APPENDIX A6.1.3.14 N-NITROSONORNICOTINE (NNN) - FACTSHEET

	HPHC	BoExp assessed by PMI
IUPAC name	3-[(2S)-1-nitrosopyrrolidin-2-yl]pyridine	Total NNN
Usual abbreviation	NNN	NNN
Other names	N-Nitrosonornicotine, N-nitroso, (±)-N'-nitrosonornicotine, (±)-1'-demethyl-1'-nitrosonicotine, 3-(1-nitroso-2-pyrrolidinyl)-, (*S*)-pyridine	-
CAS number	16543-55-8	16543-55-8
Formula and molecular weight	$C_9H_{11}N_3O$ MW: 177.20	$C_9H_{11}N_3O$ MW: 177.20

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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): 92.1 ng (ISO); 276 (HCI) [9]

Smoke phase: particulate phase [10]

Presence in tobacco / pyrosynthesis (T°): 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 [12, 13]. Tobacco-specific N-nitrosamines occur in all commercially and non-commercially prepared tobacco products including cigarettes, cigars, bidis, pipe tobacco and smokeless tobacco products [14]. They are found in the urine of all tobacco products users [15].

Because most of the TSNA yield in smoke comes from unchanged transfer from the tobacco by distillation with little contribution by pyrolysis [16, 17], it is dependent on their respective content in the cigarette tobacco. Thus, the wide disparity in levels of TSNA 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. NNN, NAB and NAT are formed primarily from their corresponding secondary amines (namely nornicotine, anatabine and anabasine) in the early stages of tobacco curing and processing, whereas the majority of NNK and some NNN are formed from the tertiary amine nicotine at the later stages of tobacco curing and fermentation.

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Other sources	N-Nitrosamines occur in a wide variety of both food and non-food products, but the amounts of tobacco-specific N-nitrosamines in all tobacco products exceed the levels of other N-nitrosamines in other commercial products by several orders of magnitude. The degree of exposure to tobacco-specific N-nitrosamines depends not only on the levels of these compounds in tobacco products or smoke, but also on the manner in which the tobacco products are used [14].
Toxicity class (FDA) [3]	CA
Toxicity – CA	IARC: group 1: carcinogenic to humans [1, 18] Epidemiological studies of tobacco users, both smoked and smokeless, support a plausible association between exposure to NNN and NNK and cancer [1, 18]. NNN was associated with esophageal cancer risk [19, 20], independent of smoking intensity and duration [21]. Although less carcinogenic than NNK overall [22], NNN was reported to cause nasal tumors in rodents by the following additional routes of exposure: 1) esophageal and stomachal or dietary exposure in male rats, 2) by administration in the drinking water and by intraperitoneal injection in hamsters of both sexes, and 3) by subcutaneous injection in male rats, male hamsters, and female mink [23].
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) [24].

ADME properties

The TSNA in tobacco are primarily formed after harvesting of the leaves during drying, curing, aging, and especially during fermentation [25], and are formed nitrosation of tobacco alkaloids (Figure 1).

