/group at start Dosing regimen Duration	Lung burden	Results Target organ Incidence and/or multiplicity of tumours (%)	Significance	Comments
Mice, Snell (M, F) Leuchtenberger & Leuchtenberger (1970)	160 M, 118 F Whole-body, whole fresh cigarette smoke, concentration (NR), 2 puffs, 1 × /d, lifetime (26 mo)	Nicotine, 5 µg	M: Lung A-7/107 (6.5%) Controls-8/106 (7.5%)	NS	
			Lung AdC-11/107 (10%) Controls-5/106 (4.7%)	P = 0.15	
			F: Lung A-2/65 (3%) Controls-1/78 (1.2%)	P = 0.475	
			Lung AdC-5/65 (7.7%) Controls-3/78 (3.8%)	P = 0.035	
			M: Lung A-1/44 (2%) Controls-8/106 (7%)	NS	
	100 M, 89 F Whole-body, gas phase of fresh cigarette smoke, concentration (NR), 2 puffs, 1 × /d, lifetime (26 mo)	NR	Lung AdC-10/44 (23%) Controls-5/106 (5%)	P = 0.005	
			F: Lung A-3/44 (7%) Controls-1/78 (1%)	P = 0.15	
			Lung AdC-5/44 (11%) Controls-3/78 (4%)	P = 0.15	
			Lung A: 93/330 (28%) Sham-exposed controls-22/326 (7%)	P < 0.001	
			Bronchiolar papillomas: 15/330 (4%) Controls-0/326	P < 0.001	
Mice, B6C3F1 (F) Hutt et al. (2005)	330, 326 controls Whole-body, smoke from Kentucky 2R1 unfiltered reference cigarettes, 250 mg total particulate matter/m ³ , 6 h/d, 5 d/wk for 30 mo; 30 mo or lifetime	NR	Lung AdC: 67/330 (20%) Controls-9/326	P < 0.001	
			All lung tumours: 148/330 (45%) Controls-31/326	P < 0.001 ^b	
			Nasal cavity tumours: 20/330 (6%) Controls-0/326	P = 0.002, one-tailed Fisher	
			Squamous-cell carcinomas: 9/330 (3%) Controls-0/326		

Table 3.1 (continued)

Species, strain (sex) Reference	Animals/group at start Dosing regimen Duration	Lung burden	Results Target organ Incidence and/or multiplicity of tumours (%)	Significance	Comments
Mice, Swiss (M, F) Balansky <i>et al.</i> (2007)	38, 36 controls (neonatal mice, 21 h of age) Whole-body, cigarette smoke/air, 818 mg total particulate matter/m ³ , 65 min/d for 120 d	NR	Lung A: 15/38 (19%) Sham-exposed controls–0/36 Lung AdC: 7/38 (18%) Controls–0/38 Kidney A: 6/16 (16%) (F only) Controls–0/21 Liver carcinomas: 2/16 (5%) (F only) Controls–0/21	$P < 0.001$ $P < 0.01$ $P < 0.01$	
Rats, Wistar (F) Davis <i>et al.</i> (1975)	408, 102 untreated, 102 sham Nose-only, mixture of cigarette smoke/air (1/5), concentration (NR), 15 sec/min, 2 × 11 min/d, 5 d/wk, lifetime	NR	4/408 (1%) (1 lung C and 3 lung neoplasms of uncertain malignancy) Controls–0/102 Sham treated controls–0/102	NS	
Rats, F344 (F) Dalbey <i>et al.</i> (1980)	80, 63 untreated, 30 sham Nose-only, mixture of non-filter cigarette smoke/ air (1/10), 18.4 mg smoke particulate and 0.89 mg nicotine/cigarette, 1 cigarette/h, 7 cigarettes/d, 5 d/wk for 128 wk; 160 wk	Particulate deposition, 1.75 mg/d	10/80 (12%) (1 nasal AdC, 1 nasal C, 5 pulmonary A, 1 pulmonary C, 2 alveolar C) Controls–3/93 (3%) Subcutaneous sarcomas at forelimb ulceration sites: 21/80 (26%) Controls–0/93	$P < 0.05$ $P < 0.05$	
Rats, CDF (F344)/CrIBR (M, F) Finch <i>et al.</i> (1995)	2165 animals Whole-body, cigarette smoke/air, 100 mg (LCS) or 250 mg (HCS) total particulate matter/m ³ , 6 h/d, 5 d/ wk for 30 mo; lifetime	NR	Lung tumours: M: LCS 3/173 (2%) HCS 7/78 (9%) Filtered air 3/119 (2%) F: LCS–4/145 (3%) HCS–6/83 (7%) Filtered air–0/113	$P < 0.05$ $P < 0.05$	

Table 3.1 (continued)

Species, strain (sex) Reference	Animals/group at start Dosing regimen Duration	Lung burden	Results Target organ Incidence and/or multiplicity of tumours (%)	Significance	Comments
Rats, F344 (M, F) Mauderly et al. (2004)	M: 178 LCS, 82 HCS F: 175 LCS, 81 HCS Whole-body, smoke from Kentucky 1R3 unfiltered reference cigarettes, 100 mg (LCS) or 250 mg (HCS) total particulate matter/m ³ , 6 h/d, 5 d/wk for 30 mo; lifetime	NR	M: Lung A– LCS 4/178 (2%) HCS 2/82 (2%) Sham-exposed controls 1/118 (1%) Lung AdC– LCS 1/178 (1%) HCS 5/82 (6%) Controls 3/118 (3%) All lung tumours– LCS 4/178 (2%) HCS 7/82 (8%) Controls 4/118 Nasal cavity (all tumour types)– LCS 1/178 (1%) HCS 5/82 (6%) Controls 1/118 (1%) F: Lung A– LCS 7/175 (6%) HCS 7/81 (9%) Sham-exposed controls 0/119 AdC– LCS 4/175 (2%) HCS 4/81 (5%) Controls 0/119 All lung tumours– LCS 10/175 (6%) HCS 11/81 (13%) Controls 0/119 Nasal cavity (all tumour types)– LCS 0/175 HCS 3/81 Controls 0/119	NS NS NS; trend, $P = 0.055$ $P = 0.032$ (HCS); trend, $P = 0.010$ NS (LCS); $P < 0.001$ (HCS) NS $P = 0.023$ (LCS); $P = 0.001$ (HCS) $P = 0.020$ (HCS); trend, $P = 0.003$	

Table 3.1 (continued)

Species, strain (sex) Reference	Animals/group at start Dosing regimen Duration	Lung burden	Results Target organ Incidence and/or multiplicity of tumours (%)	Significance	Comments
Hamsters, Syrian golden (M, F) Dontenwill et al. (1972)	80 animals/sex/group Whole-body, mixture of German reference cigarette smoke/air (1/15), concentration (NR), smoke of 30 cigarettes for 7–10 min; 1, 2 or 3 × /d, 5 d/wk, lifetime	NR	Laryngeal carcinomas: 1/160 (1%), 17/160 (11%) and 11/160 (7%) Controls–0/80		
	Whole-body, mixture of dark air-cured cigarette smoke/air (1/15), concentration (NR), Smoke of 30 cigarettes for 7–10 min; twice/d, 5 d/wk, lifetime	NR	Laryngeal carcinomas: 2/160 (1%) Controls–0/80		
Hamsters, Syrian golden (M, F) Dontenwill et al. (1972)	80 animals/group Whole-body, mixture of German reference cigarette smoke/air (1/15), 1.5 mg nicotine, 0.173 mg phenol and 12.7 mL CO/cigarette, smoke of 30 cigarettes for 7–10 min; 1, 2 or 3 × /d, 5 d/wk, lifetime	NR	M: Laryngeal C–0, 4, 6 and 11%, Controls–0% F: Laryngeal C–0, 1, 2 and 7% Controls–0%		
	102 animals/group Whole-body, mixture of US reference cigarette smoke/ air (1/5), concentration (NR), duration (NR)		Inbred BIO 15.16: Laryngeal tumours–9/84 (10%) Nasopharyngeal tumours–2/84 (2%) Sham-exposed controls 0/42 Controls 0/40 Inbred BIO 87.20: Laryngeal tumours–2/87 (2%) Sham-exposed controls 0/44 Controls 0/48		
Rats, Inbred BIO 15.16 (M) Bernfield et al. (1979)	Number at start (NR) Whole-body, 11 or 22% smoke from commercial British filter cigarettes, concentration (NR), 2 × 12 min/d, 7 d/wk for 35–42 wk; up to 74–80 wk	NR	Laryngeal carcinomas: 11% smoke–3/44 (7%) 22% smoke–27/57 (47%) Sham-exposed controls 0/36; Controls 0/50		

^a Most of these lung tumours are adenomas^b Nasal cavity tumours included 14 squamous cell carcinomas (5 in situ), 5 hemangiomas, and 1 respiratory papilloma
A, adenoma; AdC, adenocarcinoma; C, carcinoma; CO, carbon monoxide; d, day or days; F, female; h, hour or hours; HCS, high cigarette smoke; LCS, low cigarette smoke; M, male;
min, minute or minutes; mo, month or months; NR, not reported; NS, not significant; sec, second or seconds; wk, week or weeks; yr, year or years

Table 3.2 Carcinogenicity in response to exposure to mainstream tobacco smoke in conjunction with exposure to known carcinogens or other agents in animals

Species, strain (sex) Reference	Animals/group at start Dosing regimen Duration	Results Target organ Incidence and/or multiplicity of tumours (%)	Significance
Rats, Wistar (F) Davis et al. (1975)	84 or 408 animals/group A single intratracheal instillation of benzo[a]pyrene (2 mg) + infusine + carbon black followed by British reference cigarette smoke/air (1/5); 1 cigarette, twice/d, 5 d/wk, lifetime	3/84 (4%, lung C), 1/84 (1%, lung C; benzo[a]pyrene alone), 4/408 (1%, 3 A + 1 malignant neoplasm; cigarette smoke only), 0/204 (controls + sham-exposed controls)	NS
Rats, Wistar (M) Gupta et al. (1990)	35 animals/group Cigarette smoke; 5 cigarettes/8.2 L air; 1 h/d during 2nd–24th wk or 10th–24th wk of the study Benzo[a]pyrene (20 mg) + Fe ₂ O ₃ ; intratracheally (3 weekly doses) during 6th–8th wk of the study; 24 wk	Conventional diet: 2nd–24th wk, 2.14 lung C/animal; 10th–24th wk, 1.33 lung C/animal; benzo[a]pyrene control, 1.22 lung C/animal. Vitamin A-deficient diet: 2nd–24th wk, 2.86 lung C/animal; 10th–24th wk, 1.67 lung C/animal; benzo[a]pyrene control, 1.83 lung C/animal	
Rats, Sprague-Dawley, sex NR Chameaud et al. (1982)	28–50 animals/group French reference cigarette smoke (9 cigarettes/500 L air); 10–15 min session, 4 d/wk for 1 yr Radon progeny (4 000, 500 or 100 WLM) Lifetime	4000 WLM: 34/50 (68%, lung C); 17/50 (34%, lung C; radon progeny alone) 500 WLM: 8/30 (27%, lung C); 2/28 (7%, lung C; radon progeny alone) 100 WLM: 1/30 (3%, lung C); 0/50 (radon progeny alone)	P = 0.0015
CDF (F344)/CrIBR (M, F) Finch et al. (1995)	Number at start (NR) Cigarette smoke/air, 100 mg (LCS) or 250 mg (HCS) total particulate matter/m ³ ; 6 h/d, 5 d/wk for 126 wk ²³⁹ PuO ₂ aerosol, 1 wk (6th wk of the study); > 52 wk	49–61% (lung tumours, LCS + ²³⁹ PuO ₂) 72–74% (HCS + ²³⁹ PuO ₂) 20–33% (²³⁹ PuO ₂) 2–3% (LCS) 7–8% (HCS)	
Syrian golden (M, F) Dontenwill et al. (1973)	80 animals/sex/group German reference cigarette smoke/air (1/15); Smoke of 30 cigarettes for 7–10 min; twice/d, 5 d/wk, lifetime DMBA (0.5 mg); intratracheally 10 d before the beginning of smoke exposure	32/160 (20%, laryngeal C), 17/160 (11%, laryngeal C; smoke only), 0/160 (DMBA alone)	

Table 3.2 (continued)

Species, strain (sex) Reference	Animals/group at start Dosing regimen Duration	Results Target organ Incidence and/or multiplicity of tumours (%)	Significance
Syrian golden, (sex NR) Hoffmann <i>et al.</i> (1979)	20 or 40 animals/group Cigarette smoke/air (1/7); Cigarette smoke 2 × 10 min/d, 5 d/wk, 48 wk DMBA (0.24 mg); intralaryngeally	3/40 (7%, laryngeal C), 0/20 (smoke only), 0/20 (DMBA alone)	
Syrian golden (M) Takahashi <i>et al.</i> (1992)	10 or 30 animals/group Cigarette smoke/air (1/7); Smoke of 30 cigarettes for 9 min; twice/d, 5 d/wk, 12 wk NDEA (100 mg/kg bw); subcutaneously	Non-filter cigarettes (2.10 ± 1.74 P+H/animal) and filter cigarettes (1.93 ± 1.55 P + H/animal) versus sham-exposed (0.97 ± 1.03 P + H/animal)	<i>P</i> < 0.01 <i>P</i> < 0.01
Syrian golden (M) Harada <i>et al.</i> (1985)	30 animals/group Non-filter cigarette smoke/air (1/7); Smoke of 30 cigarettes for 6 min: twice/d, 5 d/wk, 58 wk NDEA (10 mg/hamster); subcutaneously (12 weekly doses)	Nasal cavity tumours 14/30 (47%, smoke + NDEA), 5/30 (17%, NDEA alone)	<i>P</i> < 0.05

A, adenoma; bw, body weight; C, carcinoma; d, day or days; DMBA, 7,12-dimethylbenz[*a*]anthracene; F, female; h, hour or hours; HCS, high cigarette smoke; LCS, low cigarette smoke; M, male; min, minute or minutes; mo, month or months; NDEA, *N*-nitrosodiethylamine; NR, not reported; NS, not significant; P + H, epithelial hyperplasias and papillomas; sec, second or seconds; wk, week or weeks; WLM, work-level-months; yr, year or years

(c) *Plutonium oxide*

CDF®/CrlBR rats were exposed to either filtered air or mainstream cigarette smoke at concentrations of either 100 or 250 mg total particulate matter/m³ (LCS and HCS groups, respectively). At 12 weeks, rats were removed from smoke chambers and exposed nose-only to plutonium oxide (²³⁹PuO₂) then returned to the smoke chambers one week later for 30 months of continuous exposure to either filtered air or cigarette smoke. The incidence and multiplicity of lung tumours (adenocarcinomas, squamous-cell carcinomas, adenosquamous carcinomas) in animals exposed to both concentrations of cigarette smoke and ²³⁹PuO₂ were higher than those in animals exposed to ²³⁹PuO₂, LCS or HCS alone (Finch *et al.*, 1995).

3.2.2 Hamster

(a) 7,12-Dimethylbenz[*a*]anthracene

Groups of 160 Syrian golden hamsters received 7,12-dimethylbenz[*a*]anthracene (DMBA) intratracheally, followed by cigarette smoke for life, or treated with cigarette smoke or DMBA only. A total of 32 squamous-cell carcinomas of the larynx were observed in animals treated with both DMBA and cigarette smoke, in comparison with 17 in hamsters exposed to cigarette smoke only and none in hamsters treated with DMBA alone (Dontenwill *et al.*, 1973). Similar results were reported from other experiments in which Syrian golden hamsters were exposed to DMBA and cigarette smoke (Hoffmann *et al.*, 1979).

(b) *N*-Nitrosodiethylamine

Groups of hamsters received a single subcutaneous injection of *N*-nitrosodiethylamine (NDEA) and then were exposed to smoke from unfiltered cigarettes, filtered cigarettes and sham smoke. Controls were exposed to either unfiltered cigarette smoke, filtered cigarette smoke or sham smoke. In the NDEA-smoke-treated

groups, epithelial hyperplasias and/or papillomas of the larynx were induced at higher frequency than in controls (Takahashi *et al.*, 1992). Hamsters exposed to cigarette smoke in air also received 12 weekly subcutaneous injections of NDEA (total dose, 10 mg/hamster). Treatment with NDEA only resulted in both benign and malignant tumours of the respiratory tract, and co-exposure to cigarette smoke potentiated the development of tumours in the nasal cavity (Harada *et al.*, 1985).

3.3 Smoke condensates

Study design and results of the studies on administration of tobacco smoke condensates are summarized in Table 3.3.

3.3.1 Skin application

(a) Mouse

Cigarette-smoke condensate produces both benign and malignant tumours on mouse skin. The carcinogenic potency of the cigarette-smoke condensate depends upon tobacco variety, composition of cigarette paper and the presence of additives (Wynder *et al.*, 1957; Gargus *et al.*, 1976; Gori, 1976).

(b) Rabbit

Cigarette-smoke condensate induced skin papillomas and carcinomas when applied to the ears of rabbits for lifetime (Graham *et al.*, 1957).

3.3.2 Intrapulmonary administration

Injection of 24 mg cigarette-smoke condensate into the lungs of female Osborne Mendel rats led to the development of squamous cell carcinomas (Stanton *et al.*, 1972). These observations were confirmed by Dagle *et al.* (1978) who observed a dose-dependent incidence of lung carcinomas when cigarette-smoke condensate prepared from two types of cigarettes were given.

Table 3.3 Carcinogenicity in response to exposure to cigarette-smoke condensate in animals

Species, strain (sex) Reference	Animals/group at start Dosing regimen Duration	Results Target organ Incidence and/or multiplicity of tumours (%)	Significance
Mice CAF1 (M, F) Wynder et al. (1953)	112, 44 controls Skin painting (dorsal) of CSC, CSC/acetone solution (40 mg CSC/ application), 3 × /wk, lifetime	36/81 (44%, skin epidermoid C), 0/30 (acetone controls)	
Mice ICR Swiss (F) Gargus et al. (1976)	5200 Skin painting (dorsal) of CSC, CSC/acetone solution (150 mg or 300 mg CSC/wk), 6 × /wk, 78 wk	482/5200 (9%, skin C), 3/800 (0.4%, acetone controls) ^a	
Mice ICR Swiss (F) Gori (1976)	4900 Skin painting (dorsal) of CSC, CSC/acetone solution (25 mg or 50 mg CSC/application), 6 × /wk, 78 wk	1157/4900 (24%, skin C), 0/800 (acetone controls)	
Mice, ICR/Ha Swiss (F) Hoffmann & Wynder (1971)	30 animals/group Skin painting (dorsal) with CSC active fraction with or without subsequent painting of the skin with croton oil, CSC active fraction/acetone (2.5 mg of 0.6% CSC/application), 10 times on alternate d Croton oil (2.5%), 3 × /wk, up to 12 mo, 10 d after last CSC active fraction application; 15 mo	After 12 and 15 mo: 4/30 (13%, skin C), 0/65 (croton oil controls)	
Mice, Swiss (F) Wynder & Hoffmann (1961)	30–50 animals/group Skin painting (dorsal) of CSC with or without initiation by DMBA application; DMBA (75 µg); CSC/acetone (75 mg CSC/application, start: 1 wk after DMBA application), 2–3 × /wk, 12 mo; 15 mo	DMBA: 2/30 (7%, skin C) 2 × CSC: 1/40 (3%, skin C) DMBA + 2 × CSC: 8/30 (27%, skin C) 3 × CSC: 11/50 (22%, skin C) DMBA + 3 × CSC: 11/30 (37%, skin C)	
Mice, SENCAR (F) Meckley et al. (2004a)	40 animals/group Skin painting (dorsal) of CSC from Kentucky IR4F reference cigarettes, with or without initiation by DMBA application; DMBA (75 µg) or acetone, 1x. Then starting 1 wk after DMBA or acetone: CSC in acetone, 0, 10, 20 or 40 mg/application, 3 × /wk, 29 wk; 31 wk	Mean mice with tumours/mice per group at 31 wk: No DMBA: 0/40 acetone-acetone, 9/40 (22%) acetone-CSC 40 mg/treatment DMBA/CSC: 0/40, 3/40 (1.0), 16/40 (75 tumours/16 mice = 4.7), 32/40 (200 tumours/32 mice = 6.3)	
Mice, SENCAR (F) Meckley et al. (2004b)	40 animals/group Skin painting (dorsal) of CSC from Kentucky IR4F reference cigarettes or ECLIPSE (non-burned) cigarettes, with or without initiation by DMBA application; DMBA (75 µg) or acetone, 1 × . Then starting 1 wk after DMBA or acetone: CSC in acetone, 0, 10, 20 or 40 mg/application, 3 × /wk, 29 wk; 31 wk	No DMBA: acetone/acetone 0/40 (0); acetone/1R4F CSC 40 mg, 9/40 (1.8); acetone/ECLIPSE CSC 40 mg, 0/40 (0) DMBA/CSC: acetone, 0/40 (0); 1R4F CSC 10 mg, 6/40 (1.8); 1R4F CSC 12 mg, 28/40 (6.6); 1R4F CSC 40 mg, 36/40 (6.8); ECLIPSE CSC, 0/40 (0); ECLIPSE CSC 10 mg, 1/40 (1); ECLIPSE CSC 20 mg, 2/40 (5.5); ECLIPSE CSC 40 mg, 12/40 (2.6)	

Table 3.3 (continued)

Species, strain (sex) Reference	Animals/group at start Dosing regimen Duration	Results Target organ Incidence and/or multiplicity of tumours (%)	Significance
Mice, SENCAR (F) Hayes et al. (2007)	40 or 50 animals/group Skin painting (dorsal) of CSC from heat-exchanged flue cured tobacco (HE; low TSNA) or direct-fire (DF) cured tobacco, with or without initiation by DMBA application; DMBA (75 µg) or acetone, 1 × . Then starting 1 wk after DMBA or acetone: CSC/acetone, 0, 9, 18, or 36 mg/ application, 3 × /wk, 29 wk; 31 wk	No DMBA: acetone/acetone 0/40; acetone/DF CSC 36 mg, 5/50 (1.4); acetone/HE CSC 36 mg, 8/50 (1.3) DMBA/CSC: DF CSC, 0/40, DF CSC 9 mg, 15/40 (5.5); DF CSC 18 mg, 30/40 (10.0); DF CSC 36 mg, 43/50 (8.2); HE CSC, 0/40, HE CSC 9 mg, 17/40 (4.8); HE CSC 18 mg, 32/40 (7.3); HE CSC 36 mg, 42/50 (8.5)	$P < 0.05$ $P < 0.05$ $P < 0.05$
Mice, Swiss albino (M) Pakhale et al. (1988)	20 animals/group Oral gavage of Indian bidi smoke condensate; 1 mg bidi smoke condensate/0.1 mg DMSO, 5 d/wk, 55 wk; 90 wk	4/15 (27%, hepatic haemangiomas); 1/15 (7%, stomach papilloma); 1/15 (7%, stomach carcinoma); 1/15 (7%, oesophageal carcinoma); 0/15 (untreated or DMSO-treated controls)	
Rats, Osborne Mendel (F) Stanton et al. (1972)	Number/group at start (NR) Intrapulmonary administration of CSC pellet; CSC/ beeswax:tricaprylin (24 mg CSC/injection), up to 107 wk after implantation	14/40 ^c (35%, lung squamous-cell C), 0/63 ^c (beeswax:tricaprylin controls)	
Rats, OM/NCR (F) Dagle et al. (1978)	120 ^d Intrapulmonary administration of CSC pellet; CSC/ beeswax:tricaprylin (5, 10, 20 or 67 mg CSC/injection), 120 wk after implantation	4, 10, 20 and 42% pulmonary C prevalence; 0% C prevalence for 3 control groups of about 190 rats each	
Rabbits, Albino New Zealand (M, F) Graham et al. (1957)	38, 7 controls Skin painting of CSC (both ears); CSC/acetone solution (100 mg CSC/ application/ear), 5 × /wk, lifetime (4–6 yr)	4/38 (11%, 2 skin C + 1 skin liposarcoma + 1 skin fibrosarcoma), 0/7 (acetone controls)	

^a Skin papillomas^b Mostly adenomas^c Incidence in animals that died 43–107 weeks after injection^d 4 × 10 rats/group terminated before 120 weeks^e Total visually identified and histologically confirmed skin tumours included mostly squamous papillomas and carcinomas [Tumour incidences and multiplicities estimated from graphs]C, carcinoma; CSC, cigarette-smoke condensate; d, day or days; DMBA, 7,12-dimethylbenz[*a*]anthracene; DMSO, dimethyl sulfoxide; F, female; M, male; NR, not reported; TSNA, tobacco-specific *N*-nitrosamines; wk, week or weeks

3.3.3 Initiation-promotion skin painting studies

Cigarette-smoke condensate and its fractions can act as skin co-carcinogens in Swiss and SENCAR mice when tested in conjunction with croton oil ([Hoffmann & Wynder, 1971](#)) or DMBA ([Wynder & Hoffmann, 1961](#); [Meckley et al., 2004a, b](#); [Hayes et al., 2007](#)).

3.3.4 Bidi smoke

Swiss albino mice administered 1 mg bidi smoke condensate in dimethyl sulfoxide (DMSO) by oral gavage developed haemangiomas (4/15), stomach carcinoma (1/15), and esophageal carcinoma (1/15), whereas no tumours were observed in controls ([Pakhale et al., 1988](#)).

3.4 Synthesis

Mainstream tobacco smoke induced lung tumours in mice, lung and nasal cavity tumours in rats and laryngeal carcinomas in hamsters.

Co-administration of tobacco smoke with benzo[a]pyrene, radon progeny and plutonium resulted in higher lung tumour responses in rats than administration of either agent alone. Hamsters exposed to cigarette smoke and either DMBA or NDEA had higher lung tumour responses compared to cigarette smoke, DMBA or NDEA alone.

Topical application of cigarette-smoke condensate led to the development of skin tumours in mice and rabbits; intrapulmonary administration of cigarette-smoke condensate induced squamous cell carcinomas in rat lung.

4. Other Relevant Data

4.1 Overview of the mechanistic evidence for the carcinogenicity of tobacco

4.1.1 Conceptual model of the carcinogenesis of tobacco and tobacco smoke

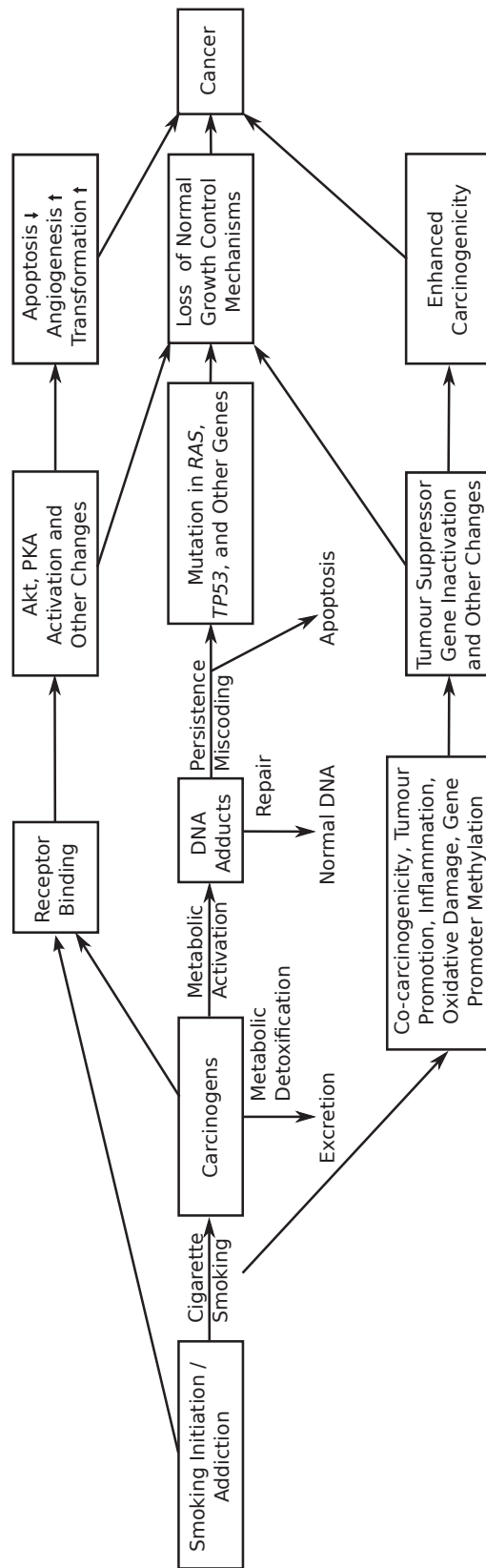
A conceptual model for understanding mechanisms by which tobacco smoke causes cancer is shown in Fig. 4.1 ([Hecht, 1999, 2003](#)). This model also applies to smokeless tobacco and other forms of smoked tobacco and, in theory, to second-hand tobacco smoke since it contains all of the same carcinogens and toxicants as mainstream cigarette smoke, although at lower doses.

