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Determination of tobacco-specific *N*-nitrosamines in urine of smokers and non-smokers

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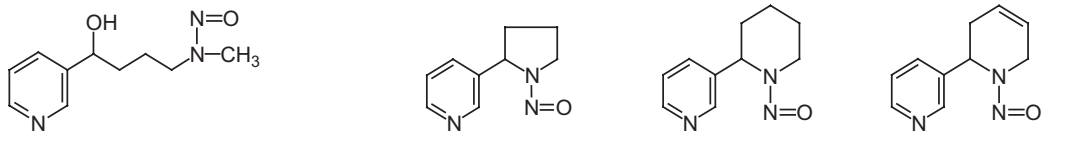
Abstract

Tobacco-specific *N*-nitrosamines (TSNA) include 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), *N′-nitroso*nornicotine (NNN), *N′-nitroso*anabasine (NAB) and *N′-nitroso*anatabine (NAT) and are found in tobacco and tobacco smoke. TSNA are of interest for biomonitoring of tobacco-smoke exposure as they are associated with carcinogenesis. Both NNK and NNN are classified by IARC as Group 1 carcinogens. Samples of 24 h urine collections ($n = 108$) were analysed from smokers and non-smokers, using a newly developed and validated LC-MS/MS method for determining total 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL, the major metabolite of NNK), and total NNN, NAB and NAT. TSNA levels in smokers' urine were significantly higher than in non-smokers. In smokers, urinary excretion of total TSNA correlated significantly ($r > 0.5$) with markers of smoking dose, such as daily cigarette consumption, salivary cotinine and urinary nicotine equivalents and increased with the ISO tar yield of cigarettes smoked. The correlation between urinary total NNN and the smoking dose was weaker ($r = 0.4$–0.5). In conclusion, this new method is suitable for assessing tobacco use-related exposure to NNK, NNN, NAB and NAT.

Keywords: *Tobacco-specific* N*-nitrosamines (TSNA); 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK); 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL);* N′*-nitrosonornicotine (NNN);* N′*-nitrosoanabasine (NAB);* N′*-nitrosoanatabine (NAT); urine*

Introduction

Tobacco-specific *N*-nitrosamines (TSNA) are formed from tobacco alkaloids during the curing process and occur in tobacco and tobacco smoke (Hecht et al. 1981, Brunnemann & Hoffmann 1991, Wiernik et al. 1995). 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is formed from nicotine, *N′-nitroso*nornicotine (NNN) is derived from nornicotine (Figure 1), which in turn can also be formed from nicotine. *N′-nitroso*anabasine (NAB) and *N′-nitroso*anatabine (NAT) are derived from anabasine and anatabine, respectively. The amount of TSNA in tobacco is significantly correlated with nitrate levels in the tobacco leaf,



4-(Methylnitrosamino)1-(3-pyridyl)-1-butanone (NNAL) | *N′*-Nitrosonornicotine (NNN) | *N′*-Nitrosoanabasine (NAB) | *N′*-Nitrosoanatabine (NAT)

Figure 1. Chemical structures of the free bases of tobacco-specific nitrosamines determined in human urine.

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and is dependent on the curing process used to prepare the tobacco (Fischer et al. 1989, Chamberlain et al. 1992, de Roton et al. 1992, Baker 1999). A recent study conducted on UK cigarette brands using ISO (International Organization for Standardization) smoking conditions gave ranges of TSNA in mainstream smoke per cigarette of 5–500 ng for NNK, 6.5–258 ng for NNN, 1.1–44.2 ng for NAB and 7.8–148 ng for NAT (Gregg et al. 2004).

Of the common TSNA, the group 1 carcinogens NNK and NNN are thought to be the most carcinogenic in rodent models (International Agency for Research on Cancer (IARC) 2007). Animal model studies suggest a link between NNK and the development of lung, pancreatic and possibly other cancers, whereas NNN may play a role in esophageal, oral and nasal cancer development in smokers (Hecht & Hoffmann 1989, Hecht 1998). NNK and NNN have also been implicated in the development of oral cancer in smokeless tobacco users (IARC 2007). By comparison, NAB and NAT have been classed as IARC group 3 chemicals (not classifiable as to its carcinogenicity to humans), with studies in rats showing NAB as a weak esophageal carcinogen, whereas NAT is inactive in rats (Hecht 1998).

