APPENDIX A6.1.3.13 4-(METHYLNITROSAMINO)-1-(3-PYRIDYL)-1-BUTANONE (NNK) - FACTSHEET

	HPHC	BoExp assessed by PMI (matrix)
IUPAC name	N-methyl-N-(4-oxo-4-pyridin-3-ylbutyl)nitrous amide	Total 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol (in urine)
Usual abbreviation	NNK	Total NNAL
Other names	4-(Methylnitrosamino)-1-(3-pyridyl)-1- butanone , 4-(N-methyl-N-nitrosamino)-1-(3-pyridyl)-1-butanone, 4-(methylnitrosoamino)-1-(3-pyridinyl)-4-(N-nitroso-N-methylamino)-1-(3-pyridyl)-1-butanone, nicotine-derived nitrosamine ketone	N-(4-hydroxy-4-pyridine-3-yl-butyl)-N-methylnitrous amide (IUPAC); CCRIS 3049, 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanol, 4-[methyl(nitroso)amino]-1-pyridine-3-yl-butane-1-ol, alpha-(3-(methylnitrosoamino)propyl)-3-pyridine-methanol, nicotine-derived nitrosamine alcohol
CAS number	64091-91-4	76014-81-8
Formula and molecular weight	$C_{10}H_{13}N_3O_2$ MW: 207.23	$C_{10}H_{15}N_3O_2$ MW: 209.24

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Main characteristics

Chemical class	N-nitrosamine (also classified as non-volatile tobacco specific nitrosamine (TSNA)) [1, 2]
HPHC lists	FDA-93 [3]; FDA-18 [4]; HC [5, 6], PMI-58; WHO-39 [7]; WHO-18 [8]; WHO-9 [8];
Source in cigarettes	Amount in MSS (3R4F): 85.5 ng (ISO); 243 (HCI) [9] Smoke phase: particulate phase [10] Presence in tobacco / pyrosynthesis (T°) [1]: Tobacco-specific N-nitrosamines, including 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), N'-nitrosonornicotine (NNN), N'-nitrosoanabasine (NAB) and N'-nitrosoanatabine (NAT), occur widely in tobacco and tobacco smoke. They are formed by the nitrosation of nicotine and other tobacco alkaloids and have been detected in green tobacco leaves from Nicotiana tabacum and N. rustica species; however, the largest quantities of tobacco-specific N-nitrosamines are formed during tobacco curing [11] and processing and additional amounts are formed during smoking. They are mainly transferred from the tobacco to smoke during smoking process [12]. Tobacco-specific N-nitrosamines occur in all commercially and non-commercially prepared tobacco products including cigarettes, cigars, bidis, pipe tobacco and smokeless tobacco products [13, 14]. Because most of the TSNA yield in smoke comes from unchanged transfer from the tobacco by distillation with little contribution by pyrolysis [15, 16], they are dependent on their respective contents in the cigarette tobacco. Thus, the wide disparity in levels of TSNA, including NNK, in the mainstream smoke of cigarettes and in smokeless tobacco products [1] is largely due to differences in the tobacco types used in any given product, agricultural practices, curing methods and manufacturing processes. The majority of NNK is formed from the tertiary amine nicotine at the later stages of tobacco curing and fermentation.
Other sources [1]	N-Nitrosamines occur in a wide variety of both food and non-food products, but the amounts of tobacco-specific N-nitrosamines including NNK in all tobacco products exceed the levels of other N-nitrosamines in other commercial products by several orders of magnitude [1]. The degree of exposure to

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	tobacco-specific N-nitrosamines depends not only on the levels of these compounds in tobacco products or smoke, but also on the manner in which the tobacco products are used. [13]. In addition to exposure by secondhand smoke, NNK is present on surfaces in most homes occupied by smokers (thirdhand smoke), indicating a potential hazard in homes that have been occupied by smokers [17].
Toxicity class (FDA) [3]	CA
Toxicity – CA	IARC: group 1: carcinogenic to humans [18] NNK is reasonably anticipated to be a human carcinogen based on sufficient evidence of carcinogenicity in experimental animals [13]. Epidemiological studies of tobacco users, both smoked and smokeless, support a plausible association between exposure to NNK and cancer [1] and demonstrate an association of NNK metabolites with lung cancer in smokers. NNK has a remarkable specificity for tumorigenesis of the lung, inducing predominantly adenocarcinomas of the lung of mice, rats, hamsters, and mink, independent of the route of administration [18]. It is, however, impossible to exclude the potential confounding effect of other carcinogens present in tobacco smoke [18]. NNK has recently been reported to be associated with lung cancer risk, independently of smoking intensity and duration, together with cotinine plus its glucuronide and phenanthrene-tetrol [19].
Toxicity – RT	-
Toxicity – CT	-
Toxicity – RDT	-
Toxicity – AD	-
Toxicity - others	All available toxicity data can be found on the Website Aggregated Computational Toxicology Resource (ACToR) [20].

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ADME properties

NNK in tobacco, like other TSNAs, is primarily formed after harvesting of the leaves during drying, curing, aging, and especially during fermentation [21], and is formed from nitrosation of tobacco alkaloids (Figure 1).

