Overview information for

Tobacco leaf

TOBACCO LEAF REFERENCES

Combined reduced expression of two gene families lowers nicotine content to ultra-low levels in cultivated tobacco.	
Kernodle SP, Webb S, Steede TM, Lewis RS.	Plant Cell Rep. 2022 Sep;41(9):1853-1862
Reduced expression of two gene families results in ultra-low nicotine accumulation in Nicotiana tabacum. The potential for mandated lowering of tobacco cigarette filler nicotine levels to below 0.4 mg g-1 is currently being discussed by regulatory and public health organizations. Commercial tobacco cultivars that would routinely meet this proposed standard do not currently exist. Inactivation or silencing of gene families corresponding to single enzymatic steps in the nicotine biosynthetic pathways have not resulted in tobacco genotypes that would meet this standard under conventional agronomic management. Here, we produced and evaluated under field conditions tobacco genotypes expressing an RNAi construct designed to reduce expression of the Methyl Putrescine Oxidase (MPO) gene family associated with nicotine biosynthesis. In a standard flue-cured genetic background, cured leaf nicotine levels were reduced to only 1.08 to 1.65 mg g-1. When MPO RNAi was combined with reduced Berberine Bridge Like (BBL) activity conferred by induced mutations, genotypes producing cured leaf nicotine levels slightly lower than 0.4 mg g-1 were generated. Past research has suggested that MPO activity may contribute to the biosynthesis of nornicotine in a route that does not involve nicotine. However, nornicotine was not reduced to zero in MPO-silenced plants that were also homozygous for induced mutations in known Nicotine Demethylase genes that are responsible for the vast majority of nornicotine accumulation.	

The Agronomic Traits, Alkaloids Analysis, FT-IR and 2DCOS-IR Spectroscopy Identification of the Low-Nicotine-Content Nontransgenic Tobacco Edited by CRISPR-Cas9.	
Zhang J, Zhou Q, Zhang D, Yang G, Zhang C, Wu Y, Xu Y, Chen J, Kong W, Kong G, Wang J.	Molecules. 2022 Jun 14;27(12):3817
In this study, the agricultural traits, alkaloids content and Fourier transform infrared spectroscopy (FT-IR) and two-dimensional correlation infrared spectroscopy (2DCOS-IR) analysis of the tobacco after Berberine Bridge Enzyme-Like Proteins (BBLs) knockout were investigated. The knockout of BBLs has limited effect on tobacco agricultural traits. After the BBLs knockout, nicotine and most alkaloids are significantly reduced, but the content of myosmine and its derivatives increases dramatically. In order to identify the gene editing of tobacco, principal component analysis (PCA) was performed on the FT-IR and 2DCOS-IR spectroscopy data. The results showed that FT-IR can distinguish between tobacco roots and leaves but cannot classify the gene mutation tobacco from the wild one. 2DCOS-IR can enhance the characteristics of the samples due to the increased apparent resolution of the spectra. Using the autopeaks in the synchronous map for PCA analysis, we successfully identified the mutants with an accuracy of over 90%.	

Genotoxic effect induced by dried nicotiana tabacum leaves from tobacco barns (kiln-houses) in chinese hamster lung fibroblast cells (V79).	
Dalberto D, Nicolau CC, Rosa De Sousa M, Garcia ALH, Boaretto F, Picada JN, De Souza GMS, Chytry P, Dias JF, Feistel CC, Ferraz ABF, Grivicich I, Da Silva J	J Toxicol Environ Health A. 2021 Sep 2;84(17):689-701.
Nicotiana tabacum is the most cultivated tobacco species in the state of Rio Grande do Sul, Brazil. Workers who handle the plant are exposed to the leaf components during the harvesting process and when separating and classifying the dried leaves. In addition to nicotine, after the drying process, other components may be found including tobacco-specific nitrosamines, polycyclic aromatic hydrocarbons, as well as pesticides	

residues. The objective of this study was to examine the genotoxicity attributed to the aqueous extract of dried tobacco leaves obtained from tobacco barns using Chinese hamster lung fibroblast cells (V79) as a model system by employing alkaline comet assay, micronucleus (MN) and Ames test. MTT assay was used to assess cytotoxicity and establish concentrations for this study. Data demonstrated cell viability > 85% for concentrations of 0.625-5 mg/ml while the comet assay indicated a significant increase in DNA damage at all concentrations tested. A significant elevation of MN and nuclear buds (NBUD) was found for 5 mg/ml compared to control and other dry tobacco leaves concentrations (0.625-2.5 mg/ml). Mutagenicity was not found using the Salmonella/Microsome test (TA98, TA100, and TA102 strains) with and without metabolic activation. The concentration of inorganic elements was determined employing the PIXE technique, and 13 inorganic elements were detected. Using CG/MS nicotine amounts present were 1.56 mg/g dry tobacco leaf powder. Due to the observed genotoxicity in V79 cells, more investigations are needed to protect the health of tobacco workers exposed daily to this complex mixture of toxic substances present in dry tobacco leaves.

Anxiolytic Effect of Two Tobacco Essential Oils (Nicotiana tabacum Linn.) on Mice.	
Xie D, Yao L, Huang Y, Wu S, Ma L, Li Y, Wang W	Molecules. 2021 Jul 9;26(14):4171.

Tobacco (Nicotiana tabacum Linn.) is a famous traditional herb used in folk medicine. The essential oils of tobacco have been demonstrated in modern studies to possess antioxidant, anti-inflammatory, and neuroprotective properties, while its anxiolytic effect has not been reported. The purpose of this study was to evaluate the anxiolytic effect of Yunnan tobacco essential oil (YTO) and Zimbabwe tobacco essential oil (ZTO) on mice. The constituents of YTO and ZTO were analyzed by GC/MS. The anxiolytic effect of YTO and ZTO (0.1%, 1%, and 10%, v/v) on male ICR mice was evaluated in the light-dark box test (LDB) and the elevated plus maze test (EPM) test via inhalation and transdermal administration. After the behavioral tests, salivary corticosterone levels in mice were measured. The behavioral analysis showed that the administration of both YTO and ZTO elevated the time that the mice spent in the light chamber in the LDB test compared to the untreated control. In the EPM test, YTO and ZTO increased the time spent in open arms and the number of entries into the open arms. In addition, both YTO and ZTO significantly decreased salivary corticosterone levels in mice ($p \leq 0.001$). In summary, our results demonstrated that inhalation and transdermal administration of both YTO and ZTO showed anxiolytic effect on male ICR mice.

The Consequence of Aqueous Extract of Tobacco Leaves (Nicotiana tabacum) on Feed Intake, Body Mass, and Hematological Indices of Male Wistar Rats fed under Equal Environmental Conditions.	
Andong FA, Okwuonu ES, Melefa TD, Okoye CO, Nkemakolam AO, Hinmikaiye FF, Nwankpo EO, Ozue CC.	J Am Coll Nutr. 2020 Jul 30:1-14.

While the dangers of consuming tobacco by smoking has been of concern, the hazardous effect of other forms of tobacco consumption (in smokeless form) on health indices are less well explored. In this paper, we explored the effect of different doses of aqueous extract of tobacco leaves on feed intake, body mass, and hematological indices of male Wistar rats under equal environmental conditions.

METHOD: Using an oral route of administration, the rats ($n = 24$; $w = 65\text{-}85$ g; 2-3 weeks old) were administered at different doses of 100, 200, 400, 0 mg/kg body weight (b.w.) per day to group A, B, C, and D for 42 days, after phytochemical and acute toxicity testing of the tobacco leaves.RESULTS: Compared to the negative control group (D), packed cell volume, hemoglobin concentration, red blood cells, and lymphocytes reduced dose-dependently in contrast to the white blood cells, neutrophils, mean corpuscular hemoglobin concentration, mean corpuscular hemoglobin, and mean corpuscular volume. As body mass and feed intake in relation to the different doses of aqueous extract of tobacco leaves reduced significantly ($p < 0.05$), the reverse was observed for body mass and feed intake in relation to room temperature.

CONCLUSIONS: By influencing hematological indices, feed intake, and body mass, the extract of tobacco leaves can be hazardous to health. However, to assess safety and to come up with a conscious conclusion, future studies should explore the effect of the extract on organs histopathology, biochemical parameters, and lipid profile of the body.

Effect of bidi cigarette smoking on interleukin-1β and 8 levels in chronic periodontitis patient.	
Nath S, Prakash J, Prajapati VK, Sharma N, Pulikkotil SJ.	Indian J Dent Res. 2020 May-Jun;31(3):433-438
INTRODUCTION: Bidi, a leaf rolled cigarette, is the most popular form of smoking in India. Bidi cigarette contains higher tar, ammonia, and nicotine content than a conventional cigarette and is more hazardous. AIM OF STUDY: The aim of this study was to determine the effect of bidi smoking on periodontitis by assessing the interleukin (IL)-1β and IL-8 from a gingival crevicular fluid (GCF). MATERIALS AND METHODS: A total of 60 patients were selected, which included 40 patients diagnosed with chronic periodontitis (20 bidi smokers and 20 non-bidi smokers) and 20 periodontal healthy controls. Diseased and healthy sites were selected from each of the chronic periodontitis subjects. Clinical parameters assessed were plaque index (PI), gingival index (GI), periodontal probing depth (PPD), recession (RC), and clinical attachment level (CAL). Pooled GCF samples were taken from the same site and analyzed for IL-1β and IL-8 using enzyme-linked immunosorbent assay. RESULTS: Bidi smokers displayed decreased levels of IL-1β and IL-8 than non-bidi smokers for both healthy and diseased sites and significantly reduced IL-8 levels among bidi smokers when compared to controls. Among bidi smokers, the diseased site had significantly higher levels of IL-8 than the healthy site. Non-smoker subjects with chronic periodontitis especially diseased sites contained significantly higher amounts of IL-1β and IL-8 than smokers and controls. The PI scores were highest among bidi smokers with reduced BOP and GI scores. CONCLUSIONS: Bidi smoking influenced the cytokine profile among periodontitis patients exhibiting decreased levels of IL-1β and IL-8.	

Determination and distribution of 210 Po in different morphological parts of tobacco plants and radiation dose assessment from cigarettes in Turkey.	
Cankurt S, Görgün AU.	. Ecotoxicol Environ Saf. 2020 Jul 1;197:110603
Both sides of tobacco leaves accumulate 210Po through their sticky hairs (trichomes) by means of diffusive deposition. It is known that tobacco leaves may contain high amounts of 210Po concentrations. However, there is less information about radionuclides in tobacco plants. In the study, the 210Po concentrations were determined monthly in different morphological parts of tobacco plants (leaf, stem, root) and soil samples and soil-to-plant transfer factor for 210Po was determined and the activity concentrations of 210Po radionuclide in 16 different popular brands of cigarettes were investigated in order to estimate the annual effective doses of 210Po to smokers. Besides the study investigated (focused on) the correlation between the amount of rainfall and 210Po concentration of tobacco leaf. The results of the present study indicated that the activity concentration of 210Po in cigarettes distributed in Turkey ranged from 16.1 ± 1.0 to 37.6 ± 2.6 mBq per cigarette and the mean value of their activity concentrations was 22.4 ± 1.5 mBq per cigarette. The mean value of annual effective doses of 210Po to smokers (20 cigarettes smoked by an individual per day) obtained from these activity concentrations was estimated to be 188.5 ± 12.4 μSv y-1.	

Chromium Levels in Tobacco,Filter and Ash of Illicit Brands Cigarettes Marketed in Brazil.	
Lisboa TP, Mimura AMS, da Silva JCJ, de Sousa RA.	J Anal Toxicol. 2020 Apr 2;44(5):514-520
Smoking is a public health problem and an important source of exposure to toxic metals. This work describes an efficient analytical method comparable to the ones based on atomic emission techniques for the determination of chromium in different constituent parts of cigarette samples (tobacco, filters and ashes) using electrothermal vaporization-atomic absorption spectrometry. The method was evaluated using 12 samples, and the results showed recovery values between 83 and 107%. The accuracy was also evaluated using a reference sample of tomato leaves (NIST SRM 1573a), which proved the efficiency of the method. The limits	

of detection of the developed method were 20.4, 75.8 and 80.7 ng g-1 for tobacco, filter and cigarette ash samples, respectively. The average chromium values found for the analyzed samples were in the range of 0.96 to 3.85 and from 0.32 to 0.80 µg/cigarette for tobacco and ashes, respectively. For most pre-burn and post-burn filter samples, the values of chromium concentration remained below limits of detection. The developed method presented adequate results about precision and accuracy, demonstrating its applicability in the determination of chromium in cigarette samples.

An electrophysiological characterization of naturally occurring tobacco alkaloids and their action on human α4β2 and α7 nicotinic acetylcholine receptors.

Alijevic O, McHugh D, Rufener L, Mazurov A, Hoeng J, Peitsch M.	Phytochemistry. 2020 Feb;170:112187**.**

Nicotinic acetylcholine receptor (nAChR) subtype-selective pharmacological profiles of tobacco alkaloids are essential for understanding the physiological effects of tobacco products. In this study, automated electrophysiology was used to functionally characterize the effects of distinct groups of tobacco alkaloids on human α4β2 and α7 nAChRs. We found that, in tobacco alkaloids, pyridine as a hydrogen bond acceptor and a basic nitrogen atom at a distance of 4-7 Å are pharmacophoric elements necessary for molecular recognition by α4β2 and α7 nAChRs with various degrees of selectivity, potency, and efficacy. While four alkaloids-nicotine, nornicotine, anabasine and R-anatabine-potently activated α4β2, they were also weak agonists of α7 nAChRs. Nicotine was the most potent agonist of α4β2, while anabasine elicited the highest activation of α7. None of the tobacco alkaloids enhanced nAChR activity elicited by the endogenous ligand acetylcholine; therefore, none was considered to be a positive allosteric modulator (PAM) of either α4β2 or α7 nAChRs. In contrast, we identified tobacco alkaloids, such as the tryptophan metabolite 6-hydroxykynurenic acid, that decreased the activity of both α4β2 and α7 nAChRs. Our study identified a class of alkaloids with positive and negative effects against human α4β2 and α7 nAChRs. It also revealed human α4β2 to be the principal receptor for sensing the most abundant alkaloids in tobacco leaves.

Changes in physiological and biochemical properties of flue-cured tobacco of different leaf ages during flue-curing and their effects on yield and quality.

Chen Y, Ren K, He X, Gong J, Hu X, Su J, Jin Y, Zhao Z, Zhu Y, Zou C. Dynamic	BMC Plant Biol. 2019 Dec 16;19(1):555

BACKGROUND: The leaf age for harvesting flue-cured tobacco leaves is closely related to the quality of tobacco leaves, so an appropriate leaf age for harvesting is important for improving yield and quality of flue-cured tobacco, however, at present, there are few studies on effects of leaf age on physiological and biochemical changes during flue-curing and there is no clear standard of proper leaf ages for harvesting in production.

RESULTS: In the Yunnan tobacco-growing area, an experiment was carried from 2016 to 2017 and different leaf ages were set. The results demonstrate that leaf age has a significant on tissue cell gap, leaf age and flue-curing stages exert significant effects on upper epidermis, palisade and spongy tissue, and leaf thickness of tobacco leaves. The thicknesses of upper and lower epidermis as well as palisade and spongy tissues at different ages show an approximately W-shaped change trend during flue-curing. With the advance of flue-curing stages, contents of starch, chlorophyll, carotenoid, and water in tobacco leaves at different leaf ages decrease, while polyphenol and malondialdehyde (MDA) contents increase. The older the leaf, the faster the chlorophyll, carotenoid, and water contents reduce, while the faster the polyphenol and MDA content rise during flue-curing. The flue-cured tobacco leaves at 116 DAT (days after transplanting) show the highest contents of total nitrogen and nicotine, followed by 123 DAT and those at 130 DAT are the lowest; however, the contents of total sugar and reducing sugar demonstrate a contrary tendency, and the starch content at 116 DAT is much lower than those in the other two treatments. The proportion of superior tobacco, average price, yield, and output value of upper tobacco leaves at different leaf ages are the highest at 123 DAT. The highest sensory evaluation score is found at 123 DAT, while that at 130 DAT is significantly lower in comparison with the other two treatments.

CONCLUSIONS: Tobacco leaves harvested at 123 DAT are mature and exhibit a low degree of membrane

lipid peroxidation, moderate chemical compositions, and high economic value. 123 DAT improves availability of tobacco leaves.

Transcription Factor Gene Expression in Commercial Flue-Cured Tobacco (*Nicotiana tabacum*).	
Liu H, Kotova TI, Timko MP. Increased Leaf Nicotine Content by Targeting	Genes (Basel). 2019 Nov 14;10(11):930.
Nicotine, the most abundant pyridine alkaloid in cultivated tobacco (Nicotiana tabacum L.), is a potent inhibitor of insect and animal herbivory and a neurostimulator of human brain function. Nicotine biosynthesis is controlled developmentally and can be induced by abiotic and biotic stressors via a jasmonic acid (JA)-mediated signal transduction mechanism involving members of the APETALA 2/ethylene-responsive factor (AP2/ERF) and basic helix-loop-helix (bHLH) transcription factor (TF) families. AP2/ERF and bHLH TFs work combinatorically to control nicotine biosynthesis and its subsequent accumulation in tobacco leaves. Here, we demonstrate that overexpression of the tobacco NtERF32, NtERF221/ORC1, and NtMYC2a TFs leads to significant increases in nicotine accumulation in T2 transgenic K326 tobacco plants before topping. Up to 9-fold higher nicotine production was achieved in transgenics overexpressing NtERF221/ORC1 under the control of a constitutive GmUBI3 gene promoter compared to wild-type plants. The constitutive 2XCaMV35S promoter and a novel JA-inducible 4XGAG promoter were less effective in driving high-level nicotine formation. Methyljasmonic acid (MeJA) treatment further elevated nicotine production in all transgenic lines. Our results show that targeted manipulation of NtERF221/ORC1 is an effective strategy for elevating leaf nicotine levels in commercial tobacco for use in the preparation of reduced risk tobacco products for smoking replacement therapeutics.	

Emission level of seven mainstream smoke toxicants from cigarette with variable tobacco leaf constituents.	
Cai B, Li Z, Wang R, Geng Z, Shi Y, Xie S, Wang Z, Yang Z, Ren X.	Regul Toxicol Pharmacol. 2019 Apr;103:181-188
Seven smoke constituents, including hydrogen cyanide (HCN), ammonia (NH3), phenol, benzo[α]pyrene (B[a]P), carbon monoxide (CO)¸ crotonaldehyde, and 4-(methylnitrosamino)-1- (3-pyridyl)-1-butanone (NNK), are proposed be the most relevant constituents for smoking-related diseases. [Methods] Different combinations of leaf stalk positions, varieties and locations were used to create variable chemistry of cigarette filler and smoke. Experimental cigarettes were measured for emission level of seven smoke toxicants and content of seventy-three filler components. [Results] The ranges of coefficient of variation (CV) for seven smoke toxicants were 15.43%-43.15%. The emission pattern of NNK and crotonaldehyde were different from that of other five smoke toxicants. Most of the seven smoke toxicants were influenced in following order: stalk position > location > variety. The leaf constitutes closely correlated with seven smoke toxicants were analyzed. [Conclusions] The results showed that seven toxicants were significantly influenced by leaf position and location, and closely correlated with leaf components, such as potassium, malate and alkaloid contents. The results provide useful and comprehensive information on the affecting factors and correlating leaf constituents for the variations of seven smoke toxicants.	

Determination of tobacco alkaloid enantiomers using reversed phase UPLC/MS/MS.	
Ji H, Wu Y, Fannin F, Bush L.	Heliyon. 2019 May 15;5(5):e01719. doi: 10.1016/j.heliyon.2019.e01719
N'-Nitrosonornicotine (NNN), a carcinogenic tobacco-specific N'-nitrosamine (TSNA), is on the FDA list of harmful and potentially harmful constituents (HPHCs). Nornicotine, a product of the demethylation of nicotine, is the immediate alkaloid precursor for NNN formation. Nicotine, nornicotine and NNN are optically active. The accumulation of the isomers of nicotine, nornicotine, and NNN impacts their biological activity. In this paper, we report the determination of tobacco alkaloid enantiomers (including nicotine, nornicotine, anabasine, and anatabine) in samples of different tobacco lines using a reversed phase ultra-performance liquid chromatography-tandem mass spectrometer (UPLC/MS/MS) method. Current method demonstates excellent	

detection capability for all alkaloid enantiomers, with correlation coefficients (r2) > 0.996 within their linear dynamic ranges. The limit of detection (LOD) and limit of quantitation (LOQ) of all analytes are less than 10 ng/mL and 30 ng/mL, respectively. In addition, their recovery and coefficient of variation (CV%) are within 100-115% and 0.2-3.7%, respectively. The method validated in this paper is simple, fast, and sensitive for the quantification of alkaloid enantiomers in tobacco leaf and has been applied to investigations of tobacco alkaloid enantiomer ratios in different tobacco lines and tobacco products.

Contribution of tobacco composition compounds to characteristic aroma of Chinese faint-scent cigarettes through chromatography analysis and partial least squares regression.	
Yin F, Karangwa E, Song S, Duhoranimana E, Lin S, Cui H, Zhang X.	J Chromatogr B Analyt Technol Biomed Life Sci. 2019 Jan 15;1105:217-227
To further explore the aroma mechanism of Chinese faint-scent cigarettes, the contribution of tobacco leaf composition, including six kinds of saccharides, eight tobacco alkaloids, seventeen kinds of organic acids, eighteen kinds of amino acids and four ions (Na+, K+, Mg2+ and Ca2+), on aroma quality characteristic (freshness, flowery and acidic notes) of faint-scent cigarettes was analyzed by chromatography and PLSR. The results showed that (i) xylose, fructose, glucose, maltose and sucrose were negatively correlated to acidic note, while galactose showed significantly positive correlation to acidic note. (ii) Phenylalanine and proline showed significant and positive correlation with characteristic aromas. Proline contributed to freshness and flowery, while leucine significantly contributed to acidic note. (iii) Most organic acids were significantly correlated to characteristic aromas. Palmitic acid and stearic acid contributed to the freshness, while dodecanoic acid and palmitic acid significantly contributed to flowery. (iv) Tobacco Na ion plays negative and significant correlation to acidic note.	

Enantiomeric composition of nicotine in tobacco leaf, cigarette, smokeless tobacco, and e-liquid by normal phase high-performance liquid chromatography.	
Zhang H, Pang Y, Luo Y, Li X, Chen H, Han S, Jiang X, Zhu F, Hou H, Hu Q.	Chirality. 2018 Jul;30(7):923-931
Evaluating the source of nicotine in e-liquid is a problem. Tobacco-derived nicotine contains predominantly (S)-(-)-nicotine, whereas tobacco-free nicotine products may not. Thus, we developed a new normal phase high-performance liquid chromatography method to determinate the enantiomeric composition of nicotine in 10 kinds of flue-cured tobacco, 3 kinds of burley, 1 kind of cigar tobacco, 2 kinds of oriental tobacco, 5 kinds of Virginia cigarette, 5 kinds of blend cigarette, 10 kinds of e-liquid, and 4 kinds of smokeless tobacco. The amount of (R)-(+)-nicotine ranged from ~0.02% to ~0.76% of total nicotine. An e-liquid sample had the highest level of (R)-(+)-nicotine. The extraction and purification processes used to obtain commercial (S)-(-)-nicotine from the tobacco do not decrease the amount of (R)-(+)-nicotine in tobacco. So the amount of (R)-(+)-nicotine in samples in our work were the same as tobacco samples.	

Effects of different growth temperatures on growth, development, and plastid pigments metabolism of tobacco (Nicotiana tabacum L.) plants.	
Yang LY, Yang SL, Li JY, Ma JH, Pang T, Zou CM, He B, Gong M.	Bot Stud. 2018 Feb 5;59(1):5. doi: 10.1186/s40529-018-0221-2
Temperature remarkably affects the growth and metabolism of plants. Tobacco is an important cash crop, and the long-term effects of different growth temperatures (18.5, 23.5 and 28.5 °C, daily average) on growth, development and plastid pigments metabolism of tobacco plants were investigated in this study. RESULTS: Compared with tobacco plants grown under 23.5 °C, treatments with 18.5 and 28.5 °C inhibited the expansion of leaves. The contents of superoxide anion (O 2·-), hydrogen peroxide (H2O2) and malonaldehyde (MDA) in the leaves were significantly increased under 28.5 °C from 0 to 60 days, which in turn accelerated the flowering and senescence of tobacco plants. By contrast, the treatment with 18.5 °C remarkably decreased O 2.- , H2O2 and MDA, and delayed the flowering and senescence. Furthermore,	

treatment with 18.5 °C significantly up-regulated the expression of glutamyl-tRNA reductase (Glu-TR) and magnesium chelatase (MgCH), and down-regulated the ferri chelatase (FeCH), protochlorophyllide oxidoreductase, chlorophyllase (CHLase), phaeophorbide a monooxygenase (PaO) and phytoene synthase (PSY), which further promoted the accumulation of chlorophyll (Chls) and reduced the carotenoids (Cars) in leaves. On the contrary, exposing to 28.5 °C remarkably down-regulated the Glu-TR and MgCH, and up-regulated the FeCH, CHLase, PaO and PSY, which in turn decreased the Chls and increased the Cars in tobacco leaves.

CONCLUSION: As compared with the plants grown under 23.5 °C, lower (18.5 °C) and higher (28.5 °C) growth temperature inhibited the growth of tobacco plants. In general, treatment with 28.5 °C accelerated the flowering and senescence of tobacco plants by enhancing the accumulation of O 2.- and H2O2 in leaves, while exposing to 18.5 °C had the opposite effects. Treatment with 18.5 °C increased the content of Chls and reduced the Cars in leaves. In contrast, Treatment with 28.5 °C decreased the Chls and increased the Cars. Moreover, both O 2.- and H2O2 took part in the breakdown of Chls in tobacco leaves to some extent. The results suggest that growth temperature could regulate growth, development, and plastid pigments metabolism, and 23.5 °C could be an optimal temperature for growth, development and metabolism of plastid pigments of tobacco plants under the experimental conditions.

Metabolic changes in primary, secondary, and lipid metabolism in tobacco leaf in response to topping.

Zhao J, Li L, Zhao Y, Zhao C, Chen X, Liu P, Zhou H, Zhang J, Hu C, Chen A, Liu G, Peng X, Lu X, Xu G.	Anal Bioanal Chem. 2018 Jan;410(3):839-851

As an important cultivation practice used for flue-cured tobacco, topping affects diverse biological processes in the later stages of development and growth. Some studies have focused on using tobacco genes to reflect the physiological changes caused by topping. However, the complex metabolic shifts in the leaf resulting from topping have not yet been investigated in detail. In this study, a comprehensive metabolic profile of primary, secondary, and lipid metabolism in flue-cured tobacco leaf was generated with use of a multiple platform consisting of gas chromatography-mass spectrometry, capillary electrophoresis-mass spectrometry, and liquid chromatography-mass spectrometry/ultraviolet spectroscopy. A total of 367 metabolites were identified and determined. Both principal component analysis and the number of significantly different metabolites indicated that topping had the greatest influence on the upper leaves. During the early stage of topping, great lipid level variations in the upper leaves were observed, and antioxidant defense metabolites were accumulated. This indicated that the topping activated lipid turnover and the antioxidant defense system. At the mature stage, lower levels of senescence-related metabolites and higher levels of secondary metabolites were found in the topped mature leaves. This implied that topping delayed leaf senescence and promoted secondary metabolite accumulation. This study provides a global view of the metabolic perturbation in response to topping. Graphical abstract Metabolic alterations in tobacco leaf in response to topping using a multiplatform metabolomics.

Polyamines delay leaf maturation in low-alkaloid tobacco varieties.

Nölke G, Volke D, Chudobová I, Houdelet M, Lusso M, Frederick J, Adams A, Kudithipudi C, Warek U, Strickland JA, Xu D, Schinkel H, Schillberg S(1)	Plant Direct. 2018 Jul 31;2(7):e00077. doi: 10.1002/pld3.77

The development of low-alkaloid (LA) tobacco varieties is an important target in the tobacco breeding industry. However, LA Burley 21 plants, in which the Nic1 and Nic2 loci controlling nicotine biosynthesis are deleted, are characterized by impaired leaf maturation that leads to poor leaf quality before and after curing. Polyamines are involved in key developmental, physiological, and metabolic processes in plants, and act as anti-senescence and anti-ripening regulators. We investigated the role of polyamines in tobacco leaf maturation by analyzing the free and conjugated polyamine fractions in the leaves and roots of four Burley 21 varieties: NA (normal alkaloid levels, wild-type control), HI (high intermediates, nic2 -), LI (low intermediates, nic1 -), and LA (nic1 - nic2 -). The pool of conjugated polyamines increased with plant age in the roots and leaves of all four varieties, but the levels of free and conjugated putrescine and spermidine were higher in the LI and LA plants than NA controls. The increase in the polyamine content correlated with delayed maturation and senescence, i.e., LA plants with the highest polyamine levels showed the most severe impaired leaf

maturation phenotype, characterized by higher chlorophyll content and more mesophyll cells per unit leaf area. Treatment of LA plants with inhibitors of polyamine biosynthesis and/or the growth regulator Ethephon® reduced accumulation of polyamines, achieving a partial amelioration of the LA phenotype. Our data show that the regulation of polyamine homeostasis is strongly disrupted in LA plants, and that free and conjugated polyamines contribute to the observed impairment of leaf maturation.

Innovative Approaches for Estimating the Levels of Tobacco-Specific Nitrosamines in Cured Tobacco Samples

Kaiser S, Soares FLF, Ardila JA, Marcelo MCA, Dias JC, Porte LMF, Gonçalves C, Pontes OFS, Sabin GP	Chem Res Toxicol. 2018 Sep 17;31(9):964-973

Tobacco-specific nitrosamines (TSNAs), mainly the 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), are known carcinogens. Part of the NNK found in smoke is provided from matrix-bound NNK, and its determination is extremely relevant. However, the reference extraction procedure of matrix-bound NNK is time-consuming and labor-intensive and has a limited analytical capacity. Three different methodologies were proposed to predict matrix-bound NNK: simple linear regression (LR) with soluble NNK; multiple linear regression (MLR) considering soluble NNK and characteristic parameters of the samples; and orthogonal partial least-squares (O-PLS) regression using high-throughput screening by flow injection analysis coupled to high-resolution mass spectrometry (HTS-FIA-HRMS) data. Simple linear regression showed a high influence of matrix and leaf origin. Although an existing linearity trend has been observed (R2 = 0.62) for the global model, higher correlation values were achieved for matrix and country segregation models. Multiple linear regression predicted matrix-bound NNK with more satisfactory efficiency than simple linear regression models. The coefficients of determination were 0.87 and 0.94 for flue-cured Virginia and air-cured Burley, respectively. However, this method has a limited application, since previous information about the sample is required. The proposed method based on HTS-FIA-HRMS and O-PLS has shown the most suitable performance in the prediction of matrix-bound NNK, with errors comparable to the reference method, and a higher throughput. In addition, this approach allows to determine other soluble nitrosamines, namely N'-nitrosoanatabine, N'-nitrosoanabasine, and N-nitrosonornicotine, with relative percentage errors between 5.25 and 11.98%. Therefore, the third approach is the best method for a large number of cured tobacco for accuracy in determination of TSNAs.

In vivo monitoring of nicotine biosynthesis in tobacco leaves by low-temperature plasma mass spectrometry

Martínez-Jarquín S, Herrera-Ubaldo H, de Folter S, Winkler R	Talanta. 2018 Aug 1;185:324-327

Low-temperature plasma (LTP) is capable of ionizing a broad range of organic molecules at ambient conditions. The coupling of LTP to a mass analyzer delivers chemical profiles from delicate objects. To investigate the suitability of LTP ionization for mass spectrometry (MS) based in vivo studies, we monitored the auxin-regulated nicotine biosynthesis in tobacco (Nicotiana tabacum) and evaluated possible biological effects. The measured nicotine concentrations in different experiments were comparable to literature data obtained with conventional methods. The observed compounds suggest the rupture of trichomes, and cell damage was observed on the spots exposed to LTP. However, the lesions only affected a negligible proportion of the leaf surface area and no systemic reaction was noted. Thus, our study provides the proof-of-concept for measuring the biosynthetic activity of plant surfaces in vivo.

Enantiomeric composition of nicotine in tobacco leaf, cigarette, smokeless tobacco, and e-liquid by normal phase high-performance liquid chromatography.

Zhang H, Pang Y, Luo Y, Li X, Chen H, Han S, Jiang X, Zhu F, Hou H, Hu Q	Chirality. 2018 Jul;30(7):923-931

Evaluating the source of nicotine in e-liquid is a problem. Tobacco-derived nicotine contains predominantly (S)-(-)-nicotine, whereas tobacco-free nicotine products may not. Thus, we developed a new normal phase high-performance liquid chromatography method to determinate the enantiomeric composition of nicotine in

10 kinds of flue-cured tobacco, 3 kinds of burley, 1 kind of cigar tobacco, 2 kinds of oriental tobacco, 5 kinds of Virginia cigarette, 5 kinds of blend cigarette, 10 kinds of e-liquid, and 4 kinds of smokeless tobacco. The amount of (R)-(+)-nicotine ranged from ~0.02% to ~0.76% of total nicotine. An e-liquid sample had the highest level of (R)-(+)-nicotine. The extraction and purification processes used to obtain commercial (S)-(-)-nicotine from the tobacco do not decrease the amount of (R)-(+)-nicotine in tobacco. So the amount of (R)-(+)-nicotine in samples in our work were the same as tobacco samples.

Simultaneous quantitative assessment of nine glycosides in tobacco by liquid chromatography-tandem mass spectrometry.	
Yuan Y, Zhou R, Li D, Luo C, Li G	J Sep Sci. 2018 Mar;41(5):1009-1016
A simple and efficient method combining ultrasound-assisted extraction, the conditions of which were optimized by response surface methodology, with liquid chromatography and tandem mass spectrometry was established and validated for the absolute quantification of nine non-volatile neutral glycosides originating from tobacco (Nicotiana tobaccum L.) leaves, comprising three phenolic glycosides, one benzanoid glycoside, and five sesquiterpene glycosides within three isomers, originating from tobacco leaves. Factors of extraction time, sample quantity, extraction solvent, liquid chromatographic conditions, and electrospray ionization parameters were carefully investigated to ensure the selectivity and sensitivity of the method. All calibration curves showed excellent coefficients of determination ranging from 0.9940 to 0.9996, within the range of tested concentrations. The limits of detection and quantification were 2.33-25.9 and 7.06-78.5 ng/mL, respectively. Satisfactory values of accuracy were between 80.1 to 107.9% among different sample matrixes. The relative standard deviations of intra- and inter-day analysis were less than 13.7 and 13.0% respectively. The developed method was successfully applied in a pilot study to determine the amounts of the nine endogenous glycosides in real flue-cured tobacco samples obtained from different habitats in China	

New isolates from leaves of Nicotiana tabacum and their biological activities.	
Shang S, Shi J, Tang J, Jiang J, Zhao W, Zheng X, Lei P, Han J, Wang C, Yuan D, Yang G, Chen Y, Miao M	Nat Prod Res. 2018 Jan 19:1-7. doi: 10.1080/14786419.2018.1425840.
Three new isolates (1-3) including one new sterol and two new flavonoids together with three known sterols (4-6) were isolated from the leaves of Nicotiana tabacum. Their structures were determined mainly by spectroscopic methods, including extensive 1D and 2D NMR techniques. All compounds were evaluated for their anti-tobacco mosaic virus and cytotoxic activities. The results showed that compounds 2 and 3 exhibited high anti-TMV activity with inhibition rate of 34.2 and 33.4%, respectively, which were roughly equivalent to that of positive control. The cytotoxicities of compounds 1 and 4-6 against five human tumour cell lines were also tested, and tested compounds showed weak inhibitory activities against some tested human tumour cell lines.	

Novel approach for selective reduction of NNN in cigarette tobacco filler and mainstream smoke.	
Lusso M, Gunduz I, Kondylis A, Jaccard G, Ruffieux L, Gadani F, Lion K, Adams A, Morris W, Danielson T, Warek U, Strickland J	Regul Toxicol Pharmacol. 2017 Oct;89:101-111. doi: 10.1016/j.yrtph.2017.07.019
Research conducted during past decades to reduce the level of the tobaccospecific nitrosamine N-nitrosonornicotine (NNN) and its precursor nornicotine in tobacco yielded identification of three tobacco genes encoding for cytochrome P450 nicotine demethylases converting nicotine to nornicotine. We carried out trials to investigate the effect of using tobaccos containing three non-functional nicotine demethylase genes on the selective reduction of NNN in cigarette tobacco filler and mainstream smoke. Our results indicate that the presence of non-functional alleles of the three genes reduces the level of nornicotine and NNN in Burley tobacco by 70% compared to the level observed in currently available low converter (LC) Burley tobacco varieties. The new technology, named ZYVERT™, does not require a regular screening process, while a yearly selection process is needed to produce LC Burley tobacco seeds for NNN reduction. The reduction of NNN observed in smoke of blended prototype cigarettes is proportional to the inclusion level of tobacco	

having ZYVERT™ technology. Inclusion of Burley tobacco possessing the new trait into a typical American blend resulted in a selective reduction of NNN in cigarette smoke, while the levels of other Harmful and Potentially Harmful Constituents (HPHC) currently in the abbreviated list provided by the US Food and Drug Administration are statistically equivalent in comparison with the levels obtained in reference prototype cigarettes containing LC Burley.

A model for evolution and regulation of nicotine biosynthesis regulon in tobacco.	
Kajikawa M, Sierro N, Hashimoto T, Shoji T	Plant Signal Behav. 2017 Jun 3;12(6):e1338225. doi: 10.1080/15592324.2017.1338225
In tobacco, the defense alkaloid nicotine is produced in roots and accumulates mainly in leaves. Signaling mediated by jasmonates (JAs) induces the formation of nicotine via a series of structural genes that constitute a regulon and are coordinated by JA-responsive transcription factors of the ethylene response factor (ERF) family. Early steps in the pyrrolidine and pyridine biosynthesis pathways likely arose through duplication of the polyamine and nicotinamide adenine dinucleotide (NAD) biosynthetic pathways, respectively, followed by recruitment of duplicated primary metabolic genes into the nicotine biosynthesis regulon. Transcriptional regulation of nicotine biosynthesis by ERF and cooperatively-acting MYC2 transcription factors is implied by the frequency of cognate cis-regulatory elements for these factors in the promoter regions of the downstream structural genes. Indeed, a mutant tobacco with low nicotine content was found to have a large chromosomal deletion in a cluster of closely related ERF genes at the nicotine-controlling NICOTINE2 (NIC2) locus.	

Effect of humic acid-based amendments with foliar application of Zn and Se on Cd accumulation in tobacco.	
Yu Y, Wan Y, Wang Q, Li H	Ecotoxicol Environ Saf. 2017 Apr;138:286-291
The smoke of tobacco is a major source of exposure to Cd in humans and therefore it is urgent to find a way to a method to reduce Cd accumulation in tobacco. A four-month tobacco pot experiment was conducted to investigate the effects of two base treatments (humic acid-based amendments) and two foliar treatments (Zn and Se) on Cd uptake by tobacco. The results showed that Cd in tobacco was mainly transferred into leaves, which could be significantly reduced by both applied amendments. The Cd contents in leaves were reduced by up to 67%. Foliar Zn alone significantly decreased Cd contents in leaves while foliar Se slightly increased them. When base and foliar treatments were combined, base treatments had dominant effects but those of foliar treatments were not distinct. The applied amendments did reduce Cd contents in all the parts of tobacco and the translocation into leaves and they were more effective than foliar Zn and Se.	

Design of experiments for amino acid extraction from tobacco leaves and their subsequent determination by capillary zone electrophoresis.	
Hodek O, Křížek T, Coufal P, Ryšlavá H.	Anal Bioanal Chem. (2017) Vol. 409(9):2383-2391
In this study, we optimized a method for the determination of free amino acids in Nicotiana tabacum leaves. Capillary electrophoresis with contactless conductivity detector was used for the separation of 20 proteinogenic amino acids in acidic background electrolyte. Subsequently, the conditions of extraction with HCl were optimized for the highest extraction yield of the amino acids because sample treatment of plant materials brings some specific challenges. Central composite face-centered design with fractional factorial design was used in order to evaluate the significance of selected factors (HCl volume, HCl concentration,sonication, shaking) on the extraction process. In addition, the composite design helped us to find the optimal values for each factor using the response surface method. The limits of detection and limits of quantification for the 20 proteinogenic amino acids were found to be in the order of 10(-5) and 10(-4) mol l(-1), respectively. Addition of acetonitrile to the sample was tested as a method commonly used to decrease limits of detection. Ambiguous results of this experiment pointed out some features of plant extract samples, which often required specific approaches. Suitability of the method for metabolomic studies was tested by analysis of a real sample, in which all amino acids, except for L-methionine and L-cysteine, were successfully	

detected. The optimized extraction process together with the capillary electrophoresis method can be used for the determination of proteinogenic amino acids in plant materials. The resulting inexpensive, simple, and robust method is well suited for various metabolomics studies in plants. As such, the method represents a valuable tool for research and practical application in the fields of biology, biochemistry, and agriculture.

Microbial Biofertilizer Decreases Nicotine Content by Improving Soil Nitrogen Supply.	
Shang C, Chen A, Chen G, Li H, Guan S, He J.	Appl Biochem Biotechnol. (2017) Vol. 181(1):1-14.
Biofertilizers have been widely used in many countries for their benefit to soil biological and physicochemical properties. A new microbial biofertilizer containing Phanerochaete chrysosporium and Bacillus thuringiensis was prepared to decrease nicotine content in tobacco leaves by regulating soil nitrogen supply. Soil NO3(-)-N, NH4(+)-N, nitrogen supply-related enzyme activities, and nitrogen accumulation in plant leaves throughout the growing period were investigated to explore the mechanism of nicotine reduction. The experimental results indicated that biofertilizer can reduce the nicotine content in tobacco leaves, with a maximum decrement of 16-18 % in mature upper leaves. In the meantime, the total nitrogen in mature lower and middle leaves increased with the application of biofertilizer, while an opposite result was observed in upper leaves. Protein concentration in leaves had similar fluctuation to that of total nitrogen in response to biofertilizer. NO3(-)-N content and nitrate reductase activity in biofertilizer-amended soil increased by 92.3 and 42.2 %, respectively, compared to those in the control, whereas the NH4(+)-N and urease activity decreased by 37.8 and 29.3 %, respectively. Nitrogen uptake was improved in the early growing stage, but this phenomenon was not observed during the late growth period. Nicotine decrease is attributing to the adjustment of biofertilizer in soil nitrogen supply and its uptake in tobacco, which result in changes of nitrogen content as well as its distribution in tobacco leaves. The application of biofertilizer containing P. chrysosporium and B. thuringiensis can reduce the nicotine content and improve tobacco quality, which may provide some useful information for tobacco cultivation.	

Comprehensive investigation of tobacco leaves during natural early senescence via multi-platform metabolomics analyses.	
Li L, Zhao J, Zhao Y, Lu X, Zhou Z, Zhao C, Xu G.	Sci Rep. (2016) Vol.6:37976.
Senescence is the final stage of leaf growth and development. Many different physiological activities occur during this process. A comprehensive metabolomics analysis of tobacco middle leaves at 5 different developmental stages was implemented through multi-platform methods based on liquid chromatography, capillary electrophoresis and gas chromatography coupled with mass spectrometry. In total, 412 metabolites were identified, including pigments, sterols, lipids, amino acids, polyamines, sugars and secondary metabolites. Dramatic metabolic changes were observed. Firstly, membrane degradation and chlorophyll down-regulation occurred after the 50% flower bud stage. Levels of major membrane lipids decreased, including those of the glycolipids in chloroplast thylakoids and phospholipids in membrane envelopes. Clear decreases in free sterols and acylated sterol glucosides were detected along with the accumulation of sterol esters. The accumulation of alkaloids was found. The amino acid levels were significantly decreased, particularly those of N-rich amino acids (glutamine and asparagine), thus reflecting N translocation. Subsequently, the antioxidant system was activated. Sugar alcohols and polyphenols accumulated when the lower leaves turned yellow. These results comprehensively revealed the metabolic changes that occur during tobacco leaf development and senescence under natural conditions.	