In terms of biomarkers for TSNA exposure, until recently, most of the scientific literature has concentrated on NNK (Hecht 2002). For measuring human exposure to this chemical, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) and its pyridine-*N*-glucuronides have commonly been analysed in urine. A number of techniques have been employed for measurement of urinary NNAL and its glucuronides including gas chromatography with a nitrosamine-specific thermal energy analyser (GC-TEA), gas chromatography–mass spectrometry (GC-MS) and liquid chromatography–tandem mass spectrometry (LC-MS/MS) which had reported limits of detection (LODs) of 0.8 pg ml^{-1}, 0.6 pg ml^{-1} and ~1.7 pg ml^{-1}, respectively (Parsons et al. 1998, Bernert et al. 2005, Xia et al. 2005). Measuring biomarkers for the other three TSNA in addition to NNAL would increase the validity of the TSNA exposure assessment, because the ratios of the amounts of TSNA in tobacco could vary, depending on type of tobacco and curing process. Furthermore, NNAL levels in urine are influenced by interindividual variations in the NNK metabolism, the influence of which might be reduced when assessing all four TSNA in urine.

Stepanov and Hecht (2005) were the first to detect NNN, NAB and NAT, as well as their corresponding pyridine-*N*-glucuronides, in the urine of smokers and smokeless-tobacco users. The method used for determining total NNN, NAB and NAT comprised enzymatic hydrolysis of the *N*-glucuronides, two liquid/liquid distributions, two solid-phase extractions and analysis with GC-TEA. Recently, a new analytical method using LC-MS/MS has been developed and validated, and has been used for the simultaneous determination of total (sum of free base and *N*-glucuronide) NNAL, NNN, NAB and NAT in the urine of cigarette smokers (Kavvadias et al. 2009).

In this study, we applied the LC-MS/MS method for urinary TSNA to test urine samples collected from a previously conducted clinical study, which were thought to represent a useful range of smoke exposures (Shepperd et al. 2009). The main objective of our study was to validate the TSNA biomarkers according to the requirements established in the Report of the Institute of Medicine (2001), i.e. to demonstrate specificity, sensitivity, reproducibility and a dose-response relationship.

Materials and methods

Chemicals and standards

4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), methyl-deuterated 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL-d_3), *N*′-nitrosonornicotine (NNN), pyridine-ring-deuterated *N*′-nitrosonornicotine (NNN-d_4), *N*′-nitrosoanabasine (NAB), pyridine-ring-deuterated *N*′-nitrosoanabasine (NAB-d_4), *N*′-nitrosoanatabine (NAT), and pyridine-ring-deuterated *N*′-nitrosoanatabine (NAT-d_4) were purchased from Toronto Research Chemicals, North York, Ontario, Canada.

Acetonitrile/0.1% formic acid (for LC-MS), hydrochloric acid (>37%), de-ionized water, de-ionized water/0.1% ammonium acetate (both irradiated at 254 nm for >12 h) were supplied by Riedel-de Haën/Sigma-Aldrich, Steinheim, Germany. Ammonium hydroxide (25% in water) was obtained from Fluka/Sigma-Aldrich, Steinheim, Germany. Dichloromethane, hexane, methanol (all nitrosamine free), were purchased from Promochem GmbH, Wesel, Germany. Disodiumhydrogen phosphate (purity ≥ 99.5%), heptane (purity ≥ 99%), toluene (purity ≥ 99.9%) and β-glucuronidase (type IX-A, *Escherichia coli*) were obtained from Sigma-Aldrich. Potassium dihydrogen phosphate was purchased from Merck, Darmstadt, Germany.

