

# DE MINIMUS NON CURAT LEX – VIRTUAL THRESHOLDS FOR CANCER INITIATION BY TOBACCO SPECIFIC NITROSAMINES – PROSPECTS FOR HARM REDUCTION BY SMOKELESS TOBACCO

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## Abstract

Whereas the impact of tobacco specific nitrosamines in smokers is obscured by the presence of numerous other carcinogens and promoters, for smokeless tobacco virtually all the carcinogenic potential is associated with 4-(nitrosomethylamino)-1-(3-pyridyl)-1-butanone (NNK) and N<sup>2</sup>-nitrosornicotine (NNN). In some countries exposure to smokeless tobacco with extremely high nitrosamine concentrations have been found to induce cancers in the head-neck region, whereas three recent large epidemiological studies failed to detect any such risk with respect to Swedish low-nitrosamine snuff.

This review deals with quantitative aspects of DNA adduct formation from NNN and NNK in relation to the background levels ubiquitously found in healthy humans without known exposures to either tobacco or alkylating agents. The lack of significant increases of pro-mutagenic O<sup>6</sup>-methylations and DNA pyridyloxobutylations seen in smokers, as well as the negative outcome of the Swedish epidemiological studies, can be expected on basis of extrapolation of the dose response relationships found in rodents to actual exposures to NNK and NNN in Swedish snuff or from smoking.

Sweden has the lowest prevalence of male smokers and smoking related diseases in the Western World, which has been ascribed to the fact that more than 20% of the grown up male population uses snuff. Smokeless tobacco represents an inexpensive and effective alternative to nicotine delivering products like nicotine patch, spray or gum. Considering that all other tobacco products are freely marketed, the ban on low-nitrosamine snuff in all countries in EU except Sweden is difficult to defend on either medical or ethical grounds.

## Key words:

Cancer, Tobacco specific nitrosamines, Smokers, Epidemiology studies

## EXPOSURE TO NITROSAMINES

The presence of nitrosamines like N-nitrosodiethylamine (NDEA) and N-nitrosodimethylamine (NDMA) in some foods and beverages as well as their formation in the acid environment of the human stomach has been a matter of considerable concern [1–3]. The finding of N-nitrosopyrrolidine in concentrations as high as 1400 ng/m<sup>3</sup> from the grilling of bacon [4] should also not be neglected at least for some exposure scenarios relevant to indoor air quality [5]. However, only in a few cases has it been possible to

provide epidemiological evidence for a causal association between nitrosamine exposure and cancer, as e.g., for nasopharyngeal carcinoma in populations consuming Cantonese-style pickled fish containing high levels of NDMA as well as NDEA [3,6–7].

The highest exposure to humans of nitrosamines is caused by tobacco. The plant genus *Nicotiana*, and to a much lesser extent related species of the Solanaceae family, contain several so called tobacco-specific alkaloids, mainly nicotine, nor nicotine, anabasine, anatabine, and

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myosmine. However, with respect to myosmine, this alkaloid has recently, in addition, been demonstrated to be present in significant quantities in a large number of foods and dietary components [8–10]. During curing and processing, tobacco-specific nitrosamines (TSNA) like 4-(nitrosomethylamino)-1-(3-pyridyl)-1-butanone (NNK), N<sup>2</sup>-nitrosornicotine (NNN), N<sup>2</sup>-nitrosoanabasine (NAB), and N<sup>2</sup>-nitrosoanatabine (NAT) are formed from the corresponding alkaloids. Myosmine is readily nitrosated giving rise to NNN or the same alkylating intermediate as is formed metabolically from NNN [11]. Small amounts of the volatile nitrosamines N-nitrosodimethylamine, N-nitrosopiperidine and N-nitrosopyrrolidine are also formed.

Nitrate is present in significant concentrations in tobacco as well as in salad and other leafy plants. Reduction of nitrate by microorganisms to nitrite during processing seems to constitute the mechanism underlying the nitrosation of amines present in tobacco. The high potency of the systemic carcinogens NNK and NNN in the rodent, inducing tumors in lung, liver nasal cavities, esophagus and exocrine pancreas, has been a matter of serious concern [12], and a quantitative risk assessment of TSNA with respect to the low-dose region constitutes the main topic for this article. Tobacco smoke contains a plethora of carcinogens other than nitrosamines, notably several potent polycyclic aromatic hydrocarbons (PAH), aryl amines, ethylene oxide, butadiene, acrylonitrile, as well as promoting agents which makes it extremely difficult to assess the relative importance of TSNA in the induction of smoking-related cancers. Thus, there is so far no adequate proof of a causal relationship between TSNA uptake and human lung cancer, and the mutation spectra of the *p53* suppressor gene in lung tumors from smokers are more consistent with the genotoxic action from polycyclic aromatic hydrocarbons (PAH) than that expected from TSNA [13–15]. In contrast to tobacco smoke, the carcinogenic potential of smokeless tobacco products (snuff and chewing tobacco) is believed to be wholly associated with TSNA, and users of this kind of tobacco products, therefore, offer a unique opportunity for the study of an association between TSNA and human cancers. This is especially so for Sweden, where more than

20% of the grown up male population uses moist oral snuff (snus), while at the same time smoking prevalence is the lowest among the industrialized countries [16]. Snuff use is also prevalent in the US, Canada, Norway, North Africa, Central and South East Asia, and to a lesser extent in Finland, Denmark and Mexico. It has been estimated that in 1991 more than 5 million US Americans were “snuff dippers” [17]. In Bavaria, parts of Austria and Slovenia there is also a limited use of nasal snuff.

In this context it is important to realize that the levels of TSNA in smokeless tobacco may differ by orders of magnitude depending on origin and manner of processing. Sudanese “Toombak”, that has been associated with cancers of the oral cavity, is prepared from locally grown *Nicotiana rustica* and may contain up to 7870 µg/g (av. 2310 µg/g) of NNK and up to 3080 µg/g (av. 1130 µg/g) dry weight of NNN [18]. These levels should be compared to the average total TSNA values for Swedish moist snuff of 18 µg/g dry weight in 1983, a value that had dropped to about 9 µg/g in 1992 [19,20], and further down to 1 µg/g in 2002 [21]. Swedish snuff (snus) is produced from unfermented tobacco by a special heat sterilization process that results in comparatively low levels of TSNA. In the US, there has been a similar development for many snuff products. In Table 1 the nitrosamine and nicotine contents of moist snuff products from various sources are listed. For the sake of comparison, the TSNA levels found in 3 commercial US brands of dry snuff as well as representative values for US cigarette tobaccos have also been included. The fact that locally grown tobacco used in many oral smokeless tobacco products that are consumed in South East Asia are mixed with areca nuts and betel leaves [12,22] – products that contain arecoline and associated nitrosamines – further underlines the serious mistake made by several reviewers, including the International Agency for Research on Cancer (IARC) [12,23], when treating snuff as a well-defined and homogeneous tobacco product. Morpholine, previously used in the manufacture and/or packaging of certain tobacco products like snuff, gave rise to the carcinogen N-nitrosomorpholine found to occur at levels up to 0.7 µg/g in some US snuff products. Similarly, the presence of N-nitrosodiethanolamine at 0.3–3.3 µg/g in tobacco

**Table 1.** Nicotine and nitrosamine content (based on dry weight) of tobacco products from various sources

Source	Nicotine (mg/g)	NNK (µg/g)	NNN (µg/g)	Total TSNA (µg/g)
Moist oral snuff products				
Sweden				
13 samples [19–21,24]		1.8 (0.59–3.8)	12.5 (4.7–77.1)	19.8 (5.5–106)
1983 (36 samples)	8–18	1.6 (0.5–2.4)	7.6 (3.2–9.4)	18 (5–102)
1986 (34 samples)		1.5	8.6	16.0
1992 (20 samples)		1.3	3.8	8.8
2002 (24 samples)		0.2	0.5	1.0 (0.2–3.1)
Norway [19]				
1983 (2 samples)		6.6 (5.4–7.8)	42 (26–58)	77 (52–102)
Sudan [18]				
Toombak		2310	1130	3740
US				
3 samples [24]		2.8	23	45.3
5 samples [25]		1.3 (0.1–3.1)	16.6 (5.8–64.1)	85.6 (9.6–288)
2 samples of a new brand [26]	1.8/2.0	7/16	37/57	94/156
Copenhagen [27]				
Skoal, Original [27]	12.0 ± 0.7	1.9 ± 0.6	8.7 ± 1.4	17.2 ± 3.0
Fine cut	11.9 ± 1.3	1.3 ± 0.1	8.2 ± 1.3	14.9 ± 2.5
Skoal, Bandits [27]				
Straight	10.1 ± 0.8	0.9 ± 0.3	5.1 ± 1.0	8.2 ± 1.7
Kodiak [27]				
Wintergreen	10.9 ± 0.8	0.6 ± 0.2	6.3 ± 1.1	11.0 ± 2.4
Hawken [27]				
Wintergreen	3.2 ± 0.2	0.2 ± 0.04	3.1 ± 0.3	4.1 ± 0.4
Canada [28]				
		3.2–5.8	50–7	209–260
Dry snuff products				
US [25]				
3 brands	13 (12–14)	8 (2–15)	46 (15–84)	78 (37–135)
Cigarette tobacco				
US [29]				
Flue cured tobacco		1.8	0.9	4
Burley tobacco		1	5	10

NNK – 4-(nitrosomethylamino)-1-(3-pyridyl)-1-butanone;

NNN – N'-nitrosornicotine;

TSNS – tobacco specific nitrosamines.

was probably due to the agricultural use of diethanolamine as solubilizer for the growth inhibitor maleic hydrazide [30,31]. The contents of volatile nitrosamines in Swedish snuff have generally been low (mean for 14 samples, 0.008 µg/g, 1982), and the finding in 1981 of two Swedish products that contained N-nitrosodimethylamine and N-nitrosopyrrolidine in levels ranging from about 0.1 to 0.2 µg/g dry weight must be regarded as an exception [32]. Today, the products found on

the US as well as on the Swedish market are practically free from these nitrosamines [33].

As for some foods, the weakly carcinogenic non-tobacco specific nitroso amino acids N-nitrososarcosine (NSAR), and 3-(methylnitrosamino)-propionic acid (MNPA), and the moderately active 4-(methylnitrosamino)-butyric acid (MNBA) are also formed in snuff upon processing and storage. However, except for the single discovery of rela-

tively high levels of MNPA and MNBA in one US brand [26], the contributions of these nitrosamines to total cancer risk seem to be negligible [19,27].

In fire-cured tobacco elevated concentrations of PAH are bound to occur. Benzo[a]pyrene (BaP), an indicator of PAH exposure, and that has a carcinogenic potency comparable to that of NNK [34], may be present in some US snuff products up to about 60 ng/g [35]. However, this exposure is negligible in comparison with that from TSNA, and the cited PAH content is actually less than for a Frankfurter sausage grilled over open fire that can boost the total PAH content of up to 1600 ng/g out of which some 212 ng/g may consist of BaP [36]. The impact of dietary PAH exposures derived from grilled meats is reflected in a marked increase in DNA adduct levels [37,38]. Finally, tobacco frequently also contains low levels of alpha particle emitters like  $^{210}\text{Po}$  mainly derived from natural radioisotopes present in bedrock or phosphate fertilizers, and the activity in Swedish moist snuff has been measured and found to be in the range of 22–120 Bq/kg dry weight. The dose of ionizing radiation from this source must be considered as totally negligible in comparison, e.g., with the natural radiation background and other sources of ionising radiation [39]. In summary, because other agents present in smokeless tobacco only contribute to a minor extent to the total potential risk, an adequate quantitative cancer risk assessment should be based on exposure to NNK and NNN.

