

4. Other Data Relevant to an Evaluation of Carcinogenicity and its Mechanisms

4.1 Absorption, distribution, metabolism and excretion

4.1.1 *Humans*

(a) *Nicotine*

There are well-documented differences in the absorption of nicotine from smoked tobacco and that from smokeless tobacco products. However, once nicotine from smokeless tobacco has been absorbed into the systemic circulation, it is assumed that it is distributed, metabolized and excreted similarly, regardless of the route of administration. The experimental designs among the many pharmacokinetic studies of nicotine differ. Some experiments involved the intravenous administration of nicotine (occasionally as a radiolabelled

compound) and subsequent sampling of blood and urine at specified intervals over a fixed time period. In other studies, nicotine was delivered from cigarette smoking and, in relatively few studies, nicotine delivery from smokeless tobacco products was studied. The route of administration is regarded here as important when considering absorption characteristics of nicotine but after nicotine enters the circulation its distribution, metabolism and excretion are regarded as independent of the route of administration (see Table 81).

Table 81. Tobacco consumption and exposure to nicotine from smokeless tobacco use and cigarette smoking

Parameter	Oral snuff	Chewing tobacco	Cigarettes
Grams of smokeless tobacco or no. of cigarettes per day	15.6 ± 5.9 ^a (6.8–22.0) ^b	72.9 ± 21.6 (33.7–103.7)	36.4 ± 10.4 (25.0–54.0)
Maximal plasma nicotine concentration (µmol/L)	0.20 ± 0.10 (0.07–0.38)	0.17 ± 0.07 (0.07–0.29)	0.19 ± 0.03 (0.14–0.22)
AUC _{nic} (µmol/L/h)	2.48 ± 1.13 (0.97–4.67)	2.06 ± 0.84 (0.83–3.69)	3.04 ± 0.69 (2.45–4.37)
Urine nicotine (µmol/24 h)	5.58 ± 5.06 (1.04–15.84)	6.45 ± 4.65 (1.29–13.68)	6.97 ± 2.87 (3.58–10.75)
AUC _{cot} (µmol/L/h)	48.50 ± 30.77 (24.79–118.26)	48.25 ± 29.66 (13.67–113.87)	46.17 ± 13.29 (29.19–64.87)
AUC _{cot} /AUC _{nic}	21.4 ± 6.2 ^c (9.7–29.6)	23.9 ± 4.6 ^c (17.8–33.6)	16.4 ± 3.0 (11.6–21.4)

Adapted from Benowitz *et al.* (1989)

AUC, area under the plasma concentration–time curve; _{nic}, nicotine; _{cot}, cotinine

^a Mean ± standard deviation

^b Range

^c $p < 0.05$ compared with cigarettes

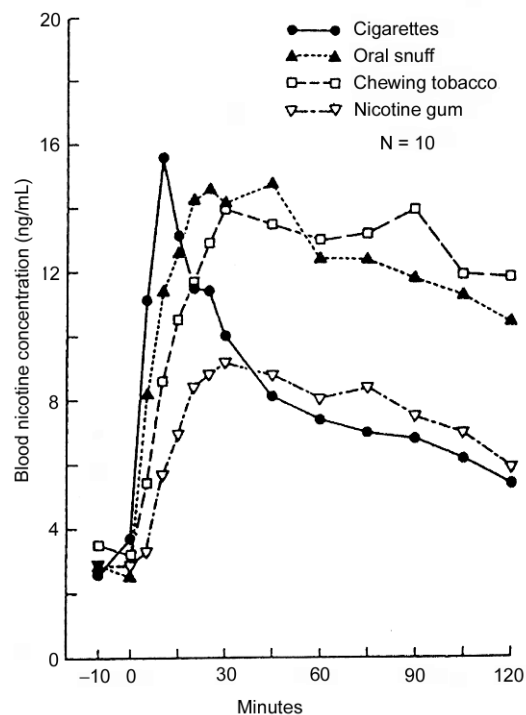
(i) Absorption

The absorption of nicotine (and possibly other components) from smokeless tobacco products is determined by several factors, such as the amount of the product used, the length of time it is kept in the mouth and the flux (movement of the product around the oral cavity) which are under the control of the consumer. Other factors, such as the concentration of nicotine in the tobacco product, the pH at the interface between the product and the buccal membrane and the particle size of the product, are not under the control of the consumer. However, as reviewed below, many studies have demonstrated the association between pH and nicotine absorption and some illustrated the importance of the particle size (cut) of the tobacco product.

Russell *et al.* (1981) demonstrated that moist snuff delivered measurable and significant quantities of nicotine to the systemic circulation. The time course of plasma nicotine levels was studied in three adults who were given 'Skoal Bandits' or nicotine chewing gum. The participants held the product in their mouths for 30 min, and blood was collected before and for up to 60 min after exposure. The peak increase in plasma levels of nicotine occurred after 30 min. Skoal Bandit increased plasma nicotine levels by 11 ng/mL over pretreatment levels, whereas 2 mg nicotine gum increased the levels by 6 ng/mL (Russell *et al.*, 1985).

In a study of 10 volunteers, Benowitz *et al.* (1988) measured plasma nicotine levels after use of moist (oral) snuff, chewing tobacco and nicotine chewing gum and cigarette smoking. After a single exposure to each product, maximal plasma levels were approximately equivalent for the cigarette and the smokeless tobacco products but, because of the prolonged exposure to the oral tobacco products, the overall amount of nicotine absorbed was twice as high as that after cigarette smoking. Nicotine gum delivered less nicotine than any of the other conditions (see Figure 6).

Figure 6. Blood nicotine concentrations during and after smoking cigarettes (average, 1 and 1/3 cigarettes), use of oral snuff (2.5 g), use of chewing tobacco (7.9 g) and chewing nicotine gum (two 2-mg pieces)



Adapted from Benowitz *et al.* (1988)

Many studies have demonstrated that the pH in the oral cavity is a major determinant of the absorption of nicotine from smokeless tobacco (Tomar & Henningfield, 1997). The unprotonated (free base) nicotine is most rapidly absorbed from the buccal cavity (Armitage & Turner, 1970), whereas the protonated molecule penetrates membranes very poorly. The percentage of unprotonated nicotine available is determined by use of the Henderson-Hasselbach equation which equates the ratio of the unprotonated and protonated molecules to the pH and the dissociation constant (pKa). Richter and Spierto (2003) compared the nicotine content, moisture and pH of 18 brands of smokeless tobacco products. The pH of the eight moist snuff products ranged from 5.35 to 8.28 and the nicotine content ranged from 4.28 to 13.54 mg/g. In the loose-leaf products (chewing tobacco), the pH ranged from 5.33 to 6.41 and the nicotine content ranged from 3.73 to 8.26 mg/g. The percentage of unprotonated nicotine in the moist snuff product ranged from 0.2 to 64.5%, whereas the loose-leaf product contained much less available nicotine (unprotonated form, 0.20–2.44%). The authors also noted that the moist snuff products with the highest levels of unprotonated nicotine were those that had the highest market share. The data suggest that the rapid availability of nicotine for absorption may be an important determinant of product appeal (discussed in the section on nicotine addiction) and supports the notion that smokeless tobacco products are used to obtain nicotine.

Henningfield *et al.* (1995) determined the aqueous pH of suspensions of four brands of moist snuff: Copenhagen, 8.6; Skoal Wintergreen, 7.6; Skoal Long Cut Cherry, 7.5; Skoal Bandits, 6.9. In a direct test of the proposal that pH determines nicotine absorption, Fant *et al.* (1999) administered these products to volunteers in a cross-over study. A different product was tested each day, and plasma nicotine levels were determined before administration, during the 30 min that the product was held in the mouth and at specified intervals for 90 min after it was removed. Plasma nicotine levels were directly related to pH. Specifically, 'Copenhagen', the product with the highest aqueous pH, delivered the highest peak levels of plasma nicotine, with an increase of 19.5 ng/mL above baseline. The products with intermediate pH ('Wintergreen' and 'Cherry') increased plasma nicotine levels by up to 12 ng/mL while 'Bandits' (lowest pH) induced an increase of 3 ng/mL only. Subjective ratings of preference and strength and increases in heart rate were also directly related to pH and plasma levels of nicotine.

It is recognized that both the saliva and the components of smokeless tobacco products have buffering capacity. These two buffering systems interact such that the pH of saliva at the buccal-product interface is maintained. Ciolino *et al.* (2001) investigated the relative buffering capacity of a series of six commercial smokeless tobacco products and 10 samples of unstimulated whole human saliva. The buffering capacity of the moist snuff products was 10–20 times greater than that of human saliva, which suggests that the pH at the buccal interface is almost entirely determined by the tobacco products.

The size of the tobacco cuttings in smokeless tobacco (fineness) also influences buccal nicotine absorption. More finely cut smokeless tobacco (smaller particle size) provides more surface area and a greater wetted surface which lead to the rapid absorption of nicotine (Connolly, 1995). Other additives that bind the tobacco cuttings together diminish absorp-

tion by decreasing the surface area. For example the 'long-cut' products use larger pieces of tobacco and a binding agent that allows the user to pack the tobacco tightly. These products release nicotine more slowly than the 'fine-cut' products (Connolly, 1995).

Another determinant of buccal nicotine absorption is the flux, i.e. the active process by the smokeless tobacco user of chewing, mastication and mixing the product with saliva. The extent to which the user 'works' the smokeless tobacco plug (quid) by chewing or moving it around the mouth may affect the speed and efficiency of nicotine absorption. As mentioned above, flux is a greater determinant of nicotine absorption from chewing tobacco than that from moist snuff products (Andersson *et al.*, 1994). Tomar and Henningfield (1997) concluded that the pH of moist snuff products are the main determinants of nicotine absorption and that rates of expectoration and oral manipulation probably have little effect on nicotine absorption.

Many moist snuff products are sold in small sachet pouches. This packaging appeals to consumers because the product is held closely together, it is provided in a unit dose and the tobacco particles do not migrate around the oral cavity. Connolly (1995) reported that the sachet packaging decreases nicotine absorption. The decrease may be due to less flux, slower and less saliva penetration (wetting) and the addition of another and limiting interface between the tobacco and the buccal membrane. The presence of the sachet seemed to slow down the release of nicotine from the tobacco to about 60% in the first minute (Nasr *et al.*, 1998). Whether the sachet package decreases the absorption and penetration of other components of the smokeless tobacco has not been determined. Both in-vitro (Nasr *et al.*, 1998) and in-vivo studies have reported that the release of nicotine from smokeless tobacco products is extremely fast. About 90% of the available nicotine was released within the first minute.

Although most absorption of nicotine from smokeless tobacco products is through the buccal mucosa and is pH-dependent, nicotine is absorbed from the intestine after oral administration (D'Orlando & Fox, 2004). Since swallowing the smokeless tobacco juice was documented in up to 48% of snuff users (Ebbert *et al.*, 2004), it is possible that some nicotine absorption from smokeless tobacco products occurs in the intestine. Oral nicotine absorption is typically slower (peak, 1–2 h) than buccal absorption (D'Orlando & Fox, 2004), which may also contribute to the sustained plasma levels of nicotine observed after the use of snuff and chewing tobacco (Figure 6).

(ii) *Distribution*

After nicotine is absorbed into the systemic circulation, it is rapidly distributed to all areas of the body. At physiological pH (7.4), about 69% of the nicotine is protonated and 31% is unprotonated; less than 5% is bound to plasma proteins (Benowitz *et al.*, 1982). The volume of distribution of nicotine averaged 180 L or about 2.5 times the body weight in kilograms. This means that, at steady state (equilibrium) levels, the amount of nicotine in the body tissue is 2.6 times that predicted by the product of blood concentration and body weight (DHHS, 1988).

Once nicotine enters the blood, it is rapidly distributed to body tissue and plasma levels fall very quickly after intravenous administration. Thus, immediately after intravenous administration, levels in the arterial blood, brain and lung are high whereas those in the muscle and adipose tissue (storage sites at steady-state concentrations) are quite low. As a result, the brain is immediately exposed to high levels of nicotine, and several animal studies have confirmed rapid uptake of nicotine into the brain (Schmiederl \ddot{o} w *et al.*, 1967; Oldendorf, 1972; Maziere *et al.*, 1976). When tobacco is smoked, the profile of nicotine distribution is very similar to that after intravenous administration: the concentration in the brain is influenced by distribution kinetics. The distribution half-life of nicotine is estimated to be 9 min (Feyerabend *et al.*, 1985), and reflects the rapid nicotine uptake into the brain after tobacco has been smoked or after intravenous administration of nicotine. However, after administration of smokeless tobacco, nicotine absorption is slower and follows a more protracted time course. For example, Benowitz *et al.* (1988) measured plasma levels of nicotine in volunteers after smoking a cigarette, and after using chewing tobacco, moist oral snuff or nicotine gum. Plasma levels increased from 3 ng/mL to about 15 ng/mL after smoking the cigarette or use of the smokeless tobacco products, respectively (levels after the use of nicotine gum were lower). However, the cigarette peak occurred early (10 min) whereas the peak after the smokeless tobacco products was closer to 30 min (see Figure 6). Furthermore, there was a clear difference in the declining slope of the plasma levels; after cigarette smoking, two phases were apparent and represented tissue distribution for the first 15 min and a second slope that represented plasma elimination kinetics (half-life, 2 h). After administration of the smokeless tobacco products, the plasma levels of nicotine declined at a slow steady rate that was parallel to the slope of the elimination phase that followed intravenous administration. The plasma levels did not decline immediately after the product was removed from the mouth, which indicates that local tissue deposition of nicotine may contribute to systemic absorption for some time after the product is removed. As a consequence of the differences in absorption and distribution of nicotine after smoking or administration of smokeless tobacco, brain tissue is confronted with a steady rate of nicotine distribution after smokeless tobacco as opposed to the pulsed increases seen after each puff of a cigarette. It is generally recognized that the speed of delivery of psychoactive drugs is an important determinant of their abuse liability (DHHS, 1988).

Swallowing the juice was more prevalent among people who use moist snuff (48% always swallowed, 15% never swallowed) than those who use chewing tobacco (31% always swallowed, 41% never swallowed) (Ebbert *et al.*, 2004). Furthermore, plasma cotinine analyses indicated that higher serum cotinine concentrations were associated with higher frequency of swallowing and the number of cans of product used per week. However, nearly 80% of nicotine that is absorbed from the intestine is metabolized (to cotinine) in the first pass through the liver and never reaches the systemic circulation. Thus, the level of plasma cotinine may not be as strong an index of consumption in users of smokeless tobacco as it is in cigarette smokers (Ebbert *et al.*, 2004).

(iii) *Metabolism*

In humans, 85–90% of a dose of nicotine is converted metabolically before its excretion, and only 5–10% is excreted unchanged in the urine. Nearly all nicotine metabolism occurs in the liver (Tricker, 2003), although one study (in dogs) showed that there is some metabolism in the lung (Turner *et al.*, 1975). The rate of hepatic metabolism is very rapid. Non-renal clearance of nicotine averaged 1100 mL/min, and it has been estimated that about 70% of nicotine in the plasma is extracted in each pass through the liver (Benowitz *et al.*, 1982; Table 82). More recent data discussed below suggest that some enzymes are capable of metabolizing nicotine in the brain (cytochrome-P450 [CYP] 2B6, CYP2E1) but it is uncertain to what extent such metabolism actually occurs.

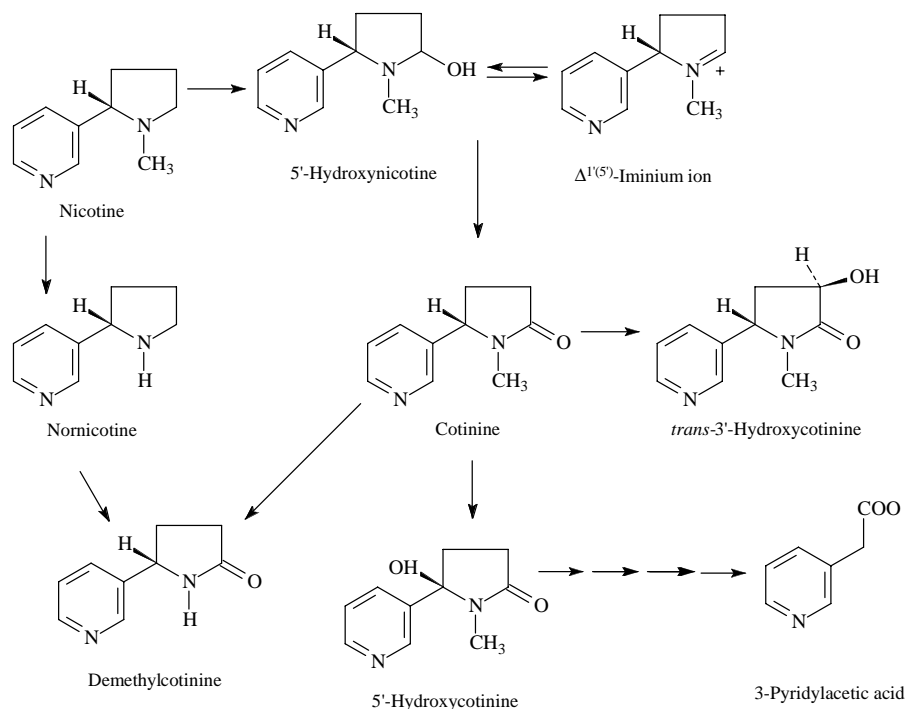
Table 82. Pharmacokinetics of nicotine and cotinine in cigarette smokers

Parameter	Nicotine	Cotinine
Half-life	120 min	18 h
Volume of distribution	180 L	88 L
Total clearance	1300mL/min	72 mL/min
Renal clearance	200–600 mL/min (acid urine) 100 mL/min (pH 5.8)	12 mL/min
Non-renal clearance	1100 mL/min	60 mL/min

Adapted from Benowitz *et al.* (1982, 1983)

A recent review by Yildiz (2004) describes the metabolism of nicotine as a two-phase process that occurs in the liver. In the first stage (microsomal oxidation), nicotine is largely (about 80% of a given dose) converted to cotinine, the major hepatic metabolite of nicotine in humans. Cotinine is formed from the oxidation of nicotine at the 5 position of the pyrrolidine ring. As illustrated below (Figure 7), this is a two-step process with the formation of 5'-hydroxynicotine and the iminium ion which is mediated by the iso-enzymes (2A6) in the CYP system. In a second step, the nicotine iminium ion is converted to cotinine by aldehyde oxidase. Cotinine itself is further metabolized at a much slower rate than nicotine (plasma half life, 18 h) and only about 17% of cotinine is excreted unchanged in the urine (Benowitz *et al.*, 1983). As shown below the major metabolites of cotinine include *trans*-3'-hydroxycotinine and 5'-hydroxycotinine and norcotinine.

In humans, the microsomal oxidation of nicotine to cotinine and from cotinine to *trans*-3'-hydroxycotinine are regulated by CYP enzymes. Several polymorphisms of these enzymes occur in humans, and some are more efficient than others at metabolizing nicotine. In the presence of less efficient enzymes, nicotine levels (after a single exposure) are higher and persist longer than when more efficient enzymes are present. Thus, polymorphism in the metabolizing enzymes may influence smoking behaviour (Nakajima *et al.*, 2001; Sellers

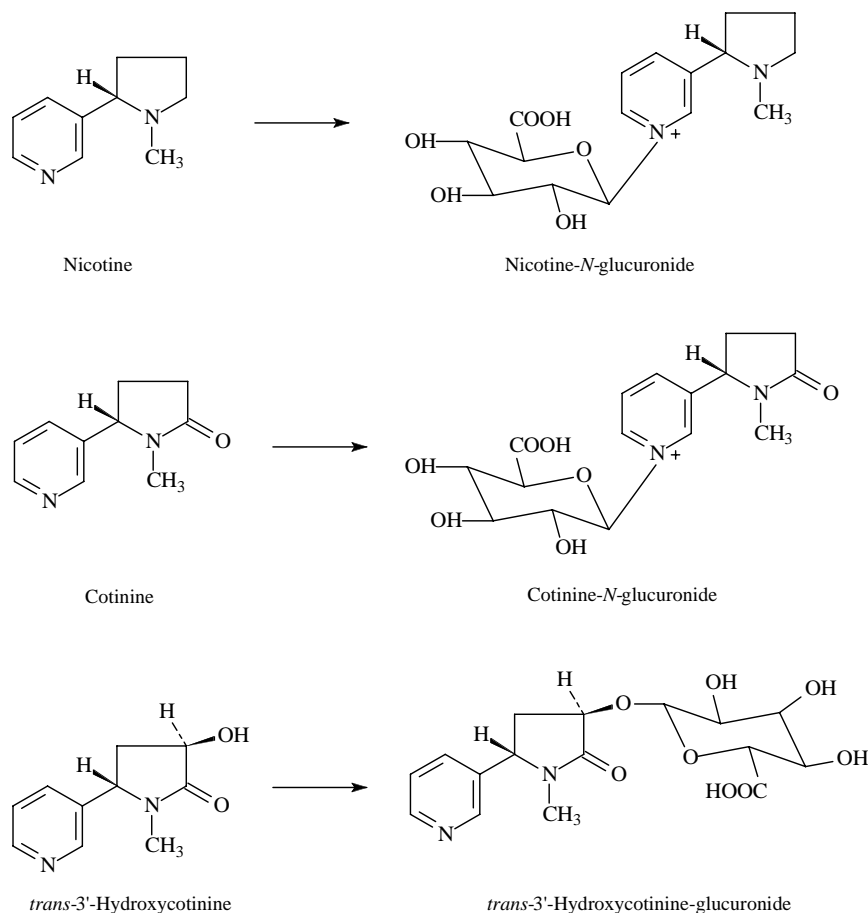
Figure 7. C-Oxidation of nicotine

Modified from Yildiz (2004)

et al., 2003). However, contradictory studies do not support the notion that polymorphisms in the *CYP2A6* and other genes that encode enzymes in the metabolic pathway of nicotine influence smoking behaviour or the health consequences of smoking (Tricker, 2003; Carter *et al.*, 2004). The influence of various polymorphisms of the *CYP2A6* gene on metabolism of nicotine from smokeless tobacco has not been studied.

The final step in nicotine metabolism is the formation of glucuronides. Glucuronidation of nicotine and cotinine results in compounds that are more water-soluble than the parent and more rapidly excreted in the urine. *N*- and *O*-Glucuronidation of nicotine and its metabolites (cotinine and *trans*-3'-hydroxycotinine) results in the formation of nicotine-*N*-glucuronide, cotinine-*N*-glucuronide and *trans*-3'-hydroxycotinine glucuronide (Figure 8 and Table 83) (Tricker, 2003; Yildiz, 2004).

Although most metabolism of nicotine occurs in the liver, enzymes present in the brain, lungs and elsewhere are also capable of metabolizing nicotine and may play a role in the health consequences of tobacco consumption. For example, *CYP2B6* metabolizes nicotine and is present in the human brain. Expression of the gene in autopsy samples of the brains of nonsmokers, smokers, alcoholics and non-alcoholics were compared. Gene expression was specific for different brain regions and occurred in both neurons and astrocytes.

Figure 8. N- and O-Glucuronidation of nicotine and cotinine

Modified from Yildiz (2004)

CYP2B6 levels were higher in the brains of smokers and alcoholics, particularly in the hippocampus and the cerebellum. The authors suggested that higher brain levels of the enzyme may alter the sensitivity to psychoactive drugs, increase susceptibility to neurotoxins and carcinogenic xenobiotics and even play a role in the tolerance to nicotine (Miksys *et al.*, 2003).

CYP2E1 is another enzyme that has been identified in the brain; it metabolizes alcohol and bioactivates tobacco-derived procarcinogens. Brain tissue from autopsied alcoholic smokers also revealed higher enzyme levels than tissue from non-alcoholic non-smokers (Howard *et al.*, 2003). Finally, nicotine induced CYP2E1 in cell cultures of human neuroblastoma cells. The results of this study also indicated that administration of nicotine influences its own metabolism and also that of other drugs (Howard *et al.*, 2003;

Table 83. Reported mean urinary excretion as a molar percentage ($\% \pm$ standard deviation) of total recovered nicotine and metabolites in the urine of smokers, smokeless tobacco users and persons who received dermal nicotine

Nicotine and metabolites	Study 1 (11 subjects)	Study 2 ^a (12 subjects)		Study 3 (54 subjects)	Study 4 (91 subjects)	Study 5 (12 subjects)	Study 6 (5 subjects)
	Smoking	Smoking	Transdermal nicotine	Smokeless tobacco	Smoking	Smoking	Smoking
Cotinine	13.2 \pm 3.9	13.3 \pm 3.1	14.9 \pm 4.6	7.9 \pm 2.2	9.2 \pm 2.6	14.8 \pm 5.9	15.2
Nicotine	10.4 \pm 3.7	10.4 \pm 4.4	11.1 \pm 4.3	8.3 \pm 5.7	9.4 \pm 5.7	7.9 \pm 4.6	9.5
<i>trans</i> -3'-Hydroxycotinine	35.2 \pm 7.4	39.1 \pm 12.5	37.0 \pm 10.8	41.6 \pm 10.6	36.1 \pm 10.6	42.4 \pm 12.8	34.1
Cotinine- <i>N</i> -glucuronide	17.5 \pm 6.3	15.8 \pm 7.8	15.4 \pm 7.9	8.9 \pm 4.6	14.0 \pm 5.4	12.1 \pm 6.0	20.1
Nicotine- <i>N</i> -glucuronide	2.8 \pm 2.2	4.6 \pm 2.9	5.3 \pm 3.3	3.0 \pm 1.8	4.5 \pm 2.5	2.6 \pm 2.1	3.7
<i>trans</i> -3'-Hydroxycotinine- <i>O</i> -glucuronide	8.5 \pm 3.8	7.8 \pm 5.9	7.9 \pm 4.7	19.4 \pm 11.0	22.8 \pm 10.0	10.3 \pm 7.6	7.4
Nicotine <i>N</i> -1'-oxide	6.8 \pm 2.9	3.7 \pm 0.9	2.7 \pm 1.2	8.6 \pm 6.9	3.0 \pm 2.1	ND	6.7
Cotinine <i>N</i> -1-oxide	3.0 \pm 1.9	4.5 \pm 1.5	5.2 \pm 1.5	2.5 \pm 2.3	0.9 \pm 0.9	ND	2.2
Normicotine	–	0.6 \pm 0.2	0.4 \pm 0.1	–	–	–	–
Norcotinine	1.5 \pm 0.5	ND	–	–	ND	ND	1.3
Others	–	–	–	–	–	10.1 ^b	–
Total	99.8	99.8	–	100.2	99.9	100.2	100.2

Adapted from Tricker (2003)

ND, not determined

Study 1, Byrd *et al.* (1992); Study 2, Benowitz *et al.* (1994); Study 3, Andersson *et al.* (1994); Study 4, Andersson *et al.* (1997); Study 5, Hecht *et al.* (1999a); Study 6, Meger *et al.* (2002)

^a In this study, 12 smokers were studied while smoking cigarettes and while receiving transdermal nicotine.

^b Sum of 4-hydroxy-4-(3-pyridyl)butanoic acid and 4-oxo-4-(3-pyridyl)butanoic acid

Miksys *et al.*, 2003). The effects of nicotine delivered from smokeless tobacco products on metabolism have not been documented but they are probably similar to those of smoke-delivered nicotine. It is also possible that smokeless tobacco products affect local (buccal) disposition of drugs and carcinogens present in the tobacco products.

The metabolism of nicotine may be influenced by the actions of other drugs. In a recent study, the effects of menthol cigarette smoking on nicotine metabolism was investigated (Benowitz *et al.*, 2004). Cigarette mentholation did not affect the intake of nicotine or carbon monoxide but nicotine metabolism was significantly slower after mentholated than after non-mentholated cigarettes. Menthol inhibited the metabolism of nicotine to cotinine and the formation of cotinine glucuronide. Menthol is a flavouring agent in many smokeless tobacco products. Although no studies are available, it is possible that menthol and other flavouring agents influence the metabolism of nicotine from smokeless tobacco products.

(iv) *Excretion*

Nicotine, cotinine and other metabolites are largely excreted in the urine. The excretion of unmetabolized nicotine (about 10% of a single dose) depends upon glomerular filtration rate and tubular secretion. The pH of the urine and urinary flow determine the amount of nicotine that is reabsorbed in the renal tubules. In an acidic pH, most of the nicotine is ionized and tubular reabsorption is low. Thus, an acidic urine increases the elimination of nicotine. Benowitz *et al.* (1983) determined that renal clearance of nicotine was 600 mL/min with acidic urine (pH 4.4) in which nicotine is largely protonated; conversely, when the urine is alkaline, nicotine is unprotonated and is reabsorbed into the circulation in the renal tubule and nicotine excretion is decreased. For example, when urine pH was adjusted to 7.0, the clearance of nicotine decreased to 17 mL/min; when urine pH was not controlled, pH averaged 5.8 and renal nicotine clearance was 100 mL/min, a rate that yielded an elimination of about 10–15% of the daily nicotine intake (Tables 82 and 83).

(b) *Smokeless tobacco constituents other than nicotine*

(i) *Absorption*

In several parts of the world, smokeless tobacco is invariably chewed with lime which is responsible for the highly alkaline pH (Nair *et al.*, 1990, 1992). Almost all smokeless tobacco products contain additives, such as ammonia, carbonate or bicarbonate, to raise the pH (Nair *et al.*, 2004). The pH of smokeless tobacco products is important, because nicotine most readily crosses the oral mucosa in the non-protonated form. Moist snuff products tested in volunteers were found to deliver high doses of nicotine to the bloodstream rapidly depending on the pH of the snuff product in aqueous solution (Fant *et al.*, 1999 ; see also Section 4.1.1(a)).

N-Nitrosamines in saliva

Carcinogens derived from smokeless tobacco products have been detected in the saliva of users of these substances. The TSNA, NNN, NNK, NAT and NAB (see sections in the

monograph on Tobacco-specific *N*-nitrosamines for the structures), as well as the volatile nitrosamines, NDMA and *N*-nitrosodiethylamine (NDEA), were detected in the saliva of tobacco chewers and snuff dippers (see Table 84). Volatile nitrosamines are probably also tobacco-derived.

High levels of TSNA (NNN, NNK, NAB) and volatile nitrosamines were detected in saliva samples collected from India. The saliva of men who chewed tobacco with lime contained higher levels of TSNA than that of men who chewed betel quid with tobacco and lime (Bhide *et al.*, 1986). NNN and NNK were also reported to be present in saliva in several other studies (Wenke *et al.*, 1984; Nair *et al.*, 1985, 1987). Volatile nitrosamines and TSNA in the saliva of chewers could derive from the leached nitrosamines present in the tobacco or could be formed endogenously from abundant precursors during chewing. The saliva of *mishri* users showed high levels of NNN (14–43 ppb [14–43 ng/mL]) and NPYR (2.2–8.3 ppb [2.2–8.3 ng/mL]) (Bhide *et al.*, 1987b).

Levels of TSNA, nicotine and cotinine were measured in the saliva of 20 snuff dippers (Inuit, Northwest Territories, Canada). Levels of NNN, NNK and NAT plus NAB found in the saliva following a 15-min period of keeping 0.5–1.5 g moist snuff in the gingival groove were considerable: NNN, 115–2601 ppb [115–2601 ng/mL]; NAT plus NAB, 123–4560 ppb [123–4560 ng/mL]; and NNK, up to 201 ppb [201 ng/mL]. The salivary levels increase with the duration of keeping snuff in the mouth. The total amount of TSNA was estimated to be 444 µg per use, a large part of which could be swallowed (Brunnemann *et al.*, 1987c).

Levels of TSNA were analysed every 10 min in the saliva of habitual snuff dippers. Detectable levels of at least two TSNA (NNN, NAT and NNK) were found in all samples collected between 10 and 30 min after the snuff had been placed in the mouth. The saliva of snuff dippers was reported to contain 57–420 ng/g NNN, up to 96 ng/g NNK and 7–470 ng/g NAT (Hoffmann & Adams, 1981). In a similar study, concentrations of 37–225, 0–61 and 48–555 ng/g NNN, NNK and NAT plus NAB were reported in snuff dippers' saliva, respectively (Palladino *et al.*, 1986), and total concentrations of TSNA up to 241 ng/g were found. Trace levels of TSNA were still found in the saliva 20 min after the snuff had been removed (Österdahl & Slorach, 1988). Enzymatically active human saliva was found to liberate up to twice the amount of the NNK than heat-treated saliva (Prokopczyk *et al.*, 1992b).

Salivary TSNA were measured in Sudanese oral snuff (*toombak*) users. NNN, NAT, NAB and NNK were measured before, during and after taking snuff. In addition, two other TSNA, NNAL and 4-(methylnitrosamino)-4-(3-pyridyl)-1-butanol (*iso*-NNAL), were detected in the saliva of tobacco chewers for the first time. Nine of 10 subjects had detectable levels of total salivary TSNA before chewing (0.01–1.0 µg/mL) and immediately following chewing (0.1–2.6 µg/mL). During dipping, TSNA concentrations in the saliva reached (µg/mL; range (number of subjects positive/total number)): NNN, 0.6–21 (12/12); NAT, 0.06–0.5 (2/12); NAB, 0.05–1.9 (12/12); NNK, 0.06–6.7 (11/12); NNAL, 0.05–3.3 (11/12); and *iso*-NNAL, 0.07–0.4 (8/12). These levels of salivary TSNA were 10–100 times

Table 84. Tobacco-specific *N*-nitrosamines (TSNA) in the saliva of tobacco chewers and snuff dippers

Smokeless tobacco	Country	No. of samples	TSNA (ng/mL saliva)			Reference
			NNN	NNK	NAT + NAB	
Snuff	USA	12	5–420	2–201	7–470	Hoffmann & Adams (1981)
	USA	30	37–225	ND–61	48–555	Palladino <i>et al.</i> (1986)
	Canada	20	115–2600	ND–201	123–4560	Brunnemann <i>et al.</i> (1987c)
	Sweden	4	3–140	ND–16	4–85	Österdahl & Slorach (1988)
Tobacco	India	7	36–130	ND	ND–380	Stich & Anders (1989)
	India	3	17–60	ND	14–52	Nair <i>et al.</i> (1985)
	India	10	10–430	ND–29	ND–133	Bhide <i>et al.</i> (1986)
<i>Khaini</i>	India	15	180–1580	ND–180	99–780	Stich <i>et al.</i> (1992)
Toothpaste containing tobacco	India	7	15–88	ND–10	10–70	Stich <i>et al.</i> (1992)
<i>Mishri</i>	India	9	14–44	ND	ND	Bhide <i>et al.</i> (1986)
<i>Toombak</i> ^a	Sudan	12	582–20 990	ND–6690	46–1940	Idris <i>et al.</i> (1992)

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

Note: Saliva samples were mostly collected a few minutes after beginning use of a new snuff dip or tobacco chew

^a Saliva also contained ND–409 ng/mL 4-(methylnitrosamino)-4-(3-pyridyl)-1-butanol (*iso*-NNAL) and ND–3270 ng/mL 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL).

those reported previously and are consistent with the unusually high levels of TSNA in Sudanese *toombak* (Idris *et al.*, 1992).