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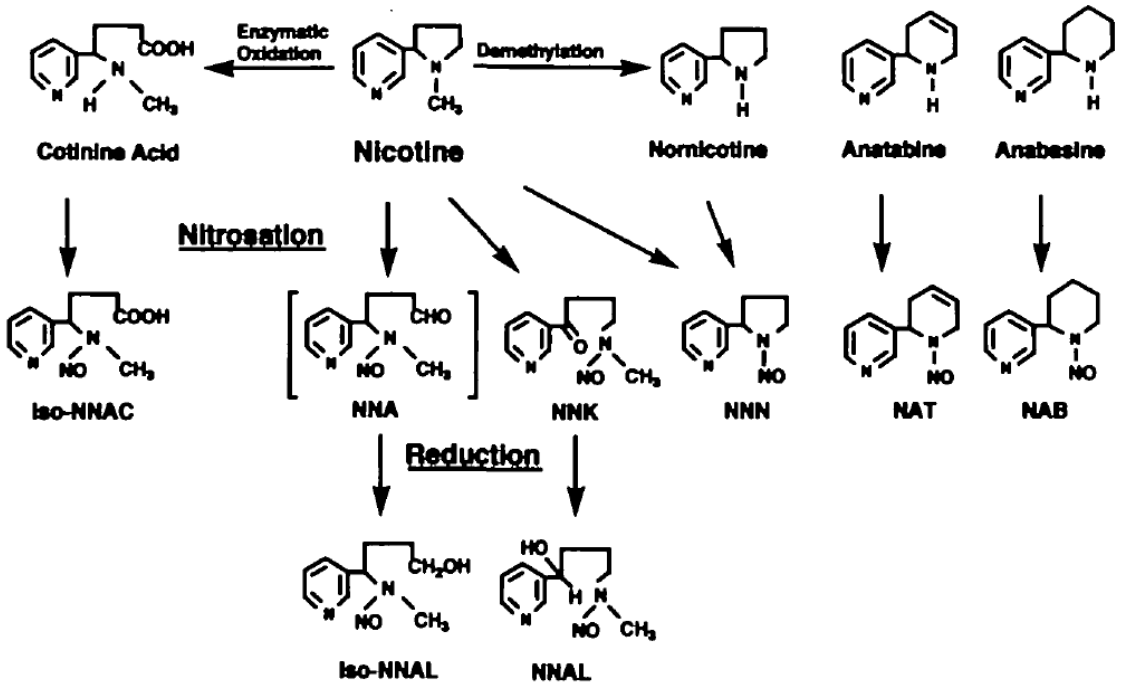


Figure 1 Formation of tobacco-specific N-nitrosamines by nitrosation of tobacco alkaloids during the curing and processing of tobacco [26]

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 [1] - Absorption of NNN has been demonstrated by detection of NNN and NNN-N-Gluc in the urine in smokeless tobacco users and smokers.

Metabolism [1, 27] - Specific pathways that have been observed in *in vitro* studies with human tissues [28] or enzymes or *in vivo* in humans are described below and schematized in Figure 2 [1]. NNN can undergo pyridine N-oxidation to yield NNN-N-oxide and pyridine N-glucuronidation to yield NNN-N-Gluc. Denitrosation and oxidation of NNN yields myosmine or norcotinine, and the latter can undergo pyridine N-oxidation to yield norcotinine-N-oxide. NNN is also metabolized to 3′-hydroxynorcotinine (3′-HOnorcotinine), but it is not clear whether this proceeds via norcotinine or by an independent pathway. 3′-HOnorcotinine undergoes glucuronidation on its hydroxyl group to yield 3′-HOnorcotinine-Gluc. Hydroxylation of the pyrrolidine ring of NNN produces 2′-hydroxy-NNN (2′- HONNN), 3'-hydroxy-NNN (3′-HONNN), 4'-hydroxy-NNN (4′-HONNN) and 5'-hydroxy-NNN (5′-HONNN). While 3′-HONNN and 4′-HONNN are stable metabolites, 2′-HONNN and 5′-HONNN, which are α-hydroxynitrosamines, are unstable. 2′-HONNN spontaneously undergoes ring opening to yield POB-DZH. POB-DZH reacts with water to form 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB). POB-DZH

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also reacts with DNA and hemoglobin to form adducts that are believed to have structures identical to those formed by the POB-DZH pathway in NNK metabolism. HPB can be further oxidized to keto acid or reduced to diol. 5′-HONNN spontaneously undergoes ring opening to produce 1-(3-pyridyl)-4-oxo-1-butanediazohydroxide (iso-POB-DZH), which reacts with water to form 5-(3-pyridyl)-2-hydroxytetrahydrofuran (lactol). Lactol can be oxidized to 5-(3-pyridyl)tetrahydrofuran-2-one (lactone) or 4-hydroxy-4-(3-pyridyl)butanoic acid (hydroxy acid). Thus, HPB and keto acid are indicative of the metabolic activation of NNN by 2′-hydroxylation whereas the lactol and the hydroxy acid are indicative of the metabolic activation of NNN by 5′-hydroxylation. Lactol, hydroxyl acid and keto acid are also metabolites of NNK, another major TSNA [1].