### METABOLIC TRANSFORMATIONS OF TSNA

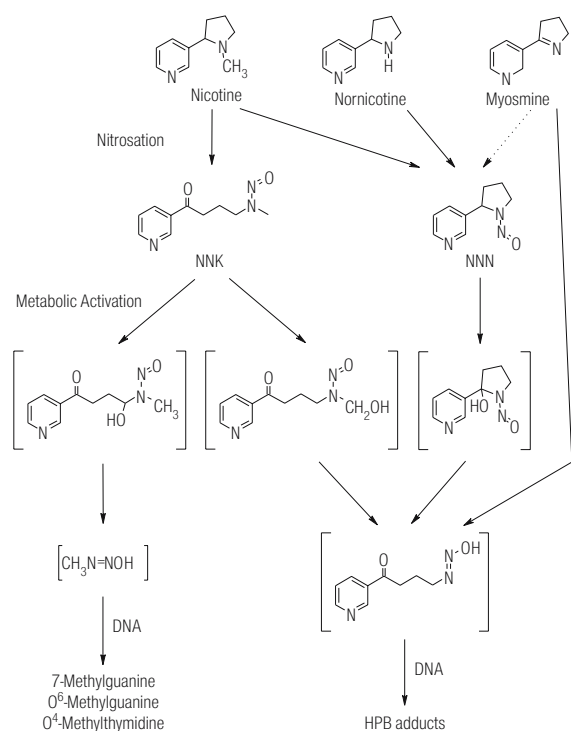
Little or no unchanged NNK or NNN is excreted with urine, and in mammals the carbonyl group of NNK is reduced to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), that is either conjugated with glucuronic acid and excreted in urine, or further metabolized by hydroxylation [40]. Like NNK, NNAL has been found to be a potent experimental carcinogen [35].

By one pathway the methylene group adjacent to the N-nitroso group of NNK is hydroxylated producing an intermediate that apparently spontaneously decomposes to the directly methylating agent, methanediazohydroxide. Upon reaction with DNA, the latter will give rise mainly to 7-methylguanine and O<sup>6</sup>-methylguanine as well as small

amounts of O<sup>4</sup>-methylthymine. Reduction of the carbonyl group of NNK leads to the formation of 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butan-1-ol (NNAL) which may form glucuronides, or undergo methylene hydroxylation like NNK [40].

A second hydroxylation pathway involves  $\alpha$ -methylhydroxylation to give  $\alpha$ -hydroxymethyl-NNK, an intermediate that is sufficiently stable to form the corresponding glucuronide. However, by the main route of conversion,  $\alpha$ -hydroxymethyl-NNK leads to the formation of 4-(3-pyridyl)-4-oxobutane-1-diazohydroxide that introduces covalently bound pyridyloxobutyl adducts at nucleophilic centers in DNA and proteins. Mild acid or alkaline hydrolysis of these adducts releases 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB), which can be derivatized and conveniently analyzed by GC-MS [40–42]. Minor metabolic pathways involve the hydroxylation of the 6-position of the pyridine ring of NNK, and pyridine-N-oxidation of NNK and NNAL. Studies using human lung and liver microsomal preparations seem to indicate that the NNAL pathway is more important, and direct hydroxylation less so in man than in rodents [40]. Similarly to rodents, NNK is rapidly and extensively metabolized by the  $\alpha$ -hydroxylation pathway in the rhesus monkey [43], whereas this metabolic pathway as well as pyridine-N-oxidation seem to be much less prominent in human tissues [44]. The inhibition of the NNK  $\alpha$ -hydroxylation pathway by nicotine, cotinine and NNN constitutes an important observation, where the suppression of metabolic activation involves a shift towards increase of detoxification. As a consequence there is an increased excretion of pyridine-N-oxides and NNAL-glucuronides as well as a reduction in the generation of HPB haemoglobin adducts [42,45]. There is also considerable species variation with respect to the metabolism of NNK and NNN. Thus, The extent of  $\alpha$ -carbon hydroxylation of NNN and NNK in human tissues was only 1/10th to 1/100th of that in animal tissues [44].

Upon oxidative metabolism NNN generates the same reactive diazohydroxide as is obtained upon  $\alpha$ -hydroxylation of the terminal methyl group of NNK, thereby inducing pyridyloxobutylation of proteins and DNA. NNN gives a complex pattern of metabolites in urine with N'-nitrososnicotine-1-N-oxide, 4-hydroxy-4-(3-pyridyl)-butyric acid, and 4-oxo-4-(3-pyridyl)-butyric acid as the main excretion products, which



HPB – 4-hydroxy-1-(3-pyridyl)-1-butanone.

**Fig. 1.** Main metabolic activation pathways of 4-(nitrosomethylamino)-1-(3-pyridyl)-1-butanone (NNK) and N'-nitrosornicotine (NNN).

also appear as main metabolites from NNK. In the exposure range 3–300 mg/kg, 86 to 91% of an i.p. administered dose is excreted as metabolites in rat urine [40,46]. The major metabolic activation pathways for NNK and NNN are summarized in Fig. 1.

## CARCINOGENICITY OF TSNA IN HUMANS

While the carcinogenic hazards of smoking are universally known, even among the medical profession there are a number of misconceptions of the cancer risks associated with smokeless tobacco. Local snuff that was produced in North Carolina between the World Wars as well as snuffs from Sudan and India with extremely high levels of TSNA have clearly been associated with cancers of the head-neck region [12,47,48]. Several later studies based on a limited material have been conducted in the US yielding conflicting results. There remains little doubt that the oral cancers observed in some studies can be ascribed to TSNA, although synergistic effects from the consumption

of alcohol and possibly also from smoking has probably contributed to the outcome of the Winn et al. study from the US. Based on this type of evidence, IARC [12,23] in Lyon classified oral snuff as a human carcinogen, and subsequently EU prohibited the marketing of oral snuff within the Community. However, in several large and well conducted epidemiological investigations that were subsequently carried out in Sweden [16,49–51], no increase in cancer risk could be detected. Sweden has the highest per capita consumption of moist oral snuff in the Western World, but the lowest incidence of cancers of the lip and oral cavity. Thus, it is 78% of that in Switzerland, 71% of that in US, 69% of that in Finland, 61% of that in Canada, 58% of that in Denmark, 45% of that in Austria, 39% of that in France and 13% of that in Sri Lanka [52].

Because of the large differences in use of snuff in males and females in Sweden (16% vs. 3% 1990), one would expect a much higher male/female ratio in oral cancers in Sweden than in other countries with low prevalence of male snuff users. However, the opposite is true. In 1990, age standardized incidence ratio figures for cancer of the lip and oral cavity in neighbouring Scandinavian countries [52] with much lower per capita consumption of smokeless tobacco were 2.0 (Sweden), 2.7 (Denmark), 2.6 (Finland), and 2.5 (Norway). It should be mentioned that a recently conducted prospective epidemiological study in 6779 American users also found no indication that snuff produced during recent decades induces either oral cancers or increases the incidence of total cancers [53].

When Sweden joined EU in 1995, this country was granted an exemption from the ban on snuff provided that the packages carried a cancer warning. However, on basis of the negative epidemiological studies carried out in this country, the Swedish Ministry of Health subsequently withdrew the cancer warning, a regulatory action that was sanctioned by the revised EU Tobacco Directive [54].

In a recent cohort study, Boffetta et al. [55] found no evidence that snuff affects the incidence of cancers of the esophagus, stomach, bladder, or kidney. On the other hand, and although the number of cases were small, they reported a statistically significant association between snuff use and risk for pancreatic cancer. However, this

study has some technical shortcomings that have been severely criticized [55]. Thus, for example, no adequate provisions had been made with respect to follow-up and confounding from alcohol abuse as well as pre-existing diabetes, both of which are important risk factors for cancer of the pancreas, and no follow-up of tobacco habits for the cohort was made. Finally, the purity of the snuff used by this cohort is unknown. Together with Iceland, Sweden has the lowest incidence of pancreatic cancer in Europe, and if at all snuff contributes to the development of this disease, its contribution is bound to be very modest. An analysis of a Swedish material is underway to assess this issue.

The Institute of Medicine, established by the US National Academy of Sciences in 1970 to examine policy matters in the area of public health, and acting as an advisory body to the US Federal Government, has stated that “Swedish snus (lower TSNA and nicotine levels than American brands) should be evaluated as a possible harm reduction product since two recent epidemiological studies have suggested that it does not increase the risk of oral cancer and has favorable cardiovascular outcomes” [56].

The Royal College of Physicians in London has expressed similar opinions [57], as have in a commentary in *The Lancet* Peter Boyle, the recently appointed new Director General of IARC, and Nigel Gray, previous president of the International Union Against Cancer [58]. Some researchers have tried to find fault with the Swedish epidemiological studies [59], but as will be discussed below, the negative outcome of the Swedish studies is actually to be expected on basis of molecular epidemiology and biochemical considerations.

### SMOKELESS TOBACCO KERATOSIS

It has been known for a long time that snuff use may induce certain soft tissue changes of the oral cavity known as “snuff dipper’s lesion”, snuff-induced leukoplakia, or smokeless tobacco keratosis [60]. Considerable confusion has been caused by equating these lesions with the preneoplastic leukoplakia induced by smoking, and that mostly involve the floor of the mouth, ventral tongue, and soft palate [61,62]. Larger studies have indicated that

whereas for smoking induced leukoplakias – where about 20% of the oral leukoplakias demonstrate some degree of dysplasia [63] – such changes are relatively rare in “snuff dipper’s lesions”. Thus, upon analyzing 114 biopsies from smokeless tobacco keratoses in a Swedish material, no single case of epithelial dysplasia was found [64]. In one prospective study in the US also no case of oral cancer was found during a ten-year period among 1550 persons with smokeless tobacco keratosis [65]. Prospective studies have also indicated that the histological changes induced by low-nitrosamine snuff are reversible upon cessation of use [66], although in rare cases progression to malignancy obviously cannot be excluded. It should be noted that the observations made in Scandinavia differ in general from the severe oral lesions that have been found in populations that use oral tobacco with high levels of nitrosamines or impure oral tobacco products that are mixed with betel leaves, areca nuts and lime [61].

### MODULATION OF THE CARCINOGENIC EFFECTS OF TSNA

The major role of diet as the most important factor in the etiology of avoidable cancers in the general population is now well established [67–69]. It has, for example, been estimated that about 25% of the incidence of colorectal cancer, about 15% of the incidence of breast cancer, and about 10% of the incidence of prostate cancer could be prevented if the population in a country like Poland would have adopted the traditional Mediterranean diet [70].

In animal experiments several compounds of plant origin, exemplified by tea polyphenols, ellagic acid, resveratrol, alpha-tocopherol, and the isothiocyanates, have demonstrated striking chemoprotective effects against the action of various potent carcinogens in rodents. As expected, the induction of tumors by TSNA represents no exception in this respect. In one experiment where NNN and NNK, with and without an extract derived from snuff (same total TSNA exposure) were swabbed in the oral cavity of rats, the yield of oral tumors was reduced from 8/30 in absence to 3/30 in presence of the extract [71]. These observations are consistent with the fact that, similarly to other plants,

tobacco contains antimutagens [72]. In particular, the benzyl as well as phenethyl isothiocyanates contained in cruciferous vegetables appear to be chemoprotective against the carcinogenic action of NNK [73–75]. The concentrations of TSNA used in the above mentioned study by Hecht and co-workers [71] – and where approximately a 60% protection was observed in presence of the snuff extract – were orders of magnitude higher than those to which users of snuff are exposed. It is therefore possible, that at more realistic exposures, and where the relation between the levels of protective agents and TSNA is more favorable, a still higher degree of protection can be attained. It is possible, though, that at least part of the inhibition can be ascribed to inhibition of  $\alpha$ -hydroxylation by nicotine and cotinine mentioned above [42,45].