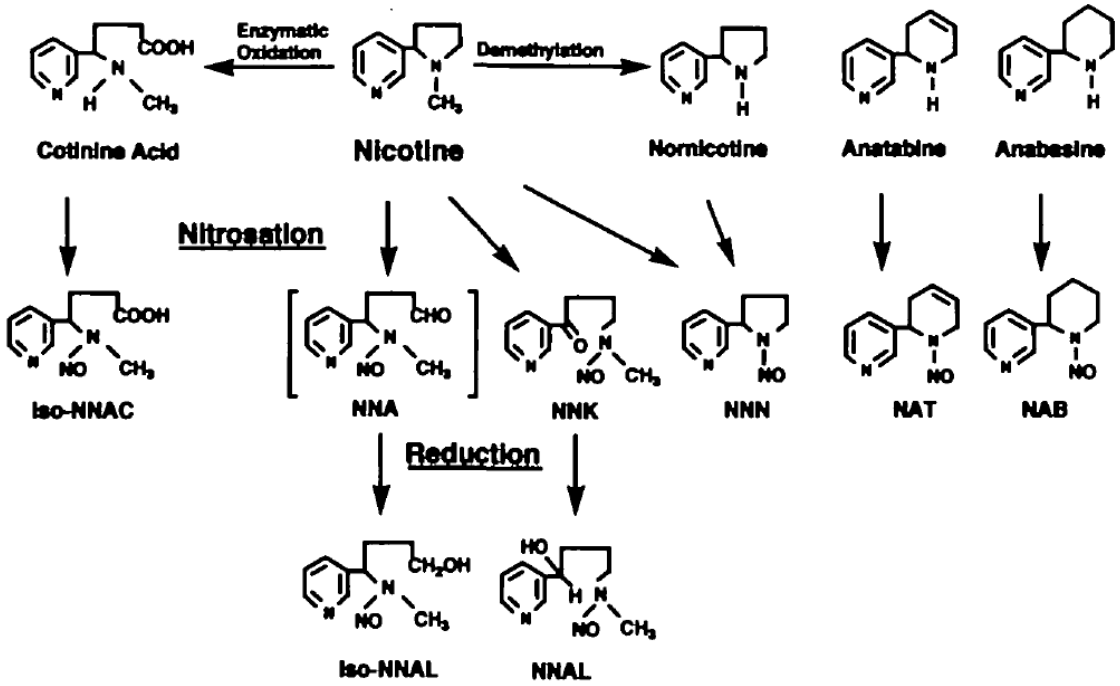


Figure 1 **Formation of tobacco-specific N-nitrosamines by nitrosation of tobacco alkaloids during the curing and processing of tobacco [22]**

Iso-NNAC = 4-(methylnitrosamino)-4-(3-pyridyl)butyric acid; NNA = 4-(methylnitrosamino)-4-(3-pyridyl)butanal; NNK = 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN = N'-nitrosonornicotine; NAT = N'-nitrosoanatabine; NAB = N'-nitrosoanabasine; Iso-NNAL= 4-(methylnitrosamino)-4-(3-pyridyl)-1-butanol; NNAL = 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol.

Figure by Hoffmann et al., J Natl Cancer Inst. 1995; 87(24):1862-9. Published with permission of Oxford University Press. Copyright © 2016 Oxford University Press.

Absorption and distribution - Absorption of NNK in tobacco product users is demonstrated by the detection of its metabolites, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) and NNAL-glucuronide (Gluc), in plasma and urine and of NNAL-N-oxide in urine [1, 23]. Although the rapid absorption and distribution of NNK and its metabolites ensures widespread and extensive distal binding in all tissues, first-pass metabolism and activation of NNK in the airway mucosa is sufficiently rapid to cause levels of binding at the site of absorption to be approximately 20-fold those of distal tissues. NNK may thus act as a site-of-entry carcinogen [24]. Systemic metabolism is also rapid, as the parent compound NNK disappears from the systemic circulation within 18 min of instillation and no NNK is found in the trachea or any distal tissue 45 min after instillation [24].

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Metabolism [1, 23]

Metabolic activation: Metabolism of NNK (Figure 2; structure 1) is complex, and metabolic pathways include carbonyl reduction of NNK to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL; Figure 2; structure 2), glucuronidation of NNAL, α-hydroxylation of NNK and NNAL, and pyridine N-oxidation of NNK and NNAL. The major metabolic pathway for NNK in most tissues is carbonyl reduction to NNAL [25]. NNK and NNAL are procarcinogens that require metabolic activation by cytochrome P450 (CYP) enzymes to express their biological activity. α-Hydroxylation of the methylene carbon atoms adjacent to the N-nitroso group is believed to result in metabolic activation of NNK and NNAL since the unstable intermediates produced decompose to methanediazohydroxide, which can methylate DNA and other cellular macromolecules [26],, which is further hydrolyzed to the corresponding acids, 4-(3-pyridyl)-4-oxobutanoic acid (keto acid; Figure 2; structure 4) and 4-(3-pyridyl)-4-hydroxybutanoic acid (hydroxy acid; Figure 2; structure 5).

Kinetic parameters (V_{max} or K_m) for NNK metabolism mediated by microsomal preparations from tissues of laboratory animals and the relevant CYP enzymes have been reported [1]. In rat, mice and monkeys, a number of CYP2A enzymes are excellent catalysts of NNK α-hydroxylation, but more research is needed to clarify the contribution of individual CYPs to microsome-mediated NNK metabolism in animals.