The influence of light quality on the accumulation of flavonoids in tobacco (Nicotiana tabacum L.) leaves	
Fu B, Ji X, Zhao M, He F, Wang X, Wang Y, Liu P, Niu L.	J Photochem Photobiol B. (2016) Vol. 162:544-9.
Flavonoids are important secondary metabolites in plants regulated by the environment. To analyze the effect of light quality on the accumulation of flavonoids, we performed a rapid analysis of flavonoids in extracts of tobacco leaves using UHPLC-QTOF. A total of 12 flavonoids were detected and identified in tobacco leaves,	

which were classified into flavonoid methyl derivatives and flavonoid glycoside derivatives according to the groups linked to the flavonoid core. Correlation analysis was further conducted to investigate the effect of different wavelengths of light on their accumulation. The content of flavonoid methyl derivatives was positively correlated with the proportions of far-red light (FR; 716-810nm) and near-infrared light (NIR; 810-2200nm) in the sunlight spectrum and negatively correlated with the proportion of ultraviolet (UV-A; 350-400nm) and the red/far-red ratio (R/FR). By contrast, the content of flavonoid glycoside derivatives was positively correlated with the proportion of UV-A and the R/FR, and negatively correlated with FR and NIR. The results indicated that light quality with higher proportions of FR and NIR increases the activity of flavonoid methyltransferases but suppresses the activity of flavonoid glycoside transferases. While a high proportion of UV-A and a high R/FR can increase flavonoid glycoside transferase activity but suppress flavonoid methyltransferase activity.

Tobacco, Microbes, and Carcinogens: Correlation Between Tobacco Cure Conditions, Tobacco-Specific Nitrosamine Content, and Cured Leaf Microbial Community.

Law AD, Fisher C, Jack A, Moe LA.	Microb Ecol. (2016) Vol. 72(1):120-129.

Tobacco-specific nitrosamines are carcinogenic N-nitrosamine compounds present at very low levels in freshly harvested tobacco leaves that accumulate during leaf curing. Formation of N-nitrosamine compounds is associated with high nitrate levels in the leaf at harvest, and nitrate is presumed to be the source from which the N-nitrosation species originates. More specifically, nitrite is considered to be a direct precursor, and nitrite is linked with N-nitrosation in many environmental matrices where it occurs via microbial nitrate reduction. Here, we initiate work exploring the role of leaf microbial communities in formation of tobacco-specific nitrosamines. Leaves from burley tobacco line TN90H were air cured under various temperature and relative humidity levels, and 22 cured tobacco samples were analyzed for their microbial communities and leaf chemistry. Analysis of nitrate, nitrite, and total tobacco-specific nitrosamine levels revealed a strong positive correlation between the three variables, as well as a strong positive correlation with increasing relative humidity during cure conditions. 16S rRNA gene amplicon sequencing was used to assess microbial communities in each of the samples. In most samples, Proteobacteria predominated at the phylum level, accounting for >90 % of the OTUs. However, a distinct shift was noted among members of the high tobacco-specific nitrosamine group, with increases in Firmicutes and Actinobacteria. Several OTUs were identified that correlate strongly (positive and negative) with tobacco-specific nitrosamine content. Copy number of bacterial nitrate reductase genes, obtained using quantitative PCR, did not correlate strongly with tobacco-specific nitrosamine content. Incomplete denitrification is potentially implicated in tobacco-specific nitrosamine levels.

Tobacco alkaloids reduction by casings added/enzymatic hydrolysis treatments assessed through PLSR analysis.

Lin S, Zhang X, Song S, Hayat K, Eric K, Majeed H.	Regul Toxicol Pharmacol. (2016) Vol. 75:27-34

Based on encouraged development of potential reduced-exposure products (PREPs) by the US Institute of Medicine, casings (glucose and peptides) added treatments (CAT) and enzymatic (protease and xylanase) hydrolysis treatments (EHT) were developed to study their effect on alkaloids reduction in tobacco and cigarette mainstream smoke (MS) and further investigate the correlation between sensory attributes and alkaloids. Results showed that the developed treatments reduced nicotine by 14.5% and 24.4% in tobacco and cigarette MS, respectively, indicating that both CAT and EHT are potentially effective for developing lower-risk cigarettes. Sensory and electronic nose analysis confirmed the significant influence of treatments on sensory and cigarette MS components. PLSR analysis demonstrated that tobacco alkaloids were positively correlated to the off-taste, irritation and impact attributes, and negatively correlated to the aroma and softness attributes. Additionally, nicotine and anabasine from tobacco leaves positively contributed to the impact attribute, while they negatively contributed to the aroma attribute ($P<0.05$). Meanwhile, most alkaloids in cigarette MS positively contributed to the impact and irritation attributes ($P<0.05$). Hence, this study paved a way to better understand the correlation between tobacco alkaloids and sensory attributes.

Arsenic Speciation and Cadmium Determination in Tobacco Leaves, Ash and Smoke

Iwai Takahiro	Analytical Chemistry, (2016) Vol. 32, No. 9, 957-62

The concentrations of arsenic (As) and cadmium (Cd) in the tobacco leaves, ash and smoke of 10 kinds of cigarettes collected from different countries worldwide were determined by ICP-MS after microwave-assisted digestion. Total As and Cd concentrations in the tobacco leaves ranged from 0.20 to 0.63 and 1.8 to 9.9 mg kg(-1), respectively. By the speciation analysis of As in tobacco leaves and ash by HPLC-ICP-MS following acid extraction, arsenite [As(III)] and arsenate [As(V)] were determined and trace amounts of monomethylarsonic acid (MMAA), dimethylarsinic acid (DMAA), trimethylarsine oxide (TMAO), tetramethylarsonium (TeMA) and some unidentified As species were also found. Arsenic speciation for smoke absorbed in an aqueous solution was carried out. The sum of the As species in tobacco leaves, ash and smoke was in good agreement with the result of total As determination in each sample, and the recoveries of speciation were 100 ± 10%. The distributions and the behaviors of As species were clarified.

Levels of heavy metals in the raw and processed Ethiopian tobacco leaves

Regassa Girma	SpringerPlus, (2016) Vol. 5, pp. 232

Tobacco (Nicotiana tabacum L.) is a commercial plant. Tobacco leaves naturally accumulate and concentrate relatively high levels of heavy metals and particular cadmium in leaves. Tobacco is one of the basic agricultural products, in Ethiopia, with social and economic importance. However, there is no report in the literature on the determination of levels of heavy metals in Ethiopian tobacco leaves. Hence this research is intended to determine the levels of heavy metals in the raw and processed Ethiopian Virginia tobacco leaves. Samples of raw Virginia tobacco leaves were collected from two different regions of Ethiopia (Billate and Shewa Robit). The three processed tobacco samples were collected from National Tobacco Enterprise, Addis Ababa, Ethiopia. The samples were wet-digested using 3 mL HNO3 (69-72 %) and 3 mL HClO4 (70 %) at 350 °C for 3.5 h and concentrations of heavy metals (Cd, Cr, Cu, Ni, Pb and Zn) in the samples were determined by flame atomic absorption spectrometer. The mean metals concentrations (in µg/g dry weight) in the raw Virginia tobacco leaves from Billate and Shewa Robit, respectively, were: Cu (4.38, 7.30), Zn (53.7, 33.2), Cd (1.20, 1.30), Cr (ND, 1.45), Ni (ND, 1.90). The mean metals concentrations (in µg/g dry weight) in the processed tobacco from Billate and Shewa Robit, respectively, were: Cu (9.80, 12.8), Ni (2.35, 2.20) Cd (1.45, 1.90), Cr (1.65, 1.75), Zn (101, 83.8). The mean metals concentrations (in µg/g dry weight) in the processed tobacco Nyala (Ethiopian cigarette leaves) were: Cu (8.95), Cd (1.55), Cr (1.62), Ni (4.70), Zn (79.3). The concentrations of Cr and Ni in tobacco leaves from Billate and Pb in all the tobacco samples were below the detection limits. This study showed that the metal contents of tobacco leaves varied with the geographical origin in which the tobacco plant grows. The metal contents of processed tobacco were higher than the corresponding raw leaves. Pb was not detected in both the raw and processed Ethiopian tobacco leaves.

Correlation between endogenous harmful components in mainstream cigarette smoke and chemical constituents in tobacco leaves

Geng ZL, Zhang J, Ge YH, Xiang ZM, Cai K, Zhu XL, Li JX, Feng YG.	Ying Yong Sheng Tai Xue Bao. (2015) Vol. 26(5):1447-53.

Correlation analysis between main chemical constituents of tobacco leaves and endogenous harmful components in mainstream cigarette smoke was conducted. Leaf stalk positions exhibited a high relation with endogenous harmful components and hazard index (H). Upper stalk position leaves had greater release of 1-butanone,4-(methylnitrosoamino)-1-(3-pyridinyl)-(NNK), B[a]P, HCN, NH3 and phenol in mainstream cigarette smoke, and a higher hazard index than middle position leaves except for crotonaldehyde, which had greater release from middle position leaves. Different endogenous harmful components in mainstream cigarette smoke presented complicated correlation with main chemical constituents in tobacco. The same type of leaf chemical constituents presented different correlations with various endogenous harmful components in mainstream cigarette smoke. Cigarette hazard index showed significantly positive correlations with contents of nicotine, protein, total nitrogen, major polyphenols and organic acids, while significantly negative correlation with potassium and carbonaceous substances, such as total sugars, reducing sugars and starch. The results suggested that properly increasing potassium content and decreasing nitrogenous constituents in cured tobacco leaf may reduce the cigarette hazard index.

Levels of nicotine in Ethiopian tobacco leaves

Tassew Zebasil, Chandravanshi Bhagwan Singh	SpringerPlus, 2015 4: 649.
Tobacco is a valuable cash crop. It is the most widely grown non-food crop in the world. Tobacco use is widespread due to its addictive nature of its main constituent nicotine. Therefore, the knowledge of nicotine level in tobacco is important to tobacco industry and in the area of toxicology to control its harmful effect on health. There is no report in the literature on nicotine level of Ethiopian raw (unprocessed) tobacco leaves. Hence, the objective of this study is to determine the levels of nicotine in the Ethiopian tobacco leaves. Samples were collected based on their leaves positions, species and place of cultivation from different regions of Ethiopia. These were Virginia type tobacco from Shewa Robit and Billate, Burley and Oriental types of tobacco from Awassa and native tobacco used as pipe smoking (Gaya) from Wollayita. The level of nicotine in four different varieties of Ethiopian tobacco leaves was determined using high performance liquid chromatography. The level of nicotine in the four different varieties of Ethiopian tobacco were Virginia tobacco (3.26 %), the native tobacco 'Gaya' (1.10 %), Burley tobacco (0.650 %), and Oriental tobacco leaves (≤0.0500 %). It was found that the nicotine level of Ethiopian Virginia tobacco leaves increases from bottom to top leaf (stalk) positions of the tobacco plant. It was also found that the nicotine level of Ethiopian tobacco leaves varies in different species and the nicotine level of the same tobacco species differ in different area of cultivation. In general, the level of nicotine in Ethiopian tobacco is comparable with that in the rest of the world.	

Spatial distribution and contamination assessment of six heavy metals in soils and their transfer into mature tobacco plants in Kushtia District, Bangladesh.	
Saha Narottam, Rahman M Safiur, Jolly Yeasmin Nahar, Rahman Atiqur; Sattar M Abdus; Hai M Abdul	Environmental science and pollution research nternational, 2016 23(4): 3414-26
Although the tobacco production and consumption rate in Bangladesh is very high and a substantial portion of premature deaths is caused by tobacco smoking, the status of heavy metals in tobacco plants has not yet determined. This study, therefore, investigated the concentrations of Cu, Ni, Cd, Pb, Cr, and Zn in tobacco plants and their surrounding agricultural soils in Kushtia District, Bangladesh. The geochemical maps showed a similar spatial distribution pattern of the analyzed metals and identified Shempur, Kharara, Taragunia, and Shantidanga as metal hot spots. Geoanalytical indexes were applied to assess the extent of soil contamination, and the results depicted that the soils of Shempur, Kharara, Taragunia, and Shantidanga were moderately contaminated where Cd contributed the most to contamination degree (C d) in spite of its relative low content. However, other five areas in Kushtia District were suggested as uncontaminated according to both C d and pollution load index (PLI). The hazard quotient (HQ) and hazard index (HI) showed no possible indication of human health risks via ingestion of agricultural soils. This study also determined that human activities such as excess application of commercial fertilizers, animal manures, and metal-based pesticides were the sources of Cu, Ni, Cd, and Cr enrichment in soils and that the metals into tobacco plants were transported from the soils. The present study conclusively suggested that regulation of improper use of agrochemicals and continuous monitoring of heavy metals in tobacco plants are needed to reduce the tobacco-related detrimental health problems in Bangladesh.	

Simultaneous determination of alkaloids and their related tobacco-specific nitrosamines in tobacco leaves using LC-MS-MS.	
Li Yong; Pang Tao; Shi Junli; Lu Xiuping; Deng Jianhua	Journal of chromatographic science, 2015 53(10): 1730-6
Tobacco alkaloids (e.g., nicotine) and their metabolized tobacco-specific nitrosamines (TSNAs) are very important compounds for tobacco quality and safety. A simple and specific liquid chromatography-tandem mass spectrometry method was developed for the simultaneous determination of eight tobacco alkaloids and their related four TSNAs in tobacco leaves. The milled tobacco was extracted using 0.1 mol/L ammonium acetate solution and purified using methanol. Mass spectrometry parameters including declustering potential and collision energy were optimized to ensure that both the TSNAs and the tobacco alkaloids have suitable responses. Recoveries for accuracy were in the range of 80.2-105.2%. Intra-day and inter-day repeatability were in the range of 1.7-12.1% and 6.4-18.7%, respectively. Limit of detection and limit of quantitation were estimated in the range of 6 ng/g-45 μg/g and 24 ng/g-90 μg/g, respectively. The established method was applied to investigate the distribution of tobacco alkaloids and TSNAs in four kinds of tobacco. The result showed that the burley and the flue-cured have the highest (0.00047%) and the lowest (0.000024%)	

percentage of transformation from alkaloids to TSNAs, respectively. Thus, this method can be used for a wide range of samples. .

Quantitative analysis of 17 amino acids in tobacco leaves using an amino acid analyzer and chemometric resolution.	
Zeng Yihang; Cai Wensheng; Shao Xueguang	Journal of separation science, 2015 38(12): 2053-8
A method was developed for quantifying 17 amino acids in tobacco leaves by using an A300 amino acid analyzer and chemometric resolution. In the method, amino acids were eluted by the buffer solution on an ion-exchange column. After reacting with ninhydrin, the derivatives of amino acids were detected by ultraviolet detection. Most amino acids are separated by the elution program. However, five peaks of the derivatives are still overlapping. A non-negative immune algorithm was employed to extract the profiles of the derivatives from the overlapping signals, and then peak areas were adopted for quantitative analysis of the amino acids. The method was validated by the determination of amino acids in tobacco leaves. The relative standard deviations (n = 5) are all less than 2.54% and the recoveries of the spiked samples are in a range of 94.62-108.21%. The feasibility of the method was proved by analyzing the 17 amino acids in 30 tobacco leaf samples.	

Investigation by microarray analysis of effects of cigarette design characteristics on gene expression in human lung mucoepidermoid cancer cells NCI-H292 exposed to cigarette smoke.	
Sekine T, Sakaguchi C, Fukano Y.	Exp Toxicol Pathol. 2015 Feb;67(2):143-51
The effects of tobacco leaf types and the presence or absence of charcoal in the cigarette filters on gene expression were investigated using cigarette prototypes made of either flue-cured (FC) leaf or burley (BLY) leaf and Kentucky Reference 2R4F as a representative blend cigarette with cellulose acetate filters or charcoal filters. NCI-H292, human lung mucoepidermoid carcinoma cell line, was exposed to the total particulate matter (TPM) and gas/vapor phase (GVP) from each prototype for 8h and then the changes in gene expression from microarray data were analyzed. A number of genes associated with oxidative stress, inflammation, DNA damage and xenobiotic response were modified by the two fractions, TPM and GVP, from the three prototypes with cellulose acetate filters. Both TPM and GVP fractions strongly enhanced the gene expression of HMOX1, which is encoding the limiting enzyme in heme degradation and a key regulator of oxidative stress and inflammatory process. Comparing the effects of TPM and GVP fraction, TPM strongly activated Nrf2 pathway-mediated anti-oxidative stress reaction, whereas GVP caused notable DNA damage response. In comparison of FC and BLY, TPM from FC more strongly induced the expression of histone family proteins than that from BLY. GVP from FC markedly induced gene expression associated with HSP70-mediated inflammation relative to that from BLY. Charcoal included in the filter strongly reduced the effects of GVP from each cigarette on gene expression. However, charcoal did not modified the effects of TPM. As a whole, charcoal is a useful material for reducing the biological effects of GVP.	
PMID: 25497788	

Combusted but not smokeless tobacco products cause DNA damage in oral cavity cells.	
Gao H, Prasad GL, Zacharias W.	Environ Toxicol Pharmacol. 2014 May;37(3):1079-89
The aim of this work was to investigate genomic DNA damage in human oral cavity cells after exposure to different tobacco product preparations (TPPs). The oral carcinoma cell line 101A, gingival epithelial cells HGEC, and gingival fibroblasts HGF were exposed to TPM (total particulate matter from 3R4F cigarettes), ST/CAS (2S3 smokeless tobacco extract in complete artificial saliva), and NIC (nicotine). Treatments were for 24 h using TPM at its EC-50 doses, ST/CAS and NIC at doses with equi-nicotine units, and high doses for ST/CAS and NIC. Comet assays showed that TPM, but not ST/CAS or NIC, caused substantial DNA breaks in cells; only the high ST/CAS dose caused weak DNA damage. These results were confirmed by	

immunofluorescence for γ-H2AX protein. These data revealed that the combusted TPP caused substantial DNA damage in all cell types, whereas the two non-combusted TPPs exerted no or only minimal DNA damage. They support epidemiologic evidence on the relative risk associated with consumption of non-combusted versus combusted tobacco products, and help to understand potential genotoxic effects of such products on oral cavity cells.

PMID: 24780532 [PubMed - in process]

A comprehensive evaluation of the toxicology of experimental, non-filtered cigarettes manufactured with different circumferences.

Coggins CR, McKinney WJ Jr, Oldham MJ.	Inhal Toxicol. 2013; 25 Suppl 2:69-72.

CONTEXT: Historical work indicates that cigarette circumference may affect the toxicological profile of experimental cigarettes.

OBJECTIVE: Studies were conducted to examine the effect of different cigarette circumferences on (1) selected mainstream smoke constituents including concentrations of tobacco specific nitrosamines (TSNA) in smoke and (2) mutagenicity and cytotoxicity of cigarette smoke condensate.

MATERIALS AND METHODS: Analytical chemistry, Salmonella mutagenicity and cytotoxicity assays were used to evaluate the composition and biological activity of mainstream smoke from experimental, non-filtered cigarettes manufactured with four different circumferences (17.0-27.1 mm).

RESULTS: Most smoke constituents, including TSNA, decreased with decreasing cigarette circumference; however, amounts of hydrogen cyanide increased in a non-circumference dependent manner. Mutagenicity and cytotoxicity also decreased slightly with decreasing cigarette circumference.

CONCLUSION: Cigarette circumference may have a minor role in the toxicological profile of experimental cigarettes, with a so-far-unidentified mechanism.

PMID: 24341849 [PubMed - indexed for MEDLINE]

A comprehensive evaluation of the toxicology of the "Deli" cast sheet process used in experimental cigarettes.

Coggins CR, Merski JA, Oldham MJ.	Inhal Toxicol. 2013;25 Suppl 2:64-8

CONTEXT: Manufacture of cigarettes results in tobacco by-products, some of which can be processed and added back to cigarettes. Such additions (known as reconstituted tobacco or reconstituted leaf) have been shown to reduce tar yields. A new process (termed "Deli" cast sheet) is a potential refinement of the reconstitution process.

OBJECTIVE: Compare toxicity of smoke from experimental cigarettes made with reconstituted leaf with that from cigarettes made with Deli cast sheet.

MATERIALS AND METHODS: Analytical chemistry, Salmonella mutagenicity and cytotoxicity assays were used to evaluate the composition biological activity of mainstream smoke from experimental cigarettes made with Deli cast sheet or with reconstituted leaf. The effect of different amounts of guar and propylene glycol in Deli cast sheet was also evaluated.

RESULTS: Small increases in the amount of nitrogen oxides were found as a result of inclusion of the Deli cast sheet when compared with reconstituted leaf; no differences in cytotoxicity or mutagenicity were found.

CONCLUSION: The Deli process neither significantly modified chemical composition of smoke nor affected its biological activity, as measured by the mutagenicity and cytotoxicity assays used here.

PMID: 24341848 [PubMed - indexed for MEDLINE]

A comprehensive evaluation of the toxicology of different cut widths of tobacco in experimental cigarettes.	
Coggins CR, Fisher MT, Patskan GJ, Oldham MJ.	Inhal Toxicol. 2013; 25 Suppl 2:46-58.
CONTEXT: Literature suggests that the width of tobacco strips in cigarettes may affect the smoke chemistry and toxicology of such products. OBJECTIVE: A comprehensive analysis of smoke from experimental cigarettes can be used to determine whether different cut widths of tobacco result in different toxicological activity. MATERIALS AND METHODS: A battery of tests was used to compare the chemistry and in vitro and in vivo toxicology of smoke from experimental cigarettes made with tobacco cut to different widths. RESULTS: Different cut widths of tobacco did not elicit consistent and significant differences in cigarette smoke chemistry, responses in in vitro mutagenicity or cytotoxicity assays or most endpoints in 90-d rat inhalation studies. Of note, however, were atypical in-life observations and slightly depressed body weights observed in two rat inhalation studies. CONCLUSION: Most of our data indicate that different cut widths of tobacco used in cigarettes are unlikely to change the toxicity of mainstream cigarette smoke; however, without additional investigation, the atypical in-life observations and depression in body weights cast doubt on the toxicological acceptability of cutting the tobacco into wider shreds.	
PMID:24341846 [PubMed - indexed for MEDLINE]	

Genetic toxicology and toxicogenomic analysis of three cigarette smoke condensates in vitro reveals few differences among full-flavor, blonde, and light products.	
Yauk CL, Williams A, Buick JK, Chen G, Maertens RM, Halappanavar S, White PA.	Environ Mol Mutagen. 2012 May; 53(4):281-96.
Cigarette smoking leads to various detrimental health outcomes. Tobacco companies produce different brands of cigarettes that are marketed as reduced harm tobacco products. Early examples included "light" cigarettes, which differ from regular cigarettes due to filter ventilation and/or differences in chemical constituents. In order to establish baseline similarities and differences among different tobacco brands available in Canada, the present study examined the cytotoxicity, mutagenicity, clastogenicity, and gene expression profiles of cigarette smoke condensate (CSC) from three tobacco products, encompassing a full-flavor, blonde, and "light" variety. Using the Salmonella mutagenicity assay, we confirmed that the three CSCs are mutagenic, and that the potency is related to the presence of aromatic amines. Using the Muta™Mouse FE1 cell line we determined that the CSCs were clastogenic and cytotoxic, but nonmutagenic, and the results showed few differences in potencies among the three brands. There were no clear brand-specific changes in gene expression; each brand yielded highly similar expression profiles within a time point and concentration. The molecular pathways and biological functions affected by exposure included xenobiotic metabolism, oxidative stress, DNA damage response, cell cycle arrest and apoptosis, as well as inflammation. Thus, there was no appreciable difference in toxicity or gene expression profiles between regular brands and products marketed as "light," and hence no evidence of reduced harm. The work establishes baseline CSC cytotoxicity, mutagenicity, and expression profiles that can be used as a point of reference for comparison with data generated for products marketed as reduced harm and/or modified risk tobacco products.	
PMID: 22431010 [PubMed - indexed for MEDLINE] PMCID: PMC3350776	

Evaluation of in vitro assays for assessing the toxicity of cigarette smoke and smokeless

tobacco.	
Johnson MD, Schilz J, Djordjevic MV, Rice JR, Shields PG.	Cancer Epidemiol Biomarkers Prev. 2009 Dec; 18(12):3263-304.
BACKGROUND: In vitro toxicology studies of tobacco and tobacco smoke have been used to understand why tobacco use causes cancer and to assess the toxicologic impact of tobacco product design changes. The need for toxicology studies has been heightened given the Food and Drug Administration's newly granted authority over tobacco products for mandating tobacco product performance standards and evaluate manufacturers' health claims about modified tobacco products. The goal of this review is to critically evaluate in vitro toxicology methods related to cancer for assessing tobacco products and to identify related research gaps. METHODS: PubMed database searches were used to identify tobacco-related in vitro toxicology studies published since 1980. Articles published before 1980 with high relevance also were identified. The data were compiled to examine (a) the goals of the study, (b) the methods for collecting test substances, (c) experimental designs, (d) toxicologic end points, and (e) relevance to cancer risk. RESULTS: A variety of in vitro assays are available to assess tobacco smoke that address different modes of action, mostly using non-human cell models. However, smokeless tobacco products perform poorly in these assays. Although reliable as a screening tool for qualitative assessments, the available in vitro assays have been poorly validated for quantitative comparisons of different tobacco products. Assay batteries have not been developed, although they exist for nontobacco assessments. Extrapolating data from in vitro studies to human risks remains hypothetical. CONCLUSIONS: In vitro toxicology methods are useful for screening toxicity, but better methods are needed for today's context of regulation and evaluation of health claims.	
PMID: 19959677 [PubMed - indexed for MEDLINE] PMCID: PMC2789344	

Review: Is lung inflammation associated with microbes and microbial toxins in cigarette tobacco smoke?	
Pauly JL, Smith LA, Rickert MH, Hutson A, Paszkiewicz GM.	Immunol Res. 2010 Mar; 46(1-3):127-36.
Chronic inflammation that has been observed for malignant and non-neoplastic lung diseases of smokers has been attributed to the numerous and diverse particulate ('tar')-phase and gas-phase chemicals in mainstream smoke, most of which arise from the burning of tobacco. The primary cell-mediator of lung inflammation is the macrophage. Most probably, inflammation is promoted also from some of the more than 50 other cell types of the lung. Cured tobacco in diverse types of cigarettes is known to harbor a plethora of bacteria (Gram-positive and Gram-negative), fungi (mold, yeast), spores, and is rich in endotoxin (lipopolysaccharide). Reviewed herein are recent observations of the authors' team and other investigators that support the hypothesis that lung inflammation of long-term smokers may be attributed in part to tobacco-associated bacterial and fungal components that have been identified in tobacco and tobacco smoke.	
PMID: 19763893 [PubMed - indexed for MEDLINE	

Cytotoxicity of eight cigarette smoke condensates in three test systems: comparisons between assays and condensates.	
Richter PA, Li AP, Polzin G, Roy SK.	Regul Toxicol Pharmacol. 2010 Dec; 58(3):428-36.

Cytotoxic properties of tobacco smoke are associated with chronic tobacco-related diseases. The cytotoxicity of tobacco smoke can be tested with short-term predictive assays. In this study, we compare eight mainstream cigarette smoke condensates (CSCs) from commercial and experimental cigarettes in three different cytotoxicity assays with unique and overlapping endpoints. The CSCs demonstrated cytotoxicity in all assays. In the multiple cytotoxicity endpoint (MCE) assay with TK-6 cells, the cigarette varieties that had the highest EC50s for reduced cell growth also showed a positive dose-response relationship for necrotic cells. In the IdMOC multiple cell-type co-culture (MCTCC) system, all CSCs reduced the viability of the cells. Low concentrations of some CSCs had a stimulatory effect in lung microvascular endothelial cells and small airway epithelial cells. In the neutral dye assay (NDA), except for a 100% flue-cured tobacco CSC, there was little consistency between CSCs producing morphological evidence of moderate or greater toxicity and the CSCs with the lowest EC50s in the MCE or MCTCC assays. Overall, cigarettes made with flue-cured tobacco were the most cytotoxic across the assays. When results were expressed on a per-mg of nicotine basis, lower tar cigarettes were the most cytotoxic in primary human respiratory cells.	
PMID: 20719243 [PubMed - indexed for MEDLINE]	

Effects of dermal exposure to Nicotiana tabacum (Jean Nicot, 1560) leaves in mouse evaluated by multiple methods and tissues.	
Da Silva FR, Erdtmann B, Dalpiaz T, Nunes E, Da Rosa DP, Porawski M, Bona S, Simon CF, Da C Allgayer M, Da Silva J.	J Agric Food Chem. 2010 Sep 8; 58(17):9868-74.
Tobacco farmers are routinely exposed to complex mixtures of the compounds present in tobacco leaves, including organic and inorganic pesticides. Penetration through skin is the most significant route of uptake in occupational exposure to chemicals, including dust and liquids containing toxic and carcinogenic substances. This study evaluates the genotoxic effect of tobacco leaves with and without dermal exposure to flumetralin in Mus musculus, determining cell damage by the micronucleus test and the Comet assay as well as antioxidant enzyme activities and hematologic parameters. Nicotine was used as positive control. Blood samples were collected for 0, 3, 24 and 48 h exposure periods, and DNA damage by Comet assay and micronucleus test was evaluated for all these periods. Bone marrow and liver cells were also evaluated for the 48 h exposure period. Significant differences between Comet assay results in blood cells from animals exposed to tobacco leaves with and without pesticide were found in 24 and 48 h exposure periods in relation to negative control. Bone marrow cells from the group exposed to leaves with pesticide (48 h) also demonstrated significant increase in DNA damage. Concerning the micronucleus test, only animals exposed to tobacco leaves without pesticide (24 h) showed increase in frequency of micronuclei when compared to the negative control. Oxidative stress activities also were demonstrated for different groups. The results demonstrate the injury effect caused by tobacco leaves in different *Mus musculus* tissues, suggesting that the effects of dermal exposure to tobacco leaves are caused by complex mixtures present in the plant, but mainly by nicotine.	
PMID: 20684553 [PubMed - indexed for MEDLINE]	

Effects of flue-curing on cigarette smoke condensates mutagenicity.	
Morin, Andre; Poirier, Nicole; Prefontaine, David; Lacasse, Martine	Beitraege zur Tabakforschung International (2010), 24(2), 72-77 CODEN: BTAID3; ISSN: 0173-783X

	2010:1295432 CAPLUS
Flue-curing is a post-harvest conditioning process which strongly affects the tobacco leaf chem., and consequently the chem. properties of tobacco smoke. Several studies identified the major changes in tobacco chem. occurring during flue-curing. It is not known how flue-curing contributes to changes in bioactivity of cigarette smoke condensate (CSC).In this study, tobacco leaves collected throughout the twelve days of flue-curing were used to prep. cigarettes that were smoked to generate CSC samples. The assessment of mutagenicity was performed using the Bacterial Reverse Mutation / Ames test with Salmonella typhimurium TA98 in the presence of S9 metabolic activation. CSC from cured leaves were significantly more mutagenic than CSC from uncured leaves. The no. of revertants was pos. influenced by the duration of the curing. The effect of the duration of curing on the no. of revertants was more pronounced with increasing CSC concn.	

Effect of a flue-curing process that reduces tobacco specific nitrosamines on the tumor promotion in SENCAR mice by cigarette smoke condensate.	
Hayes, J. R.; Meckley, D. R.; Stavanja, M. S.; Nelson, P. R.; Van Kampen, K. R.; Swauger, J. E.	Food and Chemical Toxicology (2007), 45(3), 419-430 CODEN: FCTOD7; ISSN: 0278-6915 2007:132888 CAPLUS
A 30-wk dermal tumor promotion study was conducted to evaluate the dermal tumor-promoting potential of cigarette smoke condensate (CSC) collected from cigarettes contg. flue-cured tobacco cured by a heat-exchange process (HE) relative to that of cigarettes contg. flue-cured tobacco cured by the traditional direct-fire process (DF). Heat-exchange process cured tobacco contains significantly lower concns. of tobacco specific nitrosamines (TSNAs) compared to traditional direct-fire cured tobacco. Mainstream CSCs were collected by cold trap from smoke generators using the Federal Trade Commission puffing regimen. Groups of 40 female SENCAR mice were initiated by a single application of 75 ug 7,12-dimethylbenz[a]anthracene (DMBA) to the shaved dorsal skin. CSCs were then applied to the skin three times/wk for 29 wk at 9, 18, or 36 mg tar/application. End-points included body wts., clin. observations, organ wts., dermal tumor development and histopathol. The nos. of dermal tumors and the nos. of tumor-bearing mice for each CSC were statistically different from the DMBA/acetone control group and increased with increasing dose. When corresponding doses of each CSC were compared, only the DMBA/mid-dose HE CSC group was statistically significantly different (lower) from the corresponding DMBA/mid-dose DF CSC group. In this assay, the dermal tumor-promotion potential of CSC from heat-exchange flue-cured tobacco did not differ from that of traditional direct-fire flue-cured tobacco CSC.	

The mouse lymphoma thymidine kinase assay for the assessment and comparison of the mutagenic activity of cigarette mainstream smoke particulate phase.	
Schramke, H.; Meisgen, T. J.; Tewes, F. J.; Gomm, W.; Roemer, E.	Toxicology (2006), 227(3), 193-210. Elsevier Ltd CODEN: TXCYAC; ISSN: 0300-483X 2006:989845 CAPLUS
The mouse lymphoma thymidine kinase assay (MLA) was optimized to quant. det. the in vitro mutagenicity of cigarette mainstream smoke particulate phase. To test whether the MLA is able to discriminate between different cigarette types, specially constructed cigarettes each contg. a single tobacco type - Bright, Burley, or Oriental - were investigated. The mutagenic activity of the Burley cigarette was statistically significantly lower, up to approx. 40%, than that of the Bright and Oriental cigarettes.To det. the impact of 2 different sets of smoking conditions, American-blend cigarettes were smoked under US Federal Trade Commission/International Organization for Standardization conditions and under Massachusetts Department of Public Health (MDPH) conditions. Conventional cigarettes - 8 from the US com. market plus the Ref. Cigarettes 1R4F and 2R4F - and an elec. heated cigarette smoking system (EHCSS) prototype were tested. There were no statistically significant differences between the 2 sets of smoking conditions on a per mg total particulate matter basis, although there was a consistent trend towards slightly lower mutagenic activity under MDPH conditions. The mutagenic activity of the EHCSS prototype was distinctly lower than that of the conventional cigarettes under both sets of smoking conditions. These results show that the MLA can be used	

to assess and compare the mutagenic activity of cigarette mainstream smoke particulate phase in the comprehensive toxicol. assessment of cigarette smoke.

Analysis of the expression of heme oxygenase-1 gene in human alveolar epithelial cells exposed to cigarette smoke condensate.	
Fukano, Yasuo; Oishi, Masako; Chibana, Fumika; Numazawa, Satoshi; Yoshida, Takemi	Journal of Toxicological Sciences (2006), 31(2), 99-109 Japanese Society of Toxicology CODEN: JTSCDR; ISSN: 0388-1350 2006:747364 CAPLUS
Airway epithelium is exposed to inhaled exogenous sources. Injury of the alveolar epithelium by cigarette smoking is presumed to be an important process in the pathogenesis of smoking-related pulmonary diseases.Current mechanistic assays that measure the toxicity of cigarette smoke focus on carcinogenesis. However, there is a need to design assays relevant to other disease processes. Oxidative stress is implicated in the pathogenesis of many respiratory diseases including chronic obstructive pulmonary disease. Therefore, we evaluated whether in vitro studies of cigarette smoking are appropriate to examine HO-1 mRNA expression. The human lung epithelial cell line A549 was exposed to the particulate fraction of cigarette smoke (Cigarette Smoke Condensate; CSC) and examd. for the induction of HO-1 mRNA. HO-1 gene expression by CSC is increased dose-dependently. In comparison of the induction of HO-1 mRNA by CSC prepd. from flue-cured or Burley tobacco, CSC from flue-cured tobacco seems to tend to induce an mRNA of HO-1 higher than CSC from Burley tobacco. The adaptation of HO-1 mRNA expression assay as a biol. relevant indicator of cigarette smoke-induced stress may be exemplified in this study whereby CSC derived from cigarette smoke pos.correlated with an increase in HO-1 expression and the difference of the type of tobacco can be detected.	

Approaches to identify less harmful tobacco and tobacco products	
Albino, Anthony; Jorgensen, Ellen; Traganos, Frank; Darzynkiewicz, Zbigniew; Jin, Wendy	PCT Int. Appl., 284 pp. Patent CODEN: JTSCDR; ISSN: 0388-1350 2005:1259628 CAPLUS
Aspects of the invention concern methods for detecting, identifying and evaluating tobacco and tobacco products to det. the potential that these compns. have to contribute to a tobacco-related disease. It is based, at least in part; on the discovery that exposure of pulmonary cells to smoke or smoke condensate obtained from tobacco or tobacco products induces double stranded breaks in cellular DNA, which were efficiently detected using assays that measure the presence, absence, or amt. of phosphorylation of the histone, H2AX.	

Global gene expression analysis of human bronchial epithelial cells exposed to cigarette smoke, smoke condensates, or components thereof	
Jorgensen, Ellen D.; Albino, Anthony P.; Jin, Wendy	PCT Int. Appl., 173 pp. Patent CODEN: PIXXD2 2005:1170695 CAPLUS
Aspects of the present invention concern the identification of several methods to analyze the genes that are modulated in normal human bronchial epithelial (NHBE) cells after exposure to cigarette smoke condensates (CSC) or cigarette smoke (CS). Embodiments described herein include methods to identify a gene that is modulated in response to exposure toCSC or CS, methods to identify tobacco products that have a reduced potential to contribute to tobacco-related disease, methods to make tobacco products that have a reduced potential to contribute to a tobacco-related disease, methods to identify a subject's predilection to acquire a tobacco related disease, the use of particular genes as biomarkers for tobacco-related disease, and patterns of gene expression or genetic signatures that are unique to each particular tobacco product.	

Acute and chronic health effects due to green tobacco exposure in agricultural workers	
Parikh, J. R.; Gokani, V. N.; Doctor, P. B.; Kulkarni, P. K.; Shah, A. R.; Saiyed, H. N.	American Journal of Industrial Medicine (2005), 47(6), 494-499. Wiley-Liss, Inc. CODEN: AJIMD8; ISSN: 0271-3586 2005:580330 CAPLUS
An epidemiol. study was undertaken in Guijarat, India to study the acute and chronic health effects of occupational exposure to green tobacco.Non-Flue Cured Virginia (FCV) tobacco is the main crop in many districts of Central Gujarat. Three villages were selected from Anand district for the study and a random sample of 685 exposed workers were examd. Six hundred and fifty-five control workers with the same socio-economic status were examd. from two villages where tobacco was not cultivated. The overall prevalence of green tobacco sickness (GTS) was 47.0% among tobacco workers. The prevalence in women workers was 55.7% while in men workers it was 42.66%. To detect the chronic health effects prevalence of hypertension, ECG abnormalities, and eye problems in all the workers and reproductive abnormalities in women workers, all subjects received a medical examn. The data were compared in exposed and control group but they were nonsignificant statistically. No case of tobacco amblyopia was detected. The prevalence of GTS among non-FCV tobacco workers is high. However, from viewpoint of severity it can be considered as mild acute nicotine toxicity, which is relieved without medication. Nosignificant difference is obsd. as regards to chronic health effects among tobacco workers and control workers.	

Inhibition of human MAO-A and MAO-B by a compound isolated from flue-cured tobacco leaves and its neuroprotective properties in the MPTP mouse model of neurodegeneration.	
Castagnoli, K.; Petzer, J. B.; Steyn, S. J.; Van Der Schyf, C. J.; Castagnoli, N., Jr.	Inflammopharmacology (2003), 11(2), 183-188. VSP BV CODEN: IAOAES; ISSN: 0925-4692 2003:621505 CAPLUS
Prompted by the findings that smokers have lowered brain and blood platelet monoamine oxidase-A and -B activities compared to non-smokers and that smokers have a lowered incidence of Parkinson's disease, we have examd. the neuroprotective properties of an MAO inhibitor, 2,3,6-trimethyl-1,4-naphthoquinone (TMN), which is present in the tobacco plant and smoke in the MPTP C57BL/6 mouse model of neurodegeneration. Dopamine (DA) levels in the striata of mice treated with TMN prior to the administration of MPTP were significantly higher than DA levels in the striata of mice receiving MPTP only, thus indicating a degree of neuroprotection in this model of Parkinson's disease. The potential consequences on MAO activity of long term exposure to this compd. need to be evaluated. Furthermore, there is evidence for the presence of other inhibitors in the tobacco leaf and smoke, including compds. with irreversible MAO inhibitory properties. Although there is no evidence to link the lowered activities of MAO to the lowered incidence of Parkinson's disease in smokers, the neuroprotective effects of TMN in the MPTP mouse model suggest that such a relationship is worthy of further evaluation.	

Rat Subchronic Inhalation Study of Smoke from Cigarettes Containing Flue-Cured Tobacco Cured Either by Direct-Fired or Heat-Exchanger Curing Processes	
Kinsler, Steven; Pence, Deborah H.; Shreve, W. Keith; Mosberg, Arnold T.; Ayres, Paul H.; Sagartz, John W.	Inhalation Toxicology (2003), 15(8), 819-854. Taylor & Francis, Inc CODEN: INHTE5; ISSN: 0895-8378
A subchronic, nose-only inhalation study compared the effects of mainstream smoke from a cigarette contg. 100% flue-cured tobacco cured by a direct-fired process to that of a cigarette contg. 100% flue-cured tobacco cured by a heat exchanger process. The tobaccos and mainstream smoke from tobaccos cured by the heat exchanger process were shown to have significantly lower levels of tobacco-specific nitrosamines than tobaccos cured by a direct-fired process. Male and female rats were exposed for 1 h/day, 5 days/wk, for 13 wk to	

mainstream smoke at 0, 0.06, 0.20, or 0.80 mg wet total particulate matter per L of air. Clin. signs, body and organ
wts., clin. chem., hematol., carboxyHb, serum nicotine, plethysmog., gross pathol., and histopathol. were detd. When histol. changes resulting from exposure to smoke from the 2 types of cigarettes were compared, the only
significant difference was increased epithelial hyperplasia of the anterior nasal cavity in males in the high-exposure group for the heat-exchanger cigarette. At the end of the exposure period, subsets of rats from each group were maintained without smoke exposures for an addnl.13 wk (recovery period). At the end of the recovery period, there were no statistically significant differences in histopathol. findings obsd.between the heat-exchanger-cured tobacco cigarette when compared to the direct-fired cured tobacco cigarette. The complete toxicol. assessment in this study of heat exchanger and direct-fired tobaccos suggests no overall biol. significant differences between the 2 cigarettes

Reduction in Ames Salmonella mutagenicity of mainstream cigarette smoke condensate by tobacco protein removal.	
Clapp, William L.; Fagg, Barry S.; Smith, Carr J.	Mutation Research, Genetic Toxicology and Environmental Mutagenesis (1999), 446(2), 167-174 Elsevier B.V. CODEN: MRGMFI; ISSN: 1383-5718
The mutagenic activity of cigarette smoke condensates (CSC) made from tobacco before and after removal of protein was assessed by the Ames Salmonella assay in bacterial strains TA98 and TA100. Removal of proteinand peptides from **flue**-cured **tobacco** via water extn. followed by protease digestion reduced the mutagenicity of the resultant CSC by 80% in the TA98 strain and 50% in the TA100 strain. Similarly, redns. of 81% in TA98 and 54% in TA100 were seen following water extn. and protease digestion of **burley tobacco**. The significant redns. in Ames mutagenicity following protein removal suggest that protein pyrolysis products are a principal contributor to the genotoxicity of CSC as measured in this assay.	

Isolation and Characterization of a Monoamine Oxidase Inhibitor from Tobacco Leaves.	
Khalil, Ashraf A.; Steyn, Salome; Castagnoli, Neal, Jr.	Chemical Research in Toxicology (2000), 13(1), 31-35 American Chemical Society CODEN: CRTOEC; ISSN: 0893-228X
Recent positron emission tomog. imaging studies have demonstrated a significant decrease in both monoamine oxidase A and B (MAO-A and MAO-B) activities in the brains of smokers. Normal levels of activity are obsd. in former smokers, suggesting the presence of one or more compds. in tobacco smoke that may inhibit these enzymes. In this paper, we report the results of efforts to identify compds. present in flue-cured tobacco leaves that inhibit MAO. The isolation procedure was guided by estg. the inhibitory properties of tobacco leaf exts. on the liver mitochondrial MAO-B-catalyzed oxidn. of 1-methyl-4-(1-methylpyrrol-2-yl)-1,2,3,6-tetrahydropyridine to the corresponding dihydropyridinium metabolite. Fractionation of exts. from flue-cured tobacco leaves led to the isolation of a competitive inhibitor of human MAO-A (Ki = 3 μM) and MAO-B (Ki = 6 μM), the structure of which could be assigned by classical spectroscopic anal. and confirmed by synthesis. This information may help to provide insights into some aspects of the pharmacol. and toxicol. of tobacco products.	