#### **HIGH-TO-LOW DOSE EXTRAPOLATION OF CANCER RISK BASED ON MECHANISTIC CONSIDERATIONS**

It is generally accepted that cellular DNA damage that is misrepaired, or not repaired, constitutes a necessary, although not sufficient prerequisite for the development of malignant growth. Mainly based on theoretical considerations it has been assumed that the shape of the dose response curve for genotoxic carcinogens is linear in the low-dose range that is not accessible for epidemiological or experimental investigations. However, this approach does not take into account saturation of detoxification and repair mechanisms. Thus, the exceedingly efficient and virtually error-free repair of oxidative damages by the base excision DNA repair pathway represents a life-saving mechanism that is necessary for the survival of all higher organisms, and linear extrapolation from high to low doses for agents that induce this type of DNA damages is certainly not warranted. Similar considerations apply to agents that cause cancer by an indirect mechanism, like arsenic that induces genotoxicity by inhibiting DNA repair [76]. Likewise, linear extrapolation to zero dose is also not justified for genotoxic substances like antimony trioxide, formaldehyde and propylene oxide, where tissue damage, cellular proliferation and hyperplastic reactions

are required for tumor induction. The “mega mouse experiment” with 2-acetylaminofluorene [77] indicated that whereas correlation between dose and tumor incidence may appear linear for one target organ, this may not be so for other sites. Thus, while there seemed to be a strict proportionality between toxic endpoint and dose for liver, clear non-linear relationships for bladder tumors were found where the data shows good of fit to the non-linear probit model [78]. Finally, operation of mechanisms that involve trapping of the proximate carcinogenic agent may also result in sub-linear relationships resulting in what for practical purposes can be considered as a dose threshold. The extremely rapid formation of an enzyme-substrate complex between epoxide hydrolase and directly alkylating genotoxic epoxides provides a striking example of the latter mechanism [79].

When exposure induced by a specific genotoxic agent is so low that the number of primary DNA lesions induced by the agent in question will not appreciably affect the background level of the same type of DNA damages that are normally present in the organism, this can be considered as a “virtually safe” dose threshold, irrespective of the actual shape of the dose response relationship. Based on rodent and human data, the existence of such a threshold for exposure to the two nitrosamines NNK and NNN will be demonstrated below.

#### **TYPE OF DNA ADDUCTS FROM TOBACCO SPECIFIC NITROSAMINES**

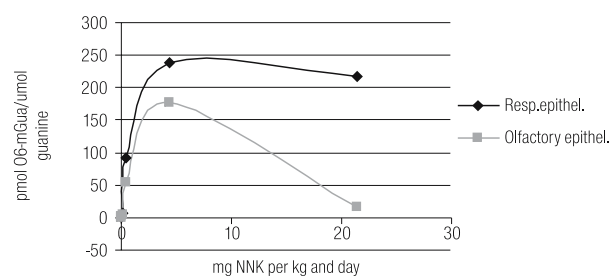
As already mentioned, NNK and NNK – the major carcinogens present in smokeless tobacco – induce two types of primary DNA lesions: nucleotide methylations as well as pyridyloxobutylations. Recently researchers at the Stockholm University have obtained evidence that NNK, in addition, induces DNA phosphate pyridyloxobutyl alkylations [80]. With respect to methylations, the highest yields of adducts in the target organs, such as lung, liver and nasal mucosa of rats exposed to NNK have been found for 7-methylguanine (7-mGua), followed by O<sup>6</sup>-methylguanine (O6-mGua), whereas very low levels of O<sup>4</sup>-methylthymidine (O4-mThd) were present [81]. O6-mGua

is, on the other hand, a highly pro-mutagenic adduct that gives rise to GC to AT transitions [82–84] of a type found in codon 12 of the Ki-ras oncogene from mouse lung tumors induced by NNK [85,86]. Some adducts from pyridyloxobutylations (HPB) have recently been identified, and include the HPB 7- and O<sup>6</sup>- guanines, as well as HPB O<sup>2</sup>-cytidine adducts [87].

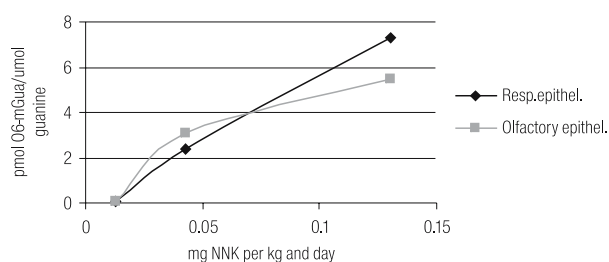
The complex kinetics of DNA adduct formation caused by NNK and NNN has been extensively studied, and reveals marked differences between tissues. After single or repeated exposures to NNK, Belinsky et al. [81,88,89] investigated the kinetics of O6-mGua, O4-mThd, and 7-mGua formation as well as secondary histopathological changes including tumor induction in the rodent liver, lung and nasal mucosa.

### O<sup>6</sup>-methylguanine (O6-mGua)

**Nasal mucosa.** When rats were given 100 mg NNK per kg/day by the i.p. route for 12 days, after an initial sharp increase during the first day after treatment, there was no further increase in the concentrations of O6-mGua as well as of 7-meGua. This effect was ascribed to cytotoxicity and necrosis rather than induction of DNA repair capacity that was found to be decreased [81]. Rats were treated during 4 weeks by s.c. injections, 3 times per week, with doses ranging from 0.03 mg to 50 mg/kg [89], i.e. corresponding to 0.013 to 21.4 mg/kg/day. The adduct levels increased rapidly in the dose range 0.13 to 0.43 mg/kg/day, followed by a decline in alkylation efficiency at higher doses (Fig. 2). An analysis of the low dose region is of particular interest in this context. Using linear dose extrapolation for the adduct data obtained in the low dose region (0.013–0.43 mg/kg), the X-axis is intercepted at about 0.01 mg/kg/day (Fig. 3), which agrees with fact that no increase in O6-mGua was detected in the respiratory epithelium at the lowest dose of 0.013 mg/kg/day, although the limit of detection for O6-mGua was stated as 0.1 pmol/μmol guanine. After a 4-week treatment a steady state for adduct formation has been reached, and it is tempting to regard a value of 10 μg/kg/day as a NOEL for O6-mGua adduct formation from NNK in the nasal epithelia of the rat.



**Fig. 2.** Induction of O6-mGua by 4-(nitrosomethylamino)-1-(3-pyridyl)-1-butanone (NNK) in the rat nasal mucosa.



**Fig. 3.** Induction of O6-mGua by 4-(nitrosomethylamino)-1-(3-pyridyl)-1-butanone (NNK) in the rat nasal mucosa – low dose range.

For the rat nasal olfactory epithelium, some necrotic changes were detected at 1 mg/kg, and further necrotic changes became increasingly severe at doses above 10 mg/kg. The respiratory epithelium was considerably less sensitive. After 20 weeks treatment a significant increase in malignant tumors was only found at 50 mg/kg. The authors therefore concluded that cell proliferation secondary to toxicity is required for tumor induction by NNK in the rodent nose [89,90].

**Liver.** Following single administration of NNK in doses between 1 to 50 mg/kg the levels of O6-mGua and 7-meGua increase in an approximately linear fashion in liver [89]. However, upon repeated administration of 100 mg NNK/kg/day over a 12 day period, after an initial sharp increase the concentration of O6-mGua as well as of 7-meGua declines markedly in liver, probably due to the induction of DNA repair enzyme O<sup>6</sup>-methylguanine-DNA methyltransferase [91]. This enzyme efficiently remove methyl groups in a second order reaction ( $k \approx 10^9$  l/m/min) [92] whereby the free enzyme is not regenerated. Also, no increase in O6-mGua could be detected one day after single s.c. injections of low doses of NNK in the range 0.03–0.3 mg/kg/day, nor at 0.43 mg/kg/day during 4 weeks, reflecting

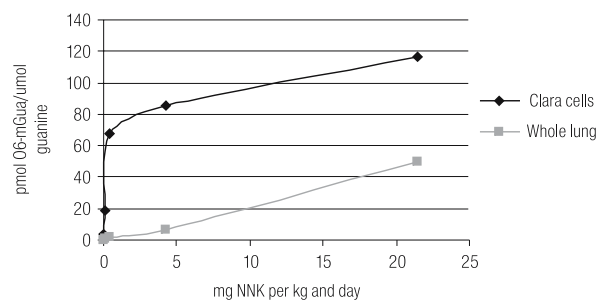


efficient removal of the adducts by the DNA methyltransferase [89]. As the dose was increased to 21.4 mg/kg/day, necrotic changes and subsequent development of hepatic neoplasia appeared after 20 weeks of treatment. Based on these data 130 µg/kg/day appears to represent the NOEL for the formation of O6-mGua in liver.

NDMA produces the same methylating alkylating intermediate as NNK, and utilizing radiolabeled nitrosodimethylamine (NDMA), the O<sup>6</sup>- and 7-methylation of guanine was studied over a wider dose range in rat liver after a single i.p. injection [93]. Alkylation increased rapidly at low doses, and is approximately linear in the dose range 1 to 50 µg/kg (alkylation efficacy ≈ 4 pmole/µmole Gua per mg NDMA). At higher doses the efficacy of alkylation levels off. These data cover doses that are orders of magnitude below those used by Belinsky et al. [89], but are difficult to extrapolate to chronic exposure conditions.

**Lung.** In contrast to liver and nasal mucosa, repeated administration of 100 mg/kg/day NNK during 12 days causes a progressive accumulation of O6-mGua and O4-mThd in the lung [81]. In this organ O6-mGua is more slowly eliminated from Clara cells than from other cell types [89], probably due to low levels of O6-mGua DNA methyltransferase [94]. It is important to note in this context, that the methyltransferase activity in lung is drastically reduced at higher exposures, an effect that is bound to augmented DNA alkylation. Thus, 12 days' treatment with 100 mg/kg/day was found to diminish the activity by 95% [81]. Using radiolabeled NNK, Murphy et al. [95] were unable to detect any increase in O6-mGua in either whole lung or liver below a dose of 0.6 mg/kg/day given by the i.p. route during 4 days.

In Figure 4, showing data obtained from rats treated for 4 weeks by s.c. injections, 3 times per week, with doses ranging from 0.1 mg to 50 mg/kg (corresponding to 0.043 to 21.4 mg/kg/day), there is a sharp increase in the yield of adducts at a dose of 0.13 mg/kg/day for Clara cells, and above 4.3 mg/kg/day for whole lung. Correspondingly, there was a non-significant increase in benign lung tumors at 0.013 mg/kg/day after 20 weeks' treatment, with a steep increase of the slope of the dose-response curve in the range 0.13–0.43 mg/kg/day. For O6-mGua there was



NNK – 4-(nitrosomethylamino)-1-(3-pyridyl)-1-butanone.

**Fig. 4.** Induction of O6-mGua in rat lung.

a very good correlation between degree of alkylation in Clara cells (less so for other cell types or whole lung) after administration of NNK and the incidence of lung tumors in the mouse [96] as well as in the rat ( $r = 0.99$ ) [89]. Unfortunately, no data were published for adduct levels in lung at the lowest dose, 0.013 mg/kg/day.

#### O<sup>4</sup>-methylthymine (O4-mT)

O4-mThd adducts are strongly pro-mutagenic. Although the concentrations induced by NNK in the rat are more than one order of magnitude below those for O6-mGua [81], they may contribute to a limited extent to the overall cancer risk from TSNA.