Levels of salivary TSNA were measured in Indian smokeless tobacco users, who place a mixture of *khaini* (tobacco and slaked lime) into the lower gingival groove, and users of tobacco-containing toothpaste (*gudhaku*) in Orissa, India. Among *khaini* tobacco chewers, up to 1580 ng/mL NNN, 690 ng/mL NAT, 90 ng/mL NAB and 180 ng/mL NNK were measured. Users of *gudhaku* showed much lower concentrations of these compounds, which may be due to the low amount of TSNA released from *gudhaku* and the short exposure time, which is restricted to the period of tooth brushing (Stich *et al.*, 1992).

(ii) *Distribution*

NNAL and NNAL-glucuronides (NNAL-Gluc) have been detected in the plasma of smokeless tobacco users, and NNAL has been detected in the plasma of smokers (Hecht *et al.*, 1999b; Hecht, 2002).

DNA and protein adducts

A ^{32}P -postlabelling assay has been explored to detect smokeless tobacco use-specific DNA adducts in the exfoliated oral mucosa cells of smokeless tobacco users. Adduct spots were detected in users as well as in non-users. ^{32}P -Postlabelling analysis of DNA from the oral cavity of these subjects did not demonstrate unique patterns or relative adduct level values. A lack of information on the structure of the majority of adducts was a serious limitation of these studies (Dunn & Stich, 1986; Chacko & Gupta, 1988).

Immunoassays for *O*⁶-methyldeoxyguanosine, a DNA adduct that could arise from NNAL and NNK, have given negative results in exfoliated oral cells from snuff dippers (Hecht *et al.*, 1987).

Haemoglobin adducts have been explored as biomarkers of exposure to and metabolic activation of TSNA. NNN and NNK form haemoglobin adducts in humans and experimental animals; these adducts release 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB) upon mild alkaline hydrolysis. HPB released from human haemoglobin can be quantified by gas chromatography–mass spectrometry (Hecht *et al.*, 1991). Levels of HPB released from haemoglobin (fmol HPB/g haemoglobin) were 517 ± 538 (mean \pm SD) in snuff dippers, 79.6 ± 189 in smokers and 29.3 ± 25.9 in nonsmokers (Carmella *et al.*, 1990). In Sudanese snuff dippers, the levels of the HPB-releasing haemoglobin adduct ranged from 68 to 323 fmol/g haemoglobin (mean \pm SD, 148 ± 104 fmol/g haemoglobin). The wide range of adduct levels observed suggests that, despite similar levels of exposure to NNK and NNN, significant differences exist in the ability of individuals in this population to activate these compounds (Murphy *et al.*, 1994). Nasal snuff users also showed high levels of haemoglobin adducts; however, HPB-releasing adducts were not correlated with the amount or type of snuff used (Schäffler *et al.*, 1993).

(iii) *Metabolism and excretion***Urinary carcinogen biomarkers**

All of studies reviewed in this section were carried out on smokeless tobacco users in the USA, unless otherwise specified.

The use of human urinary metabolites of carcinogens as biomarkers in tobacco carcinogenesis has been reviewed comprehensively (Hecht, 2002). NNK (see the monograph on Tobacco-specific nitrosamines) is metabolized to NNAL, which, similarly to NNK, is a potent pulmonary carcinogen in rodents. Glucuronidation of NNAL at the pyridine nitrogen yields NNAL-*N*-Gluc while conjugation at the carbinol oxygen yields NNAL-*O*-Gluc (Carmella *et al.*, 2002). Both NNAL and NNAL-Gluc are excreted in human urine and are very useful biomarkers because they derive from NNK that is specific to tobacco products (Hecht, 2002). In all studies to date, these biomarkers have been found to be absolutely specific to exposure to tobacco and have not been detected in the urine of non-users of tobacco unless they had been exposed to secondhand tobacco smoke. Because NNAL is not usually present in tobacco, NNAL and NNAL-Gluc in urine originate largely from the metabolism of NNK. Most investigations to date have demonstrated a correlation between NNAL plus NNAL-Gluc and cotinine (Hecht, 2002).

Seven male Sudanese *toombak* (snuff) dippers excreted exceptionally high amounts of urinary NNAL and NNAL-Gluc (0.12–0.44 mg) daily. Therefore, assuming chronic *toombak* use, the minimum daily dose of NNK to which these users were exposed was 0.12–0.44 mg. This is the highest documented uptake of a non-occupational carcinogen. The (*S*)-NNAL-*O*-Gluc:(*R*)-NNAL-*O*-Gluc ratio was 1.9. The two diastereomers of NNAL-Gluc were present in all urine samples analysed (Murphy *et al.*, 1994). The high systemic exposure to NNK suggests that these individuals may also be at risk for cancers other than those of the oral cavity.

The distribution half-lives of NNAL and NNAL-Gluc were determined in 13 male smokeless tobacco users. Baseline levels in urine as well as renal clearance of the NNK metabolites correlated with the number of tins or pouches of smokeless tobacco consumed. Ratios of (*S*)-NNAL:(*R*)-NNAL and (*S*)-NNAL-Gluc:(*R*)-NNAL-Gluc in urine were significantly higher 7 days after cessation than at baseline, which suggests a receptor site for the more carcinogenic NNAL enantiomer, (*S*)-NNAL. Urinary NNAL plus NNAL-Gluc also provides a good approximation of the dose of carcinogen taken in by snuff dippers. A correlation between the number of tins or pouches of smokeless tobacco consumed per week and NNAL plus NNAL-Gluc in the urine was observed, as well as a correlation between urinary cotinine and NNAL plus NNAL-Gluc in the urine of smokeless tobacco users (Hecht, 2002; Hecht *et al.*, 2002).

Urinary NNAL and NNAL-Gluc levels in 39 male smokeless tobacco users were similar to those in smokers. The ratio of NNAL-Gluc:NNAL was higher in snuff dippers than in tobacco chewers. A significant association between levels of NNAL plus NNAL-Gluc in the urine of smokeless tobacco users and the presence of oral leukoplakia was

observed, which supports the potential role of NNK as a causative factor for this lesion (Kresty *et al.*, 1996).

NNAL, NNAL-*N*-Gluc and NNAL-*O*-Gluc were analysed in the urine of 14 smokeless tobacco users. NNAL-*N*-Gluc in the urine comprised $24 \pm 12\%$ of total NNAL-Gluc and demonstrated that NNAL-*N*-Gluc contributes substantially to NNAL-Gluc in human urine (Carmella *et al.*, 2002).

Pyridine-*N*-oxidation of NNK and its major metabolite, NNAL, produces NNK-*N*-oxide and NNAL-*N*-oxide, respectively, which are detoxification products of NNK metabolism and are excreted in the urine of rodents and primates. Analysis of the urine of smokeless tobacco users for NNAL-*N*-oxide showed its presence at lower concentrations than that of NNAL. Thus, pyridine-*N*-oxidation is a relatively minor detoxification pathway of NNK and NNAL in humans (Carmella *et al.*, 1997).

Although tobacco products with reportedly reduced carcinogen content are on the market (see Section 1), carcinogen uptake in people who use these products has not been assessed systematically. In one study, 54 users of smokeless tobacco were randomly assigned to one of two groups. One switched to Swedish snuff (*snus*), while the other quit and used medicinal nicotine (the nicotine patch). All participants were assessed for urinary levels of total NNAL and NNAL-Gluc. Total NNAL levels were statistically significantly lower in users of smokeless tobacco after they had switched to Swedish moist snuff or to a nicotine patch than before the switch, although the overall mean total level of NNAL among subjects who used the nicotine patch was statistically significantly lower than that among those who used moist snuff (mean, 1.2 and 2.0 pmol NNAL/mg creatinine, respectively; mean difference, 0.9 pmol NNAL/mg creatinine; 95% CI, 0.2–1.5; $p = 0.008$) (Hatsukami *et al.*, 2004a).

Absorption of NNN by smokeless tobacco users has been demonstrated by detection of NNN and NNN-*N*-glucuronide (NNN-*N*-Gluc) in urine. Levels in 11 users were 0.03–0.58 pmol/mg creatinine (mean \pm SD, 0.25 ± 0.19 pmol/mg) NNN and 0.091–0.91 pmol/mg creatinine (mean \pm SD, 0.39 ± 0.27 pmol/mg) NNN-*N*-Gluc (Stepanov & Hecht, 2005).

NAB and NAB-*N*-glucuronide were excreted in the urine of smokeless tobacco users. Levels in 11 users ranged from not detectable to 0.11 pmol/mg creatinine (mean \pm SD, 0.037 ± 0.034 pmol/mg) NAB and 0.020–0.44 pmol/mg creatinine (mean \pm SD, 0.19 ± 0.16 pmol/mg) NAB-*N*-glucuronide (Stepanov & Hecht, 2005).

Absorption of NAT by smokeless tobacco users and smokers has been demonstrated by detection of NAT and NAT-*N*-glucuronide in urine. Levels in 11 smokeless tobacco users were 0.020–0.15 pmol/mg creatinine (mean \pm SD, 0.069 ± 0.046 pmol/mg) NAT and 0.08–2.78 pmol/mg creatinine (mean \pm SD, 1.36 ± 1.06 pmol/mg) NAT-*N*-glucuronide (Stepanov & Hecht, 2005).

Endogenous nitrosation

Tobacco contains secondary and tertiary amines that can be nitrosated in the saliva during the chewing of tobacco when they react with available nitrite in the presence of

nitrosation catalysts such as thiocyanate. The NPRO test measures the potential for intra-gastric formation of carcinogenic nitrosamines in humans. Nitrate and L-proline are administered to volunteers; non-carcinogenic NPRO is produced by an acid-catalysed reaction of proline (a model for ingested amines) with nitrate-derived nitrite in the stomach. It is then absorbed and excreted in the urine, which is analysed for NPRO (Ohshima & Bartsch, 1981).

The role of poor oral hygiene in the formation of *N*-nitroso compounds was investigated by means of the NPRO assay. Endogenous nitrosation was significantly higher in tobacco chewers with poor oral hygiene (determined by dental plaque) compared with those with good oral hygiene (Nair *et al.*, 1996). Nitrite found in human saliva is the product of the microbial reduction of nitrate released from the salivary glands. Salivary nitrite level and nitrate reductase activity, when detected, have been reported to be higher in tobacco chewers and *mishri* users from India than in controls (Murdia *et al.*, 1982). Increased formation of nitrite and nitric oxide in the mouth was found in people with dental plaque (Carossa *et al.*, 2001) and bacterial enzyme-mediated formation of nitrosamines has been reported (Calmels *et al.*, 1996). Thus, dependent on the availability of nitrosatable amines from tobacco, the formation of nitrosamines is more extensive in subjects who have poor oral hygiene if they chew tobacco.

Among subjects dosed with proline, the level of NPRO was significantly elevated in the urine of individuals who chewed tobacco plus lime than in non-users (Chakradeo *et al.*, 1994). Levels of other *N*-nitrosamino acids (NSAR, MNPA, MNBA, NTCA and MNTCA) were not significantly affected (Nair *et al.*, 1985; Chakradeo *et al.*, 1994).

Measurable concentrations of all tobacco alkaloids (nicotine, normicotine, anabasine, and anatabine) were excreted in the urine of subjects who used smokeless tobacco. These compounds could be substrates for endogenous nitrosation in tobacco chewers (Jacob *et al.*, 2002).

4.1.2 *Experimental systems*

(a) *Absorption*

Squier (1986) investigated the simultaneous penetration of nicotine and NNN across porcine skin and various regions of oral mucosa. Penetration by nicotine was as 'rapid as water'. Non-keratinized regions were also permeable to NNN, a pattern that is mimicked by the locations of tumours in the oral cavity. A later study (Du *et al.*, 2000) tested the influence of nicotine and alcohol on the permeability of the mucosal membranes to NNN *in vitro*. It was reported that alcohol and nicotine each increased the permeability of NNN and that, together, ethanol and nicotine increased the permeability to levels higher than that of ethanol alone.

(b) *Effect of smokeless tobacco on enzyme activities and metabolism*

(i) *Animal studies*

Most investigations of the effects of smokeless tobacco on enzyme activities in animals have measured changes in levels of phase I and phase II enzymes in the lung and liver of mice and rats.

While TSNA are the most abundant carcinogens in smokeless tobacco products, some forms of pyrolysed smokeless tobacco products also contain PAHs. The PAH profiles of *mishri* and snuff revealed significant amounts of carcinogenic PAH compounds. *Mishri* extract or snuff extract was given intraperitoneally to inbred male Swiss mice (eight animals per group) and the activities of hepatic microsomal cytochrome b5, CYP and benzo[*a*]pyrene hydroxylase were measured. A significant increase in levels of CYP and benzo[*a*]pyrene hydroxylase was observed as a result of both treatments (Bhide *et al.*, 1984a, 1991).

Male Sprague-Dawley rats (12 weeks of age) were fed a standard diet and were given *mishri* extract and benzo[*a*]pyrene at 75% of the dose that causes 50% lethality (LD₅₀) by intraperitoneal injection three times at 24-h intervals. An increase in the levels of the hepatic phase I activating enzymes, CYP content, benzo[*a*]pyrene hydroxylase and benzphetamine demethylase and a significant decrease in glutathione-*S*-transferase (GST) activity were observed. Depletion in glutathione (GSH) content and hepatic vitamin A pool and a concomitant increase in vitamin C content were also noted (Ammigan *et al.*, 1989a; Bhide *et al.*, 1991).

Acute exposure to smokeless tobacco and nutritional deficiency

Malnutrition is a condition that affects the population in several countries where the use of smokeless tobacco is widespread. Altered metabolism as a result of vitamin deficiency and protein-calorie malnutrition may be an important factor in susceptibility to carcinogens. The following studies have investigated the effect of smokeless tobacco in the presence of some nutritional deficiencies (obtained using a well-defined semi-synthetic basal diet) on hepatic and pulmonary carcinogen-metabolizing enzymes in male Sprague-Dawley rats.

The effects of extracts of chewing tobacco and *mishri* were evaluated in a series of experiments on nutritional deficiency. NNN and benzo[*a*]pyrene were tested at the same time as standard carcinogens using the following basic experimental protocol.

Inbred male weanling Sprague-Dawley rats (19–21 days old and weighing 35–50 g) were randomly divided into three groups of eight animals each and were fed three different dietary regimens that consisted of standard diet, control semi-synthetic diet and semi-synthetic deficient diet. In each set of experiments, the semi-synthetic diets were either adequate (control) or deficient in vitamin A (Nair *et al.*, 1991a), vitamin B complex (Ammigan *et al.*, 1990a) or protein (Ammigan *et al.*, 1989b, 1990b). At 12 weeks, tobacco extract, *mishri* extract, NNN or benzo[*a*]pyrene was administered intraperitoneally at 75% of the LD₅₀ dose, divided in three equal doses at 24-h intervals. Twenty-four hours after the last

injection, animals overnight fasted were killed and the lung and liver were excised. The hepatic and pulmonary biotransformation enzymes, CYP, cytochrome b-5, benzo[*a*]pyrene hydroxylase, benzphetamine *N*-demethylase, GST and GSH content were determined. Vitamin A and C were also measured. Smokeless tobacco extracts were found to be more toxic to animals with nutritional deficiencies. The tolerance of animals on deficient diets was lower as seen by the increase in toxicity of the test substances. In vitamin A-, vitamin B complex- or protein-deficient rats, the LD₅₀ for intraperitoneally injected tobacco extract was reduced by 32–40%, that of *mishri* extract by 43%, that of NNN by 20–24% and that of benzo[*a*]pyrene by 24% (Ammigan *et al.*, 1990b). These deficiencies also resulted in decreases in the basal levels of CYP, benzo[*a*]pyrene hydroxylase, benzphetamine demethylase, GST and GSH.

In vitamin A-sufficient and -deficient groups, treatment with *mishri* extract, benzo[*a*]pyrene (Ammigan *et al.*, 1990c), tobacco extract and NNN (Nair *et al.*, 1991a) significantly increased the phase I activating enzymes in all groups. A greater increase in hepatic and pulmonary phase I activities was observed in deficient animals compared with sufficient groups. An increase in GSH and GST levels was observed in the sufficient group following treatment, but exposure to the test substances caused further suppression of the hepatic and pulmonary GSH/GST system in the deficient animals.

Groups of Sprague-Dawley rats fed low-protein (5% casein) or vitamin B complex-deficient diets were exposed to tobacco extract, *mishri* extract, NNN or benzo[*a*]pyrene according to the same protocol. All animals showed a significant increase in phase I enzymes with concurrent inhibition of GSH and GST levels compared with corresponding control groups on high-protein (20% casein) or vitamin B complex-sufficient diets (Ammigan *et al.*, 1989b, 1990a).

Smokeless tobacco (50 or 100 mg/kg bw per day) was shown to modify the activity of phytic acid (1000 mg/kg bw per day by gavage) and butylated hydroxyanisole (1% w/w in diet) both directly and translactationally by significantly inhibiting the phytic acid-induced hepatic GST and GSH levels and further augmenting phytic acid- or butylated hydroxyanisole-induced microsomal cytochrome b5 and CYP in lactating dams and suckling pups of mice (Singh & Singh, 1998).

Chronic exposure to smokeless tobacco

The effects of chronic exposure to 10% *mishri* in a standard diet for 20 months on the activities of several activating enzymes, GST and GSH levels were measured in several organs of three rodent species: Swiss mice, Sprague-Dawley rats and Syrian golden hamsters. At 20 months, the upper alimentary tract, tongue, oesophagus, stomach, liver and lung were excised. Significant increases in activities of phase I activating enzymes and decreases in the phase II detoxification system were observed in most extrahepatic tissues of the treated animals of all three species. These observations suggest that prolonged exposure to smokeless tobacco extract affects the drug-metabolizing enzymes of the gastrointestinal tract, which may be an important factor that determines the susceptibility of different organs to exposure to carcinogens (Nair *et al.*, 1991b). The base levels

of enzymes in proximal, medium and distal parts of the intestine in the three species were similar. However, the levels of CYP, benzo[*a*]pyrene hydroxylase and GST were highest in hamsters followed by rat and mice. In the exposed groups, a significant induction of CYP and benzo[*a*]pyrene hydroxylase and depletion of GSH and GST levels were observed only in the proximal and distal parts of the intestine of the three species, which suggests the importance of proximal and distal parts of the rodent intestine in metabolism and susceptibility to intestinal xenobiotic exposure (Nair *et al.*, 1991c).

Chronic exposure to smokeless tobacco and vitamin A deficiency

Chronic exposure was investigated in two long-term studies in Sprague-Dawley rats that were fed standard vitamin A-sufficient and -deficient diets and were administered daily oral doses of 3 mg tobacco extract or *mishri* extract over a period of 21 months. Pulmonary and hepatic carcinogen-metabolizing enzymes, both phase I (CYP, cytochrome b5, benzo[*a*]pyrene hydroxylase, benzphetamine demethylase) and phase II (GST), as well as levels of vitamin A and C in plasma and liver were measured at 12 and 21 months. Overall, the phase I enzyme activities were significantly higher in vitamin A-sufficient than in vitamin A-deficient rats at both 12 months and 21 months. Treatment with tobacco extract increased the activity of hepatic and pulmonary phase I enzymes but decreased the GSH/GST system at both time points. The vitamin A-deficient *mishri*-treated animals also showed a decrease in the GSH/GST detoxification system after 12 and 21 months while the converse was observed in vitamin A-sufficient group after 21 months. Similar to short-term exposure studies, treatment with tobacco extract and *mishri* extract significantly lowered the hepatic and circulating levels of vitamin A, while a concurrent increase was observed in the level of vitamin C. The data showed that chronic exposure to smokeless tobacco together with vitamin A deficiency renders the rats more susceptible to smokeless tobacco (tumorigenicity data in Section 3.1), partly due to the augmented carcinogen activation together with depletion of the detoxifying GSH/GST system (Ammigan *et al.*, 1991; Bhide *et al.*, 1991).

(ii) *Cell culture systems*

Indian snuff extract showed an overall inhibitory effect on cell count, [³H]thymidine uptake and ornithine decarboxylase and aryl hydrocarbon hydroxylase activities when incubated either alone or in combination with NNN or NNK in in-vitro cultures of embryonic mouse tongue primary epithelial cells (Gijare *et al.*, 1989). Cultures treated with snuff extract in combination with DMBA also showed inhibition of cell proliferation and a decrease in ornithine decarboxylase and aryl hydrocarbon hydroxylase activities compared with control, DMBA- and DMBA plus TPA-treated cultures (Gijare *et al.*, 1990b).

(c) *Smokeless tobacco and reactive oxygen species*

(i) *Animal studies*

Oxidative stress and reactive oxygen species can play a significant role in the cytotoxic effects induced by smokeless tobacco products. Acute and subchronic administra-

tion of smokeless tobacco extract in phosphate buffer to rats induced hepatic mitochondrial and microsomal lipid peroxidation, hepatic DNA single-strand breaks, significant increases in urinary excretion of malondialdehyde, formaldehyde, acetaldehyde and acetone, and significant increases of nitric oxide production in peritoneal macrophages, which suggest the involvement of oxidative stress in the toxicity of smokeless tobacco extract (Bagchi *et al.*, 1994; Hassoun *et al.*, 1995; Bagchi *et al.*, 1998).

(ii) *In-vitro studies*

Application of smokeless tobacco resulted in generation of reactive oxygen species in in-vitro experiments with peritoneal macrophages, and in hepatic mitochondria and microsomes from female Sprague-Dawley rats and that of nitric oxide in the peritoneal macrophage J774A.1 cells in culture (Bagchi *et al.*, 1995; Hassoun *et al.*, 1995; Bagchi *et al.*, 1996).

Cultured human oral epithelial carcinoma cells produced reactive oxygen species following in-vitro incubation with an aqueous extract of smokeless tobacco (Bagchi *et al.*, 1996). Smokeless tobacco extracts significantly induced the production of superoxide anion, and increased lipid peroxidation, DNA fragmentation and protein kinase C activity in primary cultures of human oral keratinocytes (Bagchi *et al.*, 1997, 2002). Using flow cytometry with the fluorescent dye, propidium iodide, a dose-dependent increase in apoptotic cell death was observed following treatment with smokeless tobacco extract which was inhibited by several antioxidants including vitamin C and vitamin E (Bagchi *et al.*, 1999).

Smokeless tobacco extract that contained an equivalent amount of nicotine was found to be more toxic than nicotine in the generation of reactive oxygen species, as assessed by the measurement of changes in GSH and malondialdehyde levels in Chinese hamster ovary cells (Yildiz *et al.*, 1999).

In summary, nutritional deficiencies that are either already prevalent or caused by the use of smokeless tobacco are often observed in a large proportion of smokeless tobacco users. The situation is aggravated by further induction of phase I enzymes and suppression of antioxidant systems, such as the GSH/GST system. Reduced plasma levels of several antioxidant vitamins have also been reported in smokeless tobacco users. The generation of reactive oxygen species and lipid peroxidation due to smokeless tobacco extracts have been reported and can accelerate different stages of carcinogenesis.

(d) *Biomarkers (including adducts) of smokeless tobacco carcinogens*

(i) *In-vitro formation of TSNA from a variety of smokeless tobacco products*

The levels of the TSNA (NAB, NAT, NNN and NNK) were determined in a variety of chewing tobacco, oral snuff, *mishri* and *zarda* samples. The potential endogenous formation of TSNA was estimated by incubation of tobacco samples at pH 2.0 for 1 h at 37 °C and over the pH range 1.0–5.5 under conditions that simulated the normal fasting stomach, with a constant nitrite concentration of 25 µM. Under the simulated gastric

conditions, NAB, NAT and NNN were formed, and maximum formation of these TSNA occurred at pH 2.5. Under the acidic simulated gastric conditions (pH 2.0), slight decomposition of NNK via transnitrosation was observed (Tricker *et al.*, 1988).

(ii) *DNA adducts*

Male Fischer 344 rats were pretreated for 2 weeks with either a solution of a snuff extract or 0.002% nicotine in the drinking-water. Subsequently, the rats were given a single dose of NNK and the effects of snuff and nicotine on the methylation of guanine by NNK in the DNA of target organs were determined. Formation of 7-methylguanine in the liver, nasal mucosa and oral cavity and of *O*⁶-methylguanine in the liver and oral cavity were much lower in the rats pretreated with snuff extract than in those that were not pretreated. In contrast, pretreatment of the rats with nicotine had no significant effect on the methylation of DNA by NNK nor on the elimination constants of NNK and its major metabolite NNAL (Prokopczyk *et al.*, 1987). The authors suggested that snuff extract contains one or more compounds other than nicotine that alter the methylation of guanine by NNK.

A ³²P-postlabelling assay to detect adducts in DNA from rat oral epithelial cells after their exposure *in vitro* to chewing tobacco extract in the presence of ethanol showed slightly higher adduct levels in treated cells than in control cells (Autrup *et al.*, 1992). However, following chronic exposure of rats to snuff in a surgically created canal in the lower lip, aromatic DNA adducts were not detected by ³²P-postlabelling. The adduction to DNA in organs of the gastrointestinal tract and the kidneys indicates that the use of snuff results in systemic exposure to carcinogens and may contribute to the incidence of neoplasms in organs outside the oral cavity (Smith *et al.*, 1997).

4.2 Toxic effects

4.2.1 Humans

(a) *Nicotine addiction*

At the Mayo Clinic, Morse *et al.* (1977) described a case of a 53-year-old man who requested treatment for dependence on chewing tobacco. The report was remarkable because the patient defined himself as addicted at a time when the medical community was reluctant to include tobacco as a dependence-producing substance. The patient exemplified the hallmarks of drug dependency — loss of control over consumption, compulsive use in the face of obvious harm, escalating patterns of use and symptoms of withdrawal on discontinuation of the drug. Despite this and other clinical cases, there was little systematic study of smokeless tobacco dependence and withdrawal until the 1980s. Nevertheless, on the basis of available evidence for all forms of tobacco use, the American Psychiatric Association included smokeless tobacco as a potential cause of dependence and withdrawal in its 1980 Diagnostic and Statistical Manual of Mental Disorders, Third Revision (American Psychiatric Association, 1980). The potential for smokeless tobacco to cause dependence and withdrawal was specifically reviewed by an advisory committee

to the US Surgeon General in 1986 (DHHS, 1986) which concluded that “The use of smokeless tobacco products can lead to nicotine dependence and addiction”. The conclusion was based on evidence from clinical and animal studies that showed that exposure to nicotine from smokeless tobacco products, either through self administration of the products in people or from administration of nicotine to animals, induced psychoactive effects in both animals and people. These conclusions were reaffirmed in 1988 (DHHS, 1988) and later (DHHS, 1995, 1996).

It has now been recognized by several governing bodies that smokeless tobacco products initiate and sustain addiction — that is, they cause physical dependence (DHHS, 1986; Henningfield *et al.*, 1997; Henningfield & Fant, 1999). The following section reviews characteristics that define drug addiction and dependence and gives information on how smokeless tobacco products fulfil these requirements.

(i) *‘Addiction’ versus ‘dependence’ and ‘withdrawal’*

Technical scientific reports and medical diagnoses have used the terms ‘dependence’ and ‘withdrawal’ to define compulsive drug-seeking behaviour and the abstinence-associated behavioural and physiological disruptions (American Psychiatric Association, 1987, 1994; WHO, 2006). However, the term ‘addiction’ is more typically used as the most universally recognized term in general communications by major health organizations, and remains the term used to describe the phenomenon of compulsively driven drug-seeking behaviour.

The criteria for a drug to be identified as addictive have been delineated (DHHS, 1988), and include a pattern of use that is either highly controlled or compulsive, which entails psychoactive effects and drug-reinforced behaviour. Additional criteria include stereotypic use patterns, use despite harmful effects, relapse following abstinence and recurrent drug cravings. Physiological manifestations of dependence-producing drugs include the development of tolerance, physical dependence manifest by withdrawal signs and symptoms upon acute discontinuation of the drug and pleasurable or euphoriant acute effects.

Clinical criteria to determine whether a person is dependent upon a drug and/or if drug abstinence has produced a withdrawal syndrome are provided by the American Psychiatric Association (1987, 1994) and by the WHO (2006). In 1980, the American Psychiatric Association acknowledged ‘tobacco dependence’ and ‘tobacco withdrawal’ syndromes. The US National Institute on Drug Abuse came to similar conclusions and further concluded that nicotine met the same criteria as a dependence-producing drug such as cocaine and morphine (DHHS, 1988).

The determination of whether or not a substance meets criteria that can produce dependence and/or withdrawal has been elaborated through laboratory studies of the pharmacological actions of the drugs (United Nations Single Convention on Narcotic Drugs, 1961; United Nations Convention on Psychotropic Substances, 1971; Balster & Bigelow, 2003; Spillane & McAllister, 2003). In practice, both laboratory data and observations of clinical dependence in users are critical in determining whether a drug is appropriately categorized as ‘dependence’-producing. It is important to note that the phenomenon of ‘withdrawal’

can be determined independently in clinical evaluations and in laboratory studies and is neither necessary (e.g. many users of heroin and cocaine use the drugs intermittently and do not show signs of withdrawal upon discontinuation of use) nor sufficient (e.g. experimental production of physical dependence in laboratory studies and production of physical dependence in patients treated for pain with analgesics does not necessarily result in drug-seeking behaviour and dependence) (for reviews, see DHHS, 1988; Feinstein *et al.*, 2000).

(ii) *Tobacco versus nicotine*

Nicotine is the drug in tobacco that defines tobacco use as an addiction since it meets independent criteria for addiction and is delivered in sufficient quantities to produce physiological and behavioural effects that comprise addiction. However, nicotine alone does not fully explain all aspects of symptoms, clinical course or need for treatment that relate to addiction. As for other addictive drugs, the prevalence of use, risk of addiction and related consequences are linked to the formulation of the drug, cost, access and social image. Tobacco addiction, prognosis and treatment have been discussed in detail elsewhere (DHHS, 1988; Royal College of Physicians of London, 2001).

(iii) *Evaluation of potential dependence-producing effects of smokeless tobacco*

The majority of data that show that tobacco products are addictive derive from studies of cigarette smoking (DHHS, 1988; Royal College of Physicians of London, 2000). However, repeated demonstrations (Gritz *et al.*, 1981; Benowitz *et al.*, 1983; Russell *et al.*, 1985; Fant *et al.*, 1999) that smokeless tobacco products rapidly deliver nicotine at levels equal to or greater than that of cigarette consumption imply that continual use of smokeless tobacco products initiates and sustains tobacco dependence. Demonstrable signs of withdrawal upon the discontinuation of regular smokeless tobacco use (Hatsukami *et al.*, 1987; Keenan *et al.*, 1989; Hatsukami *et al.*, 1992) are further evidence that smokeless tobacco products produce dependence. Finally, the reports by smokeless tobacco users that they are addicted, have tried and failed to quit use on several occasions and that the smokeless tobacco products influence occupational and social behaviours (Hatsukami & Severson, 1999; Severson, 2003) also provide direct support for the addictive potential of smokeless tobacco products.

(iv) *Patterns of use*

Dependence-producing drugs typically cause a pattern of use that is characterized by strong, almost irresistible, urges and cravings to consume the drug. Consumption is continued even when the user acknowledges that the drug has the potential to or has caused physical harm or interferes with social or occupational pursuits. The use pattern persists even when there is a strong effort to stop drug consumption. The urge to use is enduring; even after long periods of drug abstinence, cravings for the drug are evident and re-initiation of drug use (relapse) is frequent. Reports have suggested patterns of escalating dosage with duration of smokeless tobacco use and age and have shown consi-

derable evidence for relapse and inability to maintain abstinence from smokeless tobacco (Hatsukami & Severson, 1999).

The pattern of compulsive use has been amply demonstrated for cigarette smoking (DHHS, 1988) and there is substantial evidence that the case is similar for smokeless tobacco. Many (74%) first-time users of smokeless tobacco found the experience unpleasant; when used a second time, 53% found the experience to be unpleasant (Ary *et al.*, 1989). These data suggest that some tolerance to the unpleasant effects of smokeless tobacco are apparent even after a single use. As users become more tolerant, the time interval between smokeless tobacco use decreases rapidly. For example, 20% of first-time users repeated the experience within 24 h and 33% used their second dip within 24 h. Consumption of smokeless tobacco tends to increase with age; adolescents that reported daily use consumed less smokeless tobacco than young adults who reported daily use (Ary *et al.*, 1987, 1989).

When daily smokeless tobacco use is established, levels of exposure to nicotine are comparable with those of daily cigarette smokers. Gritz *et al.* (1981) examined plasma nicotine levels over the course of a day in which participants were allowed to use smokeless tobacco *ad libitum*. After overnight abstinence, plasma nicotine levels averaged 2.9 ng/mL and increased to 21.5 ng/mL after 6 h of *ad-libitum* smokeless tobacco consumption. Eight of the 12 subjects had levels of nicotine similar to those of cigarette smokers. In other studies, Hatsumaki *et al.* (1987, 1988) reported that saliva cotinine levels among regular smokeless tobacco users ranged between 255 and 280 ng/mL, a level that was similar to those seen in daily cigarette smokers.

Daily use of smokeless tobacco was associated with brand switching to products that deliver more nicotine. Tomar *et al.* (1995) observed that smokeless tobacco users switch from low nicotine-delivery products to higher delivery products twice as frequently as they switch from higher to lower delivery products. Sales of Skoal Bandit and Hawken, the lowest nicotine delivery products, constitute 3% of the market but sales of Copenhagen and Kodiak (high nicotine-delivery products) constitute 43% of sales (Hoffmann *et al.*, 1995). Similarly Henningfield *et al.* (1995) compared sales and nicotine delivery in three regions of the USA and reported that higher sales were associated with greater nicotine delivery. More recently, Richter and Spierto (2003) surveyed 18 brands of smokeless tobacco and concluded that those with the most available nicotine (as a function of pH and nicotine content) had the highest market share.