By a similar process, α-hydroxylation of the methyl group of NNK and NNAL yields unstable intermediates that decompose to formaldehyde, and to either 4-(3-pyridyl)-4-oxobutanediazohydroxide or 4-(3-pyridyl)-4-hydroxybutane-diazohydroxide, which can react with DNA to yield various adducts both in vitro [26] and in vivo [27]. The NNK-specific reactive intermediate, 4-(3-pyridyl)-4-oxobutanediazohydroxide, can pyridyloxobutylate cellular macromolecules, resulting in the formation of HPB-releasing adducts [28-31], or is hydrolyzed to 4-hydroxy-1-(3-pyridyl)-1-butanone (keto alcohol; Figure 2, structure 3, also referred to as HPB). HPB can also be further oxidized to keto acid. The reactive intermediate resulting from α-methyl hydroxylation of NNAL has the potential to pyridylhydroxybutylate DNA [30] or is hydrolysed to 4-(3-pyridyl)butane-1,4-diol (diol; Figure 2, structure 5), which can be further oxidized to hydroxy acid (Figure 2, structure 6) [32].

NNAL has a chiral center and, therefore, exists as a pair of enantiomers, (S)-NNAL and (R)-NNAL [33]. The enantiomeric ratio of metabolically formed NNAL and its glucuronide derivative may be species dependent [33]. Both (R)-NNAL and (S)-NNAL are formed in smokers and the enantiomeric distribution in smokers' urine is about 54% (R)- and 46% (S)-NNAL, while that of the glucuronide conjugate is about 68% (R)-NNAL-Gluc and 32% (S)-NNAL-Gluc [34]. Enantiomers frequently differ in their biological effects. (R)-NNAL was demonstrated to be more tumorigenic than (S)-NNAL in A/J mouse lungs; while the tumorigenicity of (R)-NNAL was the same as that of NNK [35]. The NNAL enantiomers are further metabolized to the corresponding diastereomeric glucuronides (S)-NNALGluc and (R)-NNAL-Gluc. (S)-NNAL-Gluc is not tumorigenic in mice [35].

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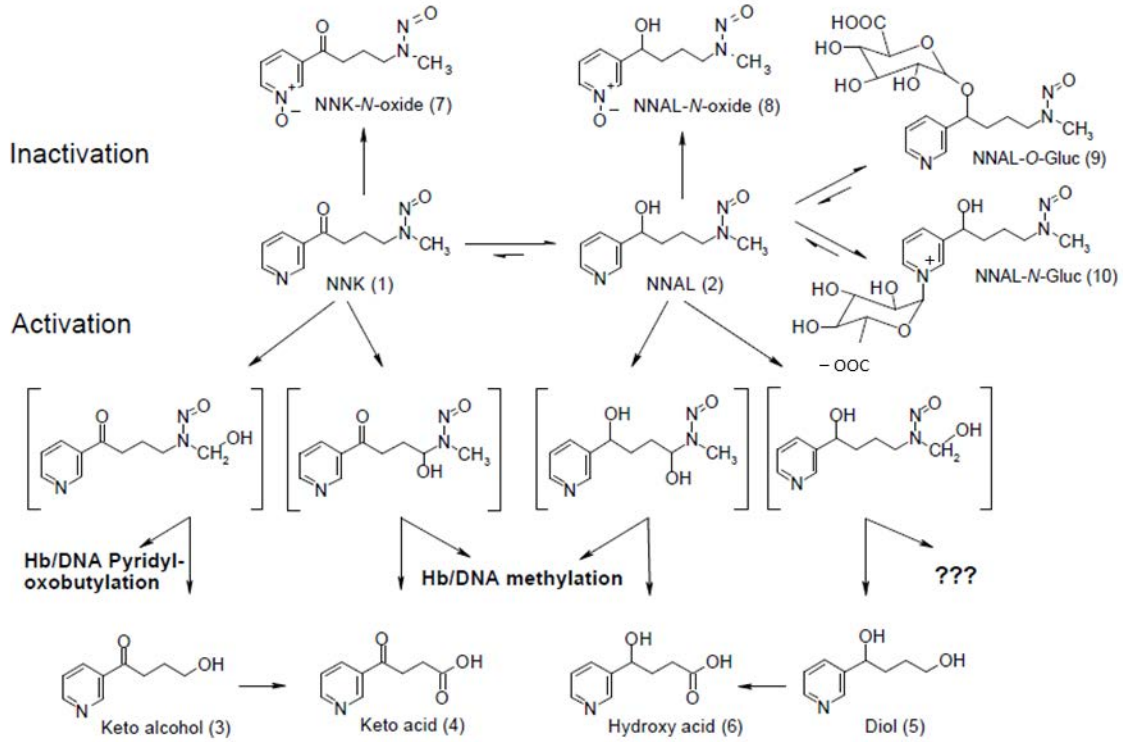


Figure 2 **Major pathways of metabolism of NNK in laboratory animals and man. Structures in brackets represent hypothetical reactive intermediates. Adapted from Richter et al. [36]**

Structure 10 was modified from the published figure as follows: a positive charge (+) was added on the pyridine-N and a negative charge (-) on the carboxyl-group of the glucuronic acid (COO-).

Figure by Richter and Tricker, Mol Nutr Food Res. 2008; 52(1):7-25. Published with permission of John Wiley & Sons, Inc. Copyright © 2016 Elsevier B.V. or its licensors or contributors.