#### 7-Methylguanine (7-mGua)

In comparison with O6-mGua, the levels of 7-mGua induced by NNK are between 4 (lung) to 8 (liver) times higher [81]. For liver and lung the dose response for formation of this adduct was studied upon i.p. administration of tritiated NNK in the dose range 0.003 to 5 mg/kg/day during 4 days [95]. Analysis of the rodent data obtained by Murphy et al. [95] gives a slope factor for induction of 7-mGua by NNK in the low dose region of approximately 50 pmol 7-mGua/µmol guanine per mg NNK per kg and day. Above 0.075 mg/kg there was steep increase in the yield of adducts that was virtually linear for liver. In this organ as well as in lung, adduct concentrations of 0.22 and 0.23 pmol 7-mGua/µmole guanine could be detected at the lowest dose. However, the background levels for 7-mGua in these organs could not be measured. By employing the <sup>32</sup>P postlabeling assay Zhao et al. [97] found

a background concentration in rats of 2.1–2.5 7-mGua/ $10^7$  nucleotides (0.8–1.0 pmol/ $\mu$ mole guanine), implying that the adduct yield for NNK at 3  $\mu$ g/kg/day approximately represents a 20% increase in the natural background, a dose that may be considered as a LOEL value slightly above a true NOEL value.

7-mGua causes insignificant distortion of the helix, and upon replication the DNA polymerases will not distinguish alkylated guanosine from the normal nucleoside. In general, this adduct is poorly repaired, but may result in spontaneous depurination, or be repaired by alkylpurine-DNA-glycosylases in the base excision pathway [98]. Apurinic sites usually undergo rapid and error-free repair, but may give rise to base-pair change mutations [99,100]. However, as compared with O6-mGua and O4-mThd, 7-mGua is a poor inducer of point mutations [84,101–103], and although the yield of 7-mGua is much higher than that of O6-mGua, 7-mGua adducts seem to be of secondary importance with respect to cancer induction by NNK. This assumption is strengthened by the observation that there is no correlation between 7-mGua adduct levels and incidence of tumors in rodent [104]. Agents that preferentially induce a high level of 7-guanine alkylation have, on the other hand, a propensity to induce chromosomal aberrations [105]. However, in view of the fact that adducts other than 7-mGua are also generated, the role of the latter adduct is somewhat difficult to assess with respect to clastogenicity.

**Oral administration of NNK.** Exposure to NNK by the oral route may result in an adduct tissue distribution that is different from that from s.c. or i.p. injection, a fact that is underlined by the finding, that in contrast to injection, pancreatic tumors can readily be induced by administering NNK by the oral route. NNAL has been suggested to induce pancreatic tumors, and one reason for this discrepancy may be a first pass metabolism in liver and small intestine yielding more NNAL.

In the study conducted by Rivenson et al. [106], 344 male Fischer rats were administered the nitrosamine in drinking water at 0.5, 1.0 or 5.0 ppm during the animals' lifetime. Clear dose response relationships were evident for tumors in lung, liver, and nasal cavities, out of which the induction

of lung tumors appears to be the most sensitive end point that could conveniently be used for high-to-low dose risk extrapolation. At the lowest dose, there was a significant increase in pancreatic tumors but not in lung. However, an anomaly was the unusually high incidence of lung tumors in controls (7.5%) as well as the fact that the pancreatic tumor incidence was less at the highest than at the lowest dose. As compared with i.p. injection, the levels of O<sup>6</sup> and 7 guanine adducts induced by NDMA in rat kidney were significantly lower upon oral administration [93].

### DNA pyridyloxobutylations

Plotting the data for dose vs. HPB released from hemoglobin in an investigation where tritiated NNK was administered to rats by 4 daily i.p. injections in the dose range 3–10 000  $\mu$ g/kg/day to rats gives a strict linear relationship. However, alkaline hydrolysis only releases between 30 to 40% of the bound tritium as HPB in the dose range 3–600  $\mu$ g/kg, and at higher doses an even lower fraction of the activity was recovered [95]. HPB phosphate adducts are quantitatively released upon hydrolysis (Törnqvist, personal communication), and the nature of the material that remains in DNA after hydrolysis is obscure. Nevertheless, HPB Hb adducts have been used for monitoring exposure of humans to TSNA (see below).

The interpretation of HPB adduct data is complicated by the fact that more than one adduct seems to be generated [107], and reliable dose response relationships in the low-dose region that can be correlated with induction of cancer do not seem to be available. When investigating HPB released from liver and lung DNA in rats given daily i.p. injections of NNK during 4 days, no increase in the adduct concentration could be detected at a dose of 3  $\mu$ g/kg/day (detection limit, 0.05 pmol HPB/ $\mu$ mol Gua). In the range 3 to 600  $\mu$ g/kg/day the dose response relationship was roughly linear, whereas a non-linear response was seen in the upper dose range, an observation that was tentatively interpreted as saturation of the metabolic activation system involved [95]. For the nasal epithelia of the rat, a single dose of 3460  $\mu$ g/kg NNK did not cause any detectable elevation of HPB adducts, neither in the respiratory nor in the olfactory mucosae [108]. The bulky HPB

adducts, that can be expected to be repaired by the nucleotide excision pathway, have been reported to induce G to A transitions and G to T transversions [86], and there is convincing evidence that HPB DNA adducts are involved in the induction of tumors of the rodent nasal epithelium and esophagus [108,109]. NNN and NNK, both of which induce HPB adducts at this site, have very similar carcinogenic potency with respect to induction of neoplasia in the rat nasal mucosa, whereas NDMA which does not induce HPB adducts, but is a potent methylator, has a very low carcinogenic potency with respect to the nasal mucosa.

With respect to HPB phosphate alkylations, although persistent, there is as yet no evidence that this type of adducts are involved in cancer initiation. Thus, there is, for example, no correlation between degree of phosphate alkylation and mutations (Dag Jenssen, personal communication).

#### UPTAKE OF TSNA FROM SMOKING AND USE OF SNUFF

**Uptake of TSNA in smokers and users of snuff.** The uptake of microgram amounts of NNK in smokers as well as from the use of American snuff has been adequately documented by recovery of the metabolites 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) and [4-(methylnitrosamino)-1-(3-pyridyl) but-1-yl]- $\beta$ -O-D-glucosiduronic acid (NNAL-Gluc) from urine. In these studies, the mean excreted amounts of NNAL-Gluc and NNAL were approximately similar in smokers and users of snuff [110,111]. Because of the appreciable variations in content of TSNA in cigarettes, that does not necessarily correlate with tar yield [112], it is difficult to estimate the actual intakes for smokers included in the investigations cited above. The same goes for consumers of US American snuff. According to Philip Morris the highest levels are found in burley tobacco with a content of about 10  $\mu$ g/g of TSNA, out of which 1  $\mu$ g/g is NNK and 5  $\mu$ g/g consists of NNN. The US flue cured tobaccos have, typically, 4  $\mu$ g/g of TSNA with 1.8  $\mu$ g/g NNK and 0.9  $\mu$ g/g NNN. Greek and Turkish oriental tobaccos are characterized by very low TSNA contents [29]. In a systematic survey of cigarettes on the

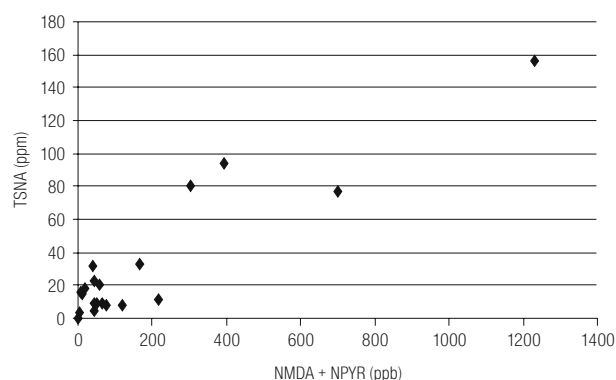
US and German markets, the French Gauloises contained 8.6  $\mu$ g/g of TSNA, whereas in US and German brands the TSNA concentrations were in the range 1.6–5.5  $\mu$ g/g, out of which 50–60% was NNN and 11–21% NNK [113]. For estimation of actual intakes, measurements of yields in mainstream smoke from modern cigarettes conducted at the Massachusetts Institute of Technology are more representative [112] and shall be used in this context. Average values based from this source indicate an uptake of about 0.25  $\mu$ g of NNN and 0.17  $\mu$ g of NNK per cigarette, assuming 100% absorption in the lung. Smoking 20 cigarettes per day will then give a dose of 0.07  $\mu$ g/kg/day of NNN and 0.05  $\mu$ g/kg/day of NNK. For moist snuff an absorption of about 60% has been demonstrated [114].

**Present time and historical exposures to TSNA from Swedish snuff.** In the case-control study from North Carolina – the only adequately performed study that has demonstrated a significant association between oral snuff and cancers in the oral cavity and pharynx – in 1980 the average age of the included white women was 69 years [47], implying that a considerable extent of the exposure had occurred before World War I. The snuff that had been consumed for decades by these women, and that in part was most probably produced by local North Carolina tobacco growers (Palmer, personal communication), was certainly characterized by a much higher content of TSNA than the snuff produced by the Swedish producers after the war. As discussed below, the lower nitrosamine content of the latter no doubt provides an explanation why the four more recent epidemiological studies from Sweden failed to detect any increase in cancer [16,49–51]. However, to assess actual exposures of the investigated Swedish cohorts, and because the Swedish epidemiological investigations also included subjects with a long history of snuff use extending over several decades, the changes in contents of TSNA in Swedish snuff over a longer period of time must be taken into consideration.

According to analyses performed by the responsible Government agency [21], Swedish moist snuff today contains on the average 0.2  $\mu$ g/g of NNK and 0.5  $\mu$ g/g of NNN (dry weight), giving a daily total intakes of 0.009–0.018 and 0.021–0.042  $\mu$ g/kg/day for NNK and NNN, respectively,

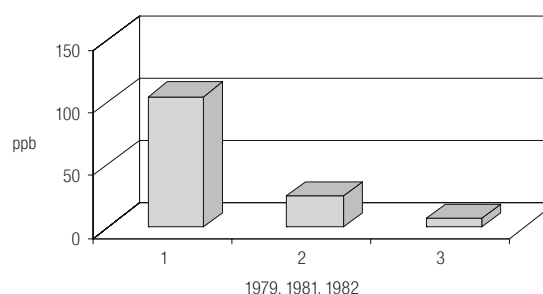
based on a daily consumption of 10–20 g of moist snuff and 60% TSNA absorption [114]. The first determinations of NNN and NNK were conducted in 1983 by the Swedish Food Administration (Table 1) [19]. During the following decade the average levels were approximately 1.6  $\mu\text{g/g}$  of NNK and 7.6  $\mu\text{g/g}$  of NNN, corresponding to daily intakes of 0.14 and 0.65  $\mu\text{g/kg/day}$  for the two nitrosamines, respectively. Hoffmann et al. [24] have, on the other hand, provided evidence that Swedish snuff prior to 1983 had higher contents of TSNA. Thus, the American Health Foundation group identified one box of snuff, where the tobacco had a total content of 106  $\mu\text{g/g}$  TSNA, out of which some 77  $\mu\text{g/g}$  was identified as NNN and 4  $\mu\text{g/g}$  as NNK. However, in view of the fact that considerably lower values were found for the other 12 analyzed samples purchased in several Swedish cities, the sample with extremely high TSNA levels might have been an outlier. The contents of NNK and NNN for Swedish snuff produced before 1983 can be roughly estimated also in the following manner.