The major accepted mechanistic pathway is summarized in the central track of Fig. 4.1. Smokers inhale carcinogens which, either directly or after metabolism, covalently bind to DNA, forming DNA adducts. DNA adducts are central to chemical carcinogenesis because they can cause miscoding and permanent mutations. If these mutations occur in critical regions of oncogenes and tumour suppressor genes, which are essential in growth control, the result can be loss of normal cellular proliferation mechanisms, genomic instability, and cancer. A study that sequenced 623 cancer-related genes in 188 human lung adenocarcinomas validated this premise by finding multiple somatic mutations in critical growth control genes, consistent with the chronic bombardment of cellular DNA by tobacco smoke carcinogens and their metabolically activated forms ([Ding et al., 2008](#)).

Each step of this conceptual model is considered in detail below.

Most people begin smoking cigarettes when they are teenagers, and become addicted to nicotine. Nicotine is not generally considered to be a carcinogen ([Schuller, 2009](#)), but it is accompanied in each puff of each cigarette by a complex

Fig. 4.1 Conceptual model for understanding mechanisms of tobacco carcinogenesis



There may be overlap between these three tracks: e.g. mutation in *TP53* leading to tumour suppressor gene inactivation.

mixture of carcinogens and toxicants. There are over 60 carcinogens in cigarette smoke that have been evaluated in the previous *IARC Monograph* as having *sufficient evidence* for carcinogenicity in laboratory animals ([IARC, 2004a](#)), sixteen of which are considered to be *carcinogenic to humans* (Group 1). There are also many other carcinogens and potential carcinogens in cigarette smoke that have not been evaluated ([Rodgman & Perfetti, 2006](#); see Section 1.1). Structures of tobacco smoke constituents and biomarkers discussed here are presented in Fig. 4.2.

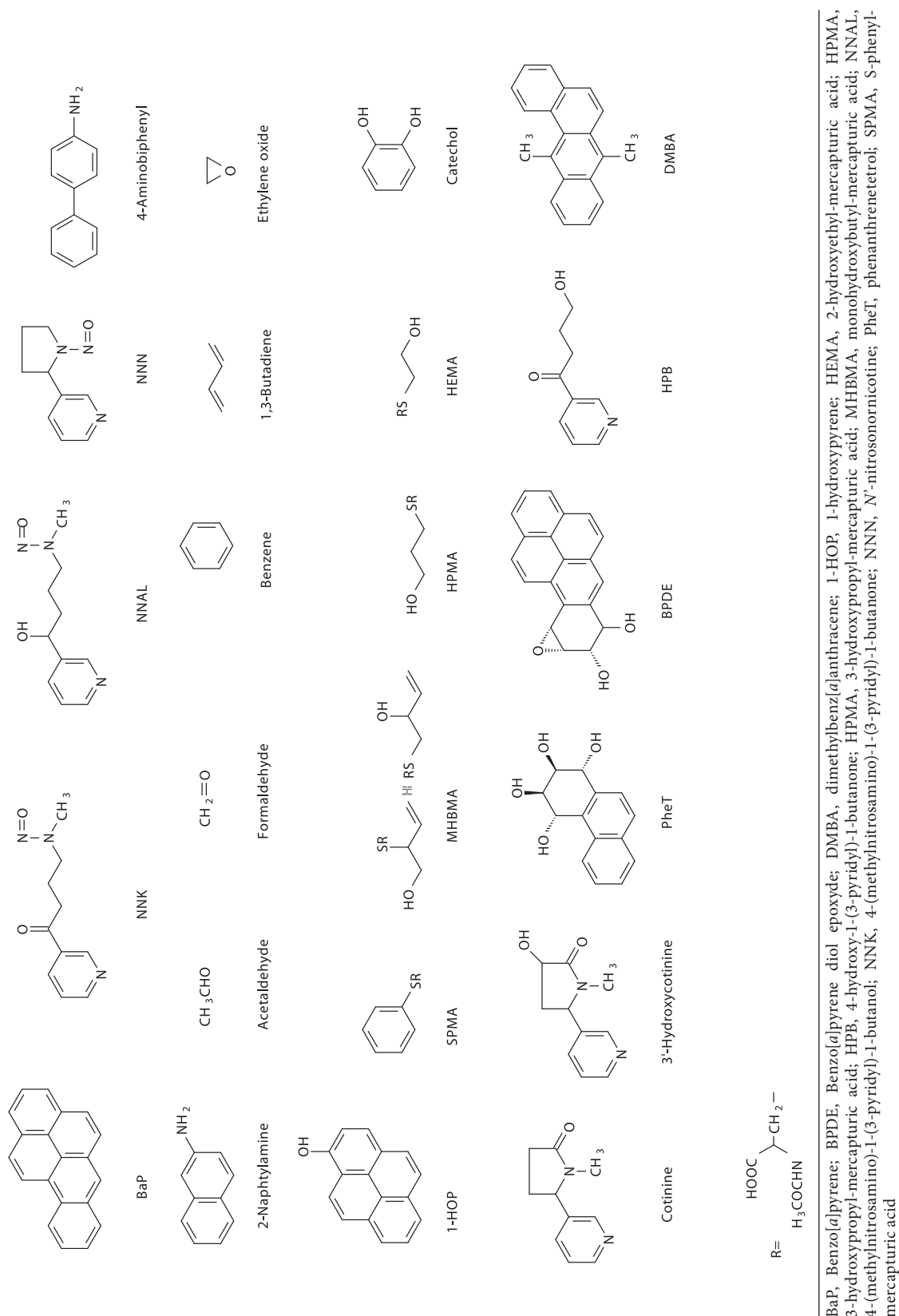
Numerous studies demonstrate the uptake of tobacco smoke carcinogens and toxicants by smokers, and showed higher levels of their metabolites in urine and blood of smokers than non-smokers (Sections 4.1.1 and 4.1.2). There are substantial differences in carcinogen exposure among people because of the number and types of cigarettes they smoke and the ways in which they smoke them. These differences can be monitored in part by biomarkers of exposure such as urinary metabolites or haemoglobin adducts (Section 4.1.2). Haemoglobin adducts of multiple aromatic amines and volatile carcinogens have been demonstrably related to tobacco ([Hatsukami et al., 2006a](#)). There may also be differences in carcinogen exposure due to genetic variations (Section 4.2).

The body's response to cigarette smoke constituents is similar to its response to pharmaceutical agents and other foreign compounds. Drug metabolizing enzymes, most frequently CYPs, convert these compounds to more water soluble forms, facilitating excretion. During this natural protective attempt, some reactive intermediates are formed. These intermediates are frequently electrophilic (electron seeking, or bearing a partial or full positive charge). Electrophilic intermediates may react with water, generally resulting in detoxification, or may covalently bind to nucleophilic (electron rich) sites in DNA, forming DNA adducts ([Guengerich, 2001](#); [Jalas et al., 2005](#)), which are

critical in the carcinogenic process (see Section 4.1.3c). CYP1A1 and CYP1B1, repeatedly shown to be inducible by cigarette smoke via interactions of smoke compounds with the aryl hydrocarbon receptor (AhR), are particularly important in the metabolic activation of PAHs, while CYP2A13 is critical for the metabolism of NNK ([Nebert et al., 2004](#); [Jalas et al., 2005](#)). The inducibility of certain CYPs may be a critical aspect of cancer susceptibility in smokers ([Nebert et al., 2004](#)). CYP1A2, CYP2A6, CYP2E1 and CYP3A4 are also important in the metabolism of cigarette smoke carcinogens to DNA binding intermediates ([Jalas et al., 2005](#)), and aldo-keto reductase enzymes, also induced by tobacco smoke ([Quinn et al., 2008](#)), are involved in the metabolism of NNK, BaP and other tobacco smoke carcinogens. Competing with this process of “metabolic activation” resulting in DNA binding is the intended metabolic detoxification, which leads to harmless excretion of carcinogen metabolites, and is also catalysed by CYPs and a variety of other enzymes including GSTs, uridine diphosphate-glucuronosyl transferases (UGTs), and arylsulfatases. The relative amounts of carcinogen metabolic activation and detoxification differ among individuals. It is widely hypothesized that this balance will affect cancer risk with those having higher activation and lower detoxification capacity being the most susceptible. This premise is supported in part by molecular epidemiologic studies of polymorphisms, or variants in more than 1% of the population, in certain genes coding for these enzymes ([Vineis et al., 2003](#); [Carlsten et al., 2008](#)).

DNA adducts are thought to be a critical lesion in carcinogenesis. Many investigations demonstrate the presence of DNA adducts in human tissues, and some of these are summarized in Section 4.1.2c. There is massive evidence, particularly from studies which use relatively non-specific DNA adduct measurement methods, that DNA adduct levels in the lung and other tissues of smokers are higher than in non-smokers, and some epidemiologic data link

Fig. 4.2 Structures of compounds discussed in the text



these higher adduct levels to increased cancer risk ([IARC, 2004b](#); [Veglia et al., 2008](#)). However, there is much more limited evidence from studies using specific carcinogen-derived DNA adducts as biomarkers ([Pfeifer et al., 2002](#)). Oxidative DNA damage has also been observed, and this may result partially from exposure to metals in cigarette smoke ([Stavrides, 2006](#)).

Cellular DNA repair systems can excise DNA adducts and restore normal DNA structure ([Christmann et al., 2003](#)). These complex multiple systems include direct base repair by alkyltransferases, removal of DNA damage by base and nucleotide excision repair, mismatch repair, and double strand repair. If these DNA repair systems are unsuccessful in fixing the damage, then the DNA adducts can persist, increasing the probability of a permanent mutation. There are polymorphisms in genes coding for some DNA repair enzymes. If these variants lead to deficient DNA repair, the probability of cancer development can increase ([Vineis et al., 2009](#)).

DNA adducts can cause miscoding during replication when DNA polymerase enzymes misread the DNA adduct and consequently insert the wrong base opposite to it. There is some specificity in the relationship between specific DNA adducts formed from cigarette smoke carcinogens and the types of mutations which they cause. G to T and G to A mutations have often been observed (Section 4.1.3) ([Hecht, 1999](#)). Extensive studies have characterized the mutations which occur because of specific carcinogen-DNA adducts ([Delaney & Essigmann, 2008](#)). Mutations have been reported in the *KRAS* oncogene in lung cancer and in the *TP53* tumour suppressor gene in a variety of cigarette smoke-induced cancers ([Ahrendt et al., 2001](#); [Pfeifer et al., 2002](#); [Ding et al., 2008](#)). The cancer causing role of these genes has been firmly established in animal studies ([Lubet et al., 2000](#); [Johnson et al., 2001](#)). A selection and promotion process may also play a role in the final mutation spectrum seen in genes

in smoking-associated tumours ([Rodin & Rodin, 2005](#); [Sudo et al., 2008](#)).

Urinary mutagenicity, sister chromatid exchanges, micronuclei in buccal cells, and other genetic effects have been consistently observed in smokers at higher levels than in non-smokers ([IARC, 2004a](#); [Proia et al., 2006](#)). In addition to mutations, numerous cytogenetic changes are observed in lung cancer, and chromosome damage throughout the field of the aerodigestive tract is strongly associated with cigarette smoke exposure. Mutations resulting from DNA adducts can cause loss of normal cellular growth control functions, via a complex process of signal transduction pathways, ultimately resulting in genomic instability, cellular proliferation and cancer ([Ding et al., 2008](#)). Apoptosis, or programmed cell death, is a protective process, and can remove cells which have DNA damage, thus serving as a counterbalance to these mutational events. The balance between apoptotic mechanisms and those suppressing apoptosis will have a major impact on tumour growth.

While the central track of Fig. 4.1 is the major pathway by which tobacco smoke carcinogens cause cancer, other mechanisms also contribute, as indicated in the top and bottom tracks ([Hecht, 2003](#)). Nicotine, NNK, and NNN bind to nicotinic and other cellular receptors, resulting in activation of serine/threonine kinase Akt (also known as protein kinase B), protein kinase A, and other changes. Nicotine and NNK increase expression of survivin, an inhibitor of apoptosis in normal human bronchial epithelial cells, and survivin mRNA is detected in bronchial brush samples from heavy smokers ([Jin et al., 2008](#)). This can cause decreased apoptosis, increased angiogenesis, and increased transformation ([Heeschen et al., 2001](#); [West et al., 2003](#)). Thus, although nicotine is not carcinogenic, it may enhance carcinogenicity in various ways ([Schuller, 2009](#)). Cigarette smoke also contains well established oxidants, co-carcinogens, tumour promoting fractions, and inflammatory agents, as well as

cilia-toxic compounds such as acrolein, which impede clearance. Many studies demonstrate the co-carcinogenic and cytotoxic effects of catechol, an important constituent of cigarette smoke. An epigenetic pathway frequently observed in tobacco-induced cancers is enzymatic methylation of promoter regions of genes such as *p16* and *FHIT* [fragile histidine triad gene, a gene coding for a dinucleoside 5', 5'''- P¹, P³-triphosphate hydrolase, a putative tumour suppressor protein] resulting in gene silencing, which are also strongly implicated in tobacco-induced lung cancer (D'Agostini *et al.*, 2006; Bhutani *et al.*, 2008). When this occurs in tumour suppressor genes, the result can be unregulated proliferation (Belinsky, 2005). Inflammation due to smoking is associated with tumour promotion and activation of factors such as NFκB. Inflammation also plays a role in chronic obstructive pulmonary disease (COPD), which in turn is an independent risk factor for lung cancer (Smith *et al.*, 2006; Turner *et al.*, 2007; Lee *et al.*, 2008a).

This conceptual model can be applied to smokeless tobacco products. Smokeless tobacco products have much lower levels of carcinogens and toxicants that result from combustion, so the effects of these agents are not seen to a significant extent. The most prevalent strong carcinogens in smokeless tobacco are the tobacco-specific nitrosamines; other nitrosamines, PAHs, aldehydes and metals are also present, and there are large amounts of some inorganic salts that may contribute to inflammation (IARC, 2007a; Stepanov *et al.*, 2008). An additional factor in carcinogenesis by betel quid with tobacco is the basic pH resulting from addition of slaked lime to the quid, leading to oxidative damage and inflammation (IARC, 2004b).

Multiple studies demonstrate that tobacco-specific nitrosamines are absorbed and metabolised in smokeless tobacco users (IARC, 2007a).

There is evidence for DNA adduct formation in oral tissues of smokeless tobacco users, and sister chromatid exchanges, chromosomal

aberrations, and micronuclei – consequences of DNA adduct formation – have been reported (Proia *et al.*, 2006; Warnakulasuriya & Ralhan, 2007). Many studies have demonstrated RAS and TP53 mutations in smokeless tobacco users (Warnakulasuriya & Ralhan, 2007) consistent with the conceptual framework.

Oxidative stress and reactive oxygen species could play a significant role in cancer induction in smokeless tobacco users, particularly at high pH (Boffetta *et al.*, 2008). Chronic local inflammation and irritation induced by smokeless tobacco and its constituents could have a tumour promoting or co-carcinogenic effect (Boffetta *et al.*, 2008). Upregulation of cyclooxygenase-2, involved in prostaglandin synthesis and inflammation, has been observed in animal studies upon exposure to smokeless tobacco (Boffetta *et al.*, 2008). Smokeless tobacco products have relatively high levels of sodium chloride (NaCl), which could contribute to inflammation, tumour promotion, and co-carcinogenesis. Cancer of the oral cavity is strongly associated with tobacco smoking (IARC, 2004a) or chewing (IARC, 2007a) and alcoholic beverage drinking (IARC, 2010a). However only a fraction of exposed subjects develop tumours, which suggests that other exposures such as HPV may be independently involved or act as cofactors. HPV is known to infect the oral cavity of healthy individuals and several HPV-related lesions have been characterized (IARC, 2007b). Herpes simplex virus has also been shown to enhance the carcinogenicity of smokeless tobacco products in animal studies (Park *et al.*, 1986). These factors may contribute significantly to the local carcinogenic effects characteristic of smokeless tobacco use.

4.1.2 Absorption, distribution, metabolism and excretion

There are examples of toxicant and carcinogen metabolism and excretion for representatives of virtually every major class of compounds;

some of these are summarized in [Table 4.1](#). Nicotine and five of its urinary metabolites – cotinine, 3'-hydroxycotinine and their glucuronides, and nicotine glucuronide – comprise about 73–96% of the nicotine dose ([Hukkanen et al., 2005](#)), and are found in blood, sweat, hair and toenails ([Al Delaimy, 2002](#); [Hukkanen et al., 2005](#); [Stepanov et al., 2007](#); [Al Delaimy & Willett, 2008](#)). Metabolites of various polycyclic aromatic hydrocarbons including pyrene, phenanthrene, fluorene, and benzo[a]pyrene have been quantified in human urine and are higher in smokers than in non-smokers ([Hecht, 2002](#); [Hecht et al., 2005a](#); [Jacob et al., 2007](#); [Hansen et al., 2008](#)). Metabolites of tobacco-specific nitrosamines – NNAL and its glucuronides (total NNAL) from NNK; and NNN and its glucuronides (total NNN) from NNN – are present in human urine ([Hecht, 2002](#); [Stepanov & Hecht, 2005](#); [Hecht et al., 2008a](#); [Stepanov et al., 2008](#)). Total NNAL has also been quantified in blood and toenails ([Hecht et al., 2002](#); [Stepanov et al., 2007](#)). Aromatic amine-haemoglobin adducts have been frequently measured in human blood, and their levels increase with smoking ([Hecht, 2002](#); [Hatsukami et al., 2006a](#)). Mercapturic acids of several tobacco smoke compounds such as benzene, 1,3-butadiene, acrolein, and ethylene oxide are present in human urine and are related to smoking ([Carmella et al., 2009](#)). Haemoglobin adducts of acrylonitrile and related compounds are elevated in smokers' blood, and levels of metals such as Cd are increased in smokers' urine ([Carmella et al., 2002](#); [IARC, 2004b](#)).

All of the metabolites listed in [Table 4.1](#) are elevated in cigarette smokers; in studies of second-hand smoke exposure, only nicotine metabolites and urinary total NNAL are consistently increased in exposed versus non-exposed subjects, although one very large study also observed an increase in PAH metabolites ([Pirkle et al., 2006](#); [Hecht, 2008](#); [Suwan-ampai et al., 2009](#)). Smokeless tobacco users have significantly raised levels of nicotine metabolites

and tobacco-specific nitrosamine metabolites compared to non-tobacco users ([Hecht et al., 2007](#)).

4.1.3 Biomarkers

Tobacco carcinogen biomarkers are quantifiable entities that can be *specifically* related to tobacco carcinogens. Specificity to a given carcinogen is critical because tobacco carcinogens vary widely in their potency and target organs.

Considering the mechanistic framework outlined in Fig. 4.1, one could visualize various types of biomarkers. Currently, biomarkers of carcinogen/toxicant dose, reflecting the second box of the central track of Fig. 4.1, are by far the most extensively used and validated. The second most common are measurements of DNA adducts (or protein adducts as their surrogates), but fewer of these have both practical utility and validation with respect to tobacco carcinogen specificity.

The use of tobacco carcinogen biomarkers bypasses many uncertainties in estimation of dose. The most commonly used estimation of dose is self-reported number of cigarettes/day, but this is not a very good marker. It may not be reported accurately and it provides no information on the way in which the cigarettes were smoked, which is critical when one considers the common phenomenon of smoker's compensation. Brand information together with machine smoking measurements of specific components is another way of obtaining a measure of dose. However, machine smoking measurements are known to have limitations and the application of a given machine smoking protocol to a given smoker requires smoking topography measurements for that smoker. A disadvantage of tobacco carcinogen biomarkers is that they are affected to some extent by individual differences in metabolism, which may complicate interpretation of dose.

Table 4.1 Examples of toxicant or carcinogen metabolites in tobacco users

Toxicant or carcinogen	Examples of metabolites in tobacco users	References
Nicotine	Cotinine, 3'-hydroxycotinine and their glucuronides in urine, blood or saliva; nicotine and cotinine in toenails	Al Delaimy (2002) , Hukkanen et al. (2005) , Al Delaimy & Willett (2008) , Stepanov et al. (2007)
Polycyclic Aromatic Hydrocarbons (PAHs)	1-hydroxypyrene, phenanthrols, phenanthrene tetraols, fluorenols, benzo[a]pyrenols, benzo[a]pyrene tetraols in urine	Hecht (2002) , Hecht et al. (2005a) , Hansen et al. (2008) , Jacob et al. (2007)
Tobacco-specific nitrosamines	NNAL and its glucuronides (total NNAL) in urine or blood, total NNN in urine; NNAL and NNN in toenails	Hecht (2002) , Hecht et al. (2002, 2008a) , Stepanov & Hecht (2005) , Stepanov et al., (2007, 2008)
Aromatic amines	Parent amines in urine and haemoglobin adducts in blood	Hecht (2002) , Hatsukami et al. (2006a)
Volatile hydrocarbons		
Benzene 1,3-Butadiene	Muconic acid and S-phenyl-mercapturic acid (SPMA) in urine; Monohydroxybutyl-mercapturic acid (MHBMA) in urine	Hecht (2002) , Carmella et al. (2009)
Acrolein	3-hydroxypropyl-mercapturic acid (HPMA) in urine	Carmella et al. (2009)
Ethylene oxide	2-hydroxyethyl-mercapturic acid (HEMA) in urine, haemoglobin adducts in blood	Bono et al. (2002) , Carmella et al. (2009)
Acrylonitrile	Haemoglobin adducts in blood	Carmella et al. (2002)
Metals	Cadmium in urine	IARC (2004a)

(a) Urinary biomarkers

Probably the most practical and, to date, the most extensively applied tobacco carcinogen biomarkers are urinary metabolites of tobacco carcinogens, and these have been comprehensively reviewed ([Hecht, 2002](#); [IARC, 2004a](#)). Advantages include the ready availability of samples, and concentrations in urine that are easily quantifiable using modern analytical chemistry methods, most frequently liquid chromatography-tandem mass spectrometry (LC-MS/MS). The urinary metabolites listed in [Table 4.1](#) have all been used as biomarkers and all are validated with respect to exposure in cigarette smokers ([Carmella et al., 2009](#)). Total nicotine equivalents (the sum of nicotine and the five metabolites in [Table 4.1](#)) is a particularly effective way of estimating nicotine dose from tobacco products.

Total NNAL, the sum of NNAL and its glucuronides, is a highly useful biomarker of NNK exposure ([Hecht, 2002, 2003](#); [Hatsukami et al., 2006a](#)). The tobacco-specificity of NNK, and therefore total NNAL, is a key feature of this biomarker because studies in which it is applied are not confounded by other environmental or dietary exposures. It also has a considerably longer half-life than cotinine and several other urinary biomarkers. Total NNAL has been used in numerous studies that estimated uptake of NNK in smokers under varying circumstances. In one example, smokers reduced their number of cigarettes smoked per day, but there was not a corresponding decrease in NNK uptake due to compensation ([Hecht et al., 2004](#)). In another study, NNK and PAH uptake, estimated by total NNAL and 1-hydroxypyrene, respectively, were compared in smokers of regular, light, and ultra-light cigarettes, and found to be similar, consistent with epidemiologic studies that

demonstrate no protection against lung cancer in smokers of light compared to regular cigarettes (Hecht *et al.*, 2005b). Other studies evaluated NNK uptake in smokers who switched from their current cigarette brand to products advertised as being less hazardous, but the results generally did not support these claims (Hatsukami *et al.*, 2004). One of the most useful applications of total NNAL has been in studies of non-smokers exposed to second-hand tobacco smoke (Hecht, 2003). The sensitivity and specificity of this biomarker are ideal for such studies, and it is the most commonly elevated tobacco carcinogen biomarker in non-smokers exposed to second-hand smoke. Total NNAL has also found utility in establishing NNK uptake in smokeless tobacco users (Hecht *et al.*, 2002, 2007, 2008a, b; Hecht, 2008).

The relationship of urinary total NNAL to lung cancer was demonstrated in a study of stored urine samples collected years before diagnosis of lung cancer from smokers in Shanghai, China and Singapore (Yuan *et al.*, 2009). There was a significant relationship between total NNAL and lung cancer incidence, after correction for numbers of cigarettes smoked per day and duration of smoking. An 8.5 fold increased risk for lung cancer was observed for those smokers in the highest tertile of total NNAL and cotinine, relative to smokers with the same smoking history but in the lowest tertiles of total NNAL and cotinine. Urinary biomarkers were also used to demonstrate higher uptake of nicotine and NNK per cigarette in smokers with polymorphisms in the nicotinic acetylcholine genes associated with lung cancer in genome-wide association studies (see Section 4.2; Le Marchand *et al.*, 2008). Collectively, these results indicate that urinary total NNAL is not only a biomarker of exposure, but also a biomarker of risk for lung cancer.

(b) Serum and saliva metabolites

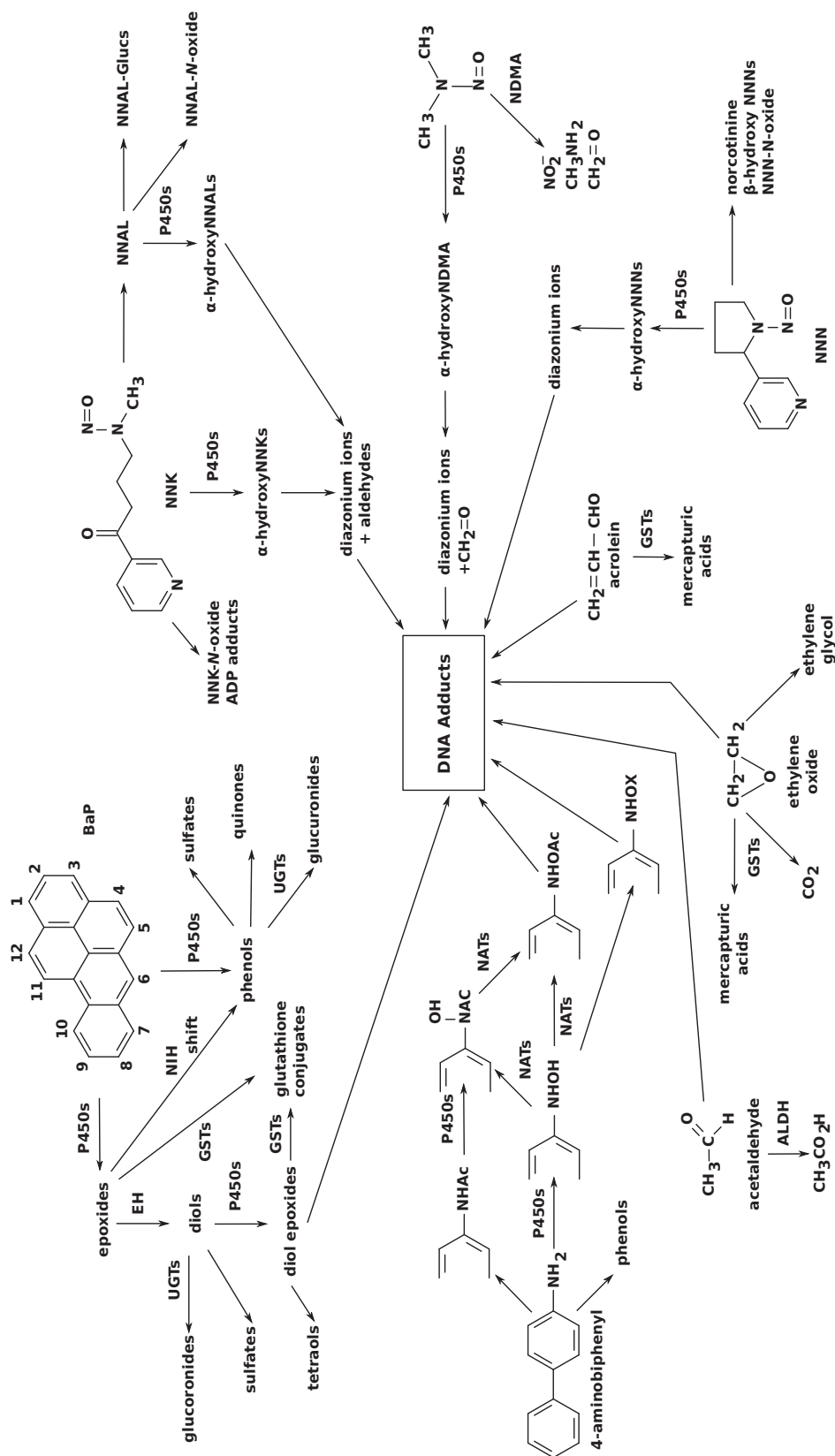
Serum and saliva metabolites have been used as biomarkers much less often than urine metabolites. The most frequently measured tobacco smoke toxicant in serum and saliva is cotinine, documented as a useful biomarker of cigarette smoking in many studies (Lee, 1999; Hukkanen *et al.*, 2005). Total NNAL can be readily quantified in serum and its levels remain relatively constant in a given smoker sampled at bimonthly intervals over a one year period. Consistent with the results described above, one study showed a significant relationship between total NNAL in prospectively collected serum samples from smokers and lung cancer risk (Church *et al.*, 2009). Other biomarkers that have been measured in serum include cadmium, benzene, styrene and *r*-1,*t*-2,3,*c*-4-tetrahydroxy-1,2,3,4-tetrahydrophenanthrene (PheT) (IARC, 2004a; Church *et al.*, 2009).

(c) DNA adducts

Fig. 4.3 presents an overview of metabolism and DNA adduct formation from eight tobacco smoke compounds (clockwise from top left): BaP, NNK, *N*-nitrosodimethylamine (NDMA), NNN, acrolein, ethylene oxide, acetaldehyde and 4-aminobiphenyl. Evidence exists for DNA adduct formation from each of these carcinogens in smokers, based on studies carried out with tissues or blood cells. DNA adduct biomarkers have been applied mainly in studies of smokers, and there is far less evidence from studies of second-hand tobacco smoke or smokeless tobacco use.