In man, the major pathway for NNK metabolism is reduction to NNAL (>95% of total metabolism) in most tissues, including lung and freshly isolated lung cells [37-39]. Similar observations have been reported for NNK metabolism in microsome preparations from various human tissues [35, 40, 41]. Pulmonary metabolism of NNK is cell-specific, including in man [38], and their profiles differ between lung microsomes and lung tissue explants from the same donor nonhuman primate [42].

Although the major pathways of metabolism observed in laboratory animal are qualitatively similar to those observed in man, the metabolism of NNK by α-hydroxylation is considerably lower in human lung as compared to that observed in rodent species, suggesting that extrapolation of in vitro rodent data to man may result in invalid conclusions about the capacity of the human lung to activate NNK under realistic conditions of NNK exposure [39].

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Detoxification: Detoxification of NNK occurs by glucuronidation of NNAL to yield 4-(methylnitrosamino)-1-(3-pyridyl)-1-(O-β-D-glucopyranuronosyl)butane (NNAL-O-Gluc; Figure 2, structure 9) [32, 43-45] and, to a lesser extent, 4-(methylnitrosamino)-1-(3-pyridyl-N-β-D-glucopyranuronosyl)-1-butanolonium inner salt (NNAL-N-Gluc; Figure 2, structure 10) [46]. Glucuronidation of 4-(hydroxymethylnitrosamino)-1-(3-pyridyl)-1-butanone, the putative reactive intermediate formed by α-hydroxylation of the methyl carbon of NNK, has also been reported [47-49]. Pyridine N-oxidation of NNK and NNAL to yield 4-(methylnitrosamino)-1-(3-pyridyl-N-oxide)-1-butanone (NNK-N-oxide, structure 7) and 4-(methylnitrosamino)-1-(3-pyridyl-N-oxide)-1-butanol (NNAL-N-oxide; Figure 2, structure 8), respectively, are considered to result in detoxification [35, 37]. Most of the above metabolic pathways have been demonstrated to occur in various rodent species [37, 40, 50] and nonhuman primates [42, 44, 51, 52]. Total NNAL corresponds to the molar sum of free NNAL plus both its O- and N-glucuronide conjugates).

Endogenous nitrosation: It has been speculated that endogenous nitrosation of nicotine may occur in vivo [53-56]. In vitro nitrosation of nicotine under near ideal conditions results only in very low yields of NNK and NNN [57]. Treatment of rats with nicotine and monitoring of NNK- and NNN-derived HPB-releasing hemoglobin adduct formation has also failed to demonstrate endogenous nitrosation of nicotine [58]. However, it has been suggested that additional pathways may exist which involve either direct oxidative nitrosation of nicotine via the formation of 4-(methylnitrosamino)-4-(3-pyridyl)butanal (NNA), or nitrosation of the major nicotine metabolite, cotinine, and its hydrolysis product, 4-(methylamino)-4-(3-pyridyl)butyric acid [59]. Both pathways would yield 4-(N-methylnitrosamino)-4-(3-pyridyl)butyric acid (iso-NNAC), a nicotine-derived N-nitrosamino acid. In an experimental study, iso-NNAC was not formed endogenously and excreted in urine of abstinent smokers and non-smokers treated orally with either nicotine or cotinine plus nitrate supplementation [60]. Kinetic considerations strongly argue against endogenous nitrosation of tertiary amines such as nicotine [61, 62] and the trace occurrence of iso-NNAC is some urine samples from smokers is thought to result from the occurrence of iso-NNAC in mainstream smoke from some commercial cigarette brands [60, 63]. There is no convincing evidence to support that endogenous formation of NNK occurs in man [64]. Studies of long-term users of nicotine replacement therapy (NRT) provide no indication that endogenous nitrosation of nicotine occurs when administered via a nicotine patch to yield either NNK [26, 65] or NNN [65].

Adducts formation: α-Hydroxylation of NNK and NNAL leads to DNA and haemoglobin adducts [1]. α-Hydroxylation of the NNK methyl group gives 4-(hydroxymethylnitrosamino)-1-(3-pyridyl)-1-butanone (α-HOMeNNK) which can be conjugated as a glucuronide, α-HOMeNNK-Gluc. α-HOMeNNK spontaneously decomposes to 4-oxo-4-(3-pyridyl)-1-butanediazohydroxide (POB-DZH) and formaldehyde. POB-DZH reacts with water to give 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB) which can be conjugated to form a glucuronide, HPB-Gluc. POB-DZH also reacts with DNA and haemoglobin to produce a series of adducts. α-Hydroxylation of the NNK methylene group produces 4-(methylnitrosamino)-1-(3-pyridyl)-1-(4-hydroxy)butanone (α-HOMethyleneNNK). This metabolite spontaneously decomposes to 4-(3-pyridyl)-4-