In vitro inhibition of catalase activity by cigarette smoke: relevance for oxidative stress	
Mendez-Alvarez, Estefania; Soto-Otero, Ramon; Sanchez-Sellero, Ines; Lamas, Manuel Lopez-Rivadulla	Journal of Applied Toxicology (1998), 18(6), 443-448 John Wiley & Sons Ltd. CODEN: JJATDK; ISSN: 0260-437X
The in vitro effects of cigarette smoke on catalase activity were investigated in biol. prepns. from rat liver and	

brain using a polarog method. In both cases cigarette smoke solns. showed a potent ability to inhibit catalase activity with a slight time dependency. The reversibility of their inhibitory activity was demonstrated by in vitro dialysis tests. The catalase inhibitory compd.(s) are formed in the smoking process, are not extd. with org. solvents and appear to have a relatively low mol. wt. We also examd. the effects obtained by using two different com. blends of tobacco, achieving a major inhibition with Burley tobacco in comparison to Bright tobacco. These data suggest that the cytotoxic and mutagenic effects of cigarette smoke may be mediated by its addnl. capacity to enhance the generation of free radicals by inhibiting catalase activity, thus contributing to cell damage particularly during oxidative stress.

Comparative cytotoxicity studies of smoke condensates from different types of cigarettes and tobaccos

Bombick, D. W.; Putnam, K.; Doolittle, D. J.	Toxicology in Vitro (1998), 12(3), 241-249 Elsevier Science Ltd. CODEN: TIVIEQ; ISSN: 0887-2333

The neutral red assay, a rapid and accurate method for estg. the cytotoxicity of chems., has been used to assess the cytotoxicity of cigarette smoke condensate (CSC), a complex chem. mixt. contg. over 3000 identified compds. The first objective was to optimize the neutral red assay for evaluation of CSCs. This study also assessed and compared the cytotoxicity of smoke condensates from three ref. cigarettes which differ in "tar" content; cigarettes of different tobacco type compn.; an ultra-low tar cigarette (R1); and an RJR test cigarette which heats but does not burn tobacco. Finally, this study investigated the cytotoxicity of a specific CSC component, nicotine, and its metabolite, cotinine. Exposure times of 24 h or longer using CHO cells provided optimal conditions for evaluation of CSC cytotoxicity. The cytotoxicity of CSCs from ref. cigarettes was similar. CSC from cigarettes comprised of flue-cured tobacco exhibited greater cytotoxicity than CSC from cigarettes comprised of burley tobacco. CSC from the R1 cigarette exhibited similar cytotoxicity compared with 1R4F CSC. The CSC from a cigarette that heated but did not burn tobacco (RJR test cigarette) demonstrated no cytotoxicity in CHO cells. Finally, nicotine and cotinine were not cytotoxic to CHO cells. The neutral red assay has been proved useful for quantifying differences in cytotoxicity of smoke condensates from cigarettes which vary in "tar" yield and for assessing specific smoke constituents.

Inhibition of brain monoamine oxidase by adducts of 1,2,3,4-tetrahydroisoquinoline with components of cigarette smoke

Mendez-Alvarez, Estefania; Soto-Otero, Ramon; Sanchez-Sellero, Ines; Lopez-Rivadulla Lamas, Manuel	Life Sciences (1997), 60(19), 1719-1727. Elsevier CODEN: LIFSAK; ISSN: 0024-3205

A series of adducts of 1,2,3,4-tetrahydroisoquinoline (TIQ) and some components of tobacco smoke were investigated for their ability to inhibit rat brain monoamine oxidase. 1-Cyano-TIQ (1CTIQ), N-(1'-cyanoethyl)-TIQ (CETIQ), N-(1'-cyanopropyl)-TIQ (CPTIQ), and N-(1'-cyanobutyl)-TIQ (CBTIQ) were found to act as competitive inhibitors for both MAO-A and MAO-B. Ki values ranged from 16.4 to 37.6 uM. N-(Cyanomethyl)-TIQ (CMTIQ) was not found to be an inhibitor (Ki>100 uM). These findings may help to explain the in vivo inhibitory effects of tobacco smoke on MAO activity and the suggested protective effect of tobacco smoking against Parkinson's disease. They also appear to reinforce the usefulness of reversible MAO inhibitors in smoking cessation and abstinence. However, different results must be expected between Burley and Bright tobacco.

The changing cigarette, 1950-1995

Hoffmann, Dietrich; Hoffmann, Ilse	Journal of Toxicology and Environmental Health (1997), 50(4), 307-364. Taylor & Francis CODEN: JTEHD6; ISSN: 0098-4108

A review with many refs. Nicotine is recognized to be the major inducer of tobacco dependence. The smoking of cigarettes as an advantageous delivery system for nicotine, accelerates and aggravates cardiovascular disease, and is causally assocd. with increased risks for chronic obstructive lung disease, cancer of the lung and of the upper aerodigestive system, and cancer of the pancreas, renal pelvis, and urinary bladder. It is also assocd. with cancer of the liver, cancer of the uterine cervix, cancer of the nasal cavity, and myeloid leukemia. In1950, the first large-scale epidemiol. studies documented that cigarette smoking induces lung cancer and described a dose-response relation between no. of cigarettes were not only confirmed by several hundreds of prospective and case-control studies but the plausibility of this causal assocn. was also supported by bioassays and by the identification of carcinogens in cigarette smoke. Whole smoke induces lung tumors in mice and tumors in the upper respiratory tract of hamsters. The particulate matter of the smoke elicits benign and malignant tumors on the skin of mice and rabbits, sarcoma in the connective tissue of rats, and carcinoma in the lungs of rats upon intratracheal instillation. More than 50 carcinogens have been identified, including the following classes of compds.: polynuclear arom. hydrocarbons (PAH), arom. amines, and N-nitrosamines. Among the latter, the tobacco-specific N-nitrosamines (TSNA) have been shown to be of special significance. Since 1950, the makeup of cigarettes and the compn. of cigarette smoke have gradually changed. In the United States, the sales-weighted av. "tar" and nicotine yields have declined from a high of 38 mg "tar" and 2.7 mg nicotine in 1954 to 12 mg and 0.95 mg in 1992, resp. In the United Kingdom, the decline was from about 32 mg "tar" and 2.2 mg nicotine to less than 12 mg "tar" and 1.0 mg nicotine per cigarette. During the same time, other smoke constituents changed correspondingly. These redns. of smoke yields were primarily achieved by the introduction of filter tips, with and without perforation, selection of tobacco types and varieties, utilization of highly porous cigarette paper, and incorporation into the tobacco blend of reconstituted tobacco, opened and cut ribs, and "expanded tobacco.". In most countries where tobacco blends with air-cured (burley) tobacco are used, the nitrate content of the cigarette tobacco increased. In the United States nitrate levels in cigarette tobacco rose from 0.3-0.5% to 0.6-1.35%, thereby enhancing the combustion of the tobacco. More complete combustion decreases the carcinogenic N-nitrosamines, esp. the TSNA in the smoke. However, all anal. measures of the smoke components have been established on the basis of standardized machine smoking conditions, such as those introduced by the Federal Trade Commission, that call for 1 puff to be taken once a minute over a 2-s period with a vol. of 35 mL. These smoking parameters may have simulated the way in which people used to smoke the high-yield cigarettes; however, they no longer reflect the parameters applicable to contemporary smokers, and esp. not those applicable to the smoking of low- and ultra-low-yield filter cigarettes. Recent smoking assays have demonstrated that most smokers of cigarettes with low nicotine yield take between 2 and 4 puffs per min with vols. up to 55 mL to satisfy their demands for nicotine. The overview also discusses further needs for reducing the toxicity and carcinogenicity of cigarette smoke. From a public health perspective, nicotine in the smoke needs to be lowered to a level at which there is no induction of dependence on tobacco.

Black (air-cured) and blond (flue-cured) tobacco cancer risk IV: molecular dosimetry studies implicate aromatic amines as bladder carcinogens

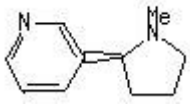
Bartsch, H.; Malaveille, C.; Friesen, M.; Kadlubar, F. F.; Vineis, P.	Eur. J. Cancer, Part A (1993), 29A(8), 1199-207 CODEN: EJCTEA

A review with 80 refs. Tobacco smoking causes a major fraction of male urinary bladder cancers and the relative risk of bladder cancer is reported to be two to three times higher for smoking of black (air-cured) than for smoking blond (flue-cured) tobacco. In mol. dosimetry studies to examine the hypothesis that arom. amines in tobacco smoke are primarily responsible for bladder cancer, the higher bladder cancer risk in smokers of black tobacco was correlated with two to five times higher exposure to carcinogenic arom. amines present in black tobacco smoke, notably 4-aminobiphenyl (ABP). For the same amt. of smoking, black tobacco smokers had levels of ABP-Hb adducts 1.5 times higher and excreted a 1.8-fold higher level of urinary mutagens. These mutagens were characterized as arom. amines, and included the heterocyclic amine PhIP, a known mutagen and multiorgan/species carcinogen. In smoking volunteers, the ABP-Hb

adduct level depended significantly on the acetylator and P-450IA2 phenotypes, being 1.3- to 1.5-fold lower in fast acetylators, slow/intermediate P-450IA2 individuals. The N-(deoxyguanosin-8-yl)-ABP adduct was a major smoking-related DNA adduct in bladder biopsies from surgical patients. It was also tentatively identified in exfoliated urothelial cells of smoking volunteers, who showed a significant and linear correlation between adduct levels of ABP and Hb and with deoxyguanosine in urothelial DNA; both were related to no. of cigarettes smoked per day. Levels of several smoking-related DNA adducts in urothelial cells were 2-20 times elevated in smokers. Similar convex dose-response relationships have been found between the no. of cigarettes smoked and the relative risk for bladder cancer and between the levels of
ABP-Hb adducts and markers of recent smoking. A possible explanation is that fast and slow acetylators have different susceptibility to arom. amine carcinogens. Case-control studies have consistently revealed annexcess of variable magnitude of slow acetylators in subgroups exposed occupationally to carcinogenic arom. amines. Altogether, results from these studies reinforce the assocn. between cigarette smoking, carcinogen-DNA adducts in urothelial cells, and implicate primary arom. and possibly heterocyclic amines as bladder carcinogens

The effects of tobacco modification on the biological response to 13-week cigarette smoke inhalation in inbred Syrian hamsters

Bernfeld, P.; Soto, E.; Tso, T. C.	Journal of the American College of Toxicology (1984), 3(4), 249-60 CODEN: JACTDZ; ISSN: 0730-0913

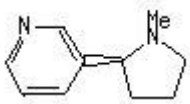


I

Twice daily exposure of 12-24 male inbred Syrian BIO 15.16 hamsters for 13 wk to maximally tolerated doses of smoke from 12 types of investigational cigarettes under exptl. conditions and with previously described instrumentation (Bernfeld et al., 1979) resulted in the following response: increased occurrence and aggregation of alveolar macrophages, increased occurrence of tracheal squamous metaplasia, and increased frequency and severity of laryngeal hyperplasia. The significance of these changes was evaluated by means of loglinear models. The effect of varying the nicotine (I) [54-11-5] level in tobacco leaf between 0.2 and 1.5% caused no significant changes in the subchronic response. Smoke from Bright tobacco with av. tar yields of 22.4-26.2 mg/cigarette caused significantly more alveolar macrophages and laryngeal hyperplasia but less tracheal squamous metaplasia than did smoke from Burley tobacco, with 9.3-10.5 mg tar/cigarette. Deproteinization of tobacco did not change the response of the hamsters to the resulting smoke. Maleic hydrazide [123-33-1]-field treatment of tobacco significantly reduced the alveolar macrophages, but spiking of tobacco with maleic hydrazide increased the response. Apparently, smoke from low-I cigarettes is not less tumorigenic in the hamster than that from high-I cigarettes, smoke from high-tar Bright tobacco does not affect the tumorigenicity of the resulting smoke, and maleic hydrazide field treatment of tobacco does not increase the tumorigenicity of the resulting smoke, whereas spiking of tobacco with maleic hydrazide might do so.

Effect of some factors on the content of substances harmful to health in cigarets. III. Effect of tobacco type

Zydron, Krystyna	Przemysl Spozywczy (1982), 36(2), 53-5 CODEN: PRSPAD; ISSN: 0033-250X

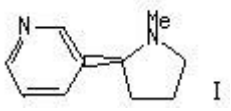


I

A stain of Virginia tobacco generated crude smoke condensate 18.36, resinous substances 11.62, total alkaloids as nicotine (I) [54-11-5] 0.66, and CO 14.82 mg/cigarette. The resp. contents for 6 other strains and

variations were 22.84-33.76, 13.25-18.98, 2.06-5.64, and 15.47-22.51 mg/cigarette. The coeffs. of crude condensate correlation with I or CO were 0.795 and 0.706, resp. The resp. coeffs. were 0.624 and 0.874 for the resinous substances. The crude condensate/resinous substances correlation coeff. was 0.927. The CO evolution also depended on paper permeability.

Genetic effects of fresh cigaret smoke in Saccharomyces cerevisiae.	
Gairola, C.	Mutation Research, Genetic Toxicology Testing (1982), 102(2), 123-36 CODEN: MRGTE4; ISSN: 0165-1218



I

The ability of fresh cigaret smoke from University of Kentucky ref. cigaret 2R1 to induce gene conversion, reverse mutation and mitotic crossing-over in strain D7 of S. cerevisiae was examd. A closed cell suspension-recycle system using 2 peristaltic pumps interconnected to a single-port reverse-phase smoking machine was developed to provide complete exposure of cells to smoke within 0.2-10 s of its generation. The exposed cells showed a dose-dependent increase in the frequency of all the 3 genetic endpoints examd. Cell age was an important factor with younger cells being more sensitive than older. Filtration studies showed that the gas phase possessed <25% of the total whole-smoke activity. Activated charcoal reduced the activity of smoke in direct proportion to its amt. in the filter. An acetate filter did not appreciably alter the activity. A comparison of whole smoke from various cigarets showed that: (1) the nicotine (I) [54-11-5] content of a cigaret does not effect the genetic activity of smoke; (2) burley and flue-cured tobaccos have differential activity in gene conversion and reverse-mutation systems; and (3) the genetic effects of whole smoke are not peculiar to tobacco pyrolysis because similar effects are produced by smokes from lettuce and other nontobacco cigarets. Apparently, the yeast D7 system can be used effectively for the quant. evaluation of genetic effects of smoke from different cigarets, and both whole cigaret smoke and its gas phase possesses mutagenic as well as recombinogenic activity that can be modified by the use of filters.

Comparative lung pathology of rats after exposure to cigarette and cigar Smoke.	
Betts, T. E.; O'Sullivan, J. P.; Elson, L. A.	British Journal of Experimental Pathology (1981), 62(4), 429-35 CODEN: BJEPA5; ISSN: 0007-1021

Rats bred and maintained under min. disease conditions suffered much less lung damage after exposure to cigar smoke than did similar animals exposed to the smoke of Virginia tobacco. Rats exposed to cigar tobacco smoke also showed a greater wt. gain and a lower acute-phase protein increase than did their Virginia tobacco counterparts. These differences in response were compounded by the results obtained from measuring changes in the epithelium of the intrapulmonary airways. The smoke of air-cured but not fermented cigar tobacco had an effect similar to the smoke of Virginia tobacco.

Inhalation toxicity studies on cigarette smoke (VI). 6-week comparative experiments using modified flue-cured cigarettes: histopathology of the lung.	
Walker D, Wilton LV, Binns R.	Toxicology. 1978 Jul;10(3):229-40.

Rats were exposed twice daily for 6 weeks to diluted smoke derived from cigarettes with a range of deliveries of particulate matter. The inhaled smoke reached the alveolar surface, increased the size and number of free macrophages and provoked epithelial metaplasia but did not appear to alter the lymphoid reaction to spontaneous infection. The hypertrophy of the macrophages was typically alveolar and their hyperplasia was directly proportional to the particulate delivery of cigarettes smoked. Alveolar metaplasia was incipient, developed characteristically near the respiratory bronchiole in close association with macrophage clusters and

only in rats exposed to smoke from cigarettes with the highest delivery of particulate matter.
PMID: 705798 [PubMed - indexed for MEDLINE]

Inhalation toxicity studies on cigarette smoke (VII). 6-week comparative experiments using modified flue-cured cigarettes: histopathology of the conducting airways.	
Walker D, Wilton LV, Binns R	Toxicology. 1978 Jul;10(3):241-59.
Rats were exposed twice daily for 6 weeks to diluted smoke derived from cigarettes with a range of deliveries of particulate matter. The inhaled smoke caused squamous metaplasia and keratinising hyperplasia in the larynx and goblet cell hyperplasia in the nasal cavity, trachea and intrapulmonary bronchus. Squamous metaplasia occurred in the larynx of almost all rats exposed to smoke but never in their tracheas or bronchi. The degree of reaction observed for the other responses, except goblet cell hyperplasia in the trachea, was positively related, by quantitative microscopy, to the particulate delivery of cigarettes. These findings, together with other smoke-induced changes which occur in the pulmonary alveoli, provide a basis for the short-term bioassay of inhalation toxicity of cigarette smoke.	
PMID: 705799 [PubMed - indexed for MEDLINE]	

Inhalation toxicity studies on cigarette smoke (VIII). 6-week comparative experiments using modified flue-cured cigarettes: general toxicology.	
Binns R, Wilton LV.	Toxicology. 1978 Nov;11(3):207-17
Details are given of studies carried out to compare the inhalation toxicity to rats of smoke from cigarettes modified to give a range of deliveries of particulate matter. Dosimetry work showed that under conditions similar to those used for subsequent toxicity experiments, smoke particulates deposited in the lower respiratory system within the approximate dose range of 500--700 microgram TPM/g lung tissue. Respiratory monitoring showed that the response of animals to a range of smoke exposure conditions was similar and did not change during the course of the experiments. This observation was confirmed by monitoring of blood carboxyhaemoglobin levels after exposure to smoke. During exposure to smoke the rate of respiration decreased to approximately 40% of the pre-exposure rate. Tidal volume, after an initial slight decrease, showed a progressive increase throughout the smoke exposure period. Bodyweight gain was reduced in those animals subjected to smoke exposures. True sham-smoked animals showed a body weight gain intermediate between that of smoke and cage control rats. The clear indications of such between-group differences in response to treatment, coupled with the indicators of consistent dosing of animals under the defined exposure conditions, form a sound basis for the interpretation of terminal pathology.	
PMID: 705798 [PubMed - indexed for MEDLINE]	

Factors affecting mutagenic activity of cigarette smoke condensate in Salmonella typhimurium TA 1538	
Mizusaki, S.; Takashima, T.; Tomaru, K.	Mutation Research, Genetic Toxicology Testing (1977), 48(1), 29-36 CODEN: MRGTE4; ISSN: 0165-1218
Smoke condensates from Burley tobacco, bright-type tobacco and various brands of commercial cigarettes were tested for mutagenicity by using a microsomal test system with Salmonella typhimurium TA 1538. Smoke condensate from Burley tobacco had much higher mutagenic activity than that from bright-type tobacco. Increased mutagenic activity was obsd. with smoke condensates from Burley tobacco grown with increasing amts. of nitrogen fertilizer, and from commercial cigarettes blended with Burley tobacco. There was a significant correlation between nitrate content of cigarette and mutagenic activity of the resulting smoke condensate. The results suggest that nitrate in cigarettes may influence the formation of potential mutagens	

during the burning of a cigarette.

Experimental studies on tumorigenic activity of cigarette smoke condensate on mouse skin. Part V. Comparative studies of condensates from different modified cigarettes.	
Dontenwill, W.; Chevalier, H. J.; Harke, H. P.; Klimisch, H. J.; Reckzeh, G.; Fleischmann, B.; Keller, W.	Zeitschrift fuer Krebsforschung und Klinische Onkologie (1976), 85(2),141-53 CODEN: ZKKOBW; ISSN: 0084-5353
The carcinogenicity to mouse skin of smoke condensates from ref. and modified cigarettes was studied. Condensates of smoke from reconstituted tobacco sheets made from a ref. cigarette blend of Virginia and Burley tobaccos and other tobacco mixts. showed decreased carcinogenicity, as did condensates from mixts. ofthe Virginia-Burley blend and 20% or 50% reconsituted tobacco sheets. Condensates from a cigar-type dark tobacco mixt. and the Virginia tobacco from the above mixt. showed no differences in biol. activity compared to the ref. blend, but condensate from the Burley tobacco of the blend showed decreased carcinogenicity. Use of nitrate [14797-55-8] additive (1.8%) produced limited redn. Of carcinogenicity. Condensate from a cigarette comprising 20% reconstituted tobacco sheets, 1.8% nitrate, and a com. used filter showed a marked decrease in carcinogenicity. Application of high doses of condensate did not produce further increases in tumor incidence, possibly because of the cytotoxicity and viscosity of the higher concn. solns.	

Evaluations of cigarets made with mold-damaged and nondamaged flue-cured tobacco	
Welty, Ronald E.; Vickroy, David G.	Beitraege zur Tabakforschung (1975), 8(2), 102-6 CODEN: BETAAY; ISSN: 0005-819X
Flue-cured tobacco damaged by species Aspergillus from com. and exptl. sources was shredded and made into cigarettes. Paired samples of nondamaged tobaccos served as controls. Subsamples of cigarettes were analyzed for viable fungal propagules/g, 39 org. and inorg. compds. in the smoke and smoke condensates, and taste perference. Principal fungi assocd. with leaves and shreds of mold-damaged tobacco were A. repens, A. ruber, and A. niger. Except for the original differences in the nicotine content and in the tar, there were no significant differences in total particulate matter nor in the amt. of specific vapor-phase components measured in the smoke from cigarettes made with mold-damaged and nondamaged tobaccos. Smoke panelists distinguished between cigarettes made with mold-damaged and nondamaged tobaccos and preferred those made with the latter. Viable fungus spores passed through the tobacco cylinder in lighted and nonlighted cigarettes. These data suggest that the use of mold-damaged tobacco in cigarette manufg. is to be avoided because of off-flavors and because the fungi isolated are common allergens to the respiratory tract of humans.	

Mainstream Smoke Chemistry and *In Vitro* and *In Vivo* Toxicity of the Reference Cigarettes 3R4F and 2R4F *

by

Ewald Roemer [1], Heike Schramke [1], Horst Weiler [2], Ansgar Buettner [3], Sandra Kausche [2], Susanne Weber [2], An Berges [4], Markus Stueber [2], Monja Muench [2], Edgar Trelles-Sticken [2], Jan Pype [4], Karola Kohlgrueber [2], Hartmut Voelkel [2], and Sandra Wittke [2]

[1] Philip Morris International Operations, Philip Morris Products S.A., Rue des Usines 90, 2000 Neuchâtel, Switzerland
[2] Philip Morris International R&D, Philip Morris Research Laboratories GmbH, Fuggerstr. 3, 51149 Cologne, Germany
[3] Histovia GmbH, Schoene Aussicht 5, 51491 Overath, Germany
[4] Philip Morris International R&D, Philip Morris Research Laboratories bvba, Grauwmeer 14, 3001 Leuven, Belgium

SUMMARY

A new reference cigarette, the 3R4F, has been developed to replace the depleting supply of the 2R4F cigarette. The present study was designed to compare mainstream smoke chemistry and toxicity of the two reference cigarettes under the International Organization for Standardization (ISO) machine smoking conditions, and to further compare mainstream smoke chemistry and toxicological activity of the 3R4F cigarette by two different smoking regimens, i.e., the machine smoking conditions specified by ISO and the Health Canada intensive (HCI) smoking conditions.
The *in vitro* cytotoxicity and mutagenicity was determined in the neutral red uptake assay, the *Salmonella* reverse mutation assay, and the mouse lymphoma thymidine kinase assay. Additionally, a 90-day nose-only inhalation study in rats was conducted to assess the *in vivo* toxicity. The comparison of smoke chemistry between the two reference cigarettes found practically the same yields of total particulate matter (TPM), 'tar', nicotine, carbon monoxide, and most other smoke constituents. For both cigarettes, the *in vitro* cytotoxicity, mutagenicity, and *in vivo* toxicity showed the expected smoke-related effects compared to controls without smoke exposure. There were no meaningful differences between the 2R4F and 3R4F regarding these toxicological endpoints. The assessments for the 3R4F cigarette by smoking regimen found as a trivial effect, due to the higher amount of smoke generated per cigarette under HCI conditions, an increased yield of toxicant and higher toxicological activity per cigarette. However, per mg TPM, 'tar', or nicotine, the amounts of toxicants and the *in vitro* toxicity were generally lower under HCI conditions, but the *in vivo* activity was not different between the two machine smoking conditions.
Overall, as the main result, the present study suggests equivalent smoke chemistry and *in vitro* and *in vivo* toxicity for the 2R4F and 3R4F reference cigarettes. [Beitr. Tabakforsch. Int. 25 (2012) 316–335]

ZUSAMMENFASSUNG

Eine neue Referenzzigarette 3R4F ist entwickelt worden, die die 2R4F Zigarette ersetzen soll, da deren Vorrat erschöpft ist. In der vorliegenden Studie sollten die Chemie des Hauptstromrauches und die Toxizität beider Referenzzigaretten unter den maschinellen Abrauchbedingungen der Internationalen Organisation für Normung (ISO) verglichen werden. Des Weiteren sollten die Chemie des Hauptstromrauches und dessen Toxizität für die 3R4F Zigarette unter den ISO-Abrauchbedingungen mit denen unter den Bedingungen, wie sie Health Canada (HCI) spezifiziert, verglichen werden.
Die *in vitro*-Zytotoxizität und -Mutagenität wurden im Neutralrot-Aufnahme-Test, dem *Salmonella*-Rückmutations-Test und dem Maus-Lymphoma-Thymidin-Kinase-Test bestimmt. Zusätzlich wurde in einer 90-Tage-Inhalationsstudie an Ratten die *in vivo*-Toxizität

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bestimmt. Der Vergleich beider Referenzzigaretten ergab keine wesentlichen Unterschiede in der Ausbeute an Partikelphase (TPM), Teer, Nikotin, Kohlenmonoxid und den meisten anderen Rauchbestandteilen. Die *in vitro*-Zytotoxizität und -Mutagenität und *in vivo*-Toxizität zeigten, verglichen mit den Kontrollen ohne Rauchexposition, für beide Zigaretten die erwarteten Raucheffekte. Es ergaben sich keine erwähnenswerten Unterschiede zwischen der 2R4F und der 3R4F Zigarette bezüglich dieser toxikologischen Endpunkte. Beim Vergleich der Abrauchbedingungen ergaben sich, bedingt durch die größere Rauchmenge, die unter HCI-Bedingungen generiert wurde, als trivialer Befund auch größere Mengen an toxischen Rauchbestandteilen und eine größere Toxizität pro Zigarette. Hingegen pro mg TPM, Teer oder Nikotin waren die Mengen an toxischen Rauchbestandteilen und die *in vitro*-Toxizität unter den HCI-Bedingungen im Allgemeinen geringer. Die *in vivo*-Toxizität war jedoch nicht unterschiedlich unter beiden Abrauchbedingungen.
Insgesamt, als Hauptergebnis, legt diese Untersuchung nahe, dass die Referenzzigaretten 2R4F und 3R4F bezüglich ihrer Rauchchemie sowie *in vitro*- und *in vivo*-Toxizität als gleichartig zu betrachten sind. [Beitr. Tabakforsch. Int. 25 (2012) 316–335]

RESUME

Une nouvelle cigarette de référence, la 3R4F, a été développée pour remplacer la 2R4F dont le stock s'épuise. La présente étude vise à comparer le courant principal de la fumée produite par ces deux cigarettes en termes de composition chimique et en termes de toxicité. Dans le cas de la 3R4F la fumée a été produite en suivant le protocole de fumage sur machine préconisé par l'Organisation Internationale de Normalisation (ISO) mais aussi suivant le protocole de fumage sur machine plus intense (HCI) spécifié par Santé-Canada. Ceci a permis de comparer la composition chimique et l'activité toxicologique du courant principal de la fumée de la 3R4F obtenu suivant chacun de ces deux régimes de fumage.
La cytotoxicité et la mutagenicité ont été mesurées *in-vitro* suivant le test de fixation du colorant rouge neutre, le test de mutation réverse de salmonelles et l'épreuve sur cellules de lymphome murin à gène TK. En outre, une étude d'inhalation de 90 jours a été effectuée chez le rat, par voies nasales uniquement, pour évaluer la toxicité *in-vivo*. Les deux cigarettes de référence donnent des fumées ayant des rendements équivalents en masse totale des particules (TPM), goudron et monoxyde de carbone, ainsi que la majorité des autres analytes. Les mesures de toxicité *in-vitro*, cytotoxicité et mutagénicité, ainsi que les tests *in-vivo* donnent les résultats attendus pour une exposition à la fumée. Aucune différence persuasive n'est observée entre 2R4F et 3R4F pour ce qui est de ces mesures de toxicité.
Comparé à un fumage selon ISO, dans le cas d'un fumage selon le protocole HCI les rendements en produits toxiques ainsi que l'activité toxique de la fumée de la 3R4F sont plus élevés lorsqu'ils sont calculés par cigarette. Ceci est trivial compte tenu de l'accroissement considérable de la quantité de fumée générée. Toutefois, exprimés par unité de masse (mg TPM, mg goudron ou mg nicotine) les rendements en composés toxiques et la toxicité *in-vitro* sont plus bas que ceux obtenus selon ISO.
Il n'y a pas de différence entre les résultats des mesures de toxicité *in-vivo* obtenus selon les 2 protocoles de fumage sur machine.
En conclusion, la présente étude suggère principalement une équivalence de la 2R4F et de la 3R4F en termes de leurs compositions chimiques et de leurs toxicités *in-vitro* et *in-vivo*. [Beitr. Tabakforsch. Int. 25 (2012) 316–335]

INTRODUCTION

Reference cigarettes play an important role in the identification and assessment of cigarette smoke-related effects. These cigarettes allow the replication and comparison of experiments performed in other laboratories. Such comparisons can be performed by setting the values from reference cigarette data to 100% and expressing the values from other experimental cigarette as a percentage of the reference value. In order to provide reference cigarettes that are easily available for all laboratories, all over the world, working in this field of research, the United States (U.S.) cigarette industry, on request of the Scientific Advisory Board of the Council for Tobacco Research, has provided such cigarettes since 1969. They resemble typical prototypes of certain market segments. Historically, these cigarettes have also provided the basis for both qualitative and quantitative comparisons of different cigarettes types (1), and they will continue to be necessary in the evaluation of future reduced harm products (2).
The University of Kentucky has provided the organizational structure for the design, development and distribution of reference cigarettes (http://www.ca.uky.edu/refcig/). These reference cigarettes differ in their design and characteristics, e.g., with and without filter, blend composition, and smoke delivery. The cigarettes are constructed to represent typical segments of the American market. One of these reference cigarettes is a "full flavor", filtered, American blended cigarette with a total particulate matter (TPM) yield of approximately 11 mg/cigarette under ISO machine smoking conditions, which is currently in its third version. The first version of this reference, called 1R4F was produced in 1983. It was later replaced in 2003 by its successor the 2R4F cigarette, which was chemically characterized and compared to the 1R4F (3, 4). Additional smoke chemistry data for the 2R4F can be found in studies by ADAM *et al.* (5) and INTORP *et al.* (6). The biological activity of its smoke has also been characterized in detail (4, 7–15). In 2008, due to diminishing stock of 2R4F, a replacement was made available. This replacement, the 3R4F cigarette has not yet been compared in the literature to the 2R4F. Evidence that these cigarettes are essentially the same regarding both their smoke chemistry and biological activities would allow for them to be used interchangeably as a comparison basis and would facilitate comparison of a larger number of studies as was the case with the previous versions, 1R4F and 2R4F (3).
In addition to reference cigarettes, the existence of generally accepted machine smoking protocols is essential to allow for the comparison of results from cigarette smoke obtained in different laboratories. Machine smoking

Table 1. Cigarette specifications.

Parameter	Cigarette	
	2R4F	3R4F
Physical data		
Cigarette length (mm)	84.0	84.0
Filter length (mm)	27.0	27.0
Circumference (mm)	24.9	24.5
Cigarette weight (g)	1.06	1.05
Filter ventilation (%)	28.0	29.0
Paper permeability (sec/50 mL)	24.0	24.0
Resistance to draw (cm H_2O)	13.4	12.8
Blend composition (%)		
Flue cured	32.5	35.4
Burley	19.9	21.6
Maryland	1.2	1.4
Oriental	11.1	12.1
Reconstituted (Schweitzer process)	27.1	29.6
Sugar (Isosweet™)	5.3	6.4
Glycerol	2.8	2.7
Filler analysis (%)		
Total alkaloids	2.3	2.1
Reducing sugars	10.7	8.7
Glycerol	2.4	2.4
Yield data from supplier		
Puff count	9.2	9.0
TPM (mg/cig)	11.7	11.0
'Tar' (mg/cig)	9.7	9.4
Nicotine (mg/cig)	0.9	0.7
Carbon monoxide (mg/cig)	13.0	12.0

protocols such as those defined by the International Organization for Standardization (16) and Health Canada (17) have been implemented by various regulatory authorities to provide consumers and regulators with data on cigarette smoke yields. It should be noted that standardized machine smoking protocols that apply either more- or less-intense smoking parameters can provide only one specific combination of possible settings of characteristics, like certain fixed puff volume, puff duration, and puff frequency, and are not meant to mimic human smoking behavior, nor could they be expected to do so, as each smoker smokes differently and as such, there is no typical human smoker (18–20).

Therefore, the objective of the present study was to compare the 3R4F cigarette with its predecessor the 2R4F cigarette in smoke chemistry and biological activity under the ISO machine smoking regimen for their interchangeability/similarity, and to further characterize the 3R4F between two smoking protocols, the ISO and HCI regimens in smoke chemistry and biological activity.

EXPERIMENTAL

Cigarettes and mainstream smoke (MS) generation

The reference cigarettes 3R4F and 2R4F were obtained from the University of Kentucky, Kentucky Tobacco Research and Development Center. Both are American blended filter cigarettes (for further details see Table 1). They were conditioned following ISO standard 3402 (21), i.e., at least 48 hours at target conditions of 22 °C ± 1 °C and a relative humidity of 60% ± 3%. MS was generated under ISO machine smoking conditions following ISO Standard 3308 (16), and under HCI smoking conditions (17). Minor deviations were necessary for technical reasons. The cigarettes were smoked on a 20-port Borgwaldt smoking machine (RM20H, Hamburg, Germany) for the *in vitro* tests, and on 30-port rotary smoking machines (15 ports blocked for HCI protocol) with an active sidestream smoke exhaust (type Philip Morris Research Laboratories (PMRL), SM2000, equipped with a programmable dual-syringe pump (22) for the *in vivo* studies. In short, puff volume, puff duration, and puff frequency for the ISO smoking conditions were 35 mL, 2 s, and 1/min. For the HCI smoking conditions, the respective values were 55 mL, 2 s, and 2/min. Under HCI smoking conditions, all cigarette filter ventilation holes were completely covered by tape.

Mainstream smoke chemistry

MS was generated for both 2R4F and 3R4F cigarettes under the ISO conditions as described above. In addition, the 3R4F was smoked according the HCI specifications.

Analytes in smoke were quantified and compared for both cigarettes according to established methodology (17, 23) as previously described (9). Total particulate matter (TPM) was determined gravimetrically from the smoke trapped on Cambridge glass fiber filters (23) which were also used for sample collection of individual particle phase analytes (see below). Nicotine was determined by gas chromatography (GC) with flame ionization detection from a 2-propanol extract of the TPM filter. Water was determined from the same 2-propanol extract by Karl Fischer titration (24). Carbon monoxide was determined by non-dispersive infra-

red photometry (25). 'Tar' yield was calculated as the TPM yield minus the nicotine and water yields (23). Aldehydes, derivatized with 2,4-dinitrophenylhydrazine and stabilized with pyridine, were determined by high-performance liquid chromatography with ultraviolet (HPLC/UV) detection using water/acetonitrile (9:1) and methanol as solvents (26). Vinyl chloride, 1,3-butadiene, isoprene, benzene, toluene, acrylonitrile, and styrene in the gas phase were trapped in three impingers containing methanol at approx. – 78 °C cooled with 2-propanole and dry ice and analyzed after addition of internal standards by GC using a CP PoraBond Q column (25 m x 0.25 mm, 3 µm) coupled to a mass spectrometer (GC-MS) with electron impact ionization in single ion monitoring mode (27). Styrene and acetamide in TPM were extracted from a glass fiber filter using acetone and analyzed after addition of internal standards by GC using a DB-WAX column (30 m x 0.25 mm, 0.25 µm) coupled to a mass spectrometer (GC/MS) with electron impact ionization in single ion monitoring mode. The analysis of acrylamide after extraction from a glass fiber filter was performed as described (28). Ethylene oxide in the gas phase was trapped in an impinger containing toluene at approx. – 78 °C (cooled with 2-propanole and dry ice) which was connected in series with a glass fiber filter as first trap. After addition of the internal standard propylene oxide-d_6, the toluene solution was analyzed by GC using a CP PoraPlot U column (25 m x 0.25 mm, 8 µm) and hydrogen as carrier gas coupled to a mass spectrometer (GC-MS) with electron impact ionization in single ion monitoring mode (29). 2-nitro-propane was determined from mainstream smoke trapped on a silica cartridge by adding 2-methyl-2-nitro-propane as internal standard, washing the cartridge with pentane and eluting the target analyte using 15% diethyl ether in n-pentane. 2-nitropropane was analyzed by GC-MS/MS in chemical ionization mode using iso-butane as ionization gas, helium as carrier gas and argon as collision gas. Aromatic amines were determined by extracting TPM-filters with dilute hydrochloric acid, followed by back extraction, derivatization, clean-up by solid phase extraction, and analysis by GC with a triple quadrupole mass spectrometer (30). Nitrogen oxides were determined by online gas phase chemiluminescence according to the CORESTA recommended method (31). Hydrogen cyanide was trapped in two impingers with sodium hydroxide solution connected in series. An aliquot was analyzed by headspace GC with nitrogen sensitive detection after acidification of the samples with phosphoric acid. Ammonia was trapped on a glass fiber filter and a wash bottle connected in series. The glass fiber filter was extracted with the content of the wash bottle, derivatized with dansyl chloride, and analyzed by HPLC with a tandem mass spectrometer (HPLC/MS-MS) (32).

Volatile *N*-nitrosamines were collected on a glass fiber filter and in two wash bottles containing a citrate/phosphate buffer solution with ascorbic acid to inhibit artificial generation of *N*-nitrosamines. The glass fiber filter was extracted with citrate/phosphate buffer solution with ascorbic acid and combined with the buffer solution of the wash bottles. The combined buffer solution was three times extracted with dichloromethane and the concentrated chloromethane phase was eluted through an alumina column. After elution with dichloromethane and another concentration step, the extract was analyzed by GC with a thermal energy analyzer. Tobacco-specific *N*-nitrosamines (TSNAs) were analyzed as published (33). TSNAs were extracted with ammonium acetate solution from TPM trapped on a glass fiber filter pad, and analyzed by HPLC/MS-MS. Phenols were extracted from a TPM filter with trichloromethane/acetone after addition of the internal standards phenol-d_6, catechol-d_6 and hydroquinone-d_6. An aliquot of the extract was derivatized with *N,O*-bis-(trimethylsilyl)-trifluoracetamide / 1% trimethyl-chloro-silane and the trimethylsilyl ethers of the phenols were analyzed by GC-MS using electron impact ionization in single ion monitoring mode. Polycyclic aromatic hydrocarbons were extracted from TPM filters with pentane/isooctane (9:1) after addition of the labeled internal standards. The sample clean-up was performed by a 2-step solid phase extraction using aminopropyl cartridges eluted with n-hexane, and octadecyl cartridges eluted with methanol. After concentration of the eluate by solvent evaporation and dissolving in isooctane, the 13 target analytes were determined by GC-MS using electron impact ionization in single ion monitoring mode. Arsenic, cadmium, chromium, nickel, lead, and selenium were trapped in quartz glass tubes using electrostatic precipitation. The condensate was dissolved with dichloro-methane/methanol mixture, and after addition of nitric acid, hydrogen peroxide, and water, the samples were subjected to microwave digestion and analyzed with atomic absorption spectroscopy. In the case of matrix inter-ferences, selenium was reanalyzed with the flow injection analysis system furnace technique. Mercury, after electrostatic precipitation of the particle phase, was trapped in 2 impingers containing potassium permanganate in sulfuric acid. For microwave digestion hydrogen peroxide was added. The digest was made up with water and an aliquot was analyzed with a mercury analyzer.

In vitro toxicity

Assessments of cytotoxicity and mutagenicity were carried out for evaluations of 3R4F and 2R4F cigarettes under ISO machine smoking conditions and the 3R4F reference cigarette was also assessed according to HCI machine smoking conditions. Cytotoxicity of TPM, and the gas vapor phase (GVP) from the 3R4F and 2R4F reference cigarettes, was assessed with the neutral red uptake (NRU) assay with mouse embryo BALB/c 3T3 cells as previously described (8). Briefly, 1.6×10^4 cells were seeded and cultivated in culture medium containing 10% fetal bovine serum (FBS; 100 µL per well). Approximately 24 hours after seeding, the cells were exposed for 24 hours to the smoke fractions, suspended or dissolved in culture medium containing 5% FBS (100 µL per well) resulting in a final concentration of 1.6% dimethyl sulfoxide (DMSO) and 8.4% phosphate buffered saline (PBS), or to solvent control (100 µL per well; culture medium containing 5% FBS, 1.6% DMSO, and 8.4% PBS). Following exposure, cells were incubated for 3 hours in culture medium containing 5% FBS and neutral red dye (100 µL per well). Cells were washed with PBS, and the neutral red dye taken up by viable cells was extracted with a destaining solution

(ethanol/acetic acid; 100 µL per well). The optical density of the neutral red, a measure for the number of viable cells, was determined photometrically at 540 nm. Cytotoxicity assessments were performed in triplicate for each smoke fraction, from both cigarettes, using 8 equidistant smoke fraction concentrations with 2–16 cig/L (TPM) and 3–24 cig/L (GVP) for the reference cigarettes smoked under ISO machine smoking conditions, and 0.7–5.6 (TPM) cig/L and 1–8 cig/L (GVP) for the 3R4F smoked according to the HCI machine smoking conditions.

The Ames *Salmonella typhimurium* reverse mutation assay was performed in general accordance to the Organization for Economic Co-operation and Development, OECD, guideline no. 471 (1997) using the five tester strains TA98, TA100, TA102, TA1535, and TA1537, with and without the S9 fraction, as previously reported (34). Three TPM concentrations per strain were used, ranging from 0.4–2.5 mg TPM/plate depending on the strain. The S9 fraction was purchased from Cytotest Cell Research (CCR, Rossdorf, Germany), and was prepared from the livers of male Sprague-Dawley rats injected with Aroclor 1254. Bacterial mutagenicity was determined for two independent TPM batches of both reference cigarettes. The number of revertants with and without the metabolic activation system was determined for each mutagenicity assay with an automatic colony counter.

The mouse lymphoma assay (MLA) for the mutagenicity of TPM was performed using L5178Y/tk+/--3.7.2C mouse lymphoma cells essentially according to OECD guideline no. 476 (1997) in the microtiter plate version (35) as previously described (10). Cells were obtained from LGC Standards, Germany (in partnership with American Tissue Culture Collection, Manassas, VA, USA). The assays were performed with two independent TPM batches, at three TPM doses with S9 metabolic activation (80, 140/150, 200/220 µg/mL TPM) and without S9 metabolic activation (25, 40, 55 µg/mL TPM). S9 metabolic activation mix was obtained from CCR, Rossdorf, Germany.