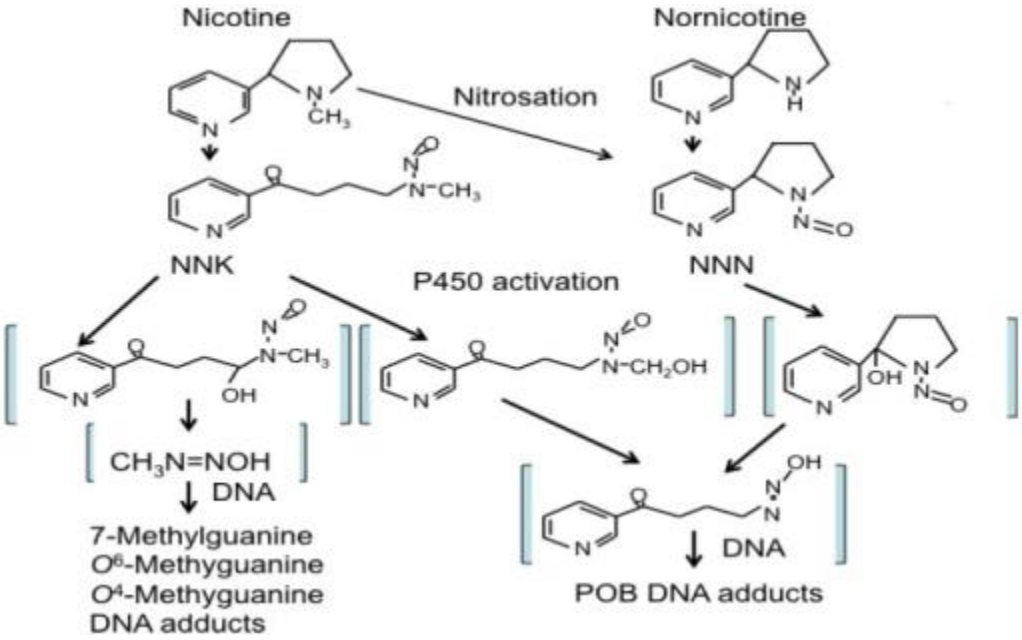


Figure 2 Schematic illustration of the pathways of NNK and NNN metabolism and DNA adduct formation as determined by studies in laboratory animals and humans [1]

NNK: 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, NNN: N'-nitrosonornicotine

Figure source: © 2014 Xue J, Yang S, Seng S. Mechanisms of Cancer Induction by Tobacco-Specific NNK and NNN. Cancers. 2014; 6(2):1138-56. http://www mdpi.com/2072-6694/6/2/1138 (licensed under Creative Commons Attribution 4.0 International licence: https://creativecommons.org/licenses/by/4.0/).

NNN-N-oxide and hydroxy acid (from 5′-hydroxylation) have been detected after incubation of cultured human tissues - buccal mucosa, trachea, oesophagus, bronchus, peripheral lung and urinary bladder - with NNN [1]. Smaller amounts of keto acid (from 2′-hydroxylation) were also observed. Cytochrome P450 (CYPs) enzymes have been identified as the major catalysts of NNN metabolic activation by α-hydroxylation. Human CYP2A6 and CYP2A13 are effective catalysts of NNN α-hydroxylation. 5′-Hydroxylation predominates in liver microsomes and both 2′-hydroxylation and 5′-hydroxylation are observed to comparable extents in oesophageal microsomes.

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Pyridyloxobutyl–hemoglobin adducts have been detected in smokeless tobacco users and smokers, and pyridyloxobutyl–DNA adducts have been detected in smokers [27]. These adducts could arise from NNN or NNK.

The metabolic pathways of NNN and DNA adduct formation are shown in Figure 2. This information is based on studies in vitro, in laboratory animals and in humans [1].