The duration of smokeless tobacco use appears to influence levels of perceived dependence. In a sample of youths, Riley *et al.* (1996) found that 37% of adolescents who used smokeless tobacco for longer than 1 year rated themselves as addicted and were 12 times more likely to report perceived addiction than adolescents who had used such products for less than 1 year.

When chronic smokeless tobacco users attempt to quit, they are frequently unsuccessful. As reviewed by Hatsukami and Severson (1999), between 75 and 100% of subjects in the control groups of seven treatment studies relapsed within 3–12 months. The studies reviewed by Hatsukami and Severson (1999) and Ebbert *et al.* (2003) suggest that,

even among volunteers in smokeless tobacco cessation treatment studies who are highly motivated to quit, successful and lasting cessation is very uncommon. These results concur with the clinical impressions that smokeless tobacco cessation is difficult — even more difficult than quitting cigarette smoking — as suggested by reports of subjects who have attempted to quit both substances (Severson, 2003).

It is clear that the risk and magnitude of dependence is directly related to the amount of nicotine ingested per day and probably to years of use (DHHS, 1988; Food and Drug Administration, 1995, 1996; Royal College of Physicians of London, 2001). It is not clear, however, what threshold dose produces dependence or, conversely, what dose would pose a negligible risk of dependence. Benowitz and Henningfield (1994) theorized that a threshold dose for nicotine dependence could be identified and that the level below which dependence was unlikely to be produced for cigarettes was 0.45 mg nicotine (less than 0.2 mg absorption per cigarette). Similar conclusions were drawn in a report commissioned and endorsed by the American Medical Association (Henningfield *et al.*, 1998). The Food and Drug Administration (1995, 1996) considered this theory and its implications for reducing the nicotine dose to a level that might be exempt from the label 'addictive'. They concluded, based in part on an advisory committee evaluation, that (a) a threshold for addiction probably exists but that the level is not known and (b) that low levels of nicotine that may not sustain addiction in adult users might pose a risk as a starter product for young people. It was concurred that even very low nicotine cigarettes should not be exempt from an addiction warning since they might promote the development of addiction (Henningfield *et al.*, 1998). The experience in the USA with very low-nicotine dose 'starter' smokeless tobacco products supports the extension of such a conclusion to smokeless tobacco products (DHHS, 1986; Connolly *et al.*, 1986).

Taken together, the data on patterns of use of smokeless tobacco support the conclusion drawn by Henningfield *et al.* (1997) that many users of smokeless tobacco are dependent but that the overall risk of dependence among users appears to be somewhat lower than that for cigarette smokers. This conclusion is consistent with pharmacokinetic data that compare cigarettes and smokeless tobacco (described in Section 4.1.1 and later in this section) and show that speed of nicotine absorption from smokeless tobacco is slower than that from inhaled cigarette smoke.

(v) *Psychoactive effects*

Dependence-producing drugs are psychoactive (i.e. they act on the brain to produce changes in mood, performance or thought). There is now abundant evidence from studies in animals and humans that administration of nicotine is associated with subjective and physiological changes due to actions on receptors in the brain (reviewed by DHHS, 1988; Henningfield *et al.*, 1993). Furthermore, there is a vast body of literature that documents the psychoactive effects of human cigarette smoking (DHHS, 1988). These studies have emphasized that the administration of intravenous or smoke-delivered nicotine is euphoric (and, in some subjects, is indistinguishable from cocaine or amphetamine). Laboratory studies have also shown that subjects will self-administer intravenous nicotine

(Henningfield *et al.*, 1983, 1985). The administration of nicotine increases subjective measures of 'feeling good', appreciation of the drug and other measures that are typically associated with administration of psychostimulants (Jasinski *et al.*, 1984).

A laboratory study of smokeless tobacco users (Fant *et al.*, 1999) administered four smokeless tobacco products and a mint snuff that did not contain nicotine. Before administration, during the time (30 min) that the subjects held the product in their mouths and for up to 60 min after it was removed, the participants answered questions on subjective effects. The high nicotine-delivery product (Copenhagen snuff) yielded higher scores on scales of strength, head rush and feeling alert than the low nicotine-delivery product (Skoal Bandit) or the placebo mint snuff.

Surveys indicate that a significant number of adolescents and adults use smokeless tobacco products because they are relaxing and calming (Ary *et al.*, 1989; Hatsukami & Severson, 1999). Gritz *et al.* (1981) studied the effects of smokeless tobacco in college students; few reported subjective effects — especially relaxation — but many reported stimulation related to the increase in plasma nicotine.

Another indicator of psychoactive effects is the circumstances that engender drug use. For example, cigarette smoking often occurs after meals, when alcohol or coffee is consumed, or in the presence of other smokers. Surveys of adolescent smokeless tobacco users demonstrate that its use is associated with situations of boredom, after meals and during sports (Gritz *et al.*, 1981; Ary *et al.*, 1989; Hatsukami & Severson, 1999). Although few data are available, one reason that smokeless tobacco products are consumed is to change mood or levels of arousal.

(vi) *Withdrawal signs and symptoms*

When psychoactive drugs are administered chronically, changes occur in brain structure and function that are referred to as neuroadaptation such that the individual functions in a relatively 'normal' state when the drug is present. When the drug is acutely withheld, a withdrawal syndrome occurs that is usually opposite to the acute drug effect. For example, withdrawal from sedatives/hypnotics is characterized by excitation and arousal whereas withdrawal from stimulants causes extended periods of lethargy. The presence of a withdrawal syndrome is neither necessary nor sufficient for a drug to be classified as dependence-producing. However, many studies have documented changes in mood or performance and physiological changes (electroencephalogram, heart rate) when chronic smokers abruptly quit smoking (DHHS, 1988). Similar changes occur when smokeless tobacco users stop using the product.

Hatsukami *et al.* (1999) reported that signs and syndromes of abstinence were regularly observed on discontinuation of smokeless tobacco. After a 24-h period of abstinence, smokeless tobacco users who volunteered for treatment experienced craving (95.7%), impatience (76.1%), irritability (73.8%), increased eating (66.3%), restlessness (65.4%), anxiety (65%), difficulty in concentrating (60.2%), depressed mood (22.5%) and disrupted sleep (17%). Approximately 69% of the subjects reported four or more of the

symptoms, which is the number of symptoms required for a diagnosis of nicotine withdrawal (American Psychiatric Association, 1994).

In a study of the effects of 24-h abstinence from smokeless tobacco in 20 users, Keenan *et al.* (1989) reported behavioural, physiological and subjective signs typical of tobacco withdrawal. Specifically, heart rate decreased, reaction time on performance task increased, craving for smokeless tobacco increased and self-rated withdrawal symptoms increased.

The withdrawal effects of smokeless tobacco and cigarettes were compared in a prospective study of 16 smokeless tobacco users and 11 smokers. All subjects used tobacco products for 3 days and abstained for 3 days (5 days for tobacco smokers) (Hatsukami *et al.*, 1987). Compared with baseline, smokeless tobacco abstinence was associated with significantly decreased heart rate and orthostatic pulse change, increased craving for tobacco, confusion, eating, number of awakenings, and total scores on both self-rated and observer-rated withdrawal checklists. The authors reported that withdrawal symptoms from cigarette smoking were more severe than those after discontinuation of smokeless tobacco.

Gire and Eissenberg (2000) examined the role of non-nicotine factors in smokeless tobacco withdrawal. Smokeless tobacco users administered their own brand of smokeless tobacco, an oral mint snuff (that contained no nicotine) or nothing each hour for 3 h. Subjects reported cravings and a desire to use smokeless tobacco; heart rate was recorded at intervals throughout the experimental session. Both the smokeless tobacco and the mint snuff significantly reduced cravings for smokeless tobacco compared with no use. Heart rate was significantly elevated in the smokeless tobacco users compared with the users of the mint snuff or no use. These data suggest that placebo smokeless tobacco can diminish some but not all signs of acute abstinence from smokeless tobacco. The duration of the effectiveness of the placebo smokeless tobacco to reduce cravings and other subjective signs of abstinence has not been determined. Similar results were observed in studies of nicotine-free cigarettes that reduced the symptoms of overnight tobacco abstinence in cigarette smokers (Robinson *et al.*, 2000).

The probability and severity of withdrawal symptoms appears to be directly related to frequency of use; however, a threshold for the product of dependence has not been determined. A study by the CDC and an analysis by the Food and Drug Administration support these conclusions. The CDC study showed that increasing signs of withdrawal and dependence were associated with an increased frequency of use (MMWR, 1994). Similar conclusions relied upon the CDC data and those from other US national surveys (Food and Drug Administration, 1995, 1996).

(vii) *Measurement of tobacco dependence in smokeless tobacco users*

A number of approaches have been developed to assess quantitatively the level of physical dependence on tobacco products. Most research has focused on the quantification of dependence in cigarette smokers but some studies have specifically addressed

the issue in smokeless tobacco users. The level of dependence has been associated with severity of tobacco withdrawal and is predictive of success in attempts at smoking cessation. Levels of dependence are also used clinically to guide prescriptions and therapeutic interventions. Thus an accurate assessment of dependence has practical and theoretical importance (Severson, 2003).

In some studies, the emergence of tobacco withdrawal symptoms and signs have been used to define tobacco dependence in smokeless tobacco users. A systematic analysis of smokeless tobacco withdrawal symptoms was published by Hatsukami *et al.* (1992). Tobacco craving, difficulty in concentration, restlessness, excessive hunger and generalized withdrawal discomfort were identified as symptoms that reliably occurred during abstinence from smokeless tobacco products.

Cotinine is the major metabolite of nicotine and its levels in plasma, saliva or urine are a marker of nicotine ingestion. Some studies have used cotinine levels in smokeless tobacco users to define dependence (Gritz *et al.*, 1981; Siegel *et al.*, 1992). In these studies, it was observed that daily users of smokeless tobacco had cotinine levels in the same range as those of cigarette smokers, which suggests that similar levels of nicotine were absorbed. A relationship between plasma cotinine levels and tobacco dependence in smokeless tobacco users was described by Hatsukami *et al.* (1992).

A widely used and broadly accepted self-reported questionnaire, the Fagerström test for nicotine dependence (Fagerström, 1978; Heatherton *et al.*, 1991), was adopted and verified for use among smokeless tobacco users (Boyle *et al.*, 1995; see Table 85). Two groups of 100 and 121 smokeless tobacco users answered questions on their use of smokeless tobacco products and the responses were correlated with cotinine levels. As shown in Table 85, the questions included amount of use, type of product and pattern of use. Analyses of the individual items in each of the questionnaires indicated that there was a low correlation between items (indicating that the items indexed different domains) but a highly significant correlation between each of the items and the total score, which suggests that each of the items was related to the overall measure of dependence. Multiple regression analyses were used to determine which items were most predictive of cotinine levels. In group 1, use within 30 min of waking, experiencing strong cravings after 2 h of tobacco abstinence and the length of time a fresh chew is held in the mouth were identified as being particularly predictive. About 33% of the variability of the cotinine level could be predicted using the scores on only these three independent items. In group 2, the items that were significantly predictive of the cotinine levels were number of tins used per week, frequency of swallowing tobacco juice and time (≤ 30 min or > 30 min) from waking to using a chew. Scores on these three variables could predict about 15% of the variability of cotinine levels.

(viii) *Treatment of smokeless tobacco dependence*

Although numerous studies have assessed behavioural and pharmacological therapy for cigarette smoking cessation (DHHS, 1996; Fiore *et al.*, 2000), relatively few studies have considered interventions for cessation of smokeless tobacco use. For example, a

Table 85. Items and scoring of dependence scale for sample 1 (100 subjects)

Questions	Answers	Points	Response (%)	r^a
1. After a normal sleeping period, do you use smokeless tobacco within 30 min of waking?	Yes	1	68	0.43 $p < 0.0001$
	No	0	32	
2. Is it difficult for you not to use smokeless tobacco where its use would be unsuitable or restricted?	Yes	1	53	
	No	0	47	
3. Do you use smokeless tobacco when you are sick or have mouth sores?	Yes	1	63	0.21 $p < 0.04$
	No	0	37	
4. What brand of smokeless tobacco do you use?	L ^b	1	4	
	M	2	40	
	H	3	56	
5. How many days does a tin/can last you?	6–7 days	1	11	
	3–5 days	2	21	
	< 3 days	3	68	
6. On average, how many minutes do you keep a fresh dip or chew in your mouth?	10–19	1	22	0.26 $p < 0.009$
	20–30	2	27	
	> 30	3	51	
7. Do you intentionally swallow tobacco juices?	Never	0	33	0.25 $p < 0.01$
	Sometimes	1	47	
	Always	2	20	
8. Do you keep a dip or chew in your mouth almost all the time?	Yes	1	61	0.36 $p < 0.0004$
	No	0	39	
9. Do you experience strong cravings for a dip/chew when you go for more than 2 h without one?	Yes	1	91	0.33 $p < 0.001$
	No	0	9	
10. On average, how many dips/chews do you take each day?	1–9	1	39	0.21 $p < 0.04$
	10–15	2	46	
	≥ 16	3	15	

Adapted from Boyle *et al.* (1995)

^a Significant correlations with the log of baseline salivary cotinine

^b Scoring for question 4 is based on nicotine content. For example: a high (H) brand is Copenhagen, a medium (M) brand is Kodiak and a low (L) brand is Hawken/Skoal Bandits (see Tilashalski *et al.*, 1994)

meta-analysis (Ebbert *et al.*, 2003) noted only 20 published reports of cessation in smokeless tobacco users that met criteria for experimental rigor, of which 14 were randomized clinical trials: eight studies compared behavioural interventions and six investigated pharmacological interventions. Treatments that have demonstrable efficacy in the cessation of cigarette smoking have been evaluated for cessation of smokeless tobacco use. Specifically nicotine replacement products, bupropion and behavioural interventions show

varying degrees of efficacy to promote cessation of smokeless tobacco use. The overall conclusion of the meta-analyses was that behavioural therapies are effective, bupropion is probably effective and nicotine replacement therapy may be effective. Among behavioural interventions, those that involve an oral examination were consistently the most effective (see Table 86). Further comment on this study and a critical review of its results are provided elsewhere (Severson, 2003). The studies on treatment of smokeless tobacco dependence indicate that people who compulsively use smokeless tobacco regard themselves as dependent and actively seek treatment. Furthermore, as shown in Table 86, treatment is seldom effective. The demand for treatment and the high rates of relapse are characteristic of dependence-producing drugs.

Table 86. Smokeless tobacco cessation rates and relative risks by type of intervention

Channel of intervention Study	No. of subjects	Rates of quitting (%)		Relative risk (95% CI)	Months to assessment
		Treatment	Control		
Dental clinics					
Severson <i>et al.</i> (1998)	633	10.21 ^a	3.3 ^a	3.2 (1.5–7.0)	12
Stevens <i>et al.</i> (1995)	518	18.4 ^a	12.5 ^a	1.6 (1.0–2.6)	12
Sports					
Walsh <i>et al.</i> (1999)	365	34.5	15.9	2.8 (1.7–4.6)	12
Group support					
Glover <i>et al.</i> (1994)	23	55.5	7.1	12.1 (1.2–123.6)	1–2
Williams <i>et al.</i> (1995)	130	14.7	10.6	1.4 (0.5–4.0)	3
Hatsukami <i>et al.</i> (1996)	210	30.5	22.9	1.5 (0.8–2.7)	12
Self-help					
Severson <i>et al.</i> (2000a)	1069	12.9 ^a	9.7 ^a	1.4 (0.9–2.0)	12
Severson <i>et al.</i> (2000b)	172	24.0 ^a	18.4 ^a	1.4 (0.7–2.9)	6

Adapted from Severson (2003)

CI, confidence interval

^a Sustained rates of quitting

(ix) Conclusions

As shown in the literature that directly tested the effects of smokeless tobacco products and from the inference of many studies on the effects of cigarette smoking, the conclusion that smokeless tobacco is an addictive and dependence-producing substance is warranted and justified.

(b) *Effects on hard and soft oral tissues*

The main categories of snuff- or smokeless tobacco-induced oral soft-tissue lesions are oral squamous-cell carcinoma, verrucous carcinoma, leukoplakia, erythroplakia, snuff dipper's lesion, tobacco and lime user's lesion, verrucous hyperplasia and snuff-induced submucosal deposits. These oral lesions are indicated by the authors' terminology and are discussed in Section 2. The pathological features of mucosal alterations due to the use of smokeless tobacco are described here. It should be noted that oral submucous fibrosis has not been found to be associated with the use of smokeless tobacco.

(i) *Pathology of leukoplakia and snuff dipper's lesions*

The histopathology of oral leukoplakia or snuff-induced lesions was reported by Greer *et al.* (1986) and Daniels *et al.* (1992) in cases from the USA, by Roed-Petersen and Pindborg (1973), Jungell and Malmström (1985) and Andersson *et al.* (1989) in cases from Scandinavia and by Idris *et al.* (1996) in cases from the Sudan.

Common epithelial changes noted were hyperorthokeratosis, hyperparakeratosis, chevron pattern keratinization, pale surface staining, koilocytosis-like changes with vacuolated cells and basal-cell hyperplasia. Dysplasia was uncommon in the Sudanese biopsies studied (Idris *et al.*, 1996) and Larsson *et al.* (1991) noted that dysplasia may occur occasionally in snuff dipper's lesions. Although dysplasia was not found in 29 snuff dipper's lesions in moist snuff users in Sweden, increased mitotic rate was found in a large majority (Larsson *et al.*, 1991). Kaugars *et al.* (1989) found that women were more liable to have moderate-to-severe epithelial dysplasia than men ($p = 0.02$), but this may be because their lesions were detected a decade or so later or because the women were older. Of all the pathological studies, that of Kaugars *et al.* (1989) recorded the highest prevalence of oral epithelial dysplasia (66.7% mild dysplasia, 5.4% severe dysplasia) but noted that 91% of these biopsies were taken from the site of tobacco quid placement. However, the majority of dysplastic changes were focal in nature. In a later study by the same group, 10 of 45 cases who had smokeless tobacco lesions were diagnosed with dysplasia (four cases were focally mild, three were mild, two were moderate and one was severe) (Kaugars *et al.*, 1992). In the USA, the use of snuff was more frequently associated with the development of oral mucosal lesions than that of chewing tobacco, and snuff appeared to cause a greater variety of epithelial changes than chewing tobacco (Daniels *et al.*, 1992). In Sweden, loose snuff users had more increased thickening than sachet snuff users who had less pronounced epithelial changes (Andersson *et al.*, 1989, 1994). In a study of biopsies from mucosal lesions in Sweden, Andersson *et al.* (1990) noted that the daily but intermittent use of snuff caused a mixed tissue reaction of injury and repair. Morphological koilocytic alterations noted in the epithelial cells in several studies (26/45 cases, Greer *et al.*, 1986; 22/141 cases, Idris *et al.*, 1996) suggested to the authors the presence of human papillomavirus (HPV) in smokeless tobacco-induced lesions (Greer *et al.*, 1986; Idris *et al.*, 1996).

(ii) *Changes in cell morphology*

Cellular atypia in buccal smears was more common in heavy users of *toombak* (≥ 11 quids per day) than in cigarette smokers with similar frequency of use (≥ 11 per day) but the authors remarked that the method is unreliable for the diagnosis of precancerous lesions, because cells are taken from the surface while abnormalities mostly occur at the base of the epithelium in the progenitor layers (Ahmed *et al.*, 2003).

Ramaesh *et al.* (1999) reported variations in cell and nuclear diameters in Sri Lankan betel quid–tobacco chewers. While the nuclear diameter was increased, the cell diameter was reduced compared with normal buccal cells, to give an increased nucleus:cytoplasm ratio in chewers. In an electron microscopic examination, widening of intercellular spaces was noted in the spinous layer in Finnish snuff dippers (Jungell & Malmström, 1985).

(iii) *Effects on keratins*

Increased expression of keratins K13 and K14 in oral squamous-cell carcinomas of Sudanese snuff dippers has been reported (Ibrahim *et al.*, 1998), which indicates dysregulation of keratinocyte maturation; a third of the lesions also expressed K19, a basal keratin, which suggests epithelial de-differentiation. Suprabasal expression of K19 was also reported by Luomanen *et al.* (1997a) in oral biopsies from snuff-affected mucosa of 11 snuff users from Sweden. The increased tenascin expression reported in biopsies of smokeless tobacco users was more manifest than that in smokers (Luomanen *et al.*, 1997b). In normal oral mucosa, tenascin was seen to underlie the epithelium as a thin band. In the biopsies, tenascin was distributed as a broad band under the epithelium into the adjacent connective tissue and suggested a marked connective tissue reaction to snuff with an epithelial–mesenchymal interaction that was either inflammatory or preneoplastic in nature.

(iv) *Snuff-induced submucosal deposits*

An amorphous deposit in the lamina propria of the oral mucosa where the snuff is habitually placed was noted in users in Denmark 40 years ago (Pindborg & Poulsen, 1962). Several investigators have subsequently commented on the presence of a similar histological phenomenon that was initially regarded as being amyloid (Lyon *et al.*, 1964) but was later thought to be non-amyloid (Archard & Tarpley, 1972; Hirsch *et al.*, 1982) or collagen (Axéll *et al.*, 1976). Idris *et al.* (1998c), using electron microscopy, later characterized an amorphous, eosinophilic, acellular deposit of varying size with a fibrillar texture at the margins in 25 oral snuff-induced lesions from the Sudan as being collagen.

(v) *Chronic oesophagitis*

Chronic oesophagitis was reported in 273 male *naswar* users from Uzbekistan following endoscopy. An increased risk was found in men who used *naswar* for more than 39 years (odds ratio, 1.6; 95% CI, 1.1–2.3), in men who started using *naswar* before the age of 24 years (odds ratio, 1.5; 95% CI, 1.03–2.1) and in men who used *naswar* 12–20 times/day (odds ratio, 1.5; 95% CI, 1.01–2.1) (Evstifeeva & Zaridze, 1992).

(vi) *Gingival recession/loss of periodontal attachment*

Gingival recessions are more common and are irreversible among users of loose snuff than among users of portion-bag packed snuff (Axéll, 1993). Robertson *et al.* (1990) reported that gingival sites adjacent to mucosal lesions in smokeless tobacco users showed significantly greater recession and loss of periodontal attachment than sites not adjacent to lesions in users or comparable sites in non-users. Recession increased by 0.36 mm within 1 year of use of smokeless tobacco. The odds of having gingival recession were estimated to be nine times higher among students who were smokeless tobacco users than among non-users, but only among those who had concurrent gingivitis (Offenbacher & Weathers, 1985).

Loss of periodontal attachment was measured over 3 years in older adults (Beck *et al.*, 1995). Among a series of variables entered into the logistic regression in this study, smokeless tobacco use was highly significant (odds ratio, 3.0; $p = 0.001$) as a predictor of new periodontal lesions.

(vii) *Wear on teeth*

Snuff and smokeless tobacco have been shown to contribute to excessive incisal and occlusal wear (tooth wear) in an adult Swedish population of 220 subjects (Ekfeldt *et al.*, 1990). This finding had been reported earlier in smokeless tobacco users in the USA (Christen *et al.*, 1979). Silica compounds contained in snuff may contribute to the abrasive effect on teeth (Dahl *et al.*, 1989).

(viii) *Dental caries*

The US Third National Health and Nutrition Examination Survey (NHANES III) reported a significantly increased incidence of root dental caries in adult tobacco chewers (odds ratio, 3.21; 95% CI, 2.0–4.98) compared with snuff users or smokers. Decayed dental surfaces matched sites of quid placement, which suggests cariogenicity of the tobacco (Tomar & Winn, 1999).

A higher prevalence of caries was found among adolescents in Göteborg, Sweden, who used smokeless tobacco (Hirsch *et al.*, 1991).

(ix) *Effects at application sites: studies in human volunteers*

Inflammatory effects

Several studies have investigated the short-term application of smokeless tobacco in humans (Johnson *et al.*, 1998; Payne *et al.*, 1998). A group of 19 men (mean age, 25 ± 1.4 years) who were regular snuff users placed moist snuff on a new mucosal site during an experiment. The authors reported erythema, ulceration and white striae where the snuff was placed as early as 2–7 days after application. By 7 days, 56% of subjects displayed white striated lesions (Johnson *et al.*, 1998). Significantly increased mucosal concentrations of interleukin (IL)-1 and prostaglandin E₂ (PGE₂), molecules that have immune and inflammatory functions, were also reported at new sites of snuff placement. These

data are similar to those reported earlier on 18 male smokeless tobacco users who exhibited increased gingival inflammation at new placement sites (Poore *et al.*, 1995).

Effect of pH and nicotine content

When the pH and nicotine content of the snuff used by 20 regular healthy volunteers were reduced, significantly fewer pronounced clinical and histological changes were noted at the sites of placement (Andersson & Warfvinge, 2003).

Effect on buccal transport of smokeless tobacco substances

Exposure of human buccal mucosa to 1.5–2.5 g smokeless tobacco (in Ringer's solution) altered barrier function and caused dilatation of intercellular spaces of the epithelium, which suggests that smokeless tobacco may facilitate buccal transport of substances at application sites (Tobey *et al.*, 1988).

Gingival blood flow was measured in 22 healthy snuff consumers from Norway (Mavropoulos *et al.*, 2001). Unilateral application of commercial snuff (500 mg that contained ~1% nicotine) caused a markedly rapid increase in gingival blood flow on the exposed side as well as on the contralateral side.

(c) *Cardiovascular system*

(i) *Epidemiological studies of clinical cardiovascular disease outcomes*

Cohort studies

Four cohort studies have investigated deaths from cardiovascular disease and use of smokeless tobacco. Details of these studies are summarized in Table 87.

Bolinder *et al.* (1994) analysed mortality data from a large cohort of Swedish construction workers. Exclusive use of smokeless tobacco was associated with a significantly increased risk for mortality from all cardiovascular diseases in comparison with never use of tobacco (relative risk, 1.4; 95% CI, 1.2–1.6), adjusted for age and region. Relative risks for ischaemic heart disease, stroke and all cardiovascular disease were higher for workers aged 35–54 years at baseline than for workers aged 55–65 years. [This pattern of decreasing relative risk with increasing age is probably attributable to the rapid increase in background rates of cardiovascular disease with increasing age. A similar pattern is seen, for example, with coronary heart disease and active smoking (DHHS, 1983). Another possible factor that could contribute to lower relative risks in the older age group is that the older workers more probably quit tobacco use during follow-up than the younger workers.] No analysis was made for duration of use of smokeless tobacco; however, 87% of the deaths from cardiovascular disease among users of smokeless tobacco were reportedly in subjects who had ≥ 15 years of use at the time they entered the study. [The strengths of this study include the prospective design, the large number of exclusive users of smokeless tobacco in the cohort, the large number of deaths from cardiovascular disease and the availability of information on a number of important risk

Table 87. Cohort studies on use of smokeless tobacco and clinical cardiovascular disease

Reference, location, name of study	Cohort description	Exposure assessment	Cardiovascular disease outcome	Exposure categories	No. of deaths	Relative risk (95% CI)	Adjustment for potential confounders	Comments	
Bolinder <i>et al.</i> (1994), Sweden, construction workers study	135 036 male construction workers ≤ 65 years old who underwent medical examinations in 1971–74 (75% response rate); 6297 exclusive smokeless tobacco users, 32 546 never users of tobacco; follow-up through to 1985	Self-reported current use assessed at baseline medical examination	Mortality from all cardiovascular diseases	Exclusive smokeless tobacco users versus never users of tobacco	220	1.4 (1.2–1.6)	Age, region of origin	Further adjustment for body mass index, blood pressure, diabetes, history of heart symptoms and blood pressure medication reportedly did not change relative risks for cardiovascular disease.	
				<i>Stratified by age at baseline (years)</i>					
				35–54	44	2.1 (1.5–2.9)			
				55–65	174	1.1 (1.0–1.4)			
				Mortality from ischaemic heart disease	35–54	35			2.0 (1.4–2.9)
Mortality from stroke	55–65	137	1.2 (1.0–1.5)						
Accortt <i>et al.</i> (2002), USA, NHANES I Epidemiological Follow-up Study (NHEFS)	6805 subjects aged ≥ 45 years at baseline (1971–75); 414 exclusive ever users of smokeless tobacco, 2986 non-users of tobacco; follow-up until 1992	Self-reported current use assessed at baseline interview for subsample; for the remainder, smokeless tobacco use inferred from 'ever use' in 1982–84 follow-up interview	All cardiovascular mortality	Exclusive ever users of smokeless tobacco versus non-users of tobacco	NR	<i>Men</i> 1.0 (0.7–1.5) <i>Women</i> 1.2 (0.7–1.9)	Age, race, poverty index ratio	Smokeless tobacco user category included former users; pipe or cigar use were not taken into account in tobacco use definitions.	
				Mortality from ischaemic heart disease					Age, race, poverty index ratio, alcohol, recreational physical exercise, fruit/vegetable intake, systolic blood pressure (ischaemic heart disease also adjusted for serum cholesterol, body mass index)
				Mortality from stroke					

Table 87 (contd)

Reference, location, name of study	Cohort description	Exposure assessment	Cardiovascular disease outcome	Exposure categories	No. of deaths	Relative risk (95% CI)	Adjustment for potential confounders	Comments
Henley <i>et al.</i> (2005), USA, CPS-I	Men aged \geq 30 years enrolled in 1959; 7745 exclusive current smokeless tobacco users (median age at enrollment, 62 years); 69 662 never users of tobacco (median age, 53 years); 12-year follow-up (11 871 deaths)	Self-reported current use of smokeless tobacco assessed at baseline	Cardiovascular disease (ICD-7 330–468) Coronary heart disease (ICD-7 420) Cerebrovascular disease (ICD-7 330–334)	Never use	6378	1.00	Age, race, educational level, body mass index, physical activity, alcoholic beverage consumption, fat consumption, fruit/vegetable intake, aspirin use	Demographically, people enrolled in CPS-I were more probably higher educated, married, middle class, and white than general US population; for full CPS-I cohort, 6.7% lost to follow-up; death certificate information obtained for 97% of known deaths; analyses of coronary heart disease excluded men with prevalent heart disease or diabetes; analyses of cerebrovascular disease excluded men with prevalent stroke.
				Current use	1399	1.18 (1.11–1.26)		
				Never use	4035	1.00		
				Current use	809	1.12 (1.03–1.22)		
Henley <i>et al.</i> (2005), USA, CPS-II	Men aged \geq 30 years enrolled in 1982; 2488 exclusive current smokeless tobacco users (median age at enrollment, 57 years); 839 exclusive former smokeless tobacco users (median age, 62 years); 111 482 never users of tobacco (median age, 56 years); 18-year follow-up (19 588 deaths)	Self-reported current or former use of chewing tobacco or snuff, as well as frequency or duration of current use, assessed at baseline; current users: 74% used chewing tobacco only, 14% used snuff only, 12% used both	Cardiovascular disease (ICD-9 390–459) Coronary heart disease (ICD-9 410–444) Cerebrovascular disease (ICD-9 430–438)	Never use	8315	1.00	Age, race, educational level, body mass index, physical activity, alcoholic beverage consumption, fat consumption, fruit/vegetable intake, aspirin use, employment status and type	Demographically, people enrolled in CPS-II were more probably higher educated, married, middle class, and white than general US population; for full CPS-II cohort, 0.2% lost to follow-up; death certificate information obtained for 98.9% of known deaths; analyses of coronary heart disease excluded men with prevalent heart disease or diabetes; analysis of cerebrovascular disease excluded men with prevalent stroke; no clear dose-response trends observed by frequency or duration of current use.
				Current use	278	1.23 (1.09–1.39)		
				Chew/never snuff	186	1.26 (1.09–1.46)		
				Snuff/never chew	36	1.38 (0.99–1.92)		
				Both	37	1.26 (0.91–1.75)		
				Chew/former snuff	9	0.87 (0.45–1.70)		
				Snuff/former chew	10	0.64 (0.33–1.24)		
				Former use	96	0.92 (0.75–1.13)		
				Never use	4920	1.00		
				Current use	172	1.26 (1.08–1.47)		
				Chew/never snuff	111	1.25 (1.03–1.51)		
				Snuff/never chew	24	1.59 (1.06–2.39)		
				Both	23	1.31 (0.87–1.98)		
				Chew/former snuff	6	1.02 (0.45–2.30)		
				Snuff/former chew	8	0.80 (0.37–1.70)		
				Former use	44	0.70 (0.52–0.95)		
Never use	1858	1.00						
Current use	71	1.40 (1.10–1.79)						
Chew/never snuff	45	1.38 (1.02–1.86)						
Snuff/never chew	4	0.62 (0.23–1.67)						
Both	17	2.57 (1.59–4.17)						
Chew/former snuff	3	1.24 (0.39–3.91)						
Snuff/former chew	2	0.68 (0.17–2.75)						
Former use	29	1.21 (0.83–1.76)						

CI, confidence interval; CPS-I, Cancer Prevention Study; CPS-II, Cancer Prevention Study II; NHANES I, US First National Health and Nutrition Examination Survey; NR, not reported

factors that was collected at baseline. Information on certain other potential confounders, such as dietary factors and family history, was not available; however, some of the main risk factors, such as age, body mass index, diabetes and blood pressure at baseline, were considered in the analyses. Furthermore, this cohort of construction workers was probably relatively homogeneous with respect to some lifestyle factors, and the relative risk analyses were based on internal comparisons. A possible upward bias of the relative risk estimates from the misclassification of smokers as exclusive users of smokeless tobacco is improbable because, as noted by the authors, an increased risk for lung cancer in smokeless tobacco users was not observed. In contrast, misclassification of tobacco users as never users of tobacco would tend to underestimate the relative risks for use of smokeless tobacco. Changes in tobacco use status after baseline would also tend to underestimate relative risks for current use, since people in these age groups would be more likely to quit using tobacco than to start or to change from smokeless tobacco use to smoking. It should be noted that follow-up was through to 1985; thus, it is possible that the results of this study may not reflect the risks of more contemporary Swedish snuff.]