Inhalation / in vivo toxicity

Two 90-day nose-only inhalation studies with male and female Sprague-Dawley rats were performed to determine the biological activity of diluted MS. The first study was conducted using an exposure regimen of 6 h/day, 7 days/week at 200 µg/L TPM of the 2R4F or 3R4F cigarette. The second study was conducted at an exposure regimen of 6 h/d, 5 days/week at three increasing concentrations of 100, 150, and 200 µg/L TPM of the 3R4F cigarette and compared between the ISO and HCI machine smoking protocols.

Generally, 10 rats/sex/group were exposed nose-only to MS or to filtered, conditioned fresh air (sham-exposure group). General conditions and animal health, as well as smoke exposure and uptake were monitored. Local effects in the respiratory tract along with systemic effects were investigated after 13 weeks of smoke exposure as previously described (36). Endpoints included all parameters specified in the OECD Guideline for Testing of Chemicals 413 (37) with an extended histopathological assessment of irritation in the respiratory tract. Histopathological changes were scored according to a defined severity scale from 0 to 5. Mean severity scores were calculated based on all rats in a group. Hereby, special histological sections were prepared for the nose according to the method of YOUNG (38), and for the larynx according to LEWIS (39). The trachea was cut frontally (at the bifurcation). One frontal section passing through the main bronchus for the left lung and one frontal section passing through a maximum number of lobes for the right lung were prepared (40). A 42-day post-inhalation period was included for the high-dose smoke exposure and the sham-exposure groups, to assess reversibility, persistence, or delayed occurrence of smoke-exposure effects (data not shown).

Statistical analysis

All tests were conducted without correction for multiple comparisons. The significance level is $\alpha \leq 0.05$, with the exception of smoke chemistry tests which were conducted at the significance levels of 0.05, 0.01, and 0.001. Generally, the mean and the standard error were given as descriptive statistics. Comparisons were made on a per cigarette, a per milligram TPM, a per milligram 'tar', and a per milligram nicotine basis.

For smoke chemistry, comparisons were performed using the t-test. For the NRU assay, the reciprocal EC_{50} ($1/EC_{50}$) was determined separately for each smoke fraction (TPM and GVP) and for each of the three batches. The mean reciprocal EC_{50} values were compared by t-test. The Ames assay data were evaluated based on the slopes of the linear dose-response curves using linear regression analysis with Poisson-weighted data excluding the 0-dose as previously described (8). Analysis of covariance (ANCOVA) was used to compare slopes. MLA assay data was compared using the dose-response curves for the mutant frequency values as calculated by nonlinear regression analysis with the power function $y = a + bx^c$ from which the smoke concentrations were calculated that resulted in a mutagenic response as high as three times the background (spontaneous) mutant frequency (C_{3B}). In addition, non-linear dose-response curves were examined using the sum of square reduction test (comparison 3R4F ISO/HCI only).

In the 90-day rat inhalation studies, MS exposure groups were compared with the sham-exposure group using one-way ANOVA, followed by the Dunnett post-hoc test to confirm exposure effects. Differences in biological activity due to the different smoking protocols for all endpoints were compared on a per milligram TPM basis using two-way ANOVA. Histopathological findings from non-respiratory organs were evaluated with a scoring system from 0 to 5 and analyzed either with the Cochran Mantel Haenszel test for overall and pair-wise comparisons or by ANOVA. Incidences were analyzed with χ^2 statistics.

RESULTS

Cigarette smoke chemistry

In the comparisons of the 3R4F with the 2R4F reference cigarette analyte, yields smoked according to ISO machine smoking conditions, the mean difference over all calculation bases was less than 3% between both cigarettes

Table 2. Relative smoke constituent yields of the 3R4F versus the 2R4F reference cigarette, when smoked according to ISO machine smoking conditions.

Parameter	3R4F to 2R4F ratio (%)			
	Per cigarette	Per mg TPM	Per mg 'tar'	Per mg nicotine
ISO Parameter				
TPM	105**	100	99	101
'Tar'	105***	101	100	101
Nicotine	104*	99	99	100
Water	100	95	95	96
CO	105**	100	99	100
Aldehydes				
Formaldehyde	109	104	103	104
Acetaldehyde	105	100	99	100
Acrolein	104	99	98	99
Propionaldehyde	103	98	98	99
Crotonaldehyde	112*	107	106	108
Aliphatic dienes				
1,3-Butadiene	99	94	94	95
Isoprene	96	92*	91*	92*
Acid derivatives				
Acetamide	102	97	97	98
Acrylamide	87**	83***	83***	84***
Acrylonitrile	100	95	95	96
Epoxides				
Ethylene oxide	97	92	92	93
Nitro compounds				
2-Nitropropane	90	86	85	86
Aromatic amines				
o-Toluidine	101	96	96	97
o-Anisidine	104	99	99	100
2-Naphthylamine	98	93*	93*	94
4-Aminobiphenyl	97	93*	92*	93
Halogen compounds				
Vinyl chloride	87*	83*	82*	83*
Inorganic compounds				
Nitrogen oxides	98	94*	93*	94*
Hydrogen cyanide	102	97	96	97
Ammonia	90	85	85	86
Monocyclic aromatic hydrocarbons				
Benzene	98	94*	93*	94
Toluene	100	95	95	96
Styrene	99	94	93	94
Volatile N-nitrosamines				
NDMA	<LOQ	<LOQ	<LOQ	<LOQ
NMEA	<LOQ	<LOQ	<LOQ	<LOQ
NDEA	<LOQ	<LOQ	<LOQ	<LOQ
NPRA	<LOQ	<LOQ	<LOQ	<LOQ
NBUA	<LOQ	<LOQ	<LOQ	<LOQ
NPY	<LOQ	<LOQ	<LOQ	<LOQ
NPI	<LOQ	<LOQ	<LOQ	<LOQ
Tobacco-specific N-nitrosamines				
NNN	84***	80***	79***	80***
NNK	78***	74***	74***	75***
NAB	103	98	98	99
NAT	106	101	100	101

Table 2. (cont.).

Parameter	3R4F to 2R4F ratio (%)			
	Per cigarette	Per mg TPM	Per mg 'tar'	Per mg nicotine
Phenols				
Phenol	108*	103	102	104
Catechol	102	98	97	98
Hydroquinone	106***	101	101	102
Polycyclic aromatic hydrocarbons				
Pyrene	108*	103	102	103
Benz[*a*]anthracene	105	101	100	101
Benzo[*b*]fluoranthene	113***	107*	107*	108*
Benzo[*j*]fluoranthene	113**	108*	107*	109*
Benzo[*k*]fluoranthene	116**	111*	110*	111*
Benzo[*a*]pyrene	108**	103	103	104*
Dibenz[*a,h*]anthracene	<LOQ	<LOQ	<LOQ	<LOQ
Dibenzo[*a,e*]pyrene	108	103	103	104
Dibenzo[*a,h*]pyrene	<LOQ	<LOQ	<LOQ	<LOQ
Dibenzo[*a,i*]pyrene	<LOQ	<LOQ	<LOQ	<LOQ
Dibenzo[*a,l*]pyrene	<LOQ	<LOQ	<LOQ	<LOQ
Indeno[*1,2,3-cd*]pyrene	113***	107**	107**	108**
5-Methylchrysene	<LOQ	<LOQ	<LOQ	<LOQ
Elements				
Cadmium	99	95	94	95
Chromium	105	100	100	101
Nickel	<LOQ	<LOQ	<LOQ	<LOQ
Lead	93	89***	89***	89**
Arsenic	109***	104*	103*	104*
Selenium	102	98	97	98
Mercury	90**	86	85	86

CO = carbon monoxide; LOQ = at least one value below limit of quantification; NBUA = *N*-nitrosodi-n-butylamine; NDMA = *N*-nitrosodimethylamine; NDEA = *N*-nitroso-n-diethylamine; NMEA = *N*-methylethanolamine; NNN = *N'*-nitrosonornicotine; NNK = 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NPI = *N*-nitrosopiperidine; NPRA = *N*-nitrosodi-n-propylamine; NPY = *N*-nitrosopyrrolidine; TPM = total particulate matter.
Statistical significances: *: 0.01<p ≤0.05, **: 0.001<p ≤0.01, ***: p ≤0.001.

(Table 2; Figure 1; Annex, Table A). The maximum differences were + 16% (benzo[*k*]fluoroanthene, per cigarette) and – 26% (NNK, per mg TPM and 'tar'). Statistically significant lower yields for the 3R4F cigarettes were found for acrylamide, ammonia, vinyl chloride, and the TSNA *N*-nitrosonornicotine (NNN) and 4-(methyl-nitro-samino)-1-(3-pyridyl)-1-butanone (NNK) using all calculation bases (per cigarette, per mg 'tar', TPM or nicotine). Statistically significant higher yields for the 3R4F reference cigarette were found for 4 PAHs and arsenic for all calculation bases. Other statistically significant differences were well within the inherent variability of the analytes yields and not observable under all calculation bases.

Further comparisons of smoke chemistry for the 3R4F reference cigarette were performed under the ISO and the HCI machine smoking conditions. Yield ratios for each calculation basis are presented in Table 3. As a trivial finding, due to the higher amount of smoke produced under the HCI machine smoking conditions, statistically significant higher yields per cigarette were found for all constituents under the HCI compared to the ISO machine smoking conditions ($p \leq 0.001$). However, when expressed per mg TPM, per mg 'tar', and per mg nicotine the yields of TSNAs, phenols, nitrogen oxide, aromatic amines, and benzene, were generally lower under HCI machine smoking conditions, as compared to when using the ISO regimen ($p \leq 0.05$; for all calculation bases). Polycyclic aromatic hydrocarbon levels were lower under the HCI compared to the ISO machine smoking conditions ($p \leq 0.01$) on a per mg TPM basis and on a per mg 'tar' basis

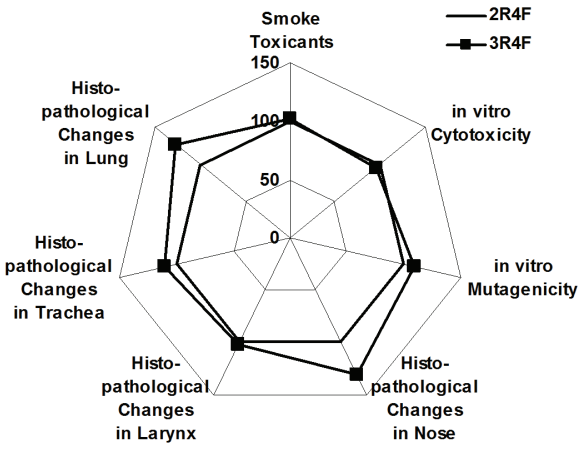

Figure 1. Summary of comparisons of the 3R4F to 2R4F reference cigarettes - means from Tables 2, 4, and 6.

Table 3. Relative smoke constituent yields of the 3R4F reference cigarette when smoked according to ISO and HCI machine smoking conditions.

Parameter	3R4F HCI to 3R4F ISO ratio (%)			
	Per cigarette	Per mg TPM	Per mg 'tar'	Per mg nicotine
ISO Parameter				
TPM	367*** [a]	100	119***	136***
'Tar'	308***	84***	100	114***
Nicotine	271***	74***	88***	100
Water	796***	217***	258***	294***
CO	279***	76***	91***	103
Aldehydes				
Formaldehyde	338***	92***	110	125***
Acetaldehyde	274*** [a]	75***	89**	101
Acrolein	298*** [a]	81***	97	110***
Propionaldehyde	267*** [a]	73***	87***	98
Crotonaldehyde	415***	113*	135***	153***
Aliphatic dienes				
1,3-Butadiene	272***	74**	88	100
Isoprene	280***	76**	91	103
Acid derivatives				
Acetamide	407***	111*	132***	150***
Acrylonitrile	273***	74***	89*	101
Nitro compounds				
2-Nitropropane	244***	67***	79**	90
Aromatic amines				
o-Toluidine	223***	61***	72***	82***
o-Anisidine	231*** [a]	63***	75***	85**
2-Naphthylamine	203***	55***	66***	75***
4-Aminobiphenyl	248***	68***	81**	92
Halogen compounds				
Vinyl chloride	249***	68***	81**	92
Inorganic compounds				
Nitrogen oxides	246***	67***	80***	91**
Hydrogen cyanide	427***	116*	139***	158***
Monocyclic aromatic hydrocarbons				
Benzene	241***	66***	78***	89*
Toluene	257***	70***	83**	95
Styrene	418*** [a]	114*	136**	154***
Volatile N-nitrosamines				
NDMA	<LOQ	<LOQ	<LOQ	<LOQ
NMEA	<LOQ	<LOQ	<LOQ	<LOQ
NDEA	<LOQ	<LOQ	<LOQ	<LOQ
NPRA	<LOQ	<LOQ	<LOQ	<LOQ
NBUA	<LOQ	<LOQ	<LOQ	<LOQ
NPY	<LOQ	<LOQ	<LOQ	<LOQ
NPI	<LOQ	<LOQ	<LOQ	<LOQ
Tobacco-specific N-nitrosamines				
NNN	249***	68***	81***	92**
NNK	268***	73***	87**	99
NAB	231***	63***	75***	85**
NAT	250***	68***	81***	92*
Phenols				
Phenol	203***	55***	66***	75***
Catenol	251***	68***	81***	93**
Hydroquinone	269*** [a]	73***	87***	99
o-Cresol	196***	53***	64***	72***
m-Cresol	196***	53***	64***	72***

Table 3. (cont.).

Parameter	3R4F HCI to 3R4F ISO ratio (%)			
	Per cigarette	Per mg TPM	Per mg 'tar'	Per mg nicotine
Phenols				
p-Cresol	207***	56***	67***	76***
Resorcinol	286***	78***	93*	106
Polycyclic aromatic hydrocarbons				
Pyrene	248***	67***	80**	91
Benz[*a*]anthracene	260***	71***	84***	96
Benzo[*b*]fluoranthene	263***	72***	85**	97
Benzo[*j*]fluoranthene	264*** [a]	72***	86**	97
Benzo[*k*]fluoranthene	262***	71***	85**	97
Benzo[*a*]pyrene	250*** [a]	68***	81***	92*
Dibenz[*a,h*]anthracene	<LOQ	<LOQ	<LOQ	<LOQ
Dibenzo[*a,e*]pyrene	253***	69***	82***	93*
Dibenzo[*a,h*]pyrene	<LOQ	<LOQ	<LOQ	<LOQ
Dibenzo[*a,i*]pyrene	<LOQ	<LOQ	<LOQ	<LOQ
Dibenzo[*a,l*]pyrene	<LOQ	<LOQ	<LOQ	<LOQ
Indeno[1,2,3-*cd*]pyrene	250***	68***	81***	92**
5-Methylchrysene	<LOQ	<LOQ	<LOQ	<LOQ
Elements				
Arsenic	378***	103	123**	139***
Cadmium	<LOQ	<LOQ	<LOQ	<LOQ
Chromium	<LOQ	<LOQ	<LOQ	<LOQ
Nickel	332***	90	108	122*
Lead	314***	85*	102	116

CO = carbon monoxide; LOQ = at least one value below limit of quantification; NAB = *N*-nitrosoanabasine; NAT = *N*-nitrosoanatabine; NBUA = *N*-nitrosodi-n-butylamine; NDMA = *N*-nitrosodimethylamine; NDEA = *N*-nitroso-n-diethylamine; NMEA = *N*-methylethanolamine; NNN = *N*'-nitrosonornicotine; NNK = 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NPI = *N*-nitrosopiperidine; NPRA = *N*-nitrosodi-n-propylamine; NPY = *N*-nitrosopyrrolidine; TPM = total particulate matter.
[a]: assumption of equality of variances not reasonable, Satterthwaites approximation of the t-test used,
Statistical significances: *: $0.01<p \leq 0.05$, **: $0.001<p \leq 0.01$, ***: $p \leq 0.001$.

but similar on a per mg nicotine basis. Yields of benzo-[*a*]pyrene, dibenzo[*a,e*]pyrene, and indeno-[1,2,3-*c,d*]-pyrene were only significantly lower on a per mg nicotine basis ($p \leq 0.05$).

Water, crotonaldehyde, acetamide, hydrogen cyanide and styrene levels were higher for the 3R4F reference cigarette smoked under HCI machine smoking conditions compared to when smoked under ISO conditions ($p \leq 0.05$) regardless of the calculation basis.

In vitro cytotoxicity (NRU assay)

For all samples there was a reproducible dose-dependent decrease in cell viability for both TPM and GVP exposure of both reference cigarettes. There were no statistically significant differences in the cytotoxicity of either the TPM or GVP smoke fractions of the 3R4F cigarette when compared to that of the 2R4F cigarette smoked under the same ISO machine smoking conditions, regardless of the calculation basis (i.e. per cigarette, per unit mass of TPM, 'tar', or nicotine). The mean difference was – 5% and the maximum difference was – 9% for GVP on a per cigarette basis (Table 4).

There were significant differences, however, in the cytotoxicity values of the TPM and GVP smoke fractions from the 3R4F reference cigarette when smoked under HCI machine smoking conditions, as compared to the ISO conditions. The expected statistically significant increase in cytotoxicity per cigarette was + 183% for the TPM and + 198% for the GVP. A statistically significant decrease in cytotoxicity of – 18% was observed for the TPM on a per mg TPM basis. The cytotoxicity of the GVP on the same calculation basis was reduced to the same extent, but the difference was not statistically significant. The other calculation bases did not reveal statistical significances (Table 5).

In vitro bacterial mutagenicity (Ames assay)

Dose-dependent increases in the number of revertants was observed in strains TA98, TA100, and TA1537, with and without S9 metabolic activation, following TPM-exposure from either reference cigarette, compared to solvent control. For these tester strains that, according to the literature (9), have been proven to be responsive to TPM and discriminative, the dose-dependent increases were in most cases statistically significant. There were no statistically significant differences, however, in the observed mutagenicity between the 2R4F and 3R4F reference cigarettes, when smoked under ISO machine smoking conditions, with and without S9 activation, regardless of the calculation basis (Table 4). On a per cigarette basis, a significantly higher mutagenicity of TPM from the 3R4F cigarette (+ 98% to + 263%) when smoked

Table 4. Relative in *vitro* toxicity of the 3R4F versus the 2R4F reference cigarette, when smoked according to ISO machine smoking conditions.

Assay, measure, smoke fraction	3R4F to 2R4F ratio (%)			
	Per cigarette	Per mg TPM	Per mg 'tar'	Per mg nicotine
Cytotoxicity, 1/EC_{50}				
TPM	96	97	98	99
GVP	91	93	93	94
Bacterial mutagenicity, TPM, Revertants per calculation basis				
TA 98, +S9	98	94	94	99
TA 100, +S9	109	104	104	106
TA1537, +S9	122	117	117	119
TA 100, -S9	125	121	119	94
Mammalian cell mutagenicity, TPM, 1/C_{3B}				
+S9	101	109	107	104
-S9	101	107	107	103

Cytotoxicity measured in the neutral red uptake assay. Bacterial mutagenicity in the *Salmonella* reverse mutation assay and mammalian cell mutagenicity in the mouse lymphoma TK assay. Statistical significances: *: $0.01<p \leq 0.05$, **: $0.001<p \leq 0.01$, ***: $p \leq 0.001$.

under HCI machine smoking conditions was observed, as compared to TPM obtained under ISO smoking conditions, reflecting the higher smoke yields produced under the HCI smoking conditions. With metabolic activation all sensitive tester strains showed decreases in the mutagenic activity per mg TPM, 'tar', or nicotine. The decreases, up to –45%, were statistically significant for the calculations based on per mg TPM.

In vitro mammalian mutagenicity (ML assay)

Under ISO machine smoking conditions, dose-dependent increases in TPM mutant frequencies from both the 2R4F and 3R4F reference cigarettes were observed, with and without metabolic activation, compared to solvent control. No significant differences in mutagenicity were observed between the reference cigarettes, regardless of the calculation basis. Comparisons of the TPM mutagenicity from the 3R4F cigarette by both machine smoking regimens found an increase in total activity of approximately + 150% per cigarette, with and without metabolic activation. On a per mg TPM basis, statistically significant decreases in activity were observed with (– 34%) and without (– 23%) metabolic activation. When expressed on a per mg 'tar' basis, the decreases were less distinct. On a per mg nicotine basis, the activities were not different between both machine smoking regimens.

Ninety-day rat inhalation

- *In life* observations and body weights

Following MS-exposure to 2R4F and 3R4F cigarette smoke generated under ISO machine smoking conditions, significant reductions in body weight development in male rats were observed, compared to sham-exposed rats ($P \leq 0.01$). In addition, Harderian gland secretion and wet fur were more frequently observed for MS-exposed groups, compared to the sham-exposed groups. The same magnitude of effects was observed for both cigarettes. There were no other significant differences observed between the smoke-exposed groups and sham-exposure groups. 3R4F reference cigarette mainstream smoke was generated under both the ISO and the HCI machine smoking regimens showed the same effect on body weight reduction, Harderian gland secretion, and the occurrence of wet fur.

- Clinical chemistry, hematology and organ weights

Following MS-exposure to both the 2R4F and 3R4F cigarettes smoked according to ISO machine smoking conditions, only expected alterations in clinical chemistry (41) were observed (e.g., decreased serum concentrations of proteins, triglycerides, cholesterol), hematology (increased hemoglobin) and organ weights (e.g., decreased thymus weight). There were no further consistent, or significant, differences for these parameters between both reference cigarettes (data not shown). Following exposure to MS of the 3R4F reference cigarette under either machine smoking condition, none of the above parameters was affected by the smoking regimen in a meaningful and consistent way (data not shown).

- Histopathology

The histopathological evaluation of the respiratory tract organs revealed qualitatively similar findings for the 2R4F and 3R4F reference cigarettes when smoked under ISO machine smoking conditions that are comparable to expected results following MS exposure reported in the literature (41). Quantitatively, there was no consistent trend (male and female rats, response at various sites) for a difference in toxicity (Table 6), although, numerically, the 3R4F reference cigarette smoke-exposed female rats showed effects that were slightly more pronounced than those in the 2R4F group.

MS exposure to the 3R4F reference cigarette smoked according to ISO and HCI machine smoking conditions did not reveal consistent and meaningful differences. A higher response in the trachea in the male rats exposed to the smoke generated under HCI conditions relative to the ISO groups is due to the fact that at very low incidence rates differences that are small on an absolute basis translate into high relative differences in percent (Table 6, Figure 2).

Table 5. Relative *in vitro* toxicity of the 3R4F reference cigarette when smoked according ISO and HCI machine smoking conditions.

Assay, measure, smoke fraction	3R4F HCI to 3R4F ISO ratio (%)			
	Per cigarette	Per mg TPM	Per mg 'tar'	Per mg nicotine
Cytotoxicity, 1/EC_{50}				
TPM	283***	82**	98	106
GVP	298***	83	102	112
Bacterial mutagenicity, TPM, Revertants per calculation basis				
TA 98, +S9	267***	75***	90	99
TA 100, +S9	258***	72**	86	96
TA1537, +S9	198***	55**	66*	74
TA 100, -S9	363***	99	120	133
Mammalian cell mutagenicity, TPM, 1/C_{3B}				
+S9	242***	66***	79***	90
-S9	276***	77**	92	103

Cytotoxicity measured in the neutral red uptake assay. Bacterial mutagenicity in the *Salmonella* reverse mutation assay and mammalian cell mutagenicity in the mouse lymphoma TK assay. Statistical significances: *: $0.01 < p \leq 0.05$, **: $0.001 < p \leq 0.01$, ***: $p \leq 0.001$.

Comparisons performed on the rats after the post-inhalation period did not show differences between the toxicity of the smoke from the 3R4F and the 2R4F reference cigarettes or between the two smoking regimens (data not shown). With the exception of thymus atrophy, tissues from non-respiratory organs were not significantly affected by the exposure to cigarette smoke.

Nineteen animals showed histo-morphological alterations that are indicative for a rat respiratory virus (RRV) infection. More sham-exposed rats than MS-exposed rats (16 vs. 3, respectively) were affected. The histopathological evaluation of the lung tissue did not appear to be compromised as the infection-related changes can be discriminated from the morphological alterations associated with MS exposure (2). Therefore, the RRV infections were not considered to interfere with the objective of the study.

DISCUSSION

In the evaluation of smoke chemistry under ISO machine smoking conditions, similar results for TPM, 'tar', and carbon monoxide were obtained for the 2R4F and 3R4F reference cigarettes. For some toxicants including the carcinogenic TSNAs and acrylamide, somewhat lower yields were observed for the 3R4F, compared to the 2R4F reference cigarette.

As previously reported from comparisons of the 2R4F and the 1R4F reference cigarette, some variation in smoke constituent yields could be expected due to year-to-year differences in the tobacco crop (3). Overall, however, the objective to produce a new reference cigarette as close as possible to the previous 2R4F reference cigarette was met. Thus, for practical purposes, smoke chemistry data of the 2R4F and the 3R4F are equivalent. Both cigarettes can be used interchangeably as references, although it is recognized that some smoke constituents as, e.g., acrylamide, NNN, and NNK, show statistically significant differences.

The two reference cigarettes also displayed similar *in vitro* cytotoxicity and mutagenicity. In addition, there were no meaningful differences in biomarkers or histopathological changes obtained in the inhalation studies in rats that would suggest any significant differences in toxicity from exposure to the smoke of the 3R4F reference cigarette, as compared to the 2R4F reference cigarette. This also holds true when the 3R4F data are compared to published data for the 2R4F reference cigarette (2, 8, 15). Accordingly, the toxicological profiles of the 2R4F and the 3R4F reference cigarettes are considered to be equivalent.

As expected e.g. (7, 8), due to the significantly higher amount of 3R4F smoke generated under the intense HCI machine smoking conditions, when expressed on a per cigarette basis, toxicants yields, *in vitro* mutagenicity and cytotoxicity, and inhalation toxicity increased compared to when smoked according to ISO machine smoking conditions.

However, as with other cigarettes, comparisons on a per mg TPM, per mg 'tar', or per mg nicotine basis, were reduced under the HCI machine smoking conditions. This observation has been previously reported or can be deduced from data presented in similar investigations of machine smoking protocols for other cigarettes (8, 9, 42, 43). One of the potential explanations for this seemingly counterintuitive observation is based on the increase in the flow of air and thus oxygen that is drawn through the burning zone of the cigarette. Specifically, the higher puff volume that, due to occluded filter ventilation, cannot bypass the burning zone of the cigarette, resulting in higher oxygen supply to the burning zone and thus to higher combustion temperatures when smoked under HCI machine smoking conditions.

It can be hypothesized that higher combustion temperatures obtained under the HCI machine smoking conditions might result in more complete combustion and thus to a lower yield of cytotoxic and mutagenic toxicants. This is consistent with the increase in water in the TPM under intense machine smoking conditions (8).

It is noteworthy that the same authors have demonstrated that these reductions in toxicant yields and cytotoxic and mutagenic activity under intense machine smoking conditions, when normalized to TPM, 'tar', or nicotine, are less apparent between different cigarettes. Differences observable for different cigarettes under ISO machine smoking conditions are also not as apparent when smoked

Table 6. Relative *in vivo* toxicity of the 3R4F versus the 2R4F reference cigarette when smoked according to ISO machine smoking conditions, and the 3R4F reference cigarette when smoked under both the HCI and ISO machine smoking conditions.

Organ, Epithelium, Finding	3R4F to 2R4F ratio (%)		3R4F HCI to 3R4F ISO ratio (%)					
	Male rats	Female rats	Male rats			Female rats		
	200 µg/L	200 µg/L	100 µg/L	150 µg/L	200 µg/L	100 µg/L	150 µg/L	200 µg/L
Nose level 1, respiratory epithelium,								
Reserve cell hyperplasia	100	100	106	100	100	100	100	100
Nasal septum, loss of goblet cells	112	108	98	100	93	100	102	98
Squamous epithelial metaplasia	100	100	111	123	100	118	103	103
Lamina propria, inflammatory cell infiltrates	95	161*	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.
Lumen								
Exudate	125	406**	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.
Nose level 2, respiratory region								
Reserve cell hyperplasia	78	139	76	124	95	77	108	100
Olfactory region								
Atrophy	128	111	30	102	100	171	100	91
Squamous epithelial metaplasia	123	100	n.r.	94	115	600*	96	90
Loss of nerve bundle	96	117	30	116	97	200	102	85
Nose level 3, olfactory epithelium								
Atrophy	129	103	n.r.	81	117	n.r.	125	94
Squamous epithelial metaplasia	138	111	n.r.	77	120	n.r.	130	82
Lamina propria, inflammatory cell infiltration	263*	142	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.
Loss of nerve bundle	150	111	n.r.	87	117	n.r.	126	100
Nose level 4 , olfactory epithelium								
Atrophy	129	107	n.r.	91	105	n.r.	129	93
Squamous epithelial metaplasia	131	100	n.r.	44	105	n.r.	144	85
Larynx, ventral depression								
Squamous epithelial metaplasia	103	100	126	139	152	55	138	109
Floor of larynx								
Squamous epithelial metaplasia	100	100	102	100	100	100	100	100
Cornification	100	88	106	100	100	107	100	100
Vocal cords, lower medial region								
Squamous epithelium hyperplasia	80	100	121	88	104	108	100	100
Cornification	103	100	195	59	143	89	110	100
Vocal cords, upper medial region								
Pseudostratified epithelium hyperplasia	89	147	171	64	117	n.r.	73	65
Cornification	85	147	n.r.	21	67	n.r.	356	156
Vocal folds								
Pseudostratified epithelium Cornification	124	96	197	94	94	100	124	98
Trachea, bifurcation								
Squamous epithelial metaplasia	50	123	n.r.	254	285	<100	n.r.	>100
Lung, alveoli								
Goblet cell hyperplasia	108	212*	93	92	96	211	125	77
Lumen								
Alveolar macrophages	81	105	79	133	100	107	110	93

Values are derived from mean scores for histopathological changes after a 90-day inhalation period. Statistical significances: *:0.01<p ≤0.05, **: 0.001<p ≤0.01, ***: p ≤0.001, n.r.: not recorded.

under more intense machine smoking regimens (44). While notable effects were observed between different machine smoking conditions for smoke chemistry and *in vitro* assessments, there were no such differences noted in the *in vivo* investigations. The results are qualitatively identical to previous results observed in smoke-exposed rats (2, 15, 41, 45, 46). There were also no quantitative differences in toxic effects detected in our study between both machine smoking conditions. Recently, distinct differences have been published regarding the *in vivo* toxicity of cigarette smoke generated under smoking regimens that were rather extreme in intensity (8). It can be argued that the *in vivo* endpoints were different from those reported here. The study published by ROEMER *et al.* deals with dermal

tumorigenicity (skin painting) in mice and not with irritative effects as presented in this publication. Due to their study design (2-stage protocol: mice initiated by dermal application of a carcinogen and subsequent application of cigarette smoke condensate) they put their emphasis of their assessment on the promoting activity (47) of cigarette smoke condensate. However, the promoting, non-genotoxic activity of cigarette smoke has been linked to its irritative capacity (48). As such, one might expect similarities in response between effects in the inhalation toxicity and the 2-stage dermal tumorigenicity study. This apparent discrepancy might be explainable by the fact, that by inhalation exposure both, the particulate as well as the gas phase constituents can exert their action. The mouse skin painting assay, in contrast, investigates only the particulate phase of cigarette smoke. Further research on the possible differences behavior of the gas/vapor phase and the particulate phase under different intensities of smoking regimens might contribute to the understanding of the inherent toxicity of cigarette smoke.

There were two exposure regimens, i.e., seven days per week at 200 µg/L TPM and five days per week at 100, 150, and 200 µg/L, all with a daily exposure duration of six hours. The smoke induced effects were for the same exposure concentration of 200 µg/L quantitatively and even qualitatively approximately the same. The group with 5 days exposure scored, as a mean, 0.6 points lower than the group with 7 days per week. The group with 150 µg/L and 5 days per week scored slightly lower, i.e., 0.8 points. This can be interpreted that the toxicity may be somewhat more dependent on the concentration than on the weekly exposure time, which is in line with results obtained for 21 inhalation toxicants (49) or with cigarette smoke (50). However, both exposure regimens, i.e., 7 days or 5 days per week, are obviously suitable methods to assess the toxicity of cigarette smoke.

Expressing smoke chemistry or toxicity data on a calculation basis other than on a per cigarette basis, i.e., a normalization to account for different yield data, as it is performed here for the comparison of the ISO and HCI machine smoking conditions, always needs to be interpreted cautiously due to nonlinear relationships and possibly unidentified measurement biases. Both complications are especially important for low delivery cigarette data (51). Nevertheless, interpretations of normalized data using all bases of calculation are useful for evaluating human exposure, even for cigarettes with low smoke deliveries, as they allow at least a meaningful estimate of the amount of toxicants or biological activity accompanied with a certain amount of nicotine, 'tar', etc. For the 2R4F and 3R4F reference cigarette data presented here, these precautions do not apply, as both cigarettes have high yields under machine smoking conditions. Standardized machine smoking conditions that apply either more or less intense smoking parameters can provide only one specific combination of possible settings of characteristics, such as fixed puff volume, puff duration, and puff frequency, and are not meant to mimic human smoking behavior, nor could they be expected to do so, as each smoker smokes differently. Accordingly, there is no typical human smoker and no typical human smoking regimen (18–20). However, the results obtained under different machine smoking conditions of different intensity can give insight into the possible spectrum of different smoke qualities and the underlying mechanisms.

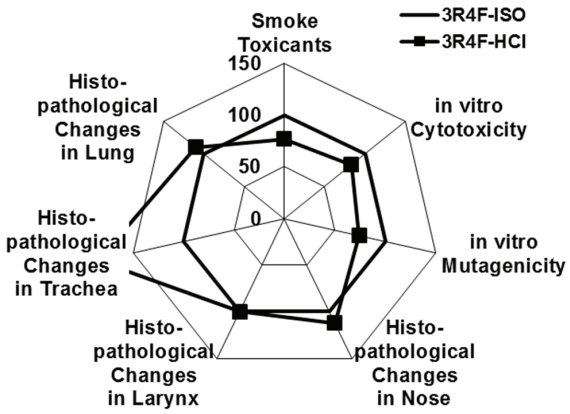

Figure 2. Summary of comparisons of the 3R4F reference cigarettes HCI to ISO machine smoking conditions - means from Tables 3, 5, and 6.

CONCLUSIONS

Overall, the results of the present study suggest equivalent smoke chemistry and toxicity for the 3R4F and 2R4F reference cigarettes when smoked under the same smoking regimen. As observed already for other cigarettes, 3R4F mainstream smoke generated under intense smoking conditions is generally less cytotoxic and mutagenic *in vitro* than the smoke generated under less intense conditions. The *in vivo* inhalation toxicity, however, seems not to be different.

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Corresponding author:

Dr. Ewald Roemer
c/o Philip Morris Intl.
Rue des Usines 90
2000 Neuchâtel
Switzerland
ewald.roemer@pmi.com

APPENDIX

Table A. Smoke constituent yields for the 3R4F and the 2R4F reference cigarettes.

Analyte	Unit per cig	3R4F ISO		2R4F ISO		3R4F HCI	
		M	SE	M	SE	M	SE
ISO Parameter							
TPM	mg	9.77	0.04	9.32	0.08	37.7	0.3
'Tar'	mg	7.98	0.03	7.57	0.05	25.5	0.2
Nicotine	mg	0.707	0.005	0.678	0.007	1.90	0.02
Water	mg	1.08	0.04	1.08	0.02	10.3	0.2
CO	mg	11.2	0.1	10.7	0.1	32.7	0.2
Aldehydes							
Formaldehyde	µg	20.0	0.7	18.4	0.4	68.1	1.4
Acetaldehyde	µg	567	10	542	5	1534	32
Acrolein	µg	56.7	1.4	54.7	0.7	155	3
Propionaldehyde	µg	48.4	0.9	47.0	0.4	124	3
Crotonaldehyde	µg	10.1	0.3	9.00	0.21	43.1	0.9
Aliphatic dienes							
1,3-Butadiene	µg	38.5	1.2	38.9	1.6	76.5	2.1
Isoprene	µg	395	11	411	5	863	27
Acid derivatives							
Acetamide	µg	4.46	0.12	4.37	0.05	15.2	0.2
Acrylamide	µg	1.37	0.02	1.57	0.03	-	-
Acrylonitrile	µg	26.4	0.7	26.5	0.5	67.0	0.8
Epoxides							
Ethylene oxide	µg	9.24	0.18	9.56	0.36	-	-
Nitro compounds							
2-Nitropropane	µg	18.3	0.3	20.4	1.7	35.9	0.6
Aromatic amines							
o-Toluidine	ng	54.1	0.7	53.5	0.7	99.3	1.4
o-Anisidine	ng	2.32	0.04	2.23	0.03	4.25	0.12
2-Naphthylamine	ng	5.69	0.07	5.83	0.12	10.1	0.4
4-Aminobiphenyl	ng	1.01	0.01	1.04	0.02	2.24	0.06
Halogen compounds							
Vinyl chloride	ng	49.7	2.0	57.2	2.1	69.7	2.1
Inorganic compounds							
Nitrogen oxides	µg	265	3	270	5	626	6
Hydrogen cyanide	µg	70.9	1.9	69.8	1.5	319	9
Ammonia	µg	11.1	0.2	12.4	0.2	-	-
Monocyclic aromatic hydrocarbons							
Benzene	µg	45.7	0.9	46.6	0.6	104	1
Toluene	µg	73.6	1.4	73.7	1.0	208	5
Styrene	µg	6.00	0.22	6.09	0.09	24.9	0.9
Volatile N-nitrosamines							
NDMA	ng	<5.00	-	<5.00	-	<10.0	-
NMEA	ng	<10.0	-	<10.0	-	<20.0	-
NDEA	ng	<7.00	-	<7.00	-	<14.0	-
NPRA	ng	<11.0	-	<11.0	-	<22.0	-
NBUA	ng	<9.00	-	<9.00	-	<18.0	-
NPY	ng	<7.00	-	<7.00	-	<14.0	-
NPI	ng	<8.00	-	<8.00	-	<16.0	-
Tobacco-specific N-nitrosamines							
NNN	ng	92.1	1.5	110	2	276	3
NNK	ng	85.5	1.8	110	2	243	6
NAB	ng	9.60	0.46	9.33	0.40	24.0	0.5
NAT	ng	92.9	4.4	87.9	3.6	251	5

Table A. (cont.).

Analyte	Unit per cig	3R4F ISO		2R4F ISO		3R4F HCI	
		M	SE	M	SE	M	SE
Phenols							
Phenol	µg	7.04	0.10	6.52	0.13	14.8	0.3
Catenol	µg	37.1	0.2	36.2	0.3	89.3	0.5
Hydroquinone	µg	29.1	0.1	27.4	0.2	75.7	1.0
Polycyclic aromatic hydrocarbons							
Pyrene	ng	38.0	0.8	35.3	0.2	92.5	1.9
Benz[*a*]anthracene	ng	11.8	0.3	11.2	0.1	29.8	0.6
Benzo[*b*]fluoranthene	ng	5.09	0.08	4.52	0.04	13.2	0.3
Benzo[*j*]fluoranthene	ng	3.24	0.07	2.86	0.04	8.35	0.19
Benzo[*k*]fluoranthene	ng	2.02	0.05	1.74	0.02	5.38	0.11
Benzo[*a*]pyrene	ng	6.73	0.11	6.21	0.07	16.2	0.4
Dibenz[*a,h*]anthracene	ng	<0.970	-	<0.970	-	<2.42	-
Dibenzo[*a,e*]pyrene	ng	0.173	0.005	0.160	0.004	0.858	0.015
Dibenzo[*a,h*]pyrene	ng	<0.230	-	<0.230	-	<0.575	-
Dibenzo[*a,i*]pyrene	ng	<0.220	-	<0.220	-	<0.550	-
Dibenzo[*a,l*]pyrene	ng	<0.190	-	<0.190	-	<0.475	-
Indeno[*1,2,3-cd*]pyrene	ng	2.87	0.04	2.55	0.02	7.37	0.10
5-Methylchrysene	ng	<0.400	-	<0.400	-	<1.00	-
Elements							
Cadmium	ng	38.3	0.3	38.5	1.2	146	3
Chromium	ng	2.48	0.09	2.36	0.12	<6.40	-
Nickel	ng	<2.10	-	<2.10	-	<8.40	-
Lead	ng	9.89	0.13	10.6	0.1	32.2	1.1
Arsenic	ng	2.81	0.01	2.58	0.01	8.62	0.38
Selenium	ng	0.621	0.018	0.606	0.017	-	-
Mercury	ng	2.81	0.1	3.13	0.15	-	-

CO = carbon monoxide; LOQ = at least one value below limit of quantification; NBUA = *N*-nitrosodi-n-butylamine; NDMA = *N*-nitrosodimethylamine; NDEA = *N*-nitroso-n-diethylamine; NMEA = *N*-methylethanolamine; NNN = *N'*-nitrosonornicotine; NNK = 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NPI = *N*-nitrosopiperidine; NPRA = *N*-nitrosodi-n-propylamine; NPY = *N*-nitrosopyrrolidine; TPM = total particulate matter.
The 3R4F reference cigarette values obtained under HCI machine-smoking conditions (3R4F-HCI) were derived in separate studies to the 2R4F (2R4F-ISO) and 3R4F (3R4F-ISO) values obtained under ISO machine smoking conditions. The calculation of 3R4F-HCI/3R4F-ISO ratios may result in slightly different values as presented in Tables 3, 5, and 6 as concurrently determined 3RF4-ISO values (not presented) are used.

Table B. *In vitro* toxicity of the 3R4F and the 2R4F reference cigarettes.

Assay, measure, smoke fraction	Unit	3R4F ISO		2R4F ISO		3R4F HCI	
		M	SE	M	SE	M	SE
Cytotoxicity, 1/EC_{50}	mL/mg TPM						
TPM		10.3	0.2	10.6	0.4	8.6	0.3
GVP		7.8	0.3	8.4	0.3	6.2	0.3
Bacterial mutagenicity, slope, TPM	Revertants/ mg TPM						
TA 98, +S9		2343	85	2507	52	1991	116
TA 100, +S9		1285	72	1232	106	986	74
TA1537, +S9		395	34	337	43	281	24
TA 100, -S9		100	17	83	19	130	25
Mammalian cell mutagenicity, 1/C_{3B}, TPM,	mL/mg TPM						
+S9		6.3	0.3	5.8	0.3	4.1	0.5
-S9		27.7	2.9	26.0	1.8	23.2	1.6

Cytotoxicity measured in the neutral red uptake assay. Bacterial mutagenicity in the *Salmonella* reverse mutation assay and mammalian cell mutagenicity in the mouse lymphoma TK assay. The 3R4F reference cigarette values obtained were obtained under HCI machine smoking conditions (3R4F-HCI). 3R4F (3R4F-ISO) and 2R4F (2R4F-ISO) values obtained under ISO machine smoking conditions. Three replicates per assay in the cytotoxicity and two in the bacterial and mammalian mutagenicity assay. M = mean, SE = standard error.

Table C. *In vivo* toxicity of the 3R4F and the 2R4F reference cigarettes when smoked under ISO machine smoking conditions.