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oxobutanal (keto aldehyde) and methanediazohydroxide (Me-DZH). Keto aldehyde is further metabolized to 4-(3-pyridyl)-4-oxobutanoic acid (keto acid) which in turn can be converted to 4-hydroxy-4-(3-pyridyl)butanoic acid (hydroxy acid). Me-DZH reacts with water to yield methanol and with DNA to produce methyl adducts. NNAL similarly undergoes α-hydroxylation at its methylene group to yield 4-(methylnitrosamino)-1-(3-pyridyl)-1-(4-hydroxy)butanol (α-HOMethyleneNNAL) and at its methyl group to yield 4-(hydroxymethylnitrosamino)-1-(3-pyridyl)-1-butanol (α-HOMeNNAL). α-HOMethyleneNNAL spontaneously decomposes to Me-DZH and 5-(3-pyridyl)-2-hydroxytetrahydrofuran (lactol), which can be converted to hydroxy acid. α-HOMeNNAL spontaneously decomposes to 4-hydroxy-4-(3-pyridyl)-1-butanediazohydroxide (PHB-DZH) and formaldehyde. PHB-DZH reacts with water to yield 4-(3-pyridyl)butane-1,4-diol (diol), cyclizes to 2-(3-pyridyl)tetrahydrofuran (pyridylTHF) and reacts with DNA and haemoglobin to produce adducts [1].

Excretion - Urine is the only reported route of excretion of NNK metabolites for which data from studies with humans are currently available [1]. Studies in laboratory animals indicate that urine is the major route of excretion of NNK metabolites. NNAL is highly tobacco specific and seems to have one of the longest half-life of the biomarkers of exposure studied thus far, not considering DNA or hemoglobin adducts. This long half-life has an advantage over the currently most widely used biomarker, cotinine, because it allows for a time-averaged assessment of exposure, which is less dependent on sampling time with respect to when the last cigarette was smoked. Hecht et al. assessed urinary levels of NNAL in a smoking cessation study, with or without the use of nicotine patch [26]. One week after smoking cessation, 34.5% of baseline NNAL plus NNAL-Gluc was detected in urine, whereas the corresponding values for cotinine and nicotine were 1.1 and 0.5%, respectively. Even 6 weeks after cessation, 7.6% of the original levels of NNAL plus NNAL-Gluc remained. In some subjects, NNAL plus NNAL-Gluc were detected 281 days after cessation. The distribution half-life for NNAL and NNAL-Gluc was 3–4 days, whereas the elimination half-life was 40–45 days [26]. In another study, total NNAL in urine was monitored for 14 days in daily smokers after stopping smoking and for up to 60 days in occasional smokers [66]. The average half-life for the terminal phase in the daily smoker group using a two-compartmental body model was 10.3 days (beta phase), and using a non-compartmental model, it was 9.1 days. In the occasional group, these values were 17.6 and 16.0 days, respectively. The alpha-phase half-lives were 14.3 and 27.8 hours for the two groups, respectively. There was very good agreement between the plasma and urinary half-lives in two subjects with plasma analyses. The terminal half-life of NNAL of 10 to 18 days indicates that this biomarker can be used to detect tobacco smoke exposure for 6 to 12 weeks after cessation of exposure and requires a similar time to assess the steady levels of NNAL after switching from one tobacco product to another.

NNAL and its conjugated detoxification products, NNAL-N-Gluc and NNAL-O-Gluc, are the major metabolites of NNK detected in human urine, and together are designated as total NNAL [14, 46, 67, 68]. NNAL-N-oxide, another detoxification product of NNK, is also found, albeit at lower concentrations [69]. NNK-N-oxide is not found in human urine [69]. Other NNK-derived metabolites observed in the urine of laboratory animals

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are not specific to NNK metabolism in man, since they are also formed by metabolism of nicotine present in both tobacco and tobacco smoke [70-73]. Unmetabolized NNK is not detected in human urine [26] and the urinary NNK metabolite profile observed in man is very different to that found in rodents [45, 74-76] and non-human primates [44, 51, 52]. It is common to measure total NNAL to assess exposure to NNK, however, free NNAL can also be measured on its own. It was observed that after adjusting for age and gender, higher free NNAL or higher free/total NNAL can be indicative of a significantly increased risk of urothelial carcinoma, in a dose-relationship, however, this was also with higher total NNAL [77].

Exposure levels in smokers and non-smokers

Total NNAL

In non-smokers	Ranges: ND [78]
In smokers	Ranges: 1.1-2.9 nmol/24h [230-606 ng/24h] [78]

Urinary levels of total NNAL was significantly higher in cases of lung cancers than controls after adjustment for self-reported smoking intensity and duration. Urinary total NNAL (and total cotinine) was shown to be independently related to lung cancer risk [79].

Findings suggest that environmental factors (urinary NNK-related metabolites levels) may play a more important role in urothelial carcinoma than CYP2A13 or UGT2B7 gene polymorphisms [77]. However, large-scale studies or studies using tag-SNPs for genotype determination are yet missing to verify the association between the single nucleotide polymorphisms of NNK metabolism-related enzymes and cancer risk.

Urine of participant of the NHANES surveys (National Health and Nutrition Examination Survey; 1988-2008) was analysed [80].The association of NNAL concentrations with tobacco exposure was supported by the regression of total urinary NNAL concentrations on serum cotinine in the same persons from the entire population, which indicated that serum cotinine concentrations were strongly predictive of total urinary NNAL levels. The concentration difference between non-Hispanic whites and Mexican Americans was significant before and after adjustment for creatinine, whereas the NNAL concentrations in non-Hispanic Whites was only significantly higher than non-Hispanic blacks when the data were adjusted for creatinine concentrations. No significant difference was found in either adjusted or unadjusted data between non-Hispanic black and Mexican-American non-smokers. Measuring NNAL is a common assessment of exposure to NNK. NNK measurement has been found to not always be detected in the urine of smokers, making it a less reliable marker than NNAL, probably because of its rapid metabolism to NNAL and other products [81] .