Accortt *et al.* (2002) analysed mortality data from a follow-up study of a national survey in the USA. No significant increases in mortality from all cardiovascular disease, stroke or ischaemic heart disease were observed in exclusive smokeless tobacco users in comparison with non-users of tobacco. [Some of the strengths of this study include the prospective design, a high follow-up rate and the availability of information on a variety of risk factors. A limitation of the study is that current smokeless tobacco use at baseline was known only for a subset of participants and, for the remainder, use of smokeless tobacco was based on 'ever use' reported 10 years later. Thus, participants who were categorized as users of smokeless tobacco would include former users as well as current users, even at baseline; this would tend to underestimate any risk from current use of smokeless tobacco. Similarly, no information on quantity of smokeless tobacco consumed was available. An additional limitation was the inclusion of pipe or cigar smokers in the non-user of tobacco referent group. Since there was probably a greater proportion of participants who smoked pipes or cigars exclusively than of participants who smoked pipes or cigars and used smokeless tobacco but did not smoke cigarettes, this would tend to underestimate any risks from use of smokeless tobacco.]

The two most recent prospective studies of cardiovascular disease and smokeless tobacco use are the mortality analyses of the large US Cancer Prevention Study (CPS-I and CPS-II) cohorts by Henley *et al.* (2005). The CPS-I and CPS-II cohorts were recruited in a similar manner but at different periods of time. Statistically significant increases in mortality from all cardiovascular disease, coronary heart disease and stroke were observed in exclusive current (at baseline) smokeless tobacco users in comparison with never users of tobacco in both cohorts. In the CPS-II cohort, additional information was available on type of smokeless tobacco used, former use, and frequency and duration of current use. Results were not significantly different for use of chewing tobacco versus snuff; however, only 14% of the current users used snuff only, thus the results for use of snuff only are less stable. No clear dose-response trends by frequency or duration of

current use were observed. For stroke, however, 79% of the deaths in current users with information on duration of use were in the > 30 years of use group (hazard ratio, 1.7; 95% CI, 1.3–2.3). Cardiovascular diseases were not associated with former smokeless tobacco use. [The strengths of these studies include their prospective design, the large numbers of exclusive smokeless tobacco users, the large numbers of deaths available for analysis and the availability of information on a large number of potential confounders. A limitation of CPS-I is that the cohort was followed from 1959 through to 1972; therefore, the results might not represent current smokeless tobacco products. In addition, the current smokeless tobacco users in both cohorts differ substantially from the never users of tobacco in terms of education level and blue-collar employment (employment data for CPS-II only) and, although the results were adjusted for these covariates as well as a number of other important potential confounders, it is possible that uncontrolled confounding related to socioeconomic status or other factors could have influenced the results. The authors note, however, that significantly increased risks for mortality from cardiovascular disease were not observed for former smokeless tobacco users and that former users were more similar demographically to current users than to never users. In contrast, CPS-I also shows statistically significantly increased risks for mortality for a number of causes that were not expected to be causally related to smokeless tobacco use, such as non-cancer diseases of the respiratory system and digestive system, which similarly raised concerns that observed increases were the result of some factor(s) other than smokeless tobacco use. Increased risks for other causes of death are not observed in CPS-II. However, increased mortality from cirrhosis was observed for current smokeless tobacco users in CPS-II (hazard ratio, 3.0; 95% CI, 1.6–5.7; based on 11 deaths in current users), which suggests the possibility of residual confounding by alcoholic beverage consumption. In addition, the risk for mortality from lung cancer was significantly increased (hazard ratio, 2.0; 95% CI, 1.2–3.2; based on 18 deaths among current users), which raises the possibility that some smokers may have been included in the smokeless tobacco user group. Such misclassification of tobacco use is hypothetical and is not indicated in the CPS-I results. It is also possible that the association with lung cancer is due to chance (given the small number of cases) or is a true effect of smokeless tobacco (see the monograph on Tobacco-specific nitrosamines). It should also be noted that any misclassification of tobacco users as never users would tend to underestimate any risks for cardiovascular disease. Similarly, any cessation of smokeless tobacco use after the baseline survey would tend to underestimate risks associated with current use.]

Case-control studies

Three case-control studies of fatal and non-fatal cardiovascular disease outcomes have examined smokeless tobacco use. Details of these studies are summarized in Table 88.

Huhtasaari *et al.* (1992) conducted a case-control study of first-time acute cases of myocardial infarction in 35–64-year-old men in northern Sweden. No increased risk for myocardial infarction in nonsmoking snuff users versus current non-users of tobacco was observed. In a multiple logistic regression model that included current smoking habits and

Table 88. Case-control studies on use of smokeless tobacco and clinical cardiovascular disease outcomes

Reference, study, location, period	Cardiovascular disease outcome (ICD code)	Characteristics of cases	Characteristics of controls	Exposure assessment	Exposure categories	Odds ratio (95% CI)	Adjustment for potential confounders	Comments
Huhtasaari <i>et al.</i> (1992), Northern Sweden MONICA Project, northern Sweden, 1989–91	First-time fatal or non-fatal acute myocardial infarction	585 men aged 35–64 years (93% of those identified in the two provinces)	589 of 750 men invited (250 in each 10-year age group; 609 participated; 20 excluded)	Self-reported; regular snuff use defined as at least once daily	Current snuff use, including former smokers, versus non-current users of tobacco, including former smokers and former smokeless tobacco users	0.89 (0.62–1.29)	Age	Only 146 nonsmoking current snuff users, including 70 former smokers; former smokers who did not use snuff had a significantly higher risk for myocardial infarction compared with snuff users who had never smoked (odds ratio, 1.8; 95% CI, 1.04–3.11).
Huhtasaari <i>et al.</i> (1999), Northern Sweden MONICA Project, northern Sweden, 1991–93	First-time fatal or non-fatal acute myocardial infarction or sudden death (ICD 410–414)	687 men identified in the northern Sweden MONICA register and validated using MONICA criteria; mean age, 55.6 years; 117 cases were fatal	687 men selected from population registers based on date of birth of index case, matched for county; mean age, 55.6 years	Self-reported in interview for live cases and matched controls or by questionnaire for next-of-kin of dead cases and matched controls; regular snuff use defined as at least once daily	Current snuff use, including former smokers; unadjusted comparison with never use of tobacco; adjusted comparisons include former smokers and former smokeless tobacco users as non-users.	<i>Fatal and non-fatal</i> 0.96 (0.65–1.41) <i>Fatal and non-fatal</i> 0.58 (0.35–0.94) <i>Fatal only</i> 1.50 (0.45, 5.03)	Matched on age, county Hypertension, education, marital status, diabetes, high cholesterol, family history	78% response rate; only 149 cases and controls were current smokeless tobacco users, including former smokers.
Asplund <i>et al.</i> (2003), nested in Northern Sweden MONICA Project and Vasterbotten Intervention Project, northern Sweden, 1985–2000	First occurrence of stroke (brain infarction or intracerebral haemorrhage; subarachnoid haemorrhage excluded), fatal or non-fatal	276 men identified in a northern Sweden MONICA register; 96% confirmed by CT scan or autopsy; mean age at risk factor survey, 54.8 years; mean age at stroke, 59.2 years; tobacco use data available for 89%; participation in MONICA, ~77%; participation in intervention project, ~60%	551 (2 per case), matched on sex, age (± 2 years), geographical area, year of baseline examination, cohort; mean age at survey, 54.7 years; tobacco use data available for 95%; participation in MONICA, ~77%; participation in intervention project, about ~60%	Tobacco use ascertained at baseline survey within cohort study; regular smokeless tobacco use defined as use at least once daily	Exclusive smokeless tobacco use versus never use of tobacco. Smokeless tobacco use including former smokers versus never use of tobacco. Smokeless tobacco use including former smokers versus non-users including former smokers and former smokeless tobacco users	1.05 (0.37–2.94) 1.16 (0.60–2.22) 0.87 (0.41–1.83)	Taking matching into account Elevated blood pressure, education, marital status, diabetes, serum cholesterol	Collection of data on risk factors was prospective because nested within two cohort studies; average follow-up, 4.5 years; subarachnoid haemorrhage excluded, but this subtype has been associated with smoking; only 42 subjects were exclusive regular smokeless tobacco users; 53 former smokers were smokeless tobacco users at baseline.

CI, confidence interval; CT, computerized tomography; MONICA, WHO Monitoring Trends and Determinants in Cardiovascular Disease Project

the potential confounders age and education, snuff use was not a significant predictor of myocardial infarction. [A limitation of this study is that it did not examine exclusive use of smokeless tobacco. Former smokers were included in the group of non-users of tobacco as well as the group of smokeless tobacco users, despite the finding that non-snuff using former smokers had a significantly higher risk for myocardial infarction than snuff users who never smoked. This 'tobacco use misclassification' would create a downward bias on the risk estimate. A further limitation of the study is the low power to observe an effect of smokeless tobacco use because of the small number of current users of smokeless tobacco among the cases (59, including 33 former smokers) and in total (146, including 70 former smokers).]

Huhtasaari *et al.* (1999) conducted a case-control study of first-time acute myocardial infarction in men aged 25–64 years in northern Sweden. No increased risk for acute myocardial infarction was associated with regular snuff use. When the analysis was restricted to fatal cases, the adjusted odds ratio was 1.50 (95% CI, 0.45–5.03). [A limitation of this study is that it did not examine exclusive use of smokeless tobacco. In contrast, the inclusion of former smokers among the smokeless tobacco users did not yield an increased crude odds ratio for non-fatal or fatal acute myocardial infarction compared with never users of tobacco. However, for the adjusted results, former smokers and former smokeless tobacco users were apparently included in the group of non-users of tobacco and any risk in these former tobacco users would result in a downward bias on the odds ratio for current smokeless tobacco use. A further limitation of the study is the low power to observe an effect of smokeless tobacco use because of the small number of current smokeless tobacco users, including former smokers, among the cases (59) and in total (149). The authors further suggest the possibility of confounding by alcoholic beverage consumption, which is associated with a decreased risk for acute myocardial infarction, if alcoholic beverage consumption in snuff users was different from that of non-users of tobacco.]

Asplund *et al.* (2003) conducted a case-control study of stroke in men, nested within two cohort studies in northern Sweden. No significant increases in risk for stroke were associated with smokeless tobacco use. [A strength of this study was the prospective collection of information on a variety of risk factors for cardiovascular disease. The average time from baseline to a stroke event was 4.5 years, so it is improbable that many risk factors, including tobacco habits, changed greatly in such a short time. A limitation of the study was the low power to observe an effect of smokeless tobacco, because of the small number (42) of exclusive smokeless tobacco users in the study. In contrast, the inclusion of former smokers (an additional 53 smokeless tobacco users) did not yield an increased odds ratio for stroke in the adjusted analysis. However, former smokers and former smokeless tobacco users were apparently included in the group of non-users of tobacco in this analysis which would tend to underestimate the odds ratio for smokeless tobacco use. Another possible limitation is the definition of stroke that was used. It is unclear why cases of subarachnoid haemorrhage were excluded from the study, since subarachnoid haemorrhage has been associated with active smoking and exhibits some of the highest relative risks that relate to stroke and

smoking (DHHS, 2004b), and why cases of intracerebral haemorrhage, for which the association with smoking is less clear, were included.]

- (ii) *Epidemiological and experimental studies of subclinical cardiovascular disease outcomes*

Atherosclerosis and thrombosis

Atherosclerosis, which is a thickening and hardening of the arteries, is a major risk factor for cardiovascular disease and plays a key role in the pathogenesis of coronary heart disease, stroke and peripheral artery disease. Smoking is associated with atherosclerosis and has been shown to affect a number of key processes in its development. Two cross-sectional studies investigated atherosclerosis and the use of smokeless tobacco. Both measured intima-media thickness in the carotid artery using ultrasound. Increased thickness of these inner layers of the artery wall is an indication of atherosclerosis.

Bolinder *et al.* (1997a) studied 143 healthy male firefighters, aged 35–60 years, in Sweden. Of these, 28 were exclusive users of smokeless tobacco (daily use for > 6 months; median, 25 years of use), 29 were smokers, 40 never used tobacco and the remainder had changed their tobacco habits at some time. Exclusive users of smokeless tobacco did not differ significantly from those who never used tobacco with regard to age, body mass index, blood pressure at rest, family history of myocardial infarction, alcoholic beverage and coffee consumption, cholesterol, triglycerides, apolipoproteins (Apo) A-1, Apo B or plasma fibrinogen, although all blood chemistry parameters had a tendency towards greater cardiovascular risk in the smokeless tobacco users. None of the measurements of intima-media thickness or lumen diameter differed significantly between smokeless tobacco users and those who never used tobacco. The largest difference was for the maximum carotid bulb thickness, which was 1.01 ± 0.18 mm in smokeless tobacco users and 0.95 ± 0.15 mm in those who never used tobacco, but this was not statistically significant. Atherosclerotic plaques were diagnosed in two smokeless tobacco users (7.1%) and none of those who never used tobacco. In smokers, the maximum carotid bulb thickness was 1.14 ± 0.34 mm ($p < 0.001$) and 11 (37.9%) had plaques. A statistically significant interaction was observed between smoking and serum cholesterol levels for increasing intima-media thickness; a similar pattern was observed for smokeless tobacco users but was not statistically significant. This study suggests that long-term smokeless tobacco use does not have a substantial impact on the progression of atherosclerosis; however, most of the measurements pointed to increased atherosclerosis in smokeless tobacco users compared with those who never used tobacco [and it is possible that a larger study might have found small but statistically significant differences].

Wallenfeldt *et al.* (2001) studied 391 healthy men, all aged 58 years, in Sweden, of whom 96 were current smokers, 152 were former smokers, 139 had never used tobacco, 48 were current smokeless tobacco users and 33 were former smokeless tobacco users. Of the current and former smokeless tobacco users, only four had never smoked. The authors concluded that smokeless tobacco use is not associated with intima-media thickness of the carotid or femoral artery. However, most of the results presented were for 'never snuff

user', 'ex-snuff user' or 'current snuff user' groups in which current and former smokers were included. Thus, in the 'never snuff user' referent group, only 45% of the men had never smoked. [The Working Group noted that this analysis is not useful to make conclusions about smokeless tobacco use and intima-media thickness, especially because these investigators reported significantly increased intima-media thickness in the femoral artery for both current and former smokers and in the carotid artery for current smokers. The groups of smokers also contained smokeless tobacco users; however, the referent group for these comparisons, i.e. 'never smokers', only contained 2/143 current smokeless tobacco users and 2/143 former smokeless tobacco users (< 3% of the total referent group) and more (171) current and former smokers were never smokeless tobacco users than current or former smokeless tobacco users who never smoked (four). Given these limitations, the multiple regression analyses that included both smoking and smokeless tobacco use would be expected to be of limited value to discern an independent effect of smokeless tobacco use.]

Two other cross-sectional studies have investigated the effects of smokeless tobacco use on endothelial function (Granberry *et al.*, 2003; Rohani & Agewall, 2004). Endothelial dysfunction is considered to be an important early event in atherosclerosis, and smoking has been linked to various adverse effects on the endothelium (DHHS, 2004b). Endothelial effects can also promote thrombus formation, and thrombosis is a key element in many cases of myocardial and cerebral infarction. Both studies measured endothelial-dependent flow-mediated dilation (FMD) in the brachial artery. Increases in blood flow were induced in the forearm by applying and then releasing a tourniquet. Normal endothelial cells react to increased blood flow by localized vasorelaxation. This response is reduced when endothelial cells are damaged. Impaired brachial artery FMD correlates with coronary artery endothelial dysfunction. Smoking is thought to affect FMD, at least in part, through the action of reactive oxygen species on nitric oxide, which is a major mediator of endothelial-dependent FMD. Impaired endothelial-dependent FMD is also considered to be a marker for other adverse changes in the endothelium. For example, endothelium-derived nitric oxide also has important anti-inflammatory and anti-thrombotic effects (Landmesser *et al.*, 2004).

Granberry *et al.* (2003) studied 17 healthy adult male volunteers in the USA; seven of the participants had not used tobacco for > 1 year (mean age, 25.6 years), five had used at least two containers of Skoal or Copenhagen smokeless tobacco per week for > 1 year (mean age, 28.8 years) and five had smoked ≥ 10 cigarettes per day for > 1 year (mean age, 21.2 years). Tobacco users were asked to abstain for 8 h before the procedure to reduce any acute effects of tobacco. Endothelial-dependent FMD was significantly impaired in both users of smokeless tobacco ($4.1 \pm 0.7\%$ dilation) and tobacco smokers ($3.9 \pm 5.1\%$) compared with the non-users of tobacco ($12.2 \pm 5.7\%$); the magnitude of the effect was similar in the smokeless tobacco users and smokers. This similarity in effect suggests that endothelial dysfunction may be attributable to nicotine, since nicotine levels are similar for users of smokeless tobacco and smokers (Benowitz *et al.*, 1989). The authors cited several studies of transdermal nicotine, nicotine gum or nicotine nasal spray

and endothelial function as support for this hypothesis. Rohani and Agewall (2004) studied the acute effects of smokeless tobacco use on brachial artery FMD in 20 healthy adult users of smokeless tobacco (18 men and two women; mean age, 34 years). FMD decreased significantly ($p = 0.004$) from $3.4 \pm 2.0\%$ (baseline) to $2.3 \pm 1.3\%$ 35 min after administration of 1 g oral moist snuff, but did not change with an oral placebo.

A number of studies that investigated the effects of smokeless tobacco use also measured serum cholesterol levels. The incorporation of lipids into the arterial intima is an important feature of atherosclerosis and is directly related to high blood levels of low-density lipoprotein (LDL) cholesterol and low levels of high-density lipoprotein (HDL) cholesterol. Smoking is associated with adverse lipid profiles, especially reduced HDL, and the association is thought to be causal (DHHS, 1990, 2004b).

The results from studies on levels of cholesterol and smokeless tobacco are summarized in Table 89. In general, no effect of smokeless tobacco use on cholesterol levels was observed. Most of the studies of smokeless tobacco use that measured serum cholesterol levels did so as part of the collection of data on potentially confounding risk factors and not as an outcome measurement; thus, these data are not adjusted for age, diet, exercise or other potential confounders. An exception is the study of Tucker (1989) on tobacco use and hypercholesterolaemia, which examined serum cholesterol levels in adult male employees who had participated in a health examination programme in the USA. The estimated relative risk for hypercholesterolaemia in smokeless tobacco users was 2.51 (95% CI, 1.47–4.29) compared with current non-users of tobacco, adjusted for age, education, physical fitness and additional tobacco use. Control for body fat had little effect; dietary differences were not taken into account.

Another exception is the study of Ernster *et al.* (1990), who measured total cholesterol and HDL in professional baseball players in the USA. No differences in total cholesterol or HDL (adjusted for age, race, smoking and serum caffeine level) were observed between current, former and non-users of smokeless tobacco. [This study investigated a relatively young and physically fit population; therefore, the results may not be applicable to the general population.] A similar study of the same population by Siegel *et al.* (1992) 1 year later also reported no differences in cholesterol levels between smokeless tobacco users and non-users of tobacco.

Two studies of smokeless tobacco use in countries other than the USA and Sweden also provided measurements of cholesterol levels (Table 90). A study from India of subjects who ate a typical Indian diet measured increased levels of total cholesterol and LDL and significantly increased levels of very low-density lipoproteins (VLDL), together with significantly decreased levels of HDL, in tobacco chewers (Khurana *et al.*, 2000). A study from Turkey reported significant increases in total cholesterol and LDL and significant decreases in HDL with the use of *maras* powder (Güven *et al.*, 2003). [The stronger findings of adverse lipid profiles in both of these studies compared with the results generally seen in the US and Swedish studies could be a result of the different smokeless tobacco products that were consumed; however, they might reflect an increased ability to

Table 89. Measurements of cholesterol levels in epidemiological and experimental studies of smokeless tobacco

Reference, location, study	Description of study population	Comparison groups	Mean results (unless stated)	Comments
Tucker (1989), USA	2840 adult men (mean age, 40.7 years); 93 smokeless tobacco users, including 10 smokers; 2179 non-users of tobacco; 429 smokers of 1–20 cigarettes/day; 139 smokers of > 20 cigarettes/day	Current non- users of tobacco	Relative risk (95% CI) 1.00	Results adjusted for age, education, physical fitness, additional tobacco use; body fat had no effect; not adjusted for dietary factors; hypercholesterolaemia defined as total serum cholesterol ≥ 6.2 mmol/L
		Smokeless tobacco users	2.51 (1.47–4.29)	
		Smokers 1–20 cigarettes/day	1.51 (1.14–2.00)	
		Smokers > 20 cigarettes/day	1.98 (1.29–3.03)	
			<i>Serum cholesterol (mmol/L)</i>	
			5.29	
	Current non-users of tobacco	5.36		
	Smokeless tobacco users	5.38		
	Smokers 1–20 cigarettes/day	5.52		
	Smokers > 20 cigarettes/day			
Ernster <i>et al.</i> (1990), USA	1109 professional baseball players (85.6% under 30 years of age); 463 current smokeless tobacco users; 4% of the subjects were current smokers, 9.1% were former smokers; these were included in the comparison groups.	Non-user of tobacco	Cholesterol (mmol/L) <i>Total HDL</i>	Adjusted for age, race, smoking, serum caffeine level; young, physically fit population; no association with duration or frequency of use, but inverse relation between levels of serum cotinine and HDL
		Former smokeless tobacco user	4.50 1.29	
		Current smokeless tobacco user	4.40 1.29	
		within a month	4.29 1.37	
		within a week	4.37 1.32	
Eliasson <i>et al.</i> (1991), Sweden	21 regular smokeless tobacco users; 18 never users of tobacco; 19 smokers; all healthy males ≤ 30 years old	Never users of tobacco	<i>Serum cholesterol (mmol/L)</i>	Unadjusted, but of similar age and body mass index; young, healthy population
		Smokeless tobacco users	4.39 2.86	
		Smokers	4.45 2.87	
			5.28* 3.16	

Table 89 (contd)

Reference, location, study	Description of study population	Comparison groups	Mean results (unless stated)	Comments
Huhtasaari <i>et al.</i> (1992), Sweden, case-control study within northern Sweden MONICA project	589 male controls aged 35–64 years; 114 non-smokeless tobacco-using smokers; 87 non-smoking snuff-users, including former smokers; participation rate, 81.2%	Snuff users	<i>Cholesterol (mmol/L)</i> 6.59	Age-adjusted; levels in non-users not reported
		Smokers	6.61	
Siegel <i>et al.</i> (1992), USA	Follow-up of same professional baseball players as in Ernster <i>et al.</i> (1990) 1 year later; 477 current smokeless tobacco users, 584 non-users of tobacco; current smokers, former smokeless tobacco users and infrequent smokeless tobacco users excluded; former smokers included; 75% smokeless tobacco users used mainly oral snuff; remainder used chewing tobacco.		Cholesterol (mmol/L)	Adjusted for age, race, alcohol, caffeine; second set of results compared snuff and chewing tobacco and adjusted also for hours of smokeless tobacco use per day, time since last smokeless tobacco use, years of use; for subjects missing data in the follow-up, values from the earlier study were used; young, fit population; authors noted data on serum cotinine indicated these were relatively light smokeless tobacco users and 50% rarely used smokeless tobacco off-season; did not confirm earlier inverse relation between serum cotinine and HDL levels.
			<i>Total</i> <i>HDL</i>	
		Non-user of tobacco	4.42 1.30	
		Current smokeless tobacco user (within 1 week)	4.39 1.31	
		Snuff user	4.34 1.33	
		Tobacco chewer	4.39 1.33	

Table 89 (contd)

Reference, location, study	Description of study population	Comparison groups	Mean results (unless stated)	Comments		
Allen <i>et al.</i> (1995), USA	56 male smokeless tobacco abstainers (mean age, 34 years) receiving nicotine gum or placebo gum in cessation study	Cholesterol (mmol/L)			Baseline measurements reflect period of regular smokeless tobacco use; later measurements are taken during period of smokeless tobacco abstinence; only successful abstainers were included.	
		<i>Nicotine gum group</i>	<i>Total</i>	<i>LDL</i>		<i>HDL</i>
		Baseline	5.1	3.4		1.1
		4 weeks	5.4	3.5		1.1
		8 weeks	5.3	3.4		1.2
		<i>Placebo gum group</i>				
		Baseline	5.1	3.2		1.2
Eliasson <i>et al.</i> (1995), Sweden, northern Sweden MONICA study	Random sample of 2000 subjects aged 25–64 years, 250 men and 250 women from each 10-year age group in 1990; participation rate, 79%; smokeless tobacco analyses further restricted to 604 men; 220 never-users of tobacco, 130 former smokers (current non-users), 124 exclusive smokers, 92 snuff users (including former smokers of > 1 year) and 38 snuff and cigarette users	Never users of tobacco Smokeless tobacco users Former smokers Smokers	Cholesterol (mmol/L)		Unadjusted; mean ages (years): never-users of tobacco, 45.3; smokeless tobacco users, 42.0; former smokers, 49.9; smokers, 46.7; mean duration of smokeless tobacco use, 17 years	
			<i>Total</i>	<i>HDL</i>		
			6.2	1.28		
			6.3	1.36		
			6.6	1.29		
6.2	1.24					

Table 89 (contd)

Reference, location, study	Description of study population	Comparison groups	Mean results (unless stated)	Comments
Bolinder <i>et al.</i> (1997a), Sweden	143 healthy male firemen, 35–60 years of age; 28 exclusive smokeless tobacco users (mean age, 44.4 years), 40 never users of tobacco (mean age, 43.1 years), 29 smokers (mean age, 48.0 years)	Never users of tobacco Smokeless tobacco users Smokers	Cholesterol (mmol/L)	Age-adjusted
			<i>Total</i> <i>LDL</i> <i>HDL</i>	
			5.2 3.4 1.4 5.3 3.5 1.3 5.8* 3.8* 1.0*	
		Never users of tobacco Smokeless tobacco users Smokers	Apolipoproteins (g/L)	
			<i>Apo A-1</i> <i>Apo B</i>	
			1.52 1.24 1.41 1.34 1.35* 1.60*	
Wallenfeldt <i>et al.</i> (2001), Sweden, Atherosclerosis and Insulin Resistance study	391 healthy men, all 58 years old; 1728 invited, 69% participated, 818 eligible after exclusions for cardiovascular disease or medications, 391 after screening for different insulin sensitivity levels; 48 current and 33 former snuff-users, only 4 had never smoked; 96 current smokers; 139 never-users of tobacco	Never users of smokeless tobacco	Cholesterol (mmol/L)	Unadjusted; smokers and exclusive smokeless tobacco users were not considered separately, i.e. all the smokeless tobacco comparison groups also contain current and former smokers, including the never users of tobacco referent group, in which only 45% had never smoked.
			<i>Total</i> <i>LDL</i> <i>HDL</i>	
		Former smokeless tobacco users	5.98 4.04 1.28	
			6.08 4.12 1.17	
		Current smokeless tobacco users	6.18 4.09 1.28	
			Apolipoproteins (g/L)	
		Never users of smokeless tobacco	<i>Apo A-1</i> <i>Apo B</i>	
			1.43 1.21	
		Former smokeless tobacco users	1.37 1.24	
			1.43 1.25	

Table 89 (contd)

Reference, location, study	Description of study population	Comparison groups	Mean results (unless stated)	Comments
Accortt <i>et al.</i> (2002), USA, NHEFS	505 exclusive ever-smokeless tobacco users (mean age, 54.0 years), 5192 non-users of tobacco (mean age, 47.8 years), 5523 exclusive smokers (mean age, 44.9 years)	Non-users of tobacco Ever smokeless tobacco users Smokers	<i>Blood cholesterol (mg/dL)</i> 237.8 228.7 235.1	Unadjusted; smokeless tobacco users category includes former users; pipe or cigar use not taken into account
Granberry <i>et al.</i> (2003), USA	Healthy male volunteers; 5 regular smokeless tobacco users (mean age, 28.8 years), 7 current non-users of tobacco (mean age, 25.6 years), 5 smokers (mean age, 21.2 years)	Non-users of tobacco Smokeless tobacco users Smokers	<i>LDL (mg/dL)</i> 114.8 99.1 91.8	Unadjusted

Apo A-1, major protein in HDL; Apo B, main protein in LDL; HDL, high-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol; NHEFS, NHANES I Epidemiologic Follow-up Study

* $p < 0.05$, compared with non-users

Table 90. Measurements of various cardiac parameters and lipid profiles in epidemiological and experimental studies of smokeless tobacco products other than US and Swedish commercial brands

Reference, location	Characteristics of study population	Comparison groups	Mean results		Comments
Nanda & Sharma (1988), India	40 healthy men (mean age, 26 years) non-users of tobacco given <i>pan</i> with 200 mg tobacco to chew for 15–20 min; 24 age- and sex-matched controls given <i>pan</i> without tobacco to chew	<i>Baseline</i>	<i>SBP/DBP</i>	<i>HR</i>	Unadjusted; similar results in 10 habitual tobacco chewers given <i>pan</i> + tobacco (data not shown); similar increases in subgroup of 10 subjects from the <i>pan</i> + tobacco group, who served as controls (each subject was given <i>pan</i> on two occasions: once without tobacco (self-control) and again <i>pan</i> + tobacco); changes in blood pressure and HR lasted 15–30 min after chewing.
		<i>Pan</i> (control)	115.8/74.5	72.3	
		<i>Pan</i> + tobacco	116.8/74.4	72.1	
		<i>After chewing</i>			
		<i>Pan</i> (control)	116.5/73.6	72.9	
		<i>Pan</i> + tobacco	132.6*/86.2*	84.3*	
Khurana <i>et al.</i> (2000), India	30 current tobacco chewers (> 10 years), 30 current smokers (> 10 years), 30 non-users of tobacco, all aged 20–60 years; subjects of normal body weight for height and eating average Indian diet; subjects with diseases and taking medication were not included.	Non-users Chewers Smokers	<i>Cholesterol (mg %)</i>		Unadjusted; no significant difference in mean age of groups (data not shown); mean duration of smoking and chewing, 21 years and 16 years, respectively
			<i>Total</i>	<i>HDL</i>	
			168.7	44.4	
		Non-users Chewers Smokers	<i>LDL</i>	<i>VLDL</i>	
			185.4	37.6*	
			181.4	39.8*	
			86.7	20.5	
			99.1	34.1*	
			94.6	39.4*	

Table 90 (contd)

Reference, location	Characteristics of study population	Comparison groups	Mean results	Comments
Güven <i>et al.</i> (2003), Turkey	45 users of <i>maras</i> powder (mean age, 45 years), 32 smokers (mean age, 47 years), 30 non-users (mean age, 44 years); average duration of <i>maras</i> powder use, 15 years; average duration of smoking, 16 years	Non-users	<i>SBP/DBP</i> <i>HR</i> 125/70 78	Unadjusted; no statistically significant differences in ventricular repolarization parameters, left ventricular dimensions or systolic function parameters; significant differences in diastolic function parameters in both <i>maras</i> powder users and smokers: decreased left ventricular early filling velocity, E/A ratios; increased atrial filling velocity, deceleration time, isovolumetric relaxation time
		Smokeless tobacco users	132/75 82	
		Smokers	130/77 85	
			<i>Cholesterol (mg/dL)</i>	
			<i>Total</i> <i>LDL</i> <i>HDL</i>	
		Non-users	175 100 42	
		Smokeless tobacco users	230* 150* 32*	
		Smokers	235* 155* 30*	
	<i>Fibrinogen (mg/dL)</i>			
	Non-users	330		
	Smokeless tobacco users	350*		
	Smokers	360*		

DBP, diastolic blood pressure (mm Hg); E/A ratios, left ventricular early filling velocity/atrial filling velocity; HDL, high-density lipoprotein cholesterol; HR, heart rate (beats/min); LDL, low-density lipoprotein cholesterol; SBP, systolic blood pressure (mm Hg); VLDL, very low-density lipoprotein cholesterol

* $p < 0.05$

observe effects of smokeless tobacco on cholesterol levels in people with different (probably lower fat, lower cholesterol) diets.]

Similarly, several studies on smokeless tobacco have measured other blood components that are thought to be related to the pathogenesis of atherosclerosis and thrombosis. Results for these components are presented in Tables 90 and 91. Fibrinogen is an acute-phase protein that increases blood coagulability and can be converted to fibrin, an essential component of blood clots. Smoking is strongly associated with increased plasma levels of fibrinogen, which is an independent risk factor for cardiovascular disease (DHHS, 2004b). Tissue plasminogen activator is a fibrinolytic protein, while plasminogen activator inhibitor has prothrombotic activity. Platelets are blood components that play a key role in blood coagulation and plaque formation, and it is well established that smoking increases platelet activation. Thromboxane A2 is an arachidonic acid derivative that is formed by activated platelets; it promotes platelet aggregation and is a vasoconstrictor. Leukocytosis, or an increased number of white blood cells, also increases blood coagulability, and leukocyte activation plays a role in atherogenesis. Smoking is associated with inflammation and increased leukocyte counts, and tobacco smoke induces leukocyte activation *in vitro*. Antioxidant vitamins may protect against smoking-related atherosclerosis, for example, by scavenging the reactive oxygen species thought to be responsible for endothelial dysfunction and by interfering with lipid peroxidation of LDL, which promotes its incorporation into foam cells and atherosclerotic plaques. Smoking is also associated with increased levels of C-reactive protein, a marker of inflammation that is also associated with cardiovascular disease.

Of the three US and Swedish studies that measured fibrinogen levels, one observed an increase of borderline significance in smokeless tobacco users, while the other two reported no increase. A Turkish study reported significantly increased fibrinogen levels in *maras* powder users (Table 90). Two studies reported no changes in tissue plasminogen activator or plasminogen activator inhibitor associated with smokeless tobacco use. Three studies observed no increase in white blood cell count, while one study measured a slight increase in platelet count in smokeless tobacco users that was not statistically significant. The one study that examined thromboxane A2 found no increase in smokeless tobacco users. In addition, one study that measured blood levels of antioxidant vitamins observed non-significant decreases in α - and β -carotene, but no decreases in α -tocopherol or ascorbate in smokeless tobacco users. Another study reported no significant change in levels of C-reactive protein in smokeless tobacco users. Interpretation of these results on blood factors is limited by the fact that many are unadjusted and are based on small numbers of subjects but, overall, they suggest that smokeless tobacco use does not cause significant changes in several known or suspected risk factors for cardiovascular disease related to atherosclerosis and/or hypercoagulability that are known or thought to be associated with smoking.