Organ, Epithelium, Finding	3R4F ISO				2R4F ISO			
	Male rats		Female rats		Male rats		Female rats	
	200 µg/L		200 µg/L		200 µg/L		200 µg/L	
	M	SE	M	SE	M	SE	M	SE
Nose level 1								
Respiratory epithelium								
Reserve cell hyperplasia	4.0	0.0	4.0	0.0	4.0	0.0	4.0	0.0
Nasal septum loss of goblet cells	3.8	0.1	4.3	0.2	3.4	0.2	4.0	0.3
Squamous epithelial metaplasia	4.0	0.0	4.0	0.0	4.0	0.0	4.0	0.0
Lamina propria inflammatory cell infiltrates	2.1	0.2	2.9*	0.4	2.2	0.4	1.8	0.2
Lumen								
Exudate	1.5	0.3	2.8	0.6	1.2	0.5	0.6	0.2
Nose level 2								
Respiratory region								
Reserve cell hyperplasia	2.8	0.4	3.2	0.4	3.6	0.4	2.3	0.5
Olfactory region								
Atrophy	4.6	0.3	4.2	0.4	3.6	0.5	3.8	0.5
Squamous metaplasia	3.2	0.5	3.0	0.6	2.6	0.6	3.0	0.5
Loss of nerve bundle	2.2	0.6	2.8	0.6	2.3	0.5	2.4	0.5
Nose level 3								
Olfactory epithelium								
Atrophy	3.6	0.4	3.1	0.6	2.8	0.6	3.0	0.5
Squamous epithelial metaplasia	3.6	0.4	3.1	0.6	2.6	0.6	2.8	0.6
Lamina propria inflammatory cell infiltration	2.1	0.3	1.7	0.4	0.8	0.3	1.2	0.4
Loss of nerve bundle	3.6	0.4	3.1	0.6	2.4	0.7	2.8	0.6
Nose level 4								
Olfactory epithelium								
Atrophy	3.6	0.4	3.1	0.6	2.8	0.6	2.9	0.6
Squamous epithelial metaplasia	3.4	0.4	2.8	0.6	2.6	0.6	2.8	0.6
Larynx								
Ventral depression								
Squamous epithelial metaplasia	3.8	0.6	4.9	0.1	3.7	0.5	4.9	0.1
Cornification	5.0	0.0	2.8	0.7	5.0	0.0	5.0	0.0
Floor of larynx								
Squamous metaplasia	5.0	0.0	5.0	0.0	5.0	0.0	5.0	0.0
Cornification	5.0	0.0	5.0	0.0	5.0	0.0	5.0	0.0
Vocal cords, lower medial region								
Squamous epithelial hyperplasia	1.7	0.2	1.9	0.1	2.1	0.2	1.9	0.1
Cornification	4.0	0.6	5.0	0.0	3.9	0.5	5.0	0.0
Vocal cords, upper medial region								
Pseudostratified epithelial hyperplasia	4.0	0.4	5.0	0.0	4.5	0.2	4.9	0.1
Cornification	2.3	0.5	4.7	0.3	2.7	0.5	3.2	0.4
Vocal folds								
Squamous epithelium								
Cornification	4.2	0.3	4.8	0.1	3.4	0.5	5.0	0.0
Trachea								
Bifurcation								
Squamous epithelial metaplasia	1.1	0.4	1.6	0.5	2.2	0.6	1.2	0.5
Lung								
Bronchioli								
Goblet cell hyperplasia	2.6	0.7	3.6	0.5	2.4	0.7	1.7	0.5
Lumen								
Alveolar macrophages	1.7	0.2	2.2	0.2	2.1	0.1	2.1	0.2
Pigmented macrophages	4.5	0.4	4.7	0.3	4.0	0.5	3.8	0.5

M = mean scores for histopathological changes after a 90-day inhalation period, SE = standard error.

Table D1. *In vivo* toxicity of the 3R4F reference cigarette when smoked under both the ISO and HCI machine smoking conditions, male rats.

Organ, Epithelium, Finding	ISO						HCI					
	100 µg/L		150 µg/L		200 µg/L		100 µg/L		150 µg/L		200 µg/L	
	M	SE	M	SE	M	SE	M	SE	M	SE	M	SE
Nose level 1												
Respiratory epithelium												
Reserve cell hyperplasia	3.5	0.2	4.0	0.0	4.0	0.0	3.7	0.2	4.0	0.0	4.0	0.0
Nasal septum loss of goblet cells	3.2	0.3	3.9	0.2	4.4	0.2	3.1	0.3	3.9	0.1	4.1	1.2
Squamous epithelial metaplasia	2.3	0.3	3.1	0.3	3.7	0.2	2.6	0.4	3.8	0.1	3.7	0.2
Nose level 2												
Respiratory region												
Reserve cell hyperplasia	1.8	0.1	2.5	0.2	3.8	0.1	1.4	0.3	3.1	0.1	3.6	0.2
Olfactory region												
Atrophy	0.4	0.2	2.4	0.6	3.6	0.5	0.1	0.1	2.4	0.6	3.6	0.6
Squamous metaplasia	0.0	0.0	1.3	0.5	2.0	0.5	0.0	0.0	1.2	0.5	2.3	0.5
Loss of nerve bundle	0.4	0.2	2.5	0.7	3.8	0.6	0.1	0.1	2.9	0.7	3.7	0.6
Nose level 3												
Olfactory epithelium												
Atrophy	0.0	0.0	1.6	0.7	2.4	0.7	0.0	0.0	1.3	0.6	2.8	0.6
Squamous epithelial metaplasia	0.0	0.0	1.3	0.6	2.0	0.6	0.0	0.0	1.0	0.5	2.4	0.5
Loss of nerve bundle	0.0	0.0	1.5	0.6	2.4	0.7	0.0	0.0	1.3	0.6	2.8	0.6
Nose level 4												
Olfactory epithelium												
Atrophy	0.0	0.0	1.1	0.5	2.0	0.6	0.0	0.0	1.0	0.4	2.1	0.5
Squamous epithelial metaplasia	0.0	0.0	0.9	0.4	1.9	0.6	0.0	0.0	0.4	0.3	2.0	0.5
Larynx												
Ventral depression												
Squamous epithelial metaplasia	0.9	0.3	1.9	0.5	2.7	0.6	1.1	0.4	2.6	0.7	4.1	0.5
Cornification	0.0	0.0	0.9	0.6	1.5	0.8	0.0	0.0	1.1	0.7	2.7	0.9
Floor of larynx												
Squamous metaplasia	4.9	0.1	5.0	0.0	5.0	0.0	5.0	0.0	5.0	0.0	5.0	0.0
Cornification	4.7	0.3	5.0	0.0	5.0	0.0	5.0	0.0	5.0	0.0	5.0	0.0
Vocal cords, lower medial region												
Squamous epithelial hyperplasia	3.1	0.3	4.0	0.0	3.0	0.3	3.8	0.2	3.5	0.3	4.0	0.0
Cornification	1.2	0.4	3.6	0.6	3.0	0.7	2.4	0.6	2.1	0.7	4.3	0.2
Vocal cords, upper medial region												
Pseudostratified epithelial hyperplasia	1.3	0.5	4.0	0.5	3.5	0.5	2.1	0.5	2.6	0.5	4.1	0.1
Cornification	0.0	0.0	2.7	1.0	1.5	0.9	0.0	0.0	0.6	0.6	1.0	0.6
Vocal folds												
Squamous epithelium												
Cornification	1.22	0.5	3.0	0.5	4.1	0.3	2.4	0.7	2.8	0.8	3.9	0.3
Trachea												
Bifurcation												
Squamous epithelial metaplasia	0.0	0.0	0.1	0.1	0.2	0.1	0.0	0.0	0.3	0.3	0.6	0.3
Lung												
Bronchioli												
Goblet cell hyperplasia	1.2	0.3	2.2	0.5	3.1	0.5	1.1	0.3	2.0	0.4	3.0	0.3
Lumen												
Alveolar macrophages	1.9	0.2	1.8	0.1	2.6	0.3	1.5	0.2	2.4	0.2	2.6	0.3
Pigmented macrophage nests	0.5	0.2	0.5	0.3	1.3	0.4	0.5	0.3	0.9	0.3	1.3	0.4

M = mean scores for histopathological changes after a 90-day inhalation period, SE = standard error.

Table D2. *In vivo* toxicity of the 3R4F reference cigarette when smoked under both the ISO and HCI machine smoking conditions, female rats.

Organ, Epithelium, Finding	ISO						HCI					
	100 µg/L		150 µg/L		200 µg/L		100 µg/L		150 µg/L		200 µg/L	
	M	SE	M	SE	M	SE	M	SE	M	SE	M	SE
Nose level 1												
Respiratory epithelium												
Reserve cell hyperplasia	3.5	0.2	4.0	0.0	4.0	0.0	3.5	0.2	4.0	0.0	4.0	0.0
Nasal septum loss of goblet cells	3.5	0.2	4.1	0.2	4.3	0.2	3.5	0.2	4.2	0.1	4.2	0.1
Squamous epithelial metaplasia	2.2	0.3	3.5	0.2	3.9	0.1	2.6	0.3	3.5	0.3	4.0	0.0
Nose level 2												
Respiratory region												
Reserve cell hyperplasia	1.3	0.2	2.5	0.2	3.2	0.2	1.0	0.2	2.7	0.2	3.2	0.2
Olfactory region												
Atrophy	0.7	0.3	3.6	0.2	4.4	0.2	1.2	0.4	3.6	0.2	4.0	0.5
Squamous metaplasia	0.1	0.1	2.5	0.4	2.9	0.2	0.6	0.4	2.4	0.3	2.6	0.3
Loss of nerve bundle	0.8	0.4	4.1	0.1	4.7	0.2	1.6	0.6	4.2	0.1	4.0	0.5
Nose level 3												
Olfactory epithelium												
Atrophy	0.0	0.0	2.4	0.5	3.6	0.4	0.3	0.2	3.0	0.4	3.4	0.4
Squamous epithelial metaplasia	0.0	0.0	2.0	0.6	3.4	0.4	0.2	0.1	2.6	0.5	2.8	0.4
Loss of nerve bundle	0.0	0.0	2.7	0.6	3.6	0.4	0.2	0.2	3.4	0.4	3.6	0.4
Nose level 4												
Olfactory epithelium												
Atrophy	0.0	0.0	1.4	0.4	2.7	0.3	0.0	0.0	1.8	0.4	2.5	0.5
Squamous epithelial metaplasia	0.0	0.0	0.9	0.4	2.7	0.4	0.0	0.0	1.3	0.5	2.3	0.5
Larynx												
Ventral depression												
Squamous epithelial metaplasia	2.0	0.7	3.3	0.6	3.9	0.6	1.1	0.5	4.6	0.4	4.3	0.5
Cornification	1.3	0.8	2.1	0.8	3.3	0.7	0.1	0.1	4.4	0.6	3.8	0.8
Floor of larynx												
Squamous metaplasia	5.0	0.0	5.0	0.0	5.0	0.0	5.0	0.0	5.0	0.0	5.0	0.0
Cornification	4.7	0.2	5.0	0.0	5.0	0.0	5.0	0.0	5.0	0.0	5.0	0.0
Vocal cords, lower medial region												
Squamous epithelial hyperplasia	3.3	0.3	4.0	0.0	4.0	0.0	3.5	0.2	4.0	0.0	4.0	0.0
Cornification	2.4	0.7	4.1	0.2	4.2	0.3	2.1	0.6	4.5	0.2	4.2	0.3
Vocal cords, upper medial region												
Pseudostratified epithelial												
Hyperplasia	2.1	0.6	4.0	0.2	3.8	0.4	1.0	0.3	4.3	0.4	3.8	0.6
Cornification	0.6	0.6	1.0	0.6	1.6	0.8	0.0	0.0	3.6	0.8	2.5	0.9
Vocal folds												
Squamous epithelium												
Cornification	2.1	0.5	3.9	0.3	4.3	0.4	2.1	0.4	4.8	0.1	4.2	0.3
Trachea												
Bifurcation												
Squamous epithelial metaplasia	0.1	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.2
Lung												
Bronchioli												
Goblet cell hyperplasia	0.6	0.4	2.0	0.2	2.9	0.3	1.3	0.5	2.5	0.4	2.2	0.3
Lumen												
Alveolar macrophages	1.5	0.2	2.1	0.2	3.0	0.2	1.6	0.2	2.3	0.2	2.8	0.2
Pigmented macrophage nests	0.3	0.2	1.4	0.4	2.5	0.3	0.6	0.2	1.5	0.5	1.7	0.4

M = mean scores for histopathological changes after a 90-day inhalation period, SE = standard error.

Arsenic Speciation in Tobacco and Cigarette Smoke *

by Chuan Liu [1], Christopher G. Wright [1], Kevin G. McAdam [1], Sutthinum Taebunpakul [2], Julian Heroult [2], Julian Braybrook [2], and Heidi Goenaga-Infante [2]

[1] *British American Tobacco, GR&D, Regents Park Road, Southampton, SO15 8TL, UK*
[2] *LGC Limited, Queens Road, Teddington, Middlesex, TW11 0LY, UK*

SUMMARY

Arsenic is one of the metals found in cured tobacco and mainstream cigarette smoke. Levels of arsenic in modern filtered cigarette smoke range from sub-ppm to a few tens of ppms. To enable accurate smoke toxicity assessment on arsenic in cigarette smoke, it is desirable to establish its chemical forms in addition to total quantities because different arsenic compounds possess different toxicological potentials.
Progress has been made on measuring the arsenic speciation in tobacco and mainstream cigarette smoke by using a combination of synchrotron-based X-ray absorption spectroscopy and high-performance liquid chromatography-inductively coupled plasma mass spectrometry (HPLC-ICP-MS). In this paper, we describe the experimental procedures developed together with the main findings. A transient redox transformation between As(V) and As(III) was confirmed in freshly generated mainstream smoke. Potential areas for future research are highlighted in order to further our understanding of the speciation mechanism for arsenic in tobacco products. [Beitr. Tabakforsch. Int. 25 (2012) 375–380]

KEY WORDS: cigarette, smoke, synchrotron, arsenic, As(III), As(V), HPLC-ICP-MS, tobacco

RESUME

L'arsenic est l'un des métaux généralement mesurés dans les tabacs séchés à l'air chaud et dans la fumée principale de cigarette. Les concentrations d'arsenic dans la fumée de cigarette à filtration moderne sont comprises entre un niveau inférieur à 1 ppm à quelques dizaines de ppm. Afin de permettre une évaluation précise de la toxicité de la fumée relative à la présence d'arsenic dans la fumée de cigarette, il est souhaitable de déterminer également ses formes chimiques en plus des quantités totales, étant donné que différents composants de l'arsenic possèdent différents potentiels toxicologiques.
Des progrès dans le domaine de la mesure de la spéciation de l'arsenic dans le tabac et dans la fumée principale de cigarette ont été obtenus en utilisant une combinaison de la spectroscopie d'absorption des rayons X par rayonnement synchrotron et la spectrométrie de masse par plasma à couplage inductif couplé avec la chromatographie en phase liquide (HPLC-ICP-MS). Dans cette étude, nous décrivons les procédures expérimentales développées et les conclusions principales établies. Une modification de l'état rédox en conditions transitoires de As(V) à As(III) a été confirmée dans l'aérosol de fumée de cigarette fraîchement émis. Les domaines potentiels pour les recherches futures sont soulignés dans le but d'approfondir notre compréhension des mécanismes de spéciation de l'arsenic dans les produits à base de tabac. [Beitr. Tabakforsch. Int. 25 (2012) 375–380]

ZUSAMMENFASSUNG

Arsen ist eines der Metalle, die routinemäßig in getrocknetem Tabak und im Hauptstromrauch von Zigaretten gemessen werden. Die Arsenkonzentration im Rauch moderner Filterzigaretten liegt zwischen unter 1 ppm und wenigen Dutzend ppm. Für eine exakte Bewertung der Rauchtoxizität in Bezug auf Arsen in Zigarettenrauch ist es wünschenswert, zusätzlich zur Gesamtmenge dessen chemische Formen festzustellen, da verschiedene Arsenverbindungen ein unterschiedliches toxisches Potenzial besitzen. Durch den Einsatz einer Kombination von synchrotronbasierter Röntgenabsorptionsspektroskopie und Hochleistungsflüssigchromatographie - induktiv gekoppelter Plasma-Massenspektrometrie (HPLC-ICP-MS) wurden Fortschritte bei der Messung der Arsenspeziierung in Tabak und im Hauptstromrauch von Zigaretten erreicht. In diesem Artikel werden die entwickelten Versuchsverfahren

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sowie die wichtigsten Erkenntnisse daraus beschrieben. Eine vorübergehende Redoxtransformation von As(V) zu As(III) wurde in frisch erzeugtem Hauptstromrauch-Aerosol bestätigt. Mögliche zukünftige Forschungsgebiete werden aufgezeigt, um unsere Kenntnisse vom Speziierungsmechanismus von Arsen in Tabakerzeugnissen weiter auszubauen. [Beitr. Tabakforsch. Int. 25 (2012) 375–380]

INTRODUCTION

Arsenic is one of the metals (Cr, Cd, Ni, Pb and Se being the rest) among the so-called Hoffmann toxicants in cigarette smoke (1). This work deals with arsenic speciation exclusively. The level of arsenic in mainstream smoke under the ISO smoking condition is ca. 10.4 ng per cigarette for 2R4F Kentucky reference cigarette (2). International Agency for Research on Cancer (IARC) classifies arsenic as Class 1 carcinogen (3). Different forms of arsenic species (either by arsenic valence or by associated anions) are known to have different toxicities (4). For example, the most toxic arsenic species are the inorganic species, i.e., arsenite ($As(III)O_3^{3-}$) and arsenate ($As(V)O_4^{3-}$). In contrast, organic arsenicals in food and plants (arsenobetaine, monomethyl arsenic acids, dimethylarsonic acids, arsenochline and arsenosugars, etc.) have little or no toxicity (4, 5). It is therefore desirable to know both the total arsenic level and the arsenic species in tobacco and any transformation upon pyrolysis and combustion during cigarette smoking for accurate risk assessment.

Based on the knowledge gained from thermal transformation of arsenic species during food cooking (6), the combustion temperature during smoking (from 800 to 950 °C) is sufficiently high to induce thermal transformation of arsenic species presented in the cut leaf. Hence it is important to track and to preserve the native redox environment during the cigarette smoking and trapping processes.

Various options were considered for an effective measurement before choosing synchrotron based X-ray absorption spectroscopy (XANES) as our preferred technique, chiefly for its ability to uncover speciation information at ppm levels *in situ*. The main challenge was to design a metal-free method to trap mainstream smoke and also to preserve the smoke samples during storage and analysis. The preliminary results indicated possible the complex redox behaviour between two dominant arsenic valencies (7), the tri- and penta-valent arsenic during smoke formation. However, the XANES signal intensity was too low to be used to identify the arsenic species within. More recently, we developed a hyphenated HPLC-ICP-MS method that was able to study the arsenic compounds in water-soluble fractions of 3R4F cut tobacco and its mainstream smoke (8). Up to six inorganic and organic arsenic species have been identified. The results obtained from the chemical and physical methods complement each other and confirm the presence of an arsenic redox reaction.

BRIEF EXPERIMENTAL CONSIDERATION

Detailed experimental protocols are published elsewhere (7, 8).

Cigarette & smoke samples

The cigarettes used were Kentucky 3R4F reference cigarettes (University of Kentucky, Kentucky Tobacco Research and Development Centre). Cut tobacco samples were taken from ten 3R4F cigarettes and milled using a titanium coated grinder to fine powder (size distribution not measured). To avoid metal contamination and to preserve species to be detected, commercial Cambridge filter pads (a glass-fibre substrate) were not used. During machine smoking (a RM20 rotary smoking machine and a single-port smoking machine were used under 35 mL puff volume, 2-sec puff duration and once every 60 seconds), the smoke passage before impaction trapping was minimised to reduce dead volume and hence smoke ageing. Smoke particulate matter was trapped onto a metal-free plastic substrate (Kapton Tape from Fisher Scientific) with its surrounding glass chamber immersed in solid CO_2 (−78 °C).

Because this trapping method is different from the room-temperature trapping by Cambridge filter pads as normally carried out, the smoke particulate matter collected may contain a higher percentage of semi-volatile species; however the net effect of this procedure is unknown. The smoke samples were kept in clean glass containers under the solid CO_2 during the storage and transport. The storage period prior to analysis was kept below one week.

X-ray Absorption Near Edge Structure (XANES) analysis

This experiment was conducted at either Station 16.5 (Daresbury Synchrotron Radiation Source, UK) or Beamline X1 (HASYLAB, Hamburg, Germany). Further experimental details and data processing protocols are available elsewhere (7).

HPLC-ICP-MS measurements

These were performed using an Agilent 1200 HPLC system in combination with an Agilent 7500ce ICP-MS for element-specific detection.

Size-exclusion separation of arsenic compounds was carried out on a Supelco TSK gel 3000XL column (250 mm x 4.6 mm id x 6 µm). Anion-exchange HPLC was performed on a Hamilton PRP-X100 column (250 mm × 4.1 mm id × 10 µm). The size-exclusion column was calibrated with a gel filtration mixed standard solution containing albumin (66 kDa), SOD (32 kDa), MT1 (~10 kDa), vitamin B12 (1.35 kDa) and glutathione (307 Da). The HPLC column was connected directly to a 100 µL min^{-1} PFA microflow concentric nebulizer of the ICP-MS *via* PEEK tubing (30 cm × 0.1 mm id).

Extraction of water-soluble arsenic from smoke condensates

5 mL of deionised water were added to each smoke condensate collected on the metal-free plastic substrate to ensure full immersion by water. Extraction was performed by sonication in a water bath for 2 h. Water extracts were separated by decantation and filtered before analysis.

Arsenic speciation by HPLC-ICP-MS

The separation and elution of the arsenic compounds was achieved using 10 mM ammonium acetate (pH 8.5) at 0.5 mL min^{-1}. Arsenic-specific detection of the chromatographic fractions with different hydrodynamic volume was performed using on-line ICP-MS detection. For anion-exchange HPLC, 100 mL of the digest was injected and elution was achieved by using 20 mM ammonium hydrogencarbonate (pH 9.0) in 1% (v/v) methanol at 1.0 mL min^{-1}. Quantification of water-soluble arsenic compounds in the extracts (relative concentrations only) was performed by anion-exchange HPLC coupled to ICP-MS and external calibration with arsenic species standards using the peak area response for ^{75}As.

MAIN EXPERIMENTAL RESULTS

It was relatively straightforward for XANES spectra to reveal the dominant arsenic species as As(V) in both ground 3R4F tobacco and the cigarette ash samples, based on the K-edge position of the As(III) and As(V) standards (7). Grinding and pelleting the cut tobacco and ash samples were useful to improve the signal-to-noise ratio. However, in both cases the signal-to-noise ratio was not sufficient for the extended absorption fine structure spectra to be carried out for speciation detection.

When the smoke particulate matter was analysed by XANES, initial samples collected using Cambridge filter pads showed inconsistent trends in signal intensities, suggesting possible presence of arsenic in the filter material. Therefore, Cambridge filter pads were not used to trap smoke particulate matter for the remaining study. After 20 cigarettes were smoked using a rotary smoking machine under ISO puffing parameters, a visible layer of particulate matter was seen to build-up under the impaction trap. Varying the number of cigarettes smoked (1 to 20) did not significantly affect the shape or the intensity of the subsequent X-ray absorbance detected.

An example of the normalised arsenic XANES spectra from four different smoke particulate matter samples together with those from sodium arsenite and arsenate standards are shown in Figure 1. The XANES spectra from the smoke samples displayed a mixed character of As(III) and As(V). Using standard spectrum modelling approach based on a linear combination of As(III) and As(V), the two synchrotron facilities estimated that relative percentages of the two arsenic valence status (not species) were approximately 50% (Table 1). In this case, the As concentration range in the smoke particulate matter is in the range of a few ppm for 3R4F cigarettes. Without knowing the exact chemical species present, the shape of the XANES spectra of the combined models can only match the experimental arsenic XANES by edge positions. Even with identical species, differences in homogeneity, particle size or degree of crystallinity of the compounds could affect the fine degree of matching. These are one of the inherent limitations of the XANES technique.

Spectra-fitting of Figure 1 shows that the "fresh" particulate matter samples collected under solid CO_2 contained a higher percentage of As(III) than the other two samples

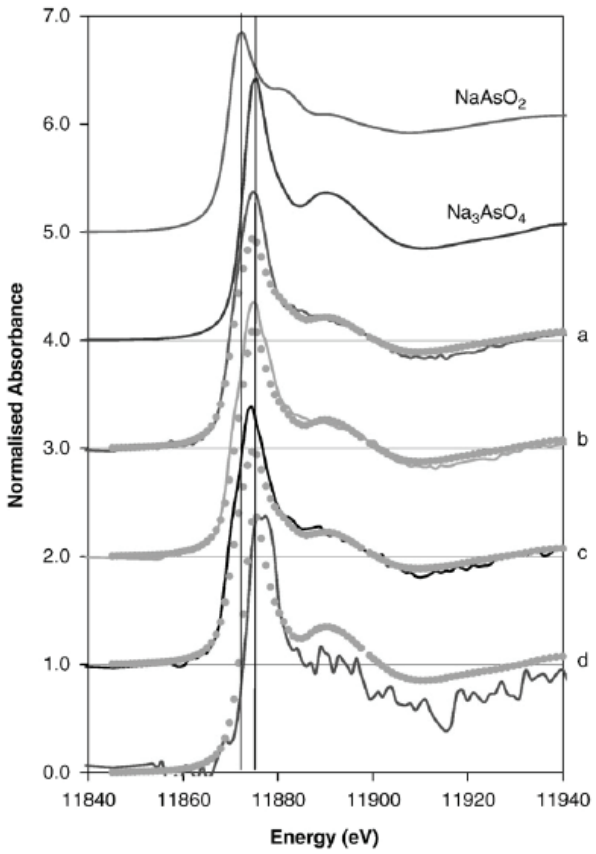

Figure 1. Normalised arsenic XANES spectra from the four different smoke particulate matter samples together with arsenite and arsenate standards. (a) 10 cigarettes on 1 filter (room temperature), (b) 20 cigarettes on 1 filter (solid CO_2), (c) 20 cigarettes on 1 filter (room temp.) and (d) ash pellet.

that were stored at room temperature. In other words, the reducing capacity during the tobacco combustion/pyrolysis converted a significant part of As(V) into As(III), and the cold trapping condition helped to stabilise the As(III) species which would otherwise be oxidised during ageing. Alternatively, when the cold-trapped smoke particulate matter was allowed to return to the room temperature, the As(III) feature was weakened (not shown). The dynamic balance between As(V) and As(III) in "fresh" smoke encouraged us to pursue the subject further, not only because it would be ideal to have an independent experimental verification of the transformation but also to gain some

Table 1. The estimated levels of arsenic species and their distributions in three types of samples from 3R4F cigarettes. The ratios of As(III)/As(V) from the XANES experiments were modelled based on linear combinations of the two As standards: $NaAs(III)O_2$ and $Na_3As(V)O_4$.

Sample	XANES Fitting		
	As(V) %	As(III) %	R* (%)
Cut tobacco	94	6	15
Room-temperature smoke condensate	53	47	—
Cryo-trapped smoke condensate	40	60	28
Cigarette ash	0	100	24

* Uncertainty values given by the modelling software.

insights into the chemical species involved. For these reasons, we pursued the chemical speciation analyses using HPLC-ICP-MS.

First we experimented using Ge as an internal standard for the accurate determination of mono-isotopic arsenic at ng g^{-1} levels in cut tobacco (8). Using this method, the cut tobacco from 3R4F cigarette was found to contain 318 ± 9 ng g^{-1} of arsenic (on a dry weight basis). The recovery of total arsenic from the NIST pine needles (1575) and rice flour (1568a) reference materials (certified reference materials for arsenic speciation analysis) was 102% and 100%, respectively. Various optimisation steps were also carried out to improve the extraction efficiency (8).

Speciation analysis of the water extract was undertaken using anion-exchange HPLC-ICP-MS. The results suggest the presence of arsenate (As(V)) (as a major species) and arsenobetaine, arsenite (As(III)) and DMA (dimethylarsenic acid, as minor species).

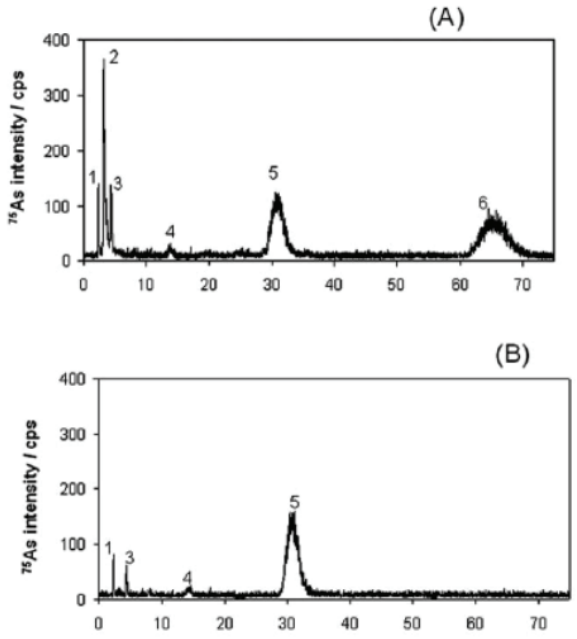

Figure 2. Arsenic speciation in the mainstream smoke particulate matter: anion-exchange HPLC-ICP-MS chromatograms of water extracts stored under (a) dry ice; and (b) room temperature.

1 - Unknown, 2 - Arsenite, 3 - Dimethylarsinic acid (DMA),
4 - Monomethylarsonic acid (MMA), 5 - Arsenate, 6 - Thioarsenate

This result agrees qualitatively with that obtained by XANES. The limits of detection obtained for arsenite and arsenate were found to be 10 and 50 pg g^{-1} As, respectively. The quantitative arsenic speciation data indicated that approximately 89% of the total water-soluble arsenic was present as inorganic arsenic (arsenite + arsenate) with the most abundant inorganic arsenic species being arsenate. The sequential extraction (after water extraction) with driselase and SDS (sodium dodecylsulfate) solutions (an enzyme preparation procedures used to break down plant structure) enabled further extraction of approximately 21% arsenic from the cell wall of the sample. The total arsenic recovery of approximately 64% (of the total arsenic in the solid) was obtained (Table 2). Due to the very low arsenic concentration in the analysed extract and the high matrix complexity, characterisation of the arsenic species detected by HPLC-ICP-MS using organic mass spectrometry was not possible.

The total concentration of water-soluble arsenic extracted from smoke condensates (mass fraction of water-soluble arsenic in smoke condensate) was found to be 0.14 ± 0.03 mg kg^{-1}. The standard deviation (18%) could be attributed to the variability of the non-standard smoke collection process and the fact that only 6 smoke condensates (6 runs of 20 cigarettes per run) were collected. For arsenic speciation in the mainstream smoke particulate matter, anion-exchange HPLC-ICP-MS chromatograms of water extracts stored under two different conditions (room temperature vs. stored at −78 °C) before analysis were compared. Figure 2A shows the profile stored at −78 °C. Retention time matching with the arsenic standards enabled the identification of major arsenite and arsenate, accounting for 51% of the total arsenic in the water extract. Figure 2A also shows the presence of arsenobetaine, DMA (dimethylarsenic acid) and MMA (monomethylarsonic acid) and of a major thioarsenite peak at the retention time of 65 min. Its peak area comprises approximately 41% of the total chromatographic peak area. In Figure 2B, almost all the As(III) species detected in Figure 2A were converted to As(V) for the smoke particulate matter stored at room temperature. This again agrees with the previous XANES results on the smoke condensate. Table 3 summarises the estimated percentages of the arsenic species based on the HPCLC-ICP-MS analysis. The important feature to note is that the peak 2 corresponding to As(III) (Figure 2B) was not seen when the smoke condensate was collected at room-temperature.

Table 2. Fractions of arsenic species in cut 3R4F tobacco.

Total arsenic in tobacco:	318 ng/g	
Water soluble extraction	134 ng/g (42%)	119 ng/g (89% water soluble): mainly inorganic (arsenite + arsenate) – As(V) being the dominant species. The remaining (11%): organic As(V): (DMA, MMA, etc)
Driselase extraction	42 ng/g (13%)	80 ~ 90% to be inorganic (arsenite + arsenate): As(V) being the major species
SDS Extraction	25 ng/g (8%)	~ 80% to be inorganic (arsenite + arsenate): As(V) being the major species

Table 3. Relative concentrations and fractions of arsenic species in mainstream smoke condensate.

Water soluble mainstream smoke condensate	
Cryo-trapped	~ 51%: inorganic (arsenite + arsenate) ~ 41%: unknown As-S species ~ 8%: organic arsenic species
Room-temperature trapped	~ 89%: inorganic (arsenite + arsenate): As(V) being the major species

Many previous publications have shown the dynamic and reactive nature of cigarette smoke (9, 10). The transformation of the arsenic species observed in this work, firstly As(V) → As(III) during the combustion/pyrolysis followed by the reverse As(III) → As(V) upon smoke ageing, adds another aspect to this complex phenomenon.

The reduction of As(V) in the cut tobacco to As(III) in smoke aerosol appears to agree with the fact that the cigarette coal is oxygen-deficient and hydrogen-rich (9, 11). This reduction is however incomplete, as a significant amount of As(V) remains to be found in the smoke particulate (Figure 3). The reducing activity is reported to increase from the 2nd puff to the last puff driven by free radical reactions (11). The "electrochemical potential" for the fresh smoke aerosol particles ranges from approx. +0.24 to +0.17 V(S.C.E., Standard Calomel Electrode), equivalent to approx. 0.00 to −0.07 V(S.H.E., Standard Hydrogen Electrode). Using a "smoke pH" value of a typical blended type of cigarette, around 5.5 to 6.5, it is also thermodynamically feasible for the As(V) /As(III) redox couple to change direction depending on the "electrochemical potential" found in cigarette smoke (12). Post smoking, the "electrochemical potential" of the smoke

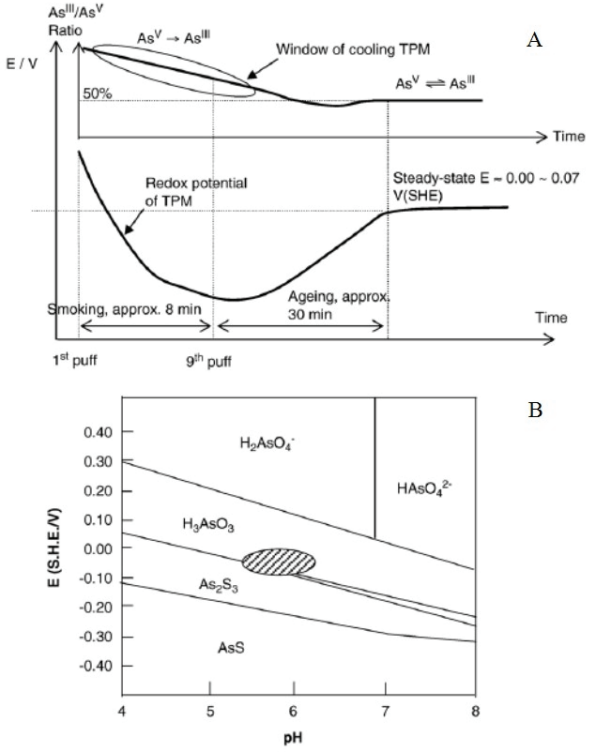

Figure 3. (a) "Electrochemical potential" of mainstream smoke aerosol as a function of puff number; (b) a region of the arsenic Pourbaix diagram.

particulate matter starts to rise gradually, possibly due to self-quenching of the radical species within. This process can be slowed down by storing the smoke sample at lower temperatures.

SUMMARY

The sequential extraction procedure (water, enzymes and surfactants) developed for cut tobacco was efficient but only able to achieve ca. 64% extraction of the total arsenic (approximately 300 ng g^{-1} As). Hence, a more thorough extraction of possibly other arsenic species other than those identified here is needed. Using AE-HPLC-ICP-MS, 89% of the water-soluble arsenic (137 ng g^{-1}) from the mainstream smoke particulate matter was found to be inorganic arsenic: among which approximately 51% was present as arsenite and arsenate, the remaining being thioarsenite species. The two arsenite (As(III)) species were only detected in "fresh" smoke particulate matter that was stored under dry ice and were not stable when the same smoke condensates was stored at room temperature. This As(III) to As(V) redox transformation was detected by both XANES and HPLC-ICP-MS.

The overall scheme of this redox processes appears to agree with the current mechanistic understanding of the combustion/pyrolysis processes responsible for mainstream smoke generation, and also the theoretical thermodynamics of arsenic in an aqueous environment. However, free radical and thermodynamic properties from modern cigarettes such as 3R4F are required to ascertain specific details, which may further enhance our understanding of this complex phenomenon. It would also be interesting to investigate the thermal transformation of endogenous and extragenous arsenic during the smoke formation.

The chemical speciation analyses developed in this work show that synchrotron-based spectroscopy is an appropriate tool to investigate the main redox species involved and any redox reactions during cigarette smoke aerosol formation. The effectiveness of this *in situ* physical detection method however depends on the concentration of the species in the sample materials. For arsenic in 3R4F cigarettes, this has proven to be a challenge. The overall results add a further dimension to the complexity of dynamic changes during cigarette smoke formation.

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Corresponding author:

Chuan Liu
British American Tobacco, GR&D
Regents Park Road
Southampton, SO15 8TL,
United Kingdom
E-mail: chuan_liu@bat.com

Effect of Sugar Content on Acetaldehyde Yield in Cigarette Smoke*

by

Xavier Cahours[1], Thomas Verron[1], and Steve Purkis[2]

[1] SEITA, Imperial Tobacco Group 48, rue Danton, 45404 Fleury-les-Aubrais, France
[2] Imperial Tobacco Limited, PO Box 525, Winterstoke Road, Bristol BS99 1LQ, UK

SUMMARY

The relationship between cigarette blend sugar and acetaldehyde formed in its smoke is a matter of current regulatory interest. This paper provides a re-analysis of data from 83 European commercial cigarettes studied in the 1970s and more modern data on sugar levels and acetaldehyde yields from a series of 97 European commercial cigarettes containing both inherent sugar and in other cases inherent and added sugar. It also provides data from 65 experimental cigarette products made from single curing grades of tobacco, having a wide range of inherent sugar levels but no added sugar.

This study has shown that there is no relationship between acetaldehyde yields and blend sugar content even if a multivariate analysis is carried out taking into account Nicotine Free Dry Particulate Matter (NFDPM) as a co-factor. Such analyses should take into consideration each of the known contributory factors in order to avoid misleading conclusions.

No distinction was found between the mainstream acetaldehyde yields from dark air-cured, flue-cured or US blended style cigarettes irrespective of their sugar content after taking account of differences in NFDPM yields. Similarly, no distinction was found between mainstream acetaldehyde yields of cigarettes made from single grades of either flue-cured, sun-cured or air-cured tobaccos with no sugar added.

This work supports the conclusion that structural material in the tobacco plant is the main source of acetaldehyde in mainstream smoke after combustion during cigarette smoking. [Beitr. Tabakforsch. Int. 25 (2012) 381–395]

ZUSAMMENFASSUNG

Das Verhältnis zwischen dem Zuckergehalt einer Zigarettentabakmischung und dem im Rauch gebildeten Acetaldehyd ist von aktuellem regulatorischem Interesse. Diese Veröffentlichung zeigt eine Reanalyse des Zuckergehaltes und Acetaldehydwerten von 83 europäischen kommerziellen Zigaretten, die in den 1970er Jahren untersucht wurden, sowie aktuelleren Daten einer Serie von 97 europäischen kommerziellen Zigaretten, die zum Teil nur tabakeigenen Zucker und in anderen Fällen tabakeigenen und zugesetzten Zucker enthalten. Zudem präsentiert diese Veröffentlichung Daten von 65 experimentellen Zigaretten, die aus einzelnen Tabaksorten bestehen und unterschiedliche Mengen an tabakeigenem Zucker, aber keinen zugesetzten Zucker, enthalten.

Diese Untersuchung zeigt, dass der Acetaldehydgehalt im Rauch nicht mit dem Zuckergehalt der Tabakmischung in Beziehung steht, auch wenn eine mulitvariate Analyse durchgeführt wird und der Wert des nikotinfreien Trockenkondensats (NFDPM - Nicotine Free Dry Particulate Matter) als Kofaktor berücksichtigt wird. Um irreführende Schlussfolgerungen bei solchen Analysen zu vermeiden, sollte jeder der bekannten, beeinflussenden Faktoren mit berücksichtigt werden.

Nachdem die unterschiedlichen NFDPM-Werte einbezogen wurden, konnte zwischen dem Acetaldehydgehalt im Hauptstromrauch der unterschiedlichen Zigaretten (Zigaretten aus dunklem luftgetrockneten Tabak, Zigaretten aus ofengetrocknetem Tabak oder US-American-Blend-Zigaretten) kein Unterschied festgestellt werden, gleichgültig wie viel Zucker sie enthalten. Genauso wird kein Unterschied zwischen dem Acetaldehydgehalt im Hauptstromrauch von Zigaretten festgestellt, die jeweils aus einzelnen Tabaksorten bestehen und denen kein Zucker zugesetzt wurde.

Diese Arbeit unterstützt die Schlussfolgerung, dass die Hauptquelle für den Acetaldehydgehalt im Hauptstromrauch, der bei der Verbrennung des Tabaks während des Rauchens einer Zigarette entsteht, das strukturelle Material der Tabakpflanze selber ist. [Beitr. Tabakforsch. Int. 25 (2012) 381–395]

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RESUME

La relation entre le sucre contenu dans les mélanges de tabac de cigarette et l'acétaldéhyde formé dans sa fumée a fait l'objet d'un intérêt constant de la part des autorités de régulation au cours des dernières décennies. Le présent document propose une nouvelle analyse des données de 83 marques de cigarettes européennes commercialisées, étudiées dans les années 1970, ainsi que de données plus récentes relatives aux concentrations en sucre et aux teneurs en acétaldéhyde pour un groupe de 97 marques de cigarettes européennes commercialisées contenant soit du sucre naturel soit du sucre naturel et du sucre ajouté. Cette étude fournit également des données concernant 65 produits de cigarette expérimentaux fabriqués à partir d'une seule qualité de séchage de tabac, contenant un large éventail de concentrations de sucre naturel, mais pas de sucre ajouté.
Cette étude a montré qu'il n'y a pas de relation entre les teneurs en acétaldéhyde et les concentrations en sucre contenu dans le mélange de tabac, même si une analyse multivariée a été menée en tenant compte de la matière particulaire anhydre et exempte de nicotine (MPAEN) en tant que co-facteur. De telles analyses devraient prendre en considération chacun des facteurs connus intervenant afin de prévenir toute conclusion erronée.
Aucune distinction n'a été détectée entre les teneurs en acétaldéhyde dans la fumée principale de cigarette des tabacs bruns séchés à l'air naturel, des tabacs séchés à l'air chaud ou des cigarettes de type " American Blend ", peu importe leur concentration en sucre respective, après avoir pris en considération les différences au niveau de teneur en MPAEN. De la même manière, aucune distinction n'a pu être établie entre les teneurs en acétaldéhyde présentes dans la fumée principale de cigarette pour des cigarettes fabriquées à partir d'une seule qualité de tabac, soit séché à l'air chaud, soit séché au soleil, soit séché à l'air naturel, sans sucre ajouté.
Le présent travail appuie les conclusions selon lesquelles de la matière structurelle dans la plante de tabac est la principale source de l'acétaldéhyde présent dans la fumée principale de cigarette après la combustion durant le fumage de la cigarette. [Beitr. Tabakforsch. Int. 25 (2012) 381–395]

INTRODUCTION

Acetaldehyde is the most abundant component in the vapour phase of cigarette mainstream smoke after oxygen, nitrogen, water, carbon monoxide and carbon dioxide. In isolation, it has been classified as an animal carcinogen (1) and may be cytotoxic or genotoxic (2).
A review of the scientific literature concluded that natural tobacco polysaccharides, such as cellulose, when combusted during smoking, are likely to be the main precursors of acetaldehyde in cigarette mainstream smoke (3).
Soluble sugars such as fructose, glucose and sucrose are natural components of tobacco (4). Sugars in tobacco are formed via enzymatic hydrolysis of starch during the period after priming and the early stages of the curing process. The sugar content of tobacco types is highly variable, but primarily depends on the method of curing. Soluble sugars are frequently added to the tobacco blend in the form of casings, typically to air-cured leaf components that have extremely low sugar contents due to losses occurring during curing. US blended style cigarettes contain blends of air-, sun- and flue-cured tobaccos, with an overall sugar content, inherent and added, that is generally similar or lower than flue-cured Virginia style cigarettes and can be evidenced by the data provided in this study. Both reducing (e.g., fructose and glucose) and non-reducing (e.g., sucrose) sugars contribute to tobacco smoke flavour and act as humectants in many tobacco products.
The relationship between cigarette blend sugar and acetaldehyde formed in its smoke is a matter of current regulatory interest (5–7). In a report requested by the European Commission, the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) concluded that acetaldehyde, given intravenously, is addictive and enhances the addictiveness of nicotine in experimental animals. They reported a hypothesis, that there is a possible action of combustion products of sugars, such as acetaldehyde and similar compounds, which enhances the addictiveness of nicotine probably by their interaction with other smoke components to form compounds such as harmans that inhibit monoamine oxidase. However, this was unsubstantiated with scientific evidence. Contrary to this hypothesis, they also reported that heavy smokers absorb only minor amounts of acetaldehyde in the blood and harman compounds are formed at very low levels in smoke.
Acetaldehyde and other aliphatic aldehydes, such as formaldehyde and acrolein, can be generated by the pyrolysis of sugar (5). Other workers (8) have reported that pyrolysis of simple sugars favours the formation of furan compounds whereas pyrolysis of cellulose favours the formation of anhydrosugars and small molecules such as acetaldehyde. Similar observations were made from data generated in our own laboratories in unpublished work. However, products formed during pyrolysis experiments either on sugars alone or even when mixed with tobacco depend on the pyrolysis conditions and generally pyrolysis does not well predict ingredient fate during combustion in a cigarette during smoking (9).
In a recent paper, the addition of sucrose at 1.5% to 4.8% to the tobacco blend of test cigarettes did not lead to any significant increases in acetaldehyde yields in smoke (7). This supports the large body of tobacco industry literature (10–14) that has demonstrated that cigarettes with or without added sugar, or indeed other additives, produce similar acetaldehyde concentrations in relation to Nicotine Free Dry Particulate Matter (NFDPM) during smoking. It may be said that, if anything, additives tend to reduce such smoke yields by decreasing the relative amount of tobacco in the blend by replacement with the additive (15). These findings concur with our own data both published (13) and unpublished at commercial sugar usage levels.
In 1975, PHILLPOTTS *et al.* (16) reported no correlation between the total aldehyde yields in cigarette mainstream smoke and tobacco blend sugar contents of 83 commercial European brands. Some authors (17) have recently re-interpreted the PHILLPOTTS data differently. They suggested applying a multivariate analysis taking into consideration NFDPM yields that gave a significant sugar-aldehyde relationship, with an R^2 correlation equal to

Table 1. Data on cigarettes made from individual tobacco grades.