In Figure 5 the correlation between total TSNA (NNK, NNN, NAT, NAB) concentrations and that of the two most important volatile nitrosamines in tobacco, N-nitrosodimethyl-amine (NDMA) and N-nitrosopyrrolidine (NPYR), are shown. Data have been derived from analysis of 24 different samples, where two outliers have been excluded [19,26,32,35,115]. The correlation coefficient is 0.92 with a beta of 0.14, i.e. 10 ppb (NMDA+NPYR) corresponds to 1.4 ppm TSNA. Figure 6 shows the levels of the two most important volatile nitrosamines in Swedish snuff during the time period 1979–1982. The sharp decline in concentrations after 1979 reflects the modernized processing introduced in the new factory of Svenska Tobaks AB, that started production in 1981–1982. Thus, the value of 209.8 ppb of volatiles for 1979 would correspond to about 29  $\mu\text{g/g}$  TSNA (CI: 27–31  $\mu\text{g/g}$ ), which is somewhat higher than for the Swedish samples that were probably collected in 1980, and that had an average TSNA content of 20  $\mu\text{g/g}$  [24]. Because the production process did not undergo appreciable changes during the decades from the time since World War II until 1981, total TSNA levels in the range 20–30  $\mu\text{g/g}$  could be considered as representative for snuff produced before this date. An average con-



NDMA – N-nitrosodimethylamine; NPYR = N-nitrosopyrrolidine (values based on dry weight) [19].

**Fig. 5.** Correlation between volatile nitrosamines and tobacco specific nitrosamines (TSNA).



**Fig. 6.** N-nitrosodimethylamine (NDMA) + N-nitrosopyrrolidine (NPYR) in Swedish snuff.

tent of 3  $\mu\text{g/g}$  of NNK and 14  $\mu\text{g/g}$  of NNN in snuff (dry weight), giving a daily total absorbed dose from 20 g moist snuff of about 0.26  $\mu\text{g/kg/day}$  of NNK and 1.2  $\mu\text{g/kg/day}$  of NNN, probably represents concentrations that are representative for Swedish snuff produced before 1980.

For Swedish snuff marketed during three decades preceding 1991, when the two major epidemiological studies were conducted, weighted data would indicate approximate average concentrations for NNK and NNN of 2.5  $\mu\text{g}$  and 12  $\mu\text{g/g}$ , respectively.

## EXTRAPOLATION OF RODENT DATA TO HUMANS

When classifying snuff (all types) as carcinogenic, IARC [12,23] has heavily relied on rodent bioassay data for NNK and NNN. In the following discussion the author of this paper will do the same, but whereas IARC unfortunately pays no attention to levels of risk, the quantitative aspects of rodent bioassays will provide an important input for

quantification of human risk associated with exposure to TSNA in tobacco products.

The appropriate manner of extrapolating target adduct concentrations induced by NNK in the rat to those expected in humans exposed to the same dose by the oral route presents several difficulties. It should be noted, however, that the carcinogenic potency of NNN and NNK for oral administration does not differ appreciably from that observed for i.p. or s.c. administration. The fact that the target tissue for carcinogenicity of TSNA in humans appears to be different from what is found in rodents is another concern. With respect to the first issue, US EPA has relied on surface based extrapolation for regulatory purposes to determine the human equivalent dose from cancer data obtained in rodents. The underlying rationale is the empirical observation that metabolic rate shows a better correlation with surface area than with body weight, and the assumption is that a similar relationship also holds for various toxicological effects [115]. For a toxicological endpoint obtained in the rat the human equivalent dose would be about 6 times lower. However, by investigating DNA alkylations in the rat, mouse, dog and monkey, we have demonstrated that this correlation does not hold for a directly alkylating agents like propylene oxide and ethylene oxide [116,117], and it is difficult to predict in which way a higher metabolic rate in the rodent would affect the balance between the various activating and detoxifying pathways for NNK.

The levels of O6-mGua and 7-mGua in liver were practically the same during 24 h post dosing in the rat and the hamster [104]. Instead of a difference of a factor of 6 as expected from surface area based extrapolation, the O6-mGua adduct level was about 40% lower in mice that received a single i.p injection of 100 mg/kg [96] as compared to rats given a single s.c. dose of 78 mg NNK per kg (Fig. 2) [118]. There is a strict linear correlation between NNK dose and HPB adducts released from Hb. As demonstrated below, HPB data from the rat agrees with those observed in humans within a factor of about two. Obviously, surface based extrapolation does not hold for DNA adduct formation from NNK.

## DNA ADDUCT LEVELS IN HUMANS RELATED TO ESTIMATED EXPOSURES AND DATA FROM RODENT STUDIES

A number of recent investigations have demonstrated that background levels of DNA adducts, probably caused by unknown dietary or endogenous sources, are ubiquitously found in healthy humans without known exposures to either tobacco or alkylating agents. In a large-scale molecular epidemiological investigation of O6-mGua using blood leukocyte DNA from subjects in 17 regions worldwide, the levels were higher in regions with higher consumption of nitrate-treated foods [119]. This observation is consistent with the hypothesis that agents formed through intragastric nitrosation contributes significantly to methylated DNA adduct formation. As mentioned above, an additional external exposure to TSNA that does not appreciably affect the "normal" background concentrations of methylated or pyridyloxobutylated DNA adducts should be considered as a "virtually safe".

## METHYLATIONS

**Adduct levels in humans.** In contrast to 7-methylguanine, relatively few studies on the background levels of O6-methylguanine have been conducted. Using a monoclonal antibody specific for O6-methyldeoxyguanosine (O6-MedGua) in a competitive enzyme-linked immunosorbent assay with a lower limit of detection of 0.5 pmol O6-mGua/ $\mu$ mol deoxyguanosine, placental DNA from smoking and non-smoking women was analyzed [120]. Two of 10 DNA samples from smoking women and three of 10 from non-smoking women had detectable concentrations of O6-MedGua. Thus, this study failed to reveal any significant differences. With the development of novel more sensitive radioimmunological techniques, the background concentrations of O6-mGua in liver was found to be in the range 0.1–0.7 pmol/ $\mu$ mol guanine. In peripheral leukocytes from healthy volunteers the median adduct concentrations were about an order of magnitude lower (range, 0.07–0.46 pmol/ $\mu$ mol Gua) than in liver [121,122] or colon [123]. Nevertheless, this adduct was found in

83–86% in samples of maternal and cord blood leukocyte DNA from healthy smoking and non-smoking women at levels up to 0.2 pmol/ $\mu$ mol guanine [124]. Interestingly, smoking status had no effect on the detected adduct levels. In normal colorectal tissues, O6-mGua was detected in 27 out of 62 samples (detection limit 0.01 pmol/ $\mu$ mol Gua) where the concentrations ranged from 0.01 to 0.94 pmol/ $\mu$ mol Gua [123].

Similar to rats treated with NNK, the concentrations of O4-mThd in human tissues appears to be low. Thus, in human liver the mean value of the ratio between O6-mGua and O4-mThd was about 6 [121].

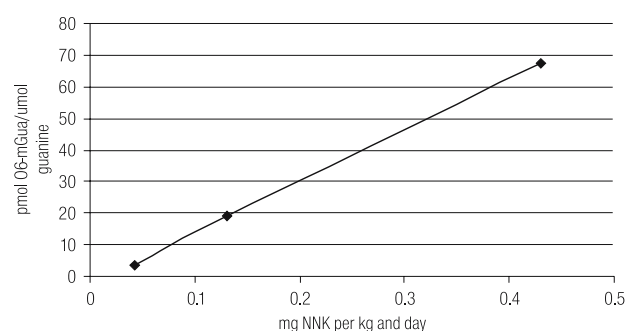
Using  $^{32}$ P-postlabeling the effect of smoking on the formation of 7-deoxymethylguanosine in human peripheral white blood cells was investigated in 10 smokers and 10 non-smokers [125]. In smokers the mean DNA adduct levels in total white blood cells, granulocytes and lymphocytes were 6.9, 4.7 and 23.6 7-mGua/ $10^7$  nucleotides to be compared with 3.4, 2.8 and 13.5 adducts/ $10^7$  nucleotides for non-smokers, a difference that was reported to be significant. Subsequently, 7-mGua adducts were determined in normal bronchial tissues and total peripheral blood lymphocytes [126]. The mean bronchial 7-mGua levels in 11 smokers and 6 non-smokers were 17.3 and 4.7 adducts/ $10^7$  nucleotides, respectively. In lymphocyte DNA, the mean levels were 11.5 for smokers and 2.3 adducts/ $10^7$  nucleotides for non-smokers. There were wide individual variations, but for bronchial adducts there was a statistical significant difference between smokers and non-smokers. In a later study performed by the same group on larynx tumor biopsies from 46 patients, the differences between smokers and non-smokers were reported as significant although less marked [127]. However, the statistical method used (t-test) is not appropriate for this data limited set. In addition, determinations of the 7-mGua adducts in human tissues obtained by thin layer chromatographic methods used in the above mentioned  $^{32}$ P-postlabeling studies are difficult to interpret because of contamination by 7-hydroxyethylguanine adducts which are present as very high background levels [128].

Using  $^{32}$ P-postlabeling 7-mdGp and 7-ethylGp DNA adducts were measured in eight separate lung segments ob-

tained by autopsy from 10 smoking and non-smoking donors that were free of cancer [129]. Adducts were detected in all samples in levels ranging from 0.3 to 11.5 adducts/ $10^7$  nucleotides with a mean of 2.5 adducts/ $10^7$  nucleotides. There were highly significant individual variations but they could not be explained by differences in smoking habits, gender, age, blood ethanol, or ventilation or perfusion variability.

**Expected adduct levels in smokers and users of snuff.** Assuming a linear relationship between absorbed NNK dose and O6-mGua adduct formation down to zero dose in rat lung Clara cells – the most sensitive biomarker relevant to cancer induction in the rat – a slope factor for adduct induction of about 160 pmol/ $\mu$ mol guanine per mg/kg/day of NNK is obtained for the low dose range (Fig. 7). Based on an intake of 0.05  $\mu$ g/kg/day of NNK for a person smoking 20 cigarettes, the expected adduct level in rat lung Clara cells would be  $0.05 \cdot 0.16 = 0.008$  pmol/ $\mu$ mol guanine, a concentration that is orders of magnitude lower than the background levels actually detected in humans. Based on rodent liver data or whole lung, the differences would be even greater.

The expected O6-mGua level in Clara cells from NNK contained in a daily dose of Swedish snuff can be estimated to be  $0.017 \cdot 0.16 = 0.0027$  pmol/ $\mu$ mol guanine. If instead, weighted representative values for snuff marketed during the decades preceding 1990 are used, a predicted O6-mGua level of  $0.26 \cdot 0.16 = 0.04$  pmol/ $\mu$ mol guanine is obtained. The extrapolated adduct value for Swedish snuffs in rat Clara cells should be compared with the 3 to 75 times natural background levels in human tissues of 0.1–0.2 pmol/ $\mu$ mol guanine. The margin of safety based on



NNK – 4-(nitrosomethylamino)-1-(3-pyridyl)-1-butanone.