Table 91. Measurements of various blood components related to cardiovascular disease in epidemiological studies of smokeless tobacco

Reference, location, study	Characteristics of study population	Comparison groups	Mean results (unless stated)	Comments
Ernster <i>et al.</i> (1990), USA	1109 professional baseball players (85.6% under 30 years old); 463 current smokeless tobacco users; 4% were current smokers and 9.1% were former smokers, and these were included in the comparison groups.	Non-user of tobacco	<i>White blood cell count</i> ($\times 10^9/L$)	Adjusted for age, race, smoking, caffeine level; young, physically fit population
		Former smokeless tobacco user	6.8	
		Current smokeless tobacco user	6.3	
		within a month	6.9	
		within a week	6.3	
Eliasson <i>et al.</i> (1991), Sweden	21 regular smokeless tobacco users, 18 never users of tobacco, 19 cigarette smokers; all healthy males ≤ 30 years old	Never users of tobacco	<i>Fibrinogen</i> (g/L)	Unadjusted, but of similar age and body mass index; young, healthy population; no consistent differences in PAI or tPA levels (data not shown)
		Smokeless tobacco users	1.78	
		Smokers	2.00 ($p = 0.07$)	
			2.12*	
		Never users of tobacco	<i>Platelets</i> ($\times 10^9/L$)	
		Smokeless tobacco users	246	
Smokers	266			
		264	<i>White blood cell count</i> ($\times 10^9/L$)	
		Never users of tobacco	4.7	
		Smokeless tobacco users	5.4	
		Smokers	7.2 ($p < 0.001$)	

Table 91 (contd)

Reference, location, study	Characteristics of study population	Comparison groups	Mean results (unless stated)	Comments
Wennmalm <i>et al.</i> (1991), Sweden	756 randomly selected 18–19-year-old men screened for compulsory military service; 577 responded and were not excluded for medication or health reasons.	Never users of tobacco (<i>n</i> = 344) Former users of tobacco (<i>n</i> = 33) Snuff only users (<i>n</i> = 127) Smokers only (<i>n</i> = 43) Cigarettes + snuff (<i>n</i> = 30)	<i>Median urinary thromboxane A2 metabolite (pg/mg creatinine)</i> 127 132 126 180 (<i>p</i> < 0.001) 187 (<i>p</i> < 0.001)	Unadjusted, but all men of same age
Siegel <i>et al.</i> (1992), USA	Follow-up 1 year later of same professional baseball players as Ernster <i>et al.</i> (1990); 477 current smokeless tobacco users, 584 non-users of tobacco; current smokers, former smokeless tobacco users and infrequent smokeless tobacco users excluded; former smokers included; 75% of smokeless tobacco users used mainly oral snuff, remainder used chewing tobacco.	Non-user of tobacco Current smokeless tobacco user (within a week) Snuff user Chewing tobacco user	<i>White blood cell count (× 10⁹/L)</i> 6.6 6.2* 6.1 6.2	Adjusted for age, race, alcoholic beverages, caffeine; second set of results compared snuff and chewing tobacco and adjusted also for hours of smokeless tobacco use per day, time since last smokeless tobacco use, years of use. For subjects for whom data were missing in the follow-up, values from the earlier study were used; young, fit population; authors noted serum cotinine data indicated these were relatively light smokeless tobacco users and 50% rarely used smokeless tobacco off-season.

Table 91 (contd)

Reference, location, study	Characteristics of study population	Comparison groups	Mean results (unless stated)	Comments
Stegmayr <i>et al.</i> (1993), Sweden, Northern Sweden MONICA Project	243 40–49-year-old men; data on food intake available for 80.7%; 150 randomly selected for vitamin study; 116 participated; 54 non-users of tobacco (or < 1 cigarette/day), 17 regular smokeless tobacco users, 26 regular smokers	Non-users of tobacco	<i>α-Tocopherol</i> ($\mu\text{mol/L}$) 26.2	Unadjusted; no significant differences in intake of fruit and vegetables, but tended to be lower in smokers; estimated intake of ascorbic acid lower in smokers but also decreased in smokeless tobacco users; β -carotene lower in both smokers and smokeless tobacco users, but differences not significant; no effect on retinol or γ -tocopherol by smoking or smokeless tobacco use; only 17 smokeless tobacco users
		Snuff users	26.0	
		Smokers	23.9*	
		Non-users of tobacco	<i>Ascorbate</i> ($\mu\text{mol/L}$) 55.0	
		Snuff users	57.3	
		Smokers	38.3*	
		Non-users of tobacco	<i>α-Carotene</i> ($\mu\text{mol/L}$) 0.069	
		Snuff users	0.053	
		Smokers	0.032*	
		Non-users of tobacco	<i>β-Carotene</i> ($\mu\text{mol/L}$) 0.37	
		Snuff users	0.31	
		Smokers	0.26*	

Table 91 (contd)

Reference, location, study	Characteristics of study population	Comparison groups	Mean results (unless stated)	Comments
Eliasson <i>et al.</i> (1995), Sweden, Northern Sweden MONICA study	Random sample of 2000 subjects aged 25–64 years in 1990, 250 men and 250 women from each 10-year age group; participation rate, 79%; smokeless tobacco analyses restricted to the 604 men; 216 never users of tobacco, 129 former smokers, 162 smokers (including 38 who also use snuff), 90 snuff users (including former smokers of > 1 year)	Never users of tobacco	<i>Fibrinogen (g/L)</i> 3.24	Unadjusted; mean ages (years): never-users of tobacco, 45.3; smokeless tobacco users, 42; former smokers, 50; smokers, 46.7; mean duration of smokeless tobacco use, 17 years; no measurement of smokeless tobacco use was related to fibrinogen, PAI or tPA levels in univariate analyses; similar results in multivariate (including age, body mass index, waist–hip ratio, height, cholesterol, triglycerides and blood pressure) analyses (data not shown).
		Smokeless tobacco users	3.16	
		Former smokers	3.45*	
		Smokers	3.58 ($p < 0.001$)	
		Never users of tobacco	<i>tPA (IU/mL)</i> 0.81	
		Smokeless tobacco users	0.90	
		Former smokers	0.76	
		Smokers	0.78	
		Never users of tobacco	<i>PAI (U/mL)</i> 5.5	
		Smokeless tobacco users	5.4	
Former smokers	6.7			
Smokers	6.4			
Bolinder <i>et al.</i> (1997a), Sweden	143 healthy male firemen, 35–60 years of age; 28 exclusive smokeless tobacco users (mean age, 44.4 years), 40 never users of tobacco (mean age, 43.1 years), 29 smokers (mean age, 48.0 years)	Never users of tobacco	<i>Fibrinogen (g/L)</i> 2.61	Age-adjusted
		Smokeless tobacco users	2.73	
		Smokers	3.20*	

Table 91 (contd)

Reference, location, study	Characteristics of study population	Comparison groups	Mean results (unless stated)	Comments
Wallenfeldt <i>et al.</i> (2001), Sweden, Atherosclerosis and Insulin Resistance study	391 healthy men, all 58 years old; 1728 invited, 69% participated, 818 eligible after exclusions for cardiovascular disease or medications, 391 after screening for different insulin sensitivity levels; of 48 current and 33 former snuff users, only 4 had never smoked; 96 current smokers; 139 never-users of tobacco	Never-user of tobacco Former smokeless tobacco user Current smokeless tobacco user	<i>C-Reactive protein (mg/L)</i> 2.47 2.27 2.64	Unadjusted; smokers and exclusive smokeless tobacco users were not considered separately, i.e. all the smokeless tobacco comparison groups also contained current and former smokers, including the never user of tobacco referent group, in which only 45% had never smoked; significant increases reported in current and former smokers (including smokeless tobacco users)

PAI, plasminogen activator inhibitor; tPA, tissue plasminogen activator; MONICA: Monitoring of Trends and Determinants in Cardiovascular Disease Project

* $p < 0.05$, compared with non-users

Blood pressure, heart rate and exercise capacity

In addition to promoting atherosclerosis and hypercoagulability, the other major ways in which smoking is thought to increase the risk for cardiovascular disease are by its haemodynamic effects, e.g. by increasing myocardial workload, and by inducing arrhythmias. It is well established that both smoking and smokeless tobacco use cause acute increases in blood pressure and heart rate. These haemodynamic effects are thought to be mediated by nicotine, which causes the release of catecholamines and other neurotransmitters. Effects on hypertension are less clear. Numerous epidemiological studies have found lower blood pressure in smokers than in nonsmokers, while ambulatory studies generally find higher daytime blood pressure levels in smokers. Smoking has also been shown to reduce exercise capacity. Smoking-reduced exercise tolerance is believed to be attributable, at least in part, to carbon monoxide, which reduces oxygen delivery to the myocardium and other tissues. Studies of smokeless tobacco that examined blood pressure, heart rate and exercise capacity are summarized in Tables 90 and 92, except for two studies that were reviewed previously, both of which observed "a significant increase in pulse rate and blood pressure after tobacco chewing" (IARC, 1985).

The large cross-sectional study of Swedish construction workers by Bolinder *et al.* (1992) warrants a detailed discussion. Both smokers and smokeless tobacco users reported a greater prevalence of cardiovascular symptoms. Compared with those who never used tobacco, age-adjusted odds ratios in smokeless tobacco users were 1.4 (95% CI, 1.3–1.6) for breathlessness on slight effort, 1.2 (95% CI, 1.1–1.4) for chest pain walking uphill and 1.3 (95% CI, 1.1–1.5) for pain in the leg while walking, which could be a sign of peripheral vascular disease. In the tobacco groups analysed, 1370 were disability pensioners. Compared with those who never used tobacco, odds ratios in smokeless tobacco users were 1.6 (95% CI, 0.7–3.5) and 1.5 (95% CI, 1.1–1.9) for a cardiovascular diagnosis as the cause of disability pension in the 46–55-year age group and in the 56–65-year age group, respectively, and 3.0 (95% CI, 1.9–4.9) for hypertension as the cause of disability pension in the combined 46–65-year age group. For hypertension as the cause of disability pension, there was no excess risk for smokers.

Of the many studies of blood pressure, heart rate and/or exercise capacity summarized in Table 92, more than half a dozen examined the acute effects of smokeless tobacco use; subjects acted as their own controls. These studies consistently observed increased systolic and diastolic blood pressure and heart rate after consumption of smokeless tobacco. A study from India reported similar results from chewing *pan* plus tobacco (Nanda & Sharma, 1988) (Table 90). Two of the studies also measured stroke volume and reported significantly decreased levels following smokeless tobacco use (Hirsch *et al.*, 1992; van Duser & Raven, 1992).

Approximately 10 studies measured random resting or baseline blood pressure and/or heart rate levels. These generally showed no difference between smokeless tobacco users and non-users of tobacco. A study from Turkey reported similar results for users of *maras* powder (Table 90). The major exception is the large study of Bolinder *et al.* (1992), which reported significantly increased odds ratios for systolic blood pressure > 160 mm Hg,

Table 92. Measurements of blood pressure, heart rate and exercise capacity in epidemiological and experimental studies of smokeless tobacco

Reference, location, study	Description of study population	Comparison groups	Mean blood pressure (SBP/DPB)	Mean heart rate (beats/min)	Comments
Squires <i>et al.</i> (1984), USA	20 healthy male college students (mean age, 20 years); refrained from tobacco use for 72 h then used 2.5 g oral snuff for 20 min	Baseline Average over 20 min with oral snuff	118/72 129*/79*	69 89.3*	10 non-users of tobacco, 10 smokeless tobacco users; changes reported similar for both groups (data not shown); similar increases in 10 anaesthetized dogs treated with oral smokeless tobacco
Schroeder & Chen (1985), USA	1663 male and female volunteers, ≥ 18 years old	<i>Men, 18–25 years</i> Non-users of tobacco 19 smokeless tobacco users 23 smokers	131.6/72.8 143.7/80.7 127.7/70.0	NR	Unadjusted; values only reported for 18–25-year-old men, the group with most pronounced results
Ksir <i>et al.</i> (1986), USA	5 male college baseball players, 18–22 years old, 160–202 lbs; all current users of ‘Copenhagen’ moist snuff; bicycle exercise test (4 min each at 3 increasing levels of intensity then 15 min recovery) for each subject on different days with and without smokeless tobacco (abstained during mornings of test)	<i>Before exercise</i> Without smokeless tobacco With smokeless tobacco <i>During exercise</i> Without smokeless tobacco With smokeless tobacco <i>After recovery</i> Without smokeless tobacco With smokeless tobacco	– DBP: no change SBP: + 4 mm Hg* – DBP: no change SBP: higher* – SBP: NS change	+ 10* – Higher* at levels 1 and 2; + 6 (NS) at level 3 – + 20.9	Effects of exercise and smokeless tobacco use on heart rate appeared additive except at the highest level of exercise

Table 92 (contd)

Reference, location, study	Description of study population	Comparison groups	Mean blood pressure (SBP/DPB)	Mean heart rate (beats/min)	Comments
Bahrke & Baur (1988), USA	112 male soldiers, mean age, 28 years, 58 smokeless tobacco users (mostly chewing tobacco) and 54 non-users of tobacco; both groups of similar mean height and weight; performance on US Army physical fitness test was measured.	Smokeless tobacco user	<i>Push-ups</i> 55.91	<i>Sit-ups</i> 63.16	Unadjusted; statistically significant ($p < 0.05$) correlation coefficients between years of smokeless tobacco use and poorer performance on push-ups, sit-ups and the 2-mile run
		Non-user of tobacco	56.43	63.46	
Benowitz <i>et al.</i> (1988), USA	10 healthy men, smokers with previous experience of oral snuff and chewing tobacco, aged 24–61 years; experiments on subsequent mornings after overnight abstinence	Average baseline for all tobacco users	121/70	58	The maximal increase in heart rate was similar for all forms of tobacco and significantly less for nicotine gum; responses to smoking were maximal when nicotine levels were maximal; peak responses to smokeless tobacco and gum preceded maximal blood nicotine levels; despite plateau or slight decline in nicotine, responses returned nearly to baseline after 120 min (i.e. tolerance acquired).
		9 min of smoking	<i>Max. change</i> 18.6/12.2	<i>Max. change</i> 26.0	
		2.5 g oral snuff for 30 min	15.6/11.4	18.2	
		7.9 g chewing tobacco for 30 min	18.6/14.4	19.0	
		2 × 2-mg pieces nicotine gum	16.0/10.4	13.6 ($p < 0.05$ compared with other forms of tobacco)	

Table 92 (contd)

Reference, location, study	Description of study population	Comparison groups	Mean blood pressure (SBP/DPB)	Mean heart rate (beats/min)	Comments	
Benowitz <i>et al.</i> (1989), USA	8 healthy men, heavy smokers with previous experience of oral snuff and chewing tobacco, aged 27–61 years (mean, 49 years); 3–4 day experimental blocks in which subjects abstained or consumed cigarettes, chewing tobacco or oral snuff as desired	Abstinence	<i>Average</i> 116.1/64.7	<i>Average</i> 62.7	Heart rate and blood pressure measured every 4 h during the day; rate-pressure products reflect myocardial work and oxygen demand; subjects consumed hospital diet without additional salt; sodium absorption was 26 and 41 mmol/day from oral snuff and chewing tobacco, respectively, and may contribute to elevation of blood pressure.	
		Smoking	121.4/67.7	69.9*		
		Chewing tobacco	119.2/67.2	70.9*		
		Oral snuff	118.9/66.7	66.7		
			<i>Rate-pressure product (mmHg/min)</i>			
		Abstinence	7285			
		Smoking	8480*			
		Chewing tobacco	8456*			
Ernster <i>et al.</i> (1990), USA	1109 professional baseball players (85.6% under 30 years old); 463 current smokeless tobacco users; 4% of the subjects were current smokers and 9.1% were former smokers, and these were included in the comparison groups.	Non-user of tobacco	118/73	67	Adjusted for age, race, smoking, caffeine level; to exclude effects of physical activity, only included subjects who did not come to examination directly after playing; young, physically fit population	
		Former smokeless tobacco user	119/73	65		
		Current smokeless tobacco user				
		within a month	114/72	60		
		within a week	116/71	67		
Westman & Guthrie (1990), USA	74 men recruited at 3 county fairs in Kentucky; 27 non-users of tobacco, 25 leaf chewers (≤ 1 pouch/day), 7 chewers (≤ 1 pouch/day), 15 snuff users (> 1 pouch/day)	Non-users	124.2/74.0	NR	Unadjusted; mean ages: 27.5, 30.3, 37.3 and 26.1 years, respectively	
		≤ 1 pouch/day	122.9/79.3			
		> 1 pouch/day	139.3*/83.1			
		Snuff users	125/74.3			

Table 92 (contd)

Reference, location, study	Description of study population	Comparison groups	Mean blood pressure (SBP/DPB)		Mean heart rate (beats/min)		Comments
			Before	After	Before	After	
van Duser & Raven (1992), USA	15 male volunteers, 18–31 years old, mean weight 81.8 kg; all habitual smokeless tobacco users; 12 h abstinence; 30 min smokeless tobacco (2.5 g) (or placebo) during seated rest, then removal of smokeless tobacco, 20-min on treadmill at 60% of pre-determined maximal steady state, then increasing incline until maximal exercise reached	Placebo	Stroke volume (mL/beat)		66	64	Effect of smokeless tobacco NS on oxygen uptake at rest, or at 60% or 85% or maximal exercise or on cardiac output at rest, at 60% or 85%; cardiac output (and stroke volume) could not be measured at maximal exercise; smokeless tobacco significantly increased plasma lactate at 60% and 85% levels but increase NS at maximal exercise; authors postulate greater demand for glycolytic energy due to decreased muscle blood flow; authors noted increased heart rate with presumed increased SBP implies increased cardiac work.
			Smokeless tobacco	84			
		Smokeless tobacco	87	89*	66	82*	
			60%	85%	60%	85%	
Placebo	126	127	153	171	no significant difference in maximum		
	Smokeless tobacco	119*	119*	164*		177*	
Hirsch <i>et al.</i> (1992), Sweden	9 healthy volunteers (8 men), 25–31 years old; 8 were habitual smokeless tobacco users; abstained 9 h before test. Phase 1: subjects rested (supine) 30 min after starting smokeless tobacco (2.5 g), then 3 min exercise test with handgrip, 30 min rest, 3 min cold pressor test, 30 min rest, oral cavity rinse; Phase 2: 1 h later started second smokeless tobacco dose, then workload test with bicycle, 15 min recovery. All experiments were carried out on two different experiment days separated in time by 2–3 weeks; one experimental day involved snuff intake whereas the alternative day served as a control day.	During rest periods of Phase 1: smokeless tobacco day versus control day	SBP and DBP increased after smokeless tobacco intake*; SBP remained elevated during 140 min of Phase 1*, DBP increase of shorter duration; cardiac output higher but NS; stroke volume significantly decreased at 30 and 110 min readings*.		Increased about 25%* 15–30 min after smokeless tobacco intake; increase remained during Phase 1		Handgrip test: blood pressure, but not heart rate higher* after tobacco smokeless at start (sitting) ($p < 0.05$, data not shown); heart rate response to exercise slightly higher after smokeless tobacco; differences in blood pressure tended to disappear. Cold pressor test: increased heart rate with smokeless tobacco remained (data not shown). Plasma epinephrine significantly increased at 30 min at 200 W workload but not at the lower workloads; no significant differences in norepinephrine or neuropeptide-Y. Authors suggest that pressor response, stroke volume and cardiac output response indicate that total peripheral resistance was increased.
			Phase 2: smokeless tobacco day versus control day	DBP, but not SBP, increased*, at low workloads only.		Increased* at low workloads; also higher* after recovery	

Table 92 (contd)

Reference, location, study	Description of study population	Comparison groups	Mean blood pressure (SBP/DPB)	Mean heart rate (beats/min)	Comments
Huhtasaari <i>et al.</i> (1992), Sweden, case-control study within Northern Sweden MONICA Project	589 control men aged 35–64 years; 114 smoking non-users of smokeless tobacco; 87 non-smoking snuff users, including former smokers; 177 never used tobacco.	Snuff users Smokers Non-tobacco users	133/86 131/83 NR		Age-adjusted
Siegel <i>et al.</i> (1992), USA	Follow-up 1 year later of same professional baseball players as Ernster <i>et al.</i> (1990); 477 current smokeless tobacco users, 584 non-users of tobacco; current smokers, former smokeless tobacco users and infrequent smokeless tobacco users excluded; former smokers included; 75% smokeless tobacco users used mainly oral snuff, remainder used chewing tobacco.	Non-user of tobacco Current smokeless tobacco user (within a week) Snuff user Chewing tobacco user	117.1/72.1 117.1/71.5 115.3/71.9 119.3/70.9	65.6 65.4 64.5 65.4	Adjusted for age, race, alcohol, caffeine; second set of results also adjusted for hours of use per day, time since last use, years of use; for subjects for whom data were missing in the follow-up, values from the earlier study were used; young, fit population; authors noted serum cotinine data indicated these were relatively light smokeless tobacco users and 50% rarely used smokeless tobacco off-season. Higher mean serum cotinine levels were associated with higher DBP ($p = 0.02$).

Table 92 (contd)

Reference, location, study	Description of study population	Comparison groups	Mean blood pressure (SBP/DPB)	Mean heart rate (beats/min)	Comments
Allen <i>et al.</i> (1995), USA	56 male smokeless tobacco abstainers (mean age, 34 years) received nicotine gum or placebo gum in cessation study.	<i>Nicotine gum</i>			Baseline measurements reflect period of regular smokeless tobacco use; later measurements were taken during period of smokeless tobacco abstinence; only successful abstainers were included.
		Baseline	128.8/84.1	76.5	
		4 weeks	125.7/83.2	70.6	
		8 weeks	123.9/89.2	66.6	
		<i>Placebo gum</i>			
		Baseline	126.0/83.9	73.8	
Eliasson <i>et al.</i> (1995), Sweden, Northern Sweden MONICA study	Random sample of 2000 subjects aged 25–64 years in 1990, 250 men and 250 women from each 10-year age group; participation rate, 79%; smokeless tobacco analyses further restricted to 604 men; 220 never users of tobacco, 130 former smokers (current non-users), 124 exclusive smokers, 92 snuff users (including former smokers of > 1 year) and 38 snuff users and smokers	Never users of tobacco	130/82.4		Unadjusted; mean ages (years): never users of tobacco, 45.3; smokeless tobacco users, 42.0; former smokers, 49.9; smokers, 46.7; mean duration of smokeless tobacco use, 17 years; anti-hypertensive medication used by 12.2% of smokers and 4.5% of smokeless tobacco users and never users of tobacco
		Smokeless tobacco users	129/82.9		
		Former smokers	132/84		
		Smokers	130/82.1		
		Never users of tobacco	121/76	57	
		Smokeless tobacco users	122/77	58	
Bolinder <i>et al.</i> (1997a), Sweden	143 healthy male firefighters, 35–60 years of age; 28 exclusive smokeless tobacco users (mean age, 44.4 years), 40 never users of tobacco (mean age, 43.1 years), 29 smokers (mean age, 48.0 years)	Smokers	122/78	62	Unadjusted

Table 92 (contd)

Reference, location, study	Description of study population	Comparison groups	Mean blood pressure (SBP/DPB)	Mean heart rate (beats/min)	Comments	
Bolinder <i>et al.</i> (1997b), Sweden	144 healthy male firefighters, 35–60 years of age; response rate, 75%; 48 smokeless tobacco users (median 25 years of use), 65 non-users of tobacco, 31 smokers; mean age about 45 years; underwent bicycle exercise test; 79% of smokeless tobacco users and 77% of smokers had consumed tobacco less than 2 h before the exercise test.	<i>At rest</i>				
		Non-users	124/79	57	Appears to be the same population as that studied by Bolinder <i>et al.</i> (1997a); physically fit population; all results adjusted for age, body mass index, waist/hip ratio, alcohol consumption, physical demands of job, leisure-time exercise ^a $p < 0.05$ smokeless tobacco use < 2 h before test versus smokeless tobacco use > 2 h before test Smokeless tobacco user maximal workloads and maximal oxygen uptake similar to non-users in all age groups, but significantly lower for smokers in all age groups; in smokeless tobacco users, no correlation between maximal workload and amount of smokeless tobacco consumed. Electrocardiogram recordings were normal in 80% of non-users, 73% of smokeless tobacco users and 71% of smokers. Cardiac-ST-segment depressions < 1 mm were observed in 8% of non-users, 15% of smokeless tobacco users and 23% of smokers.	
		Smokeless tobacco users	126/76	54		
		Smokeless tobacco > 2 h before test	116/68	52		
		Smokeless tobacco < 2 h before test	126 ^a /75	56		
		Smokers	123/80	61		
			<i>Max. O₂ uptake (mL/min/kg)</i>	<i>Max. workload (W)</i>		
		<i>Exercise</i>				
		Non-users	42.4	325		
		Smokeless tobacco users	43.9	320		
		Smokeless tobacco > 2 h before test	45.2	305		
		Smokeless tobacco < 2 h before test	45.1	310		
		Smokers	38.3 ($p < 0.001$)	266 ($p < 0.001$)		
		<i>At 190 W</i>	<i>SBP</i>			
		Non-users	184	130		
		Smokeless tobacco users	191	130		
Smokeless tobacco > 2 h before test	178	126				
Smokeless tobacco < 2 h before test	194	133				
Smokers	198*	139*				
<i>10 min after work</i>						
Smokeless tobacco > 2 h before test	115/72	77				
Smokeless tobacco < 2 h before test	124 ^a /78 ^a	85 ^a				

Table 92 (contd)

Reference, location, study	Description of study population	Comparison groups	Mean blood pressure (SBP/DPB)		Mean heart rate (beats/min)		Comments
Bolinder & de Faire (1998), Sweden	135 healthy, normotensive male firefighters, aged 35–60 years; 47 smokeless tobacco users (median 25 years of use), 29 smokers, 59 non-users of tobacco; mean age about 45 years; none used hypertensive medication; underwent 24-h monitoring.	<i>Non-ambulatory baseline</i>					
		Non-users	124/78		57		Appears to be the same population as that studied by Bolinder <i>et al.</i> (1997b); some subjects' measurements were of inadequate technical quality, leaving 135; adjusted for age, body mass index, waist–hip ratio, physical fitness level, alcoholic beverage intake; strong correlation between blood cotinine levels and 24-h SBP and DBP values in smokeless tobacco users, but not in smokers In smokeless tobacco users, all daytime 1-h mean heart rate values were significantly higher than in non-users; in smokeless tobacco users ≥ 45 years, all daytime DBP and most daytime SBP 3-h mean values were significantly elevated compared with non-users.
		Smokeless tobacco users	123/78		60		
		Smokers	119/78		58		
			<i>24 h average</i>		<i>24 h average</i>		
		Non-users	123/77		62		
Smokeless tobacco users	127*/79		65*				
		Smokers	128*/81		69*		
			<i>Day</i>	<i>Night</i>	<i>Day</i>	<i>Night</i>	
		Non-users	126/79	108/66	63	54	
		Smokeless tobacco users	131*/81	106/67	69*	56*	
		Smokers	131*/83*	110/68	74*	58*	
Fant <i>et al.</i> (1999), USA	10 male volunteers, mean age 32 years, who were daily smokeless tobacco users (range of duration of use, 2–26 years), recruited to test effects of four commercial moist snuff brands; abstained 3 h before baseline, then took 2 g snuff for 30 min.	Baseline	124.4/72.9 <i>Max. increase</i> SBP 9.4–16.8 DBP 10.3–14.2		72.2 <i>Max. increase</i> 4.7–17.9		Unadjusted

Table 92 (contd)

Reference, location, study	Description of study population	Comparison groups	Mean blood pressure (SBP/DPB)	Mean heart rate (beats/min)	Comments
Mavropoulos <i>et al.</i> (2001), Norway	22 healthy volunteers (20 men), infrequent tobacco users, age range 19–39 years; ab-stained for at least 8 h before experiment; then 500 mg pouch of Swedish snuff kept in mouth 5 min; then 10 min recovery	Baseline Maximum	<i>Arterial</i> 91.2 94.9*	61.0 66.4*	Unadjusted
Wallenfeldt <i>et al.</i> (2001), Sweden, Atherosclerosis and Insulin Resistance study	391 healthy men, all 58 years old; 1728 invited, 69% participated, 818 eligible after exclusions for cardiovascular disease or medications, 391 after screening for different insulin sensitivity levels; of 48 current and 33 former snuff users, only 4 had never smoked, 96 current smokers, 139 never users of tobacco	Snuff–years Cigarette–years	<i>Spearman's r-value for SBP</i> 0.08 0.10*		Unadjusted; smokers and exclusive smokeless tobacco users were not considered separately, i.e. 'snuff–years' includes smokers and 'cigarette–years' includes smokeless tobacco users.
Accortt <i>et al.</i> (2002), USA, NHEFS	505 exclusive ever users of smokeless tobacco (mean age, 54.0 years), 5192 non-users of tobacco (mean age, 47.8 years), 5523 exclusive smokers (mean age, 44.9 years)	Non-users of tobacco Ever users of smokeless tobacco Smokers	<i>SBP</i> 142.3 147.8 136.6		Unadjusted; smokeless tobacco user category includes former users; pipe or cigar use not taken into account
Rohani & Agewell (2004), Sweden	20 healthy volunteers, 18 men and 2 women, mean age, 34 years; all smokeless tobacco users; measurements taken before and then 20 and 35 min after administration of 1 g oral snuff	Baseline 20 min after snuff 35 min after snuff	109/74 111*/78* 110/76	55 59* 58*	SBP, DBP and heart rate unchanged with placebo 'snuff'

CI, confidence interval; DBP, diastolic blood pressure; MONICA, WHO Monitoring of Trends and Determinants in Cardiovascular Disease Project; NHEFS, NHANES I Epidemiologic Followup Study; NR, not reported; NS, not statistically significant; SBP, systolic blood pressure

* $p < 0.05$, compared with non-users or baseline

diastolic blood pressure > 90 mm Hg and heart rate > 80 beats per min in the 45–65-year age groups, but not in the younger age groups. [Many of the studies for which no effects were observed evaluated primarily younger subjects and others did not stratify by age; thus, if there is an interaction with age, most of the studies were not designed to detect it. Alternatively, the results of Bolinder *et al.* (1992) may reflect the use of smokeless tobacco products from 30 years ago or uncontrolled confounding.]

In contrast to most of the data on resting blood pressure and heart rate, but consistent with similar studies of smokers, the two studies that took measurements throughout the day reported higher blood pressure and heart rate associated with smokeless tobacco use than with no tobacco use. Benowitz *et al.* (1989) measured blood pressure and heart rate every 4 h during the day in eight smokers who abstained, smoked or used chewing tobacco or snuff on different days. They observed higher average blood pressure, heart rate and heart rate \times systolic blood pressure (cardiac work) associated with each type of tobacco use. The increases in heart rate and heart rate \times systolic blood pressure were significant for smoking and use of chewing tobacco. Bolinder and de Faire (1998) monitored 24-h blood pressure and heart rate among non-users of tobacco, smokeless tobacco users and smokers. No significant differences were observed between the groups in the resting baseline measurements; however, 24-h average systolic blood pressure and heart rate were significantly higher in smokeless tobacco users and smokers. In addition, in smokeless tobacco users \geq 45 years old, all 3-h mean values of daytime diastolic blood pressures and most of those for daytime systolic blood pressure were significantly higher than those of non-users of tobacco.

The four studies generally showed increased systolic blood pressure during exercise with smokeless tobacco use (Ksir *et al.*, 1986; Bahrke & Baur, 1988; Hirsch *et al.*, 1992; Bolinder *et al.*, 1997b). Heart rates were generally increased at the lower workloads with smokeless tobacco use, but the maximum heart rate did not typically differ from that in non-users of tobacco. One study that measured stroke volume found a significant decrease during exercise in smokeless tobacco users (Hirsch *et al.*, 1992). Increased heart rate with increased systolic blood pressure is an indication of increased cardiac work and oxygen demand. Some studies also noted that the pattern of responses observed suggested decreased blood flow to the muscle or increased total peripheral resistance (van Duser & Raven, 1992; Hirsch *et al.*, 1992).

It is apparent from the results of several studies that the effects of smokeless tobacco use on heart rate last longer than those on blood pressure (Hirsch *et al.*, 1992; Rohani & Agewell, 2004). In addition, the results of a few studies that compared users of chewing tobacco with users of snuff suggest that greater effects on blood pressure and heart rate are obtained from the use of chewing tobacco, although this is based on limited data (Benowitz *et al.*, 1989; Westman & Guthrie, 1990; Siegel *et al.*, 1992).

(iii) *Insulin resistance and diabetes as risk factors for cardiovascular disease*

Some additional risk factors for atherosclerotic cardiovascular disease that are associated with smoking are insulin resistance and diabetes. Moreover, smoking aggravates insulin resistance and further increases the risk for cardiovascular disease in people with diabetes (Targher *et al.*, 1997; Eliasson, 2003). Smokeless tobacco use and insulin resistance/diabetes is covered in more detail below (Section 4.2.1(d)). The information is very limited, but an association is plausible. In addition, some evidence shows that exposure to nicotine may aggravate insulin resistance in people who already have diabetes (Axelsson *et al.*, 2001), thus diabetics may be at particular risk for cardiovascular effects from smokeless tobacco use.

(iv) *Evidence from studies of nicotine or nicotine replacement therapy*

Because nicotine is presumably the major cardiovascular toxicant in smokeless tobacco, it may be informative to consider the results of studies of nicotine or nicotine replacement therapy on various cardiovascular outcomes. It must be noted, however, that the pharmacokinetics of nicotine delivery may not be the same for smokeless tobacco use and nicotine replacement therapy; for example, Benowitz *et al.* (1988) measured higher peak levels of blood nicotine from the use of oral snuff and chewing tobacco than from the use of nicotine gum. Therefore, while positive findings in the studies of nicotine replacement therapy would suggest that similar or greater risks from smokeless tobacco might be expected, negative findings in these studies would not necessarily exonerate smokeless tobacco. In addition, smokeless tobacco contains constituents other than nicotine that may contribute to cardiovascular risk.

The epidemiological studies of nicotine replacement therapy show little evidence of increased risk for morbidity or mortality from cardiovascular disease. However, these studies are somewhat limited to be able to draw inferences about smokeless tobacco use. For example, Greenland *et al.* (1998) conducted a meta-analysis of results reported from 34 clinical trials of nicotine patches that involved 5687 patch recipients and 3752 placebo recipients. However, most of these trials excluded subjects who had cardiovascular disease or major risk factors; thus, few cardiovascular disease outcomes were observed and the results might not be relevant to the general population. In addition, for a given cardiovascular disease outcome such as myocardial infarction or stroke, no more than four studies reported results for that outcome. Another issue with nicotine replacement therapy is that it is often for a short period of time for smoking cessation in contrast to smokeless tobacco use which is long-term. One large clinical trial of nicotine gum followed subjects for 5 years, at the end of which, 20% of them were still using the gum (Murray *et al.*, 1996). Another clinical trial only studied a 10-week course of nicotine patch use (Joseph *et al.*, 1996). Furthermore, these studies compared nicotine replacement therapy with non-nicotine replacement therapy in concomitant smokers and recent former smokers. Thus, the background rates for cardiovascular disease were relatively elevated and an effect of

nicotine, if one exists, would be more difficult to see than with background rates of cardiovascular disease for non-users of tobacco.