Curing type [a]	Country of origin	Total sugar [b] %	Acetaldehyde yield (µg/cig)	NFDPM yield [c] (mg/cig)	Acetaldehyde / NFDPM ratio (x 1000)
Sun	China	4.4	538.9	10.7	50.3
Sun	China	12.9	658.3	12.5	52.7
Sun	Greece	3.6	592.8	13.1	45.2
Sun	Greece	6.9	771.0	18.5	41.7
Sun	Greece	12.0	697.5	18.4	38.0
Sun	Lebanon	7.6	634.7	19.6	32.4
Sun	Lebanon	12.3	683.0	18.4	37.1
Sun	Macedonia	8.9	689.8	19.0	36.3
Sun	Macedonia	9.6	676.2	18.0	37.7
Sun	Syria	5.8	591.2	19.5	30.3
Sun	Turkey	15.0	693.8	17.0	40.7
Sun	Turkey	4.0	588.5	16.3	36.1
Sun	Turkey	9.0	547.9	14.1	38.8
Sun	Turkey	15.3	672.6	18.8	35.8
Sun	Turkey	12.9	622.5	18.1	34.4
Sun	Bulgaria	12.2	458.7	20.5	22.3
Air	Argentina	0.0	574.2	11.1	51.8
Air	Brazil	0.0	567.6	12.5	45.6
Air	China	0.0	658.4	10.6	62.3
Air	China	0.0	612.1	12.4	49.4
Air	China	0.0	662.5	14.9	44.6
Air	China	0.1	477.3	7.1	67.6
Air	China	0.0	507.8	12.8	39.8
Air	France	0.0	567.0	9.3	61.0
Air	France	0.3	630.4	7.4	85.5
Air	France	0.0	493.8	10.9	45.3
Air	France	0.0	601.0	12.1	49.6
Air	France	0.0	506.9	7.1	71.3
Air	India	0.0	574.9	11.4	50.4
Air	India	0.0	483.9	12.5	38.7
Air	India	0.0	577.4	15.2	38.1
Air	Italy	0.2	432.3	9.9	43.6
Air	Italy	0.0	606.0	10.5	57.9
Air	Korea	0.0	532.0	12.2	43.5
Air	Malawi	0.0	633.0	11.4	55.5
Air	Malawi	0.0	630.4	10.4	60.9
Air	Malawi	0.0	578.4	15.3	37.8
Air	Malawi	0.0	589.4	10.4	56.9
Air	Thailand	0.0	501.0	12.6	39.9
Air	Thailand	0.0	621.7	17.2	36.3
Air	Thailand	0.0	470.8	17.4	27.0
Air	Thailand	0.0	537.8	7.8	69.4
Air	USA	0.0	668.8	12.6	53.1
Air	USA	0.0	644.6	9.7	66.4
Air	Zimbabwe	0.0	670.9	9.7	69.4
Flue	Argentina	8.8	596.9	12.8	46.5
Flue	Brazil	1.3	609.3	16.2	37.6
Flue	Brazil	7.0	671.2	17.2	38.9
Flue	Brazil	2.3	548.4	8.1	67.5
Flue	China	14.3	593.0	18.2	32.6
Flue	China	7.7	613.6	19.7	31.2
Flue	China	17.1	665.5	18.5	36.0
Flue	Spain	9.1	578.7	21.2	27.4
Flue	China	10.3	551.2	9.2	60.0
Flue	France	23.7	609.6	12.8	47.5
Flue	France	23.1	683.9	13.6	50.4
Flue	France	9.8	497.8	8.9	55.8

Table 1. (cont.).

Curing type [a]	Country of origin	Total sugar [b] %	Acetaldehyde yield (µg/cig)	NFDPM yield [c] (mg/cig)	Acetaldehyde / NFDPM ratio (x 1000)
Flue	India	2.5	619.9	10.6	58.7
Flue	India	13.1	710.5	13.7	51.8
Flue	India	2.0	586.1	22.3	26.2
Flue	India	7.1	706.0	20.6	34.3
Flue	India	1.3	659.1	11.6	56.7
Flue	Kenya	9.9	635.2	12.1	52.6
Flue	Tanzania	6.1	618.5	11.9	52.1
Flue	Zimbabwe	12.0	547.6	12.2	45.0

[a] Sun = sun-cured tobaccos; air = air-cured tobaccos; flue = flue-cured tobaccos

[b] Total sugar = (fructose + glucose + sucrose)

[c] NFDPM = Nicotine Free Dry Particulate Matter

34%, and they concluded that normalising for NFDPM may obscure a sugar-aldehyde relationship. This current paper describes some further analysis to examine the claims made by these authors.

This paper also provides more up-to-date data on sugar levels and acetaldehyde yields from a series of commercial products and from cigarettes made from single curing grades of tobacco, having a wide range of inherent sugar levels but no added sugar. Similar multivariate analyses were also undertaken on data from these cigarettes to investigate relationships between these blend sugars and smoke acetaldehyde yields.

EXPERIMENTAL

Analyses carried out by PHILLPOTTS et al. in 1975

Blend sugars levels and total aldehyde yields in cigarette smoke were measured by colorimetric methods. PHILLPOTTS *et al.* briefly described how total sugars were estimated by hydrolysis of tobacco with mineral acid at 95 °C to give the reducing sugars. These were dialysed then oxidised with alkaline potassium ferricyanide. The colour loss was then measured. Total volatile aldehydes were determined by a procedure based on the Tobacco Research Council (18) standard method after smoking according to the puffing parameters later set out in the ISO 3308 standard (19). Whole smoke was collected at −80 °C and extracted with ethanol.

Aldehydes were reacted with 3-methyl-2-benzothiazolone hydrazone hydrochloride and ferric chloride to give a blue colour. Reaction and colour measurements for both blend sugars and total volatile aldehydes smoke were performed in a Technicon AutoAnalyser.

Preparation of experimental cigarettes

Experimental cigarettes were made from single tobacco grades, having a wide range of inherent sugar levels but having no added sugar. The experimental cigarettes were constructed using product design characteristics similar to brands of commercial cigarettes.

Each of the cigarettes were cellulose acetate filtered cigarettes (cigarette length: 84 mm and filter length: 21 mm) and with a filter ventilation level at 15%. The cigarettes were made to the same pressure drop (50 mm WG (water gauge)). To keep this constant, target tobacco weights were adjusted according to the tobacco density.

Commercially available cigarettes

Five data sets were obtained in 5 different years (2001, 2005, 2006, 2008, and 2010) on 97 commercial brands from the EU market: 12 from Eastern Europe (Poland, Hungary, Ukraine, and Russia); 32 from France; 14 from Germany; 8 from Spain, and 31 from UK. These products reflected the different design features used in products made by the Imperial Tobacco Group (ITG). These products also include some major brands made by other manufacturers. It can be noted that the 2001 products were identical to those studied in the UK smoke constituent study (20).

Analyses

In this work, glucose, fructose, and sucrose were extracted from cigarette filler with water and determined using a continuous flow procedure. This analysis was divided into three steps.

(1) The amount of glucose was determined. Glucose was phosphorylated by adenosine triphosphate (ATP) in a reaction catalysed by hexokinase. Glucose-6-phosphate (G6P) was then oxidised to 6-phosphogluconate in the presence of nicotinamide adenine dinucleotide (NAD) with catalysis by glucose-6-phosphate dehydrogenase (G6PDH). During this oxidation, an equimolar amount of NAD was reduced to NADH. The consequent increase in absorbance at 340 nm was directly proportional to the glucose concentration.

(2) Fructose was also phosphorylated by ATP and converted to glucose 6-phosphate by phosphoglucose isomerase (PGI). This step determined the sum of glucose and fructose concentrations.

(3) Sucrose was hydrolysed using β-fructosidase. Glucose and fructose resulting from this degradation were analysed as described above. This last step determined the sum of glucose, fructose and sucrose concentrations. By subtraction of results obtained in the different steps, the

Table 2. Data on commercial cigarette brands.

Country of sale [a]	Brand Name	Year	Blend type [b]	Total Sugar [c] %	Acetaldehyde Yield	NFDPM Yield [f]
F	Benson & Hedges [d]	2006	US	5.3	413.0	8.3
UK	Benson & Hedges KS [d]	2001	UK	8.5	720.0	9.9
UK	Berkeley Superkings [d]	2001	UK	7.7	705.0	9.9
SP	BN Clásico	2010	Dark	0.3	420.0	6.5
UK	Camel Ultra Lights [d e]	2001	US	8.0	167.0	2.7
UK	Consul Menthol [d]	2001	UK	8.7	514.0	7.3
G	Davidoff Magnum	2005	UK	12.4	513.0	11.1
SP	Ducados Azul	2010	Dark	0.3	480.3	9.2
F	Dunhill [d]	2006	UK	7.5	517.0	9.6
F	Fine 120 Menthol	2010	UK	6.2	547.5	9.2
F	Fine Bleu Super Slims	2010	UK	6.8	358.1	7.5
EE	Fine King Size Filter	2006	US	10.7	867.0	12.2
F	Fortuna Rouge	2006	US	9.2	548.0	9.9
SP	Fortuna Azul	2010	US	6.4	458.3	7.5
G	Fortuna Red	2010	US	5.9	594.6	9.7
SP	Fortuna Rojo	2010	US	6.9	588.6	10.0
SP	Fortuna Silver	2010	US	6.0	306.5	4.1
P	Fox	2010	US	3.0	587.9	10.9
F	Gauloise Blonde Bleu	2006	US	9.1	590.0	10.4
F	Gauloise Blonde Blanc Bleu	2006	US	9.5	292.0	4.7
F	Gauloise Blonde Rouge	2006	US	8.8	412.0	6.8
F	Gauloise Blonde Jaune	2006	US	9.5	251.0	2.4
F	Gauloise Blonde Jaune International	2010	US	7.1	273.4	3.7
F	Gauloise Blonde Bleu International	2010	US	5.9	716.1	10.2
F	Gauloise	2010	Dark	0.3	591.4	10.7
F	Gauloise Brunes	2006	Dark	0.9	585.0	9.9
F	Gauloise Selection Red	2010	US	6.5	431.4	6.2
UK	Gitanes	2001	Dark	1.6	687.0	11.9
F	Gitanes Blondes Blanc Bleu	2010	US	6.2	493.7	7.3
F	Gitanes Filter	2010	Dark	0.3	641.5	8.5
F	Gitanes Maïs	2010	Dark	0.3	664.3	8.6
F	JPS	2006	US	9.0	535.0	9.5
UK	L&B Gold	2008	UK	10.1	339.2	5.0
UK	L&B KS	2008	UK	11.6	598.8	9.9
UK	L&B KS	2001	UK	8.8	758.0	12.0
UK	L&B Lights KS [e]	2001	UK	7.9	463.0	5.9
UK	L&B Ultra Lights [e]	2001	UK	8.6	108.0	1.8
UK	L&B White	2008	UK	10.0	89.8	1.9
F	Lucky Strike Red [d]	2006	US	6.0	486.0	10.0
F	Lucky Strike Silver [d]	2006	UK	6.0	366.0	6.8
F	Marlboro [d]	2006	US	9.4	527.0	9.5
UK	Marlboro KS [d]	2001	US	9.5	716.0	12.4
UK	Marlboro Light [d e]	2001	US	9.7	402.0	5.7
G	Marlboro Gold [d]	2006	US	9.5	466.0	7.8
G	Marlboro Blend 29 [d]	2006	US	8.8	425.0	7.1
F	Marlboro Menthol White [d]	2006	US	9.4	452.0	7.4
UK	Mayfair Lights [d e]	2001	UK	7.5	520.0	7.4
UK	Mayfair Menthol [d]	2001	UK	6.5	349.0	8.4
F	MonteCristo	2006	US	7.4	590.0	10.1
SP	Nobel Triple Filter	2010	US	5.3	340.1	5.9
F	Peter Stuyvesant Bleu	2006	US	7.4	232.0	4.2
F	Peter Stuyvesant Silver	2006	US	5.3	59.0	1.3
F	Peter Stuyvesant Menthol	2006	US	9.4	529.0	9.7
F	Philip Morris Crème [d]	2006	US	9.5	449.0	7.3
F	Philip Morris One [d]	2006	US	8.9	75.0	1.3
SP	Popularne	2010	US	4.6	521.0	11.2
EE	Prima Lux Red	2005	US	7.1	510.1	10.8
EE	Prima Lux Red	2008	US	7.3	511.6	10.8

Table 2. (cont.).

Country of Sale [a]	Brand Name	Year	Blend type [b]	Total Sugar [c] %	Acetaldehyde Yield	NFDPM Yield [f]
EE	Prima Lux Blue	2008	US	7.2	333.6	7.2
EE	Prima Lux Menthol	2008	US	7.3	388.3	7.2
EE	Prima Lux Zolota	2008	US	7.6	283.4	3.9
EE	Prima Oval	2008	US	5.7	563.8	13
EE	Red & White	2006	US	8.0	544.0	9.8
G	R1 Blue	2008	US	7.5	42.0	1.0
G	R1 Light Flavour	2005	US	7.8	90.9	2.0
G	R1 Red	2008	US	7.5	87.1	2.0
G	R1 Red	2010	US	5.7	123.8	1.8
UK	Red Band Lights [e]	2001	UK	9.2	351.0	6.1
UK	Regal Filter	2001	UK	8.2	631.0	11.6
UK	Regal KS	2001	UK	9.7	872.0	12.6
G	Roth Haendle	2005	Dark	2.5	326.1	10.5
G	Roth Haendle	2008	Dark	2.6	325.8	10.6
UK	Roth Red	2001	UK	8.9	661.0	10.1
UK	Roth Red 120s	2001	UK	9.8	567.0	9.3
F	Royale Anise	2010	US	11.5	284.3	4.8
F	Royale Menthol	2006	US	6.3	526.0	10.2
F	Royale Menthol	2010	US	7.0	581.5	9.6
F	Royale Silver	2006	US	4.3	144.0	1.2
UK	Senior Service	2001	UK	8.8	523.0	12.5
UK	Silk Cut Extra Mild [d e]	2001	UK	10.2	183.0	2.6
UK	Silk Cut KS [d]	2001	UK	10.1	367.0	4.8
UK	Silk Cut Ultra Light [d e]	2001	UK	9.4	73.0	1.3
UK	Superkings	2001	UK	8.6	778.0	11.5
UK	Superkings Lights [e]	2001	UK	7.6	505.0	8.3
UK	Superkings Ultra Lights [e]	2001	UK	8.1	268.0	2.6
UK	Superkings	2005	UK	11.2	453.3	9.3
UK	Superkings Blue	2008	UK	9.3	392.6	8.8
UK	Superkings White	2005	UK	7.0	209.9	3.8
EE	Tiger	2006	US	5.6	506.0	9.7
UK	Vogue [d]	2001	US	7.7	346.0	7.6
G	West Ice	2005	US	8.9	298.5	6.7
EE	West Stream Tec Silver	2008	US	7.8	382.2	6.3
G	West Red	2010	US	5.58	524.8	8.7
G	West Red	2008	US	9.8	526.4	10.2
G	West Red 100	2008	US	8.8	435.2	10.3
G	West Silver	2008	US	8.9	336.9	6.8
EE	West Silver Multifilter	2008	US	10.6	218.9	6.9

[a] Countries of sale: EE = Eastern Europe; F = France; G = Germany; SP = Spain; UK = United Kingdom

[b] Blend style: Dark = contains high proportion of dark air-cured tobacco; UK = contains mainly flue-cured tobacco; US = contains a blend of sun-cured, air-cured and flue-cured tobaccos

[c] Total sugar = fructose + glucose + sucrose

[d] Other manufacturer brands (non ITG)

[e] These cigarettes were manufactured and tested before EU legislation banned the descriptors such as “light”, “mild” and “ultra-light” 2003 (28).

[f] NFDPM = Nicotine Free Dry Particulate Matter

concentrations of glucose, fructose, and sucrose in analysed tobacco were obtained (21). Two replicate determinations were made.

Total sugar was estimated as the sum of sucrose, glucose, and fructose. Other sugars are present in tobacco but only at very low levels (22).

NFDPM measurement

In each of the studies the NFDPM yields were conditioned and measured using the appropriate ISO standards (23–26) according to the puffing parameters set out in the ISO 3308 (19). Two replicates of 20 cigarettes were smoked for each cigarette type.

Acetaldehyde measurement

Acetaldehyde analysis was carried out on commercial cigarettes containing the blends analysed for sugar and on cigarettes made from individual tobacco grades, collecting smoke using the ISO smoking regime (19) according to an in-house method involving derivatisation to the

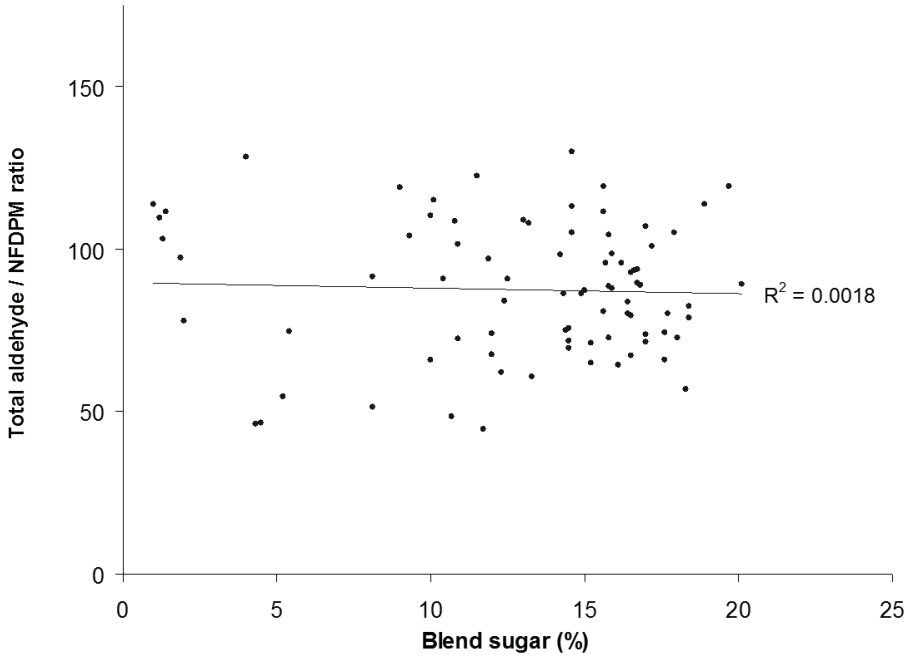

Figure 1. Relationship between total aldehydes / NFDPM ratio in cigarette smoke and blend sugar content from all brands studied by PHILLPOTTS *et al.* (16). NFDPM = Nicotine Free Dry Particulate Matter

dinitrophenylhydrazone, separation by high performance liquid chromatography and ultra violet detection, similar to a method described in the literature (27). Two replicates of 2 cigarettes were smoked for each cigarette type.

RESULTS

Analysis of commercial European brands in 1975

PHILLPOTTS *et al.* (16) reported total volatile aldehyde yields and blend sugar contents measured by colorimetry. Their data was used for the statistical analyses described in this paper.

Analysis of tobacco grades

Sixteen sun-cured grades, 29 air-cured grades and 20 flue-cured grades were studied with no added sugar. Table 1 lists the countries of origin and curing types studied along with their sugar content. Air-cured grades ranged in sugar content from 0.0–0.3% sun-cured grades from 3.6–15.3%, and flue-cured grades from 1.3–23.7%. The NFDPM and acetaldehyde yields and acetaldehyde / NFDPM ratios of cigarettes made from the studied grades are also listed in Table 1.

In spite of the similar cigarette constructions, tobaccos had different densities and weights and this resulted in the different NFDPM yields from the finished experimental cigarettes.

Analysis of current commercial products

The sugar content of the commercial products depends on curing types used in the cigarette blends and levels are variable as shown in Table 2. Dark air-cured blends contained typically much less than 2% total sugar. Flue-cured blends had no added sugar and contained 6–13% inherent sugar. US blended style tobacco blends had a mixture of inherent sugar and sugar added to the air-cured tobacco component. Even so, the total sugar content of US blended style products still tended to be lower (3–12%) than flue-cured products. The NFDPM and acetaldehyde yields are also listed in Table 2.

DISCUSSION

In 1975, PHILLPOTTS *et al.* (16) reported no correlation (R^2 = 0.0018) between the ratio of total aldehyde to NFDPM in smoke and the tobacco blend sugar content from 83 commercial European brands as shown in Figure 1. These colorimetric measurement methods were unspecific but it was estimated from in-house experiments that about 85% of the measured aldehydes was due to acetaldehyde. In 1982, a published study (29), involving 25 different experimental cigarettes, had shown a correlation between, in this case, acetaldehyde in mainstream smoke and total reducing sugars in the tobacco blend. The relationship is given in Figure 2.

However, the NFDPM yields of the studied cigarette ranged from 4–26 mg/cig and it was therefore necessary to normalise the mainstream smoke acetaldehyde yields by dividing by the NFDPM yields of each cigarette to make

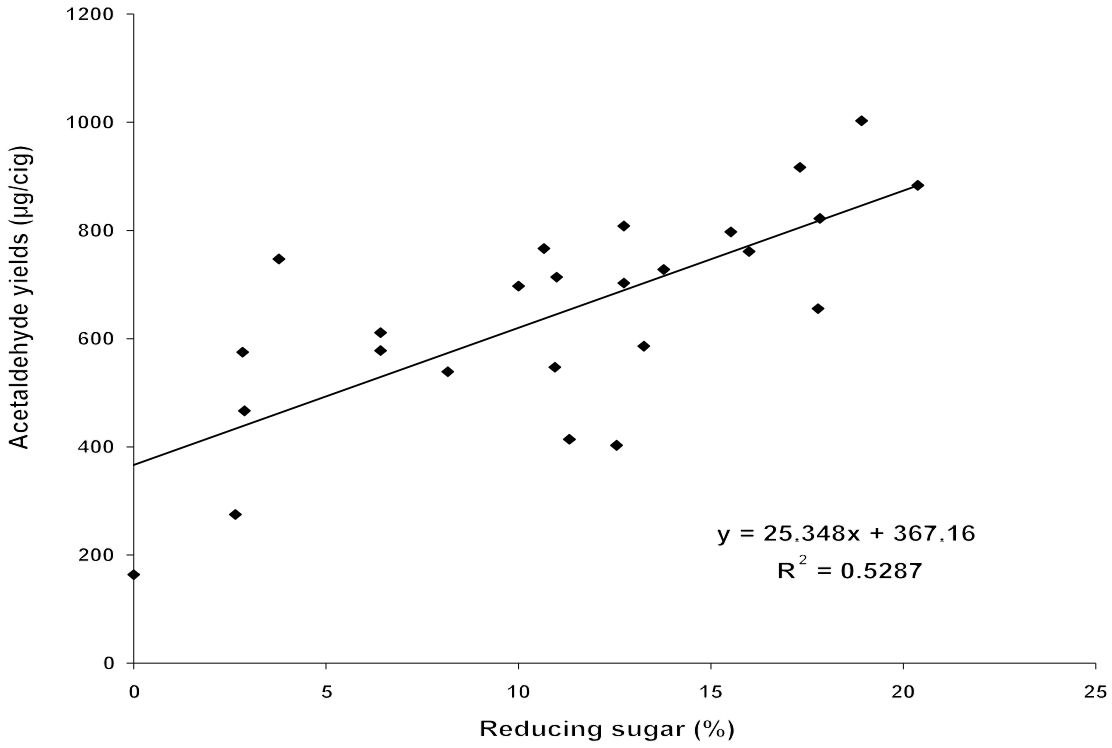

Figure 2. Relationship between acetaldehyde in cigarette smoke and blend reducing sugar content from data by ZILKEY *et al.* (29).

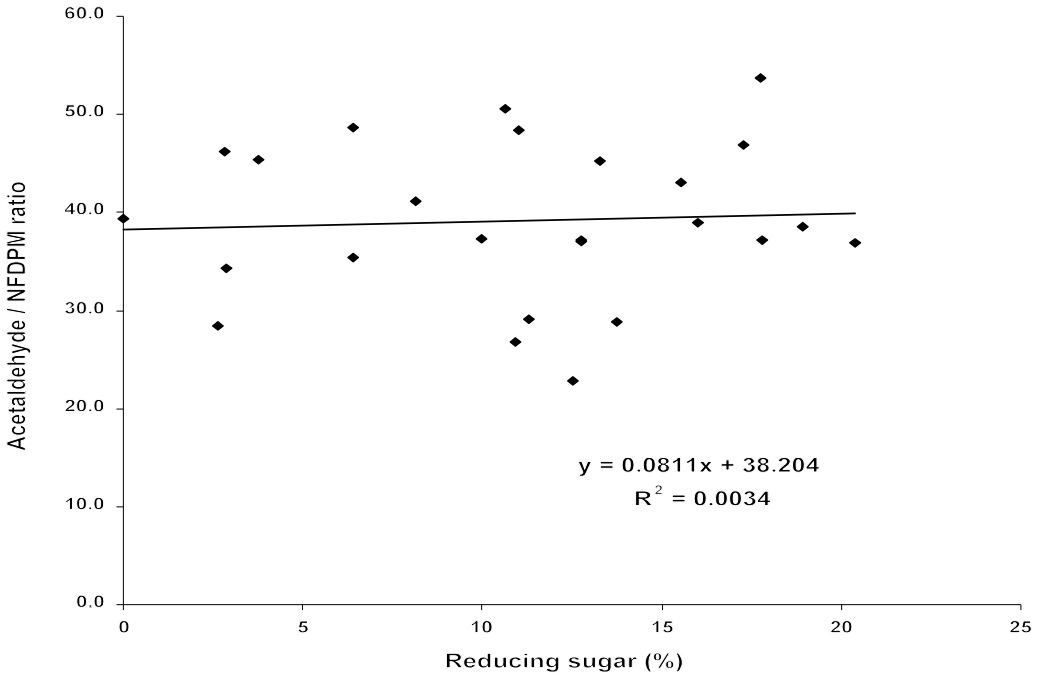

Figure 3. Relationship between acetaldehyde / NFDPM ratio and blend reducing sugar content from data by ZILKEY *et al.* (29).
NFDPM = Nicotine Free Dry Particulate Matter

Table 3. Correlation of reducing sugars in the blend with smoke acetaldehyde yields and with smoke acetaldehyde / NFDPM ratios from SEEMAN *et al.* (30).

Year	Number of brands	Correlation (R^2) of reducing sugars with:	
		Acetaldehyde	Acetaldehyde / NFDPM [b]
1985	135	0.0899	0.0000
1986	142	0.0715	0.0000
1987	185	0.0872	0.0004
1988	176	0.2349	0.0074
1989	4	ND [a]	ND [a]
1990	116	0.1633	0.0206
1991	264	0.1387	0.0004
1992	420	0.0847	0.0541
1993	102	0.0436	0.0209

[a] ND = not determined due to small sample size
[b] NFDPM = Nicotine Free Dry Particulate Matter

comparisons. After normalisation, this study also showed no significant correlation ($R^2 = 0.0034$) between the ratio of mainstream smoke acetaldehyde / NFDPM and total reducing sugar content as shown in Figure 3.

In 2003, a benchmark study (30) on a large number of US cigarettes (gathered over the time period 1985–1993) had shown that the concentration of reducing sugars in the tobacco was not correlated to the acetaldehyde yield or its concentration in NFDPM in mainstream smoke as shown in Table 3. These authors concluded that mainstream smoke acetaldehyde was mainly derived from polysaccharides, such as cellulose.

In 2009, O'CONNOR and HURLEY (17) re-investigated and interpreted the data of PHILLPOTTS *et al.* (16) differently. The authors suggested applying a multivariate analysis to provide the relationship between total smoke aldehydes and blend sugars taking into account the NFDPM yields. They reported that if NFDPM is first forced into the model it accounts for 23% of variance in aldehyde yield. If sugar content is added to the model then it is a significant predictor and accounts for an additional 11% of variance in aldehydes. In the current paper, these figures have been derived again in the way described below with some further perspective put on the analyses and conclusions from the data.

A simple linear regression, as used by PHILLPOTTS, named a bivariate model, involves two variables, one is a dependent variable, in this case total volatile aldehydes, and one is an independent variable, in this case NFDPM.

$$\text{Total volatile aldehydes} = \alpha + \beta \times \text{NFDPM}$$

In their re-analysis, these authors (17) decided to use only

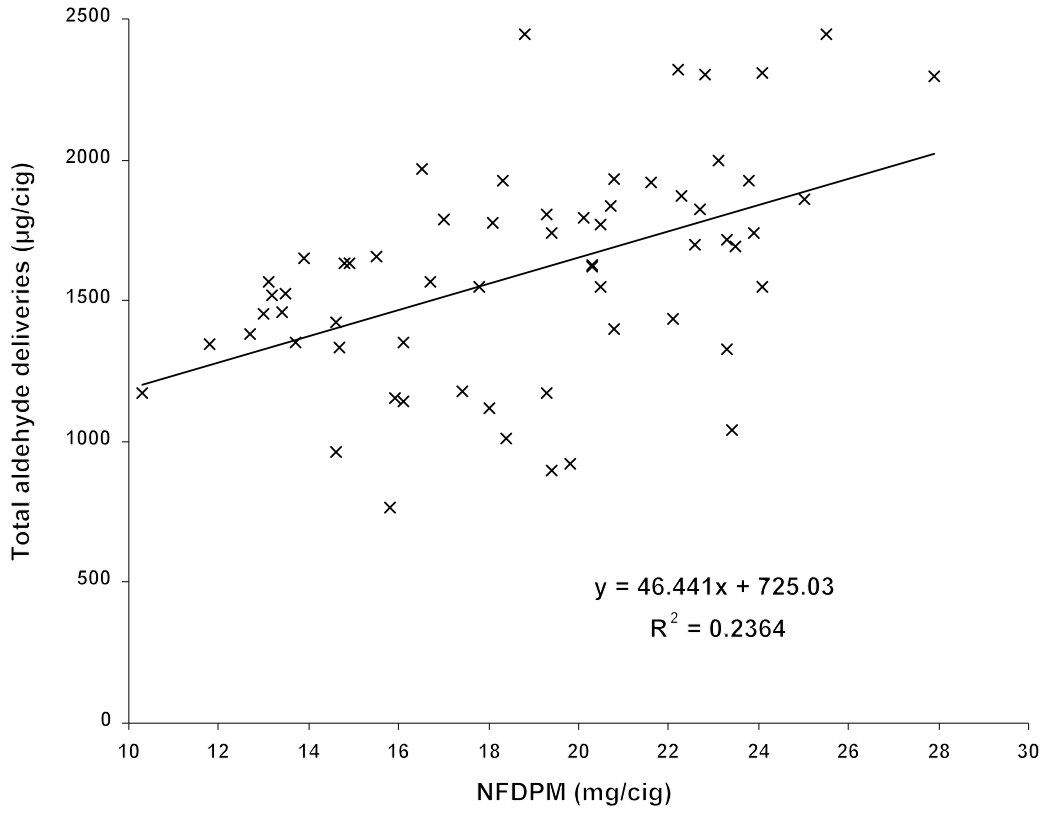

Figure 4. Relationship between total aldehyde yields in cigarette smoke and NFDPM yields from filter cigarettes studied by PHILLPOTTS *et al.* (16). NFDPM = Nicotine Free Dry Particulate Matter

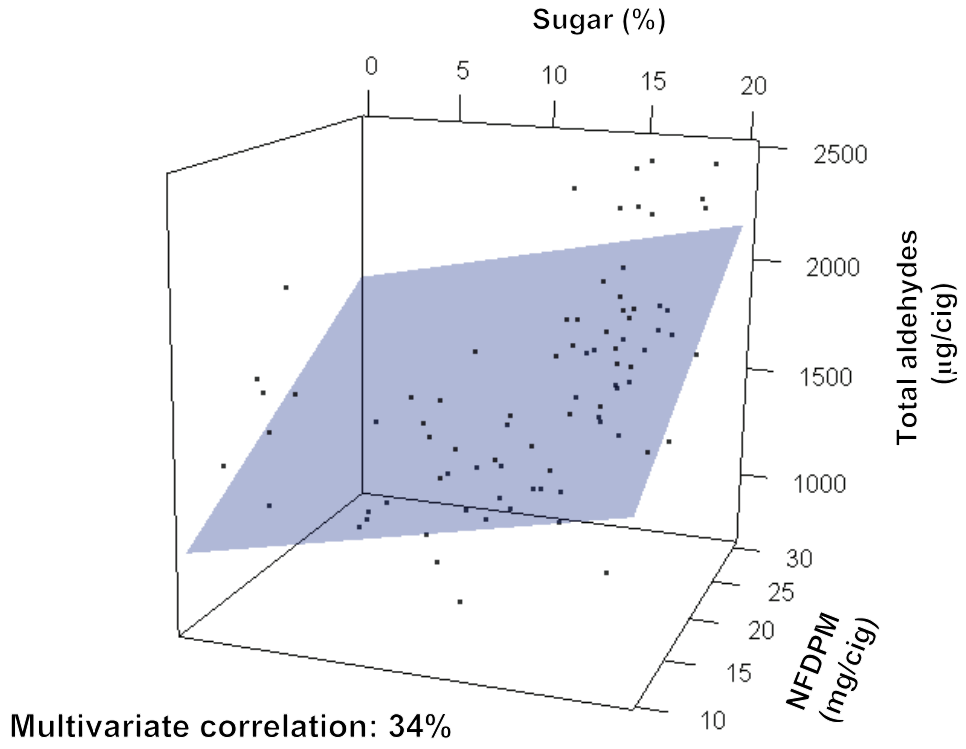

Figure 5. Multivariate analysis of total smoke aldehyde yields, NFDPM smoke yields and blend sugar content from filter cigarettes studied by PHILLPOTTS *et al.* (16). NFDPM = Nicotine Free Dry Particulate Matter

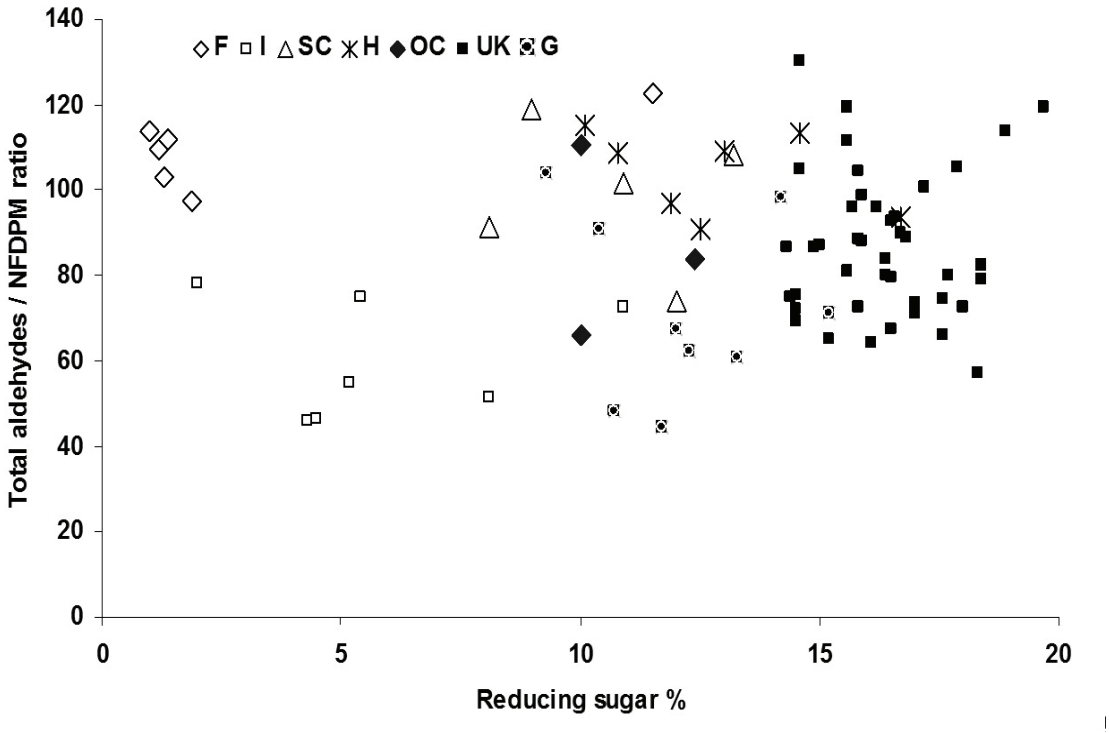

Figure 6. Relationship between total aldehyde / NFDPM ratios and blend sugar contents using data from PHILLPOTTS *et al.* (16) separated by different countries of sale.
F = France; I = Italy; Sc = Scandinavia (Denmark, Norway); H = Holland; OC = Other countries (Belgium, Luxembourg, Switzerland); UK = United Kingdom; G = Germany;
NFDPM = Nicotine Free Dry Particulate Matter

Table 4. Multivariate analysis of the effects of various factors on total aldehyde yields using data from PHILLPOTTS *et al.* (16).

Factors	Sum of squares	Degrees of freedom	Mean squares	F ratio	P value	Significance
Filter or plain	124805	1	124805	1.86	0.1772	NO
Country of sale	2.86515 E6	6	477525	7.11	0.0000	YES
NFDPM [a]	876612	1	876612	13.05	0.0006	YES
Sugar	5697.82	1	5697.82	0.08	0.7717	NO
Residue	4.83815 E6	72	67196.5			
Total	1.23991 E7	81				

[a] NFDPM = Nicotine Free Dry Particulate Matter

the data from filter cigarettes and derived an R^2 correlation of 0.24% from the bivariate model as reproduced in Figure 4. In other words, NFDPM accounts for 24% of the variance in total aldehyde yields. However, to be complete, a qualitative factor with both modalities (filter or plain cigarettes) should have been added to the model.
A multiple regression is a multivariable model, with one dependent variable and multiple independent variables.

$$\text{Total volatile aldehydes} = \alpha + \beta_1 \times \text{NFDPM} + \beta_2 \times \text{Sugar}$$

A multivariate analysis was carried out in the same way described by O'CONNOR and HURLEY (17) and is depicted in Figure 5, NFDPM and sugar take account of 34% of the variance in aldehyde yields leading to their conclusion that sugar accounts for an additional 11% in variance in aldehydes.
However, multivariate analysis should be based on the statistical principle of observation and analysis of more than one statistical variable at a time. For acetaldehyde yields, the technique should take account of all variables that might affect yields in order to avoid any misleading conclusions. PHILLPOTTS *et al.* (16) had pointed out that Italian brands had low sugar and low aldehyde yield whilst French brands had even lower sugar but higher aldehyde yield. They concluded that the low aldehyde yield measured in Italian brands could be due to other properties rather than their low sugar content. At that time, the style of products (based on tobacco curing types used in blends) smoked in those countries was substantially different to those, for example, smoked in the UK. Tobacco curing types in the blends in certain countries, used by other manufacturers, were not known by PHILLPOTTS *et al.*.
If the "country" factor is added to the analysis then a lack of correlation with different countries of sale is found as depicted in Figure 6.
Therefore, the multivariate analysis used by O'CONNOR and HURLEY (17) was incomplete and could have generated misleading conclusions. In order to further investigate this statistical approach, a multivariate analysis based on the General Linear Model (GLM) was carried out. This GLM study has been applied using each of the factors described in the paper by PHILLPOTTS *et al.* (16); that is, sugar, NFDPM, filter or plain cigarette type and country of sale.

$$\text{Acetaldehyde} = \alpha + \beta_1 \times \text{Filter or Plain} + \beta_2 \times \text{Country} + \beta_3 \times \text{NFDPM} + \beta_4 \times \text{Sugar}$$

Table 4 shows that the country and NFDPM yield have a significant effect on total volatile aldehyde yield whereas the cigarette design with filter or without (plain) and the level of blend sugar do not affect the yield.
As the country effect is significant and the global effect of sugar is not significant, the model has been refined by nesting the sugar factor in the country factor in order to assess whether there is a significant effect of sugar for each country.

$$\text{Acetaldehyde} = \alpha + \beta_1 \times \text{Country} + \beta_2 \times \text{NFDPM} + \beta_3 \times \text{Sugar (Country)}$$

Table 5. Multivariate analysis of the effects of various factors on total aldehyde yields with the sugar factor nested in the country factor using data from PHILLPOTTS *et al.* (16).

Sugar factor nested in (Country)	Model coefficient (β3)	Confidence interval at 95%		Significance
		Lower limit	Upper limit	
France	4	-25.0	34.7	NS [a]
Holland	3.2	-94.9	101.2	NS [a]
Italy	6.9	-69.5	83.4	NS [a]
Scandinavia [b]	-0.4	-129.5	128.8	NS [a]
United Kingdom	-14.8	-77.3	47.6	NS [a]
Germany	2.9	-99.0	104.9	NS [a]
Other countries [c]	-7.7	-65.4	50.0	NS [a]

[a] NS = non significant
[b] Scandanavia = Norway and Denmark
[c] Other countries = Belgium, Luxemburg and Switzerland

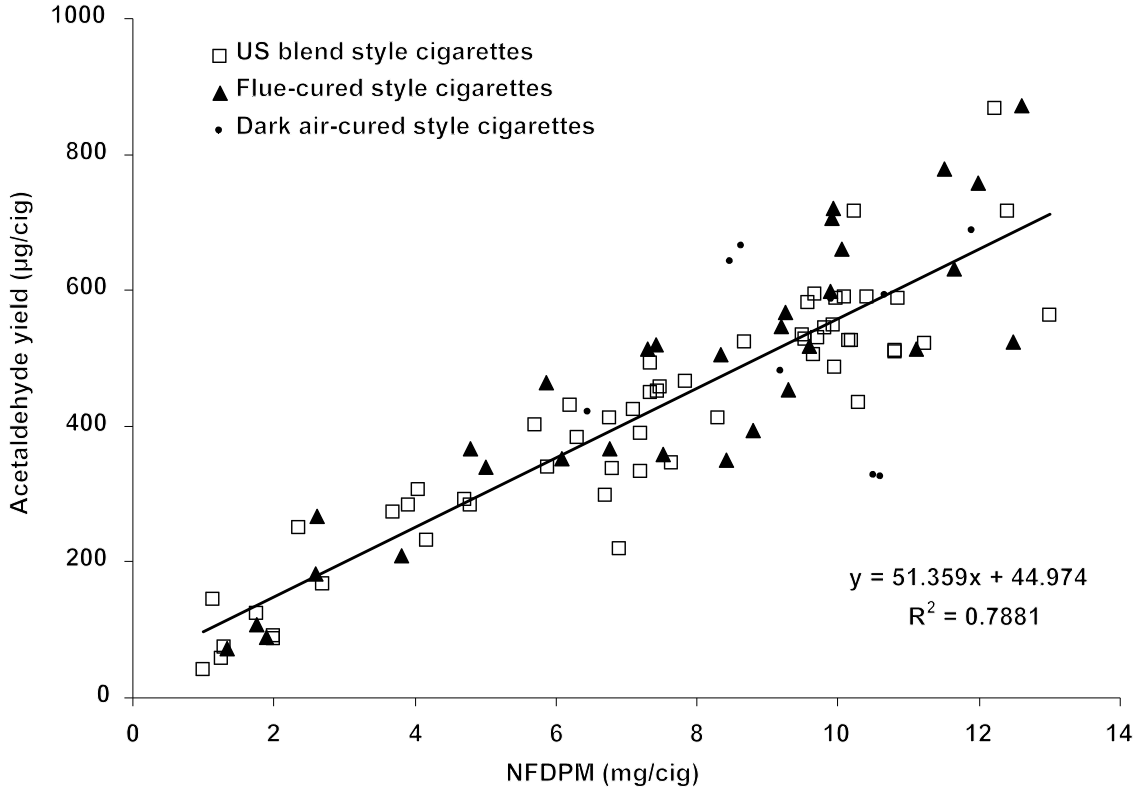

Figure 7. Relationship between acetaldehyde and NFDPM yields in current commercial cigarettes. NFDPM = Nicotine Free Dry Particulate Matter

As the design factor (filter or plain) was not significant, it was not included in the analysis.