NNAL and NNAL-Gluc are excreted in the urine more slowly than would be expected, based on their structures, after cessation of smoking or smokeless tobacco use (Figure 3) [26]. One week after smoking cessation, 34.5% of baseline NNAL plus NNAL-Gluc was detected in urine, whereas the corresponding values for the structurally related compounds cotinine and nicotine were 1.1 and 0.5%, respectively. Even 6 weeks after

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cessation, 7.6% of the original levels of NNAL plus NNAL-Gluc remained. The distribution half-life of NNAL and NNAL-Gluc was 3–4 days, while the elimination half-life was 40–45 days. Total body clearance of NNAL was estimated to be 61.4 ± 35.4 mL/min, and the volume of distribution in the β-phase was estimated to be 3800 ± 2100 L, which indicates substantial distribution into tissues [82]. After cessation of smokeless tobacco use, the distribution half-lives of NNAL (1.32 ± 0.85 versus 3.35 ± 1.86 days) and NNAL-Gluc (1.53 ± 1.22 versus 3.89 ± 2.43 days) were significantly shorter than those in smokers. There were no significant differences in the terminal half-lives. Ratios of (S)-NNAL:(R)-NNAL and (S)-NNAL-Gluc:(R)-NNAL-Gluc in urine were significantly (3.1–5.7 times) higher 7 days after cessation than at baseline in both smokeless tobacco users and smokers, which indicates stereo-selective retention of (S)-NNAL in humans [67]. From these results, the authors suggest that there is a receptor in the body, possibly in the lung, for (S)-NNAL [83].

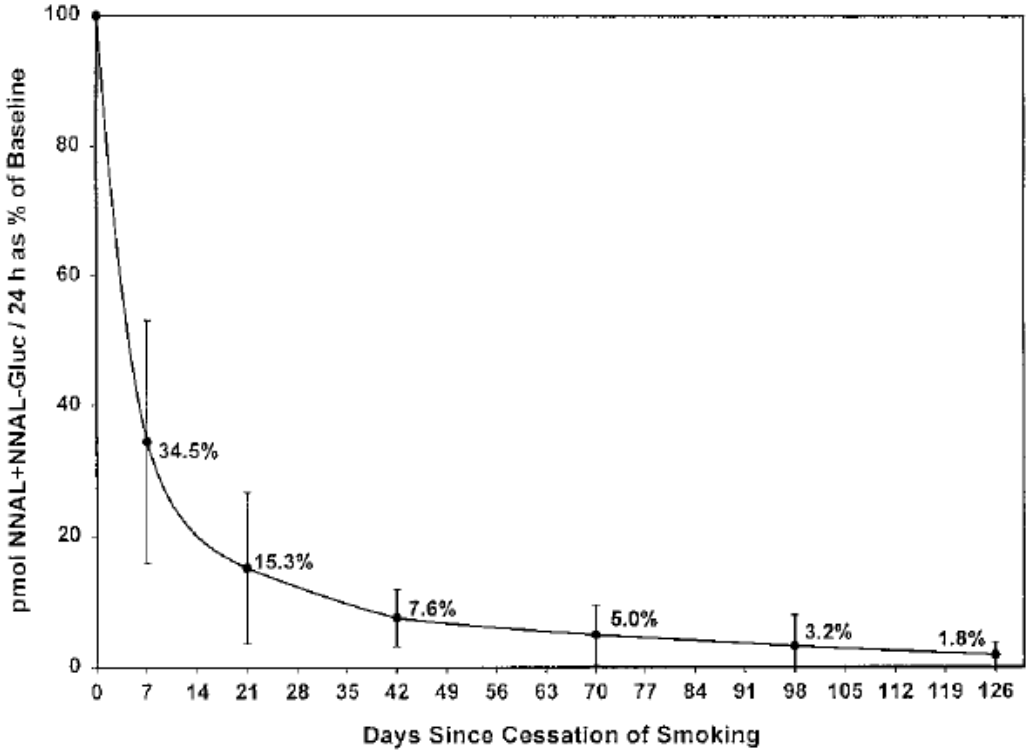


Figure 3 Mean levels of total NNAL (percentage of mean baseline levels (pmol per 24h) [26]

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NNAL-N-Gluc comprised 50 ± 25% of total NNAL-Gluc in the urine of smokers and 24 ± 12% in snuff-dippers [46]. Toombak users excreted exceptionally high levels of NNAL

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and NNAL-Gluc (0.12 to 0.14 mg per day), which demonstrated a higher uptake of NNK by humans than of any other non-occupational carcinogen [84].