In experimental studies, nicotine impairs endothelial-dependent vasodilation in humans and may also have other effects on the endothelium, e.g. altering the release of various mediator substances (Institute of Medicine, 2001; Benowitz, 2003). Studies of the effects of nicotine on other cardiovascular end-points are less numerous. Those that investigated lipoprotein profiles generally showed improved profiles in smokers who switched to nicotine replacement therapy or no differences in subjects who were experimentally exposed to nicotine in comparison with controls, although this was not consistent with some animal models that showed adverse effects on lipid profiles in response to nicotine (Allen *et al.*, 1994; Kilaru *et al.*, 2001). Similarly, studies that investigated risk factors for thrombogenesis typically reported no significant differences in platelet responses or fibrinogen levels (e.g. Benowitz *et al.*, 2002). One small study suggested that long-term (> 11 months) use of nicotine gum is associated with insulin resistance, which is a risk factor for cardiovascular disease that is also associated with smoking (Eliasson *et al.*, 1996). Finally, in addition to effects on blood pressure and heart rate, nicotine has been associated with a variety of cardiac arrhythmias, including ventricular arrhythmias (Benowitz & Gourlay, 1997). Wang *et al.* (2000) reported that nicotine may act directly on the potassium channels in the heart that maintain the hyperpolarization potential of the resting membrane, which may contribute to the ability of nicotine to promote cardiac arrhythmias independent of its role in the induction of catecholamine release.

(v) *Conclusions*

A limited number of epidemiological studies of clinical outcomes of cardiovascular disease, such as myocardial infarction or sudden cardiac death, is available to assess the potential risks for cardiovascular disease from the use of smokeless tobacco. The four cohort and three case-control studies from Sweden and the USA may not reflect the risks entailed by the use of other smokeless tobacco products in other countries. Of the seven studies, three cohort studies reported statistically significantly increased risks for cardiovascular death from smokeless tobacco use, while the other four reported no significantly increased risks for certain cardiovascular disease outcomes; however, most of these studies suffer from critical limitations that undermine their usefulness to address the issue. In the large Swedish cohort study of Bolinder *et al.* (1994), a statistically significant increased risk for mortality from cardiovascular disease was observed in male exclusive users of smokeless tobacco compared with those who never used tobacco (age-adjusted relative risk, 1.4; 95% CI, 1.2–1.6); adjustment for age, region, body mass index, blood pressure, diabetes, history of heart symptoms and medication for blood pressure reportedly did not change the relative risks. There is no apparent upward bias that would explain this observation, although one cannot rule out uncontrolled confounding. In two large US prospective studies based on the CPS-I and CPS-II cohorts (Henley *et al.*, 2005), statistically significant increases in mortality from all cardiovascular disease, coronary heart disease and stroke were observed in male exclusive users of smokeless tobacco in

comparison with those who never used tobacco in both cohorts, with hazard ratios ranging from 1.12 to 1.46, adjusted for age, race, level of education, body mass index, physical activity, alcoholic beverage consumption, fat consumption and several other potential confounders. Information on frequency and duration of use was available for CPS-II, but no clear dose–response trends were observed.

The four studies that reported no significant increases for cardiovascular diseases were typically limited by several factors, such as the inclusion of former smokers and small numbers of smokeless tobacco users, which would undermine the ability to observe any effect of smokeless tobacco use. In one study (Huhtasaari *et al.*, 1999), when the analysis was restricted to fatal cases, the adjusted odds ratio was 1.50 (95% CI, 0.45–5.03), which is consistent with the increased relative risk estimates for cardiovascular deaths observed by Bolinder *et al.* (1994) and Henley *et al.* (2005), although based on small numbers. It is plausible that an association between smokeless tobacco and cardiovascular disease may be primarily for fatal events because of the ability of nicotine to trigger catecholamine release, which could contribute to arrhythmias (Benowitz & Gourlay, 1997).

In terms of the subclinical effects on cardiovascular disease that have been investigated, increased carotid and femoral arterial intima-media thickness, a sign of atherosclerosis, is the outcome that represents the most advanced progression of chronic disease. In the study of Bolinder *et al.* (1997a), a suggestion of slightly increased atherosclerosis was observed in smokeless tobacco users, but none of the results were statistically significant in this small study (28 exclusive smokeless tobacco users). In the only other study of this end-point (Wallenfeldt *et al.*, 2001), the results pertaining to smokeless tobacco use were largely uninformative because all but four of the smokeless tobacco users were also current or former smokers. Both studies observed strong evidence of an increased incidence of atherosclerosis in smokers, however, and, if there is an actual effect of smokeless tobacco on arterial thickening, it would appear to be of relatively small magnitude.

Endothelial dysfunction, which is thought to be an early event in atherogenesis, was found to be significantly increased in both studies of endothelial-dependent flow-mediated dilation in smokeless tobacco users. Impaired endothelial-dependent vasodilation has also been observed in experimental studies of nicotine administration. Correct endothelial function is also important for maintaining the haemostatic balance in the blood, e.g. to prevent hypercoagulation.

Several studies of smokeless tobacco measured cholesterol levels, the results of which generally showed little, if any, association between smokeless tobacco use and altered cholesterol levels, although some of these were not even age-adjusted, which limits their interpretation. An exception is the study of Tucker (1989), which reported a statistically significant increased risk for hypercholesterolaemia in smokeless tobacco users in the USA, after adjustment for several risk factors. It is unclear whether there is another explanation for this finding, although uncontrolled confounding, e.g. by dietary factors, is always a possibility. Other notable exceptions are an Indian study of smokeless tobacco chewers (Khurana *et al.*, 2000) and a Turkish study of *maras* powder users (Güven *et al.*, 2003), both of which reported significantly more adverse lipid profiles associated with smokeless

tobacco use. This could be a reflection of the different smokeless tobacco products or of the effects being more apparent in populations who have different basic diets.

With respect to acute cardiovascular events, smokeless tobacco use can potentially increase risks. It does not appear from the available studies that smokeless tobacco use has much impact on blood factors that increase hypercoagulation, which could result in thrombus formation; however, the well-established haemodynamic effects of smokeless tobacco could contribute to acute events. For example, recurrent episodes of coronary vasoconstriction and increases in blood pressure, which can occur from smokeless tobacco use, may augment haemodynamic stress and cause vulnerable plaques to rupture in people who have underlying atherosclerotic disease, and lead to emboli (Institute of Medicine, 2001). Use of smokeless tobacco has also been shown to increase the myocardial workload. Especially during exercise, this could raise the risk for cardiovascular disease in people who have pre-existing conditions, although, in the absence of carbon monoxide and the thrombogenic features of smoking, the impacts of increased cardiac work are less than those from smoking. In addition, because of the nicotine in smokeless tobacco, users may be at increased risk for sudden death from ventricular arrhythmias. Further studies to compare deaths from cardiovascular disease in long-term users of smokeless tobacco and never users of tobacco, or at least long-term non-users of tobacco, could be valuable to elucidate the risks for cardiovascular disease of smokeless tobacco use; however, such studies would have to be fairly large to be useful, and care would have to be taken to minimize potential confounding.

In summary, the evidence on the risk for cardiovascular disease from smokeless tobacco use is limited, although a small increase in risk is clearly possible. Because of the high background rates of cardiovascular disease, even a small increase in risk could represent a large public health impact in countries that have a high prevalence of smokeless tobacco use.

(d) *Diabetes*

Smoking is a risk factor for insulin resistance, which can lead to diabetes, and can aggravate insulin resistance in people with diabetes. Insulin resistance is a condition that is characterized by an inability of the body to use insulin correctly. As a consequence, the pancreas secretes additional insulin in an attempt to maintain normal glucose levels. Hyperinsulinaemia (e.g. high fasting levels of insulin) can be a marker for insulin resistance, the most accurate measurement of which is by the euglycaemic hyperinsulinaemic clamp technique. If normal glucose levels cannot be maintained, impaired glucose tolerance can result and is characterized by blood glucose levels that range between normal and diabetic. Impaired glucose tolerance is determined by an oral glucose tolerance test, which is considered to be a more reliable indicator of glucose intolerance than measurement of the fasting level of glucose. Both impaired fasting glucose and especially impaired glucose tolerance are signs of 'pre-diabetes' and are risk factors for the development of both type 2 diabetes and cardiovascular disease. Eventually, the pancreas can lose its ability to secrete enough insulin and result in type 2 diabetes, which is itself a major risk factor

for cardiovascular disease. Several prospective studies have found that smoking is an independent risk factor for diabetes (Will *et al.*, 2001).

(i) *Studies of smokeless tobacco use and insulin resistance, glucose intolerance and diabetes*

Four studies investigated smokeless tobacco use and type 2 diabetes or glucose intolerance. Two were prospective studies from the USA (Henley *et al.*, 2005), one was a cross-sectional study from Sweden (Persson *et al.*, 2000) and one was a Swedish cross-sectional study with a prospective component (Eliasson *et al.*, 2004); all four are summarized in Table 93. Henley *et al.* (2005) reported results from two prospective studies of the large US CPS-I and CPS-II cohorts that included analyses of mortality from diabetes. In CPS-I, no increased risk for mortality from diabetes was observed in current smokeless tobacco users compared with never users of tobacco. In CPS-II, no significantly increased risks were found for mortality from diabetes for either current or former users. [The strengths of these studies include their prospective design, the large numbers of exclusive smokeless tobacco users and the availability of information on a number of potential confounding factors, including body mass index and physical activity. A major limitation with respect to assessment of diabetes is that these were mortality studies and data on incidence were not available. Furthermore, prevalent cases were excluded at baseline (3.2% of current users and 2.3% of never users in CPS-I; 6% of current and former users and 5% of never users in CPS-II). Thus, the results on mortality were based on a small number of deaths attributed to diabetes on death certificates (20 in current users in CPS-I; eight in current users in CPS-II). It should also be noted that significantly increased risks for mortality from cardiovascular disease in current smokeless tobacco users were observed in both studies (Section 4.2.1(c)) and, since diabetes is a risk factor for cardiovascular disease, it could have been a contributing cause of death in some of those cases. An additional limitation is that any cessation of smokeless tobacco use after the baseline survey would tend to underestimate risks associated with current use.]

In the study of Persson *et al.* (2000), impaired glucose tolerance and type 2 diabetes were determined by an oral glucose tolerance test. Insulin resistance was based on homeostasis model assessment, a method to estimate insulin sensitivity from fasting blood sample results. The adjusted odds ratio for prevalence of type 2 diabetes was 2.7 (95% CI, 1.3–5.5) for the use of ≥ 3 boxes snuff per week in current users compared with never use of smokeless tobacco. For smokers, the odds ratio for diabetes was statistically significant only for the group who smoked ≥ 25 cigarettes per day (odds ratio, 2.6; 95% CI, 1.1–5.8). An interaction between smoking and family history of diabetes was apparent; no such interaction was seen for smokeless tobacco use. No significant increases in the prevalence of impaired glucose tolerance was observed in current or former smokeless tobacco users or in current or former smokers. In subjects with impaired glucose tolerance, no increased risk for insulin resistance was associated with smokeless tobacco use; the adjusted odds ratio for impaired insulin secretion was 1.2 (95% CI, 0.5–2.8) for current smokeless tobacco users and 2.2 (95% CI, 1.1–4.4) for former users. Insulin secretion was signifi-

Table 93. Epidemiological studies of diabetes, impaired glucose tolerance and insulin resistance in users of smokeless tobacco

Reference, location, name of study	Description of study population	Exposure assessment	Outcome	Exposure categories	No. of cases/No. of subjects	Relative risk estimate (95% CI)	Adjustment for potential confounders	Comments
Persson <i>et al.</i> (2000), Sweden	Cross-sectional; 3128 men, aged 35–56 years, 52% with a family history of diabetes (subjects were originally selected for a study focused on family history of diabetes) in 1992–94; known cases of diabetes excluded; participation rate, 70% among subjects selected based on a prescreening questionnaire; health examination after overnight fasting and abstinence from smoking, including OGTT	Self-reported; smokeless tobacco use categories	Prevalence of impaired glucose tolerance	Never user of smokeless tobacco	121/2070	1.0	Age, body mass index, family history of diabetes, leisure physical activity, alcoholic beverage consumption	Smokeless tobacco use categories include current and former smokers; adjustment for smoking reportedly did not change results (not shown).
				Smokeless tobacco user	26/531	0.8 (0.5–1.3)		
			Prevalence of diabetes	Former smokeless tobacco user	19/400	0.7 (0.4–1.2)		
				Never user of smokeless tobacco	34/2070	1.0		
				Smokeless tobacco user	13/531	1.5 (0.8–3.0)		
				Former smokeless tobacco user	5/400	0.8 (0.3–2.0)		
				Smokeless tobacco user ≤ 2 boxes/week	1/246	1.0		
				≥ 3 boxes/week	12/283	0.2 (0.0–2.0)		
				Never user of tobacco	9/959	2.7 (1.3–5.5)		
			Prevalence of insulin resistance	Exclusive smokeless tobacco user (current)	4/131	1.0		
				Never user of smokeless tobacco	37	3.9 (1.1–14.3)		
				Current smokeless tobacco user	9	1.0		
			Prevalence of impaired insulin secretion	Former smokeless tobacco user	3	0.9 (0.4–2.0)		
				Never user of smokeless tobacco	28	0.4 (0.1–1.3)		
Current smokeless tobacco user	8	1.0						
Former smokeless tobacco user	12	1.2 (0.5–2.8)						
Ever smokeless tobacco user	13	2.2 (1.1–4.4)						
	≤ 2 boxes/week	7	2.1 (1.1–4.1)					
	≥ 3 boxes/week	7	1.2 (0.5–2.9)					

Table 93 (contd)

Reference, location, name of study	Description of study population	Exposure assessment	Outcome	Exposure categories	No. of cases/No. of subjects	Relative risk estimate (95% CI)	Adjustment for potential confounders	Comments		
Eliasson <i>et al.</i> (2004), Sweden, Northern Sweden, MONICA study	Cross-sectional with follow-up; 3384 men, aged 25–64 years at 1986 or 1990 surveys or 25–74 years at 1994 or 1999 surveys; average survey response rate, 76%; OGTT results available for 1158 of the subjects without diagnosed diabetes at baseline; follow-up information in 1999 on 1275 men free from diabetes at baseline (from first 3 surveys; response rate, 69%); OGTT for 513 men with normal OGTT at baseline	Self-reported <i>smus</i> use at baseline survey and follow-up; 1201 never-users of tobacco, 314 exclusive current smokeless tobacco users, 161 exclusive former smokeless tobacco users	Prevalence of diabetes diagnosis	Never user Smokeless tobacco user Former smokeless tobacco user	29/1201 6/314 5/161	1.0 1.1 (0.4–2.6) 1.5 (0.5–3.9)	Age, waist circumference	Confirmed diagnoses of diabetes for incident cases; in 1990 survey, plasma nicotine and cotinine were measured in small subsample of participants to validate tobacco habit self-reports; further adjustment for alcoholic beverage consumption and leisure time physical activity reportedly did not substantially change results.		
			Prevalence of impaired glucose tolerance or diabetes by OGTT	Never user Smokeless tobacco user Former smokeless tobacco user	35/429 5/100 6/59	1.0 1.1 (0.5–2.2) 1.48 (0.6–3.8)	Age, waist circumference			
			Incidence of diabetes diagnosis	Never user Smokeless tobacco user Former smokeless tobacco user	6/585 0/103 1/73	1.0 (0 cases) 1.7 (0.2–14.8)	Age, follow-up, percentage weight gain			
			Impaired glucose tolerance by OGTT	Never user Smokeless tobacco user Former smokeless tobacco user	32/255 1/40 2/25	1.0 0.2 (0.03–1.8) 0.8 (0.2–3.6)	Age, waist circumference, follow-up			
				Diabetes by OGTT	Never user Smokeless tobacco user Former smokeless tobacco user	6/255 1/40 3/25	1.00 0.9 (0.1–8.0) 4.0 (0.9–18.3)		Age, waist circumference, follow-up	
					Diabetes by OGTT	Never user Smokeless tobacco user Former smokeless tobacco user	6/255 1/40 3/25		1.00 0.9 (0.1–8.0) 4.0 (0.9–18.3)	Age, waist circumference, follow-up
						Diabetes by OGTT	Never user Smokeless tobacco user Former smokeless tobacco user		6/255 1/40 3/25	1.00 0.9 (0.1–8.0) 4.0 (0.9–18.3)
			Henley <i>et al.</i> (2005), USA, CPS-I	Prospective study; men aged ≥ 30 years enrolled in 1959; 7745 exclusive smokeless tobacco users (median age at enrolment, 62 years); 69 662 never users of tobacco (median age, 53 years); 12-year follow-up (11 871 deaths)	Self-reported current use of smokeless tobacco assessed at baseline	Diabetes death (ICD-7 code 260)	Never use Current use		97 20	1.0 0.9 (0.5–1.5)

Table 93 (contd)

Reference, location, name of study	Description of study population	Exposure assessment	Outcome	Exposure categories	No. of cases/No. of subjects	Relative risk estimate (95% CI)	Adjustment for potential confounders	Comments
Henley <i>et al.</i> (2005), USA, CPS-II	Prospective study; men aged ≥ 30 years enrolled in 1982; 2488 exclusive smokeless tobacco users (median age at enrolment, 57 years); 839 exclusive former smokeless tobacco users (median age, 62 years); 111 482 ever users of tobacco (median age, 56 years); 18-year follow-up (19 588 deaths)	Self-reported current or former use of chewing tobacco or snuff assessed at baseline; current users: 74% chewing tobacco only, 14% snuff only, 12% both	Diabetes death (ICD-9 code 250)	Never use Current use Former use	250 8 6	1.0 1.1 (0.6–2.3) 2.2 (0.95–4.9)	Age, race, educational level, body mass index, physical activity, alcoholic beverage consumption, fat consumption, fruit/vegetable intake, aspirin use, employment status and type	Demographically, people enrolled in CPS-II more liable to be more educated, married, middle class and white than general US population; for full CPS-II cohort, 0.2% lost to follow-up, death certificate information obtained for 98.9% of known deaths; analyses of diabetes excluded men with prevalent diabetes at baseline.

CI, confidence interval; CPS-I, Cancer Prevention Study I; CPS-II, Cancer Prevention Study II; MONICA, WHO Monitoring of Trends and Determinants in Cardiovascular Diseases project; OGTT, oral glucose tolerance test

cantly impaired in current smokers (odds ratio, 2.4; 95% CI, 1.1–5.2) and the odds ratio for insulin resistance was 1.5 (95% CI, 0.7–3.6) for smokers of ≥ 25 cigarettes per day. No significant associations were found between tobacco use and insulin resistance or impaired insulin secretion in subjects who had normal glucose tolerance (results not shown). [The strengths of this study include oral glucose tolerance test results, insulin resistance estimates and information on important potential confounders for such a large group of subjects. Major limitations of this study are the cross-sectional design and the inclusion of current and former smokers in the smokeless tobacco use categories. With such a design, there is uncertainty about the tobacco use status of the subjects prior to disease onset. At the time of the onset of disease, the men classified as current smokers or snuff users may have started using tobacco at the onset of glucose intolerance and some of those classified as former tobacco users may have been current smokers or snuff users at the time of the onset of glucose intolerance. In addition, the authors noted that their findings on insulin resistance and secretion are surprising. They reported that studies of acute smoking or nicotine administration observed insulin resistance without impaired insulin secretion. The impact of excluding people with known diabetes is unclear. This could create a bias, for example, if tobacco users were more liable to have a more severe, and thus more probably known disease. It is also unclear whether the over-sampling for subjects with a family history of diabetes impacted the results. Although no interaction between smokeless tobacco use and family history was observed, subjects with a strong family history of diabetes may be more susceptible to other risk factors for diabetes.]

Eliasson *et al.* (2004) investigated snuff use and the prevalence and incidence of type 2 diabetes and impaired glucose tolerance in men in northern Sweden. From the baseline information, no significant increases in the odds ratios for prevalent diagnosed diabetes or pathological glucose intolerance (i.e. diabetes or impaired glucose tolerance) were observed for current or former smokeless tobacco users compared with never users of tobacco; nor were dose–response relationships found for smokeless tobacco use (data not shown). For exclusive smokers, the adjusted odds ratio for prevalent diagnosed diabetes was 1.6 (95% CI, 0.9–3.1) for current smokers and 1.9 (95% CI, 1.1–3.2) for former smokers; the adjusted odds ratio for pathological glucose intolerance was 0.9 (95% CI, 0.5–1.9) for current smokers and 1.5 (95% CI, 0.8–2.6) for former smokers. For the 1275 subjects who had follow-up information (average duration of follow-up, 8.5 years), 27 incident cases of diagnosed diabetes occurred, none of which were in consistent exclusive smokeless tobacco users. For consistent exclusive smokers, the adjusted odds ratio for incident diagnosed diabetes was 4.6 (95% CI, 1.4–15.5). Based on the follow-up oral glucose tolerance test results in the 513 subjects with normal levels at baseline, the adjusted odds ratio for diabetes was 0.9 (95% CI, 0.1–8.0) for consistent exclusive users of smokeless tobacco and 4.0 (95% CI, 0.9–18.3) for former smokeless tobacco users. In consistent and former exclusive smokers, the adjusted odds ratios were 0.7 (95% CI, 0.1–5.6) and 1.3 (95% CI, 0.5–3.3), respectively. No increases in impaired glucose tolerance were found in current or former exclusive users of smokeless tobacco or in current or former exclusive smokers at follow-up. [Some of the strengths of this study were the

definitions of tobacco use, which considered exclusive smokeless tobacco use, the availability of information on a number of potential confounders and the availability of follow-up data to examine incident cases. As noted by the authors, cross-sectional studies can be prone to underestimate the risk for diabetes from tobacco use, because once people are diagnosed with diabetes, they will probably be encouraged to quit. In addition, this study used the oral glucose tolerance test to identify undiagnosed cases of diabetes and impaired glucose tolerance. A limitation of the study was the small number of incident cases that occurred during follow-up; additional follow-up time may help to accrue more cases. Furthermore, the number of subjects who used smokeless tobacco and had follow-up oral glucose tolerance test results was small (38 current, 20 former).]

Three other studies on smokeless tobacco, all from Sweden, measured serum insulin and/or blood glucose levels, and are summarized in Table 94. In a cross-sectional study, Eliasson *et al.* (1991) observed increased fasting levels of serum insulin in smokers and smokeless tobacco users; however, in their slightly larger study, Eliasson *et al.* (1995) did not replicate these findings. In a study of the acute effects of smoking or snuff use in a group of healthy smokers, Attvall *et al.* (1993) concluded that smoking, but not snuff use, acutely impairs the action of insulin. They further suggested that the increased levels of growth hormone (an insulin antagonist) observed during smoking could be a reason for the decreased sensitivity to insulin but that the smaller increase observed during snuff use may be inadequate to induce insulin resistance.

Several studies of nicotine replacement therapy or experimental exposure to nicotine suggested that nicotine may be associated with insulin resistance. For example, in a small cross-sectional study of healthy men, Eliasson *et al.* (1996) found that long-term use of nicotine gum was associated with hyperinsulinaemia and insulin resistance. In an experimental exposure study, Axelsson *et al.* (2001) observed that nicotine aggravated insulin resistance in type 2 diabetics but had no effect on insulin sensitivity in the age- and body mass index-matched non-diabetic subjects. Catecholamines and other hormones, the release of which is stimulated by nicotine, can act as antagonists to insulin (Eliasson, 2003).

(ii) *Conclusions*

The data on smokeless tobacco use and insulin resistance, glucose intolerance and diabetes are very limited and the results are inconsistent. Two prospective studies found no increased risks for mortality from diabetes associated with smokeless tobacco use; however, these were mortality studies that were not designed to investigate risks for diabetes and the number of deaths from diabetes was small. One cross-sectional study provided suggestive evidence of an increased prevalence of diabetes in heavy users of smokeless tobacco but not of impaired glucose tolerance or insulin resistance, while another cross-sectional study that included a follow-up component observed no significant increased risks for the prevalence or incidence of impaired glucose tolerance or diabetes. Both of the cross-sectional studies also suffer from critical limitations. Three other studies measured serum insulin and/or blood glucose levels. One of these observed a significant increase in mean serum insulin in smokeless tobacco users, while the other

Table 94. Measurements of serum insulin and blood glucose in epidemiological studies of smokeless tobacco users

Reference, location, study	Characteristics of study population	Comparison groups	Mean results	Comments
Eliasson <i>et al.</i> (1991), Sweden	21 regular smokeless tobacco users, 18 never users of tobacco, 19 cigarette smokers; all healthy men ≤ 30 years old; examination after overnight fasting and abstention from tobacco and 24-h abstention from alcoholic beverages	Never users of tobacco Smokeless tobacco users Smokers	<i>Blood glucose</i> 4.4 mmol/L 4.3 mmol/L 4.4 mmol/L	Unadjusted, but of similar age and body mass index; young, healthy population
		Never users of tobacco Smokeless tobacco users Smokers	<i>Serum insulin</i> 3.6 mU/L 5.5 mU/L* 8.6 mU/L*	
Attvall <i>et al.</i> (1993), Sweden	7 healthy smokers (4 women, 3 men), aged 24–35 years, no family history of diabetes or hypertension; each underwent 3 studies: (1) smoked 1 cigarette/h for 6 h, (2) 48 h abstinence, then 1 portion-bag of snuff/h for 6 h, (3) 48 h abstinence then abstinence during 6 h experiment; during the 6 h, insulin sensitivity measured by glucose-clamp technique	Abstinence Smokeless tobacco use Smoking	<i>Blood glucose during the clamps</i> 5.0 mmol/L 4.9 mmol/L 5.0 mmol/L	All subjects had moderate alcoholic beverage consumption; free fatty acid levels not significantly different before clamps and decreases were not significantly different during the clamps; rate of glucose appearance (mostly representing liver production) was similar before clamps, and decrease during clamps was similar (data not shown); basal glucose utilization similar before clamps, but increase was significant for abstinence (7.5 mg/kg/min) and smokeless tobacco use (7.7) versus smoking (6.9) for last 3 h of clamps.
		Abstinence Smokeless tobacco use Smoking	<i>Fasting insulin before clamps</i> 6.8 mU/L 7.1 mU/L 6.5 mU/L	
		Abstinence Smokeless tobacco use Smoking	<i>Insulin AUC during clamps</i> 76.1 mU/L/6 h 79.6 mU/L/6 h 79.6 mU/L/6 h	
		Abstinence Smokeless tobacco use Smoking	<i>Mean growth hormone levels during clamps</i> 2.4 nmol/L 5.7 nmol/L* 7.3 nmol/L*	

Table 94 (contd)

Reference, location, study	Characteristics of study population	Comparison groups	Mean results	Comments
Attvall <i>et al.</i> (1993) (contd)		Abstinence Smokeless tobacco use Smoking	<i>Mean glucose infusion rate during clamps</i> 6.6 mg/kg/min 6.6 mg/kg/min 5.9 mg/kg/min*	
Eliasson <i>et al.</i> (1995), Sweden, Northern MONICA study	Random sample of 2000 subjects aged 25–64 years in 1990, 250 men and 250 women from each 10-year age group; participation rate, 79%; 754 randomly selected for OGTT after overnight fasting; smokeless tobacco analyses restricted to men; of the men with OGTT: 125 never users of tobacco, 73 former smokers, 80 smokers (including snuff users), 42 snuff users (including former smokers of > 1 year)	Never users of tobacco Smokeless tobacco users Smokers Former smokers Never users of tobacco Smokeless tobacco users Smokers Former smokers	<i>Fasting serum insulin (mU/L)</i> 6.2 5.8 6.1 6.5 <i>Post-load insulin</i> 25.0 20.6 20.3 24.9	Unadjusted; fasting plasma glucose and post-load glucose for men did not differ by tobacco use (data not shown).

AUC, area under the curve; MONICA, Monitoring of Trends and Determinants in Cardiovascular Disease Project; OGTT, oral glucose tolerance test
* $p < 0.05$, compared with non-users

two showed no effects. All seven studies were conducted in Sweden or the USA and no information on diabetes, glucose intolerance or insulin resistance in relation to smokeless tobacco use in other countries was available. In addition, several studies of nicotine replacement therapy or experimental exposure to nicotine suggested that nicotine may be associated with insulin resistance, and catecholamines and other hormones, the release of which is stimulated by nicotine, can act as antagonists to insulin. Thus, effects on insulin sensitivity and glucose tolerance and risk for diabetes from smokeless tobacco use are plausible, and diabetic smokeless tobacco users, in particular, may be at increased risk for aggravated insulin resistance.

(e) *Other effects*

(i) *Smokeless tobacco and inflammation*

Some constituents of tobacco are known to cause inflammation, DNA damage and cell death. The modulation of inflammatory mediators by smokeless tobacco has been purported to play a role in the development of oral cancer. Gingival recession and white mucosal lesions frequently occur at sites of placement of smokeless tobacco. The etiology of these alterations is presumably related to the effects of tobacco components. PGE₂ and IL-1 are inflammatory mediators that are involved in periodontal destruction and keratinocyte proliferation.

(ii) *Effect on enzyme activities*

GST/GSH status

Ambient monitoring was undertaken to assess the extent of exposure to tobacco dust and biological alterations among *bidi* tobacco processing plant workers (Bhisey *et al.*, 1999). GSH levels were significantly lower among the worker group who did not have any use of tobacco while GST activity was significantly lower in the lymphocytes of workers who did or did not use tobacco.

In 32 male tobacco chewers, a reduction in the GST activity of lymphocytes was observed although the levels of GSH were similar to those in controls. However, no correlation was observed between GST activity and *GSTM1* null genotype (Mahimkar *et al.*, 2001).

Aldehyde dehydrogenase

The activity of aldehyde dehydrogenase (ALDH) in peripheral blood cells was found to be inhibited by 8% in Swedish moist snuff users compared with the value in non-users, although this difference did not reach statistical significance (Helander & Curvall, 1991).

4.2.2 *Experimental systems*

(a) *Human studies*

Exposure of keratinocyte cultures established from healthy tissues to low concentrations of smokeless tobacco extract did not affect cell numbers or viability, but significantly increased PGE2 and IL-1 levels (Johnson *et al.*, 1996). IL-1 and PGE2 levels were determined by enzyme immunoassay in specimens from soft-tissue biopsies of white mucosal lesions at habitual placement sites, in normal alveolar mucosal tissue at non-placement sites of 18 smokeless tobacco users and in normal alveolar mucosal biopsies from 15 non-users. PGE2 levels were lower in both regions in the smokeless tobacco users compared with non-users of tobacco, but values did not vary significantly between the regions at placement and non-placement sites. Both IL-1 α and IL-1 β were significantly elevated in smokeless tobacco lesions compared with either non-placement sites in smokeless tobacco users or non-users of tobacco (Johnson *et al.*, 1994). Thus, these mediators that are released as a result of smokeless tobacco-induced irritation may play a role in the development of oral mucosal lesions at habitual tobacco placement sites in smokeless tobacco users.

(b) *Animal studies*

(i) *Effects on oral mucosa*

Chen (1989) reported the effects of smokeless tobacco following weekly application to the buccal mucosa of rats for 1 year. No cancers were found but most epithelial changes noted were similar to snuff-induced lesions described earlier in humans. The subepithelial hyalinization noted in humans (Section 4.2.1(b)) was also found in rat mucosa. An interesting finding was a change of ploidy status; 25% of buccal epithelial cells of tobacco-treated rats were tetraploid and 5% were octaploid, which suggests that the mitotic process could be altered (Chen, 1989) (see also Section 4.4.2(a)). These results are of relevance because ploidy status has been reported (Sudbo *et al.*, 2001) to be a significant putative marker for dysplasia with potential for malignant transformation.

Summerlin *et al.* (1992) examined the histological effects of smokeless tobacco and alcohol on the pouch mucosa and organs of Syrian hamsters. In the group treated with smokeless tobacco (20 animals) acanthosis (epithelial hyperplasia) was found after 26 weeks but no cancers developed at the test site. Numerous alterations were also found in organs, notably the forestomach, but the findings were not significantly different from those in other treatment groups (alcohol group or alcohol + smokeless tobacco group).

Cyclooxygenase-2 (COX-2), an inducible enzyme that is responsible for prostaglandin (PGE2 and 6-keto-PGF1a) synthesis, plays an important role in inflammatory diseases and carcinogenesis. It is upregulated in human squamous-cell carcinoma cells and primary tumour tissue from head and neck cancers (Chan *et al.*, 1999; Dannenberg *et al.*, 2001). Exposure to aqueous extract of smokeless tobacco (snuff) caused loss of the anti-inflammatory activity of annexin I in the golden Syrian hamster cheek pouch and up-regulation of the pro-inflammatory COX-2 in hamster cheek pouch carcinoma (HCPC-1)

cells (Vishwanatha *et al.*, 2003) (see Section 4.2.2(a)). The dual effect of these regulatory events could lead the cells down the carcinogenic pathway.

(ii) *Cardiovascular effects*

Squires *et al.* (1984) studied haemodynamic parameters in 10 anaesthetized dogs that had 2.5 g US commercial moistened snuff (1.2% nicotine) placed in the buccal cavity for 20 min. They observed significant increases in heart rate, blood pressure, left ventricular pressure, left ventricular end diastolic pressure and dP/dt (first derivative of left ventricular pressure) and significant decreases in the coronary circumflex, renal and femoral arteries.

Suzuki *et al.* (1996) exposed hamster cheek pouch oral mucosa to an aqueous smokeless tobacco extract (US moist snuff) *in situ* and observed impairment of endothelium-dependent vasodilation elicited by two different agonists, acetylcholine and bradykinin.