**Fig. 7.** Induction of O6-mGua in rat lung.

**Table 2.** Dose response for O6-methylguanine formation and tumor induction in liver from Fischer rats treated s.c. 3 times per week up to 20 weeks, with doses from 0.03 to 50.0 mg/kg

Dose (mg/kg/day)	O6-methylguanine (pmol/ $\mu$ mol guanine)		No. of animals	Benign + malignant tumors
	1 day	4 weeks		
Vehicle				
0.03	ND	ND	60	0
0.1	ND	ND	60	1
0.3	ND	ND	60	0
1.0	2.3 $\pm$ 1.1	ND	30	2
10.0	13.4 $\pm$ 2.2	1.9 $\pm$ 0.1	30	3
50.0	86.7 $\pm$ 7.5	4.4 $\pm$ 0.6	62	51

ND < 0.1 pmol/ $\mu$ mol guanine.

background levels of O6-mGua found in whole lung would be even higher.

In Table 2, O6-mGua adduct concentrations in rat liver together with data for tumor induction are given for different doses of NNK [89]. After one day's treatment a significant increase in adduct concentrations is only apparent at a dose of 1000  $\mu$ g/kg. Thus, to achieve an adduct concentration in rat liver hepatocytes one day after a single dose, and that corresponds to the normal background levels found in human liver (0.1–0.7 pmol/ $\mu$ mol Gua), this would require a dose in the range 300–1000  $\mu$ g/kg, i.e. an intake that is orders of magnitude above the daily NNK dose consumed by a Swedish snuff user.

For 7-mGua Zhao et al. [97] found a background concentration in rats of about 2.3 adducts per  $10^7$  nucleotides (0.9 pmol/ $\mu$ mol guanine), which is about the same as for human white blood cells. Relying on the slope factor of 50 pmol/ $\mu$ mol per 1000  $\mu$ g NNK for induction of 7-mGua based on the Murphy et al. [95] study, one would have expected to find an increase in 7-mGua of 0.005 pmol/ $\mu$ mol guanine in a smoker with a NNK intake of 0.05  $\mu$ g/kg/day (0.005 adducts per  $10^7$  nucleotides), an increase that is obviously far too low for detection.

## PYRIDYLOXOBUTYLATIONS

**Hemoglobin adduct levels in humans.** In a study by Hecht et al. [130], the mean HPB hemoglobin adduct levels were  $517 \pm 538$  (SD),  $79.6 \pm 189$  and  $29.3 \pm 25.9$  fmol HPB/g

hemoglobin for users of snuff, smokers and non-smokers, respectively. However, the increase in HPB adducts exhibited large individual variations, where some non-smokers had higher HPB values than the mean value for smokers. The average cigarette consumption was reported as 21.8 cigarettes per day, estimated to have yielded an inhaled dose of NNK and NNN of about 5  $\mu$ g/day (0.07  $\mu$ g/kg/day). Although not measured, the TSNA content of the snuff used by the subjects in the US study was reported to give an intake of 0.7  $\mu$ g/g NNK and 9  $\mu$ g/g NNN (dry weight). This most probably underestimates actual exposures. Thus, although no systematic analyses of marketed products had been undertaken, at this time US snuffs could contain up to 44  $\mu$ g/g NNK and 73  $\mu$ g/g NNN (Table 1). As late as 2001 a survey commissioned by the Massachusetts Department of Health, and reported by Brunnemann et al. [131], found TSNA levels varying from 7.5 to 130  $\mu$ g/g in US moist snuffs. The authors assumed a daily consumption of about 5 g wet snuff, which is about four times lower than a typical intake by a Swedish consumer [60]. When based solely on these data the claim that snuff induces HPB levels in Hb that are much higher in users of snuff than in smokers at similar exposures to TSNA, therefore, seems unwarranted. On the other hand, Falter et al. [132] found significantly elevated levels of HPB-releasing Hb adducts in users of nasal dry snuff, and in view of the rather low amounts of nasal snuff that is normally used, and unless the TSNA level were significantly elevated in the products in question, for obscure reasons these findings seem to reflect a higher impact of the use of nasal snuff on pyridyloxobutylations of hemoglobin than from smoking. One possible reason is the high activity of CYP2A13 found in human nasal mucosa leading to effective activation of NNK, but because these metabolic competent cells are rapidly sloughed off, this may have little impact for the development of nasal tumors.

Utilizing an analytical procedure for the simultaneous determination of human hemoglobin adducts from aromatic amines and TSNA, Falter et al. [132] found only marginal differences in Hb HPB-releasing adducts in smokers and non-smokers, although the levels of aminobiphenyl adducts in smokers were drastically increased. In a larger

material, comprising 47 smokers and 93 non-smokers, significantly higher adduct levels were found in smokers ( $96 \pm 102$  fmol/g), than in non-smokers ( $57 \pm 53$  fmol/g) although the difference was only about twofold [133].

In a study sponsored by IARC in 18 smokers and 52 never-smokers [134], HPB-Hb adduct levels were significantly higher ( $P = 0.02$ ) in smokers ( $26 \pm 13$  fmol HPB/g Hb) than in never-smokers ( $20 \pm 8$  fmol HPB/g Hb). The 20% higher Hb levels found in smokers could possibly be attributed to a much higher delivery of TSNA from some types of French cigarettes in comparison with US brands [113].

#### Expected adduct levels in smokers and users of snuff.

Based on data from Murphy et al. [95], in Figure 8, the relationship between HPB adduct formation and amount of administered NNK (i.p.) daily for 4 days is shown for the dose interval 3 to 600  $\mu\text{g}/\text{kg}/\text{day}$  giving a slope factor of about 720 fmol HPB/g globin per  $\mu\text{g}$  of NNK per day. In the studies by Atawodi et al. [134] and Richter et al. [133], differences between smokers and non-smokers were in the range 10–40 fmol HPB/g globin, whereas an increase amounting to 86 fmol HPB/g globin would have been expected based on rodent data, and assuming an intake of 0.12  $\mu\text{g}/\text{kg}/\text{day}$  of NNK and NNN. A likely explanation for the modest discrepancy between expected and measured HPB Hb adduct concentrations in smokers is the less efficient metabolic activation by the  $\alpha$ -hydroxylation pathway in humans as compared with rodents, in combination with

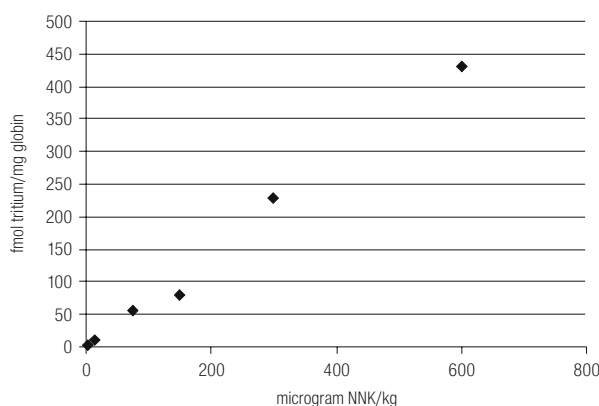


Fig. 8. Relation between NNK dose and amount of radiolabeled HPB released from hemoglobin in the rat [95].

an inhibition of the same caused by high levels of nicotine and cotinine.

For HPB DNA adducts, the data from Murphy et al. [95] for the dose range 3–600  $\mu\text{g}$  NNK gives a slope factor of about 2.0 pmol HPB/ $\mu\text{mol}$  guanine per mg of NNK. Thus, a heavy smokers' intake of 0.12  $\mu\text{g}/\text{kg}/\text{day}$  of NNN and NNK would be expected to give an HP DNA adduct level of 0.0024 pmol HPB/ $\mu\text{mol}$  guanine, a concentration that would be far below the detection limit.

**HPB adducts in human DNA.** Although HPB Hb adducts can obviously be used as a measure of exposure, the HPB releasing DNA adducts constitute the relevant biomarkers for induction of cancer. Foiles et al. [135] reported differences between 9 smokers and 8 non-smokers by measuring the release by acid hydrolysis of HPB DNA adducts from human peripheral lung and tracheobronchial tissues collected at autopsy. However, the employed methodology was not sufficiently sensitive to permit any definite conclusions. Thus, in tissues from 2 smokers and 7 non-smokers, no adducts could be found.

Employing an improved analytical method Richter's group conducted a series of investigations on sudden death victims from the Munich area [136,137]. In non-smokers a mean HPB DNA adduct level of  $50 \pm 42$ ,  $130 \pm 148$ , and  $130 \pm 110$  fmol HPB/mg DNA (corresponding to 0.6, 1.6 and 1.6 pmol HPB/ $\mu\text{mol}$  guanine), was detected in lung, esophagus and cardia, respectively. Although the average concentration of DNA HPB adducts in lung were somewhat increased in 49 smokers ( $91 \pm 133$  fmol HPB/mg) as compared with 34 non-smokers ( $50 \pm 42$  fmol HPB/mg), this difference was not statistically significant. The level predicted from animal experiments for a person smoking 20 cigarettes per day is more than 2 orders of magnitude lower. The considerably higher levels found in esophagus and cardia were unrelated to smoking status, and whereas there was a highly significant correlation between adducts in esophagus and cardia, no correlation was found between lung and esophagus. Esophagal mucosa taken close to the esophagogastric junction from 40 smokers had  $143 \pm 29$  fmol HPB/mg DNA, and in non-smokers  $130 \pm 30$  fmol HPB/mg DNA. On the other hand, a significantly higher level of HPB-releasing adducts was found



in lung DNA from smoking as compared to non-smoking patients undergoing lung cancer surgery. However, the interpretation of the latter data in relation to a “natural” adduct background is complicated by impairment of DNA repair systems that may occur in cancerous tissues. It is, for instance, well known that the O6-methylguanine-DNA methyltransferase gene is epigenetically silenced in various human tumors by hypermethylation of the promoter CpG island [138].

**Expected adduct levels in smokers.** Murphy et al. [95] determined HPB released from lung as well as liver DNA from rats treated with NNK (i.p.) in the dose range 0.003 – to 5 mg/kg/day during 4 days. In the low dose region, the amount released was similar for the two tissues and characterized by a slope factor of approximately 3 pmol HPB/ $\mu$ mol guanine per mg/kg/day of NNK (250 fmol/mg DNA). Assuming an intake of 0.12  $\mu$ g/kg/day of NNK and NNN by a smoker consuming 20 cigarettes per day, the expected HPB DNA adduct concentration in rat lung and liver would be about 0.03 fmol HPB/mg DNA, i.e. more than three orders of magnitude below the background levels determined in humans.

#### COMPARISON OF RODENT CANCER BIOASSAYS WITH EPIDEMIOLOGICAL DATA

By comparing the accumulated life time intake of 1.4 mg NNK in heavy smokers with the lowest accumulated life-time dose that induces lung tumors in rats treated for up to 100 weeks (1.8 mg/kg; from [89]), Hecht et al. [41] implicated a major role for NNK in the induction of human lung cancer. Apart from neglecting target organ species differences, the shape of the dose response curve, etc., the authors violated basic principles of toxicology when equating doses in rodents and humans. If instead the appropriate comparisons are made based on dose per kg body weight and day for humans vs. rats, a heavy smoker (40 cig/day) will receive an equivalent dose of NNK that is about 50 times lower than the lowest dose that was found to induce tumors in the rat.

Using the linearized multistage non-threshold model [139] based on oral administration to rats, Nilsson [34] obtained

a maximal carcinogen potency (slope) factor of 0.086 and 0.029 (mg/kg and day)<sup>-1</sup> for NNK and NNN, respectively (expressed as annual cancer risk). By applying these factors the extra yearly cancer incidence (per 100 000 and year) for consumption of 20 g daily of Swedish snuff marketed today will be:

$$\begin{aligned} \text{NNK} &= (0.017)10^{-3}\text{mg} \cdot 0.086 \text{ mg}^{-1} \cdot 100,000 = 0.16 \text{ cases} \\ \text{NNN} &= (0.042)10^{-3}\text{mg} \cdot 0.029 \text{ mg}^{-1} \cdot 100,000 = \underline{0.12 \text{ cases}} \\ &0.28 \text{ cases} \end{aligned}$$

Thus, on the basis of rodent data, an increased incidence of less than half a case per 100 000 consumers and year can be expected. For Swedish snuff products marketed during the decade preceding 1990, similar calculations give an expected yearly incidence of 3.1 cases, whereas consumption of snuff produced before 1981 would be expected to have resulted in an incidence of 5.6 cases per 100 000 consumer per year. Assuming an average duration for snuff consumption of 30 years for the subjects included in the Swedish cohorts, and relying on weighted TSNA levels estimated for the time before 1991, the expected number of additional cancers would be about 140 cases for a 30-year period.