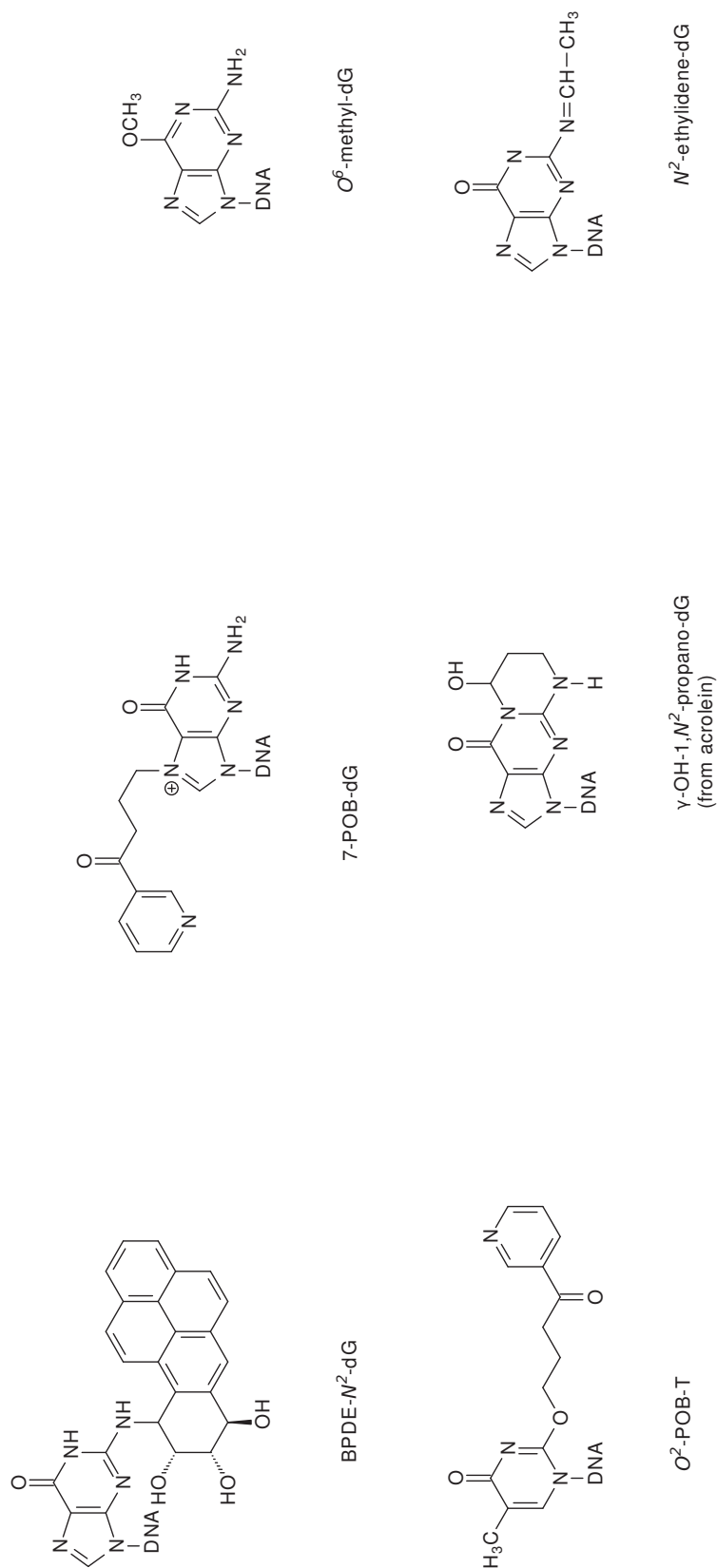
The structures of DNA adducts of tobacco smoke carcinogens have been characterized in detail, but a complete description of these structures is beyond the scope of this section. Selected DNA adduct structures are shown in Fig. 4.4. A major DNA adduct of BaP results from *trans*- addition of the benzo[*a*]pyrene diol epoxide (BPDE) to the *N*²-position of dG (Szeliga

Fig. 4.3 Overview of metabolism and DNA adduct formation from eight tobacco smoke constituents



4-ABP, 4-aminobiphenyl; AC, acetyl; ADP, adenosine diphosphate; ALDH, aldehyde dehydrogenase; AKR, aldo-ketoreductase; B[a]P, benzo[a]pyrene; EH, epoxide hydrolase; Gluc, glucuronide; GSTs, glutathione S-transferases; NATs, N-acetyltransferases; NDMA, N-nitrosodimethylamine; NIH shift, phenomenon of hydroxylation-induced intramolecular migration; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, N'-nitrosaminopyridine; P450s, cytochrome P450 enzymes; UGTs, uridine-5'-diphosphate-glucuronosyl transferases
Adapted from Cooper *et al.* (1983); Preussmann & Stewart (1984); Kadlubar & Beland (1985); Hecht (1998, 1999); Penning & Drury (2007); IARC (2008, 2010b).

Fig. 4.4 Structures of some DNA adducts of tobacco smoke constituents



BPDE-*N*²-dG, benzo[*a*]pyrene diol epoxide-*N*²-deoxyguanosine; 7-POB-dG, pyridyloxobutyl-deoxyguanosine; *O*²-POB-T, *O*²-pyridyloxobutyl-thymidine

& Dipple, 1998). Pyridyloxobutyl (POB)-DNA adducts of NNK and NNN are formed at the 7- and O^6 -positions of deoxyguanosine dG, the O^2 -position of thymidine, and the O^2 -position of deoxycytidine (Hecht, 2008). They can be measured in part as 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB) released upon hydrolysis. Metabolic activation of NNK also leads to 7-methyl-dG and O^6 -methyl-dG, identical to the DNA adducts formed from NDMA and other DNA methylating agents (Hecht, 2008). Ethylating agents and ethylene oxide in cigarette smoke also alkylate dG (Zhao *et al.*, 1999; Singh *et al.*, 2005). Acrolein and crotonaldehyde react with DNA to produce exocyclic 1, N^2 -dG adducts, while acetaldehyde forms a Schiff base adduct with the exocyclic N^2 amino group of dG. There is evidence for the presence of all these DNA adducts in tissues or blood cells of smokers, but there are also many studies in which these specific adducts have been sought but not found (Boysen & Hecht, 2003).

Measurement of these DNA adducts as biomarkers potentially can provide the most direct link between cellular exposure and cancer, because DNA adducts are so critical in carcinogenesis. However, it is challenging because their levels are extremely low, frequently ranging from 1 per 10^6 to 1 per 10^8 normal bases, and the tissue or blood samples containing them are usually available in only small quantities. Fortunately, the routine detection ofamol levels [attomole, equivalent to 10 moles] of DNA adducts by conventional LC-MS/MS techniques is now feasible (Singh & Farmer, 2006). There are still relatively few examples of quantitation of *specific* DNA adducts of tobacco carcinogens in tissues of smokers using mass spectrometry, high pressure liquid chromatography (HPLC)-fluorescence, HPLC with electrochemical detection, or postlabelling techniques (Pfeifer *et al.*, 2002). A much larger body of work has used the highly sensitive, but relatively non-specific ^{32}P -postlabelling and immunoassay methods of

DNA adduct detection. Although the adducts detected using ^{32}P -postlabelling are often referred to as “aromatic DNA adducts,” there is strong evidence that they are not related to PAHs (Arif *et al.*, 2006). Adduct levels are generally higher in lung tissues of smokers than non-smokers while studies using blood DNA have produced varied results. Adducts have also been detected in the larynx, oral and nasal mucosa, bladder, cervix, breast, pancreas, stomach, placenta, foetal tissue, cardiovascular tissues, sputum, and sperm of smokers (IARC, 2004a). A meta-analysis of the relationship of DNA adduct levels in smokers to cancer, as determined by ^{32}P -postlabelling in the majority of studies or enzyme linked immunosorbent assay (ELISA), demonstrated a positive relationship in current smokers (Veglia *et al.*, 2003; 2008).

(d) Protein adducts

Carcinogen-haemoglobin (Hb) and serum albumin adducts are regarded as surrogates for DNA adduct measurements. Although these proteins are not targets for carcinogenesis, virtually all carcinogens that react with DNA will also react with protein. Advantages of haemoglobin adducts include the ready availability of haemoglobin from blood and the relatively long lifetime of the erythrocyte in humans – 120 days –, which provides an opportunity for adducts to accumulate. Studies on protein adducts in smokers have been comprehensively reviewed (IARC, 2004a).

Haemoglobin adducts of aromatic amines are a highly informative type of carcinogen biomarker, with levels that are consistently higher in smokers than non-smokers, particularly for 3-aminobiphenyl and 4-aminobiphenyl-Hb adducts. Haemoglobin binds aromatic amines efficiently because heme accelerates the rate of nitrosoarene formation from the hydroxylamine, which is produced metabolically from the aromatic amine by CYP1A2 (Fig. 4.3; Skipper & Tannenbaum, 1990). Binding of the nitrosoarene occurs at the β -93 cysteine residue of human

haemoglobin; the adduct is hydrolysed releasing the free amine, which is quantified by GC-MS (Skipper & Tannenbaum, 1990). Adduct levels are clearly related to cigarette smoking (Skipper & Tannenbaum, 1990). Adducts that form at the terminal valine of haemoglobin are also useful biomarkers: examples include those derived from ethylene oxide, acrylonitrile and acrylamide (Bergmark, 1997; Fennell *et al.*, 2000). Ethylated N-terminal valine of haemoglobin is also higher in smokers than in non-smokers (Carmella *et al.*, 2002).

HPB-releasing Hb adducts of NNK and NNN have been quantified in studies of smokers and smokeless tobacco users (IARC, 2004a, 2007a). These adducts are thought to be tobacco-specific, but some studies report their presence in non-smokers (Falter *et al.*, 1994; Schlöbe *et al.*, 2008).

4.1.4 Genetic and related effects

(a) Mutagenicity and cytogenetic effects

Tobacco smoke and its condensates are mutagenic in a wide variety of test systems from bacteria to mammalian cells in culture to rodents and humans (DeMarini, 2004; IARC, 2004a; Husgafvel-Pursiainen, 2004). In bacterial systems, the heterocyclic amines and aromatic amines in condensates account for much of the frameshift mutagenicity, whereas the PAHs and nitrosamines may account for some of the base-substitution mutagenicity (DeMarini *et al.*, 1995). G to T is the predominant class of base-substitution mutation induced by condensates in experimental systems and found in oncogenes and tumour-suppressor genes in smoking-associated lung tumours (IARC, 2004a). The genotoxic potencies of a variety of condensates in several genotoxicity assays likely have only qualitative value with regard to health risk assessment (DeMarini *et al.*, 2008). This is consistent with findings that smokers of low- or high-tar cigarettes have similar urinary levels of lung carcinogens (Hecht *et al.*, 2005b; Hatsukami *et al.*,

2006b) and similar risks for lung cancer (Harris *et al.*, 2004).

In rodents, cigarette smoke induces sister chromatid exchange and micronuclei in bone marrow and lung cells. Human newborns of smoking mothers have increased frequencies of *HPRT* mutations, chromosomal translocations, and DNA strand breaks. Sperm of smokers has increased frequencies of aneuploidy, DNA adducts, strand breaks, and oxidative damage. Cigarette smoke also causes germ-cell mutations in mice (Yauk *et al.*, 2007). Collectively, these data suggest that smoking is likely a germ-cell mutagen in humans. Smoking produces mutagenic urine and somatic-cell mutations in humans, including *HPRT* mutations, sister chromatid exchange, microsatellite instability and DNA damage in a variety of tissues. Genotoxic effects have been found in eight organ sites at which tobacco smoke causes cancer in humans (DeMarini, 2004; IARC, 2004a).

(b) Mutations in *TP53*, *KRAS* and related genes

Gene mutation data from a variety of databases, including the IARC Cancer *TP53* Mutation Database (<http://www-p53.iarc.fr/>), have been collated in the Genetic Alterations in Cancer (GAC) database (<http://dir-apps.niehs.nih.gov/gac/>) so that mutations in a variety of genes in various cancerous tissues can be compared. An assessment of the Gene Alterations in Cancer database showed that at least three genes were mutated more frequently in lung tumours from smokers than non-smokers (Lea *et al.*, 2007): *TP53* (39 versus 26%), *K-RAS* (20 versus 3%), and loss of heterozygosity at *FHIT* (57 versus 27%). Thus, genes in the cell cycle (*TP53*), cell signalling (*KRAS*) and apoptotic (*FHIT*) pathways are mutated more frequently in smoking- rather than in nonsmoking-associated lung tumours. Genomic sequencing of lung tumours has identified other mutated genes that are associated with smoking; ten times more genes are mutated in

lung tumours from smokers compared to non-smokers ([Ding et al., 2008](#)).

GC to TA transversions were the predominant class of base-substitution mutation found in *TP53* and *KRAS* genes in lung tumours from smokers, with the frequency of this mutation in *TP53* being 30% in smokers versus 22% in non-smokers. In smoking-associated oral cancers, the percentage of GC to TA mutations in *TP53* was 15% versus 2%, respectively. This mutation spectrum is consistent with that produced by a variety of known carcinogens present in tobacco smoke ([IARC, 2004a](#)). At the codon level, the most frequently mutated codons in *TP53* in lung tumours of smokers were 157, 175, 245, 248, and 273, all of which occur in the DNA-binding domain of the protein; among these codons, only 273 was mutated in lung tumours from non-smokers. Only three of these codons (157, 245 and 273) were mutated in smoking-associated larynx tumours, and only codon 157 was mutated in smoking-associated oral tumours. Thus, the mutational specificity at *TP53* is different among smoking- and nonsmoking-associated tumours and among smoking-associated tumours at various organs ([Lea et al., 2007](#)). Thus, different pathways are involved in the development of different types of tumours ([Le Calvez et al., 2005](#); [Mounawar et al., 2007](#); [Subramanian & Govindan, 2008](#)).

4.1.5 Effects on gene expression profile

As indicated in a review by [Sen et al. \(2007\)](#) involving microarray analysis of 18 studies in human smokers, 7 in smoke-exposed rodents, and 3 in condensate-exposed mammalian cells, smoking generally upregulated a wide variety of genes, especially those involved in the stress response, phase I metabolism, and immune response. Genes that were consistently expressed differentially in smokers (as assessed in alveolar macrophages, lung cells or peripheral lymphocytes) included metallothioneins, heat-shock

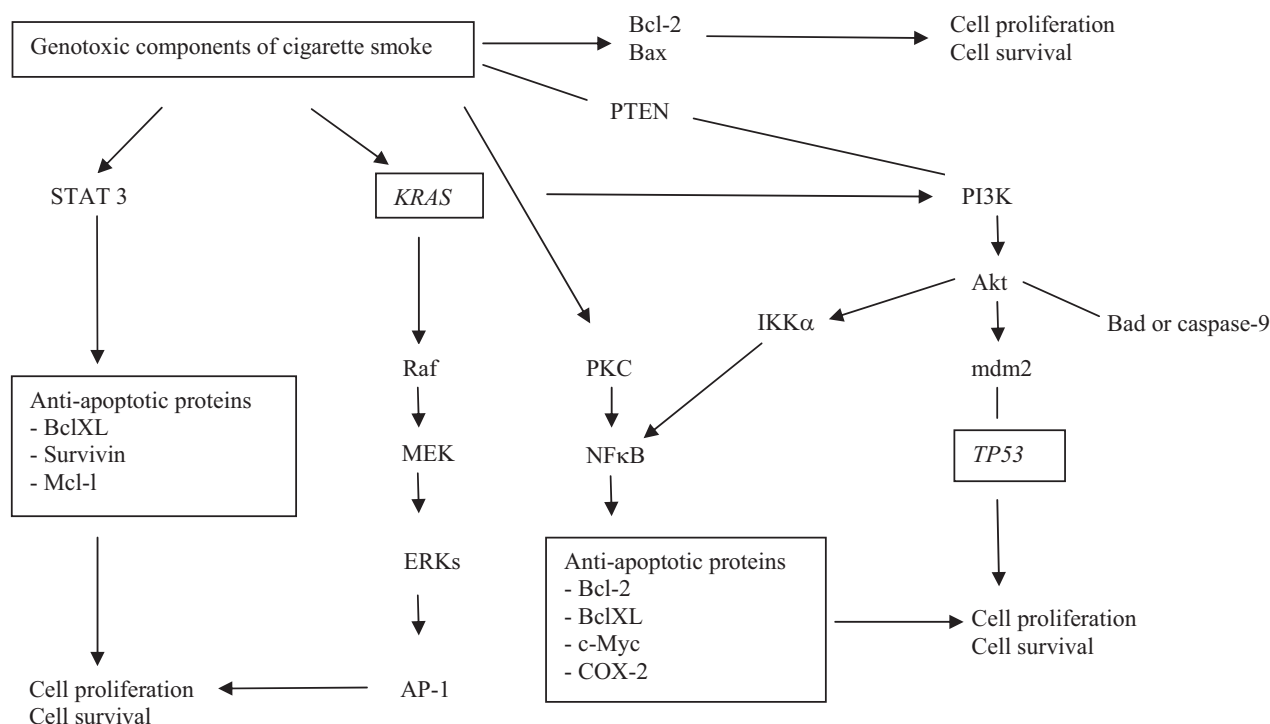
proteins, superoxide dismutase, glutathione transferase, heme oxygenase, *CYP* genes (*1A2*, *1A1* and *1B1*), interleukins and chemokines.

[Spira et al. \(2004\)](#) analysed global gene expression in bronchial epithelial cells and found that the expression levels of metabolizing and antioxidant genes had reverted to control levels after two years of smoking cessation. However, expression of potential oncogenes and tumour-suppressor genes never reverted to never-smoker levels even after years of smoking cessation. Consistently, expression of microRNAs is generally downregulated by cigarette smoke ([Izzotti et al., 2009](#)). As discussed below, smoking also altered methylation patterns and gene expression in smoking-associated tumours.

4.1.6 Other effects associated with carcinogenesis

(a) Proliferation, differentiation, apoptosis, and inflammation

As noted above, the signal-transduction pathways in lung tumours from smokers are distinctly different from those of non-smokers ([Mountzios et al., 2008](#)). Fig 4.5 shows details of signalling pathways that are deregulated by tobacco smoke. The involvement of high frequencies of mutated *K-RAS* and *TP53* genes in smoking-associated lung tumours results in altered regulation of cell proliferation, differentiation, cytoskeletal organization and protein trafficking. Cigarette smoking activates *NF- κ B*, which induces pro-inflammatory cytokine expression and induces growth factors and proliferative signals ([Mountzios et al., 2008](#)). This gene also influences the expression of the anti-apoptotic gene *BCL2* and pro-apoptotic gene *BAX*. Smoking produces chronic inflammation, which promotes cancer ([Walser et al., 2008](#)). Smoking results in high levels of reactive oxygen species, which damage epithelial and endothelial cells and impair their function. In smoking-associated lung cancer, elevated levels of cyclooxygenase-2 (COX-2) and

Fig. 4.5 General scheme of some cell-signalling pathways that are deregulated by tobacco smoke in lung carcinogenesis

Akt, serine/threonine protein kinase; ERKs, extracellular regulated kinases; MEK, mitogen-activated protein kinase; Bad, Bcl2-associated agonist of cell death; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; NF-κB, nuclear factor κB; IKKα, inhibitor of nuclear factor κ-B kinase; PTEN, phosphatase and tensin homologue; STAT3, signal transducer and activator of transcription, COX-2, cyclooxygenase-2

prostaglandin (PGE₂) indicate apoptosis resistance, proliferation, immunosuppression, angiogenesis, invasion, and epithelial-mesenchymal transition (Walser *et al.*, 2008).

(b) Endogenous nitrosation

Intragastric formation of *N*-nitroso compounds, measured using urinary nitrosamino acids excreted in urine, was increased in smokers compared to non-smokers (Hoffmann & Brunnemann, 1983). Two recent studies demonstrated that NNN forms endogenously in some users of nicotine replacement therapy products (Stepanov *et al.*, 2009a, b).

(c) Hormonal changes

These are described in Section 4.3.2a.

4.2 Polymorphisms in carcinogen-metabolizing genes

4.2.1 Introduction

It has been long proposed that the known variation among individuals in their capacity to activate and detoxify carcinogens may be associated with increased susceptibility to cancer, and that polymorphisms of carcinogen-metabolising genes may play a significant role. The most intensively studied genes involved in the metabolism of carcinogens include the various *CYP* genes, the *GST* genes and the *NAT* genes. Other relevant xenobiotic-metabolising genes, such as *EPHX*, sulfotransferase (*SULT*), *UGT*, myeloperoxidase (*MPO*), and NAD(P)H quinone oxidoreductase-1 (*NQO1*) genes, have also been studied. Recently, extensive pooled studies and

reviews have been published on polymorphisms of carcinogen-metabolising genes and their role in cancer susceptibility, especially in tobacco-related lung cancer and cancers at other sites. Similarly, various biomarkers of exposure and genotoxicity that are presumed to provide a mechanistic basis for such associations have been comprehensively investigated in relation to these polymorphisms. A brief overview based largely on reviews and the meta- and pooled analyses is presented here.

4.2.2 Genetic polymorphisms of carcinogen metabolism: some central genes

(a) CYP genes

CYPs comprise the principal enzyme system catalysing various phase I oxidation reactions, including metabolic activation and detoxification of many carcinogenic substances in tobacco smoke such as PAHs. Of the various CYP enzymes expressed in humans, many of those belonging to CYP1 to CYP3 families play a role in carcinogen metabolism, producing highly reactive DNA-damaging metabolites as well as detoxified metabolites ([Guengerich & Shimada, 1998](#); [Lang & Pelkonen, 1999](#); [Ingelman-Sundberg, 2004](#)). CYPs have evolved into a wide superfamily with close to 60 different active genes currently identified; most of these genes exhibit polymorphism (www.cypalleles.ki.se).

(i) CYP1A1

Several allelic variants of the human *CYP1A1* gene are currently known (www.cypalleles.ki.se). The major variant forms of the *CYP1A1* gene (wildtype allele *CYP1A1*1*) mostly frequently studied for association to cancer susceptibility include the following two alleles: (i) *CYP1A1*2A* allele (m1 allele; *Msp* I) and (ii) *CYP1A1*2B* ([Cascorbi et al., 1996](#)) or *CYP1A1*2C* (www.cypalleles.ki.se) allele (m2 allele; Ile⁴⁶²Val). Importantly, the *CYP1A1* m1 allele and m2 allele are in complete linkage disequilibrium in

Caucasians ([Kawajiri, 1999](#); [Bartsch et al., 2000](#)). In addition, *CYP1A1*4* allele (m4; Thr⁴⁶¹Asn) ([Cascorbi et al., 1996](#)), and *CYP1A1*3* (m3) allele found in African-Americans but not in Caucasians or Asians ([Crofts et al., 1993](#)) are included in some studies ([Bartsch et al., 2000](#)).

In smoking-related lung cancer, the various *CYP1A1* polymorphisms as well as the differences in the frequencies of the rare variant alleles between ethnicities contribute to the differences in findings. There are collective analyses of data predominantly indicating an overall mild to moderate effect of *CYP1A1* polymorphisms on lung cancer risk ([Kawajiri, 1999](#); [Bartsch et al., 2000](#); [Houlston, 2000](#); [Le Marchand et al., 2003](#); [Vineis et al., 2003](#); [Vineis et al., 2004](#); [Lee et al., 2008a](#); [Shi et al., 2008](#)). In many reviews and meta- or pooled analyses the increased risk associated with *CYP1A1* polymorphism has most clearly been seen in Asian populations ([Kawajiri, 1999](#); [Le Marchand et al., 2003](#); [Vineis et al., 2003](#); [Lee et al., 2008a](#); [Shi et al., 2008](#)).

Multiple studies have also analysed the gene-gene interactions between *CYP1A1*, *GSTM1* and *GSTT1* polymorphisms and lung cancer ([d'Errico et al., 1999](#); [Houlston, 1999](#); [Benhamou et al., 2002](#); [Bolt & Thier, 2006](#); [Raimondi et al., 2006](#); [Ye et al., 2006](#); [Carlsten et al., 2008](#)). Some of the analyses have indicated that the elevated risk for lung cancer may be more pronounced for some *CYP1A1/GSTM1* null genotype combinations ([Le Marchand et al., 1998](#); [Bartsch et al., 2000](#); [Vineis et al., 2004, 2007](#); [Lee et al., 2008a](#); [Shi et al., 2008](#)).

(ii) CYP1A2

CYP1A2 is highly inducible and metabolises, including deacetylation reactions, many tobacco smoke carcinogens such as aromatic and heterocyclic amines and nitro-aromatic compounds, and tobacco-specific nitrosamines such as NNK ([Nebert et al., 2004](#); [Jalas et al., 2005](#); [IARC, 2007a](#)). A few major variant alleles have been described (www.cypalleles.ki.se), some of which

may have been reported to influence inducibility ([Nakajima et al., 1999](#); [Ingelman-Sundberg et al., 2007](#)). Overall, the phenotype-genotype relations have not been well established for *CYP1A2*, although current evidence points towards contribution of genetic variation ([Murayama et al., 2004](#); [Ingelman-Sundberg et al., 2007](#)); data on possible associations with tobacco related cancer are sparse ([Agundez, 2004](#); [Nebert & Dalton, 2006](#)).

(iii) *CYP2A6*

Several aspects of smoking behaviour are likely to be influenced by *CYP2A6* genetic variation, which influences nicotine metabolism ([Malaiyandi et al., 2005](#); [Mwenifumbo & Tyndale, 2007](#)). The most important functionally altered allele is *CYP2A6**4 (gene deletion), which confers a poor-metabolizer phenotype in homozygous individuals ([Malaiyandi et al., 2005](#); [Ingelman-Sundberg et al., 2007](#); [Mwenifumbo & Tyndale, 2007](#)). In some studies, polymorphic variants of *CYP2A6* gene have been implicated in susceptibility to smoking-related cancers ([Gambier et al., 2005](#); [Malaiyandi et al., 2005](#); [Nakajima, 2007](#)). In line with this, the accumulated data have suggested that *CYP2A6* polymorphism may affect cancer risk in smokers but not in non-smokers ([Tan et al., 2001](#); [Kamatani et al., 2005](#); [Malaiyandi et al., 2005](#); [Canova et al., 2009](#)).

(iv) *CYP2A13*

From human CYPs, *CYP2A13* is the primary form involved in the metabolic activation of the tobacco-specific nitrosamines NNK and NNN ([Jalas et al., 2005](#); [IARC, 2007a](#)). The *CYP2A13* gene exhibits polymorphism in humans ([Zhang et al., 2002](#); [Jalas et al., 2005](#)), and experimental studies suggest that some of the polymorphisms may affect the hydroxylation of NNN and NNK ([Jalas et al., 2005](#); [Schlicht et al., 2007](#)). However, the data on possible effects of these polymorphisms on the risk of tobacco-related cancers in

humans are still limited ([Wang et al., 2003](#); [Song et al., 2009](#); [Timofeeva et al., 2009](#)).

(v) *CYP2D6*

The *CYP2D6* gene shows high variability in expression. The enzyme is not inducible, and therefore genetic variation largely contributes to the interindividual variation in enzyme activity. Currently, more than 100 different functional *CYP2D6* gene variants have been described, and these are divided into alleles causing abolished, decreased, normal, and ultrarapid enzyme activity ([Ingelman-Sundberg, 2005](#); [Ingelman-Sundberg et al., 2007](#)). The most important null alleles leading to poor-metabolizer phenotype are *CYP2D6**4 (splice defect) and *CYP2D6**5 (gene deletion) ([Ingelman-Sundberg, 2005](#); [Ingelman-Sundberg et al., 2007](#)).

A large series of studies have been carried out over the past 20 years on the association between *CYP2D6* polymorphism and susceptibility to lung cancer and to some other tobacco-related cancers ([Wolf & Smith, 1999](#)). Despite some indication of an association between *CYP2D6* poor-metabolizer and decreased risk for lung cancer, no major role for *CYP2D6* in carcinogen metabolism or a molecular basis for such an association have been discovered ([Wolf & Smith, 1999](#); [Ingelman-Sundberg, 2005](#)).

(vi) *Other CYP genes*

CYP1B1 allelic variants that affect the catalytic activity have been described but they have been studied to a lesser extent for the association with susceptibility to smoking-related cancers ([Thier et al., 2003](#)). Some positive findings have been reported on head and neck cancer ([Ko et al., 2001](#)), and lung cancer ([Zienolddiny et al., 2008](#)).

Several polymorphisms have been characterized in the *CYP2E1* gene and several positive associations with the risk of different cancers have been reported, in particular for cancers of the upper aerodigestive tract, lung and gastrointestinal tract (Section 2.19). *CYP2E1* may also

play an important role in the interaction of the carcinogenic effects of alcohol and tobacco (Section 4.4).

From the human *CYP3A* locus (*CYP3A4*, *CYP3A5* and *CYP3A7*), the *CYP3A4*1B* allele has been associated with lung cancer and prostate cancer in some studies but not in all ([Dally et al., 2003](#); [Rodriguez-Antona & Ingelman-Sundberg, 2006](#)). However, the role of these variants in relation to tobacco smoking is unknown.