The GLM analysis estimates the effect (model coefficients: β_3) of blend sugar for each country. The effect of sugar for a country is significant when the estimated value is different from zero. On the other hand, an estimated value is not different from zero when the effect is non-significant. Table 5 shows the model coefficients and their confidence intervals for each country estimated by the GLM analysis. Each of the confidence intervals, the difference between the upper and lower limits, includes the zero value indicating that the model coefficients are not significant. Therefore, there is no effect of blend sugar content on aldehyde yields whatever the country.

Analysis of tobacco grades and commercial brands

The PHILLPOTTS data was obtained for products on sale 35 years ago. The next part of this study provides an additional data analysis on modern commercially available products. Five data sets were obtained by our laboratory. A total of 97 commercial brands from the EU market included data on 9 dark air-cured, 31 flue-cured and 57 US blended style products. Figure 7 shows the relationship obtained between acetaldehyde yields and NFDPM yield based on these three blend styles.

These data sets were obtained using similar measurement methods but at different points in time. Even so, the overall correlation between NFDPM and smoke acetaldehyde was high ($R^2 = 0.79$), in spite of the fact that there will be some variability due to temporal measurement differences as well as product differences. R^2 correlation values for the individual data sets are given in Table 6 and are similar even though each data set contained a mixture of blend styles.

In this part of the work, the blend sugar content is defined as the sum of sucrose, fructose, and glucose. Brands studied in the 2001, 2005, 2006, 2008, and 2010 data sets were put through the multivariate analysis as previously described.

$$\text{Acetaldehyde} = \alpha + \beta_1 \times \text{Blend} + \beta_2 \times \text{NFDPM} + \beta_3 \times \text{Sugar}$$

The conclusions from this statistical analysis are given in Table 7. Only NFDPM was significantly related to acetaldehyde yields.

There was no relationship found between the acetaldehyde to NFDPM ratio and blend sugar for these commercial products as depicted in Figure 8.

Some of the most compelling evidence that there is no relationship between blend sugar and acetaldehyde yields generated in cigarette smoke is depicted in Figure 9 for cigarettes made from single tobacco grades. Even though the air-cured tobaccos had less than 0.5% sugar, when made into cigarettes, they produced acetaldehyde to NFDPM ratios that were similar to those from cigarettes made from the single flue-cured or sun-cured grades containing up to 23% sugar.

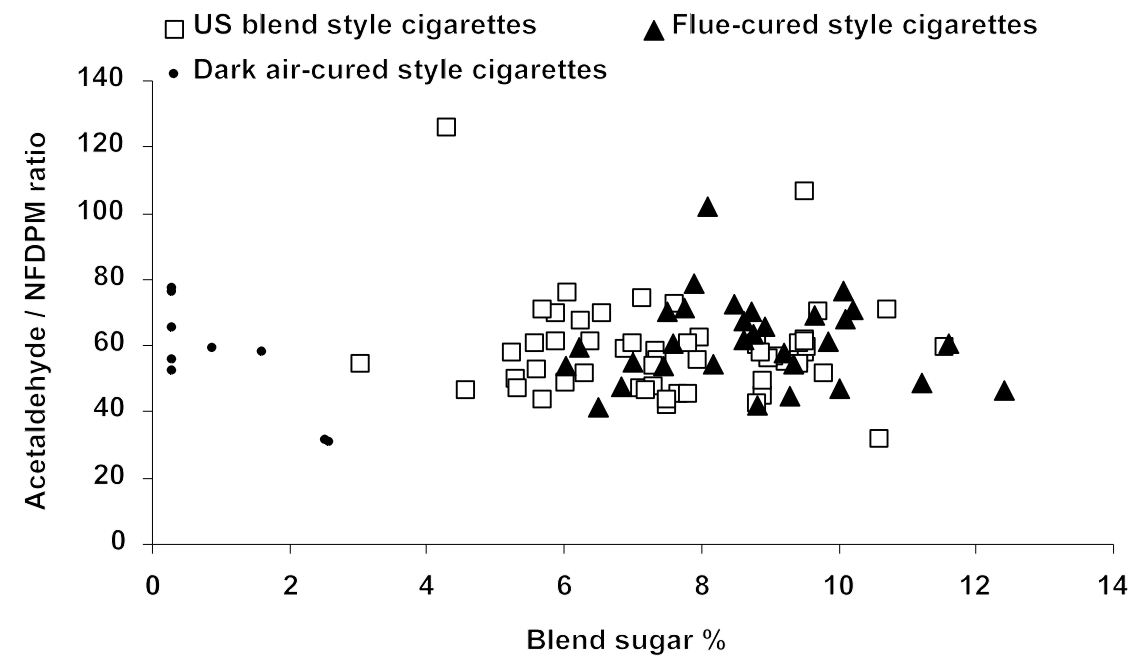

Figure 8. Relationship between the acetaldehyde / NFDPM ratio and blend sugar content for current commercial cigarettes. NFDPM = Nicotine Free Dry Particulate Matter

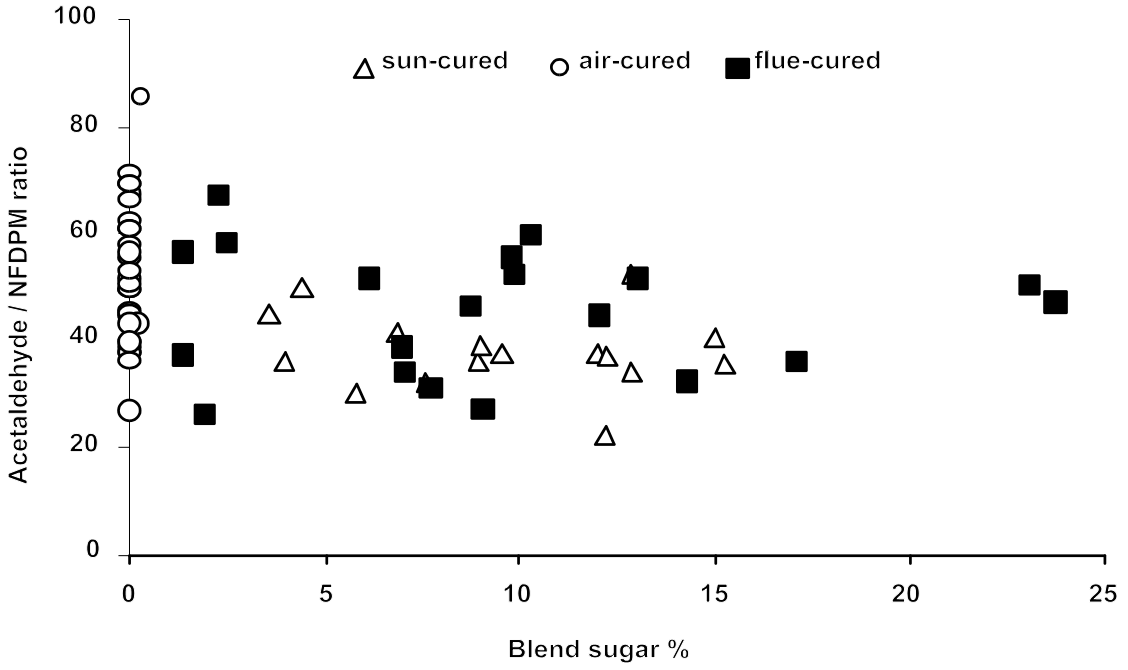

Figure 9. Relationship between the acetaldehyde / NFDPM ratio and blend sugar content for experimental cigarettes made with individual tobacco grades. NFDPM = Nicotine Free Dry Particulate Matter

Table 6. Correlation of mainstream smoke acetaldehyde with NFDPM yields for the studied data sets of commercial brands.

Datasets by year	Number of brands	R^2 Correlation
Overall	97	0.79
2001	25	0.84
2005	7	0.85
2006	26	0.92
2008	17	0.79
2010	22	0.79

NFDPM = Nicotine Free Dry Particulate Matter

CONCLUSIONS

Using several different data sets and product types, this study has shown that there is no relationship between acetaldehyde yields and blend sugar content even if a multivariate analysis is carried out taking into account NFDPM as a co-factor.

The suggestion (17) to use multivariate analysis instead of the normalisation by dividing by NFDPM yields is very relevant. Nevertheless, it is important to take into consideration each of the factors which can have an impact before drawing conclusions. In the case of the PHILLPOTTS data on 83 European brands, it has been clearly shown that the "country" factor had a large impact on the mainstream smoke acetaldehyde yields and that no relationship between blend sugars and cigarette mainstream acetaldehyde yields has been found even when using multivariate analysis.

No distinction was found between the mainstream acetaldehyde yields from 97 dark air-cured, flue-cured or US blended commercial cigarette types irrespective of their sugar content after taking account of differences in NFDPM yields. Similarly, no distinction was found between mainstream acetaldehyde yields from 65 experimental cigarette types made from either flue-cured, sun-cured or air-cured tobaccos with no sugar added.

This work supports the conclusions that structural material in the tobacco plant is the main source of acetaldehyde in mainstream smoke after combustion during cigarette smoking.

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Table 7. Multivariate analysis of the effects of various factors on acetaldehyde yields using data from commercial cigarettes.

Factors	Sum of squares	Degrees of freedom	Mean squares	F ratio	P value	Significance
Blend	21238.7	2	10619.3	1.42	0.2471	NO
NFDPM [a]	2.53128 E6	1	2.53128 E6	338.30	0.0000	YES
Sugar	2105.03	1	2105.03	0.28	0.5971	NO
Residue	688368	92	7482.26			
Total	3.34789 E6	96				

[a] NFDPM = Nicotine Free Dry Particulate Matter

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Corresponding author:

Steve Purkis
Imperial Tobacco Limited
PO Box 244, Southville,
Bristol BS99 7UJ, UK
E-mail: steve.purkis@uk.imptob.com

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IARC Monographs on the Evaluation of Carcinogenic Risks to Humans

Volume 83

Tobacco Smoke and Involuntary Smoking

Summary of Data Reported and Evaluation

Last updated: 24 July 2002

TOBACCO SMOKING AND TOBACCO SMOKE
(Group 1)

5. Summary of Data Reported and Evaluation

For definition of groups, see Preamble.

VOL.: 83 (2002)

5.1 Exposure data

Smoking of tobacco is practised worldwide by over one thousand million people. However, while smoking prevalence has declined in many developed countries, it remains high in others and is increasing among women and in developing countries. Between one-fifth and two-thirds of men in most populations smoke. Women's smoking rates vary more widely but rarely equal male rates.

Tobacco is most commonly smoked as cigarettes, both manufactured — which are a highly sophisticated nicotine delivery system — and hand-rolled. Pipes, cigars, bidis and other products are used to a lesser extent or predominantly in particular regions. Cigarettes are made from fine-cut tobaccos which are wrapped in paper or a maize leaf. Cigars consist of cut tobacco filler formed in a binder leaf and with a wrapper leaf rolled spirally around the bunch. Bidis contain shredded tobacco wrapped in non-tobacco leaves, usually dried *temburni* leaves.

The chemical composition of tobacco smoke, although influenced by the specific manner in which individuals smoke, is primarily determined by the type of tobacco. It is also influenced by the design of the smoking device or product and, for cigarettes, by the presence or absence of filters, and by other factors including ventilation, paper porosity and types of additives. As a result, concentrations of individual chemicals in smoke vary. Analysis of the ways in which people smoke modern cigarettes shows that actual doses of nicotine, carcinogens and toxins depend on the intensity and method of smoking and have little relation to stated tar yields. The total volume of smoke drawn from cigarettes as a result of specific smoking patterns is the principal determinant of dose to the smoker. All presently available tobacco products that are smoked deliver substantial amounts of established carcinogens to their users.

The yields of tar, nicotine and carbon monoxide from cigarettes, as measured by standard machine-smoking tests, have fallen over recent decades in cigarettes sold in most parts of the world, but have remained higher in some countries. The tar and nicotine yields as currently measured are misleading and have only little value in the assessment of human exposure to carcinogens.

The regulation of smoking and smoke yields varies widely around the world in scope and degree of enforcement. Certain regulatory actions, such as taxes and workplace smoking bans, are effective in reducing smoking rates and protecting nonsmokers.

5.2 Human carcinogenicity data

In the previous 1986 *IARC Monograph* on tobacco smoking, cancers of the lung, oral cavity, pharynx, larynx, oesophagus (squamous-cell carcinoma), pancreas, urinary bladder and renal pelvis were identified as caused by cigarette smoking. Many more studies published since this earlier Monograph support these causal links. In addition, there is now sufficient evidence for a causal association between cigarette smoking and cancers of the nasal cavities and nasal sinuses, oesophagus (adenocarcinoma), stomach, liver, kidney (renal-cell carcinoma), uterine cervix and myeloid leukaemia.

In cancer sites that were causally linked to cigarette smoking in the previous *IARC Monograph* on tobacco smoking, the observed relative risks ranged generally from approximately 3 for pancreatic cancer to more than 20 for lung cancer. For those cancer sites that were now also linked to cigarette smoking in this Monograph,

generally two- to threefold increased risks were observed.

Cigarettes

Lung

Lung cancer is the most common cause of death from cancer in the world. The total number of cases is now estimated to be 1.2 million annually and is still increasing. The major cause of lung cancer is tobacco smoking, primarily of cigarettes. In populations with prolonged cigarette use, the proportion of lung cancer cases attributable to cigarette smoking has reached 90%.

The duration of smoking is the strongest determinant of lung cancer in smokers. Hence, the earlier the age of starting and the longer the continuation of smoking in adulthood, the greater the risk. Risk of lung cancer also increases in proportion to the numbers of cigarettes smoked.

Tobacco smoking increases the risk of all histological types of lung cancer including squamous-cell carcinoma, small-cell carcinoma, adenocarcinoma (including bronchiolar/alveolar carcinoma) and large-cell carcinoma. The association between adenocarcinoma of the lung and smoking has become stronger over time. The carcinogenic effects of cigarette smoking appear similar in both women and men.

Stopping smoking at any age avoids the further increase in risk of lung cancer incurred by continued smoking. The younger the age at cessation, the greater the benefit.

Urinary tract

Tobacco smoking is a major cause of transitional-cell carcinomas of the bladder, ureter and renal pelvis. Risk increases with the duration of smoking and number of cigarettes smoked. As for lung cancer, stopping smoking at any age avoids the further increase in risk incurred by continued smoking.

Evidence from several cohort and case–control studies published since the previous *IARC Monograph* on tobacco smoking has indicated that renal-cell carcinoma is associated with tobacco smoking in both men and women. The association is not explained by confounding. 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.

Oral cavity

Tobacco smoking, including cigarette smoking, is causally associated with cancer of the oral cavity (including lip and tongue) in both men and women. Since the previous *IARC Monograph* on tobacco smoking, evidence from many more studies has accumulated that further confirms this association. Use of smokeless tobacco and/or alcohol in combination with tobacco smoking greatly increases the risk of oral cancer. Risk increases substantially with duration of smoking and number of cigarettes smoked. Risk among former smokers is consistently lower than among current smokers and there is a trend of decreasing risk with increasing number of years since quitting.

Nasal cavity and paranasal sinuses

An increased risk of sinonasal cancer among cigarette smokers has been reported in all nine case–control studies for which results are available. Of seven studies that have analysed dose–response relationships, a positive trend was found in five and was suggested in the other two. In all the five studies that have analysed squamous-cell carcinoma and adenocarcinoma separately, the relative risk was clearly increased for squamous-cell carcinoma.

Nasopharynx

An increased risk for nasopharyngeal cancer among cigarette smokers was reported in one cohort study and nine case–control studies. Increased relative risks were reported in both high- and low-risk geographical regions for nasopharyngeal cancer. A dose–response relationship was detected with either duration or amount of smoking. A reduction in risk after quitting was also detected. The potential confounding effect of infection with Epstein–Barr virus was not controlled for in these studies; however, such an effect was not considered to be plausible. No important role was shown for other potential confounders.

Oropharynx and hypopharynx

Oropharyngeal and hypopharyngeal cancer are causally associated with cigarette smoking. The risk increased with increased duration of smoking and daily cigarette consumption and decreased with increasing time since quitting.

Oesophagus

Tobacco smoking is causally associated with cancer of the oesophagus, particularly squamous-cell carcinoma. Tobacco smoking is also causally associated with adenocarcinoma of the oesophagus. In most of the epidemiological studies, the risk for all types of oesophageal cancer increased with numbers of cigarettes smoked daily and duration of smoking. However, risk for oesophageal cancer remains elevated many years after cessation of smoking.

Tobacco and alcohol in combination with tobacco smoking greatly increase the risk for squamous-cell carcinoma of the oesophagus. In India, use of smokeless tobacco in combination with smoking also greatly increases the risk.

Larynx

Laryngeal cancer is causally associated with cigarette smoking. The risk increases substantially with duration and number of cigarettes smoked. Use of alcohol in combination with tobacco smoking greatly increases the risk for laryngeal cancer. A few studies also reported that relative risks for cancer of the larynx increased with decreasing age at start of smoking. The relative risk decreased with increasing time since quitting smoking.

Pancreas

Cancer of the pancreas is causally associated with cigarette smoking. The risk increases with duration of smoking and number of cigarettes smoked daily. The risk remains elevated after allowing for potential confounding factors such as alcohol consumption. The relative risk decreased with increasing time since quitting smoking.

Stomach

The data available in 1986 did not permit the earlier IARC Working Group to conclude that the association between tobacco smoking and stomach cancer was causal. Since that time, further studies have shown a consistent association of cancer of the stomach with cigarette smoking in both men and women in many cohort and case–control studies conducted in various parts of the world. Confounding by other factors (e.g. alcohol consumption, *Helicobacter pylori* infection and dietary factors) can be reasonably ruled out. Risk increases with duration of smoking and number of cigarettes smoked, and decreases with increasing duration of successful quitting. In studies that had adequate numbers, the relative risks for men and women were similar.

Liver

In the previous *IARC Monograph* on tobacco smoking, a causal relationship between liver cancer and smoking could not be established, chiefly due to possible confounding from alcohol intake and hepatitis B and hepatitis C virus infections. Many cohort studies and case–control studies have provided additional information on smoking and liver cancer since then. Most of the cohort studies and the largest case–control studies (most notably those that included community controls) showed a moderate association between tobacco smoking and risk of liver cancer. In many studies, the risk for liver cancer increased with the duration of smoking or the number of cigarettes smoked daily. Former smokers who had stopped smoking for more than 10 years showed a decline in liver cancer risk. Confounding from alcohol can be ruled out, at least in the best case–control studies, by means of careful adjustment for drinking habits. An association with smoking has also been demonstrated among non-drinkers. Many studies, most notably from Asia, have shown no attenuation of the association between smoking and liver cancer after adjustment/stratification for markers of hepatitis B/hepatitis C virus infection. There is now sufficient evidence to judge the association between tobacco smoking and liver cancer as causal.

Cervix

An association of invasive cervical squamous-cell carcinoma with smoking has been observed in the large number of studies reviewed. The most recent studies have controlled for infection with human papillomavirus, a known cause of cervical cancer. The effect of smoking was not diminished by the adjustment for human papillomavirus infection, or analysis restricted to cases and controls both positive for human papillomavirus (as ascertained by human papillomavirus DNA or human papillomavirus serological methods). There is now sufficient evidence to establish a causal association of squamous-cell cervical carcinoma with smoking. In the small number of studies available for adeno- and adeno-squamous-cell carcinoma, no consistent association was observed.

Leukaemia

Myeloid leukaemia in adults was observed to be causally related to smoking. Risk increased with amount of tobacco smoked in a substantial number of adequate studies. No clear evidence of any risk was seen for lymphoid leukaemia/lymphoma.

Support 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 sufficient amounts to account for up to half of the estimated excess of acute myeloid leukaemia.

Colorectal cancer

There is some evidence from prospective cohort studies and case–control studies that the risk of colorectal cancer is increased among tobacco smokers. However, it is not possible to conclude that the association between tobacco smoking and colorectal cancer is causal. Inadequate adjustment for various potential confounders could account for some of the small increase in risk that appears to be associated with smoking.

Female breast

Most epidemiological studies have found no association with active smoking, after controlling for established risk factors (e.g. age at time of first birth, parity, family history of breast cancer and alcohol). The large multicentre pooled analysis of the association of smoking with breast cancer in non-drinkers confirms the lack of an increased risk of breast cancer associated with smoking.

Endometrium

Cigarette smoking is not associated with an increased risk for endometrial cancer.

An inverse relationship of cigarette smoking with endometrial cancer is observed consistently in most case–control and cohort studies, after adjustment for major confounders. This pattern is stronger in post-menopausal women.

Prostate

No clear evidence of any risk for prostate cancer is seen in case–control studies or in studies of incident cases in cohort studies. The small excess observed in some analytical mortality studies can reasonably be explained by bias in the attribution of the underlying cause of death.

Other

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

Cigars and pipes

Cigar and/or pipe smoking is strongly related to cancers of the oral cavity, oropharynx, hypopharynx, larynx and oesophagus, the magnitude of risk being similar to that from cigarette smoking. These risks increase with the amount of cigar and/or pipe smoking and with the combination of alcohol and tobacco consumption. Cigar and/or pipe smoking is causally associated with cancer of the lung and there is evidence that cigar and/or pipe smoking are also causally associated with cancers of the pancreas, stomach and urinary bladder.

Bidi

Bidi smoking is the most common form of tobacco smoking in India and is also prevalent in other south-Asian countries and an emerging problem in the USA. Bidi smoke was considered as carcinogenic in the earlier *IARC Monograph* on tobacco smoking, and later studies have provided further evidence of causality. Case–control studies demonstrated a strong association at various sites: oral cavity (including subsites), pharynx, larynx, oesophagus, lung and stomach. Almost all studies show significant trends with duration of bidi smoking and number of bidis smoked.

Synergy

For public health purposes, synergy should be characterized as a positive departure from additivity. The epidemiological literature often inadequately describes combined effects of smoking with co-exposures to other carcinogenic agents and in many studies power is limited for characterizing combined effects. The issue of synergistic effects can be appropriately addressed by epidemiological studies that show stratified analysis and have sufficient power. The studies reviewed found evidence of synergy between smoking and several occupational causes of lung cancer (arsenic, asbestos and radon), and between smoking and alcohol consumption for cancers of the oral cavity, pharynx, larynx and oesophagus and between smoking and human papillomavirus infection for cancer of the cervix. Data were inadequate to evaluate the evidence for synergy between smoking and other known causes of cancer (e.g. hepatitis B and alcohol for liver cancer).

5.3 Animal carcinogenicity data

Cigarette smoke has been tested for carcinogenicity by inhalation studies in rodents, rabbits and dogs. The model systems for animal exposure to tobacco smoke do not fully simulate human exposure to tobacco smoke, and the tumours that develop in animals are not completely representative of human cancer. Nevertheless, the animal data provide valuable insights regarding the carcinogenic potential of tobacco smoke.

The most compelling evidence for a positive carcinogenic effect of tobacco smoke in animals is the

reproducible increase observed in several studies in the occurrence of laryngeal carcinomas in hamsters exposed to whole tobacco smoke or to its particulate phase. In four of five studies in rats, exposure to whole smoke led to modest increases in the occurrence of malignant and/or benign lung tumours. Similarly, in four of eight studies in mice of varying susceptibility to lung tumour development, exposure to whole smoke led to a modest increase in the frequency of lung adenomas. An increased incidence of lung 'tumours' has also been reported in dogs exposed to tobacco smoke, but it is uncertain whether the histopathological features of the lesions are consistent with malignancy. In hamsters exposed to both cigarette smoke and chemical carcinogens (*N*-nitrosodiethylamine and 7,12-dimethylbenz[*a*]anthracene), the tumour response in the respiratory tract was higher than in hamsters exposed to either agent alone. The same is true in rats exposed simultaneously to cigarette smoke and radionuclides (radon progeny and plutonium oxide).

Cigarette smoke condensate both initiates and promotes tumour development in animals. It reproducibly induces both benign and malignant skin tumours in mice following topical application. Similarly, it produces skin tumours in rabbits following topical application. Topical application to the oral mucosa also produced an increased incidence of lung tumours and lymphomas in mice. In rats, cigarette smoke condensate produced lung tumours after intrapulmonary injection. In initiation/promotion assays in mouse skin, a single topical application of cigarette smoke condensate followed by application of croton oil was sufficient to initiate both benign and malignant skin tumours. Smoke condensates of Indian bidi administered to mice by gavage were found to induce tumours in a number of organs. Collectively, these data provide evidence of the carcinogenic effect of mainstream tobacco smoke in experimental animals.

5.4 Other relevant data

Causal associations have been clearly established between active smoking and adverse reproductive outcomes and numerous non-neoplastic diseases, including chronic obstructive pulmonary disease and cardiovascular diseases.

Tobacco smoking is addictive, and nicotine has been established as the major addictive constituent of tobacco products. Measurement of the nicotine metabolite, cotinine, in human blood, urine or saliva provides a specific and sensitive test for exposure to tobacco smoke and can be used to distinguish active and passive smokers from nonsmokers.

Active smoking raises the concentrations of carbon monoxide, benzene and volatile organic compounds in exhaled air. The concentrations of urinary metabolites of some important tobacco smoke carcinogens and related compounds are consistently higher in smokers than in nonsmokers. These include metabolites of benzene, a known carcinogen in humans, as well as metabolites of several carcinogens that cause lung tumours in rodents. Covalent binding to blood proteins by carcinogens present in tobacco smoke has been demonstrated to occur at significantly higher levels in smokers than in nonsmokers. The adducts are derived from various compounds including aromatic amines (e.g. 4-aminobiphenyl), polycyclic aromatic hydrocarbons (e.g. benzo[*a*]pyrene), tobacco-specific nitrosamines (e.g. 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone), benzene, acrylamide and acrylonitrile.

Smoking-related DNA adducts have been detected by a variety of analytical methods in the respiratory tract, urinary bladder, cervix and other tissues. In many studies the levels of carcinogen-DNA adducts have been shown to be higher in tissues of smokers than in tissues of nonsmokers. Some but not all studies have demonstrated elevated levels of these adducts in the peripheral blood and in full-term placenta. Smoking-related adducts have also been detected in cardiovascular tissues. Collectively, the available biomarker data provide convincing evidence that carcinogen uptake, activation and binding to cellular macromolecules, including DNA, are higher in smokers than in nonsmokers.

The exposure of experimental animals, primarily rodents, to mainstream tobacco smoke results in a number of biological effects that include (i) increases or decreases in the activities of phase I and phase II enzymes involved in carcinogen metabolism, (ii) increases in the activation of antioxidant enzymes, (iii) increased expression of nitric oxide synthase and of various protein kinases and collagenase, (iv) the formation of tobacco smoke-related DNA adducts in several tissues and (v) reduced clearance of particulate material from the lung.

Smoking is known to have inhibitory or inducing effects on the activities of many enzymes in human tissues. These include xenobiotic metabolizing enzymes, which affect drug and carcinogen metabolism. Numerous studies have reported effects on enzymes in cells treated in culture with tobacco smoke or tobacco smoke condensates.

In humans, smoking produces gene mutations and chromosomal abnormalities. Urine from smokers is mutagenic. Relative to nonsmokers, lung tumours of smokers contain higher frequencies of *TP53* and *KRAS* mutations, and the spectrum of mutations has unique features. Most of the genetic effects seen in smokers are also observed in cultured cells or in experimental animals exposed to tobacco smoke or smoke condensate. Tobacco smoke is genotoxic in humans and in experimental animals.

5.5 Evaluation

There is *sufficient evidence* in humans that tobacco smoking causes cancer of the lung, oral cavity, naso-, oro- and hypopharynx, nasal cavity and paranasal sinuses, larynx, oesophagus, stomach, pancreas, liver, kidney (body and pelvis), ureter, urinary bladder, uterine cervix and bone marrow (myeloid leukaemia).

There is *evidence suggesting lack of carcinogenicity* of tobacco smoking in humans for cancers of the female breast and endometrium.

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

Overall evaluation

Tobacco smoking and tobacco smoke *are carcinogenic to humans (Group 1).*

For definition of the italicized terms, see Preamble.

INVOLUNTARY SMOKING
(Group 1)

For definition of groups, see Preamble.

VOL.: 83 (2002)

5. Summary of Data Reported and Evaluation

5.1 Exposure data

Involuntary (or passive) smoking is exposure to secondhand tobacco smoke, which is a mixture of exhaled mainstream smoke and sidestream smoke released from the smouldering cigarette or other smoking device (cigar, pipe, bidi, etc.) and diluted with ambient air. Involuntary smoking involves inhaling carcinogens, as well as other toxic components, that are present in secondhand tobacco smoke. Secondhand tobacco smoke is sometimes referred to as 'environmental' tobacco smoke. Carcinogens that occur in secondhand tobacco smoke include benzene, 1,3-butadiene, benzo[*a*]pyrene, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and many others.

Secondhand tobacco smoke consists of a gas phase and a particulate phase; it changes during its dilution and distribution in the environment and upon ageing. The concentrations of respirable particles may be elevated substantially in enclosed spaces containing secondhand tobacco smoke. The composition of tobacco smoke inhaled involuntarily is variable quantitatively and depends on the smoking patterns of the smokers who are producing the smoke as well as the composition and design of the cigarettes or other smoking devices. The secondhand tobacco smoke produced by smoking cigarettes has been most intensively studied.

Secondhand tobacco smoke contains nicotine as well as carcinogens and toxins. Nicotine concentrations in the air in homes of smokers and in workplaces where smoking is permitted typically range on average from 2 to 10 micrograms/m^3.

5.2 Human carcinogenicity data

Lung cancer

Involuntary smoking involves exposure to the same numerous carcinogens and toxic substances that are present in tobacco smoke produced by active smoking, which is the principal cause of lung cancer. As noted in the previous *IARC Monograph* on tobacco smoking, this implies that there will be some risk of lung cancer from exposure to secondhand tobacco smoke.

More than 50 studies of involuntary smoking and lung cancer risk in never-smokers, especially spouses of smokers, have been published during the last 25 years. These studies have been carried out in many countries. Most showed an increased risk, especially for persons with higher exposures. To evaluate the information collectively, in particular from those studies with a limited number of cases, meta-analyses have been conducted in which the relative risk estimates from the individual studies are pooled together. These meta-analyses show that there is a statistically significant and consistent association between lung cancer risk in spouses of smokers and exposure to secondhand tobacco smoke from the spouse who smokes. The excess risk is of the order of 20% for women and 30% for men and remains after controlling for some potential sources of bias and confounding. The excess risk increases with increasing exposure. Furthermore, other published meta-analyses of lung cancer in never-smokers exposed to secondhand tobacco smoke at the workplace have found a statistically significant increase in risk of 12–19%. This evidence is sufficient to conclude that involuntary smoking is a cause of lung cancer in never-smokers. The magnitudes of the observed risks are reasonably consistent with predictions based on studies of active smoking in many populations.

Breast cancer

The collective evidence on breast cancer risk associated with involuntary exposure of never-smokers to tobacco smoke is inconsistent. Although four of the 10 case–control studies found statistically significant increases in risks, prospective cohort studies as a whole and, particularly, the two large cohort studies in the USA of nurses and of volunteers in the Cancer Prevention Study II provided no support for a causal relation between involuntary exposure to tobacco smoke and breast cancer in never-smokers. The lack of a positive dose–response also argues against a causal interpretation of these findings. Finally, the lack of an association of breast cancer with active smoking weighs heavily against the possibility that involuntary smoking increases the risk for breast cancer, as no data are available to establish that different mechanisms of carcinogenic action operate at the different dose levels of active and of involuntary smoking.

Childhood cancer

Overall, the findings from studies of childhood cancer and exposure to parental smoking are inconsistent and are likely to be affected by bias. There is a suggestion of a modest association between exposure to maternal tobacco smoke during pregnancy and childhood cancer for all cancer sites combined; however, this is in contrast with the null findings for individual sites. Studies on paternal tobacco smoking suggest a small increased risk for lymphomas, but bias and confounding cannot be ruled out.

Other cancer sites

Data are conflicting and sparse for associations between involuntary smoking and cancers of the nasopharynx, nasal cavity, paranasal sinuses, cervix, gastrointestinal tract and cancers at all sites combined. It is unlikely that any effects are produced in passive smokers that are not produced to a greater extent in active smokers or that types of effects that are not seen in active smokers will be seen in passive smokers.

5.3 Animal carcinogenicity data

Secondhand tobacco smoke for carcinogenicity studies in animals is produced by machines that simulate human active smoking patterns and combine mainstream and sidestream smoke in various proportions. Such mixtures have been tested for carcinogenicity by inhalation studies in rodents. The experimental model systems for exposure to secondhand tobacco smoke do not fully simulate human exposures, and the tumours that develop in animals are not completely representative of human cancer. Nevertheless, the animal data provide valuable insights regarding the carcinogenic potential of secondhand tobacco smoke.

A mixture of 89% sidestream smoke and 11% mainstream smoke has been tested for carcinogenic activity in mouse strains that are highly susceptible to lung tumours (strains A/J and Swiss). In strain A/J mice, this mixture consistently produces a significant, modest increase in lung tumour incidence and lung tumour multiplicity when the mice are exposed for 5 months followed by a 4-month recovery period. These lung tumours are predominantly adenomas. Continuous exposure of strain A/J mice to the above mixture of mainstream and sidestream tobacco smoke for 9 months with no recovery period did not increase the incidence of lung tumours. In Swiss strain mice, the same mixture induced lung tumours by both protocols, i.e. when the animals were exposed for 5 months followed by a 4-month recovery period and when they were exposed continuously for 9 months with no recovery period. In addition, exposure of Swiss mice to the tobacco smoke mixture for a shorter period was sufficient to induce lung tumours.

Condensates of sidestream and of mainstream cigarette smoke have been tested for carcinogenicity. Both kinds of condensates produced a spectrum of benign and malignant skin tumours in mice following topical application, and the sidestream condensate exhibited higher carcinogenic activity. Sidestream smoke condensate was shown to produce a dose-dependent increase in lung tumours in rats following implantation into the lungs.

Increased relative risks for lung and sinonasal cancer have been reported in companion animals

(dogs) exposed to secondhand tobacco smoke in homes.

5.4 Other relevant data

Involuntary smoking has been associated with a number of non-neoplastic diseases and adverse effects in never-smokers, including both children and adults. Epidemiological studies have demonstrated that exposure to secondhand tobacco smoke is causally associated with coronary heart disease. From the available meta-analyses, it has been estimated that involuntary smoking increases the risk of an acute coronary heart disease event by 25–35%. Adverse effects of involuntary smoking on the respiratory system have also been detected. In adults, the strongest evidence for a causal relation exists for chronic respiratory symptoms. Some effects on lung function have been detected, but their medical relevance is uncertain.

Data on the hormonal and metabolic effects of involuntary smoking are sparse. However, female involuntary smokers do not appear to weigh less than women who are not exposed to secondhand tobacco smoke, a pattern that contrasts with the findings for active smoking. No consistent association of maternal exposure to secondhand smoke with fertility or fecundity has been identified. There is no clear association of passive smoking with age at menopause.

Maternal cigarette smoking has repeatedly been associated with adverse effects on fetal growth; full-term infants born to women who smoke weigh about 200 g less than those born to nonsmokers. A smaller adverse effect has been attributed to maternal passive smoking.

Cotinine, and its parent compound nicotine, are highly specific for exposure to secondhand smoke. Because of its favourable biological half-life and the sensitivity of techniques for quantifying it, cotinine is currently the most suitable biomarker for assessing recent exposure to secondhand tobacco smoke uptake and metabolism in adults, children and newborns.

Several studies in humans have shown that concentrations of adducts of carcinogens to biological macromolecules, including haemoglobin adducts of aromatic amines and albumin adducts of polycyclic aromatic hydrocarbons, are higher in adult involuntary smokers and in the children of smoking mothers than in individuals not exposed to secondhand tobacco smoke. Protein adduct concentrations in fetal cord blood correlate with those in maternal blood but are lower. Fewer studies have investigated DNA adduct levels in white blood cells of exposed and unexposed nonsmokers, and most studies have not shown clear differences.

In studies of urinary biomarkers, metabolites of the tobacco-specific carcinogen, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, have been found to be consistently elevated in involuntary smokers. Levels of these metabolites are 1–5% as great as those found in smokers. The data demonstrating uptake of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, a lung carcinogen in rodents, by nonsmokers are supportive of a causal link between exposure to secondhand tobacco smoke and development of lung cancer.

The exposure of experimental animals, primarily rodents, to secondhand tobacco smoke has several biological effects that include (i) increases or decreases in the activity of phase I enzymes involved in carcinogen metabolism; (ii) increased expression of nitric oxide synthase, xanthine oxidase and various protein kinases; (iii) the formation of smoke-related DNA adducts in several tissues; and (iv) the presence of urinary biomarkers of exposure to tobacco smoke.

In adult experimental animals, sidestream tobacco smoke has been found to produce changes that are similar to those observed with exposure of humans to secondhand tobacco smoke. These include inflammatory changes in the airways and accelerated formation of arteriosclerotic plaques. Although the changes are often comparatively minor and require exposure to rather elevated concentrations of sidestream smoke, they support the results of human epidemiological studies. During pre- and postnatal exposure, sidestream smoke produces intrauterine growth retardation, changes the pattern of metabolic enzymes in the developing lung, and gives rise to hyperplasia of the pulmonary neuroendocrine cell population. In addition, it adversely affects pulmonary compliance and airway responsiveness to pharmacological challenges.

In humans, involuntary smoking is associated with increased concentrations of mutagens in urine. Some studies have shown a correlation of urinary mutagenicity with concentrations of urinary cotinine. Increased levels of sister chromatid exchanges have not been observed in involuntary smokers; however, there is some indication of elevated levels in exposed children. Lung tumours from nonsmokers exposed to tobacco smoke contain *TP53* and *KRAS* mutations that are similar to those found in tumours from smokers. The genotoxicity of sidestream smoke, 'environmental' tobacco smoke, sidestream smoke condensate or a mixture of sidestream and mainstream smoke condensates has been demonstrated in experimental systems *in vitro* and *in vivo*.

5.5 Evaluation

There is *sufficient evidence* that involuntary smoking (exposure to secondhand or 'environmental' tobacco smoke) causes lung cancer in humans.

There is *limited evidence* in experimental animals for the carcinogenicity of mixtures of mainstream and sidestream tobacco smoke.

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

In addition, the Working Group noted that there are published reports on possible carcinogenic effects of secondhand tobacco smoke in household pet dogs.

Overall evaluation

Involuntary smoking (exposure to secondhand or 'environmental' tobacco smoke) is *carcinogenic to humans (Group 1)*.

For definition of the italicized terms, see Preamble.

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 μm (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 910–920 °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*′-Nitrosonornicotine (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).

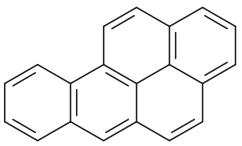
Volatile hydrocarbons in cigarette smoke include 1,3-butadiene, a powerful multi-organ carcinogen in the mouse, and benzene, a known human leukaemogen. 1,3-Butadiene (20–40 µg/cigarette) and benzene (12–50 µg/cigarette) are two of the most prevalent strong carcinogens in cigarette smoke (IARC, 2004a).

In summary, cigarette smoke is an exceedingly complex mixture which contains over 5300 compounds including multiple toxicants and carcinogens.

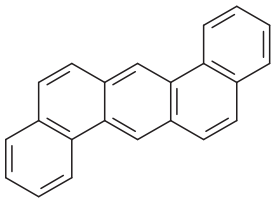
1.2.2 Smoke from other tobacco products

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).