Excretion - NNN and NNN-N-Gluc have been detected in the urine of smokeless tobacco users and smokers [29]. Considerable differences exist between NNN metabolism in rodents and patas monkeys [30]. In rodents, the major urinary metabolites of NNN are the keto acid and hydroxy acid, resulting from 2′-hydroxylation and 5′-hydroxylation. Relatively small amounts of norcotinine and NNN-N-oxide are observed, whereas the other metabolites are barely detectable in rodent urine [27]. A common feature of rodent and monkey metabolism of NNN is the relatively large amount of the hydroxy acid in urine. However, norcotinine-1N-oxide and 3′-hydroxynorcotinine are found in patas monkey urine but not in rodent urine. In addition, the amount of norcotinine in patas monkey urine is considerably greater than that observed in rodent urine [30]. Finally, there is less of the keto acid in patas monkey urine than in rodent urine [30]. In a human study, the recovered NNN dose as total urinary NNN is really low, and was described to be of about 1% during the four days of the study, where inhalation of six cigarettes was allowed per day [31]. The study had been preceded by a washout period of four days. Recovery was evaluated by the amount excreted over 24h over the total daily uptake [31].

In rabbits, analysis of pharmacokinetic parameters demonstrated that NNN was rapidly eliminated from the body in an apparent monoexponential manner with an elimination half-life of 18.5 min [32]. The major metabolite in rabbit plasma was the hydroxyl acid, as indicated by the area under the curve (AUC). NNN was rapidly metabolized to the hydroxy acid, with its maximal plasma concentration (tmax) occurring at 17 min. The biological half-life of NNN in mouse, rat, hamster, rabbit and baboon ranged from 0.24 h to 3.06 h [32, 33]. Data from humans indicate only that half-life is less than 24h [34]. A pharmacokinetic study in patas monkeys after intravenous injection of [5-^{3}H]NNN showed a half-life of 45 min [30].

NNN exists as (S)- and (R)-enantiomers, the major form being (S)-NNN. In a study using rats, (S)-NNN was found to be the most carcinogenic of both forms. (R)-NNN was weakly active, but synergistically enhanced the carcinogenicity of (S)-NNN [35]. (S)-NNN is the predominant NNN enantiomer in moist snuff, novel smokeless tobacco products, and cigarettes currently marketed. It has thus been suggested that efforts toward the reduction of NNN in tobacco products should take into account its enantiomeric composition, with particular focus on (S)-NNN as a causative agent for esophageal and oral cancers associated with tobacco use [36].

Exposure levels in smokers and non-smokers

Total NNN in urine

In non-smokers	Ranges: ND [37]
In smokers	Ranges: 0.049-0.24 nmol/24h [8.7-42.5 ng/24h] [37]

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Human exposure to NNN can be measured via quantitation of unchanged NNN (also called free NNN) and its detoxification product NNN-pyridine-N-glucuronide (NNN-N-Gluc) in urine (Table 1). The sum of free NNN and NNN-N-Gluc in urine is referred to as total NNN [21]. It has been estimated that unchanged NNN accounts for 0.63% of all urinary NNN metabolites [31].

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 NNN (i.e. 21.2, 16.0, 9.7 and 4.0 ng/24 ng/24h, respectively) [38].

NNN-N-Gluc has been detected in the urine of smokeless tobacco users and smokers. One smoking cessation study over 56 days showed drastic reduction of eight urinary biomarkers of exposure, but the results obtained for NNN were not part of this publication. They were, however, reported online [39], and show that NNN did not decrease, unlike NNAL (Figure 3). Because subjects were allowed to use nicotine replacement therapy (NRT), the only possible source of maintained levels of NNN is from these NRT, indicating possible endogenous nitrosation of nicotine [34, 40].