(b) *GSTM1* and other *GST* genes

Polymorphic *GST* genes have long been proposed to modify susceptibility to lung cancer ([Seidegård et al., 1986](#); [Ketterer et al., 1992](#)). The polymorphic genes encoding the various classes of cytosolic *GST* enzymes include the *GSTM1* and *GSTM3* genes (mu class), the *GSTP1* gene (pi class), and the *GSTT1* gene (theta class). The gene deletion (null) allele of the *GSTM1* gene (*GSTM1*0*) and of the *GSTT1* gene (*GSTT1*0*) have been the most intensively studied polymorphisms in relation to increased susceptibility to cancer ([Strange et al., 2001](#); [Bolt & Thier, 2006](#); [McIlwain et al., 2006](#)). For the *GSTP1* gene, the form most abundantly present in lung tissue, genetic variation in exon 5 (*GSTP1*2*; *Ile¹⁰⁵Val*), in exon 6 (*Ala¹¹⁴Val*), as well as a combination of these, are the variations most frequently studied for cancer susceptibility ([Watson et al., 1998](#); [Cote et al., 2009](#)).

Numerous reviews, meta- and pooled analyses have been published over the past 15 years or so for the *GST* genes with systematic assessments covering altogether tens of thousands of cases and controls. For the *GSTM1* null genotype, such analyses have largely provided negative, suggestive or at most moderately positive results for an association with an increased risk for lung cancer ([d'Errico et al., 1999](#); [Houlston, 1999](#); [Benhamou et al., 2002](#); [Ye et al., 2006](#); [Carlsten et al., 2008](#)). The larger the studies, the less significant the estimates for the role of *GSTM1* emerge in systematic analysis ([Ye et al., 2006](#); [Carlsten](#)

[et al., 2008](#)). Also the varying allele frequencies related to ethnic background affect the findings for *GSTM1* as well as for many other genes ([Garte et al., 2001](#); [Ye et al., 2006](#); [Carlsten et al., 2008](#); [Lee et al., 2008a](#)).

In a meta-analysis of the association between the *GSTT1* gene polymorphism and lung cancer no association between *GSTT1* null genotype and risk for lung cancer in Caucasians was observed, but a positive association was found for Asians ([Raimondi et al., 2006](#)). A significant association for either Caucasians or Asians was also not found in a pooled analysis ([Raimondi et al., 2006](#)). A meta-analysis found no significant association between lung cancer risk and the *GSTP1 Ile¹⁰⁵Val* polymorphism; but the pooled analysis suggested an overall statistically significant mild association between lung cancer and homozygosity or heterozygosity for the *Val¹⁰⁵* allele ([Cote et al., 2009](#)).

A recent body of epidemiologic data suggests an inverse association between cruciferous vegetables/isothiocyanates intake and cancers of the colorectum, lung and breast; the studies also provide evidence that this protective effect is greater among individuals who possess the *GSTM1* or *T1* null genotype, who would be expected to accumulate higher levels of isothiocyanates at the target tissue level, a pre-requisite for their enzyme-inducing effects ([Seow et al., 2005](#)). The association between isothiocyanates and cancer, and its modification by *GSTM1* and *GSTT1* status, is most consistent for lung cancer and appears to be strongest among current smokers who possess the combined *GSTM1* and *GSTT1* null genotypes ([London et al., 2000a](#); [Spitz et al., 2000](#); [Zhao et al., 2001](#); [Brennan et al., 2005](#); [Seow et al., 2005](#)).

(c) *NAT1* and *NAT2* genes

The pooled and meta-analyses carried out on *NAT1* and *NAT2* polymorphisms and bladder cancer risk have consistently reported significantly increased risk for *NAT2* slow acetylators

([Dong et al., 2008](#); [Malats, 2008](#); see also Section 2.9). Data on *NAT1* fast acetylators are inconsistent, as are the studies suggesting an increased risk for *NAT2* rapid acetylator status. Additionally, genotypes for other genes, specially *GSTM1*, have also been implicated ([Vineis et al., 2001](#); [García-Closas et al., 2005](#); [Hein, 2006](#); [Sanderson et al., 2007](#); [Dong et al., 2008](#); [Malats, 2008](#)).

In a recent large study on tobacco-related lung cancer and upper aerodigestive cancers, the *NAT* genes, in particular *NAT*10* haplotype, emerged from a set of 16 genes as involved in the risk ([McKay et al., 2008](#)). When more than one hundred single nucleotide polymorphisms for 31 genes involved in phase I or phase II metabolism or in antioxidant defence were investigated, only four of the previously reported polymorphisms of the *GSTP1*, *EPHX1* and superoxide dismutase *SOD2* genes and the *NAT1* fast acetylator phenotype remained significantly associated with risk of non-small cell lung cancer after correction for multiple testing ([Zienolddiny et al., 2008](#)).

In breast cancer, several recent meta-analyses of epidemiological studies have suggested increased risk among smokers with the *NAT2* slow acetylator genotype; such an association has been observed especially among long-term smokers and post-menopausal women ([Terry & Goodman, 2006](#); [Ambrosone et al., 2008](#); [Ochs-Balcom et al., 2007](#); [Baumgartner et al., 2009](#)).

In all, the role of the *NAT* gene polymorphisms in tobacco-related cancers, with the exceptions of increased risk of bladder cancer and possibly breast cancer in *NAT2* slow acetylators, remains largely open due to the incomplete understanding of phenotype-genotype relationships, and the interplay between these two genes and their polymorphisms ([Hein, 2002, 2006](#)).

(d) Others

Genes coding for *EPHX*, *UGT* and *SULT* enzymes, mainly but not exclusively involved in detoxification reactions, exhibit polymorphisms with numerous gene variants discovered

([Mackenzie et al., 1997](#); [London et al., 2000b](#); [Glatt et al., 2001](#); [Burchell, 2003](#)). Additional polymorphic genes studied for their significance in cancer susceptibility are the *NQO1* and *MPO* genes, with *NQO1* playing a dual role in the detoxification and activation of procarcinogens, and *MPO* converting lipophilic carcinogens into hydrophilic forms ([Nebert et al., 2002](#)). All these genes have been studied for their possible association with tobacco-related cancer risk to a varying extent and with variable outcomes ([London et al., 2000b](#); [Bamber et al., 2001](#); [Garte, 2001](#); [To-Figueras et al., 2001](#); [Tiemersma et al., 2002b](#); [Guillemette, 2003](#); [Wells et al., 2004](#); [Kiyohara et al., 2005](#); [Moreno et al., 2005](#); [Nagar & Remmel, 2006](#); [Gallagher et al., 2007](#)).

4.2.3 Biomarkers of tobacco carcinogenesis and polymorphic genes of carcinogen metabolism

A myriad of studies have investigated association between various biomarkers of tobacco-related carcinogenesis and genetic variation of genes involved in carcinogen metabolism. For involvement in increased cancer susceptibility, a large variety of intermediate biomarkers have been studied, including PAH metabolites in urine, urinary mutagenicity, DNA and protein adducts, cytogenetic alterations, *HPRT* mutant lymphocytes, as well as somatic mutations of the tumour suppressor gene *TP53* and *KRAS* oncogene occurring in cancer tissue.

(a) PAH metabolites and mutagenicity in urine

(i) PAH metabolites in urine

Increased excretion of 1-hydroxypyrene in urine in association with the *GSTM1* null genotype has been reported in many studies on individuals with occupational or environmental exposure to PAHs ([Yang et al., 1999](#); [Alexandrie et al., 2000](#); [Lee et al., 2001](#); [Kuljukk-Rabb et al., 2002](#); [Kato et al., 2004](#)). The associations seen between *GSTM1* polymorphism and the

PAH metabolites are somewhat more variable. Similarly, the joint effect of *GSTM1* and *GSTT1* null genotypes, as well as the effects of some other genes of xenobiotic metabolism, such as *EPHX*, *CYP1A1*, *CYP1A2* and the aryl hydrocarbon receptor (*AhR*) gene have been either positive or negative ([Yang et al., 1999](#); [Alexandrie et al., 2000](#); [Lee et al., 2001](#); [Zhang et al., 2001](#); [Kuljukkka-Rabb et al., 2002](#); [Yang et al., 2003](#); [Chen et al., 2007](#); [Cocco et al., 2007](#); [Bin et al., 2008](#)).

Another PAH metabolite studied in this context is phenanthrene, the simplest PAHs with a bay region, a feature closely associated with carcinogenicity. A study quantified ratios of urinary products of metabolic activation (such as PhET) and detoxification (such as phenanthrols, HOPhe) of phenanthrene in 346 smokers, who were also genotyped for 11 polymorphisms in genes involved in PAHs metabolism, including the *CYP1A1* and *GSTM1* genes. A significant association between the presence of the *CYP1A1* Ile⁴⁶²Val polymorphism and high PhET/3-HOPhe ratios was found, particularly in combination with the *GSTM1* null polymorphism ([Hecht et al., 2006](#)).

Overall, the data on the influence of genetic variation in PAHs metabolism on the levels of the urinary metabolite biomarkers are variable, and currently inconclusive.

(ii) Urinary mutagenicity

One relatively early line of research investigated the relationship between urinary mutagenicity and genetic variation in activation or detoxification genes. These studies, however, have seldom been focused on smokers only but rather on other sources of exposure ([Pavanello & Clonfero, 2000](#)).

In some studies, *NAT2* slow acetylator genotype either alone or in combination with *GSTM1* null genotype has been associated with increased urinary mutagenicity in the *Salmonella* test in individuals with occupational, environmental or

medicinal PAH-related exposure, or in smokers ([Vineis & Malats, 1999](#); [Pavanello & Clonfero, 2000](#)). In another study, *CYP1A2* activity, but not *NAT2*, *GSTM1* or *GSTT1* genotypes influenced urinary mutagen excretion in smokers ([Pavanello et al., 2002](#)). A further study also suggested contribution of the *CYP1A2* gene variation to increased urinary mutagenicity in heavy smokers ([Pavanello et al., 2005](#)). Associations with variants of other xenobiotic-metabolising genes (such as *EPHX1*) have also been reported, with somewhat complex results ([Kuljukkka-Rabb et al., 2002](#)).

(b) DNA adducts

The relationship between the variants of polymorphic genes of carcinogen metabolism and tobacco smoke-related DNA adduct formation has been addressed in an abundant number of studies among smokers, occupationally exposed groups, and patients with smoking-related cancer. In addition, multiple *in vitro* studies on this relationship have been carried out ([Bartsch et al., 2000](#); [Pavanello & Clonfero, 2000](#); [Alexandrov et al., 2002](#); [Wiencke, 2002](#)).

The intensive efforts to study the relationship between *CYP1A1* and *GSTM1* gene polymorphism and the level of aromatic-hydrophobic/bulky PAH-DNA adducts in human lungs have so far provided little evidence for a role of a single metabolic genotype or their combinations on DNA adduct formation, with largely weak, non-significant or contradictory results. However, a trend of increasing adduct levels in subjects with the *CYP1A1**2-*GSTM1**0 genotype combination has been observed, which was reinforced when BPDE-DNA adducts were specifically assessed. These results suggest a gene-gene interaction, supported by biological data from other studies ([Bartsch et al., 2000](#); [Alexandrov et al., 2002](#); [Wiencke, 2002](#)). Such gene-gene interaction lends support to the increased risk for lung cancer found in carriers of these genotypes in Japanese, among whom the frequency of the

variant *CYP1A1* allele is much higher ([Bartsch et al., 2000](#); [Alexandrov et al., 2002](#)).

A wide selection of genes and genotypes included in the various studies have made it difficult to assess the overall role of the polymorphisms of *GSTM1* and other genes alone or in combination. Differences between the studies in the types of adducts determined, the various tissues, cell types and cancers studied, detection methods, variation in sources and types of exposure, sample size, gender differences, and sometimes poor knowledge regarding the alleles, genotypes and haplotypes under study also contribute to the large variability seen in these studies ([d'Errico et al., 1999](#); [Hemminki et al., 2001](#); [Alexandrov et al., 2002](#); [Wiencke, 2002](#)).

(c) Cytogenetic biomarkers of genotoxicity

(i) Chromosome aberrations and sister chromatid exchanges

Early studies investigating whether homozygosity for the *GSTM1* null allele affects prevalence of cytogenetic changes in lymphocytes of smokers reported positive results ([Seidegård et al., 1990](#); [van Poppel et al., 1992](#); [Cheng et al., 1995](#)). Since then, studies have investigated the association between genetic polymorphisms of xenobiotic-metabolising genes and cytogenetic biomarkers in smokers and in some occupational groups ([Rebbeck, 1997](#); [Autrup, 2000](#); [Pavanello & Clonfero, 2000](#); [Norppa, 2003, 2004](#)).

Collectively, the reported findings are in support of increased susceptibility of smokers to chromosomal effects in association with *GSTM1* and *GSTT1* null variants deficient in detoxification of tobacco smoke carcinogens. Exposure to genotoxins generated from other environmental sources (e.g. polluted air, diet, endogenous sources such as reactive oxygen species) may contribute to the observed associations, and it is likely that other polymorphic metabolic genes such as *NAT2* may be involved ([Pavanello & Clonfero, 2000](#); [Norppa, 2001, 2003](#)).

(ii) Micronucleus induction

The relationship between formation of micronuclei and genetic polymorphisms of carcinogen metabolism has been addressed in a wide range of human population studies ([Norppa, 2003, 2004](#)). Induction of micronuclei in smokers may be little, if at all, affected by *GSTM1*, *GSTT1* or *NAT2* genotypes. In contrast, the *NAT1* rapid genotype appears to show an association with increased susceptibility to smoking-related micronuclei ([Norppa, 2004](#)).

A recent review evaluated more than seventy human studies on genetic polymorphisms and micronucleus frequency detected either in peripheral blood lymphocytes or exfoliated cells in populations exposed to various genotoxic agents. There were no significant genotype effects involved in micronucleus induction in smokers ([Iarmarcovai et al., 2008](#)). The relationship between genetic polymorphisms and micronucleus formation is complex, and is influenced to a variable extent by several genes of xenobiotic metabolism and DNA repair, as well as the variety of chromosomal alterations known to contribute to micronucleus formation ([Iarmarcovai et al., 2008](#)).

(iii) Chromosomal damage induced in vitro

The effects of genotypes or genotype combinations *in vitro* on the induction of various cytogenetic endpoints by tobacco-smoke carcinogens and their metabolites have been studied, initially focused on the *GSTM1* and *GSTT1* null genotypes ([Norppa, 2001, 2004](#)). In a study investigating NNK *in vitro*, lymphocytes from *GSTM1* null donors were more sensitive to induction of chromosomal aberrations and sister chromatid exchanges by NNK than lymphocytes from *GSTM1* positive donors ([Salama et al., 1999](#)).

(d) *Gene mutations*

(i) *HPRT mutant lymphocytes*

Associations between the frequencies of *HPRT* mutant T-lymphocytes in populations exposed to genotoxic agents, such as smokers, and the polymorphism of xenobiotic-metabolising genes have been studied. In the early studies, positive, weak, or negative associations were reported for *GSTM1* null genotype, and negative findings were published for *NAT2* slow acetylator genotype in occupationally exposed or non-exposed subjects (Rebbeck, 1997; Vineis & Malats, 1999). When healthy, non-smoking and occupationally non-exposed young adults were studied for *HPRT* mutant frequency and polymorphisms in *CYP1A1*, *GSTM1* and *NAT2* genes, none of these polymorphisms, analysed individually, were found to influence the *HPRT* mutant frequency (Davies *et al.*, 1999). A significant interaction between the *GSTM1* null genotype and *NAT2* slow acetylator was associated with higher mutant frequency, but no other genotype combinations (Davies *et al.*, 1999). Some later studies have reported variable associations between *HPRT* mutant frequency and polymorphisms for either individual genes (*GSTM1*, *GSTT1* or *EPHX1*) or some of the genotypes in combination among exposed (Viezzler *et al.*, 1999; Abdel-Rahman *et al.*, 2001, 2003).

(ii) *Mutations of the TP53 gene and other cancer-related genes*

Whether the frequency of somatic mutations detected in tumour tissue in cancer-related genes, primarily the *TP53* tumour suppressor gene and *KRAS* oncogene, may be modified by polymorphisms in carcinogen metabolizing genes was first investigated assessing the effects of the *GSTM1* genotype, alone or in combination with other genetic polymorphisms. Several, but not all, such studies showed significant association between *GSTM1* null genotype and either the frequency or type of *TP53* mutations in

smoking-induced lung cancer or other cancer type (Rebbeck, 1997; Vineis & Malats, 1999; Autrup, 2000). Fewer studies examined the association between *TP53* mutations and *GSTT1* polymorphism, and some results suggested the involvement of both null genotypes (Vineis & Malats, 1999; Autrup, 2000).

In smokers with non-small cell lung cancer, the risk of mutation was found to be the highest among the homozygous carriers of the *CYP1A1* rare allele *CYP1A1 MspI* (Ile⁴⁶²Val) who also exhibited the *GSTM1* null genotype (Kawajiri *et al.*, 1996). Similarly, positive associations between *K-RAS* mutations and homozygosity for the *CYP1A1* rare allele were observed; the risk of mutation was enhanced when the *CYP1A1* susceptible genotype was combined with *GSTM1* null genotype (Kawajiri *et al.*, 1996). In another study, also carried out in a Japanese study population, *K-RAS* mutations occurred with greater frequency in lung adenocarcinoma smoking patients and of the *GSTM1* null genotype as compared with the *GSTM1* positive genotype (Noda *et al.*, 2004).

Many of the studies that assessed *NAT2* acetylator genotypes have found non-significant associations with the frequency or type of *TP53* mutation in bladder, lung, or other cancers (Vineis & Malats, 1999; Autrup, 2000). A study on bladder cancer did not find an overall association between *TP53* mutation frequency and *GSTM1*, *GSTT1*, *GSTP1* or *NAT2* genotypes. However, among patients with *TP53* mutations, transversion mutations were more frequent in those with *GSTM1* null genotype as compared to those with *GSTM1* positive genotype; no significant associations were found for the *NAT2* gene (Ryk *et al.*, 2005).

In rectal cancer, overall negative results for an association between *TP53* or *KRAS* mutations and *GSTM1* and *NAT2* polymorphisms among smokers and non-smokers exposed to tobacco smoke were found (Curtin *et al.*, 2009). An interaction of second-hand tobacco smoke and *NAT2*

was found in *TP53* mutation positive tumours but not in smokers ([Curtin et al., 2009](#)). Earlier, an increased risk of *TP53* transversion mutations among *GSTM1* positive individuals who smoked cigarettes was found in colon cancer ([Slattery et al., 2002](#)).

A statistically significant association was observed between the *GSTT1* null genotype and *TP53* mutation status of breast tumour in one study ([Gudmundsdottir et al., 2001](#)), while in another larger study none of the genotypes for *CYP1B1*, *GSTM1*, *GSTT1* and *GSTP1* genes alone were associated with somatic *TP53* mutations ([Van Emburgh et al., 2008](#)).

In summary, data from various cancer types on the association between genetic polymorphisms of carcinogen-metabolizing genes and somatic mutations of the *TP53* and *K-RAS* genes vary widely and do not permit to conclude ([Rebbeck, 1997](#); [Vineis & Malats, 1999](#); [Autrup, 2000](#)).

4.3 Site-specific mechanisms of carcinogenicity of tobacco smoke

4.3.1 Sites with sufficient evidence of carcinogenicity of tobacco smoking

(a) Lung

The conceptual model presented in Section 4.1 (Fig. 4.1) depicts the main mechanistic steps by which cigarette smoke causes cancer. Smokers inhale into their lungs carcinogens which, either directly or after metabolism, covalently bind to DNA, forming DNA adducts (see Section 4.1, Fig. 4.3). Tobacco smoke contains multiple strong lung carcinogens such as NNN, NNK, PAHs, 1,3-butadiene and cadmium. Levels of tobacco smoke-related DNA adducts, mainly ³²P-postlabelled aromatic-hydrophobic/PAH-related bulky DNA adducts, in the lung are higher in smokers than in non-smokers ([Phillips, 2002](#); [IARC, 2004a](#); [Hecht, 2008](#)). Higher levels

of DNA adducts have further been linked to increased risk for cancer in pooled and meta-analyses ([IARC, 2004a](#); [Veglia et al., 2008](#)).

Mutations in *TP53* and *K-RAS* genes, two central genes of human carcinogenesis, are more frequently mutated in smokers' lung cancer as compared to lung cancer from non-smokers ([DeMarini, 2004](#); [IARC, 2004a](#); [Lea et al., 2007](#); [Ding et al., 2008](#); see Section 4.1.3). In particular, *TP53* but also to some extent *K-RAS* mutations found in smoking-associated lung tumours exhibit mutational specificity that is consistent with the pattern produced by PAH diol epoxides in experimental studies and different from that observed in non-smokers' lung cancer ([Pfeifer et al., 2002](#); [DeMarini, 2004](#); [IARC, 2004a](#); [Le Calvez et al., 2005](#); Section 4.1.3). Keeping with such exposure-specific mutation profile, lung cancer in non-smokers exposed to second-hand tobacco smoke shows mutational similarity to smokers' lung cancer, although less data are available ([Husgafvel-Pursiainen, 2004](#); [IARC, 2004a](#); [Le Calvez et al., 2005](#); [Subramanian & Govindan, 2008](#)). The different pathways of lung carcinogenesis for smokers and non-smokers are likely to involve somatic mutations and other genetic alterations in a larger set of genes that are critical in controlling normal cellular growth via signal transduction ([Bode & Dong, 2005](#); [Lea et al., 2007](#); [Ding et al., 2008](#)).

Smoking-related lung carcinogenesis also involves a multitude of other alterations influencing the complex pathogenic pathways involved in lung cancer development, such as increased inflammation, aberrant apoptosis, increased angiogenesis, tumour progression and tumour metastasis ([Wolff et al., 1998](#); [Heeschen et al., 2001](#); [Schuller, 2002](#); [West et al., 2003](#); [Smith et al., 2006](#); [Lee et al., 2008b](#); Section 4.1.5). Continued exposure to toxicants, genotoxins, carcinogens, co-carcinogens and tumour promoters present in tobacco smoke has major effects on biological processes at all steps of multistep tumourigenesis of human lung ([Hecht,](#)

2003, 2008; Section 4.1). For example, nicotine in tobacco smoke is currently not described as a full carcinogen, but it exerts its biological effects via binding to nicotinic and other cellular receptors and likely enhances cell transformation and carcinogenicity through mechanisms not yet defined (Heeschen *et al.*, 2001; West *et al.*, 2003).

Numerous studies have provided evidence that the human genome may contain one or several loci that confer susceptibility to lung cancer. There are low-penetrance genes involved in the metabolism of tobacco smoke carcinogens, DNA repair and cell cycle control that may influence individual susceptibility to lung cancer (Spitz *et al.*, 2006). The role of the polymorphisms of these various classes of genes in lung carcinogenesis requires a systematic evaluation of the genetic evidence with stringent criteria (Ioannidis, 2008; Risch & Plass, 2008; Vineis *et al.*, 2009; Sections 4.1 and 4.2). Recently, genome-wide association studies have identified a susceptibility locus at chromosome 15q25.1 (Amos *et al.*, 2008; Hung *et al.*, 2008; Thorgeirsson *et al.*, 2008). The identity or function of the gene is not yet known, nor is the mechanism through which it may predispose to lung cancer. It is however likely that lung cancer susceptibility is related to the nicotine receptor gene residing at 15q25.1, and there is some evidence suggesting that it may be related to increased uptake of nicotine and NNK per cigarette (Le Marchand *et al.*, 2008).

In addition to genetic alterations, a growing body of evidence shows that epigenetic mechanisms, such as aberrant DNA methylation, histone modifications and RNA-mediated gene silencing are involved in cancer development (Jones & Baylin, 2007; Cortez & Jones, 2008). In lung carcinogenesis, gene promoter-associated (CpG island-specific) hypermethylation is an early and frequent event causing transcriptional inactivation of genes involved in regulation of cellular growth and differentiation (Belinsky, 2004). For example, several studies have indicated that the tumour suppressor gene *p16*

(*p16^{INK4a/CDKN2A}*), a cell cycle regulator, is among the genes most frequently inactivated by aberrant methylation in lung cancer from smokers (Belinsky, 2004), with differences seen between smokers and never-smokers (Toyooka *et al.*, 2006). Significant associations have been established between smoking and promoter hypermethylation of tumour suppressor genes in lung tumours from smokers, and in plasma, serum or sputum DNA from cancer-free smokers (Belinsky, 2004; Belinsky *et al.*, 2005, 2006; Toyooka *et al.*, 2006).

(b) Oral cavity

PAHs can be carcinogenic at the site of application, which could include the human oral cavity. DMBA, a highly carcinogenic PAH not present in tobacco or tobacco smoke, is a standard model compound for induction of oral tumours in the hamster cheek pouch; less is known about the effects on the oral cavity of PAHs that do occur in tobacco products (Shklar, 1972; Rao, 1984; Vairaktaris *et al.*, 2008). A mixture of NNN and NNK induced oral tumours in rats when applied locally (Hecht *et al.*, 1986), and DNA adduct formation from NNN, NNK and NNAL has been observed in the rat oral cavity (Zhang *et al.*, 2009a, b). HPB-releasing DNA adducts from NNK and/or NNN have been reported in exfoliated oral cells from smokers and smokeless tobacco users (Heling *et al.*, 2008) and HPB-releasing haemoglobin adducts are elevated in smokeless tobacco users (IARC, 2007a). Unidentified DNA adduct levels are consistently elevated in oral cells and tissues from smokers compared to non-smokers (IARC, 2004a). Mutations in the *TP53* gene have been observed in oral tumours from smokers and smokeless tobacco users (IARC, 2006b, 2007a; Warnakulasuriya & Ralhan, 2007). Tobacco-associated genetic mutations including micronuclei, gene mutations, DNA polymorphisms, and chromosomal abnormalities have been reported in studies of buccal cells from smokers and smokeless tobacco users (Proia *et al.*, 2006). The use of lime by betel quid chewers is associated

with enhanced oxidative damage that could play a role in inflammation or tumour promotion ([IARC, 2004b](#)).

(c) *Larynx and nasopharynx*

Hamsters exposed to cigarette smoke by inhalation consistently developed benign and malignant tumours of the larynx; tumours were produced by inhalation of the particulate phase, but not the gas phase of cigarette smoke ([IARC, 1986](#)). In related studies in which hamsters were treated with DMBA by intratracheal instillation followed by exposure to cigarette smoke, a significantly higher incidence of laryngeal tumours was observed than in hamsters exposed only to cigarette smoke or to DMBA ([IARC, 1986](#)). Collectively, these results indicate an initiation-promotion mechanism for the production of laryngeal tumours, and are consistent with the results of experiments in which tobacco smoke condensate is applied to mouse skin ([IARC, 1986](#)). The combined data implicate PAHs and tumour promoters in tobacco smoke as potential etiological agents for cancer of the larynx in hamsters. Levels of DNA adducts measured by non-specific methods were higher in larynx tissue from smokers than from non-smokers ([IARC, 2004a](#)). Analyses of mutations in the *TP53* gene from tumours of the larynx in smokers show a pattern similar to that observed in lung tumours, and both are consistent with the pattern produced by PAH diol epoxides ([IARC, 2006b](#)). The available data are consistent with the conceptual framework illustrated in Fig. 4.1 ([Szyfter et al., 1999](#)).

Formaldehyde, a constituent of cigarette smoke, causes nasopharyngeal cancer in humans ([IARC, 2006a](#)). A recent study demonstrates a 10-fold higher level of the formaldehyde-DNA adduct N⁶-hydroxymethyldeoxyadenosine in leukocytes of smokers compared to non-smokers, suggesting its possible involvement in nasopharyngeal cancer in smokers ([Wang et al., 2009](#)). Acetaldehyde, another carcinogenic constituent of tobacco smoke, which also

forms genotoxic adducts (Section 4.1), may also contribute to the development of these forms of head and neck cancer.

(d) *Oesophagus*

Nitrosamines are probably the most effective oesophageal carcinogens known, with particularly strong activity in the rat ([Lijinsky, 1992](#)). NNN and NDEA are both present in cigarette smoke, and levels of NNN greatly exceed those of NDEA ([IARC, 2004a](#)). NNN is also present in considerable quantities in smokeless tobacco and betel quid containing tobacco ([IARC, 2004a, 2007a](#)). Thus, NNN is a likely candidate as a causative agent for esophageal cancer in smokers, smokeless tobacco users, and chewers of betel-quid with tobacco. While considerable mechanistic data are available from studies of NNN in laboratory animals ([Hecht, 1998](#); [Wong et al., 2005](#); [Lao et al., 2007](#); [Zhang et al., 2009a](#)), there are little comparable data in humans.

Increased acetaldehyde production derived both from tobacco smoke and from microbial alcohol oxidation may play a role in the synergistic carcinogenic action of alcohol and smoking on oesophagus, as well as on other upper aerodigestive locations ([Homann et al., 2000](#); [Salaspuro & Salaspuro, 2004](#); [Lee et al., 2007a](#)).