#### RISK REDUCTION IN SMOKERS WHO SWITCH TO SNUFF

It has been estimated that there are about 100 million smokers in the member states of the European Union [140]. The staggering burden imposed on public health derives mainly from three groups of smoking related diseases; cancers, cardiovascular disease and chronic obstructive pulmonary disease. Many smokers, in particular heavy smokers, have great problems quitting smoking, and the use of alternative nicotine delivering devices, including Swedish “snus”, may be of considerable assistance to promote abstinence, a strategy that is supported by responsible authorities like the US Institute of Medicine [56] as well as the Royal College of Physicians in the UK [57]. In this context it is noteworthy that the cost of nicotine chewing gum, nicotine patch as well as nicotine spray, registered as pharmaceutical products, are unacceptably high

for the majority of citizens in Central and Eastern Europe. Low nitrosamine snuff, for which the kinetics of nicotine delivery is more similar to that of smoking, offers another possibility for nicotine replacement therapy [140]. When making an assessment of the gains obtained by switching from smoking to oral snuff, data on risk reduction from long-term follow-up studies in cohorts of ex-smokers must be relied upon. For lung cancer information from a number of such investigations are available, whereas the data base for cardiovascular disease and chronic obstructive pulmonary disease is less precise.

**Number of annual deaths in 100 million EU smokers.** The average mortality in lung cancer for Swedish never-smokers from three studies gave an estimate of 6 per 100 000 individuals per year [68 – see Table 4:5, p. 148]. The widely cited evaluation by Sir Richard Doll and Richard Peto commissioned by the Office of Technology Assessment of the US Congress [67 – see Table 11, p. 1223] reported an average increased relative risk for smokers with respect to lung cancer of 10. Although both higher and lower estimates have been published for various populations, the US estimate can be used as a reasonable benchmark. Thus in a population of 100 million smokers, the expected yearly mortality for smoking induced lung cancer will be about 60 000. Doll and Peto ascribes an additional contribution of deaths from tobacco induced cancers of the oral cavity, larynx, pharynx, esophagus, pancreas and urinary tract that is 30% of the smoking induced lung cancers. Pooled data from various European countries found an increasing risk of bladder cancer with increasing duration of smoking, ranging from approximately a two-fold increased risk for a duration of less than 10 years to over a four-fold increased risk for a duration of greater than 40 years [141]. Assuming a total of 20 additional smoking induced deaths annually due to neoplasia at other sites than lung in 100 000 smokers, therefore, represent a reasonable estimate, i.e. corresponding to a total of 20 000 among 100 million smokers. After cessation of smoking, the risk for lung cancer will rapidly decrease, but will approach a level that is approximately twice the background found in never-smokers, i.e. representing about 6000 remaining casualties per million [68 – see p. 151]. The marked effect of cessation of expo-

sure can be ascribed to the important role of unspecific promotive action as the major determinant of human cancer risk from tobacco smoke [142].

Some data exist for the impact of smoking cessation with respect to other types of cancers. A study conducted by the Mayo Clinic, NY, found a decrease in risk for renal carcinoma between 20–30%, 10–20 years after cessation, and after 30 years or more the risk was similar to that found in never smokers [143]. The pan-European assessment cited above indicated an immediate decrease in risk of bladder cancer was observed for those who gave up smoking. This decrease was over 30% in the immediate 1–4 years after cessation, and continued to decrease with time, although even after 25 years the decrease in risk did not reach the level of never-smokers [141]. For pancreatic cancer, a Japanese collaborative study indicated an approximately 50% decrease in risk after smoking cessation [144]. For the purpose of this evaluation, an overall reduction in cancer mortality of 40% will be used to estimate the remaining cancer risk after cessation of smoking, implying that about 12 additional cases of cancers at other sites than lung per 100 000 smokers and year will still remain after cessation of smoking ( $12\ 000/10^8$ ).

**Cancers caused by use of Swedish oral snuff.** For year 1979 the Swedish Cancer Committee [68 – see Tables 4:6a, 4:6b] reported a total of 625 cases for men, and 155 for women relative to cancers of the lip, oral cavity, larynx and pharynx. Out of these, 500 cases among men and 80 among women were ascribed to smoking in combination with alcohol. In absence of smoking, these figures would then correspond to an incidence of 2.9 cases per 100 000 for men and 1.8 for women. For the purpose of this assessment a background morbidity in non-exposed of 2 cases per 100 000 and year is considered to represent a reasonable estimate.

As mentioned before, in several recent large Swedish case-control studies covering separate geographic regions, use of snuff was not found to be associated with any increased risk for cancers in the head neck region. No strict analyses of the power of resolution of these studies were published, but considering the confidence intervals, pooled data from the three studies would have a sufficient power of resolu-

tion to detect a 50% increase in the background incidence. In other words, for a snuff that is estimated to have contained an average of 2.5 µg NNK and 12 µg/g NNN, the maximum number of additional cases of cancers in the head neck region is about 1 additional case per 100 000 user of Swedish snuff and year. This incidence could, of course, also be lower. The prognosis for oral cancers is considerably better than for lung cancers, and a mortality of 50% will be assumed here based on a Mayo clinic material [145].

**Chronic obstructive pulmonary disease.** Chronic obstructive pulmonary disease (COPD) causes significantly more mortality and morbidity than other causes of airflow limitation in adults [146], but is underdiagnosed and under-recognised. The World Health Organization (WHO) estimates that chronic obstructive pulmonary disease is the fourth leading cause of death worldwide, with 2.74 million deaths in 2000, and this burden is growing rapidly [147]. In a Swedish study up to 50% of elderly smokers had developed COPD [148]. It has been demonstrated that smokers with airflow obstruction benefit greatly from quitting, despite previous heavy smoking [149].

Quantification of the reduction in mortality from COPD as a result of cessation of smoking has been hampered by lack of large prospective studies. However, in a comprehensive prospective study of 19 732 persons carried out by the Copenhagen Centre for Prospective Population Studies between 1978 and 2000, the yearly age-adjusted mortality rate per 100 000 individuals for COPD in never smokers was 70 for men and 40 for women [150]. WHO has given a somewhat lower over-all age standardized annual death rate from COPD for the whole Danish population of 56 for men, and 33 for women [151]. In ex-smokers from the Danish study the corresponding values were 100 for men and 30 for women, and cessation of smoking was associated with an over-all adjusted decrease in mortality of about 25%. However, due to the low number of deaths, these estimates are inexact, and the 25% risk reduction did not reach statistical significance. Heavy smoking men and women had, on the other hand, a mortality rate of 220 and 230, respectively, demonstrating a significant elevation of mortality in comparison with ex-smokers that

could indicate up to a 50% reduction in COPD mortality by quitting smoking. The latter estimate is supported by other evidence. Thus, in a cohort of 19 709 subjects followed for 14 years, the hospital admission frequency for COPD among heavy smokers was reduced by about half after cessation of smoking [152]. Further, a low FEV<sub>1</sub> (forced expiratory volume in 1 second) predicts not only an increased rate of decline in FEV<sub>1</sub> but also morbidity and mortality from COPD [153]. The subsequent rate of decline in FEV<sub>1</sub> among sustained quitters of smoking was found to be half the rate among continuing smokers, and comparable to that of never-smokers [149].

For Spain smoking prevalence has been reported to be 44% in 1993 for men in the age group 45- to 65, a subsection of the population where COPD is prone to become clinically manifest. Smoking was far less common among women over the age of 45 years, with only 9% of women smoking (although the prevalence in younger ages is considerably higher). The corresponding age standardized annual death rate per 100 000 in COPD as listed by WHO was 55 for men, and 14 for women, indicating the strong association of COPD with smoking. In Iceland the 1994 adult (age 15 to 89 years) smoking prevalence was 28% for women and 31% for men. This similarity between genders in smoking habits is reflected in the corresponding death rates for COPD, which was 21 and 29 respectively [151]. Assuming a highly plausible minimum standardized annual death rate for COPD within EU of 30 cases that can be attributed to smoking per 100 000 smokers, and a risk reduction of 50% from cessation of smoking, changing from cigarettes to snuff, would – after a transition period of 5–10 years – save another 15 000 lives from death caused by chronic obstructive pulmonary disease per year in EU among 100 million smokers.

**Cardiovascular disease (CVD).** Active smoking causes a moderate increase in risk for cardiovascular disease. However, because CVD has a high prevalence in the general population, even a modest increase translates into a high total impact. In a representative study of relative risks with respect to CVD conducted by Paffenberger et al. [154], hypertension (RR = 2.2; CI: 1.94–2.42) carried the highest risk, followed by cigarette smoking (RR = 1.84;

CI: 1.64–2.04). This estimate for risk associated with smoking agrees well with the results obtained from a very large Swedish cohort study for which the cause-specific mortality was followed during a 12-year period [49].

In the above mentioned prospective Danish study, the age-adjusted mortality rate per 100 000 per year for non-smoking men and women were 740 and 490 respectively, compared to 850 and 380 for ex-smokers, representing a non-significant difference. For cigarette smokers (average of heavy, light smokers, and reducers of smoking) the mortality rates were 1177 for men and 600 for women. In a follow up-study on 10 956 men and 8467 women, the Danish group could subsequently confirm the positive effects from cessation of smoking, and found that individuals who stopped smoking reduced their risk of myocardial infarction by 30% [155].

Due to a number of modulating factors, including diet, age-standardized annual death rates for cardiovascular disease (ischemic heart disease and cerebrovascular disease) show marked variations between the various EU member states, with a low of 154 per 100 000 for men and 84 for women in France, and a high of 553 and 325 for Hungarian men and women, respectively. It is therefore difficult to give an exact estimate for the impact of smoking on CVD that would be valid for the whole of EU. A reasonable approximation can, nevertheless, be made based on the assumption of an average EU CVD mortality rate per 100 000 in never-smokers of 200 that is increased to 360 in smokers (RR = 1.8). The Danish study cited above indicated a reduction of mortality by 30% upon cessation of smoking, which would translate into a decrease in the number of deaths by around 48 deaths per 100 000, i.e. corresponding to 48 000 for 100 million smokers.

Bolinder's group has claimed that smokeless tobacco constitutes a risk factor for sudden death in cardiovascular disease [49], a claim that has not been supported by other large studies where no association between use of snuff and cardiovascular disease has been found [156–159]. Further, whereas in the WHO Northern Sweden MONICA study, the number of cigarettes smoked was found to be quantitatively related with higher plasma fibrinogen levels, no such relation was found for snuff dip-

pers, nor were several key fibrinolytic variables affected in the latter group [160]. Thromboxane A2 is a powerful platelet aggregatory and adhesive agent as well as a strong vasoconstrictor. In an investigation covering 577 randomly selected Swedish men aged 18–19 years, out of which 7.5% were cigarette smokers and 22% snuff dippers, cigarette smoking – but not the use of snuff – significantly promoted the production of thromboxane A2 [161]. Further, the use of snuff does not decrease the plasma levels of antioxidants in a manner similar to active smoking [162].