(c) *Studies in vitro*

(i) *Effects on proliferation, differentiation and apoptosis*

Cell survival and DNA repair capacity

Significant *O*⁶-methylguanine–DNA methyltransferase (MGMT) activities (which catalyse the repair of promutagenic *O*⁶-methylguanine lesions in isolated DNA *in vitro*) were demonstrated in normal, non-tumorous human buccal mucosa, cultured buccal epithelial cells and fibroblasts from buccal tissue specimens. Lower MGMT activity than normal in two transformed buccal epithelial cell lines, SVpgC2a and SqCC/Y1, correlated with decreased MGMT mRNA and lack of functional p53 protein. Exposure of human buccal fibroblasts in culture to various organic or water-based extracts of products related to the use of tobacco and betel quid decreased both cell survival and MGMT activity. Organic extracts of *bidi*-smoke condensate and betel leaf showed higher potency than those of tobacco and snuff. An aqueous snuff extract decreased both parameters (Liu *et al.*, 1997).

Cell growth and differentiation

The effects of snuff extract on epithelial growth and differentiation were studied in HaCaT cells grown *in vitro* (Merne *et al.*, 2004). Cultures exposed to snuff did not show any increase in cell proliferation as measured by Ki-67 staining but showed a disturbance in the differentiation process by a decrease of CK 10 and filaggrin expression. Murrah *et al.* (1993), however, demonstrated that smokeless tobacco extracts increase cell proliferation and growth effects of human oral epithelial cells in culture similar to the proliferative effects shown in human oral mucosa in tobacco users (Warnakulasuriya & MacDonald, 1995). Wang *et al.* (2001) reported increased proliferation of cultured human keratinocytes induced by low doses of smokeless tobacco and that of fibroblasts in organotypic culture induced by both low and high doses.

HCPC-1 cells treated with aqueous smokeless tobacco extract have shown significantly increased DNA synthesis as assessed by bromodeoxyuridine (BrdU) incorporation (Rubinstein, 2000).

Apoptosis

A dose-dependent induction of apoptosis mediated by nitric oxide was observed in HCPC-1 cells treated with smokeless tobacco extracts (Mangipudy & Vishwanatha, 1999).

Fox *et al.* (1995) demonstrated that cell death following long-term snuff exposure of human fibrosarcoma (HT-1080) cells *in vitro* is not a result of apoptosis but is related to epithelial–mesenchymal interactions that result in the loss of cell adhesion.

(ii) *Smokeless tobacco and inflammation*

Activation of complement was demonstrated *in vitro* using aqueous extracts of loose-leaf chewing tobacco, dry snuff and moist snuff (Chang *et al.*, 1998). This may contribute to local inflammation at sites where snuff is placed and result in gingivitis, periodontitis or focal inflammation of mucosal tissue.

Furie *et al.* (2000) exposed cultured human umbilical vein endothelial cells to extracts of smokeless tobacco (US chewing tobacco, dry snuff and moist snuff) and observed increased production of compounds that promote the recruitment of leukocytes as well as increased migration of neutrophils across the endothelial cell monolayers. These investigators also reported that bacterial lipopolysaccharide in the smokeless tobacco extracts accounts for part, but not all, of the pro-inflammatory effect.

Increased PGE₂ secretion was seen when peripheral blood mononuclear cells were cultured with 1% smokeless tobacco extracts (nicotine concentration, 117.5 µg/mL) (Bernzweig *et al.*, 1998) relative to control cultures, although gingival mononuclear cells were not further activated. When subjected to 5 or 10% smokeless tobacco extracts (nicotine concentration, 560 or 1118 µg/mL), oral keratinocytes grown from healthy gingival sites were found to produce increased amounts of PGE₂ and IL-1β (Johnson *et al.*, 1996). The levels of IL-1 were not as high as those of PGE₂. PGE₂ is a regulator of keratinocyte proliferation and these experimental findings may also indicate host mechanisms for cell injury.

(iii) *Effects on collagen synthesis*

The effects of smokeless tobacco on bone glucose metabolism (oxygen consumption and lactate production) and collagen synthesis (³H]proline hydroxylation) were tested *in vitro* using cultures of tibiae from chick embryos. The smokeless tobacco extract contained 104–125 µg/mL nicotine. At concentrations found in the saliva of smokeless tobacco users, smokeless tobacco extract stimulated glycolysis and markedly inhibited bone collagen synthesis and mitochondrial activity (Galvin *et al.*, 1988). Smokeless tobacco extract also inhibited the hydroxylation of proline and the synthesis of collagenase-digestible protein in isolated osteoblast-like cells (Galvin *et al.*, 1991). Prolyl hydroxylase activity of chick embryos was inhibited by smokeless tobacco extract, but not by nicotine or anabasine (Galvin *et al.*, 1992). Smokeless tobacco contains an inhibitor of prolyl hydroxylase activity which is present in methanol extracts. This was tested on several collagen-producing cells and tissues other than bone. Results revealed that inhibition of collagen synthesis by smokeless tobacco extract is not specific for bone,

that other collagen-producing cells are directly affected and that recovery is not immediate (Lenz *et al.*, 1992). Thus, this phenomenon could contribute to the periodontal disease that is frequently seen in users of smokeless tobacco.

4.3 Reproductive, developmental and hormonal effects

4.3.1 *Humans*

(a) *Effects on pregnancy*

The rate of still births in Indian women who chewed tobacco (50–100 g per day) was 50 per 1000 live births (11/220) compared with only 17 per 1000 live births (20/1168) in women who did not chew tobacco. The mean birth weight of the offspring of tobacco chewers was approximately 100–200 g lower than that of offspring of non-chewers. This change was associated with a decrease in the mean gestation period. The sex ratio (male:female) of the offspring was 80:100 in the chewers compared with 108.5:100 in non-chewers (Krishna, 1978). The Council on Scientific Affairs of the American Medical Association reviewed the health effects of smokeless tobacco and, confirming the study of Krishna (1978), concluded that use of smokeless tobacco adversely affects pregnancy outcome [“babies of women who chewed tobacco during their pregnancies weighed an average of 100 to 200 g less at birth than did the babies of nonchewers”] (American Medical Association, 1986). It was noted in another review that this weight reduction at birth was mainly attributable to the proportion of chewers who delivered at 36 weeks or earlier, and that other potential confounders were not considered (Critchley & Unal, 2003).

The mean weight of the placenta from 48 Indian mothers who took tobacco (in 83% of the cases as a mixture of tobacco and lime) was 15% greater than that from 48 controls (Agrawal *et al.*, 1983). Re-analysis of the 48 case–control pairs reported previously (Agrawal *et al.*, 1983) with the paired *t* test showed an overall 65.4-g increase in the mean weight of placentas from smokeless tobacco users which was significantly different ($p < 0.001$) from those of non-users. The increase was 70 g in consumers of smokeless tobacco for 6 years or more compared with those who used it for a shorter period of time ($p < 0.001$) and in regular compared with intermittent and occasional users ($p < 0.001$), but the increase in placental weight was only significant in women who used tobacco with lime (Krishnamurthy, 1991). The mean weight of newborn babies of 70 Indian tobacco users (the tobacco was either chewed or ingested alone or mixed with betel leaf or with lime) was 14% less than that of the babies of 70 matched controls (Verma *et al.*, 1983).

Birth weights, pre-term delivery and pre-eclampsia, pregnancy outcomes that have consistently been shown to be affected by cigarette smoking, were evaluated in Swedish women who used snuff and who delivered singleton, live-born infants without major congenital malformations from 1999 through to 2000. For each snuff user, 10 cigarette smokers and 10 non-users were randomly selected from the Swedish Birth Registry. After exclusions, 789 snuff users, 11 240 smokers and 11 495 non-users remained. Compared

with non-users, adjusted mean birth weight was reduced by 39 g (95% CI, 6–72 g) in snuff users, by 172 g (95% CI, 158–185 g) in light smokers and by 224 g (95% CI, 207–240 g) in moderate-to-heavy smokers. In women who were known to have continued using tobacco in late pregnancy, the adjusted mean birth weight was reduced by 93 g (95% CI, 38–147 g) in 268 snuff users, by 213 g (95% CI, 193–234 g) in 2821 light smokers and by 250 g (95% CI, 225–275g) in 1638 moderate-to-heavy smokers. Snuff use was not associated significantly with newborns that were small for gestational age. The risk for pre-eclampsia was reduced in smokers but increased in snuff users, who had a 60% increased risk (odds ratio, 1.6; 95% CI, 1.1–2.3) which was unchanged after stratification by parity. Both snuff use and cigarette smoking were associated with an approximately twofold increased risk for pre-term delivery. This risk was comparable in both snuff users and smokers. For 752 snuff users (versus 11 152 non-users), the odds ratio for pre-term delivery (after exclusion of women with pre-eclampsia) was 1.8 (95% CI, 1.3–2.5) (England *et al.*, 2003).

A population-based cohort study was conducted in Mumbai, India, to determine the effect of using smokeless tobacco during pregnancy on birth weight and gestational age at birth for singleton infants. A total of 1217 women who were 3–7 months pregnant and who planned to deliver in the study area were identified, of whom 1167 (96%) were followed up. Individuals who used *mishri* (as a dentifrice) and/or chewed betel quid with tobacco, *gutka* or *pan masala* at least once daily were considered to be smokeless tobacco users. Smokeless tobacco use was associated with an average reduction of 105 g in birth weight (95% CI, 30–181 g) and a reduction in gestational age of 6.2 days (95% CI, 3.0–9.4 days). The odds ratio for low birth weight was 1.6 (95% CI, 1.1–2.4) adjusted by logistic regression for maternal age, education, socioeconomic status, weight, anaemia, antenatal care and gestational age. The adjusted odds ratio for pre-term delivery (< 37 weeks) was 1.4 (95% CI, 1.0–2.1). The odds ratios increased for delivery before 32 weeks (4.9; 95% CI, 2.1–11.8) and before 28 weeks (8.0; 95% CI, 2.6–27.2) (Gupta & Sreevidya, 2004).

(b) *Effects on male fertility*

Semen samples were collected from 626 men, 20–35 years of age, in Ahmedabad, India, who attended a clinic for idiopathic infertility and had no history of systemic disease, genital tract disorder, varicocele, genital infections or genital surgical operations, hormonal abnormalities or treatments, exposure to radiation or alcoholism or drug abuse. These included 288 non-users of any form of tobacco (mean age, 26.5 years), 119 addicted tobacco chewers (> 10 helpings per day; mean age, 26.2 years) and 219 cigarette smokers (> 10 cigarettes per day; mean age, 26.7 years). Mild or occasional tobacco users and former users were not included in the study. Both tobacco smokers and chewers had a slightly smaller ejaculate volume than non-users and a non-significant decrease in sperm density and total sperm count (Student *t* test, $p > 0.05$). No significant difference was observed among any of the groups for sperm motility or proportion of morphologically normal spermatozoa (Dikshit *et al.*, 1987).

Semen samples were obtained over a 1-year period from 165 men, aged 27–44 years, in Calcutta, India. Samples from men with a history of systemic disorders, genital tract infections, operations, varicocele, drug or hormone treatment, exposure to radiation, heavy alcoholic beverage drinking or sperm density below 1 000 000/mL were excluded. The remaining men included 21 never users of tobacco, 29 tobacco (*zarda*) chewers, 40 cigarette smokers and eight who were addicted to multiple forms of tobacco use. The mean age of never users was 36.2 years and that of all users combined was 34.3 years. Semen volume, sperm density, total sperm count and motility were significantly ($p < 0.05$, ANOVA) reduced in tobacco users (all groups combined) and the frequency of abnormal spermatozoa was significantly greater ($p < 0.05$) than in never users. Sperm density and total sperm count were more significantly reduced among tobacco chewers than in all other groups ($p < 0.05$). Sperm density and motility were not significantly lower among cigarette smokers than among never users, but total sperm count was significantly reduced and the frequency of abnormal sperm was higher ($p < 0.01$) in this group than in either tobacco chewers or never users. The frequency of abnormal spermatozoa was related to the level of tobacco consumption and was highest in the group with multiple addictions (Banerjee *et al.*, 1993).

4.3.2 *Experimental systems*

(a) *Reproductive toxicity*

Female CD-1 mice received an aqueous extract of standard reference moist snuff tobacco (University of Kentucky Tobacco and Health Research Institute, USA) at dose levels that supplied either 12 or 20 mg/kg bw nicotine at each application. Tobacco extract was administered by gavage thrice daily for 2 weeks before breeding, during breeding and during gestational days 0–16. These doses resulted in plasma nicotine levels of 363 and 481 ng/mL and 9.6 and 28% mortality among the dams, respectively. Controls received distilled water instead of tobacco extract; none of the control dams died during treatment. Surviving mice were killed on gestational day 17. No significant differences in litter sizes or in the ratios of total implantation sites to live fetuses were observed. Placental weights were increased ($p < 0.05$) in mice that had received tobacco extract at the lower dose; at the higher dose, resorptions were increased, fetal weights were reduced and fetal skeletal ossification was decreased; the lower dose had a negligible effect on maternal weight gain and fetal weights. The higher dose resulted in fetal growth retardation ($p < 0.05$), increased embryotoxicity and decreased fetal skeletal ossification ($p < 0.05$) (Paulson *et al.*, 1991).

(b) *Prenatal developmental toxicity*

Aqueous extracts of a commercial brand of moist snuff (USA) were administered to pregnant CD-1 mice by subcutaneously implanted minipumps at doses that maintained plasma nicotine levels in the range of 29–44 ng/mL during gestation days 7–14 or, using double the concentration of tobacco extract, in a higher range of 34–75 ng/mL. Dams were

killed on day 17. The main effect on fetuses was reduction of body weight; body weight was significantly lower than that of controls at the higher dose (8.6% reduction, $p < 0.0001$) and was accompanied by an increase in the number of fetal deaths ($p < 0.03$). The lower dose produced an increase in the incidence of haemorrhages and supernumerary ribs in fetal mice and a significant delay ($p < 0.05$) in ossification of the supraoccipital bone, the sacrococcygeal vertebrae and the bones of the feet. Weights of dams were significantly reduced only at the higher dose level (Paulson *et al.*, 1988).

Aqueous extracts of a reference standard moist snuff (University of Kentucky Tobacco and Health Research Institute, USA) were administered by gavage to pregnant CD-1 mice thrice daily on days 1–16 of gestation, in weight-adjusted volumes that contained 4, 12 or 20 mg/kg bw nicotine and generated plasma nicotine concentrations of 99, 398 and 623 ng/mL, respectively. Solvent controls received distilled water. Mice were killed on day 17 of gestation. The number of resorptions increased in a dose-related manner and resorptions were more frequent in all treated groups than in solvent controls. The two higher doses caused increasing numbers of maternal deaths (31% at the highest dose). Fetal weights were reduced only in the highest-dose group. Placental weights were not affected. Internal malformations included a significant increase in the incidence of minor heart defects (Paulson *et al.*, 1989).

Pregnant CD-1 mice received an aqueous extract of ethanol (1.8 g/kg bw), an aqueous solution of D-glucose of equal caloric value or an aqueous extract of standard moist snuff tobacco (University of Kentucky Tobacco and Health Research Institute, USA) equivalent to 8 mg/kg bw nicotine plus either ethanol or D-glucose by gavage thrice daily on days 6–15 of gestation and were killed on day 17 of gestation. No significant differences were observed in maternal weight gain, litter size or incidence of resorptions, fetal deaths or malformations. Fetal weights were reduced in all treatment groups, with the greatest reduction in the tobacco extract-treated group followed by the ethanol-treated group and finally the combined tobacco extract and ethanol-treated group. Placentas of the tobacco extract-treated group weighed significantly less than those of controls. Ossification of the fetal skeleton was affected to the greatest extent in the tobacco extract-treated group, followed by the ethanol-treated and combined tobacco extract and ethanol-treated groups. Craniofacial measurements were significantly affected in all three treatment groups. No interactive effect of ethanol and tobacco extract was observed on fetal growth or development (Paulson *et al.*, 1992).

Pregnant Sprague-Dawley rats received aqueous extracts of reference standard moist snuff (Code 1S3, University of Kentucky Tobacco and Health Research Institute, USA) by gavage thrice daily during days 6–18 of gestation at doses that provided 1.33 or 6 mg/kg bw nicotine and generated mean plasma nicotine levels of 283 and 846 ng/mL, respectively. Controls received distilled water. Rats were killed on gestational day 19. Weight gain of dams was reduced in both treatment groups in comparison with controls ($p < 0.05$) but fetal weights were reduced only at the higher dose. Placental weights, litter size, resorptions, deaths and malformations were not significantly affected by treatment

with tobacco extract. Decreased levels of ossification in the fetal skeletons were seen at the higher-dose level ($p < 0.05$) (Paulson *et al.*, 1994a).

(c) *Postnatal developmental toxicity*

Lactating random-bred Swiss albino mice received 100 μ L freshly prepared aqueous extract of smokeless tobacco powder (Vadakkan variety, Mysore, India) by gavage daily on days 1–21 after delivery. Litters were normalized to five pups by redistribution before treatments began. Dams were distributed to groups that received extracts of 50 or 100 mg/kg bw tobacco per day, alone or together with 1% *tert*-butylated hydroxyanisole (BHA) in the diet or phytic acid (1000 mg/kg bw per day) by gavage throughout lactation. Dams and pups were then killed and livers processed for enzyme and thiol measurements. Hepatic GST levels and thiol content were depressed in dams and pups by the higher dose of tobacco extract, but the increased levels of GST and thiol content induced by BHA or phytic acid alone were only slightly reduced in dams and pups that received combined treatment with one of these substances plus tobacco extract. Hepatic cytochrome b_5 and CYP levels were increased in dams at both doses of smokeless tobacco extract and in pups at the higher-dose level only. Combined exposures to tobacco extracts and either BHA or phytic acid resulted in increased cytochrome b_5 and CYP levels that were comparable with or higher than those in dams or pups given BHA or phytic acid alone (Singh & Singh, 1998).

Pregnant Sprague-Dawley rats received aqueous extracts of reference standard moist snuff (Code 1S3, University of Kentucky Tobacco and Health Research Institute, USA) by gavage thrice daily on days 6–20 of gestation at doses that provided 1.33 or 4 mg/kg bw nicotine. Controls received distilled water. Immediately after parturition, offspring were fostered to control mothers and litters were culled to 4 ± 1 male and 4 ± 1 female offspring. Prewaning behavioural tests, including surface righting, swimming development, negative geotaxis and open-field activity, were conducted during postnatal days 1–21 and post-weaning tests, including open-field activity, active avoidance shuttle box and Cincinnati water maze, were conducted during postnatal days 22–67. Maternal weight gain and mean pup weights at birth were lower at the higher-dose level. No significant treatment-related differences were observed in postnatal development of physical landmarks, activity levels or learning development (Paulson *et al.*, 1993).

Pregnant Sprague-Dawley rats received aqueous extracts of reference standard moist snuff (Code 1S3, University of Kentucky Tobacco and Health Research Institute, USA) by gavage thrice daily on days 6–20 of gestation at doses that provided 1.33, 4 or 6 mg/kg bw nicotine. Controls received distilled water. Litters were culled to 4 ± 1 male and 4 ± 1 female offspring and raised by their natural mothers. Weights, physical landmark development and behavioural performance of pups were monitored during pre- and post-weaning periods. Behavioural tests included surface righting, negative geotaxis, swimming development, open-field activity and active avoidance in a shuttle box. The two higher doses caused reduced maternal weight gain and significant reductions in pup weight that persisted during the early postnatal period, and infant mortality increased with increasing

exposure to tobacco extract in a dose-dependent manner. A decreased success rate for surface righting was noted for pups exposed to tobacco extract. Variable results were obtained with other measures of behavioural development: pups exposed to the intermediate dose of tobacco extract performed best in swimming development; highest-dose pups were most active during the pre-weaning period while lowest-dose pups were most active during the post-weaning period; no treatment-related differences were noted in the active avoidance shuttle box (Paulson *et al.*, 1994b).

4.4 Genetic and related effects

4.4.1 Humans

In the previous monograph on 'Betel-quid and areca-nut chewing' (IARC, 2004a), the following studies on the genetic and related effects in humans exposed to these substances with or without tobacco were included: Saranath *et al.*, 1991; Kaur *et al.*, 1994; Kuttan *et al.*, 1995; Heinzl *et al.*, 1996; Munirajan *et al.*, 1996; Baral *et al.*, 1998; Kaur *et al.*, 1998; Munirajan *et al.*, 1998; Murti *et al.*, 1998; Pande *et al.*, 1998; Agarwal *et al.*, 1999; Patnaik *et al.*, 1999; Ravi *et al.*, 1999; Saranath *et al.*, 1999; Chakravarti *et al.*, 2001; Pande *et al.*, 2001; Ralhan *et al.*, 2001; Tandle *et al.*, 2001; Nagpal *et al.*, 2002a,b; Chakravarti *et al.*, 2003). [The Working Group noted that, in the absence of detailed information on the smokeless tobacco consumption of oral squamous-cell carcinoma patients, the data from these studies and others (Lazarus *et al.*, 1996a; Ralhan *et al.*, 1998) are not included here.]

(a) Mutagenicity and genotoxicity

(i) Mutagenicity

The mutagenicity of urine samples from six *mishri* users and six non-users was similar, when tested in the *Salmonella*/microsome assay in the presence of β -glucuronidase or nitrite plus metabolic activation. However, the samples from *mishri* users showed stronger mutagenicity in TA98 in the presence of β -glucuronidase plus metabolic activation ($0.82 \pm 0.41 \times 10^6$ revertants per mol creatinine) compared with those from non-users ($0.11 \pm 0.11 \times 10^6$ rev/mol creatinine) (Govekar & Bhisey, 1993).

Mutagen levels in concentrates of urine from Swedish wet snuff users, cigarette smokers and non-tobacco users were compared. The concentrates were assayed for mutagenicity towards *S. typhimurium* TA98, both in the presence and absence of a metabolic activation system (the postmitochondrial liver fraction from Aroclor-1254-induced rats). The mean mutagenic activity in urine concentrates from smokers, found only in the presence of metabolic activation (8.6×10^3 revertants per 24 h), was significantly higher than that in the urine from snuff users (1.3×10^3 revertants per 24 h), abstinent snuff users (1.3×10^3 revertants per 24 h) and non-users (0.9×10^3 revertants per 24 h). No significant difference in mutagenic activity was found between the urine from snuff users, whether using or abstaining from snuff use, and that from non-tobacco users (Curvall *et al.*, 1987).

Niphadkar *et al* (1994) determined the bacterial mutagenicity of gastric fluid samples from chewers of tobacco and from non-users in *Salmonella* strains TA98, TA100 and TA102. While all six gastric fluid samples from non-chewers were not mutagenic, samples from nine chewers were mutagenic, either directly or upon nitrosation, in the three tester strains and in TA102 in the presence of activating enzymes.

(ii) *Micronuclei, chromosomal aberrations and sister chromatid exchange*

Several studies have shown a relationship between snuff user's hyperkeratosis and elevated frequencies of micronucleated cells and/or chromosomal aberrations and sister chromatid exchange in snuff users compared with non-user controls (Livingston *et al.*, 1990; Tolbert *et al.*, 1991, 1992; Roberts, 1997). These studies are summarized in Table 95. Swedish moist snuff users showed increased mitotic rate, increased cell density and loss of cell cohesion (Larsson *et al.*, 1991).

Higher frequencies of micronucleated cells and/or chromosomal aberrations and/or sister chromatid exchange were also reported in smokeless tobacco consumers and in patients with oral squamous-cell carcinoma, in comparison with non-user controls (see Table 95; Stich *et al.*, 1982a,b; Nair *et al.*, 1991; Das & Dash, 1992; Kayal *et al.*, 1993; Trivedi *et al.*, 1995; Ozkul *et al.*, 1997).

Elevated levels of micronucleated cells (% , with range) were found in the oral mucosa of *khaini* chewers from Bihar, India (2.1%; 0.8-4.9%), *gudhaku* chewers from Orissa, India (0.7%; 0.3-1.8%) and *naswar* users from Uzbekistan (former Soviet Union) (4.1%; 2.7-5.7%) compared with controls (non-chewers, nonsmokers) from various locations (0.5%; 0.0-1.0) (Stich & Anders, 1989). Localized micronucleus formation in the oral mucosa was described in *khaini* chewers; 2% of the cells in the gingival groove showed micronuclei (Stich *et al.*, 1992). In another study from India, 6.3% of the cells were micronucleated in chewers of tobacco with lime (Ghose & Parida, 1995).

In a study designed to monitor genotoxicity in the *bidi* industry that included tobacco-processing plant workers and *bidi* rollers who did not use tobacco, the mean frequency of micronucleated cells in the buccal epithelium was significantly higher among *bidi* rollers and plant workers than among non-exposed controls (Bagwe & Bhisey, 1993).

(iii) *Genomic instability*

Genomic instability, as reflected by microsatellite alterations in specific target regions in tobacco chewers with primary oral squamous-cell carcinoma (77 cases), was analysed by Mahale and Saranath (2000). Using a panel of 11 microsatellite markers on chromosome 9, 48/77 (62%) patients demonstrated microsatellite alterations of which 27% had microsatellite instability and 52% had loss of heterozygosity. A majority of the alterations occurred on the p arm at 9p21-23 that may be indicative of involvement of the multiple tumour-suppressor 1 (*MTS1*) p16 (*CDKN2*) gene on chromosome 9p21 in a subset of chewing tobacco-induced oral cancers. [The Working Group noted the lack of information on the consumption pattern of tobacco in this study.]

Table 95. Micronucleated cells and sister chromatid exchange in oral mucosa of non-users and users of smokeless tobacco products

Study location	No. and definition of non-users	No. of users and type of smokeless tobacco used	Micronucleated cell frequency (mean/100 or 1000 cells)		Sister chromatid exchange (mean frequency/cell \pm SE)		Reference
			Non-user	User	Non-user	User	
India	10 non-chewers	27 <i>khaini</i> (tobacco + lime) users	<i>Mean per 100 cells</i>		NT	NT	Stitch <i>et al.</i> (1982a,b)
			0.49	2.2 (front) 2.7 (right) 3.04 (left)			
USA	24 non-users	24 snuff users	0.27	2.22	6.78 \pm 0.15	6.70 \pm 0.18	Livingston <i>et al.</i> (1990)
India	27 non-users	35 tobacco + lime users	<i>Mean per 1000 cells</i>		NT	NT	Nair <i>et al.</i> (1991d)
			2.59 \pm 0.37	5.20 \pm 0.66			
USA	15 female non-users	38 female snuff users	1.58 \pm 0.58	3.79 \pm 0.56	NT	NT	Tolbert <i>et al.</i> (1991, 1992)
India	102 non-users	120 <i>gudhaku</i> (tobacco paste) users	<i>Mean per 100 cells</i>		NT	NT	Das & Dash (1992)
			0.35	2.06 (users for more than 20 years)			

Table 95 (contd)

Study location	No. and definition of non-users	No. of users and type of smokeless tobacco used	Micronucleated cell frequency (mean/100 or 1000 cells)		Sister chromatid exchange (mean frequency/cell \pm SE)		Reference
			Non-user	User	Non-user	User	
India (Gujarat, Maharashtra)	Gujarat 15 non-users	20 <i>mawa</i> (healthy) users	<i>Mean per 1000 cells</i>		NT	NT	Kayal <i>et al.</i> (1993)
		21 <i>mawa</i> (OSMF) users	1.90 \pm 0.19	6.9 \pm 0.54			
		14 tobacco + lime (healthy) users		7.05 \pm 0.75			
		12 dry snuff users (healthy)		5.9 \pm 0.49			
India	13 non-users	Maharashtra 16 <i>mishri</i> users (healthy)	1.00 \pm 0.32	3.19 \pm 0.63	NT	NT	Trivedi <i>et al.</i> (1995)
		40 non-users	40 chewing tobacco (healthy)	NT	NT	Mitomycin C-induced	
		40 chewing tobacco (oral cancer)			20.29 \pm 0.519	21.29 \pm 0.587	
						23.14 \pm 0.428	
Turkey	15 non-users nonsmokers	25 maras powder (smokeless tobacco) users	<i>Mean per 100 cells</i>		NT	NT	Ozkul <i>et al.</i> (1997)
			0.84 \pm 0.22	1.86 \pm 0.26			
USA	19 non-users	22 snuff users	<i>Mean per 1000 cells</i>		NT	NT	Roberts (1997)
			1.8	2.52			

NT, not tested; OSMF, oral submucosal fibrosis; SE, standard error

(b) *Alterations in TP53, K-RAS and related genes* (Figure 9)

(i) *TP53, K-RAS and H-RAS*

[The Working Group noted that details on (smokeless) tobacco use are often not or inadequately provided in the studies that were reviewed. Tobacco chewing in combination with betel quid is a common habit in the Indian population. As detailed data on these mixed exposures were not given in Saranath *et al.* (1992), Kannan *et al.* (2000), Tandle *et al.* (2000), Krishnamurthy *et al.* (2001) or Teni *et al.* (2002), these studies are not included in the review below.]

Analyses of *TP53* mutations in oral carcinomas associated with only tobacco chewing are limited. These studies report *TP53* mutations in oral cancers in the USA, Sudan, Norway and Sweden and are summarized in Table 96 (Lazarus *et al.*, 1995, 1996a,b; Xu *et al.*, 1998; Ibrahim *et al.*, 1999; Kannan *et al.*, 1999; Saranath *et al.*, 1999; Schildt *et al.*, 2003). In a limited study of *toombak* users, four head and neck squamous-cell carcinomas from three patients who used *toombak* and one patient who did not use *toombak* were screened for *TP53* mutations (Lazarus *et al.*, 1996a,b). Mutations were found in tumours resected from two of three *toombak* users, one at codon 282 (C→T) and the other in intron 6 (AT→GC). No *K-RAS* (codons 12 and 13) or *H-RAS* (codon 12) mutations were found in tumours that harboured *TP53* mutations and the other tumours.

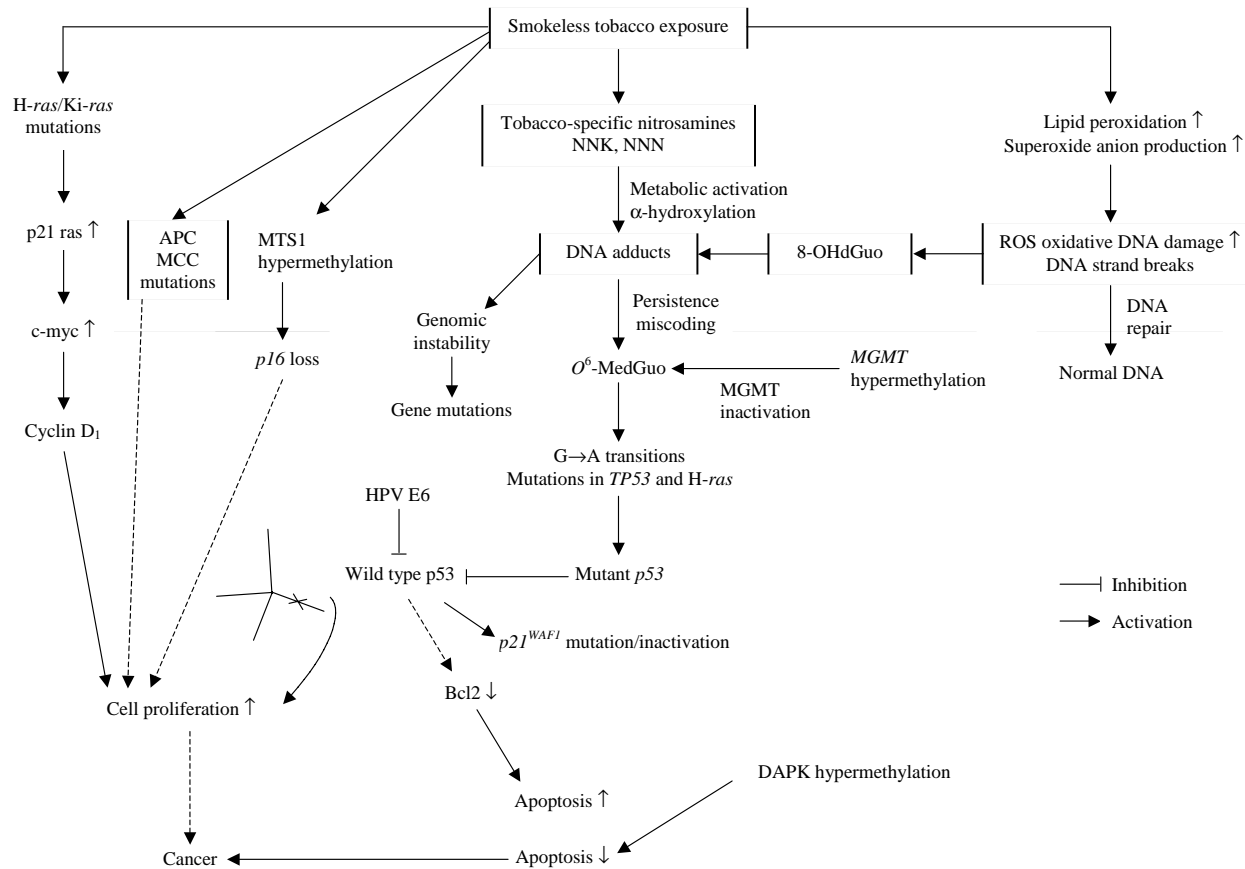
A high incidence of *H-RAS* mutations (codons 12, 13 or 61) was reported in oral cancers from India, the majority of which were in tobacco chewers (Saranath *et al.*, 1991) (see Table 97). [The Working Group noted that details on tobacco chewing were not mentioned in this study.]

Xu *et al.* (1998) analysed four oral squamous-cell carcinomas from users of snuff or chewing tobacco and 16 oral squamous-cell carcinomas from smokers only. Two of the tumours from users of snuff or chewing tobacco showed *TP53* mutations, while p53 protein accumulation was observed in all four tumours. No *H-RAS* mutation was observed in any of the tumours. No differences were observed in the p53, cyclin D1 and Rb profiles of users of smokeless tobacco and cigarette smokers.

TP53 mutations in 56 oral squamous-cell carcinomas from Sudanese *toombak* dippers and non-dippers and from Scandinavian non-dippers were analysed (Ibrahim *et al.*, 1999). No *TP53* mutations were found in non-malignant oral lesions from *toombak* dippers or non-dippers from Sudan. *TP53* mutations in exons 5–9 were found in 13/14 (93%) *toombak* dippers compared with 8/14 (57%) non-dippers from Sudan and 17/28 (61%) non-dippers from Scandinavia. Mutations G:C→A:T; C:G→T:A; G:C→T:A which are known to be associated with TSNA were found to be most common in oral squamous-cell carcinomas from *toombak* dippers which suggests a possible role of TSNA in the induction of *TP53* mutations in these tumours. [The Working Group noted that the information on cigarette smoking in Sudanese non-dippers and on alcohol drinking in 19 of the 28 Scandinavian patients were not available in this study.]