(e) *Stomach*

Hypermethylation of the E-cadherin 1 gene (*CDH1*) was observed preferentially in gastric tumours from smokers rather than non-smokers ([Poplawski et al., 2008](#)). *CDH1* can act as a tumour-suppressor gene, preventing cells from growing and dividing in an uncontrolled way to form a cancerous tumour. Because the protein encoded by this gene helps cells stick together, altered regulation may lead to metastasis.

[Boccia et al. \(2007\)](#) found an increased risk for stomach cancer among smokers who had the *SULT1A1 His* genotype, and [Lee et al. \(2006\)](#) found an increased risk for those who had the *m2* allelic variant of *CYP1A1*. A nested case-control

study found that smokers had an increased risk of gastric cancer if they carried at least one variant allele A in Ex7+129 C > A (*Thr⁴⁶¹Asn, m4*) of *CYP1A1* ([Agudo et al., 2006](#)). Stomach cancer tissue from smokers had higher levels of stable DNA adducts than did those from non-smokers; however, the number of non-smokers was quite small ([Dyke et al., 1992](#)).

(f) *Pancreas*

NNK and its metabolite NNAL are the only pancreatic carcinogens known to be present in tobacco and tobacco smoke. NNK was detected in the pancreatic juice of 15 of 18 samples from smokers, at levels significantly higher than in non-smokers; NNAL and NNN were also detected in some samples ([Prokopczyk et al., 2002](#)). DNA adducts of NNK and NNAL were present in pancreatic tissue of rats treated with these nitrosamines ([Zhang et al., 2009b](#)), but were not detected in most human pancreatic tissue samples ([Prokopczyk et al., 2005](#)).

(g) *Colorectum*

Tobacco smoke contains heterocyclic amines, such as 2-amino-1-methyl-6-phenylimidazo[4,5,6]pyridine (PhIP), which are intestinal carcinogens in rats and mutate the adenomatous polyposis coli (*Apc*) gene in mice ([Møllersen et al., 2004](#)). The *APC* gene is frequently mutated and has altered expression in human colon cancer ([Samowitz et al., 2007](#); [Samowitz, 2008](#)). A recent model of colon cancer by [Sweeney et al. \(2009\)](#) suggests that this disease can develop via at least three independent mechanistic pathways. One pathway is initiated by methylation of *MINT* (methylation in tumour) markers that proceeds down a pathway predisposing to microsatellite instability, followed by methylation of the mismatch repair gene mutL homologue 1 (*MHL1*) and the tumour-suppressor gene *TP16*, followed by mutation in *BRAF* (a homologue of a viral raf oncogen). A second independent pathway is initiated with a mutation in the *APC*

gene, followed by a mutation in the *TP53* gene. A third independent pathway involves only *KRAS2* mutations. One study found BPDE-DNA adducts at a higher frequency in colon DNA from smokers than from non-smokers ([Alexandrov et al., 1996](#)). Mutations or epigenetic changes in some or all of these genes have been found in smoking-associated colon or colorectal tumours.

Microsatellite instability, which is the expansion or contraction of short nucleotide repeats, occurs in approximately 10–15% of sporadic colorectal cancer, and is usually associated with smoking and hypermethylation of the promoter of the mismatch repair gene *MLH1* ([Samowitz, 2008](#)). Smoking-associated colorectal tumours also have high frequencies of methylation at CpG islands ([Samowitz, 2008](#)).

In a case-control study of colorectal cancer, [Kasahara et al. \(2008\)](#) found that the genetic polymorphism *APEX1/APE1* (apurinic/apyrimidinic endonuclease-1) Asp¹⁴⁸Glu, which is a gene involved in DNA repair, was associated with risk for colorectal cancer among smokers but not non-smokers. Other studies have also found associations between polymorphisms in the DNA repair genes *XRCC1* and smoking and risk for colorectal cancer ([Stern et al., 2007](#); [Campbell et al., 2009](#)).

(h) *Liver*

Tobacco smoke contains liver carcinogens such as furan and certain nitrosamines. Liver tumours exhibit increased expression of *C-MYC*, *P16^{INK4A}*, epidermal growth factor receptor (*EGFR*), telomerase, transforming growth factor- α (*TGF- α*), insulin-like growth factor-2 (*IGF-2*) and *RAF* oncogene ([Abou-Alfa, 2006](#)). Smokers show altered expression of some of these genes or of genes in the same or similar pathways ([Sen et al., 2007](#)). A genome-wide association study found that SNP rs1447295 in the 8q24 chromosome was positively associated with liver cancer among ever-smokers ([Park et al., 2008](#)). Thus, tobacco smoke appears to have epigenetic

effects on the liver that may contribute to hepatocellular carcinoma.

(i) *Urinary bladder*

Tobacco smoke contains aromatic amines such as 4-aminobiphenyl and 2-naphthylamine, which are human bladder carcinogens (see [IARC, 2012a](#)). In bladder tumours, smoking was associated with a more than twofold increase risk of methylation of the promoter region of the *PI6^{INK4A}* gene and of the soluble Frizzled receptor protein (*SFRP*) gene ([Marsit et al., 2006](#)). In addition, [Tang et al. \(2009\)](#) suggested that epigenetic silencing of *Wnt* antagonists through hypermethylation may play a role in smoking-related invasive bladder cancer ([Tang et al., 2009](#)). SNP rs6983267 of the 8q24 chromosome was inversely associated with bladder cancer among ever-smokers ([Park et al., 2008](#)). Smokers generally have mutagenic urine and smoking is associated with specific cytogenetic changes and DNA breaks in bladder tumours ([DeMarini, 2004](#)). Smoking-associated stable DNA adducts have been found in bladder tissue or exfoliated urothelial cells, supporting a role for DNA damage in smoking-associated bladder cancer ([Phillips, 2002](#)).

(j) *Cervix*

The cervical mucus of smokers is more mutagenic than that of non-smokers, and cervical epithelia of smokers have higher frequencies of micronuclei than those of non-smokers ([DeMarini, 2004](#)). Several studies have found increased levels of DNA adducts in cervical tissue from smokers relative to non-smokers, suggesting a role for smoking-associated DNA damage in cervical cancer ([Phillips, 2002](#)).

(k) *Ovary*

It has been observed that the inverse associations reported for serous and endometrioid tumours with respect to parity and oral contraceptives did not hold for the mucinous tumours.

Based on these observations, [Risch et al. \(1996\)](#) suggested that mucinous ovarian tumours may be etiologically unrelated to the other types of epithelial tumours. Whereas mucinous elements such as gastric or intestinal type glands may be seen in mature teratomas, a form of germ cell neoplasia, overall mucinous tumours are classified as surface epithelial tumours because transitions among the subtypes may be observed. The major difference between mucinous and serous tumours is their biologic behaviour. Mucinous carcinomas of the ovary are slow growing tumours that appear to develop from their benign counterparts. The fact that the transitions between the benign, borderline, and malignant form of the disease can be seen in the same tumour suggests that over time, there is a progression from benign to malignant ([Riopel et al., 1999](#)). *K-ras* mutational analysis, for example, demonstrates a heterogeneous distribution of the mutation within different parts of the same neoplasm, suggesting that acquisition of the *K-ras* mutation occurs in malignant transformation ([Mandai et al., 1998](#)). Serous carcinomas seem to develop de novo rather than from a benign pre-existing lesion; alternatively, the rate of progression is rapid and the precursor lesion is obliterated before the detection of the tumour. In some data, current smoking is associated with a shorter interval to detection of mucinous than non-mucinous tumours. Because the mucinous tumour is slow growing, smoking could contribute to the malignant progression of the adenoma-carcinoma sequence, as the benign form of the tumour may have been present for some time.

(l) *Leukaemia*

Tobacco smoke contains known leukaemogens such as benzene, 1,3-butadiene and formaldehyde ([IARC, 2012a](#)). The mechanisms of leukaemogenesis are currently not well understood. Data indicate that leukaemogenic agents, such as benzene, cause toxicity to the

haematopoietic system, as well as genotoxicity at low levels, and that genetic polymorphisms may be involved in these processes ([Aksoy, 1989](#); [Lan et al., 2004](#); [Garte et al., 2008](#); [Hosgood et al., 2009](#); [Lau et al., 2009](#); [Rappaport et al., 2009](#)). Recent studies suggest the importance in carcinogen-related leukaemogenesis of damage to haematopoietic stem/progenitor cells circulating in the peripheral blood, or, alternatively, damage to primitive pluripotent progenitor cells present in other tissues ([Zhang et al., 2009c](#)). In these two models, damaged stem/progenitor cells would then travel to the bone marrow and become initiated leukaemic stem cells. Mechanisms considered central in these models are: disruption of bone marrow DNA, through e.g. formation of DNA adducts, DNA-protein crosslinks, the action of free radicals or active states of oxygen; intercalation of metals within the DNA structure; or inhibition of enzymes involved in cell division ([Zhang et al., 2007, 2009c](#)).

4.3.2 Sites with limited evidence of carcinogenicity or evidence suggesting lack of carcinogenicity

(a) Breast

(i) Carcinogenic pathway

Carcinogens found in tobacco smoke pass through the alveolar membrane and into the blood stream, by means of which they can be transported to the breast via plasma lipoproteins ([Yamasaki & Ames, 1977](#); [Shu & Bymun, 1983](#); [Plant et al., 1985](#)). Tobacco smoke contains known rodent mammary carcinogens, including PAHs and aromatic amines ([IARC, 1986, 2004a](#); [el-Bayoumy, 1992](#); [Ambrosone & Shields, 1999](#); [Ambrosone, 2001](#); [Hoffmann et al., 2001](#)) which, due to their lipophilicity, can be stored in breast adipose tissue ([Obana et al., 1981](#); [Morris & Seifter, 1992](#)) and then metabolized and activated by human mammary epithelial cells ([MacNicoll et al., 1980](#)). Tobacco smoke constituents reach

the breast as demonstrated by the detection of cotinine in breast fluid ([Petrakis et al., 1978](#)). There is evidence suggesting the presence of mutagenic arylamines ([Thompson et al., 2002](#)) and PAHs ([Zanieri et al., 2007](#)) in human breast milk. Cigarette smoke condensate has been shown to transform normal human breast epithelial cells *in vitro* ([Narayan et al., 2004](#)), perhaps by blocking long-patch base excision repair ([Kundu et al., 2007](#)). Transformation and cytogenetic effects have been observed in human mammary epithelial cells after exposure to chemical carcinogens such as PAHs or arylamine ([Mane et al., 1990](#); [Eldridge et al., 1992](#); [Calaf & Russo, 1993](#)).

The formation of specific adducts from PAHs and aromatic amines has been observed in human breast epithelial cells *in vitro*, and unspecified-DNA adducts have been found in exfoliated ductal epithelial cells in human breast milk ([Gorlewska-Roberts et al., 2002](#); [Thompson et al., 2002](#)).

Mutations in the *TP53* tumour suppressor gene have been found in 15–30% of breast cancers ([Goldman & Shields, 1998](#); [Olivier & Hainaut, 2001](#)). An increased prevalence and altered spectrum of *TP53* mutations in breast tumours have been observed among current smokers compared with never smokers ([Conway et al., 2002](#)). The breast tumours with the most pronounced smoking-related mutational pattern (for example, a greater number of G:C→T:A transversions) were from women who had smoked for more than 20 years, although total *TP53* mutations were not associated with smoking duration ([Conway et al., 2002](#)). This increased frequency of G to T transversions in smokers versus non-smokers is also observed in the IARC *TP53* database ([IARC, 2006b](#); [Van Emburgh et al., 2008](#)).

Recent meta-analyses of epidemiological studies tend to show positive associations of breast cancer with long-term smoking among *NAT2* slow acetylators, especially among post-menopausal women (who are more likely than pre-menopausal women to be very long-term

smokers). [Firozi et al. \(2002\)](#) showed that breast tissue from NAT2 slow acetylators had significantly higher levels of the diagonal radioactive zone (smoking-related) DNA adduct pattern than that from fast acetylators.

High rates of breast cancer in women exposed to ionizing radiation during adolescence (aged 10–19 years at exposure) ([Tokunaga et al., 1987](#)) suggested that the adolescent breast may also be sensitive to the DNA-damaging effects of other exposures. This might also be true for the genotoxic compounds contained in tobacco smoke. Although some studies have supported such association, the results have been sparse and mixed. In addition, it is difficult to separate the effects of early life exposure to tobacco and smoking duration ([Terry & Rohan, 2002](#)).

Early age at first full-term pregnancy has been associated with reduced breast cancer risk ([Kelsey et al., 1993](#)), hypothetically due to terminal differentiation of the breast epithelium that occurs late in the first trimester. It has been suggested that in the early stages of pregnancy, when growth-promoting hormone levels are high, but before terminal differentiation ([Montelongo et al., 1992](#)), the breast may be particularly susceptible to the cancer-promoting chemicals in tobacco smoke. Several epidemiological studies compared measures of smoking before and after a first full-term pregnancy. Although suggestive, the data did not consistently show an increased risk for breast cancer among women who smoked before a first full-term pregnancy ([Adami et al., 1988](#); [Hunter et al., 1997](#); [Band et al., 2002](#); [Egan et al., 2003](#); [Gram et al., 2005](#); [Li et al., 2005](#); [Olson et al., 2005](#); [Cui et al., 2006](#)). Smoking was associated with a 50% increased risk among women with slow NAT2 acetylation genotype ([Egan et al., 2003](#)). Overall, studies of risk in association with the timing of smoking relative to a first pregnancy are inconclusive; nevertheless, the breast tissue appears to have a greater susceptibility to the carcinogenic chemicals in tobacco smoke

before compared to after terminal differentiation of breast epithelium.

(ii) *Estrogenic pathway*

The “anti-estrogenic” mechanism through which tobacco smoking may inhibit breast cancer progression is unclear. Estrogen is a known risk factor for breast cancer and several hypotheses have been proposed: earlier age at menopause among smokers, a reduction in the gastrointestinal absorption or distribution of estrogen, enhanced metabolism of estradiol to inactive catechol estrogens, increased binding of estrogens by serum sex hormone-binding globulin, lowered levels of estrogen derived from adipose tissue ([Baron, 1984](#); [Baron et al., 1990](#); [Terry & Rohan, 2002](#)). Several studies of cigarette smoking and mammographically-defined breast density showed lower measures of breast density in current smokers than in non-smokers ([Sala et al., 2000](#); [Vachon et al., 2000](#); [Warwick et al., 2003](#); [Jeffreys et al., 2004](#); [Modugno et al., 2006](#); [Bremnes et al., 2007](#); [Butler et al., 2008](#)). Since exposure to estrogen has been associated positively with breast density, a strong risk factor for breast cancer ([McCormack & dos Santos Silva, 2006](#)), the results of these studies are consistent with an anti-estrogenic effect of cigarette smoking. Although smokers and non-smokers may have the same concentrations of estrogens overall, it may be the type rather than the absolute levels of circulating estrogens that is important. Smokers might have a lower concentration of more biologically active estrogens, primarily 16- α -hydroxyestrone (16 α -OHE1) ([Michnovicz et al., 1986, 1988](#); [Berta et al., 1992](#); [Berstein et al., 2000](#); [Terry et al., 2002b](#)). Estrogen can be metabolized along three pathways, to 16 α -OHE1 or to 2-OHE1 or to 4-OHE1. 16 α -OHE1 and 4-OHE1 have been observed to increase mammary epithelial cell proliferation rates in experimental studies ([Schütze et al., 1993, 1994](#); [IARC, 2007c](#)). In contrast, 2-OHE1 might decrease epithelial cell proliferation rates ([Bradlow et al., 1996](#);

[Muti et al., 2000](#)). If cigarette smoking increases estradiol 2-hydroxylation, as has been suggested ([Michnovicz et al., 1986](#)), thereby increasing the ratio of 2-OHE1:16- α -OHE1, an inverse association between smoking and breast cancer risk might be observed. However, only one study has directly examined 2-hydroxylation in relation to cigarette smoking ([Michnovicz et al., 1986](#)). Using injected radiolabelled estradiol, a 50% increased estradiol 2-hydroxylation was found in premenopausal women who smoked at least 15 cigarettes/day compared with non-smokers. Two studies of urinary estrogens found increased excretion of 2-OHE1 and decreased excretion of estriol among smokers ([Michnovicz et al., 1988](#); [Berstein et al., 2000](#)), which may also support the hypothesis that smoking decreases the formation of active estrogen metabolites along the 16 α -hydroxylation pathway. However, the ratio of urinary 2-OHE1:16 α -OHE1 was not related to breast cancer risk in the one case-control study that examined the association ([Ursin et al., 1999](#)). The 4-hydroxylation of estrogens is catalysed by CYP1B1, which is induced by tobacco smoke ([Nebert et al., 2004](#)). This has been postulated as an additional pathway that could lead to formation of DNA adducts via catechol estrogen-quinones ([Gaikwad et al., 2008](#)) and oxidative/DNA damage via redox-cycling ([Zhu & Conney, 1998](#)). The ratio of 2-OHE1:4-OHE1 has been studied in relation to breast cancer risk and smoking in one study ([Berstein et al., 2000](#)). Smokers carrying the CYP1B1 Val allele [associated with high hydroxylation activity] had a significantly higher risk for breast cancer compared to never smokers with the Leu/Leu [wildtype] genotype ([Saintot et al., 2003](#)).

(b) Endometrium

Exogenous estrogens unopposed by progesterone have been shown to increase the risk for endometrial cancer through increased mitotic activity of endometrial cells, increased number of DNA replication errors, and somatic mutations

resulting in the malignant phenotype ([IARC, 2007c, 2012c](#)). Hence, factors associated with estrogen absorption or metabolism may alter the risk of this malignancy. Several investigators have hypothesized that cigarette smoking might have anti-estrogenic effects, and through this mechanism reduce the risk of endometrial cancer ([Baron, 1984](#); [Baron et al., 1990](#); [Terry et al., 2002b, 2004a](#)).

Whether mediated through changes in the amount of adipose tissue, altered age at menopause, or anti-estrogenic effects, blood hormone concentrations might be an important link between smoking and the reduced risk of endometrial cancer observed in most epidemiological studies. The estrogens that have typically been studied in relation to cigarette smoking include estrone, sex hormone binding globulin (SHBG)-bound estradiol, and estriol. Blood concentrations of androgens, typically androstenedione and dehydroepiandrosterone sulfate (DHEAS), have also been studied, because these are biological precursors of estrone. Studies that have examined blood concentrations of SHBG are less common, and studies of unbound (free) estradiol are scarce.

Studies of cigarette smoking and blood hormone concentrations have been conducted mostly among post-menopausal women who were not taking HRT. Of these studies, nine examined serum ([Friedman et al., 1987](#); [Cauley et al., 1989](#); [Slemenda et al., 1989](#); [Schlemmer et al., 1990](#); [Cassidenti et al., 1992](#); [Austin et al., 1993](#); [Law et al., 1997](#)) or plasma ([Khaw et al., 1988](#); [Longcope & Johnston, 1988](#)) estrone, ten examined serum ([Friedman et al., 1987](#); [Cauley et al., 1989](#); [Slemenda et al., 1989](#); [Schlemmer et al., 1990](#); [Key et al., 1991](#); [Cassidenti et al., 1992](#); [Austin et al., 1993](#); [Law et al., 1997](#)) or plasma ([Khaw et al., 1988](#); [Longcope & Johnston, 1988](#)) estradiol, and two examined serum ([Cassidenti et al., 1992](#)) or plasma ([Longcope & Johnston, 1988](#)) free estradiol. These studies consistently showed little or no association between smoking

and blood estrogen concentrations among post-menopausal women who were not taking hormone replacement therapy. Among pre-menopausal women, three studies ([Longcope & Johnston, 1988](#); [Key et al., 1991](#); [Berta et al., 1992](#)) found no clear association between cigarette smoking and estrogen concentrations. Studies that adjusted hormone measurements for the effects of BMI (and other covariates) showed similar results to those that did not, suggesting that BMI is not a strong confounder of this association.

In two studies the association between cigarette smoking and blood estrogen concentrations after randomization of women to groups receiving either estradiol or placebo were examined ([Jensen & Christiansen, 1988](#); [Cassidenti et al., 1990](#)). In a small study of 25 post-menopausal women, unbound estradiol was significantly lower among smokers than non-smokers both at baseline and shortly after taking micronized estradiol orally ([Cassidenti et al., 1990](#)). No important differences were observed between smokers and non-smokers in serum concentrations of either estrone or bound estradiol. In contrast, in a study in which 110 post-menopausal women were randomized to take hormones (either orally or percutaneously) or a placebo ([Jensen & Christiansen, 1988](#)), smokers had lower concentrations of both estrone and bound estradiol than non-smokers after oral (but not percutaneous) hormone treatment for at least one year (concentrations of free estrogens were not examined). These results indicate that smoking might affect the absorption or metabolism of hormones used in replacement therapy.

Of the five studies that have examined the association between cigarette smoking and serum ([Lapidus et al., 1986](#); [Cassidenti et al., 1992](#); [Law et al., 1997](#)) or plasma ([Khaw et al., 1988](#); [Longcope & Johnston, 1988](#)) SHBG, none found any clear association. However, one of these studies ([Khaw et al., 1988](#)) found an inverse association between smoking and the ratio of bound estradiol to SHBG, a measure of estrogen

activity. In this context, [Cassidenti et al. \(1990\)](#) found unbound (but not SHBG-bound) estradiol was significantly lower among smokers than non-smokers both at baseline and after taking oral estradiol, suggesting an increased SHBG-binding capacity in the women who smoked.

In post-menopausal women, androgens are the major source of estrone, converted through aromatization in fat deposits. Thus, adiposity is positively correlated with estrogen concentrations in post-menopausal women. Of the nine studies in which blood concentrations of androstenedione were examined in smokers ([Friedman et al., 1987](#); [Khaw et al., 1988](#); [Longcope & Johnston, 1988](#); [Cauley et al., 1989](#); [Slemenda et al., 1989](#); [Schlemmer et al., 1990](#); [Cassidenti et al., 1992](#); [Austin et al., 1993](#); [Law et al., 1997](#)), higher circulating concentrations were found among current than among never or former smokers in all studies. However, there was no clear variation in blood estrone concentrations by smoking status, suggesting a reduced conversion of androstenedione to estrone among smokers. Of the five studies where cigarette smoking and DHEAS concentrations were examined, three ([Khaw et al., 1988](#); [Cassidenti et al., 1992](#); [Law et al., 1997](#)) found increased blood concentrations among current smokers, one ([Friedman et al., 1987](#)) found also an increase that was not statistically significant, whereas another ([Key et al., 1991](#)) found no clear differences according to smoking status.

Cigarette smoking and urinary estrogen concentrations have been examined in seven studies ([MacMahon et al., 1982](#); [Michnovicz et al., 1986](#); [Trichopoulos et al., 1987](#); [Michnovicz et al., 1988](#); [Berta et al., 1992](#); [Key et al., 1996](#); [Berstein et al., 2000](#)). Of these, three found no major differences according to smoking status ([Trichopoulos et al., 1987](#); [Michnovicz et al., 1988](#); [Berta et al., 1992](#)). The remaining four studies all showed lower urinary estriol concentrations among smokers than among non-smokers, but mixed results for urinary estrone and estradiol.

Two of these studies ([Michnovicz et al., 1988](#); [Berstein et al., 2000](#)) showed higher concentrations of 2-hydroxyestrone among smokers, than non-smokers but only after estrogen treatment in [Berstein et al. \(2000\)](#).

Age at natural menopause varies substantially under the influence of genetic and environmental factors ([McKinlay, 1996](#)). A relatively early age at menopause has been associated with reduced risk of endometrial cancer ([Kelsey et al., 1982](#); [Baron, 1984](#); [Baron et al., 1990](#); [Akhmedkhanov et al., 2001](#)). A one year decrease in age at menopause has been associated approximately with a 7% decrease in risk ([Kelsey et al., 1982](#)). It has been proposed that cigarette smoking decreases the age at natural menopause ([Baron et al., 1990](#)), more clearly with qualitative than quantitative smoking measures ([Parente et al., 2008](#)), and thus might reduce endometrial cancer risk through reduced exposure to endogenous estrogens. On average, smokers have menopause approximately 1 to 1.5 years earlier than non-smokers ([Terry et al., 2002b, 2004a](#)). Adjustment for obesity and other covariates did not alter the results ([Terry et al., 2002b](#)).

4.4 Mechanistic considerations of the interaction of ethanol and tobacco carcinogens

The combined effects of alcoholic beverages and tobacco on the risk for cancer incidence and mortality have been widely studied in human populations. When tested for multiplicative and additive interactions, synergistic effects of alcoholic beverages and tobacco have been found, especially for oropharyngeal and oesophageal cancers ([Homann et al., 2000](#); [Castellsagué et al., 2004](#); [Salaspuro & Salaspuro, 2004](#); [Lee et al., 2005a](#); [Lee et al., 2007b](#)).

Data support at least four possible mechanisms for the modifying effects of alcoholic beverages on cancer risk due to tobacco.

1. Alcohol may have a local permeabilizing effect on penetration of the oral mucosa by tobacco carcinogens ([Du et al., 2000](#)), particularly important in the case of oropharyngeal and oesophageal cancer.
2. CYP2E1 and other CYPs may both activate and detoxify carcinogens present in tobacco smoke, including NDMA, NDEA, NNK, benzene and other tobacco-derived carcinogens in two ways: CYP induction increases metabolic activation of tobacco carcinogens leading to enhanced formation of proximate reactive chemical species at target sites; and alteration of phase II conjugation/detoxification enzymes by ethanol may also occur, changing the effective dose at the target site.
3. Competitive inhibition of CYP metabolism leads to reduced central hepatic and gastrointestinal clearance thus increasing dose delivery of carcinogens to peripheral target tissues (reviewed in [Meskar et al., 2001](#)).
4. Effects of acetaldehyde derived by microbial alcohol oxidation and from the tobacco smoke ([Homann et al., 2000](#); [Salaspuro & Salaspuro, 2004](#)).

Supportive evidence for ii) and iii) is briefly presented below.

4.4.1 Effects of induction of CYPs by ethanol

(a) CYP2E1

Ethanol induces CYP2E1 in the human liver and in all species tested. Over 70 substrates of CYP2E1 have been compiled ([Raucy & Carpenter, 1993](#); [Guengerich et al., 1994](#); [Djordjević et al., 1998](#); [Klotz & Ammon, 1998](#); [Cederbaum, 2006](#)). Among those are tobacco carcinogens such as benzene, vinyl chloride, NDMA, NDEA and *N*-nitrosopyrrolidine, as well as many low-molecular-weight compounds. Induction of CYP2E1 by ethanol generated increased levels of toxic metabolites from the metabolism of many of these chemicals ([Novak & Woodcroft, 2000](#)). Pyridine, a constituent of tobacco smoke and

substrate of CYP2E1, generates DNA damaging products by redox-cycling ([Kim & Novak, 1990](#)).

In humans, in addition to the prominent CYP2E1 expression in the centrilobular regions of the liver, the enzyme is also detectable in the kidney cortex and, at lower levels, in organs such as the oropharynx, nasal mucosa, ovary, testis, small intestine, colon and pancreas ([Ingelman-Sundberg et al., 1994](#); [Lieber, 1999, 2004](#)).

In rats, ethanol induced CYP2E1 in epithelia of the cheek, tongue and oesophagus ([Shimizu et al., 1990](#)). As a result of CYP2E1 induction by ethanol in the upper respiratory tract and possibly of inhibition of carcinogen clearance, hamsters had a significant increase of nasal cavity and tracheal tumours after intraperitoneal injection of *N*-nitrosopyrrolidine ([McCoy et al., 1981](#)). Thus, induction of CYP2E1 by ethanol may participate in the genesis of cancers at several sites via metabolic activation of tobacco carcinogens into reactive species in target tissues.

(b) Other xenobiotic-activating CYPs

In addition to CYP2E1, several CYPs, including CYP3A4 and probably CYP1A2 in humans, and CYP1A1, 2B1 and 3A in rat liver, may be induced by ethanol. Of particular interest are members of the CYP3A family, which have wide substrate specificity and have been implicated in the activation of several known or suspected human carcinogens, including those derived from tobacco ([Wojnowski & Kamdem, 2006](#)). Both CYP3A4 and CYP1A2 metabolize NNK ([Jalas et al., 2005](#)). Based on the Michaelis constant (*K_m*) data ([IARC, 2007a](#)), the relative efficiencies in NNK metabolism by human CYP are (from greatest catalyst to least): 2A13 > 2B6 > 2A6 > 1A2 ~1A1 > 2D6 ~2E1 ~3A4. As the amount of CYP enzymes with overlapping substrate specificity that participate in nitrosamine metabolism varies according to organ and species, it is difficult to determine their individual contribution at target sites.

4.4.2 Effects of inhibition of CYPs by ethanol

Ethanol is a competitive inhibitor of CYP2E1 (reviewed in [Anderson, 1992](#)). It also inhibits the activities of CYP1A1, 2B6 and 2C19 but not those of CYP1A2.

Direct inhibition of CYPs by ethanol in target tissues may reduce metabolic activation of xenobiotics and hence local toxic and tumorigenic effects. Thus CYP inhibition in the liver could increase extrahepatic exposure to genotoxic metabolites from tobacco carcinogens that are substrates for these CYP enzymes. This mechanism is supported by several studies.