Analytical method

The analytical method was performed as described previously (Kavvadias et al. 2009), and validated according to the US Food and Drug Administration (FDA) guidelines for bioanalytical methods (FDA 2001). In brief, after addition of the internal standards NNAL-d_3, NNN-d_4, NAB-d_4 and NAT-d_4 to 6 ml urine, the TSNA conjugates were split by treatment with β-glucuronidase at 37°C overnight. The hydrolysate was applied to a TSNA-molecular imprinted polymer (MIP) cartridge (Supelco,

Taufkirchen, Germany), washed and eluted with dichloromethane/toluene (1:1). The eluate dissolved in phosphate buffer was applied to a cation-exchange cartridge (Oasis MCX, Waters GmbH, Darmstadt, Germany) washed and eluted with methanol/ammonium hydroxide 25% (9:1). Ten microlitres of the eluate dissolved in 0.1% ammonium acetate/0.1% formic acid (9:1) was injected into the LC-MS/MS system, consisting of a Model 1100 HPLC device (Agilent Technologies, Waldbronn, Germany) and an atmospheric pressure ionization triple quadrupole mass spectrometer (Sciex API 4000; Applied Biosystems, Darmstadt, Germany). Chromatography was performed on a Luna C18 column (250 × 3.0 mm, 3 µm particle size; Phenomenex, Aschaffenburg, Germany). Positive electrospray ionization (ESI+) was applied, and the MS/MS system was run in the multireaction monitoring (MRM) mode.

Urine samples

One hundred and eight 24-h urine samples stored at -25°C were taken from a previous study, as described by Shepperd et al. (2009). These samples were obtained from 83 healthy smoking volunteers and 25 healthy non-smokers. The smokers belonged to three groups, smoking American Blend cigarettes with 10 mg ($n = 25$), 4 mg ($n = 33$), and 1 mg ($n = 25$) tar as nicotine-free dry particulate matter (NFDPM), determined according to the ISO standard smoking regime. The selection per group was at random.

In this study, smokers were enrolled for 19 days and smoked a supplied brand of cigarette of similar blend style and tar and nicotine yields to their normal brand for the duration of the study. In addition, subjects kept a diary that recorded daily cigarette consumption, diet, exercise and general health status. The non-smoking group was enrolled for a total of 12 days (also recording diet, exercise and health details). The 24-h urine samples used for this study were taken on day 19 for the smoking group, and day 12 for the non-smoking groups. Data on mouth level exposure to nicotine, urinary total nicotine equivalents (Tneq) and salivary cotinine were available from this study (Shepperd et al. 2009).

The study protocol and informed consent forms were approved by the ethics committee of the Ärztekammer Hamburg, Germany and the clinical study was conducted in accordance with the World Medical Association Declaration of Helsinki and International Conference on Harmonisation (ICH) Guidelines for Good Clinical Practice (GCP).

Statistics

All statistical analysis was carried out with MINITAB v15.1 (MINITAB Inc., Quality Plaza, State College, PA, USA). Three extreme outliers were identified (general linear model) in the NNN data, one in each smoking group. Generally, MINITAB identifies outliers on box plots by labelling observations that are at least 1.5 times the interquartile range (Q3–Q1) from the edge of the box.

Summary statistics were computed for each TSNA, and other markers of smoke exposure (salivary cotinine, Tneq and cigarettes per day) taken from the study by Shepperd et al. 2009. A Pearson correlation matrix was produced to test relationships across the different TSNA and markers of exposure for smokers only. For the calculation of means and standard deviations (SDs), actually measured TSNA levels were used, even when they were < LOQ and < LOD.

Analysis of covariance was carried out, with ISO tar yield and mouth level exposure (MLE) to nicotine as factors (data taken from Shepperd et al. 2009), followed by post-ANOVA comparisons (Tukey's HSD test). Urinary TSNA biomarkers, in contrast to MLE to nicotine, were not normally distributed and, therefore, log transformed.

Results

Urinary excretion of total NNAL, NNN, NAB and NAT

All urinary TSNA data in this study are expressed as 'total' NNAL, NNN, NAB or NAT, which comprises the molar sum of free and conjugated TSNA. Means, SD, medians and ranges of TSNA in the urine of 25 non-smokers and 83 smokers are shown in Table 1. Smokers excreted significantly higher amounts of NNAL, NNN, NAB and NAT compared with non-smokers. Total NNAL in 25 urine samples of non-smokers was quantifiable in 18 samples (73%), four samples were < LOD (= 2.0 ng $24\,h^{-1}$), three samples were < LOQ (= 5.0 ng $24\,h^{-1}$). Total NNN was quantifiable in five urine samples of non-smokers (20%), with 17 samples < LOD (= 0.8 ng $24\,h^{-1}$) and three samples < LOQ (= 2.0 ng $24\,h^{-1}$). Total NAB was not quantifiable in the 25 urine samples of non-smokers, 21 samples were < LOD (= 1.1 ng $24\,h^{-1}$) and four samples were < LOQ (= 5.0 ng $24\,h^{-1}$). Total NAT was < LOD (= 0.7 ng $24\,h^{-1}$) in all 25 urine samples of non-smokers.