The deleterious effect on the cardiovascular system claimed by Bolinder et al. [49] has been ascribed to the effects of nicotine. This claim is difficult to maintain in view of the fact that in users of other nicotine deliver products like nicotine gum, patch or nasal spray, no such adverse effects have been adequately documented [56 – see pp. 252–253;163–165], not even in patients with pre-existing cardiovascular disease [166–168].

Similarly the claim that snuff constitutes a risk factor for type II diabetes [169] remains unsubstantiated [170], and the findings of Bolinder et al. [49] can most probably be ascribed to residual confounding by failure to adjust for geographically skewed life style factors in the studied cohort of construction workers.

In summary, the number of deaths avoided per year in a population of 100 million smokers turned to snuff users can be calculated as follows:

■ Mortality in smoking induced cancers (lung, oral cavity, pharynx, larynx, esophagus, urinary tract, pancreas)	≈ 80 000
■ Minus mortality that remains after cessation of smoking (6000 + 12000)	≈ -18 000
■ Minus maximum cancer mortality from use of snuff = (0.5 • 2 000 • 0.5)	≈ -500
■ COPD mortality	≈ 15 000
■ CVD mortality	≈ 48 000

Total no. of lives saved	123 500
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\* Considering that the TSNA content has been reduced by a factor of 10 in comparison with the historical levels relevant for the Swedish studies, this mortality estimate can be expected to be lower.

Thus, cessation of smoking in favor of snuff usage would after a transition period of 5–10 years, save approximately 124 000 lives from death caused by smoking.

## DISCUSSION AND CONCLUSIONS – MARGINS OF SAFETY

Carcinogenic nitrosamines remain a justified health concern, where the highest exposures are clearly associated with tobacco specific nitrosamines (TSNA). Whereas the impact of TSNA in smokers is obscured by the presence of other carcinogens and promoters, for smokeless tobacco virtually all of the carcinogenic potential is associated with NNK and NNN. There is general agreement that the level of risk for consumers of smokeless tobacco is largely determined by its content of these TSNA. Also, there remains no doubt that exposure to impure products with extremely high TSNA concentrations, typified by Sudanese toombak, snuff produced in North Carolina before World War II, as well as oral snuffs mixed with areca nuts from South East Asia, are associated with a markedly increased risk for cancers in the head-neck region.

Cellular DNA damage that is misrepaired, or not repaired, constitutes a necessary, although not sufficient prerequisite for induction of cancer. There is ample evidence from the study of a number of methylating agents, that the strongly promutagenic DNA adduct O6-mGua plays a major role in initiation of cancer. In comparison with this adduct, methylation of position 7 in guanine is assumed to be less important. Although additional data points in the low dose region are required for improved resolution, the dose-response relationship with respect to the pro-mutagenic adduct O6-mGua appears linear down to the lowest tested dose for Clara cells from the rodent lung, and this seems also to be the case for induction of lung tumors. However, due to the fact that the majority of lung tumors begin as proliferative changes of Type II cells with subsequent progression to adenomas and carcinomas within the hyperplastic area [89], one may well argue that the dose-response relationship displaying a much lower level of adduct formation for this cell type is more relevant. The formation of this adduct in rat liver as well

as tumor induction from repeated exposure to NNK is, on the other hand, characterized by a clear dose-threshold evidently due to efficient DNA repair, and this seems to be true also for the nasal mucosa. For higher doses, an adaptive response of the alkyl transferase will undoubtedly have an impact in causing supra-linear responses. Nevertheless, when extrapolated to actual uptakes of TSNA from snuff, and compared with the “natural” background found in rodents as well as in humans, the expected levels of pro-mutagenic DNA adducts are insignificant.

When exposure from NNK and NNN is so low, that the number of DNA methylations or pyridyloxobutylations will not appreciably affect the level of such DNA lesions that are normally present in human tissues from individuals that have no known exposure to TSNA, this can be considered as a “virtually safe” dose threshold, irrespective of the actual shape of the dose response relationship. Based on analyses of various tissues using sufficiently sensitive techniques, the levels of O6-meGua DNA adducts in smokers are not significantly elevated above background levels. The use of today’s Swedish snuff, where the total intake of NNK and NNN ( $\approx 0.04 \mu\text{g}/\text{kg}/\text{day}$  per 20 g snuff) is about 10 times lower than from smoking 20 cigarettes per day (total intake  $\approx 0.4 \mu\text{g}/\text{kg}/\text{day}$ ), can be expected to result in an even lower impact on the existing adduct background.

The elevation of 7-guanine levels in smokers seen in some small studies indicates an elevated exposure to methylating agents, but the results are difficult to interpret in view of the simultaneous presence of 7-hydroxyethyl guanine adducts. In addition, results from cohorts involving a larger number of subjects would seem desirable. Because of modulation by genetic factors [171] in combination with high and variable backgrounds of methylated adducts from unknown sources, the risk for confounding in studies involving a small number of samples is substantial. Data from rodents predict an insignificant impact from smoking on the level of 7-mGua in humans. However, rates of DNA repair vary greatly between tissues, and comparison of adduct levels in the rodent liver with, e.g., human white blood cells is misleading because of the low repair rate in the latter.

HPB hemoglobin adducts constitute markers of exposure. However, although HPB adducts appear to inhibit the activity of alkylguanine-DNA-alkyltransferases [172], and measurement of urinary metabolites indicate striking differences between users of tobacco and non-exposed, the measured increase in HPB hemoglobin adducts have been modest, and appears to be elevated above background only in a subset of individuals [40]. However, although considerably lower, measured concentrations of HPB hemoglobin adducts in humans agree with the levels expected from rodent studies within an order of magnitude.

As discussed above, HPB DNA adducts seem to be involved in the induction of tumors of the rodent nasal epithelium and esophagus [108], and it is very likely that these bulky adducts could also be important for the induction of human cancer. However, pyridyloxobutylations evidently induce several types of DNA lesions including phosphate alkylations. This implies that only a certain fraction of all DNA adducts released as HPB are important in causing mutations. The low impact from TSNA in tobacco smoke on HPB DNA adducts as predicted by rodent studies seem to be substantiated by analysis of human biopsies of lung, esophagus and cardia, where the levels of O6-meGua DNA adducts in smokers were not significantly elevated above the background found in non-smokers. With a lower exposure to TSNA in comparison to smoking, intake of currently marketed Swedish brands of snuff can be expected not to increase the background levels of HPB DNA adducts. An important observation in virtually all the studies, where HPB adducts have been determined in humans, is the presence of appreciable levels of HPB releasing adducts in hemoglobin as well as in DNA from non-exposed subjects, indicating that sources for HPB adducts other than tobacco are important, and where myosmine that is present in various food products represents a possible candidate [11,45]. Finally, it should be kept in mind, that smokers in general tend to consume a less healthier diet containing lower levels of chemoprotective agents from vegetables than non-smokers [173], a factor that may be a source for confounding of the adduct measurements.

The Lewin et al. study [50] – the best conducted of the Swedish studies – included approximately 2 million per-

son-years at risk. Relying on the rodent data above, the additional cancer incidence for this population would be expected to be about 140 cases, i.e. representing a major part of all cancer cases in the head-neck region that were reported to the central Swedish cancer registry from the Stockholm County and the southern healthcare region of Sweden during the time period January 1988 through January 1991. In reality, no increased risk associated with snuff was found (OR = 1.0; CI: 0.7–1.6). With respect to the risk for pancreatic cancer, the low incidence of pancreatic cancer in the Swedish population does not seem to support the findings of Boffetta et al. [55]. However, a small cancer risk from Swedish snuff cannot be excluded, and the lower limit of detection for the pooled Swedish studies can be estimated at half the background yearly incidence of about 2 cases per 100 000. Nevertheless, the rodent data obviously overestimates real risk. There may be several reasons for this discrepancy. In the rat studies lung represents the most sensitive target organ for NNK, and data from this organ was used in the calculation of cancer potency to obtain an upper limit of risk related to oral administration [34]. However, the lung is obviously not a target organ in human users of snuff. Further, lung cancer in non-exposed humans is a relatively rare event, and an incidence of lung tumors in non-exposed rats that was 7.5% in the Rivenson et al. study [106], indicates that in comparison to humans, the rat lung is far more sensitive to induction of tumors, and therefore less appropriate for the purpose of risk extrapolation to humans. Thus, there is, e.g., a lower bioactivation of NNK in human lung compared to rodents [174,175]. For the estimation of a carcinogen potency factor for NNN, data relating to the nasal cavities were employed [176]. However, bioassay and histopathological data indicate that cell proliferation secondary to toxicity is required for tumor induction by NNK in the rodent nasal epithelium as well as liver, resulting in sublinear relationships between dose and tumor incidence [89,90,106]. This implies that for this type of rodent tumors the linearized multistage model may overestimate cancer risk considerably.

As mentioned above, investigations of the genotoxicity of extracts from Swedish snuff have provided support for the

presence of antimutagenic compounds [72], and the experiments with induction of tumors in the oral cavity of rats [71] likewise indicated the presence of chemoprotective agents, although the inhibitory effects on  $\alpha$ -hydroxylation by nicotine and cotinine could also be important in this rodent study.

Taking into consideration that IARC has classified wood dust as a human carcinogen [177], it is perhaps not surprising that this organization has reached the same conclusion with respect to oral snuff (all kinds). However, misinterpretation of the significance of the IARC classification system has had some unfortunate consequences for EU legislation. In line with US regulatory tradition, the author of this paper has analyzed the potential risks from TSNA, that in contrast to IARC's approach, is based on estimation of the level of risk, and where the mechanism of action has also been taken into consideration. The outcome of this analysis is that smokers' exposure to NNK and NNN seems to play a minor role compared with other carcinogens and promoters present in tobacco smoke. Taking into consideration that the levels of these nitrosamines in the currently marketed brands of Swedish snuff have decreased by a factor of 20, one may further safely assume that use of today's snuff products entail a negligible risk, a conclusion that is well supported by rodent studies on induction of the main pro-mutagenic DNA adducts as well by data on background DNA adduct levels in non-exposed humans.

It has been estimated that there are about 100 million smokers in the member states of the European Union [140]. The burden imposed on public health derives mainly from three groups of smoking related diseases; cancers, cardiovascular disease and chronic obstructive pulmonary disease. It can be demonstrated that for 100 million smokers turned to "snus" users, the number of deaths avoided in cancer, chronic obstructive pulmonary and cardiovascular diseases will reach about 124 000 annually after a period of 5 to 10 years. Nicotine products such as nicotine spray or patch are not affordable for large population groups in Central and Eastern Europe. Snuff, for which the kinetics of nicotine delivery is more similar to that of smoking, and that has been successfully used for the purpose of smoking cessation, offers an inexpensive alternative. In its recent

yearly report, the Swedish Government National Agency for Public Health concluded [178]:

"Everyone agrees that the health hazards of snus are minor compared with those of smoking. During 2005, a number of Swedish studies have been published showing that snus does not increase the risks of myocardial infarction morbidity". "By using panel data from Statistics Sweden's Living Conditions Surveys, in which the same people were interviewed in 1988–89 and 1996–97, we have demonstrated that for every person who progressed from snus to smoking, there were some four who switched from smoking to snus. Evidently, many people have used snus as a means to give up smoking. The risk that young adults (aged 16–44 years) will progress from snus to smoking is also far smaller than the risk that a non-smoker will take up smoking."

Considering that cigarettes, cigars, pipe tobacco, nasal snuff and chewing tobacco are freely marketed, the ban on low-nitrosamine oral snuff in all countries of the European Union except Sweden can be defended neither on medical nor ethical grounds.

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