Ethanol caused a fivefold increase in oesophageal DNA adducts in rats induced by NDEA ([Swann, 1984](#)). In monkeys, *O*⁶-methylguanine-DNA adducts after an oral dose of NDMA with or without ethanol were increased by co-exposure to ethanol in all tissues except the liver ([Anderson et al., 1996](#)). Effects were seen in the oesophagus (17-fold increase), colonic mucosa (12-fold), pancreas (sixfold), urinary bladder (11-fold), ovary (ninefold), uterus (eightfold), brain (ninefold), spleen (13-fold) and nasal mucosa (fivefold). In these studies, ethanol treatment was acute, so that enzyme induction was improbable, and the oesophagus was not directly exposed to either ethanol or carcinogen. This indicates that a systemic interaction, most likely inhibition of hepatic carcinogen clearance, was responsible for the observed effects in the oesophagus and other extrahepatic tissues. The 17-fold increase in DNA adducts in the monkey oesophagus is similar to the 18-fold increased risk for human oesophageal cancers in tobacco smokers combined with heavy alcohol drinking ([Tuyens et al., 1977](#)).

The relevance of increased genotoxic effects in extrahepatic target sites by ethanol is confirmed by many rodent experiments. Oral dosing of mice with NDMA in ethanol resulted in nasal cavity tumours (olfactory neuroblastoma) that were not seen with NDMA or ethanol alone ([Griciute](#)

[et al., 1981](#)). Ethanol in the drinking-water led to a ninefold increase in oesophageal tumours in rats induced by NDEA ([Aze et al., 1993](#)). Ethanol given by gavage to nursing dams together with NDMA or NNK ([Chhabra et al., 2000](#)) increased O⁶-methylguanine-DNA adducts in maternal mammary glands, by 10-fold with NDMA and to a lesser extent with NNK. In the suckling infants, DNA adducts were detected in the lungs and kidneys after maternal exposure to NDMA and increased about fourfold after maternal co-treatment with ethanol. In mice, ethanol given with NDMA in the drinking-water resulted in a fourfold increase in lung tumours, but had no significant effect when NDMA was given intragastrically, intraperitoneally, subcutaneously or intravenously ([Anderson, 1992](#)). These negative findings support that direct inhibition of hepatic carcinogen clearance by ethanol is the main operative mechanism.

There is indirect evidence that ethanol can inhibit the *in vivo* clearance of the carcinogen NDMA in humans: individuals with chronic renal failure showed detectable blood and urine levels of NDMA, which were increased by consumption of ethanol ([Dunn et al., 1990](#)). Other studies that involved sources of NDMA from tobacco smoke, diet or pharmaceuticals are consistent with ethanol reducing its clearance rate in humans ([Anderson, 1992](#)).

Other possible modifying effects of ethanol in tobacco-related tumorigenesis are presented in Section 4 of the *Monograph on Consumption of Alcoholic Beverages* in this Volume.

4.5 Synthesis

4.5.1 Mechanisms of tobacco-related carcinogenesis

The pathways by which tobacco products cause cancer essentially recapitulate established mechanisms of carcinogenesis by individual compounds, which were elaborated by landmark

studies during the second half of the 20th century. These studies demonstrate that most carcinogens, either directly or after metabolism catalyzed by multiple cytochrome P450 enzymes, react with nucleophilic sites in DNA to form covalent binding products called adducts (a contraction for “addition products”). These DNA adducts, if left unrepaired by cellular DNA repair enzymes, persist and cause mistakes during DNA replication leading to incorporation of the wrong base in a DNA strand and consequent permanent mutations. If these permanent mutations occur in important regions of critical growth control genes such as the oncogene *KRAS* or the tumor suppressor gene *p53*, cellular growth processes can become severely unregulated and cancer can result. Multiple studies of mutations in *KRAS*, *p53*, and other growth control genes in lung tumours from smokers, some of which report thousands of mutations, are fully consistent with this overall concept.

It is the complexity of tobacco carcinogenesis which challenges investigators to identify specific mechanisms that fully explain the ways in which tobacco products cause each type of cancer. There are over 70 established carcinogens in cigarette smoke, and analyses of smokers’ urine and blood clearly demonstrate higher uptake of these compounds in smokers than in non-smokers. The urine of smokers is consistently mutagenic. Similar considerations apply to smokeless tobacco users, although there are fewer identified carcinogens. Multiple DNA adducts are present in the lungs and other tissues of smokers, and sister chromatid exchanges as well as other genetic effects are consistently observed. But much less is known about the specifics of the process. Only relatively few DNA adducts in smokers’ lungs have been structurally characterized and the relationship between specific adducts and the consequent mutations in critical genes is still somewhat unsettled.

There are other processes which contribute to cancer induction by tobacco products, based on

multiple studies in both laboratory animals and humans. These include inflammation, tumor promotion, oxidative damage, co-carcinogenesis, and direct activation of cellular growth pathways by constituents of smoke. Many studies demonstrate the involvement of these processes in tobacco carcinogenesis but the details by which they interact with the DNA damage pathways and their roles in specific cancers caused by tobacco products are still not fully understood.

4.5.2 Genetic polymorphisms

Multiple studies have been carried out on the role of genetic polymorphisms of xenobiotic metabolism in smoking-related carcinogenesis in humans. These studies have covered various cancer types, with lung cancer representing one of the most intensively studied. The polymorphic genes, their variant forms, and the genotype combinations investigated in these studies have similarly been numerous. In addition to the associations with increased risk of cancer, much data have accumulated on relationships between the polymorphisms and the various biomarkers of tobacco carcinogenesis in non-cancer control populations, whether smokers or non-smokers, in subjects with work-related exposure or in patients with other cancers.

Despite the massive body of research, many observations remain ambiguous. Some associations between genetic polymorphism and increased risk for cancer, such as for the *GSTM1* null genotype, alone or in combination with *CYP1A1* polymorphism, in lung cancer, or the *NAT2* slow acetylator genotype in bladder cancer and breast cancer appear stronger and more consistent, but not without controversies. Similarly, the data on the various biomarkers of tobacco-related carcinogenesis exhibit inconsistencies.

The variability in the data is at least partially likely due to differences between the studies in the genes and gene variants included (many of

which are still of unknown functional or regulatory consequence), in the types of cancer studied, in levels and sources of exposure, in ethnic backgrounds, in sex, in histological types and in the features of the genome such as haplotype blocks and copy number variation resulting in linkage disequilibrium. In addition, gene-gene interactions and gene-environment interactions are likely to contribute to the discrepancies in current data. Mechanisms of tobacco-related carcinogenesis also involve genes from numerous other classes, such as those encoding for DNA repair proteins and many other regulators of cell cycle and growth. In addition; there are well described mechanistic pathways of carcinogenesis mediated via epigenetic alterations and genetic instability, to mention a few.

4.5.3 Site-specific mechanisms

The Working Group reviewed the mechanistic evidence relative to specific target sites for which there is sufficient evidence of carcinogenicity in humans, i.e. lung, oral cavity, oesophagus, larynx and nasopharynx, pancreas, stomach, liver, urinary bladder, leukaemia, cervix and ovary. Genotoxic effects have been found in eight organ sites at which tobacco smoke causes cancer in humans ([DeMarini, 2004](#)).

Sites with limited evidence of carcinogenicity or evidence suggesting lack of carcinogenicity in humans include the breast and the endothelium and relevant mechanisms are presented below.

Breast — There are several plausible mechanisms by which smoking may increase breast cancer risk, and some data support such an effect, including the increased risk among long-term smokers with *NAT2* slow genotype. Despite the overall lack of clear association in epidemiological studies, and the potential anti-estrogenic effects of smoking, the possibility that smoking increases breast cancer risk is biologically plausible.

Endothelium — The mechanisms by which cigarette smoking reduces the risk for endometrial cancer among current smokers, mainly among postmenopausal, remain unclear.

4.5.4 Interaction of ethanol and tobacco carcinogens

Data in rodents and non-human primates on the relationships between a) inhibition of hepatic clearance of nitrosamines by ethanol, b) the formation of promutagenic DNA adducts and c) tumours in extra-hepatic targets, likely also pertain in humans.

5. Evaluation

There is *sufficient evidence* in humans for the carcinogenicity of tobacco smoking.

Tobacco smoking causes cancers of the lung, oral cavity, naso-, oro- and hypopharynx, nasal cavity and accessory sinuses, larynx, oesophagus, stomach, pancreas, colorectum, liver, kidney (body and pelvis), ureter, urinary bladder, uterine cervix and ovary (mucinous), and myeloid leukaemia. Also, a positive association has been observed between tobacco smoking and cancer of the female breast. For cancers of the endometrium (post-menopausal) and of the thyroid, there is *evidence suggesting lack of carcinogenicity*.

There is *sufficient evidence* in humans for the carcinogenicity of parental smoking. Parental smoking causes hepatoblastoma in children. Also, a positive association has been observed between parental smoking and childhood leukaemia (particularly acute lymphocytic leukaemia).

There is *sufficient evidence* in experimental animals for the carcinogenicity of tobacco smoke and of tobacco smoke condensates.

Tobacco smoking is *carcinogenic to humans* (Group 1).

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Ingredients and Emissions

Smokeless tobacco and some tobacco-specific N-nitrosamines, IARC Monograph 89, 2007

**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

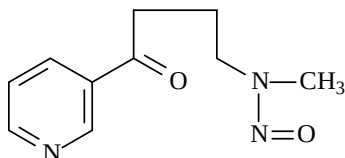
1.1.1 Synonyms and trade names

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*C₁₀H₁₃N₃O₂

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'*-Nitrosornornicotine (NNN)*1.1 Chemical and physical data**1.1.1 *Synonyms and trade names*

Chem. Abstr. Services Reg. Nos: 80508-23-2; 16543-55-8¹; 84237-38-7²

Chem. Abstr. Names: Pyridine, 3-(1-nitroso-2-pyrrolidinyl)-; pyridine, 3-(1-nitroso-2-pyrrolidinyl)-,(S)-¹; pyridine, 3-(1-nitroso-2-pyrrolidinyl)-, (+,-)-²

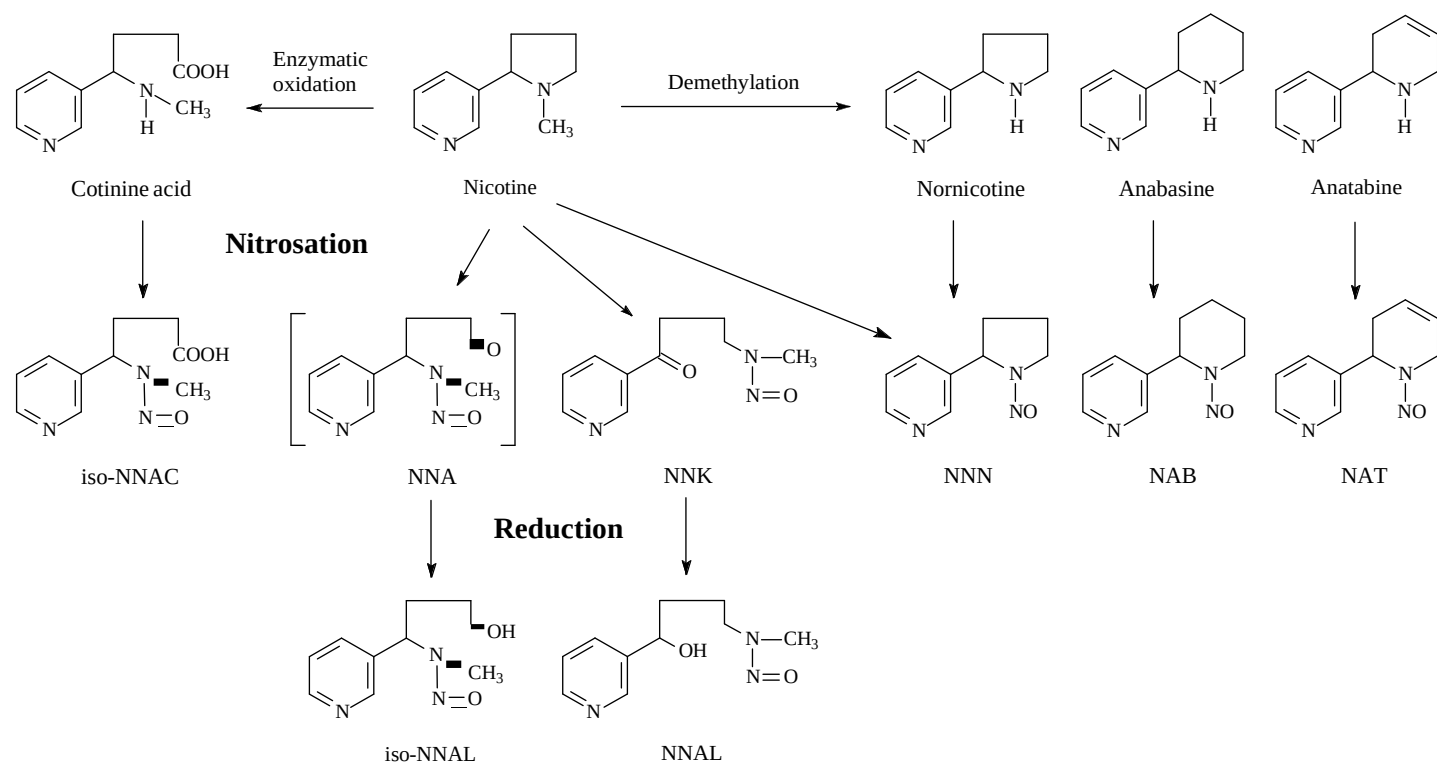
IUPAC Systematic Name: 1'-Demethyl-1'-nitrosonicotine

Synonyms: 1'-Demethyl-1'-nitrosonicotine; 1'-desmethyl-1'-nitrosonicotine; 1'-nitroso-1'-demethylnicotine; nitrosornornicotine; *N*-nitrosornornicotine; 1'-nitrosornornicotine; 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

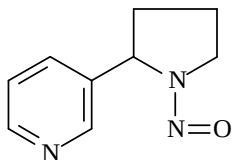


SOME TOBACCO-SPECIFIC *N*-NITROSAMINES

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'*-nitrosornicotine

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* $\text{C}_9\text{H}_{11}\text{N}_3\text{O}$

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; 1133-64-8¹; 84237-39-8²

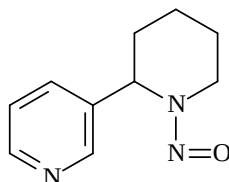
Chem. Abstr. Names: Pyridine, 3-(1-nitroso-2-piperidinyl)-; pyridine, 3-(1-nitroso-2-piperidinyl)-(S)-¹; pyridine, 3-(1-nitroso-2-piperidinyl), (+,-)-²

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

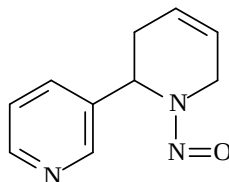
N'*-Nitrosoanatabine (NAT)*1.1 Chemical and physical data**1.1.1 *Synonyms and trade names*

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 & Spiegelhalter, 1989; Fischer *et al.*, 1989a; Spiegelhalter *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 TSNA 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 (Spiegelhalter & 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 <i>et al.</i> (1991); Morgan <i>et al.</i> (2004)
	Wet	ND–70 ^c	20–460			20–200	Fischer <i>et al.</i> (1989a)
Bright (Virginia)	Dry	160–2690	240–1700	20–150	280–6670		Djordjevic <i>et al.</i> (1989b); Risner <i>et al.</i> (2001); Morgan <i>et al.</i> (2004)
	Wet	30–1100	10–600			30–950	Fischer <i>et al.</i> (1989a)
Burley (laminae)	Dry	ND–1370	1070–8620	200–223	4270–19 700		MacKown <i>et al.</i> (1988); Burton <i>et al.</i> (1989a); Risner <i>et al.</i> (2001); Morgan <i>et al.</i> (2004)
	Wet	100–1400	1300–8850			500–3600	Fischer <i>et al.</i> (1989a)
Burley (stems/midrib)	Dry	ND–6660	1500–9080	155	1800–20 940		MacKown <i>et al.</i> (1988); Burton <i>et al.</i> (1989a); Morgan <i>et al.</i> (2004)
Reconstituted tobacco sheets	Dry	490	3400		2500		MacKown <i>et al.</i> (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 <i>et al.</i> (2005)
Australia	Dry	490–1193	420–2888	NQ ^b –207	715–2366		Counts <i>et al.</i> (2005)
Austria	ng/cig	92–310	306–1122				Fischer <i>et al.</i> (1990a)
Belgium	ng/cig	219–594	504–1939				Fischer <i>et al.</i> (1990a)
Canada	ng/cig	447–884	259–982			564–1017	Fischer <i>et al.</i> (1990b)
CEMA	Dry	NQ ^b –1127	1094–3739	NQ ^b –322	1014–2989		Counts <i>et al.</i> (2005)
European Union	Dry	NQ ^b –860	332–2736	NQ ^b –262	423–2253		Counts <i>et al.</i> (2005)
France	Dry ng/cig	260–1530 57–990	4770–18 600 120–6019	100	1200–9970		Djordjevic <i>et al.</i> (1989a); Ohshima <i>et al.</i> (1985); Fischer <i>et al.</i> (1990a)
Germany	ng/cig Dry	ND–1120 ^c 445–469	45–5340 1355–1361	NQ ^b	1142–1207	ND–2490 ^c	Fischer <i>et al.</i> (1989a, 1990a); Tricker <i>et al.</i> (1991); Counts <i>et al.</i> (2005)
India	Dry ng/cig	40–4800 19–174	1300–58 000 68–730		800–15 100	98–519 ^c	Nair <i>et al.</i> (1989); Kumar <i>et al.</i> (1991)
Italy	ng/cig	153–10 745	632–12 454				Fischer <i>et al.</i> (1990a)
Japan	Dry	190–1171	360–3892	NQ ^b –320	300–3139		Djordjevic <i>et al.</i> (2000a); Counts <i>et al.</i> (2005)
Malaysia	Dry	434–923	2223–3350	NQ ^b –176	1302–2170		Counts <i>et al.</i> (2005)
Moldova	Wet	104–942	93–2090	ND–75	55–1290		Stepanov <i>et al.</i> (2002)
Netherlands	ng/cig	105–587	58–1647				Fischer <i>et al.</i> (1990a)
Norway	Dry	1124	3736	379	2945		Counts <i>et al.</i> (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 <i>et al.</i> (1990a); Djordjevic <i>et al.</i> (2000b)
Sweden	ng/cig	192–569	544–1511				Fischer <i>et al.</i> (1990a)
Switzerland	ng/cig	450–554	1280–2208				Fischer <i>et al.</i> (1990a)
Taiwan	Dry	1194	3769	195	2883		Counts <i>et al.</i> (2005)
United Kingdom	ng/cig	92–433	140–1218				Fischer <i>et al.</i> (1990a)
USA	Dry	420–1270	880–7900	ND–212	880–5810		Ohshima <i>et al.</i> (1985); Djordjevic <i>et al.</i> (1990); Fischer <i>et al.</i> (1990a); Djordjevic <i>et al.</i> (2000a); Stepanov <i>et al.</i> (2002); Counts <i>et al.</i> (2005)
	Wet	1410–1760	2590–4300	100–140	1610–2660		
	ng/cig	433–733	993–1947				
Former USSR	Dry	ND–40 ^d	20–420		20–170		Fischer <i>et al.</i> (1990a); Djordjevic <i>et al.</i> (1991)
	ng/cig	ND–150 ^c	60–850				
Venezuela	Dry	591	2170	244	1339		Counts <i>et al.</i> (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 <i>et al.</i> (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) (55)	ng/cig	Blend	F	100–410	400–1390			220–1340	Tricker <i>et al.</i> (1991)
		Blend	NF	270–500	660–2670			460–1110	
		Dark	NF	800–960	4500–5340			1650–2330	
		Oriental	F + NF	ND–177	45–432			ND–575	Fischer <i>et al.</i> (1989a,b)
		Virginia	F + NF	170–580	133–330			253–630	
		American blend	F	160–696	500–2534			440–2490	
Japan (6)	Dry	Dark	NF	370–1120	3660–5316			266–315	
		Low yield	F	190–330	810–1110	30–60	410–660		Djordjevic <i>et al.</i> (2000a)
USA (13)	Dry	Medium yield	F	200–320	360–1040	30–70	300–620		
		Ultra-low yield (AB)	F	500–580	1750–1980	ND	970–1080		Djordjevic <i>et al.</i> (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		
(11)	Wet	High yield (AB)	NF	770–920	1290–2160	40–110	920–1170		
		Quest 1,2,3 ^c	F	54–190	820–930	3–13	43–310		Stepanov <i>et al.</i> (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 <i>et al.</i> (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 *et 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 occurrence 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 <i>et al.</i> (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 <i>et al.</i> (2005) King <i>et al.</i> (2007)
Austria	12–100	42–172				Fischer <i>et al.</i> (1990a)
Belgium	29–150	38–203				Fischer <i>et al.</i> (1990a)
Canada	6–97 50.9 ^{a,c}	4–37 25.6 ^{a,c}			9–82	Fischer <i>et al.</i> (1990b) King <i>et al.</i> (2007)
CEMA	18.0–75.0	42.5–147.5	6.0–18.5	38.6–129.2		Counts <i>et al.</i> (2005)
European Union	12.7–78.4	14.8–126.0	2.0–17.9	18.0–106.8		Counts <i>et al.</i> (2005)
Germany	ND ^d –470	5–855	6.6–9.0	39.9–52.6	6.6–520	Fischer <i>et al.</i> (1989b, 1990a); Tricker <i>et al.</i> (1991); Counts <i>et al.</i> (2005)
France	18.3–498	11–1000	15.4–18.7	127–182		Djordjevic <i>et al.</i> (1989a); Fischer <i>et al.</i> (1990a)
India	TR–73	6–401		3.8–99.4	18–146	Nair <i>et al.</i> (1989); Kumar <i>et al.</i> (1991)
Italy	8–1749	21–1353				Fischer <i>et al.</i> (1990a)
Japan	18.6–87.1	36–146	6.8–21	40.3–138		Djordjevic <i>et al.</i> (1996); Counts <i>et al.</i> (2005)
Malaysia	45.0–87.3	63.4–195.3	7.8–28.5	55.7–153.3		Counts <i>et al.</i> (2005)
Netherlands	5–102	9–163				Fischer <i>et al.</i> (1990a)
Norway	103.9	189.4	18.6	160.4		Counts <i>et al.</i> (2005)
Poland	36–990	68–2830				Fischer <i>et al.</i> (1990a); Gray <i>et al.</i> (1998); Djordjevic <i>et al.</i> (2000b)

Table 4 (contd)

Country	NNK	NNN	NAB	NAT	NAB + NAT	Reference
Sweden	27–84	44–141				Fischer <i>et al.</i> (1990a)
Switzerland	69–124	121–226				Fischer <i>et al.</i> (1990a)
Taiwan (China)	96.2	161.2	24.7	142.8		Counts <i>et al.</i> (2005)
Thailand	16–369	28–730			43.5–483	Brunnemann <i>et al.</i> (1996)
United Kingdom	5.2–500	6.5–257.6	1.1–44.2	7.8–148		Fischer <i>et al.</i> (1990a); Gregg <i>et al.</i> (2005)
USA	3–425	6–1007	ND–34.7	6–250	102–744	Adams <i>et al.</i> (1987a); Fischer <i>et al.</i> (1990a); Djordjevic <i>et al.</i> (1990); Brunnemann <i>et al.</i> (1994); Djordjevic <i>et al.</i> (1996)
Former USSR	4–55	23–389		71–196		Fischer <i>et al.</i> (1990a); Djordjevic <i>et al.</i> (1991)
Venezuela	64.2	100.1	13.3	78.6		Counts <i>et al.</i> (2005)

Adapted from IARC (2004)

CEMA, Central Europe, Middle East, Africa; NAB, *N'*-nitrosoanabasine; NAT, *N'*-nitrosoanatabine; ND, not detected; NNK, 4-(methyl-nitrosamino)-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

^aThe measurements are the averages of seven replicates within each machine-smoking method.

^bHealth Canada machine-smoking method; ISO, International Standardization Organization machine-smoking method; MDPH, Massachusetts Department of Public Health machine-smoking method

^cCigarettes 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 TSNA 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 NNN 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/m³ and those of NNK from not detected to 13.5 ng/m³ (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-m² 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/m³ and NNK concentrations ranged between 1.4 and 29.3 ng/m³ (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 <i>et al.</i> (1985)
Canada	Moist snuff	Dry	3200–5800	50 400–79 100	4000–4800	152 000–170 000	Brunnemann <i>et al.</i> (1985)
	Chewing tobacco	Dry	240	2090	100	1580	
Denmark	Chewing tobacco	Wet	19–1900	80–1600	30	180–2900	Österdahl <i>et al.</i> (2004)
Germany	Chewing tobacco	Dry	30–300	1420–2300	30–50	330–3700 ^b	Brunnemann <i>et al.</i> (1985); Tricker <i>et al.</i> (1988)
	Dry snuff	Dry	580–6430	2390–18 750	NR	1030–7830	Tricker & Preussmann (1991);
		Wet	100	680	NR	310	Österdahl <i>et al.</i> (2004)
India	Moist snuff	Wet	240	560	20	380	Stepanov <i>et al.</i> (2005)
	Chewing tobacco	Dry	130–600	470–850	30–70	300–500 ^b	Brunnemann <i>et al.</i> (1985); Tricker <i>et al.</i> (1988)
		Wet	2700–6500	15 300–24 400	NR	10 000–44 600	Nair <i>et al.</i> (1989)
	<i>Zarda</i>	Dry	220–24 100	400–79 000	NR ^b	780–99 100 ^c	Tricker & Preussmann (1988); Tricker <i>et al.</i> (1988)
		Wet	1070–3090	4810–19 900	190–1190	640–1980	Stepanov <i>et al.</i> (2005)
		NR		6550–7360			Gupta (2004)
	<i>Mishri</i>	Dry	294–1100	300–6995	NR	488–14 151	Tricker <i>et al.</i> (1988); Nair <i>et al.</i> (1987)
		Wet	870	4210	150	2550	Stepanov <i>et al.</i> (2005)
		NR		4020–4470			Gupta (2004)
	<i>Khiwam</i>	Dry	100–1030	2500–8950	NR ^b	1830–10 360 ^c	Tricker & Preussmann (1989)
	<i>Khaini</i>	Dry	110–5290	25 800–40 000	1240–2480	660–18 800	Stich <i>et al.</i> (1992)
		Wet	2340–28 400	39 400–76 900	3870–8830	4830–13 800	Stepanov <i>et al.</i> (2005)
	<i>Gutka</i>	Wet	40–430	90–1090	ND–50	10–80	Stepanov <i>et al.</i> (2005)
		NR	10 680–11 510	1870–5730	5750–6890	6270–6530	Gupta (2004)
	<i>Supari</i>	Wet	ND	ND	ND	ND	Stepanov <i>et al.</i> (2005)
		NR	4900–11 580	1920–2450	11 580–12 580	3600–4570	Gupta (2004)

Country	Type of product	Reported as ^a	NNK	NNN	NAB	NAT	References
India (contd)	Creamy snuff/toothpaste	Wet	1310–12 500	2520–48 700	70–110	530–26 600	Stepanov <i>et al.</i> (2005); Nair <i>et al.</i> (1989)
			4380–4880				Gupta (2004)
	Snuff for inhalation	Wet	245 500	1 356 000	NR	1 857 000	Nair <i>et al.</i> (1989)
	Toothpowder	Wet	ND	ND–40	ND	ND	Stepanov <i>et al.</i> (2005)
	<i>Tuibur</i>	NR		19 650–20 120			Gupta (2004)
	Other tobacco	Wet	80–2610	1740–19 200	12–1570	350–11 900	Stepanov <i>et al.</i> (2005)
Norway	Moist snuff	Wet	3300	21 000	1700	13 000	Österdahl <i>et al.</i> (2004)
South Africa	Low-TSNA moist snuff	Dry	270–290	1050–2070	90–110	890–1520	Brunnemann <i>et al.</i> (2004)
Sudan	<i>Toombak</i>	Dry	188 000–7 870 000	141 000–3 080 000	13 900–2 370 000	20 000–290 000	Idris <i>et al.</i> (1991); Prokopczyk <i>et al.</i> (1995)
Sweden	Moist snuff	Dry	190–2950	1120–154 000	40–1700	900–21 400 ^c	Brunnemann <i>et al.</i> (1985); Ohshima <i>et al.</i> (1985); Tricker <i>et al.</i> (1988); Hoffmann <i>et al.</i> (1991); Tricker & Preussmann (1991); Brunnemann & Hoffmann (1992); Djordjevic <i>et al.</i> (1993a); Connolly (2001)
		Wet	190–1300	490–4400	30–170	320–3500	Österdahl & Slorach (1988); Österdahl <i>et al.</i> (2004)
	Low-TSNA moist snuff	Wet	30–360	150–2300	10–130	60–980	Österdahl <i>et al.</i> (2004); Stepanov <i>et al.</i> (2006a)
	Chewing tobacco	Wet	10–460	700–1700	ND	1100–2100	Österdahl <i>et al.</i> (2004)
	Chewing tobacco	Dry	100	500	NR ^b	500 ^c	Tricker <i>et al.</i> (1988)
Thailand							