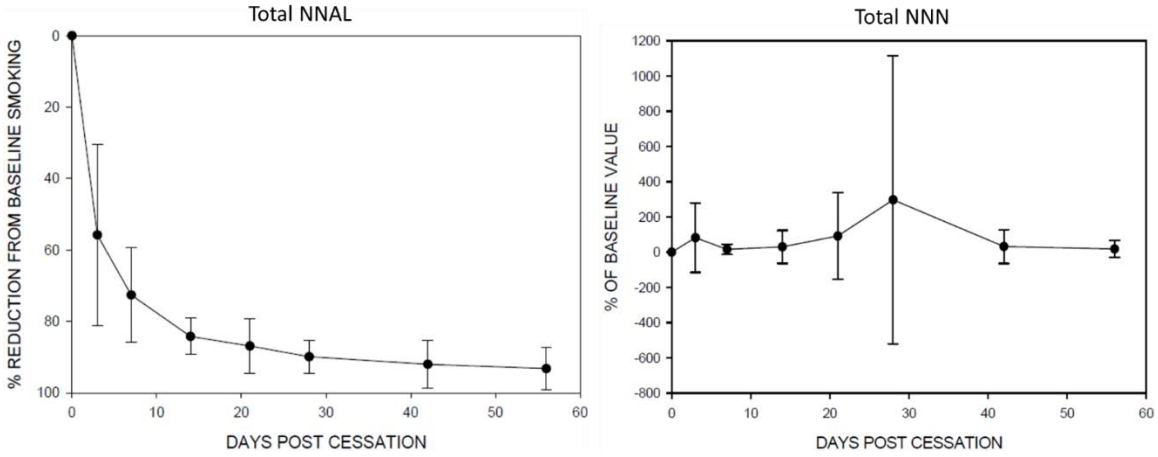


Figure 3 Percent reduction from baseline of NNAL and NNN at various intervals after cessation. Values are means ± SD [39, 41, 42]

Figure source: Hecht S. Endogenous formation of the carcinogen N′-nitrosonornicotine in some users of oral nicotine replacement therapy products [Internet]. Available from: www fda.gov/downloads/Drugs/NewsEvents/UCM232146.pdf (Accessed on 22 July 2015) (original paper: permission to reprint from Carmella SG, Chen M, Han S, Briggs A, Jensen J, Hatsukami DK, Hecht SS. Effects of smoking cessation on eight urinary tobacco carcinogen and toxicant biomarkers Chem Res Toxicol. 2009; 22(4):734-41. Erratum in Chem Res Toxicol. 2012 Mar 19;25(3):763. Copyright © 2016 American Chemical Society)

Another smoking cessation study, also showed relatively maintained levels of NNN, especially when oral nicotine was used, and not so much with a patch (Table 2) [34, 40].

Because significant amounts of NNN are excreted occasionally in some users of oral NRT, endogenous formation of NNN has been hypothesized [34, 40]. However, limited evidence has been presented to suggest that endogenous formation of NNN from

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nornicotine may occur in man [43] and in laboratory animals treated with nicotine and sodium nitrite [44, 45]. Some data suggest that possible artefactual formation of NNN may occur during sampling, storage and analysis of the urine samples. For example, storage of urine samples at room temperature for only 2 h in the presence of the precursors was found to lead to measurable NNN formation, which increased over time [31]. NNN levels might also potentially be confounded by diet, through the exposure to myosmine, which is readily nitrosated to produce NNN [46].

Keto acid and the enantiomers (R)- and (S)-hydroxy acids have been quantified in the urine of smokers and abstinent smokers who used nicotine replacement therapy [1]. The amount of (S)-hydroxy acid was far greater than that which could be formed from NNK and NNN. Both (S)-hydroxy acid and keto acid were formed in substantial amounts from nicotine, which precludes their use as biomarkers of NNN uptake in smokers. HPB-releasing hemoglobin adducts and DNA adducts have been detected in tobacco users [47]. These adducts can be formed either from NNK or from 2′-hydroxylation of NNN, thus are not specific to either compound.

In a smoking cessation study over 56 days, with or without NRT, urinary total NNN declined considerably after smoking cessation for the majority of the subjects [34]. However, in some subjects, total NNN at one or more time points after smoking cessation was comparable with or higher than baseline levels. In another smoking cessation study, over 28 weeks, also with or without NRT, the overall decline of urinary total NNN was less drastic than that of total NNAL. These results support the hypothesis that endogenous NNN formation occurs in some nicotine patch users [40]. The half-life of NNN can be estimated from the study of Stepanov et al. to be of less than 24 hours in humans [34], but no study with a calculated half-life of NNN in humans could be located. Another study looked at the levels of urinary levels of NNN in smokers who switched either to snus or NRT [48]. Subjects using snus had no significant decrease of NNN after four weeks.