CYP2A6 is one of the main catalysts of NNK α-hydroxylation. The relative efficiencies in NNK metabolism by human CYPs are (from greatest catalyst to least): 2A13 > 2B6 > 2A6 > 1A2 ~ 1A1 > 2D6 ~ 2E1 ~ 3A4

Because NNK is tobacco-specific, total NNAL is not expected to be found in non-smokers that are not exposed to secondhand smoke. A few studies were found where NNAL could be detected in the urine of non-smokers, but no speculation on the source of this NNAL was made (Table 1). Levels of total NNAL in the urine of nonsmokers exposed to secondhand tobacco smoke are typically about 1–5% of those in smokers [1]. An exhaustive review of 35 studies presenting data of urinary total NNAL in smokers has been published by Appleton et al. [85]. Their analysis of the data suggest that exposure to TSNAs has declined in smokers over the last 18 years, which seems to correlate with another observation they made, i.e. that TSNAs levels in mainstream smoke follow a downward trend since 10 years [86]. This, however, remains controversial, as others state that there is a lack of any reduction in the levels of known human carcinogens in products being sold to millions of US customers [87]. A cross-sectional study in the US showed that levels of total NNAL increased with the number of cigarettes smoked per day, but not in a linear fashion, particularly at higher amounts of cigarette consumption, where the levels seem to reach a plateau (at around 25 to 35 cigarettes per day) [88]. A recent study in which smokers consuming 20 cigarettes per day (cpd) or more were forced to smoke exactly 20, 15, 5 or 0 cpd during a 5-day confinement period showed a dose-relationship between the number of cigarettes and the levels of urinary NNAL (i.e. 624, 549, 422 and 311 ng/24h, respectively) [89]. Furthermore, a significant correlation between urinary NNAL levels and cigarette consumption was observed by Lindner et al. in European smokers, increasing with higher consumption [90].

Reduction in smoking showed significant reductions in the levels of urinary NNAL, even though reductions for most smokers were modest, with mean reductions in NNAL plus NNAL-Gluc of 29% (week 4), 33% (week 6), 37% (week 8), and 29% (week 12), when mean decreases in cigarettes per day were 53%, 74%, 75%, and 74% [91]. This can likely be explained by compensation mechanisms, such as higher inhalation volumes, or by inaccurate self-reporting of cigarette consumption. Because of the long half-life of NNAL, estimated to be between 10 and up to 45 days [26, 66], NNAL levels in smoking cessation/abstinence studies of short duration do not reach the levels of non-smokers, and decrease by around 3- to 4-fold in studies lasting between 3 to 8 days (Table 2). In a smoking cessation study lasting up to 56 days, levels of total NNAL reached levels described for non-smokers at the end of the study (i.e. 0.13 nmol/24h at day 56 vs 2.70 nmol/24h at baseline), and seemed to remain above these levels after 28 and 42 days (i.e. 0.26 and 0.2 nmol/24h, respectively) [92].

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Table 1 Representative urinary total NNAL levels in smokers and non-smokers

Author (year)	Levels in smokers	Levels in non-smokers (or ex-smokers)	Units; variability
Scherer (2007) [93]	1538 ± 1715 [321.81 ± 358.85]	NM	pmol/24h [ng/24h]; mean ± SD
Lindner (2011) [90]	189.5 ± 161.1	3.9 ± 9.4	ng/24h; mean ± SD
Mendes (2008) [94]	582.4 ± 270.0	NM	ng/24h; mean ± SD
Shepperd (2013) [95]	287 ± 164 (13-678) [60.05 ± 34.32 (2.72-141.86)]	22 ± 61 (1-411) [4.60 ± 12.76 (0.21-86)]	pmol/24h [ng/24h]; LS means ± SD (ranges)
Kavvadias (2009) [96]	204.20 ± 146.90	16.06 ± 11.99	ng/24h; mean ± SD
Lowe (2009) [97]	0.26 ± 0.10 [260 ± 100]	0.01 ± 0.01 [10 ± 10]	ng/mg creat [pg/mg creat]; mean ± SD
Lowe (2009) [97]	199.50 ± 153.90 (≤10 cpd) 362.10 ± 150.60 (≥20 cpd)	8.23 ± 6.52	ng/24h, mean ± SD
Melikian (2007) [98]	1.68 (1.41-2.0) [351.52 (295.03-418.48)]	NM	pmol/mg creat [pg/mg creat]; geometric mean (95% CI)
Mendes (2008) [94]	595.0 ± 279.9	NM	ng/24 h; mean ± SD
Sarkar (2008) [99]	320.68 ± 185.62	NM	ng/g creat [pg/mg creat]; mean ± SD
Strasser (2011) [100]	1.47 ± 0.79 [307.58 ± 165.30]	NM	pmol/mg creat [pg/mg creat];mean ± SD
Chung (2013) [77]	0.41 ± 0.09 [410 ± 90]	0.40 ± 0.07 [400 ± 70]	µg/g creat [pg/mg creat]; mean ± SD
Hecht (1999) [26]	0.601 ± 0.366	0.016 ± 0.029	pmol/ml; mean + SD
Sarkar (2008) [99]	320.68 ± 185.62	NM	ng/g creat [pg/mg creat]; mean ± SD
Lee (2008) [101]	3.16 ± 0.99 [3160 ± 990]	1.49 ± 0.28 [1490 ± 280]	µg/g creat [pg/mg creat]; mean ± SE
Calapai (2009) [102]	89.62 (61.03-131-59) [a]	6.13 (4.78-7.87)	ng/g creat [pg/mg creat]; mean (95% CI)
Calapai (2009) [102]	191.69 (143.65-255.79) [b]	6.13 (4.78-7.87)	ng/g creat [pg/mg creat]; mean (95% CI)
Morin (2011) [103]	213 (178-248) [c]	10 (9-11) [o]	ng/24h; mean (95% CI)
Morin (2011) [103]	176 (147-204) [d]	10 (9-11) [o]	ng/24h; mean (95% CI)
Morin (2011) [103]	252 (220-284) [e]	10 (9-11) [o]	ng/24h; mean (95% CI)
Hou (2012) [104]	12.25 (1.10-58.10) [f]	3.19 (0-17.29)	µg/mol creat; mean (range)
Hou (2012) [104]	12.74 (1.28-60.22) [g]	3.19 (0-17.29)	µg/mol creat; mean (range)
Hou (2012) [104]	11.83 (0.97-49.13) [h]	3.19 (0-17.29)	µg/mol creat; mean (range)