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 <i>et al.</i> (1988); Brunnemann & Hoffmann (1992)
	Chewing tobacco	Dry	300	900	NR	1500 ^c	Tricker <i>et al.</i> (1988)
	Dry snuff	Dry	580–4300	2390–16 000	NR	1030–7830	Tricker & Preussmann (1991); Brunnemann & Hoffmann (1992)
USA	Moist snuff	Wet	260	1800	NR	820	Österdahl <i>et al.</i> (2004)
		Dry	ND–18 000	ND–147 000	20–10 670	240–339 000	Brunneman <i>et al.</i> (1985); Ohshima <i>et al.</i> , 1985; Adams <i>et al.</i> (1987b); Brunnemann <i>et al.</i> (1987a,b); Chamberlain <i>et al.</i> (1988); Tricker <i>et al.</i> (1988); Andersen <i>et al.</i> (1989); Djordjevic <i>et al.</i> (1989a); Hoffmann <i>et al.</i> (1991); Brunnemann & Hoffmann (1992); Prokopczyk <i>et al.</i> (1992); Djordjevic <i>et al.</i> (1993a); Hoffmann <i>et al.</i> (1995); Prokopczyk <i>et al.</i> (1995); Connolly (2001); Brunnemann <i>et al.</i> (2002, 2004)
	Low-TSNA moist snuff	Wet	60–13 000	710–63 000	14–2800	240–83 000	Österdahl <i>et al.</i> (2004); Stepanov <i>et al.</i> (2006a)
		Wet	32–33	620–640	17–18	310–320	Stepanov <i>et al.</i> (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 <i>et al.</i> (1985); Chamberlain <i>et al.</i> (1988); Andersen <i>et al.</i> (1989); Djordjevic <i>et al.</i> (1989a); Brunnemann & Hoffmann (1992)
		Wet	80–110	250–1100	20	150–940	Österdahl <i>et al.</i> (2004)
	Dry snuff	Dry	880–84 400	9370–116 100	520–1530	11 200–238 800 ^c	Adams <i>et al.</i> (1987); Brunnemann <i>et al.</i> (1987a); Andersen <i>et al.</i> (1989); Djordjevic <i>et al.</i> (1989a)
	Compressed tobacco lozenges	NR	37–43	19–56	7–8	12–17	Stepanov <i>et al.</i> (2006a)
Uzbekistan	<i>Naswar</i>	Dry	20–130	120–520	8–30	32–300	Brunnemann <i>et al.</i> (1985)

NAB, *N'*-nitrosoanabasine; NAT, *N'*-nitrosoanatabine; ND, not detected; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, *N'*-nitrosoanornicotine; 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 nicotine (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'*-nitrososornicotine

^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 <i>et al.</i> (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 <i>et al.</i> (1986)	India	Betel quid with tobacco	ng/g saliva	–	3–85.7	ND–40	–
		<i>Mishri</i>		ND	23.7 (14.3–43.5)	ND	–
		<i>Khaini</i>		ND–28.5	91.9 (10.0–430)	–	ND–133.0
Brunnemann <i>et al.</i> (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 <i>et al.</i> (1992)	Sudan	<i>Toombak</i>	ng/mL saliva	ND–6689	582–20988	46–1944	ND–471
Stich <i>et al.</i> (1992)	India	<i>Khaini</i>	ng/mL saliva	ND–180	150–1580	13–90	86–690
		<i>Gudhaku</i>	ng/mL saliva	ND–10	15–88	1–15	9–55
		<i>Chutta</i> (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 pancreatic 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'-Nitroso-nornicotine (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 $\pm$ 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 $\pm$ SD, 0.096 ± 0.11 pmol/mL). The corresponding values in 11 smokeless tobacco users were 0.03–0.58 pmol/mg creatinine (mean $\pm$ SD, 0.25 ± 0.19 pmol/mg) and 0.091–0.91 pmol/mg creatinine (mean $\pm$ 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 $\pm$ SD, 0.003 $\pm$ 0.006 pmol/mg) NAB and from not detected to 0.14 pmol/mg creatinine (mean $\pm$ SD, 0.038 $\pm$ 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 $\pm$ SD, 0.037 $\pm$ 0.034 pmol/mg) and 0.028–0.44 pmol/mg creatinine (mean $\pm$ SD, 0.19 $\pm$ 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 $\pm$ SD, 0.067 $\pm$ 0.104 pmol/mg) NAT and from not detected to 0.43 pmol/mg creatinine (mean $\pm$ SD, 0.12 $\pm$ 0.11 pmol/mg) NAT-*N*-Gluc. Corresponding levels in 11 smokeless tobacco users were 0.020–0.15 pmol/mg creatinine (mean $\pm$ SD, 0.069 $\pm$ 0.046 pmol/mg) and 0.08–2.78 pmol/mg creatinine (mean $\pm$ SD, 1.36 $\pm$ 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
<i>NNK</i>										
Mouse	x					x		x		x
Rat	x	x	x ^a				x	x		
Hamster	x	x		x			x ^c		x ^c	
Mink	x	(x)								
<i>NNN</i>										
Mouse	x					x				
Rat		x	x ^b		x					
Hamster		x		x						
Mink		(x)								

NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, *N'*-nitrosomornicotine

^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 μ mol/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 [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 μ mol/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 μ mol. 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 intra-peritoneal 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-Wallis 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 thrice-weekly 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/m³) 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 μ mol and 50 μ mol 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 μ mol NNK and 20 mice injected with 20 μ mol 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 μ mol 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 $\mu\text{mol}/\text{mouse}$ NNAL in 100 μL acetone every other day (10 doses; total dose, 28 $\mu\text{mol}/\text{mouse}$). 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 NNN 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 μ mol/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 μ mol or 200 μ mol 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 μ mol NNK and 20 mice injected with 20 μ mol 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 μ mol, 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 $\mu\text{g}/\text{mouse}$ 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).

logical evaluation. The Student's *t*-test was used to determine statistical significance. 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 μ g) 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 μ mol 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) μ mol 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 μ mol NNK) and 100% [20/20] with a tumour multiplicity of 9.2 ± 6.3 tumours per mouse (20 μ mol 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 $50 \pm 25\%$ of total NNAL-Gluc in the urine of smokers and $24 \pm 12\%$ 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).

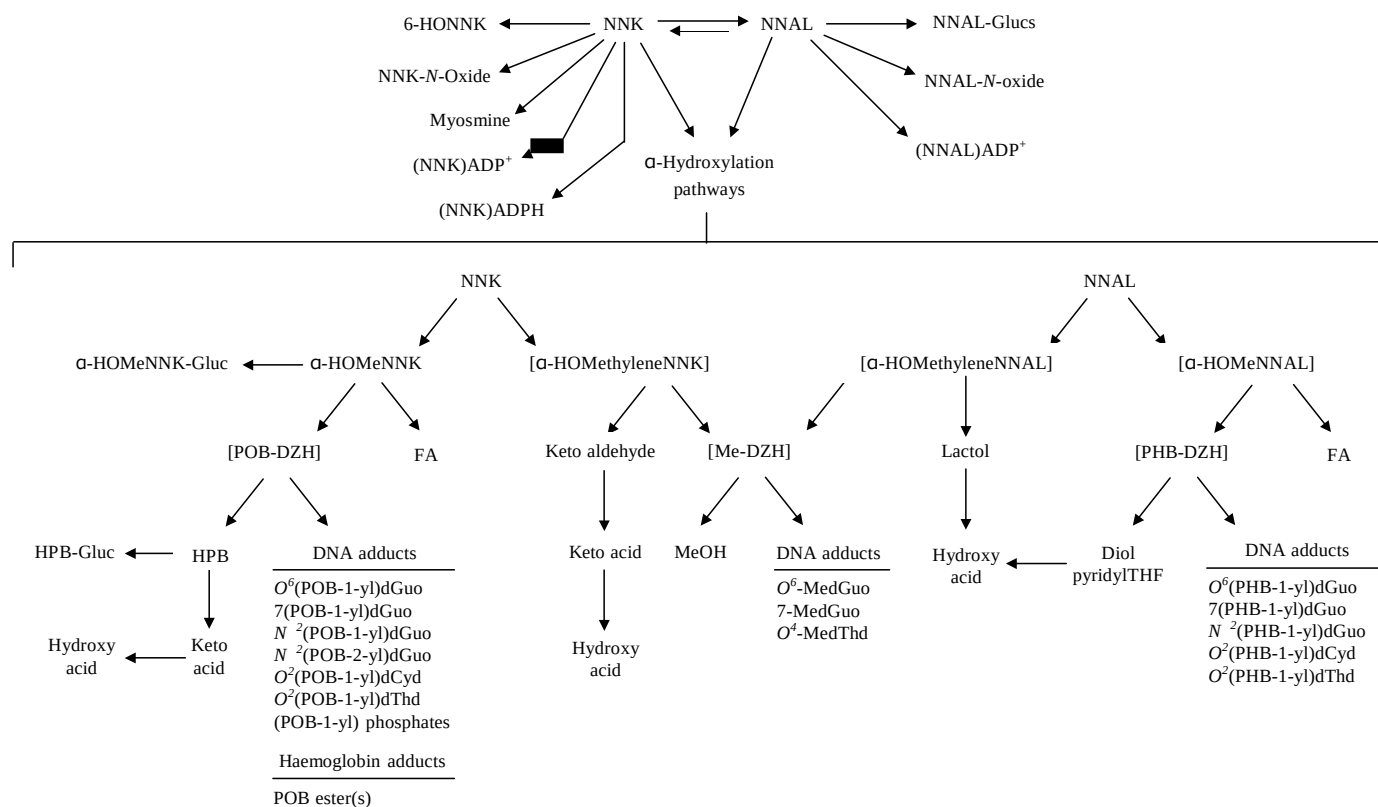
NNAL and NNAL-Gluc are excreted in the urine more slowly than would be expected, based on their structures, after cessation of smoking or smokeless tobacco use (Hecht *et al.*, 1999a, 2002). One week after smoking cessation, 34.5% of baseline NNAL plus NNAL-Gluc was detected in urine, whereas the corresponding 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

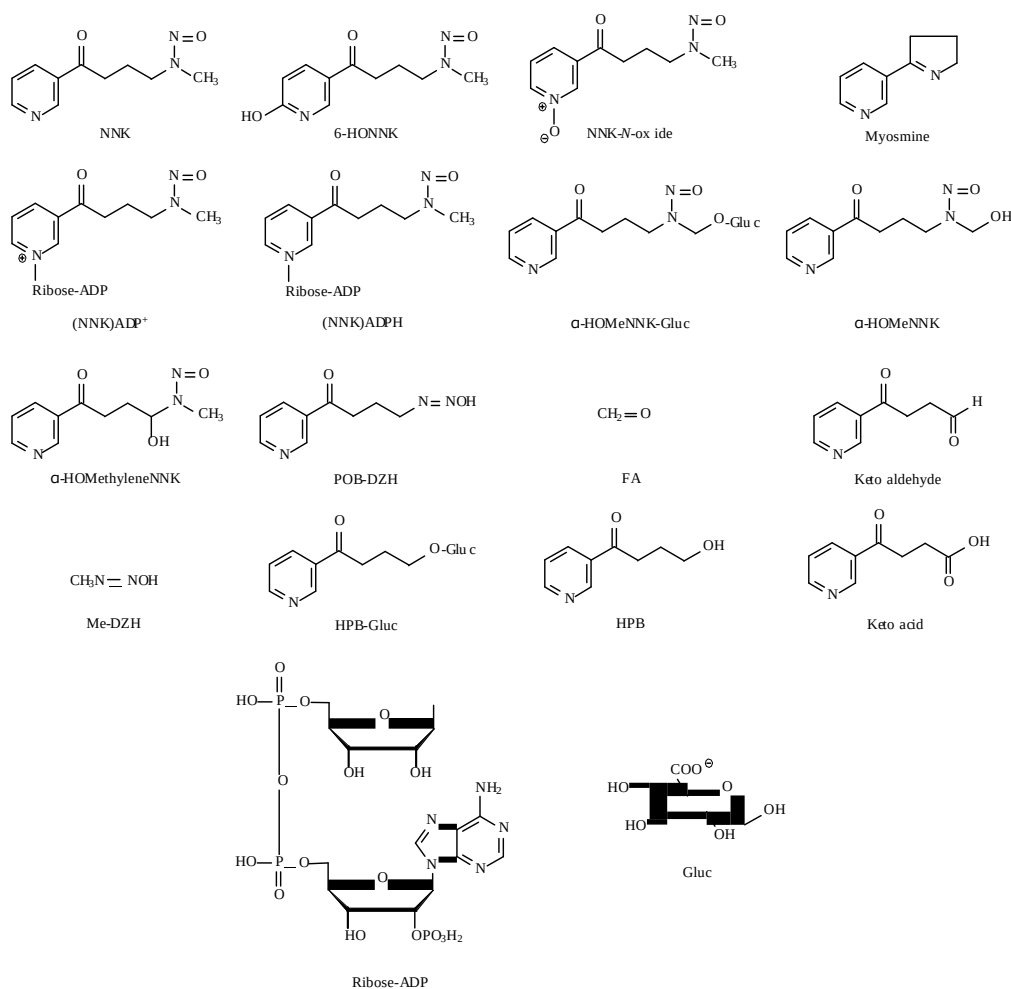
Figure 2. Metabolism of NNK and NNAL and formation of adducts, based on studies in laboratory animals and humans



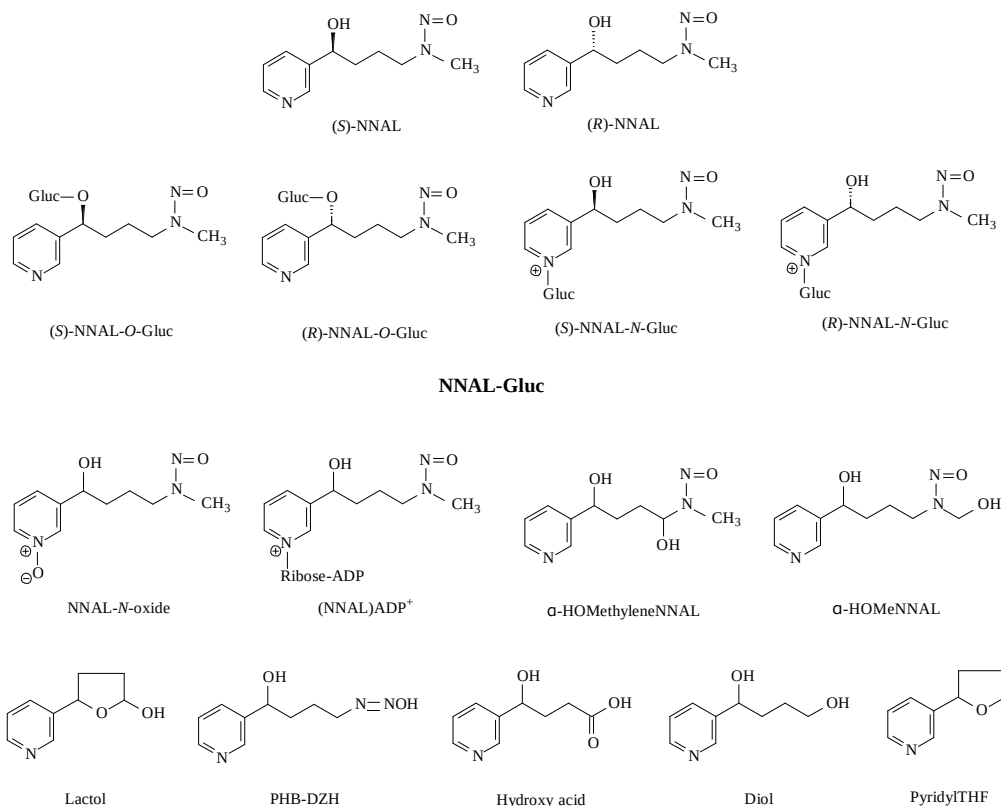
ADP⁺, adenosine dinucleotide phosphate; ADPH, adenosine dinucleotide phosphate (reduced form); dCyd, deoxycytidine; dGuo, deoxyguanosine; dThd, deoxythymidine; DZH, diazohydroxide; Gluc, glucuronide; FA, formaldehyde; HO, hydroxy; HOMe, hydroxymethyl; HOMethylene, hydroxymethylene; HPB, 4-hydroxy-1-(3-pyridyl)-1-butanone 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



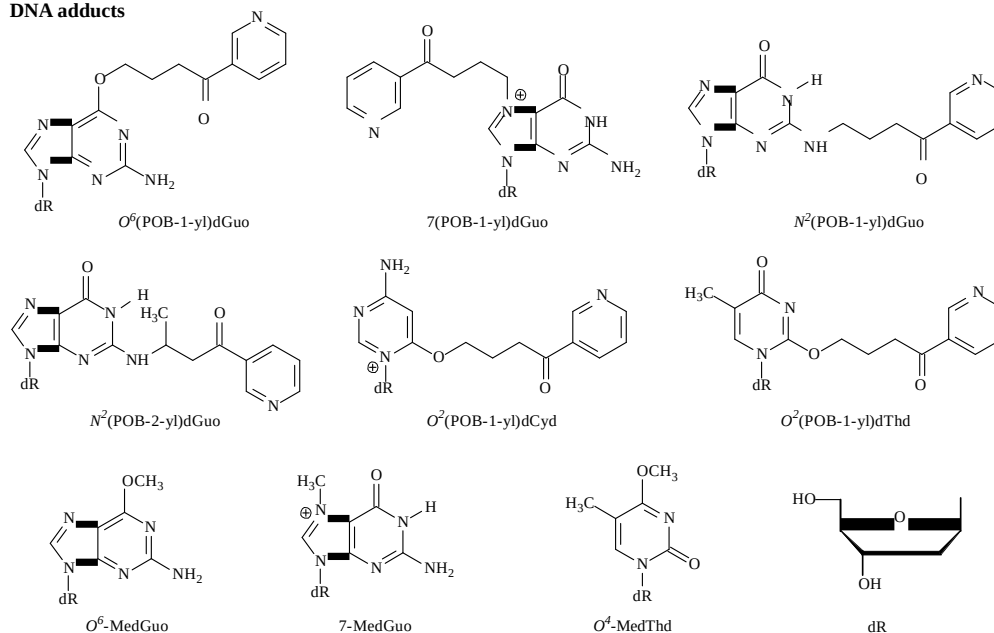
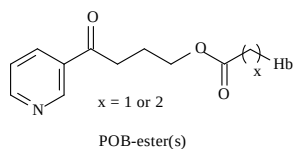
DZH, diazohydroxide; FA, formaldehyde; Gluc, glucuronide; HO, hydroxy; HOMe, hydroxymethyl; HOMethylene, hydroxymethylene; HPB, 4-hydroxy-1-(3-pyridyl)-1-butanone or keto alcohol; Me-DZH, methaneDZH; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; (NNK)ADP⁺, (NNK)adenosine dinucleotide phosphate; (NNK)ADPH, (NNK)adenosine dinucleotide phosphate (reduced form); POB-DZH, 4-oxo-4-(3-pyridyl)-1-butanoneDZH

Figure 4. Structures of NNAL and metabolites

DZH, diazohydroxide; Gluc, glucuronide; HMe, hydroxymethyl; HOMethylene, hydroxymethylene; NNAL, 4-(methyl-nitrosamino)-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)-1-(*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 lipoxxygenase 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 μ M and 6.5 ± 1.1 μ M, respectively) and antibody inhibition studies indicated that CYP2A13 (K_m , 2.8–11.3 μ M; 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 <i>et al.</i> (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 as above, DMSO control as above, but with 50 nM PEITC added as above, but with 100 nM PEITC added as above, but with 200 nM PEITC added	Smith <i>et al.</i> (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	$4.9 \times 10^{-3} \pm 2 \times 10^{-4}$		
			2.09 ± 0.20	760 ± 10	$2.8 \times 10^{-3} \pm 3 \times 10^{-4}$		
			2.06 ± 0.21	820 ± 20	$2.5 \times 10^{-3} \pm 3 \times 10^{-4}$		
			2.05 ± 0.25	1240 ± 70	$1.7 \times 10^{-3} \pm 2 \times 10^{-4}$		
Human CYP1A2 ^d	10–350	HPB	4.2 ± 0.2	309 ± 16	0.014 ± 0.001	Hep G2 cell lysate	Smith <i>et al.</i> (1992a)
Human CYP2A6	5–2000	Keto aldehyde	0.437	392	1.11×10^{-3}	Baculovirus-infected <i>Spodoptera frugiperda</i> (Sf9) cells	Patten <i>et al.</i> (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 <i>et al.</i> (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 <i>et al.</i> (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 <i>et al.</i> (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 <i>et al.</i> (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 <i>et al.</i> (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 $\pm$ 0.01	33 $\pm$ 0.7	$5.5 \times 10^{-3} \pm 3 \times 10^{-4}$	BD Biosciences Discovery Labware Supersomes	Dicke <i>et al.</i> (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 <i>et al.</i> (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 <i>et al.</i> (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 <i>et al.</i> (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 <i>et al.</i> (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 <i>et al.</i> (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 <i>et al.</i> (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 <i>et al.</i> (1992)
Rabbit CYP2G1	2.9–154	Keto aldehyde HPB	0.735 ND	186	3.95×10^{-3}	Purified, reconstituted protein	Hong <i>et al.</i> (1992)
Rat CYP1A1	1–5000	Keto aldehyde HPB	2.2 $\pm$ 0.1 0.68 $\pm$ 0.03	180 $\pm$ 34 140 $\pm$ 26	0.013 $\pm$ 0.002 $4.9 \times 10^{-3} \pm 9 \times 10^{-4}$	Gentest Supersomes	Jalas <i>et al.</i> (2005)
Rat CYP1A2	1–5000	Keto aldehyde HPB	5.0 $\pm$ 0.1 6.1 $\pm$ 0.09	180 $\pm$ 22 200 $\pm$ 11	0.028 $\pm$ 0.003 0.034 $\pm$ 0.002	Gentest Supersomes	Jalas <i>et al.</i> (2005)
Rat CYP2A3	0.25–50	Keto aldehyde HPB	10.8 $\pm$ 0.4 8.2 $\pm$ 0.3	4.6 $\pm$ 0.5 4.9 $\pm$ 0.5	2.3 $\pm$ 0.3 1.7 $\pm$ 0.2	Baculovirus-infected Sf9 cells	Jalas <i>et al.</i> (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	0.0897 ± 0.003	191 ± 20	4.70 × 10 ⁻⁴ ± 5.2 × 10 ⁻⁵	Purified, reconstituted protein	Guo <i>et al.</i> (1991a)
		HPB	0.333 ± 0.016	318 ± 5	1.05 × 10 ⁻³ ± 5 × 10 ⁻⁵		
		NNK- <i>N</i> -oxide	0.295 ± 0.006	131 ± 10	2.25 × 10 ⁻³ ± 1.7 × 10 ⁻⁴		
Rat CYP2C6	1–5000	Keto aldehyde	2.5 ± 0.1	1300 ± 130	1.9 × 10 ⁻³ ± 2 × 10 ⁻⁴	Gentest Supersomes	Jalas <i>et al.</i> (2005)
		HPB	16 ± 0.5	1400 ± 89	0.011 ± 0.0008		
		NNK- <i>N</i> -oxide	1.5 ± 0.06	1100 ± 110	1.4 × 10 ⁻³ ± 4 × 10 ⁻⁴		
Mouse CYP2A4	1–5000	Keto aldehyde	190 ± 23	3900 ± 995	0.049 ± 0.014	Baculovirus-infected Sf9 cells	Felicia <i>et al.</i> (2000)
Mouse CYP2A4	0.5–3000	Keto aldehyde	7.3 ± 0.4	97 ± 19	0.075 ± 0.015	Baculovirus-infected Sf9 cells	Jalas <i>et al.</i> (2003b)
		HPB	1.8 ± 0.1	67 ± 17	0.027 ± 0.007		
Mouse CYP2A5	0.25–100	HPB	4.0 ± 0.4	1.5 ± 0.6	2.7 ± 1.8	Baculovirus-infected Sf9 cells	Felicia <i>et al.</i> (2000)
Mouse CYP2A5	0.25–50	Keto aldehyde	2.0 ± 0.1	4.3 ± 0.8	0.47 ± 0.09	Baculovirus-infected Sf9 cells	Jalas <i>et al.</i> (2003b)
		HPB	6.5 ± 0.2	4.5 ± 0.4	1.4 ± 0.1		

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

^aUnits are pmol product/min/(pmol CYP).

^bUnits are pmol product/min/(pmol CYP)/μM.

^cV_{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).

^dThe 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).

^eCytochrome b₅ was added at a 3:1 b₅:CYP molar ratio. b₅:CYP molar ratio is corrected according to the original reference (Patten *et al.*, 1996).

^fHBP: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 μ M, 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 <i>toombak</i> users	1270	NR	NR	Murphy <i>et al.</i> (1994)
39 smokeless tobacco users	NR	4.4	NR	Kresty <i>et al.</i> (1996)
13 smokeless tobacco users	4.22	3.55	6.60	Hecht <i>et al.</i> (2002)
10 snuff dippers	4.20	NR	NR	Carmella <i>et al.</i> (2002)
55 snuff dippers	NR	3.25	NR	Carmella <i>et al.</i> (2003)
41 snuff dippers	NR	3.0	NR	Hatsukami <i>et al.</i> (2004)
54 snuff dippers	NR	3.3	NR	Lemmonds <i>et al.</i> (2005)
Smokers				
11 smokers	NR	NR	11.4	Carmella <i>et al.</i> (1993)
61 smokers	NR	3.76	NR	Carmella <i>et al.</i> (1995)
11 smokers	NR	NR	3.28	Hecht <i>et al.</i> (1995)
20 smokers	NR	NR	3.22	Meger <i>et al.</i> (2000)
13 smokers	NR	3.90	NR	Taioli <i>et al.</i> (1997)
27 smokers	1.95	2.70	3.14	Hecht <i>et al.</i> (1999a)
18 smokers	NR	3.69	NR	Hurt <i>et al.</i> (2000)
10 smokers	1.22	NR	NR	Carmella <i>et al.</i> (2002)
99 smokers	NR	2.07	NR	Hecht <i>et al.</i> (2004a)
41 smokers	NR	2.60	NR	Carmella <i>et al.</i> (2003)
34 smokers	NR	3.5	NR	Hughes <i>et al.</i> (2004)
38 smokers	NR	2.3	NR	Hatsukami <i>et al.</i> (2004)
84 smokers	NR	1.6	NR	Hecht <i>et al.</i> (2004b)
7 smokers	2.36	NR	5.32	Byrd & Ogden (2003)
12 smokers	2.8	NR	NR	Breland <i>et al.</i> (2003)
11 smokers	NR	1.34	NR	Sellers <i>et al.</i> (2003)
Nonsmokers exposed to secondhand tobacco smoke				
5 exposed men	NR	NR	0.127	Hecht <i>et al.</i> (1993a)
9 exposed hospital workers	0.059	NR	NR	Parsons <i>et al.</i> (1998)
29 exposed nonsmokers	NR	NR	0.027	Meger <i>et al.</i> (2000)
23 exposed women	0.050	NR	NR	Anderson <i>et al.</i> (2001)
74 exposed children	0.056	NR	NR	Hecht <i>et al.</i> (2001)
16 casino patrons	0.033	0.037	NR	Anderson <i>et al.</i> (2003)
19 restaurant workers	0.066	0.070	0.107	Tulunay <i>et al.</i> (2005)
27 newborns of smoking mothers	0.14	NR	NR	Lackmann <i>et al.</i> (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 $\mu\text{mol/kg}$ bw over 30 years of snuff dipping/chewing and 2.5 $\mu\text{mol/kg}$ 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 $\mu\text{mol/kg}$ 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 $\mu\text{mol/kg}$ 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 $\mu\text{mol/kg}$ bw (9.04 g/kg bw). These estimated total doses of NNK (1440 $\mu\text{mol/kg}$ bw) and NNN (43 200 $\mu\text{mol/kg}$ bw) can be compared with the total doses of NNK (240 $\mu\text{mol/kg}$ bw) and NNN (1400 $\mu\text{mol/kg}$ 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 abstinence 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 μ mol (2.4 μ mol/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 μ mol/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 2¹-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 O⁶-methyldeoxyguanosine (O⁶-MedGuo), 7-MedGuo, O⁴-methyldeoxythymidine (O⁴-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*¹-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*

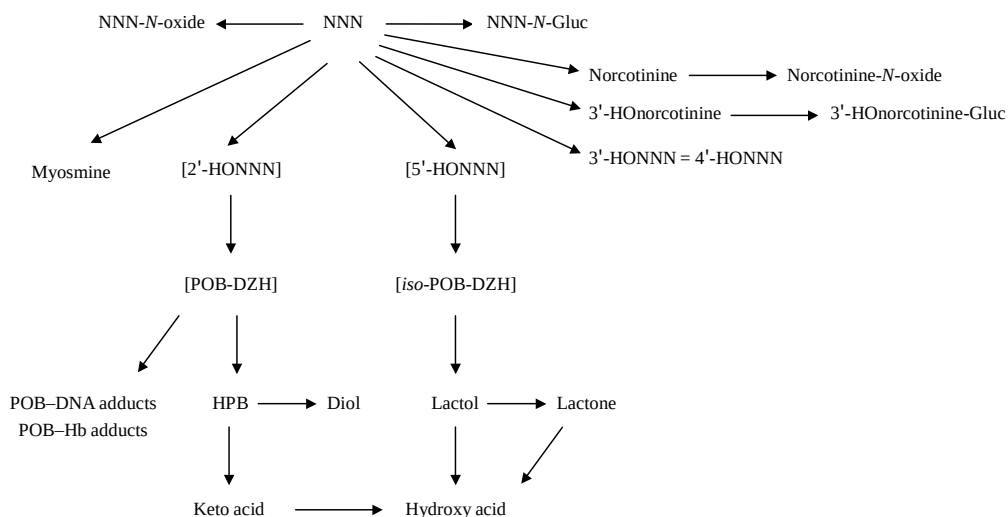
NNN was detected in two of 17 samples of pancreatic juice from smokers at levels of 68.1 and 242 ng/mL juice. It was not detected in the pancreatic juice from nine nonsmokers (Prokopczyk *et al.*, 2002). Pyridyloxobutyl-haemoglobin adducts have been detected in smokeless tobacco users and smokers, and 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.