CYP2A6 is the main activator of the carcinogenic TSNAs. CYP2A6 is a highly polymorphic gene and substantial ethnic variations exist in the population frequencies of CYP2A6 alleles [49], thus the metabolism of TSNAs, including NNN, will consequently also vary within different ethnicities, because of these genetic differences in CYP2A6 activities. Other influencing factors of CYP2A6 activity, and thus of NNN metabolism, include gender, drug intake, age and diet [50]. A study in Alaska native smokers has shown that in smokers with reduced CYP2A6 activity, the urinary levels of NNN were increased by about 1.7-fold, likely due to lower NNN clearance because of a lower bioactivation [51].

Because NNN is tobacco-specific, not many measurements have been performed in non-smokers. Two studies were found where NNN could be detected in the urine of non-smokers, but no speculation on the source of this NNN was made [46, 52].

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

Author (year)	Levels in smokers	Levels in non-smokers	Units; variability
Shepperd (2013) [52]	13.9 ± 11.4 (0.4-55.7)	0.9 ± 0.9 (0.3-5.3)	ng/24h; LS Means ± SD (ranges)
Kavvadias (2009) [46]	24.50 ± 35.83	0.49 ± 0.53	ng/24h; mean ± SD
Stepanov (2005) [29]	0.086 ± 0.12 [15.24 ± 21.26]	NM	pmol/mg creat [pg/mg creat]; mean ± SD
Yuan (2011) [19]	43.3 (33.7–55.6) [7.67 (5.97-9.85)]	NM	fmol/mg creat [pg/mg creat]; geometric mean (95% CI),
Zhu (2013) [51]	0.182 ± 0.390 [32.25 ± 69.11] [a] 0.110 ± 0.200 [19.49 ± 35.44] [b]	NM	pmol/mg creat [pg/mg creat]; mean ± SD

[a] Slow CYP2A6 metabolizers:
[b] Fast CYP2A6 metabolizers

Table 2 Representative urinary NNN levels after smoking abstinence

Author (year)	Levels in smokers	Levels in ex-smokers or switchers	Units; variability
Stepanov (2009) [34]	0.140 ± 0.194 [24.81 ± 34.38]	0.080 ± 0.142 [14.18 ± 25.16] [a]	pmol/mg creat [pg/mg creat]; mean ± SD
Urban (2009) [31]	3.1 ± 0.8	< LLOQ (2 pg/ml) [b]	pg/mL; mean ± SD
Theophilus (2015) [38]	24.6 ± 26.3	4.0 ± 3.0 [c]	ng/24h; mean ± SD

[a] Smoking cessation for 56 days, with use of NRT
[b] Washout period of 5 days
[c] Smoking cessation for 5 days

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List of abbreviations

3R4F	Kentucky 3R4F reference research cigarette [53]
ACToR	Aggregated Computational Toxicology Resource
AD	Addictive
ADME	Absorption and Distribution, Metabolism, and Excretion
AUC	Area under the curve
CA	Carcinogen
CAS number	Chemical Abstracts Service number
CI	Confidence interval
Creat	Creatinine
CT	Cardiovascular toxicant
CYP	Cytochrome P450
FDA	Food and Drug Administration
HC	Health Canada
HCI	Health Canada Intense regime [54]
HPB	4-hydroxy-1-(3-pyridyl)-1-butanone
HONNN	Hydroxy-NNN
IARC	International Agency for Research on Cancer
ISO	International Organization for Standardization smoking regime [55]
IUPAC	International Union of Pure and Applied Chemistry
LLOQ	Lower limit of quantification
LS mean	Least square mean
MSS	Main stream smoke
MW	Molecular weight
N/A	Not applicable
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
TSNA	Tobacco specific nitrosamine
WHO	World Health Organization

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