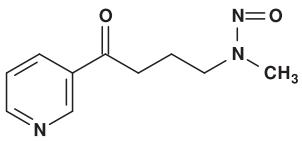
Fig. 1.1 Structures of some representative tobacco smoke carcinogens



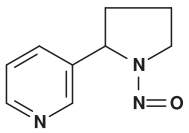
benzo[*a*]pyrene (BaP)



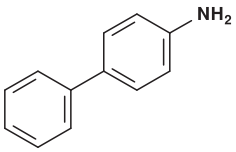
dibenz[*a,h*]anthracene



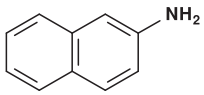
4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)



N'-nitrosonornicotine (NNN)

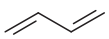


4-aminobiphenyl



2-naphtylamine

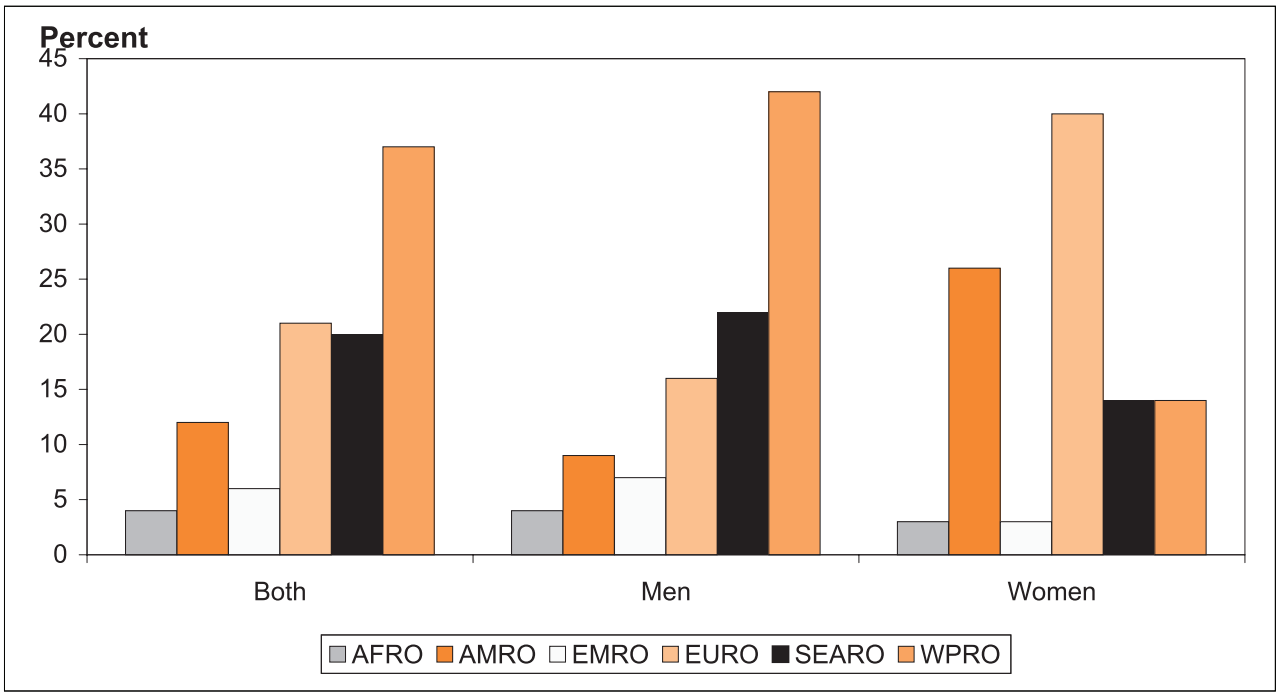
benzene



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

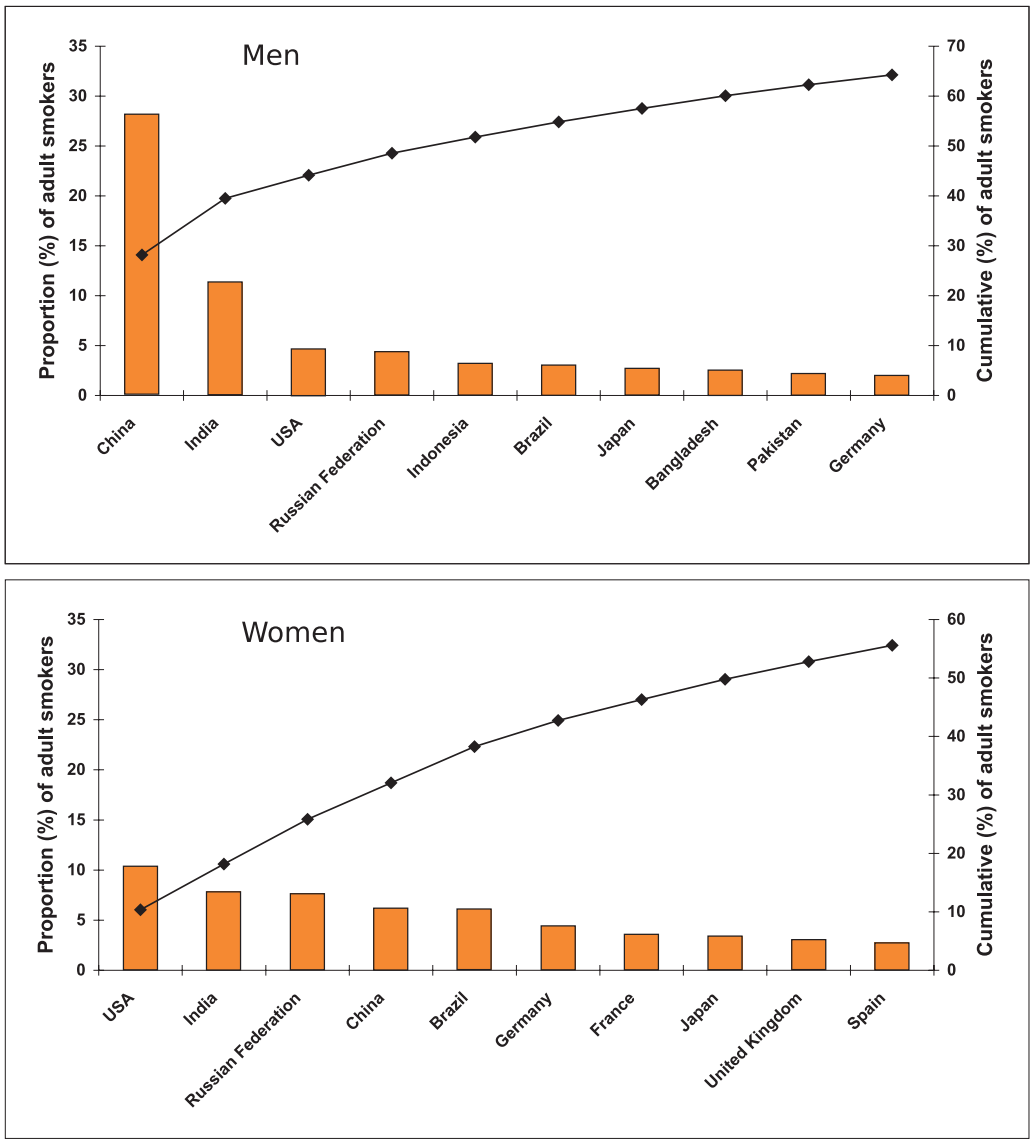
Data on smoking tobacco are available from WHO's Global Infobase (www.who.int/infobase) and the WHO Global Health Observatory (www.who.int/gho/en) – repositories of information on tobacco use and other risk factors in young people (13–15 years old) and adults (aged 15 years and over). The data span several 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). 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).

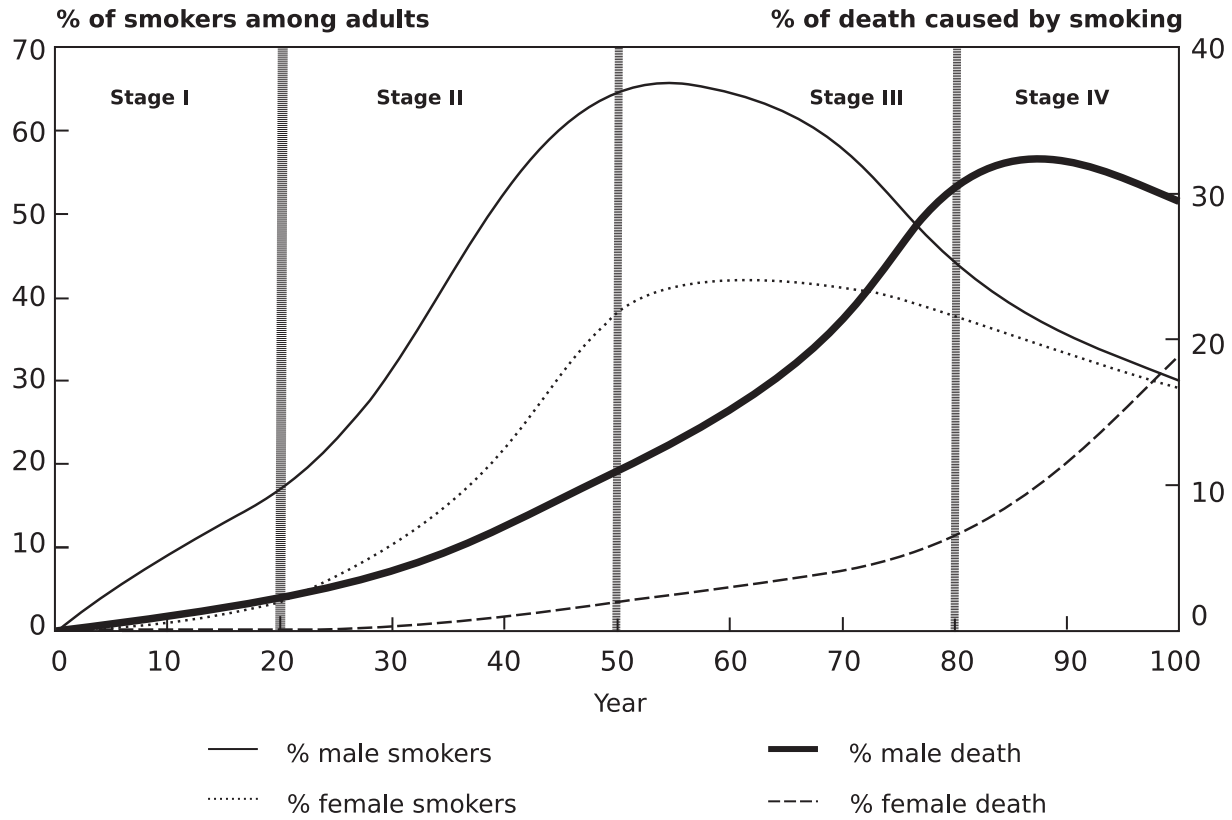
Stage 1 is characterized by low smoking prevalence in men (less than 15%) and very low in women (less than 10%). Death and disease from smoking are not apparent in this phase, as nearly all health effects from smoking are related to past smoking habits and their cumulative effects rather than current 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

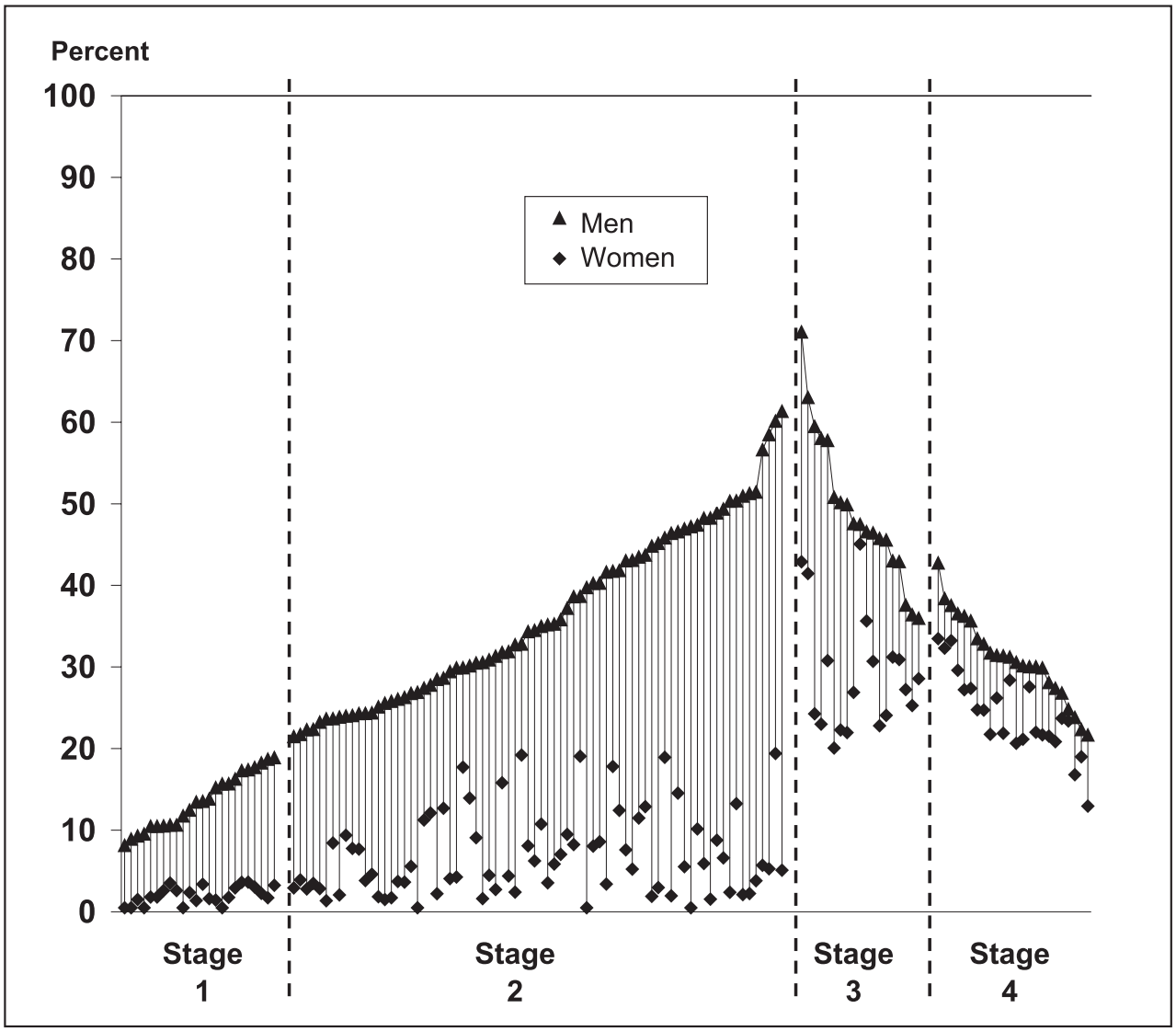
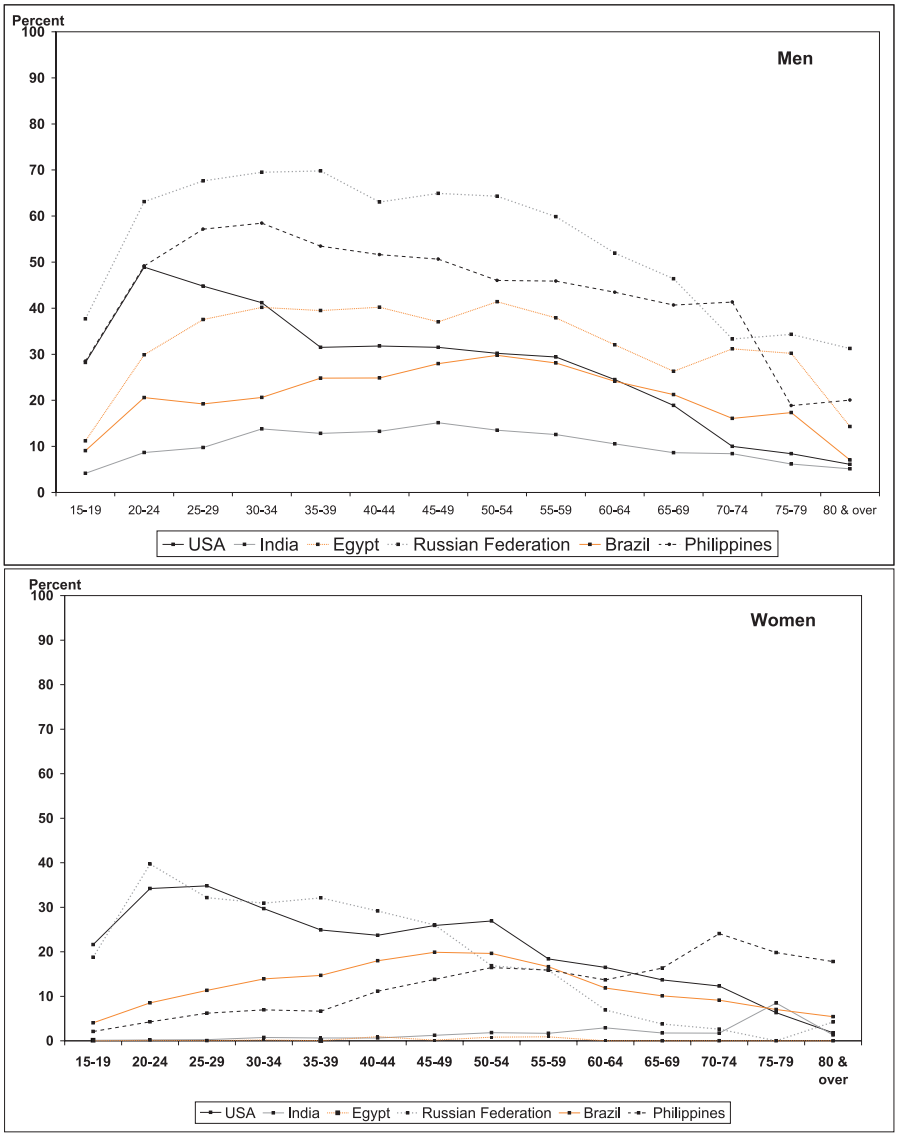


Fig. 1.6 Age-specific rates of smoking prevalence, in men and women in 2009



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 multilateral evidence-based treaty on tobacco control

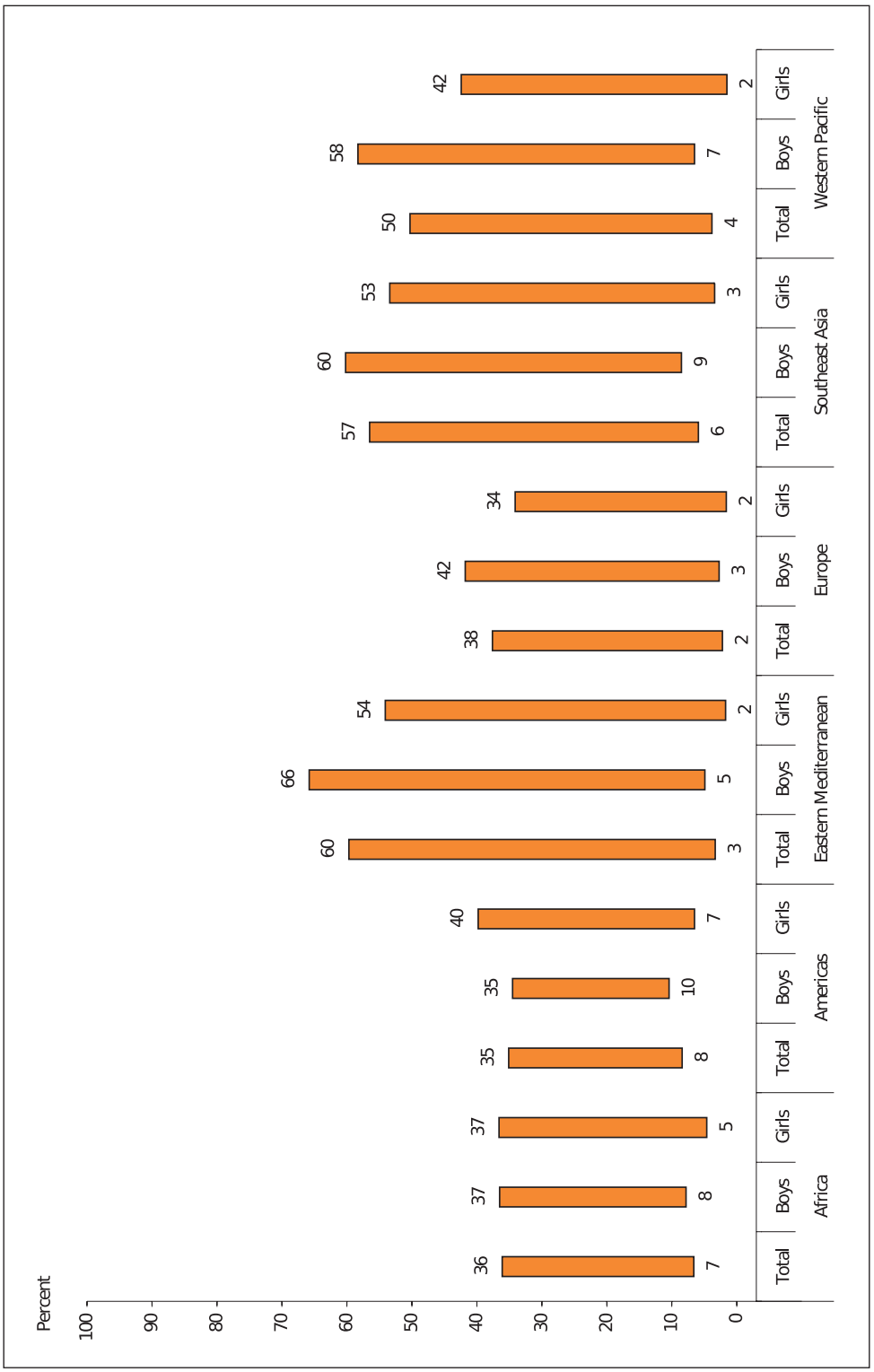
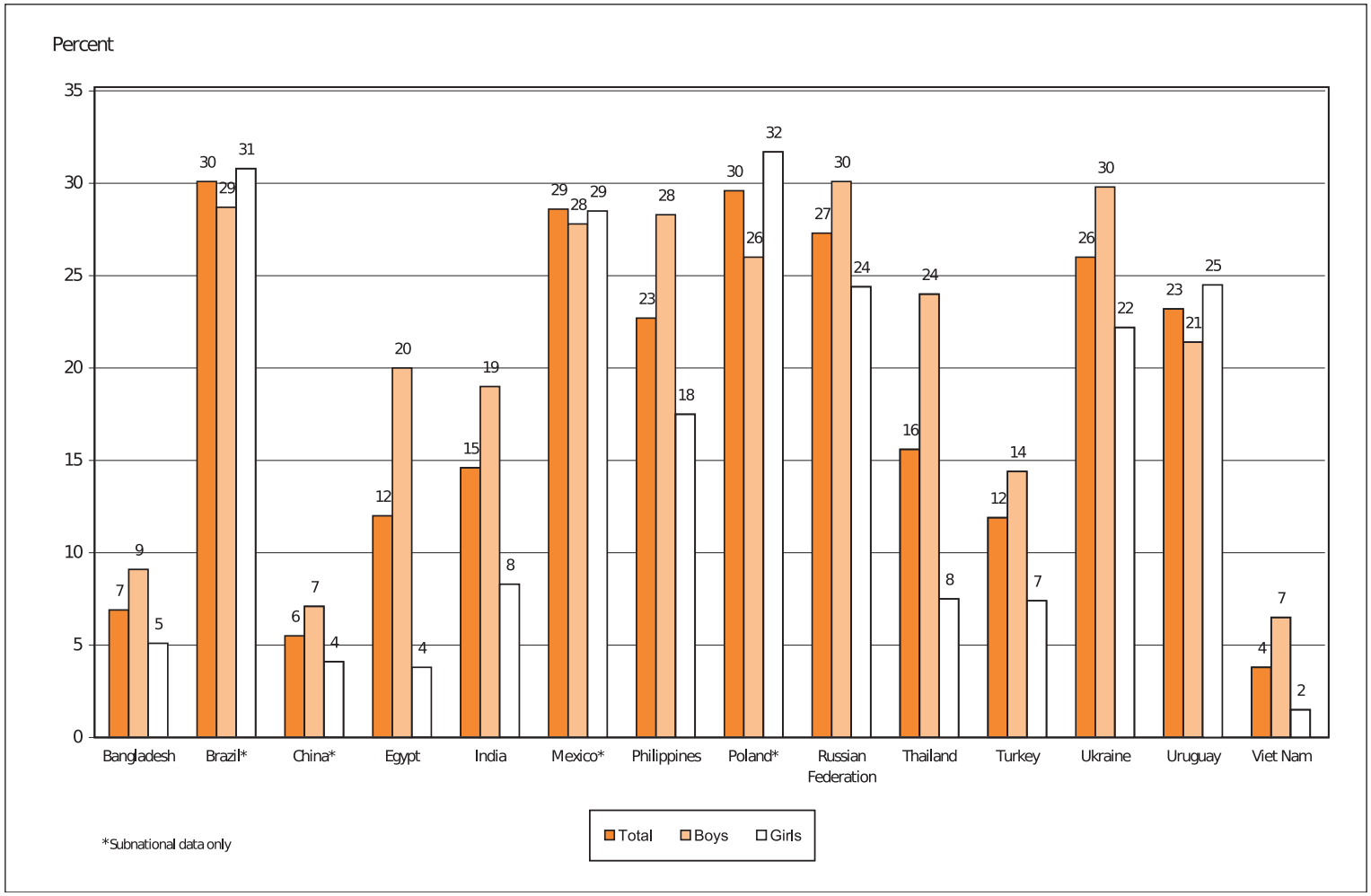


Fig. 1.7 Prevalence of current tobacco use in youth for selected countries, 2005–2009

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 polycylic 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 Ile^{462}Val was associated with lung cancer susceptibility among Caucasians or African Americans. Among Caucasians, however, CYP1B1 Leu^{432} 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 Leu^{432} 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 (re2909430), 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/m^3. The absolute risks of lung cancer by age 75 years at radon concentrations of 0, 100, and 400 Bq/m^3 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 CY1A1*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 (α(1)ATD) is one of the most common genetic disorders, especially among European descendents. Recent results suggest that α(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/mm^3), 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 sinonasal 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. Friborg *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; Friborg *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 underreport 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 hepatis 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 (Kiemeney *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 (Linet *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 (McDivit *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 Malmø, 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 progestogen 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 our 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 (Ile^{143}Val) 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) reviewd 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 lifestyle 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, socioeconomic 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 areodigestive 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 $B6C3F_1$ 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/m^3 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	Alveologenic adenocarcinomas: M–4/100 (alveologenic AdC) Controls–0/100 F–4/100 (alveologenic AdC) 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)F_1 (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%)	$P = 0.035$	
	100 M, 89 F Whole-body, gas phase of fresh cigarette smoke, concentration (NR), 2 puffs, 1 × /d, lifetime (26 mo)	NR	M: Lung A–1/44 (2%) Controls–8/106 (7%)	NS	
			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%)	$P = 0.15$	
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 A: 93/330 (28%) Sham-exposed controls–22/326 (7%)	$P < 0.001$ $P < 0.007$	
			Bronchiolar papillomas: 15/330 (4%) Controls–0/326	$P < 0.001$	
			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 *et al.* (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	$P < 0.001$	
			Lung AdC: 7/38 (18%) Controls–0/38	$P < 0.01$	
			Kidney A: 6/16 (16%) (F only) Controls–0/21	$P < 0.01$	
			Liver carcinomas: 2/16 (5%) (F only) Controls–0/21		
Rats, Wistar (F) Davis *et al.* (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 *et al.* (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 alveologenic C) Controls–3/93 (3%)	$P < 0.05$	
			Subcutaneous sarcomas at forelimb ulceration sites: 21/80 (26%) Controls–0/93	$P < 0.05$	
Rats, CDF® (F344)/CrlBR (M, F) Finch *et al.* (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%)	$P < 0.05$	
			F: LCS–4/145 (3%) HCS–6/83 (7%) Filtered air–0/113	$P < 0.05$	

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%)	NS	
			Lung AdC– LCS 1/178 (1%) HCS 5/82 (6%) Controls 3/118 (3%)	NS	
			All lung tumours– LCS 4/178 (2%) HCS 7/82 (8%) Controls 4/118	NS; trend, $P = 0.055$	
			Nasal cavity (all tumour types)– LCS 1/178 (1%) HCS 5/82 (6%) Controls 1/118 (1%)	$P = 0.032$ (HCS); trend, $P = 0.010$	
			F: Lung A– LCS 7/175 (6%) HCS 7/81 (9%) Sham-exposed controls 0/119	NS (LCS); $P < 0.001$ (HCS)	
			AdC– LCS 4/175 (2%) HCS 4/81 (5%) Controls 0/119	NS	
			All lung tumours– LCS 10/175 (6%) HCS 11/81 (13%) Controls 0/119	$P = 0.023$ (LCS); $P = 0.001$ (HCS)	
			Nasal cavity (all tumour types)– LCS 0/175 HCS 3/81 Controls 0/119	$P = 0.020$ (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.* (1973)	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.* (1977)	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%		
Rats, Inbred BIO 15.16 & Inbred BIO 87.20 (M) Bernfeld *et al.* (1974)	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) Bernfeld *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_2O_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)/CrlBR (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 *et al.* (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 *et al.* (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)	$P < 0.01$ $P < 0.01$
Syrian golden (M) Harada *et al.* (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)	$P < 0.05$

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/m^3 (LCS and HCS groups, respectively). At 12 weeks, rats were removed from smoke chambers and exposed nose-only to plutonium oxide ($^{239}PuO_2$) 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 $^{239}PuO_2$ were higher than those in animals exposed to $^{239}PuO_2$, 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 1R4F 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[e]: 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 1R4F 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

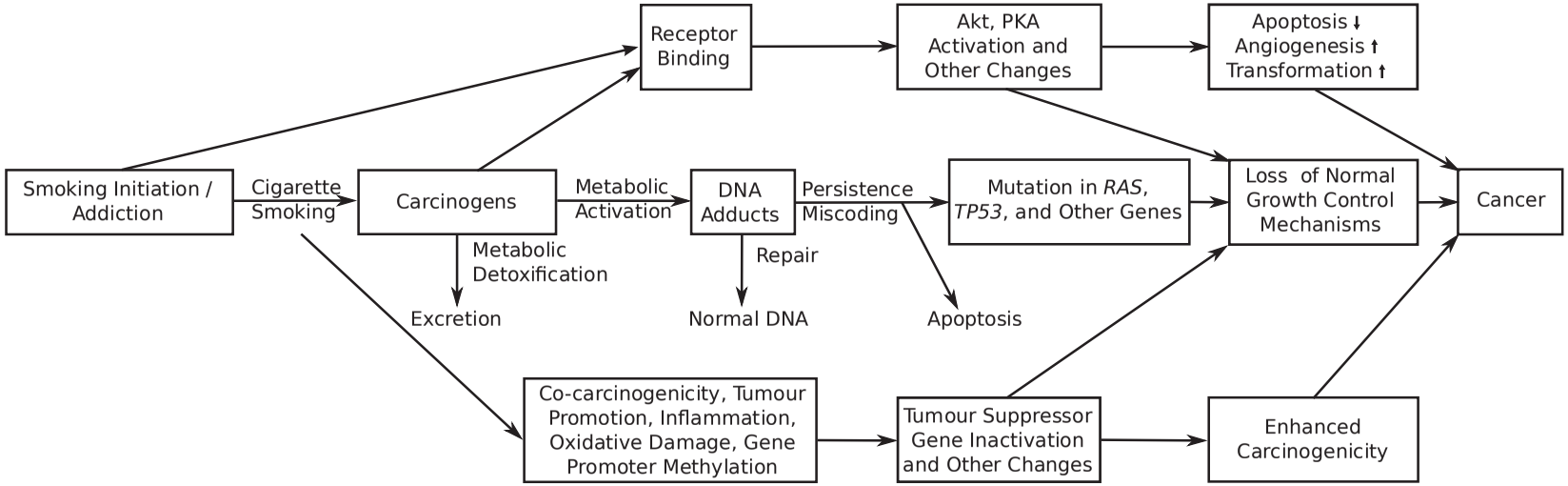
A conceptual model for understanding mechanisms by which tobacco smoke causes cancer is shown in Fig. 4.1 (Hecht, 1999, 2003). This model also applies to smokeless tobacco and other forms of smoked tobacco and, in theory, to second-hand tobacco smoke since it contains all of the same carcinogens and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

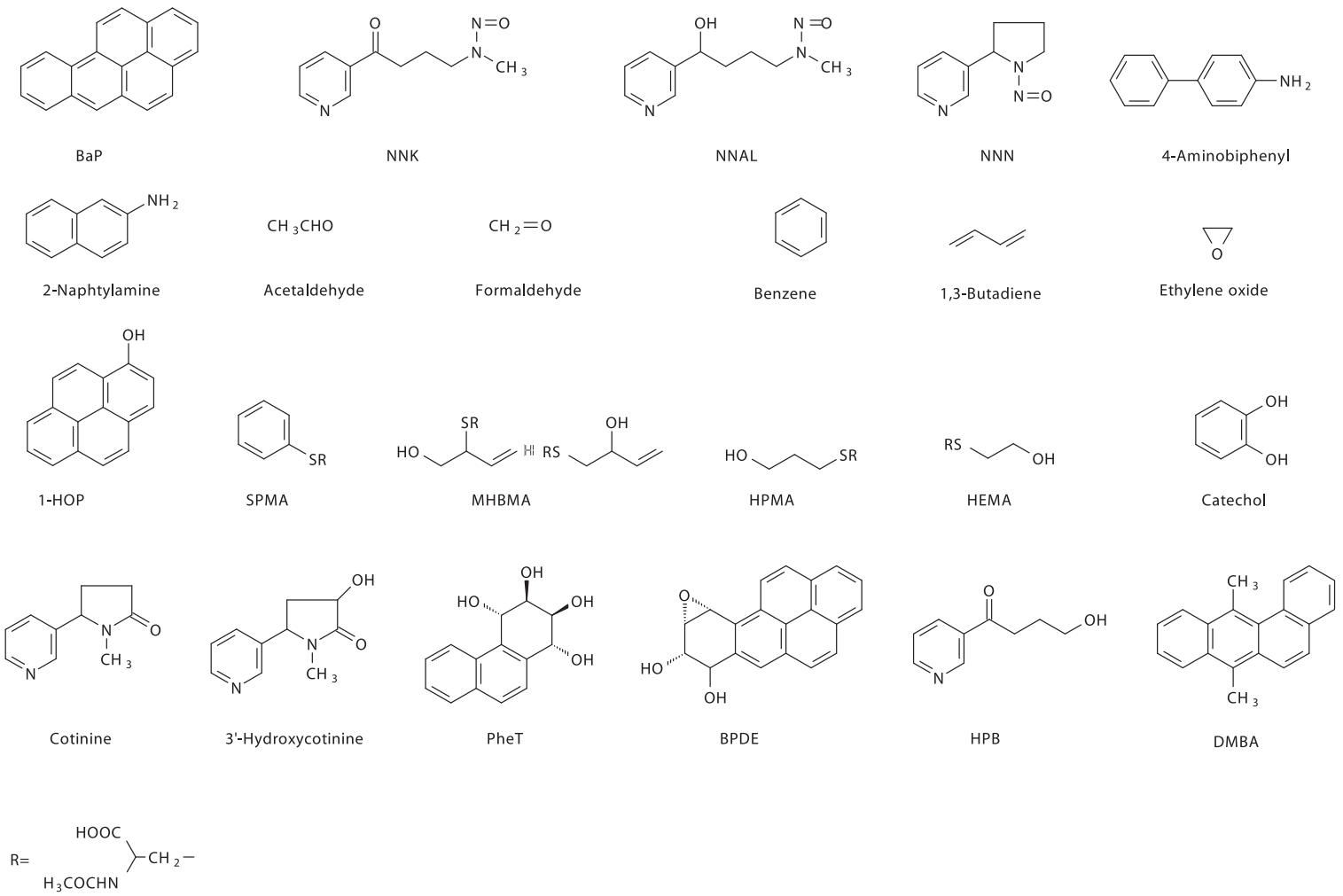
mixture of carcinogens and toxicants. There are over 60 carcinogens in cigarette smoke that have been evaluated in the previous *IARC Monograph* as having *sufficient evidence* for carcinogenicity in laboratory animals (IARC, 2004a), sixteen of which are considered to be *carcinogenic to humans (Group 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



BaP, Benzo[*a*]pyrene; BPDE, Benzo[*a*]pyrene diol epoxyde; DMBA, dimethylbenz[*a*]anthracene; 1-HOP, 1-hydroxypyrene; HEMA, 2-hydroxyethyl-mercapturic acid; HPMA, 3-hydroxypropyl-mercapturic acid; HPB, 4-hydroxy-1-(3-pyridyl)-1-butanone; HPMA, 3-hydroxypropyl-mercapturic acid; MHBMA, monohydroxybutyl-mercapturic acid; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, *N*'-nitrosonornicotine; PheT, phenanthrenetetrol; SPMA, S-phenyl-mercapturic acid

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‴- P^1, P^3-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

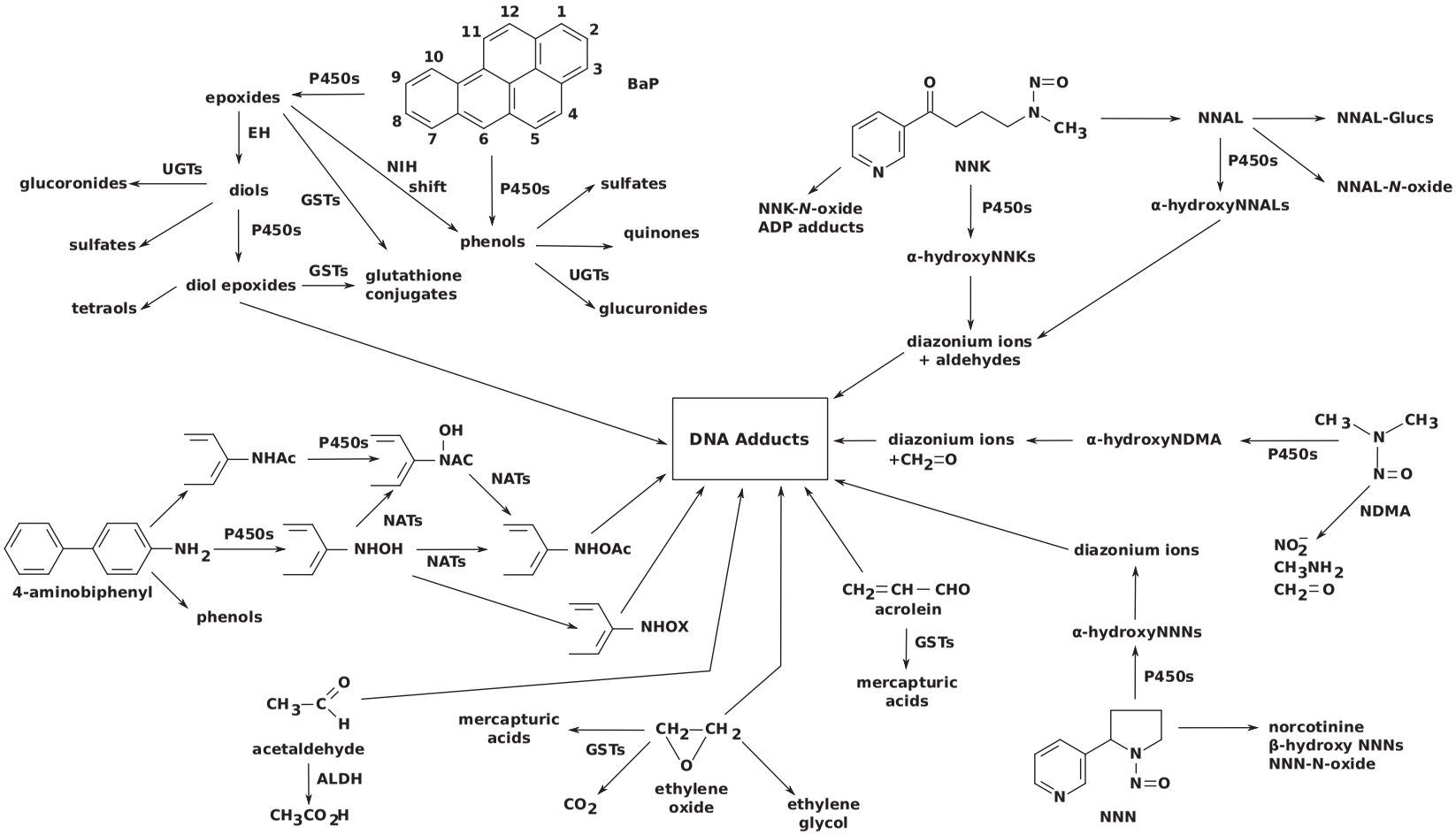
Serum and saliva metabolites have been used as biomarkers much less often than urine metabolites. The most frequently measured tobacco smoke toxicant in serum and saliva is cotinine, documented as a useful biomarker of cigarette smoking in many studies (Lee, 1999; Hukkanen *et al.*, 2005). Total NNAL 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

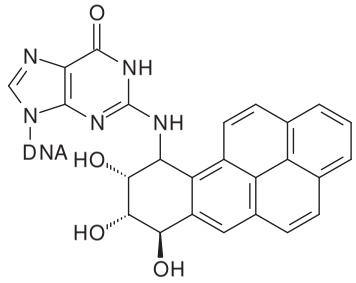
Fig. 4.3 Overview of metabolism and DNA adduct formation from eight tobacco smoke constituents



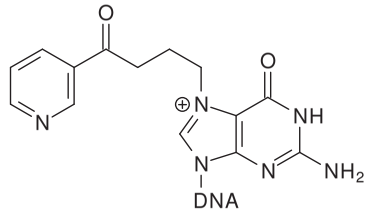
4-ABP, 4-aminobiphenyl; AC, acetyl; ADP, adenosine diphosphate; ALDH, aldehyde dehydrogenase; AKR, aldo-ketoreductase; B[*a*]P, benzo[*a*]pyrene; EH, epoxide hydrolase; Gluc, glucuronide; GSTs, glutathione *S*-transferases; NATs, *N*-acetyltransferases; NDMA, *N*-nitrosodimethylamine; NIH shift, phenomenon of hydroxylation-induced intramolecular migration; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, *N*'-nitrosonornicotine; 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).

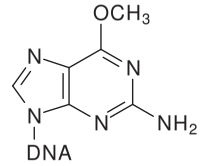
Fig. 4.4 Structures of some DNA adducts of tobacco smoke constituents



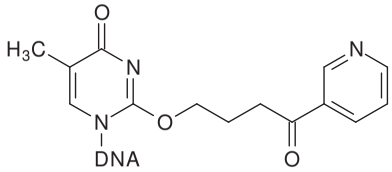
BPDE-N^2-dG



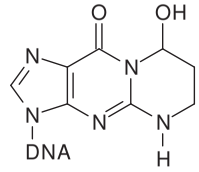
7-POB-dG



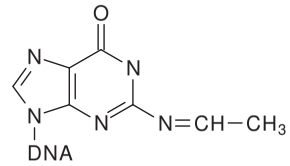
O^6-methyl-dG



O^2-POB-T



γ-OH-1,N^2-propano-dG
(from acrolein)



N^2-ethylidene-dG

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 of amol 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.

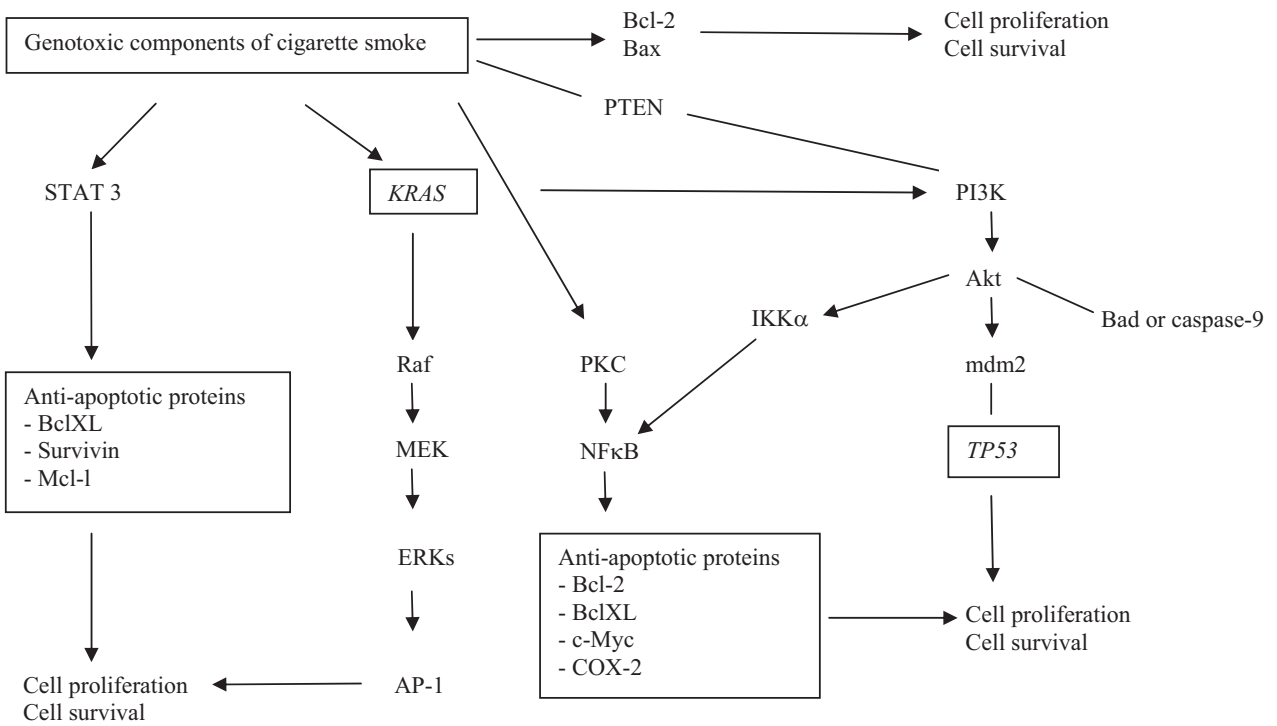
Spira *et al.* (2004) analysed global gene expression in bronchial epithelial cells and found that the expression levels of metabolizing and antioxidant genes had reverted to control levels after two years of smoking cessation. However, expression of potential oncogenes and tumour-suppressor genes never 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-κB*, 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 (PGE_2) 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; Ile^{462}Val). 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; Thr^{461}Asn) (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; Kamataki *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*; $Ile^{105}Val$), in exon 6 ($Ala^{114}Val$), 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* $Ile^{105}Val$ polymorphism; but the pooled analysis suggested an overall statistically significant mild association between lung cancer and homozygosity or heterozygosity for the Val^{105} 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 polymophisms 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 biomarker 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; Kuljukka-Rabb *et al.*, 2002; Kato *et al.*, 2004). The associations seen between *GSTT1* 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; Kuljukka-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* $Ile^{462}Val$ 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 (Kuljukka-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 genotoxicants 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 (Viezzer *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* (*Ile*^{462}*Val*) 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 ^{32}P-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, genotoxicants, 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 heamoglobin 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 etiologic 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 N^6-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 (*Thr*^{461}*Asn*, *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) Asp^{148}Glu, 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 *P16*^{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

haemotopoietic 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 (16α-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 16α-OHE1 or to 2-OHE1 or to 4-OHE1. 16α-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 16α-hydroxylation pathway. However, the ratio of urinary 2-OHE1:16α-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 be 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 (Km) 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 (Tuyns *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 O^6-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 accesory 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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