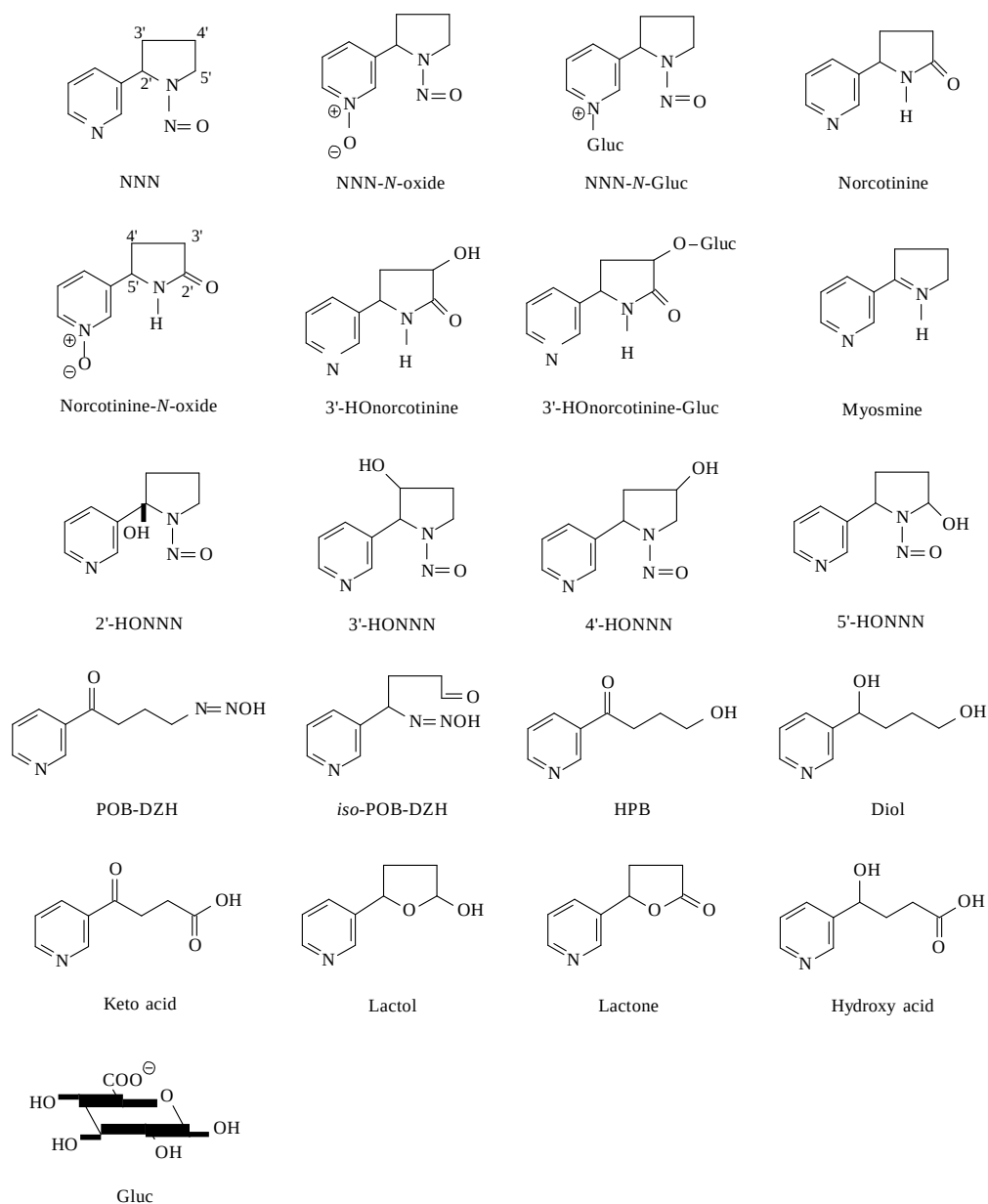
Figure 6. Metabolism of NNN and formation of adducts, based on studies in laboratory animals and humans



DZH, diazohydroxide; Gluc, glucuronide; Hb, haemoglobin; HO, hydroxy; HPB, 4-hydroxy-1-(3-pyridyl)-1-butanone or keto alcohol; NNN, *N*-nitrosornicotine; 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*'-nitrosanornicotine; 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 6 β -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_m s 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_m s 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 throughout 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*

Autoradiographic 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 - ^3H]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 μ M. 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 <i>et al.</i> (1997)
	HPB	19.1 ± 0.8	4.9 ± 0.2	3.9 ± 0.2		
	NNK- <i>N</i> -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 <i>et al.</i> (1992a)
	HPB	14.6 ± 1.0	7.0 ± 0.5	2.1 ± 0.2		
	NNK- <i>N</i> -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 <i>et al.</i> (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 <i>et al.</i> (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- <i>N</i> -oxide	140 ± 10	57 ± 9	2.5 ± 0.4	As above, but animals were treated with pregnenolone 16α-carbonitrile.	
	Keto aldehyde	550 ± 44	133 ± 21	4.1 ± 0.7		
	HPB	247 ± 18	187 ± 24	1.3 ± 0.2		
Male Sprague-Dawley rat nasal mucosa	NNK- <i>N</i> -oxide	167 ± 15	103 ± 21	1.6 ± 0.4	1–100 μM NNK, 10-min incubation, 0.013 mg/mL microsomal protein.	Smith <i>et al.</i> (1992a)
	Keto aldehyde	2833 ± 81	9.6 ± 0.3	295 ± 12		
	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 $\pm$ 2.2	5.6 $\pm$ 0.9	10.2 $\pm$ 1.7	1–20 μM NNK, 30-min incubation, 0.25 mg/mL microsomal protein.	Smith <i>et al.</i> (1990)
	HPB	56.0 $\pm$ 3.8	5.6 $\pm$ 0.9	10.0 $\pm$ 1.7		
	Keto acid	4.2 $\pm$ 0.5	9.2 $\pm$ 1.0	0.456 $\pm$ 0.074		
	NNK- <i>N</i> -oxide	54.2 $\pm$ 1.3	4.7 $\pm$ 0.9	11.5 $\pm$ 2.2	0.5–100 μM NNK, 30-min incubation, 0.25 mg/mL microsomal protein.	Peterson <i>et al.</i> (1991a)
	NNAL ^c	1322 $\pm$ 10	2541 $\pm$ 15	0.52 $\pm$ 0.005		
	Keto aldehyde	58.9 $\pm$ 2.6	23.7 $\pm$ 2.6	2.5 $\pm$ 0.3		
Female A/J mouse lung	HPB	32.5 $\pm$ 2.5	3.6 $\pm$ 0.9	9.0 $\pm$ 2.4		
	Keto aldehyde	34.0	4.9	6.9	1–10 μM NNK, 30-min incubation, 0.25 mg/mL microsomal protein.	Smith <i>et al.</i> (1993)
	HPB	38.1	2.6	15		
	NNK- <i>N</i> -oxide	60.0	1.8	33		
	Keto aldehyde	31.8	5.0	6.4	As above, but animals were treated with PEITC (1 $\mu\text{mol/g}$ diet).	
	HPB	35.1	2.9	12		
	NNK- <i>N</i> -oxide	51.0	1.8	28		
	Keto aldehyde	25.7	4.7	5.5	As above, but animals were treated with PEITC (3 $\mu\text{mol/g}$ diet).	
	HPB	23.0	2.4	9.6		
	NNK- <i>N</i> -oxide	36.7	1.6	23		
	Keto aldehyde	84.7 $\pm$ 2.8	4.5 $\pm$ 0.4	19 $\pm$ 2	0.25–20 μM NNK, 30-min incubation, 0.25 mg/mL microsomal protein.	
	HPB	62.8 $\pm$ 1.3	1.9 $\pm$ 0.2	33 $\pm$ 4		
	NNK- <i>N</i> -oxide	83.3 $\pm$ 3.6	2.0 $\pm$ 0.3	42 $\pm$ 7		
	Keto aldehyde ^d	89.2 $\pm$ 6.3	24.0 $\pm$ 2.7	3.7 $\pm$ 0.5	As above, but with PEITC (400 nM) added to the incubation mixtures.	Smith <i>et al.</i> (1993)
	HPB ^d	60.4 $\pm$ 1.8	14.9 $\pm$ 0.8	4.1 $\pm$ 0.2		
	NNK- <i>N</i> -oxide ^d	85.8 $\pm$ 4.6	17.9 $\pm$ 1.7	4.8 $\pm$ 0.5		
	Keto aldehyde	71 $\pm$ 3	4.8 $\pm$ 0.7	15 $\pm$ 2	0.25–50 μM NNK, 15-min. incubation, 0.25 mg/mL microsomal protein.	Jalas <i>et al.</i> (2003b)
	HPB	93 $\pm$ 3	3.0 $\pm$ 0.4	31 $\pm$ 4		
	NNK- <i>N</i> -oxide	109 $\pm$ 4	2.1 $\pm$ 0.3	52 $\pm$ 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 $\pm$ 17	24 $\pm$ 4	10 $\pm$ 2	0.5–100 μM NNK, 15-min	Nunes <i>et al.</i> (1998)
	HPB	100 $\pm$ 7	18 $\pm$ 4	5.6 $\pm$ 1.3	incubation, 0.25 mg/mL microsomal protein.	
	Keto aldehyde ^d	213 $\pm$ 41	23 $\pm$ 12	9.3 $\pm$ 5.2	As above, but with 2.5 μM 4-HPO	
	HPB ^d	77 $\pm$ 13	17 $\pm$ 8	4.5 $\pm$ 2.3	added	Nunes <i>et al.</i> (1998)
	Keto aldehyde ^d	210 $\pm$ 16	24 $\pm$ 4	8.8 $\pm$ 1.6	As above, but with 5.0 μM 4-HPO	
	HPB ^d	69 $\pm$ 11	17 $\pm$ 8	4.1 $\pm$ 2.0	added	
	Keto aldehyde ^d	170 $\pm$ 14	22 $\pm$ 5	7.7 $\pm$ 1.9	As above, but with 10 μM 4-HPO	Nunes <i>et al.</i> (1998)
	HPB ^d	71 $\pm$ 4	18 $\pm$ 3	3.9 $\pm$ 0.7	added	
	Keto aldehyde ^d	78 $\pm$ 1	22 $\pm$ 8	3.5 $\pm$ 1.3	As above, but with 20 μM 4-HPO	
	HPB ^d	44 $\pm$ 5	18 $\pm$ 5	2.4 $\pm$ 0.7	added	Peterson <i>et al.</i> (1991a)
	Keto aldehyde	173 $\pm$ 6	19.1 $\pm$ 2	9.1 $\pm$ 0.7	1–100 μM NNK, 10-min incubation,	
	HPB	239 $\pm$ 11	73.8 $\pm$ 6.8	3.2 $\pm$ 0.3	0.25 mg/mL microsomal protein.	
	Keto aldehyde	132 $\pm$ 11	5.5 $\pm$ 0.3	24 $\pm$ 2	1–10 μM NNK, 10-min incubation,	Smith <i>et al.</i> (1993)
	HPB	60.4 $\pm$ 3.1	5.1 $\pm$ 0.2	11.8 $\pm$ 0.8	0.50 mg/mL microsomal protein.	
	NNK-N-oxide	8.0 $\pm$ 0.6	8.8 $\pm$ 0.5	0.91 $\pm$ 0.09		
	Keto aldehyde	77.0 $\pm$ 9.3	5.4 $\pm$ 0.4	14.3 $\pm$ 2.0	As above, but animals were treated	
	HPB	39.3 $\pm$ 2.5	5.3 $\pm$ 0.3	7.4 $\pm$ 0.6	with PEITC (3 $\mu\text{mol/g}$ diet).	
	NNK-N-oxide	5.6 $\pm$ 0.7	9.1 $\pm$ 0.3	0.62 $\pm$ 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 $V_{\max}^i$, K_m^i , and $V_{\max}^i/K_m^i$, respectively, due to the presence of an inhibitor in the incubation mixtures.

determined (Hong *et al.*, 1992). In the absence of exogenous cytochrome b₅, 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 16 α -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 ($\pm$)-, (*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 μ M for NNK formation in the lung to 1624 μ M 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 μ M)	Intermediate K_m (50–500 μ M)	High K_m (> 500 μ M)	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 $\approx$ 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- <i>N</i> -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- <i>N</i> -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- <i>N</i> -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 $\mu\text{g/kg}$ 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 pharmacokinetics 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 $\mu\text{mol/kg}$ 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 <i>et al.</i> (2003a)
		Diol	0.79 ± 0.02	40 ± 3	0.020 ± 0.002		
		NNAL- <i>N</i> -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	Baculovirus- infected Sf9 cells	Jalas & Hecht (2003)
		Diol	0.98 ± 0.02	16 ± 1	0.061 ± 0.004		
		NNAL- <i>N</i> -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- <i>N</i> -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	Baculovirus- infected Sf9 cells	Jalas & Hecht (2003)
		Diol	1.2 ± 0.03	11 ± 0.8	0.11 ± 0.008		
		NNAL- <i>N</i> -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	Baculovirus- infected Sf9 cells	Jalas & Hecht (2003)
		Diol	0.17 ± 0.01	1.3 ± 0.4	0.13 ± 0.04		
		NNAL- <i>N</i> -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 μ mol/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 μ mol). 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 [5-³H] NNAL (8.5 μ mol/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 [5-³H] (*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 μ mol) 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 [³H]-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, O⁶-MedGuo and O⁴-MedThd. Other adducts are probably also produced, based on studies of other methylating nitrosamines and nitroso-ureas (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 O²(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 O⁶-alkylguanine–DNA alkyltransferase (AGT), the enzyme responsible for the repair of O⁶-MedGuo. Since O⁶-MedGuo is also formed by metabolic activation of NNK, this phenomenon is probably important in the persistence of O⁶-MedGuo in NNK-exposed tissues (Peterson *et al.*, 1993).

In-vivo studies

Since O⁶-methylguanine (O⁶-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 O⁶-MeGua, which are about 10 times greater than those of O⁴-MedThd (Belinsky *et al.*, 1986; Staretz *et al.*, 1997c). Consistently, the highest levels of O⁶-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 O⁶-MeGua. Pyridyloxobutyl adduct concentrations increase at low doses of NNK, which may lead to greater inhibition of AGT and consequent higher levels of O⁶-MeGua (Peterson *et al.*, 1993).

During chronic treatment with high doses of NNK, O⁶-MeGua increases consistently in the rat lung (Belinsky *et al.*, 1986). At lower doses over a 4-day period, O⁶-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-D₂]NNK is a stronger nasal carcinogen than either [methyl-D₃]NNK or NNK (Hecht *et al.*, 1987; Trushin *et al.*, 1994). Moreover, DNA pyridyloxobutylation by [methylene-D₂]NNK exceeds that by NNK while levels of O⁶-MeGua from [methylene-D₂]NNK are significantly lower than those from NNK or [methyl-D₃]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 O⁶-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 O⁶-MeGua are probably due to differences in repair (Belinsky *et al.*, 1986, 1990; Peterson *et al.*, 1991b). During chronic NNK treatment, O⁶-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 O⁶-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 O⁶-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 O⁶-MeGua, which in turn exceed those of HPB-releasing adducts (Peterson & Hecht, 1991). Levels of 7-MeGua and O⁶-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-D₂]NNK is significantly less tumorigenic than NNK or [methyl-D₃]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 > O⁶-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 O⁶-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 O⁶-MeGua are similar in rat and hamster liver after a single dose of NNK (Liu *et al.*, 1992). However, O⁶-MeGua is repaired more rapidly in rats (half-time, 12 h), while only 14% of the initial O⁶-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 O⁶-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-oxo-deoxyguanosine 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 $\mu\text{g/kg}$) 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'-¹⁴C]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'-¹⁴C]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 [5-³H]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'-¹⁴C]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 [5-³H]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 μ M (Murphy & Spina, 1994).

Products of 2'-hydroxylation exceeded those of 5'-hydroxylation 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 μ M). 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 μ M. 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 μ M). Mouse CYP2A4 is a poorer catalyst with K_m values of 54.1–68.5 μ M. Rat CYP2A3 and mouse CYP2A5 catalyse 2'-hydroxylation of (R)-NNN with K_m values of 0.73–1.64 μ M, while the K_m for mouse CYP2A4 is 66 μ M. 2'-Hydroxylation of (S)-NNN was not observed in studies with these enzymes (Wong *et al.*, 2005b).

compound to pass freely across biological membranes and distribute evenly in the intra-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 (LD₅₀) 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 LD₅₀ 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'-¹⁴C]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'-¹⁴C]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 Bowman's glands and caused degeneration of the olfactory epithelium at intraperitoneal doses of 4.8–48 $\mu\text{mol/kg}$ 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 $\mu\text{g/mL}$ (Zhu *et al.*, 1991) and in human–hamster hybrid A_L cells at a dose of 500 $\mu\text{g/mL}$ (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 LD₅₀ 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 LD₅₀ 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 vitamin B- and protein-deficient diets (Ammigan *et al.*, 1990b).

(c) *NAB*

The subcutaneous LD₅₀ 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		
<i>Salmonella typhimurium</i> TA100, TA1535, reverse mutation	NT	+	2 µmol/plate [414 µg/plate]	Hecht <i>et al.</i> (1983c)
<i>Salmonella typhimurium</i> TA100, TA1535, reverse mutation	NT	+	2 µmol/plate ^c [418 µg/plate]	Hecht <i>et al.</i> (1983c)
<i>Salmonella typhimurium</i> TA100, TA1535, reverse mutation	NT	–	4 µmol/plate ^d [840 µg/plate]	Hecht <i>et al.</i> (1983c)
<i>Salmonella typhimurium</i> TA100, reverse mutation	–	+	1000 µg/plate	Padma <i>et al.</i> (1989b)
<i>Salmonella typhimurium</i> TA100, reverse mutation	–	–	2000 µg/plate	Yim & Hee (2001)
<i>Salmonella typhimurium</i> TA1535, reverse mutation	–	+	1000 µg/mL	Padma <i>et al.</i> (1989b)
<i>Salmonella typhimurium</i> TA1535, reverse mutation	–	–	2500 µg/plate	Zhu <i>et al.</i> (1991)
<i>Salmonella typhimurium</i> TA1535, reverse mutation	–	+	500 µg/plate	Zhu <i>et al.</i> (1991)
<i>Salmonella typhimurium</i> TA1538, reverse mutation	–	–	1000 µg/mL	Padma <i>et al.</i> (1989b)
<i>Salmonella typhimurium</i> TA98, reverse mutation	–	–	1000 µg/plate	Padma <i>et al.</i> (1989b)
<i>Salmonella typhimurium</i> TA98, reverse mutation	–	+	20 µM [4.2 µg/mL]	Kolar & Lawson (1997)
<i>Salmonella typhimurium</i> YG7108, reverse mutation	–	NT	0.7 mM [145 µg/mL]	Kushida <i>et al.</i> (2000a,b)
<i>Salmonella typhimurium</i> YG7108 -2A6/OR ^e , reverse mutation	+	NT	0.1 mM [20.7 µg/mL]	Kushida <i>et al.</i> (2000a,b)
<i>Salmonella typhimurium</i> YG7108 -2E1/OR ^f , reverse mutation	–	NT	0.7 mM [145 µg/mL]	Kushida <i>et al.</i> (2000a,b)
<i>Salmonella typhimurium</i> TA7004, reverse mutation	–	+	2000 µg/plate	Yim & Hee (2001)
DNA strand breaks, primary rat hepatocytes <i>in vitro</i>	+	NT	5 mM [1035 µg/mL]	Liu <i>et al.</i> (1990)
DNA strand breaks, primary rat hepatocytes <i>in vitro</i>	+	NT	6.25 µmol/mL [1297 µg/mL]	Pool-Zobel <i>et al.</i> (1992)
DNA strand breaks, isolated rabbit lung cells (Type II and Clara) <i>in vitro</i>	+	NT	30 µM [6.22 µg/mL]	Becher <i>et al.</i> (1993) DNA
strand breaks, isolated rabbit alveolar macrophages <i>in vitro</i>	–	NT	300 µM [62.2 µg/mL]	Becher <i>et al.</i> (1993)
Unscheduled DNA synthesis, freshly isolated rat hepatocytes <i>in vitro</i>	+	NT	1 mM [207.5 µg/mL]	Williams & Laspia, 1979
Unscheduled DNA synthesis, rabbit lung cells <i>in vitro</i>	+	NT	2 mM [414.5 µg/mL]	Dahl <i>et al.</i> (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, <i>Hprt</i> locus, <i>in vitro</i>	–	+	10 mM [2072 µg/mL]	Swedmark <i>et al.</i> (1994)
Sister chromatid exchange, Chinese hamster lung fibroblastic V79 cells <i>in vitro</i>	–	+	20 µg/mL	Zimonjic <i>et al.</i> (1989)
Sister chromatid exchange, Chinese hamster lung fibroblastic V79 cells <i>in vitro</i>	–	+	20 mM [4145 µg/mL]	Alaoui Jamali <i>et al.</i> (1988)
Sister chromatid exchange, Chinese hamster ovary (CHO) cells <i>in vitro</i>	NT	+	0.1 mM [20.7 µg/mL]	Lee <i>et al.</i> (1996)
Micronucleus formation, Chinese hamster lung fibroblastic V79 cells <i>in vitro</i>	–	+	5 mM [1036 µg/mL]	Alaoui Jamali <i>et al.</i> (1988)
Micronucleus formation, rat tracheal epithelial cells <i>in vitro</i>	+	NT	50 µg/mL	Zhu <i>et al.</i> (1991)
Chromosomal aberrations, Chinese hamster lung fibroblastic V79 cells <i>in vitro</i>	+	–	20 mM [4145 µg/mL]	Alaoui Jamali <i>et al.</i> (1988)
DNA strand breaks, human MRC-5 fetal lung cells <i>in vitro</i>	+	NT	5 mM [1035 µg/mL]	Weitberg & Corvese (1993, 1997)
Gene mutation, human lymphoblastoid cells, <i>HPRT</i> locus, <i>in vitro</i>	+	NT	1 µg/mL	Krause <i>et al.</i> (1999)
Sister chromatid exchange, human peripheral blood lymphocytes <i>in vitro</i>	+	NT	[0.48 mM]	Padma <i>et al.</i> (1989b)
			100 µg/mL	
Sister chromatid exchange, human peripheral blood lymphocytes <i>in vitro</i>	+	+	[0.096 mM]	Zimonjic <i>et al.</i> (1989)
			20 µg/mL	
Micronucleus formation, human AGT repair-deficient fibroblasts <i>in vitro</i>	+	NT	0.05 mM [10.4 µg/mL]	Pohlmann <i>et al.</i> (1992)
Micronucleus formation, human AGT repair-proficient fibroblasts <i>in vitro</i>	–	NT	1 mM [207.5 µg/mL]	Pohlmann <i>et al.</i> (1992)
Micronucleus formation, human-derived hepatoma HepG2 and Hep3B cells <i>in vitro</i>	–	NT	5 mM [1036 µg/mL]	Majer <i>et al.</i> (2004)
Chromosomal aberrations, human peripheral blood lymphocytes <i>in vitro</i>	+	NT	0.48 mM [100 µg/mL]	Padma <i>et al.</i> (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 <i>in vivo</i>	+		12.5 mg/kg bw po 1× [60 µmol/kg bw]	Pool-Zobel <i>et al.</i> (1992)
DNA strand breaks, rat and hamster hepatocytes <i>in vivo</i>	+		0.39 mmol/kg bw [81 mg/kg bw] ip 1×	Jorquera <i>et al.</i> (1994)
Gene mutation, rat splenic T lymphocytes, <i>Hprt</i> locus, <i>in vivo</i>	—		150 mg/kg bw ip 1×	Jansen <i>et al.</i> (1996)
Micronucleus formation, Swiss male mice bone marrow <i>in vivo</i>	+		250 mg/kg bw/d ip 2×	Padma <i>et al.</i> (1989b)
Micronucleus formation, rat tracheal epithelial cells <i>in vivo</i>	+		150 mg/kg bw/d ip 3×	Zhu <i>et al.</i> (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 (MutaTMMouse) 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 (MutaTM 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 O⁶-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*)F₁ 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*)F₁ 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-epithelialized 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 fore-stomach 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 fore-stomach 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 E₂ (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 μ mol/plate [5.5–42 μ g/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 N¹-nitrosonornicotine (NNN)

Test system	Result ^a		Dose ^b (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
<i>Salmonella typhimurium</i> TA100, reverse mutation	–	+	2.5 µmol/plate [443 µg/plate]	Bartsch <i>et al.</i> (1980)
<i>Salmonella typhimurium</i> TA100, reverse mutation	–	+	250 µg/plate	Padma <i>et al.</i> (1989b)
<i>Salmonella typhimurium</i> , TA100, TA7004, reverse mutation	+	–	2000 µg/plate	Yim & Hee (2001)
<i>Salmonella typhimurium</i> TA100, TA7004, reverse mutation	+	–	500 µg/plate	Yim & Hee (2001)
<i>Salmonella typhimurium</i> TA1530, reverse mutation	+	NT	1000 µg/plate	Andrews <i>et al.</i> (1978)
<i>Salmonella typhimurium</i> TA1535, reverse mutation	–	+	1000 µg/plate	Padma <i>et al.</i> (1989b)
<i>Salmonella typhimurium</i> TA1538, TA 98, reverse mutation	–	–	1000 µg/plate	Padma <i>et al.</i> (1989b)
<i>Salmonella typhimurium</i> YG7108, reverse mutation	–	NT	0.7 mM	Kushida <i>et al.</i> (2000a); Fujita & Kamataki (2001)
<i>Salmonella typhimurium</i> YG7108 -2A6/OR ^c , reverse mutation	+	NT	0.2 mM [35.5 µg/mL]	Kushida <i>et al.</i> (2000a); Fujita & Kamataki (2001)
<i>Salmonella typhimurium</i> YG7108 -2E1/OR ^d , reverse mutation	–	NT	0.7 mM [124 µg/mL]	Kushida <i>et al.</i> (2000a); Fujita & Kamataki (2001)
DNA strand breaks, primary rat hepatocytes <i>in vitro</i>	+	NT	5 mM [886 µg/mL]	Liu <i>et al.</i> (1990)
DNA strand breaks, isolated rabbit lung cells (Type II and Clara) and alveolar macrophages <i>in vitro</i>	–	NT	3 mM [531 µg/mL]	Becher <i>et al.</i> (1993)
DNA strand breaks, rat hepatocytes <i>in vitro</i>	–	NT	25 mM [4430 µg/mL]	Pool-Zobel <i>et al.</i> (1992)
Unscheduled DNA synthesis, freshly isolated rat hepatocytes <i>in vitro</i>	+	NT	1 mM [177 µg/mL]	Williams & Laspia (1979)
Unscheduled DNA synthesis, rabbit lung cells <i>in vitro</i>	–	NT	2 mM [354 µg/mL]	Dahl <i>et al.</i> (1990)
Gene mutation, Chinese hamster V79 cells, Hprt locus, <i>in vitro</i>	–	+	10 mM [1770 µg/mL]	Swedmark <i>et al.</i> (1994)
DNA strand breaks, human MRC-5 fetal lung cells <i>in vitro</i>	+	NT	5 mM [886 µg/mL]	Weitberg & Corvese (1993, 1997)
Sister chromatid exchange, human peripheral blood lymphocytes <i>in vitro</i>	–	NT	100 µg/mL	Padma <i>et al.</i> (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 <i>in vitro</i>	—	NT	100 µg/mL	Padma <i>et al.</i> (1989b)
DNA strand breaks, Sprague-Dawley rat liver <i>in vivo</i>	+		100 mg/kg bw po x 1 [565 µmol/kg bw]	Pool-Zobel <i>et al.</i> (1992)
Micronucleus formation, Swiss male mice bone marrow <i>in vivo</i>	+		250 mg/kg bw ip x2	Padma <i>et al.</i> (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 (MutaTMMouse) (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 reversion 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 propranolol, 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_7 nAChR) 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_7 nAChR 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_7 nAChR 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 $p16^{\text{INK4a}}$ ($p16$) 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 ($\text{RAR-}\beta$), which encodes one of the primary receptors for retinoic acid, is down-regulated by methylation in human lung cancer (Vuilleminot *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 (Vuilleminot *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_7 nAChR. 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'-Nitrosornicotine (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'*-nitrosornicotine (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 subcutaneously 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.

commercially and non-commercially prepared tobacco products including cigarettes, cigars, 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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