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Author (year)	Levels in smokers	Levels in non-smokers (or ex-smokers)	Units; variability
Radwan (2013) [105]	0.96 ± 0.07 [i]	0.05 ± 0.21	pmol/ml; geometric mean ± SD
Radwan (2013) [105]	1.35 ± 0.51 [j]	0.05 ± 0.21	pmol/ml; geometric mean ± SD
Radwan (2013) [105]	1.00 ± 0.36 [k]	0.05 ± 0.21	pmol/ml; geometric mean ± SD
Zhu (2013) [106]	1.1 ± 0.8 [230.16 ± 167.39] [l] 1.4 ± 1.2 [292.94 ± 251.09] [m]	NM	pmol/mg creat [pg/mg creat]; mean ± SD
Hu (2014) [81]	0.17 ± 0.14 (0.005–0.86) [170 ± 140 (5-860)]	NM	ng/mg creat [pg/mg creat]; mean ± SD (range)
Al Ali (2015) [107]	46.8 (27.6-79.3)	3.9 (1.6-7.9)	pg/mL; mean (range)
Miura (2015) [108]	187 ± 100	3.40 ± 3.62	ng/24h; mean ± SD

[a] 10.8 cigarettes per day (mean)

[b] 20.2 cigarettes per day (mean)

[c] 4 cigarettes per day

[d] 9 cigarettes per day

[e] 11 cigarettes per day

[f] 8 mg tar yield

[g] 10 mg tar yield

[h] 13 mg tar yield

[i] < 15 cigarettes per day

[j] 16-20 cigarettes per day

[k] > 20 cigarettes per day

l Slow CYP2A6 metabolizers:

[m] Fast CYP2A6 metabolizers

[o] >90% of these results reported as below limit of quantitation (LOQ). ½ LOQ used in calculations.

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Table 2 Representative urinary of total NNAL levels after smoking abstinence or switching to a reduced toxicant prototype

Author (year)	Levels in smokers	Levels in ex-smokers or switchers	Units; variability
Roethig (2007) [109]	NM	– 73 ± 13 [a]	% change from BL; mean ± SD
Frost-Pineda (2008) [110]	528.8 ± 221.7	117.8 ± 60.4 [a]	ng/24h; mean ± SD
Carmella (2009) [92]	2.70 ± 2.03 [564.95 ± 424.76]	0.935 ± 0.496 [195.64 ± 103.78] [b]	nmol/24h [ng/24h]; SD
Stepanov (2009) [65]	1.07 ± 0.8	0.15 ± 0.10 [c]	pmol/mL; mean ± SD
Stepanov (2009) [111]	1.20 ± 0.688 [251.09 ± 143.96]	0.264 ± 0.166 [55.24 ± 34.73] [d]	pmol/mg creat [pg/mg creat]; mean ± SD
Stepanov (2009) [111]	1.20 ± 0.688 [251.09 ± 143.96]	0.154 ± 0.112 [32.22 ± 23.43] [e]	pmol/mg creat [pg/mg creat]; mean ± SD
Tricker (2012) [112]	221 ± 154	80 ± 69 [f]	ng/24h; mean ± SD
Tricker (2012) [113]	231.0 ± 112.8	59.2 ± 31.4 [a]	ng/24h; mean ± SD
Tricker (2012) [114]	146.34 ± 86.08	45.06 ± 37.82 [a]	ng/24h; mean ± SD
Tricker (2012) [115]	237 ± 192	83 ± 82 [a]	ng/24h; mean ± SD
Theophilus (2015) [89]	731 ± 399	311 ± 265 [f]	ng/24h; mean ± SD

[a] After 8 days of smoking cessation
[b] After 3 days of smoking cessation
[c] After 2 weeks of NRT (patch)
[d] After 2 weeks of NRT (patch, gum or lozenge)
[e] After 6 weeks of NRT (patch, gum or lozenge)
[f] After 5 days of smoking cessation

List of abbreviations

3R4F	Kentucky 3R4F reference research cigarette [116]
AD	Addictive
ADME	Absorption and Distribution, Metabolism, and Excretion
BL	Baseline
CA	Carcinogen
CAS number	Chemical Abstracts Service number
Creat	Creatinine
CT	Cardiovascular toxicant
FDA	Food and Drug Administration

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HC	Health Canada
HCI	Health Canada Intense regime [117]
IARC	International Agency for Research on Cancer
ISO	International Organization for Standardization smoking regime [118]
IUPAC	International Union of Pure and Applied Chemistry
LS	Least square
MW	Molecular weight
ND	Not Determined
NM	Not Measured
NRT	Nicotine replacement therapy
PMI	Philip Morris International
RDT	Reproductive or developmental toxicant
RT	Respiratory toxicant
SD	Standard deviation
SE	Standard error
TSNA	Tobacco-specific nitrosamines
WHO	World Health Organization

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