Saranath *et al.* (1999) reported *TP53* mutations in 14/83 (17%) oral squamous-cell carcinoma patients from India, the majority of whom were tobacco chewers, and multiple

Figure 9. Molecular targets affected by smokeless tobacco



APC, adenomatous polyposis coli; DAPK, death-associated protein kinase; HPV, human papillomavirus; MCC, mutated in colon cancer; MGMT, O⁶-methylguanine–DNA methyltransferase; MTS, methyl transferase; NNK, 4-methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, N'-nitrosornicotine; O⁶-MedGuo, O⁶-methyldeoxyguanosine; 8-OHdGuo, 8-hydroxydeoxyguanosine; ROS, reactive oxygen species

Table 96. Use of smokeless tobacco and *TP53* mutations in human oral premalignant and malignant lesions

Study location	Total no. of cases	Exons analysed	Total no. of lesions with <i>TP53</i> mutations	Chewing tobacco/snuff		<i>TP53</i> mutational analysis Codon/mutations	Reference
				Total cases	No. of cases with <i>TP53</i> mutation		
USA	14 oral cavity premalignant lesions (dysplasia)	5–9	4 premalignant lesions	1 dysplasia (snuff)	1	Exon 5 163/A:T→G:C and 155/A:T→C:G (same case)	Lazarus <i>et al.</i> (1995)
Sudan	4 HNSCCs	5–9	2	3 HNSCCs (<i>toombak</i> dipper) 1 HNSCC (non-dipper)	2 0	Intron 6, nt 553/A:T→G:C (1 case) Exon 8 codon 282/C:G→T:A (1 case)	Lazarus <i>et al.</i> (1996)
USA	29 oral cancers	5–8	17	4 oral cancers (chewing tobacco/snuff)	2	Exon 6 +220/TAT→TGT (1 case) ^s 191/CCT→ACT and 192/CAG→CAT (1 case)	Xu <i>et al.</i> (1998)
Sudan, Norway, Sweden	56 OSCCs	5–9	38	14 (<i>toombak</i> dipper)	13	Exon 5 Sudanese <i>toombak</i> dippers 130/CTC→CTT (1 case) ^s 166/TCA→GCA* (1 case) 132/AAG→ATG* 133/ATG→GATG ⁱ 148/GAT→TGA* (All 3 from 1 case) 139/AAG→GAG 142/CCT→CTT (1 case) Sudanese non-dippers 132 [^] /AAG→ATG* (2 cases) Scandinavian non-dippers 130 [^] /CTC→CTT (2 cases) ^s , 136 [^] /CAA→CCA* (1 case), 146 [^] /TGG→TAG (stop) (1 case)	Ibrahim <i>et al.</i> (1999)

Table 96 (contd)

Study location	Total no. of cases	Exons analysed	Total no. of lesions with <i>TP53</i> mutations	Chewing tobacco/snuff		<i>TP53</i> mutational analysis Codon/mutations	Reference
				Total cases	No. of cases with <i>TP53</i> mutation		
Sudan, Norway, Sweden (contd)				14 (Sudanese non-dippers)	8	Exon 6 Sudanese toombak dippers 190/CCT→CCG* (1 case)*, 216 [^] /GTG→GGG* (1 case) Exon 7 Sudanese toombak dippers 229/TGT→TGG* (1 case), 229/TGT→TA del (1 case), 237/ATG→ATA (1 case), 239/AAC→TAA* (stop) (1 case), 242/TGC→TAC (1 case), 249/AGG→AAG (2 cases), 245/GGC→GAA (1 case), 252/CTC→CAC* (2 cases) Sudanese non-dippers 240 [^] /AGT→CCC* and 249/AGG→AAG (both in 1 case), 244/GGC→TGA* (2 cases), 254 [^] /ATC→GTC (1 case)	Ibrahim <i>et al.</i> (1999) (contd)
				No data on cigarette smoking for Sudanese non-dippers			

Table 96 (contd)

Study location	Total no. of cases	Exons analysed	Total no. of lesions with <i>TP53</i> mutations	Chewing tobacco/snuff		<i>TP53</i> mutational analysis Codon/mutations	Reference
				Total cases	No. of cases with <i>TP53</i> mutation		
Sudan, Norway, Sweden (contd)				28 (Scandinavian non-dippers) 11/17 Norwegian and 8/11 Swedish non-dippers reported cigarette smoking.	17	Scandinavian non-dippers 238/TGT→CGT (1 case) 246/ATG→ATA (3 cases), 248/CGG→CGA (2 cases), 248/CGG→CAA (1 case), 244/GGC→GAA (1 case) 239 [^] /AAC→ACC (1 case), 246 [^] /ATG→ATA (1 case), 249 [^] /AGG→AAG (1 case) Exon 8 Sudanese toombak dippers 299/CTG→CTTG (1 case) [†] , 305/AAG→TAA* (stop) (1 case) Scandinavian non-dippers 279/GGG→GAG (1 case), 281 [^] /GAC-GCC* (1 case), 299 [^] /CTG→CTTG [†] and 305 [^] /AAG-TAA* (stop) (2 cases), 306 [^] /CGA→TGA (stop) (1 case)	Ibrahim <i>et al.</i> (1999) (contd)

Table 96 (contd)

Study location	Total no. of cases	Exons analysed	Total no. of lesions with <i>TP53</i> mutations	Chewing tobacco/snuff		<i>TP53</i> mutational analysis Codon/mutations	Reference
				Total cases	No. of cases with <i>TP53</i> mutation		
Sudan, Norway, Sweden (contd)						Exon 9 Sudanese toombak dippers 310/AAC→TTG* (1 case), 310/AAC→ACC* (2 cases), 312/ACC→AGC* (1 case) Sudanese non-dippers 322 ^Δ /CCA→CGA and 323 ^Δ /CTG→CG del (1 case), 323 ^Δ /CTG→GGG* (1 case) Scandinavian non-dippers 308/CTG→CTA 315/TCT→TGT* 308/CTG→CTC* (1 case), 309 ^Δ /CCC→TCC and 323 ^Δ /CTG→CGG* (1 case), 322 ^Δ /CCA→CGA (1 case)	Ibrahim <i>et al.</i> (1999) (contd)

Table 96 (contd)

Study location	Total no. of cases	Exons analysed	Total no. of lesions with <i>TP53</i> mutations	Chewing tobacco/snuff		<i>TP53</i> mutational analysis Codon/mutations	Reference
				Total cases	No. of cases with <i>TP53</i> mutation		
India	72 OSCCs	4-9	15	72 (All patients were tobacco chewers for > 10 years.)		Exon 5 139/AAG→AAT (1 case) 141/TGC→TAC (1 case) 179/CAT→TAT (1 case) Exon 7 237/ATG→ATA (1 case) 248/CGG→CAG (1 case) Exon 6 213/CGA→CGG (1 case) 213/CGA→CGG (1 case) Exon 8 266/GGA→GAA (1 case) 272/GTG→TTG (1 case) 272/GTG→TTG (1 case) 273/CGT→CAT (1 case) 273/CGT→TGT (2 cases) 282/CGG→TGG (1 case) Exon 9 307/GCA→GCG (1 case) Exon 5 176/TGC→TTC and (Exon 8) 266/GGA→GTA (1 case) Exon 6 190/CCT→CTT and 213/CGA→CGG (1 case) Exon 6 213/CGA→CGG and Exon 7 237/ATG→ATA (1 case)	Kannan <i>et al.</i> (1999)

Table 96 (contd)

Study location	Total no. of cases	Exons analysed	Total no. of lesions with <i>TP53</i> mutations	Chewing tobacco/snuff		<i>TP53</i> mutational analysis Codon/mutations	Reference
				Total cases	No. of cases with <i>TP53</i> mutation		
India	83 OSCCs 22 leukoplakias	5-9	14 and p53 protein over-expression in 23/62 OSCCs and in 6/22 leukoplakias	105 (All patients of OSCCs/leukoplakia were habitual tobacco chewers for a minimum duration of 10 years.)	2	Exon 5 146/TGG→TAG (1 case) 154 ^Δ /GGC→GTT (1 case) 141 ^Δ /TGC→TGT and 175/CGC→CAC (1 case) 152 ^Δ /CCG→CTG (1 case) 153 ^Δ /CCC→CCT (1 case) 175 ^Δ /CGC→CAC (1 case) Exon 6 188/GTC→GTA and 205/TAT→TAC (1 case) 205 ^Δ /TAT→TGT (1 case) 212 ^Δ /TTT→TTG (1 case) 194 ^Δ /CTT→ATT (1 case) 205 ^Δ /TAT→TGT (1 case) Exon 7 239/AAC→GAC (1 case) 231/ACC→ACA (1 case) 248/CGG→TGG (2 cases) 249 ^Δ /del G and 290/CGC→TGC (1 case) all the above in OSCCs. Leukoplakias NA for <i>p53</i> point mutations	Saranath <i>et al.</i> (1999)
Sweden	114 OSCCs	5-9	41 and p53 protein over-expression in 72 tumours	12 (never smokers)	2 (9 patients with p53 positive by IHC)	Exon 8 (details on mutations not reported)	Schildt <i>et al.</i> (2003)

HNSCCs: head and neck squamous-cell carcinomas; IHC, immunohistochemistry; NA, not analysed due to insufficient DNA; OSCCs, oral squamous-cell carcinomas; nt, nucleotide

del, deletions; * transversions

^Δp53 positive by IHC

¹ Insertions leading to frame shift and stop codon

^Δ Silent mutation

Table 97. Analysis of cancer-related gene and protein alterations in oral premalignant and malignant lesions of tobacco users

Study location	Gene/protein	Total no. of cases	Tobacco (chewers/smokers)		Tobacco (without betel quid and smokers)		Alterations Codon/mutation	Reference
			Total no. of cases	No. of cases altered	Total no. of cases	No. of cases altered		
India	H-RAS	57 OSCCs	–	–	57 chewers	20	12.2/GGC→GTC (5 cases) 12.2/GGC→GTC and 61.2/CAG→CGG (2 cases) 12.1/GGC→AGC (1 case) 13.2/GGC→GAC (1 case) 61.2/CAG→CGG (7 cases) 61.2/CAG→CTG (1 case) 61.3/CAG→CAT (3 cases)	Saranath <i>et al.</i> (1991)
USA	Cyclin D1 Rb	29 oral cancers			4 chewers 4 chewers	1 1	Overexpression Absence of expression	Xu <i>et al.</i> (1998)
Sudan, Scandinavia, USA/UK	<i>p21^{WAF1}</i> (Exon 2)	90 OSCCs	14 Sudanese <i>toombak</i> dippers 14 Sudanese non-dippers 27 Scandinavian non-dippers 35 US/UK non-dippers	6 2 6 5	–	–	Sudanese <i>toombak</i> dippers 6/GGG→GAG 7/GAT→GGT 35/GAT→TGA* (stop codon) All from 1 case 30/CTG→TTG ^s (1 case) 10/CAG→CAA ^s (1 case) 68/GTG→GTA ^s 83/CGG→CGA ^s Both from 1 case 138/CAG→TAG (stop codon) 144/CAG→TAG (stop codon) Both from 1 case 84/CGA→TGA (stop codon) 98/TCA→TAA* (stop codon) 106/GCA→GTA 107/GAG→CAG* All from 1 case	Ibrahim <i>et al.</i> (2002)

Table 97 (contd)

Study location	Gene/protein	Total no. of cases	Tobacco (chewers/smokers)		Tobacco (without betel quid and smokers)		Alterations Codon/mutation	Reference
			Total no. of cases	No. of cases altered	Total no. of cases	No. of cases altered		
Sudan, Scandinavia, USA/UK (contd)							Sudanese non-dippers 107/GAG→CAG* 114/TCA→CCA 116/TCT→CCT and 125/GAG→AGA All from 1 case 20/CGC→CGT* 92/GGC→GGT* 94/CGG→CGTG* (1) 95/CCT→GTC* and 117/TGT→TAG (stop codon) All from 1 case Scandinavian non-dippers 138/CAG→TAG (stop codon) 31/AGC→AGA* Both from 1 case 31/AGC→AGA* (1 case) 8/GTC→ATC 62/GAC→AAC 117/TGT→TAT 137/TCT→TGT* All from 1 case 126/CAG→CAC* 140/CGA→CGC* ^s 142/CGG→ACG* All from 1 case 29/CAG→CAC* 52/GAC→CAC* 88/GAG→TGA* (stop codon) All from 1 case	Ibrahim <i>et al.</i> (2002) (contd)

Table 97 (contd)

Study location	Gene/protein	Total no. of cases	Tobacco (chewers/smokers)		Tobacco (without betel quid and smokers)		Alterations Codon/mutation	Reference
			Total no. of cases	No. of cases altered	Total no. of cases	No. of cases altered		
Sudan, Scandinavia, USA/UK (contd)							US/UK non-dippers 32/CGC→ACGC* ⁱ 35/GAT→TGA* (stop codon) Both from 1 case 19/CGC→CGA* ^s 20/CGC→CGT ^s 64/GCC→GTC 79/CCC→ACC* All from 1 case 95/CCT→ACT* 98/CGC→TGA (stop codon) Both from 1 case 95/CCT→TCT 98/TCA→TAA* (stop codon) Both from 1 case 31/AGC→AGA* (1 case)	Ibrahim <i>et al.</i> (2002) (contd)

OSCC, oral squamous-cell carcinoma

*Tranversions

^s, Silent mutationⁱ, Insertion leading to frameshift resulting in stop codon

mutations were seen in 5/14 (35%) cancer tissues. Ten of 14 mutations were at cytosines. TP53 expression was found in 23/62 (37%) oral squamous-cell carcinomas. TP53 inactivations that included point mutations, protein overexpression and/or presence of HPV were observed in 38/83 (46%) patients with oral cancer; 17/38 (45%) patients showed mutation/overexpression of TP53 (altered TP53) and no detectable HPV, and 21/38 (55%) were HPV 16-positive; while 13/38 (34%) HPV 16-positive patients had no mutation/overexpression of TP53 (unaltered TP53) and 8/38 (21%) HPV 16-positive patients had mutation/overexpression of TP53 (altered TP53). HPV 18 was not detected in any of the samples. [The Working Group noted that the details of tobacco chewing consumption were not mentioned in this study.]

Discordant results on TP53 immunopositivity, assessed by immunohistochemistry, have been reported in studies of snuff-induced oral lesions. Low expression levels of p53 protein were reported by Ibrahim *et al.* (1996) and Merne *et al.* (2002), while Wood *et al.* (1994) and Wedenberg *et al.* (1996) observed elevated expression of p53 in snuff-induced lesions. The accumulation of p53 protein in leukoplakia of snuff users was higher than that in mucosa that appeared normal from snuff users or from non-user controls (Wood *et al.*, 1994).

Oral squamous-cell carcinomas from Sudanese snuff dippers showed p53 expression in 3/14 (21%) patients while 9/14 (64%), 39/60 (65%) and 28/41 (68%) oral squamous-cell carcinomas from non-snuff users from Sudan, Sweden and Norway expressed p53 protein, respectively (Ibrahim *et al.*, 1996). [The non-user patients from Sudan did not use any other form of tobacco and only eight patients from Sweden and 11 patients from Norway reported cigarette smoking. No data on alcoholic beverage consumption were available.]

In a Swedish study, Schildt *et al.* (2003) reported p53 mutations in 41/114 (36%) oral squamous-cell carcinomas, 34 of which were localized in exon 8, and p53 protein expression in 72/114 (63%) tumours. No clear pattern in relation to the expression of the biological markers p53, PCNA, Ki-67 and bcl-2 emerged in oral squamous-cell carcinomas from snuff users; however, very few snuff users (12) were included in this study.

Studies on genetic alterations in other cancer-related genes in users of smokeless tobacco are summarized in Table 97 and Figure 9.

(ii) p21^{WAF1} and S100A4

Mutations in exon 2 of p21^{WAF1}, the cyclin-dependent kinase inhibitor gene, were found in oral squamous-cell carcinomas in 6/14 (43%) *toombak* users compared with 13/76 (17%) non-users of snuff, the majority of whom were smokers (Ibrahim *et al.*, 2002). Nucleotide changes differed in *toombak* dippers [codons 10, 30, 68 and 83 in 3/14 (21%) cases] versus non-users of snuff [codons 19, 20, 92 and 140 in 6/76 (8%) cases]. These differences were not statistically significant. In the appropriate oral squamous-cell carcinomas, the presence of p21^{WAF1} exon 2 mutation coincided with the detection of a mutation in the TP53 gene exon 5 to 9 (Ibrahim *et al.*, 1999) (see Table 96).

Mutations in the metastasis-inducing S100A4 gene, a member of the S100 family of the calcium binding proteins, were found in three oral squamous-cell carcinomas (one

from a *toombak* dipper and two from non-users of snuff). The oral squamous-cell carcinoma from the *toombak* dipper had four mutations (one transition, three transversions), while those from non-users of snuff showed three mutations each (one transition, two transversions). All of these three cases were negative for mutations in *p21^{WAF1}* and *TP53* genes (Ibrahim *et al.*, 2002). No mutations were found in the non-malignant oral lesions from snuff-dippers/non-users. No correlation was found between *S100A4* mutations and *p21^{WAF1}* and/or *TP53* mutations. [The Working Group noted that the sample size in this study was small and that information on alcohol drinking was unavailable.]

(iii) *Adenomatous polyposis coli (APC) and mutated in colon cancer (MCC) genes*

Loss of heterozygosity (LOH) at *APC* and *MCC* genes was studied in 40 oral squamous-cell carcinomas and 57 leukoplakia patients from eastern India (Sikdar *et al.*, 2003). Among the oral squamous-cell carcinomas, 58% were tobacco chewers, while only 10% of leukoplakia patients were tobacco chewers. Four of 16 (25%) oral squamous-cell carcinomas, three of which were from tobacco chewers, and 1/29 (3%) leukoplakias, also from a tobacco chewer, demonstrated LOH at *APC* and were positive for HPV 16. None of the oral squamous-cell carcinomas or leukoplakias showed any LOH at the *MCC* gene.

(iv) *p16, death-associated protein kinase (DAPK), MGMT and GSTP1 genes*

Kulkarni and Saranath (2004) studied concurrent hypermethylation of tumour-suppressor gene *p16*, *DAPK*, *MGMT* and *GSTP1* genes in 60 primary oral tumours from habitual tobacco chewers and corresponding adjacent clinically and histopathologically normal mucosa as well as buccal epithelial scrapings from normal mucosa of 20 healthy non-users of tobacco. Fifty-two of 60 (86.7%) oral squamous-cell carcinomas and 46/60 (76.7%) adjacent mucosa showed hypermethylation in the promoter regions of *p16*, *MGMT* and *DAPK* genes. However, none of the tissues analysed showed promoter hypermethylation in the *GSTP1* gene. None of the tissues from tobacco non-user controls showed any hypermethylation. Promoter hypermethylation was observed in 40/60 (66.7%), 41/60 (68.3%) and 31/60 (51.7%) oral squamous-cell carcinomas in *p16*, *DAPK* and *MGMT* genes, respectively. Among the samples of adjacent mucosa analysed, 30/60 (50%), 36/60 (60%) and 16/60 (26.7%) tissues demonstrated promoter hypermethylation in *p16*, *DAPK* and *MGMT* genes, respectively. Thus the percentage of hypermethylation of *p16* and *MGMT* genes was significantly higher in tumour tissues than in corresponding adjacent mucosa. The *DAPK* methylation profile in both kinds of tissue was similar. No correlation could be established between hypermethylation of these genes and clinico-pathological parameters in patients.

(v) *BAX/BCL2/Ki-67*

Loro *et al.* (2000) reported a higher rate of apoptosis and a higher expression of BAX in oral squamous-cell carcinomas from Norway compared with those from Sudan. No

significant differences were observed in apoptosis, BAX, BCL-2 or Ki-67 in oral squamous-cell carcinomas from Sudan in relation to *toombak* use or *TP53* gene status.

(c) *Polymorphisms in carcinogen-metabolizing enzymes*

Several isozymes of CYP, e.g. CYP1A1, CYP2D6, CYP2E1, CYP2A6 and CYP2A13, are involved in the metabolic activation of the carcinogens present in smokeless tobacco — TSNA (major class) and PAHs (in some products). The initial steps usually carried out by CYP enzymes involve oxygenation of the carcinogens. Other enzymes such as lipoxygenases, cyclooxygenases, myeloperoxidase and monoamine oxidases may also be involved, but less commonly. When the oxygenated intermediates formed in these initial reactions are electrophilic, they may react with DNA or other macromolecules to form covalent binding adducts. Alternatively, these metabolites may undergo further transformations that are catalysed by GST, uridine-5-diphosphate-glucuronosyltransferases, epoxide hydrolase, *N*-acetyltransferases, sulfotransferases and other enzymes. Such reactions frequently, but not always, result in detoxification. The balance between activation and detoxification can affect the development of cancer. Numerous alleles that cause extinguished, defective, qualitatively altered, diminished or enhanced rates of metabolism have been identified for several phase I and phase II enzymes and can result in marked interindividual differences in carcinogen metabolism (reviewed in Vineis *et al.*, 1999; Nair & Bartsch, 2001; Wu *et al.*, 2004). An interaction between genetic polymorphisms and smokeless tobacco in the causation of cancer in humans is plausible: possible mechanisms of interaction include the activation or detoxification of carcinogens present in the tobacco. However, the effects are probably complex as frequently simultaneous exposure to smokeless tobacco with cigarette smoking and/or alcoholic beverage consumption occurs.

The GSTs are a large family of enzymes that protect DNA against damage and adduct formation by the conjugation of GSH to electrophilic substances, which predominantly creates hydrophilic, less reactive metabolites that can be excreted. Several GST families (alpha, mu, pi, theta) exist and show genetic polymorphisms associated with large variations in enzyme activities (Hayes & Pulford, 1995).

The impact of *GST* genotypes on the risks for pre-cancer and cancer have been investigated in Indian users of smokeless tobacco quids.

The influence of *GSTM1* and *GSTT1* genotypes on risk for oral leukoplakia was investigated using genomic DNA from biopsies from 98 oral leukoplakia patients and exfoliated cells from 82 healthy controls from India (Nair *et al.*, 1999). Most cases of leukoplakia were heavy chewers (15–20 quids of tobacco with or without betel quid per day), whereas the chewers among controls were regular but light chewers (1–2 quids per day). A significantly increased risk for oral leukoplakia was associated with *GSTM1* null (odds ratio, 22; 95% CI, 10–47) or *GSTT1* null (odds ratio, 11; 95% CI, 5–22) genotypes. Combined null genotypes of *GSTM1* and *GSTT1* prevailed in 60.2% of the cases but none were detected in controls.

The impact of *GSTM1* null genotype on the risk for oral cancer was also analysed in separate groups of individuals from India who used different types of tobacco (297 cancer patients and 450 healthy controls). The odds ratios associated with *GSTM1* null genotype was 3.7 (95% CI, 2.0–7.1) in chewers of tobacco with lime or with betel quid. Increased lifetime exposure to tobacco chewing was associated with a twofold increase in the risk for oral cancer in *GSTM1* null individuals. The risk for cancer associated with *GSTM1* null genotype increased from 2.5 (95% CI, 0.9–7.1) among chewers with less than median lifetime exposure to 4.6 (95% CI, 1.9–11.4) in chewers with more than median lifetime exposure (Buch *et al.*, 2002). [The gene–environment interaction was not estimated for chewing betel quid with or without tobacco.]

In a study of 211 cases of oral cancer and 160 controls from India, no significant differences in the distributions of *GSTM1*, *GSTM3* or *GSTT1* genotypes was observed between cases and controls. However, an increased risk for oral cancer was reported for heavy chewers with the *GSTT1* null genotype (odds ratio, 3.0; 95% CI, 1.0–9.8) and for light chewers who had *GSTP1 ile/ile* at codon105 (odds ratio, 2.0; 95% CI, 1.0–3.7) (Sikdar *et al.*, 2004). [The Working Group noted that 30% of chewers also smoked tobacco.]

Few studies are available that looked specifically into host genotype and exposure to smokeless tobacco, and were too small to provide clear evidence. Carefully designed studies of sufficient size targeted specifically on smokeless tobacco users need to be conducted to enable a clear picture of the relationship between genotype, smokeless tobacco use and cancer risk to be formed. At present, due to the incomplete nature of the literature, firm conclusions cannot be drawn regarding the modulating effects of polymorphisms on smokeless tobacco-associated cancers.

4.4.2 *Experimental systems*

(a) *Mutagenicity and genotoxicity of various types of smokeless tobacco*

(i) *In-vivo studies*

Urine samples were collected from groups of Sprague-Dawley rats that were maintained on semi-synthetic diets sufficient or deficient in vitamin A, B complex or protein. Each dietary group was exposed to tobacco extract, *mishri* extract, benzo[*a*]pyrene, NNN or NDEA (see Section 4.1.2). Urine was tested for mutagenic activity using the *Salmonella*/microsome (metabolic activation) assay. Higher mutagenic activity of urine was observed in all exposed groups. The order of mutagenicity of all treatments was deficient diet > standard laboratory diet > nutritionally sufficient diet (Ammigan *et al.*, 1990c).

As noted in Section 4.2.2(a), a change of ploidy status has been shown in buccal epithelial cells of rats treated with smokeless tobacco by application to the buccal mucosa weekly for 1 year: 25% of buccal epithelial cells were tetraploid and 5% were octaploid which suggests that the mitotic process could be altered (Chen, 1989).

[These results are relevant because ploidy status has been reported (Sudbo *et al.*, 2001) to be a significant marker for dysplasia with potential for malignant transformation.]

(ii) *In-vitro studies*

Hannan *et al.* (1986) showed the mutagenic potential of *shammah* (Yemeni snuff used in Yemen and some parts of Saudi Arabia) by use of the *S. typhimurium*/microsome mutagenicity assay (Ames test), aberrant colony formation and mitogenic gene conversion in yeast, tryptophan gene conversion in the D7 diploid strain of *Saccharomyces cerevisiae* and in-vitro oncogenic transformation in C3H mouse embryo 10T1/2 cells.

Jansson *et al.* (1991) investigated the genotoxicity of aqueous and methylene chloride extracts of Swedish moist oral snuff. Methylene chloride extract contained much more nicotine (9.1 mg/mL) than the aqueous extract (2.4 mg/mL). The aqueous extract was found to induce sister chromatid exchange in human lymphocytes *in vitro* and chromosomal aberrations in V79 Chinese hamster ovary cells *in vitro* (both with and without metabolic activation). However, no induction of mutation was observed in *Salmonella* or V79 cells, and no micronuclei were found in mouse bone marrow cells. The authors speculated that the induction of chromosomal aberrations without metabolic activation may have been due to a high concentration of salt in the extract, and that the potentially clastogenic agent(s) in the extract required metabolic activation. Methylene chloride extract gave positive results in the Ames test, and induced chromosomal aberrations and sister chromatid exchange (only in the presence of a metabolic activation system). However, no induction of mutation was observed in V79 Chinese hamster ovary cells. The in-vivo administration of methylene chloride extract did not cause micronucleus formation in mouse bone marrow cells or sex-linked recessive lethal mutations in *Drosophila melanogaster*.

The mutagenicity was determined of extracts of two leading brands of American chewing tobacco, treated with or without sodium nitrite under acidic conditions. Mutagenic activity was found only for nitrite-treated extract in *S. typhimurium* tester strains TA98 and TA100, independently of metabolic activation (Whong *et al.*, 1985). However, in a previous study, these authors had also reported the mutagenic activity of tobacco snuff treated under acidic conditions in *Salmonella* with and without a microsomal activation system (Whong *et al.*, 1984).

The mutagenic potential of an aqueous extract of *mishri*, chewing tobacco alone and a mixture of chewing tobacco with lime was tested in the *Salmonella* mutagenicity assay. *Mishri* extract was mutagenic in strain TA98 with metabolic activation by Arochlor 1254-induced rat liver microsomes. A nitrosated aqueous extract of *mishri* was mutagenic in strains TA100 and TA102. While the aqueous extract of 'Pandharpuri' — a brand of chewing tobacco — was mutagenic to TA98 and TA100 with nitrosation only, the aqueous extract of the same tobacco with lime was directly mutagenic in strains TA98, TA100 and TA102 (Niphodkar *et al.*, 1996).

Aqueous extracts of two commercial brands of smokeless tobacco were found to be clearly mutagenic (dose range, 1–3 mg/mL extractable solids) in human lymphoblastoid TK-6 cells, which do not express CYP. In human lymphoblastoid AHH-1 cells, which constitutively express CYP1A1, a similar result was found for both products tested. The mutagenicity of both extracts in TK-6 and AHH-1 cells was markedly decreased by treatment at neutral pH with sodium nitrite (0.25 mM) or by acid treatment (2 h; pH 3.0).

Since these two cell lines were found to be equally sensitive to the induction of mutation by aqueous tobacco extracts, the authors concluded that these brands of tobacco contain precursors for the formation of mutagens, the activity of which is not CYP-mediated (Shirnamé-Moré, 1991).

(b) *Effects on p53 and other genes*

Bagchi *et al.* (2001) demonstrated that treatment of human oral keratinocytes with smokeless tobacco extract (200 mg/mL) resulted in increased *TP53* mRNA expression and decreased *Bcl-2* mRNA expression. At higher concentrations, the expression of *TP53* mRNA decreased confirming an increase in apoptotic cell death as reported earlier (Bagchi *et al.*, 1999).

The human oral squamous carcinoma cell line (Amos III) established from a smokeless tobacco consumer was shown to harbour a *TP53* mutation (an insertion of C at nucleotide 13197, resulting in a termination codon at 180) and accumulation of p53 protein. Accumulation of cyclin D1, bcl-2, p21^{waf1} and mdm2 and no detectable expression of p16, pRb and RAR β proteins suggested deregulation of cell cycle and apoptotic pathways (Kaur & Ralhan, 2003).

(c) *Effect on viruses*

Smokeless tobacco extracts and TSNA have been shown to enhance cell transformation by HSV-1 (Park *et al.*, 1991), increase cell longevity (Murray *et al.*, 1993) and to inhibit replication of virus in the oral cavity (Hirsch *et al.*, 1984b; Park *et al.*, 1988). Smokeless tobacco extracts also increased survival of ultraviolet-irradiated HSV in monkey kidney CV-1 cell cultures (Dokko *et al.*, 1991).

Smokeless tobacco extracts did not activate latent Epstein-Barr virus into lytic replication (Jenson *et al.*, 1999a), but decreased the rate of lymphocyte population doubling (Jenson *et al.*, 1999b).

The inhibitory effects of snuff extract and the TSNA NNN and NNK on HSV-1 replication *in vitro* and on HSV-1 protein synthesis in infected cells were analysed. Addition of snuff extract after adsorption of the virus to cell membranes resulted in significantly reduced production of the virus at low multiplicities of infection, but, at high multiplicities of infection the inhibitory effect was less pronounced. Smokeless tobacco extracts increased the production of immediate-early (α) infected cell proteins (ICPs) 4 and 27, and early (β) ICPs 6 and 8 and decreased production of late (γ) ICPs 5, 11 and 29. Snuff extract has been proposed to block the replicative cycle of HSV at an early stage, which results in enhanced production of early ICPs and in prolonged maintenance of cellular functions that may be important for HSV-induced cell transformation (Larsson *et al.*, 1992).

Demirci *et al.* (2000) showed that smokeless tobacco extracts modulate exogenous reporter gene expression under control of the cytomegalovirus immediate enhancer and promoter in early passage transfected and cultured human oral epithelial cells, which suggests an influence of smokeless tobacco extracts on viral enhancers/promoters.

4.5 Mechanistic considerations

TSNA such as NNK and NNN are the most abundant strong carcinogens in smokeless tobacco products. Data on biomarkers such as measurements of NNK and NNN in the saliva and of the NNK metabolites NNAL and NNAL-Gluc in urine clearly demonstrate the uptake and metabolism of these carcinogens in smokeless tobacco users. The levels reported are sometimes extraordinarily high; for example, *toombak* users represent the highest reported human exposure to a non-occupational *N*-nitrosamine carcinogen. NNK and NNN are metabolically activated by α -hydroxylation in rodent and human tissues. This process leads to DNA and haemoglobin adducts. The DNA adducts are critical in the carcinogenesis of NNK and NNN. The haemoglobin adducts of NNK and/or NNN have been detected in the blood of smokeless tobacco users in three studies, which demonstrates the metabolic activation of NNK and/or NNN in these humans. Although NNK and/or NNN DNA adducts have been detected in tissues of smokers, no studies have been reported in smokeless tobacco users.

Elevated micronucleus formation and chromosome breaks have been reported in oral exfoliated cells in smokeless tobacco users. The frequency of cytogenetic alterations was significantly elevated in peripheral blood lymphocytes in smokeless tobacco users compared with those of non-users.

Smokeless tobacco-associated oral premalignant and malignant lesions have been shown to harbour mutations in various genes that play a pivotal role in carcinogenesis. Accumulation of p53 protein and *TP53* mutations G:C→A:T, C:G→T:A and G:C→T:A have been reported in oral squamous-cell carcinomas from *toombak* dippers and tobacco chewers. A high incidence of G→T and G→A mutations has been observed in oral squamous-cell carcinomas from a tobacco-chewing Indian population. Inactivation of the *p16* tumour-suppressor gene and of *MGMT* and *DAPK* by promoter hypermethylation has been observed in oral carcinomas from tobacco chewers. Aberrant levels of expression of bcl-2 and bax, as well as mutations in the *p21^{waf1}* gene have been observed in oral cancers in chewers.

In summary, multiple features of the carcinogenic process have been observed to occur *in vitro* and *in situ* in the oral cavity of smokeless tobacco chewers and in experimental animals treated with smokeless tobacco. Collectively, the available data on biomarkers provide convincing evidence that carcinogen uptake, activation and binding to cellular macromolecules are higher in smokeless tobacco users than in non-users. Smokeless tobacco is genotoxic in humans and in experimental animals. Tumours of smokeless tobacco users contain mutations in oncogenes and tumour-suppressor genes. Most of the genetic effects seen in smokeless tobacco users are also observed in cultured cells or in experimental animals exposed to smokeless tobacco.