Urinary excretion of TSNA in relation to ISO tar yields and smoking status is shown in Table 1. With the exception of NNN, urinary excretion of TSNA increased with increasing nominal tar yields. Differences in excretions of NNAL, NAB and NAT were significant between smokers of 10 and 1 mg tar cigarettes, and between 10 and 4 mg tar cigarettes, whereas NNN showed significant differences between smokers of 4 and 1 mg tar cigarettes only. Increases in urinary TSNA biomarker levels were subproportional compared with the increase in nominal tar yields. For example, the increase was about twice for

Table 1. Excretion of tobacco-specific nitrosamine(TSNA) and exposure markers in urine of smokers and non-smokers.

						Tar band comparisons	
Variable	Tar band (mg)	*n*	Mean	SD	Median	Tar Band	*p*-Value
Cigarettes/day	Non-smoker	25	0.00	0.00	0.00		
	1	25	21.76	8.61	21.00	1 vs 4	0.42
	4	33	24.27	7.30	25.00	1 vs 10	0.64
	10	25	23.68	6.39	23.00	4 vs 10	0.95
NAB ng 24 h^{-1}	Non-smoker	25	1.21	0.69	1.03		
	1	25	50.32	38.77	41.77	1 vs 4	0.48
	4	33	83.20	83.50	55.00	1 vs 10	< 0.01
	10	25	115.40	89.50	76.60	4 vs 10	0.02
NAT ng 24 h^{-1}	Non-smoker	25	0.04	0.11	0.00		
	1	25	211.50	165.60	160.30	1 vs 4	0.37
	4	33	422.60	389.30	270.30	1 vs 10	< 0.01
	10	25	493.60	424.00	299.60	4 vs 10	< 0.01
NNN[a] ng 24 h^{-1}	Non-smoker	25	0.49	0.53	0.34		
	1	24	8.67	11.17	5.90	1 vs 4	0.01
	4	32	24.50	35.83	11.00	1 vs 10	0.30
	10	24	12.26	8.59	10.56	4 vs 10	0.30
NNAL ng 24 h^{-1}	Non-smoker	25	16.06	11.99	13.03		
	1	25	138.70	94.90	117.40	1 vs 4	0.50
	4	33	204.20	146.90	162.70	1 vs 10	< 0.01
	10	25	288.70	179.30	253.20	4 vs 10	< 0.01
Tneq mg 24 h^{-1}	Non-smoker	25	0.02	0.01	0.02		
	1	25	9.17	4.61	8.78	1 vs 4	< 0.01
	4	33	14.62	7.49	12.39	1 vs 10	< 0.01
	10	25	17.86	6.01	16.36	4 vs 10	0.01
Salivary cotinine ng ml^{-1}	Non-smoker	25	0.25	0.00	0.25		
	1	25	161.60	101.40	145.00	1 vs 4	0.34
	4	33	218.70	102.80	210.00	1 vs 10	0.00
	10	25	281.00	139.60	267.00	4 vs 10	0.03

[a]One extreme outlier each excluded in all three tar band categories.

Table 2. Pearson correlation matrix between smoker tobacco-specific nitrosamine (TSNA) (ng 24 h^{-1}) and markers of smoke exposure.

	$\log_{24hNAB}$	$\log_{24hNAT}$	$\log_{24hNNN^*}$	$\log_{24hNNAL}$	$\log_{24hTNeq}$	$\log_{SalCot0700}$	MLE (nicotine mg daily)
$\log_{24hNAT}$	0.978						
$\log_{24hNNN}$[a]	0.747	0.776					
$\log_{24hNNAL}$	0.837	0.826	0.634				
$\log_{24hTneq}$	0.718	0.751	0.506	0.781			
$\log_{Saliva\ Cotinine}$	0.523	0.521	0.381	0.654	0.776		
MLE (nicotine mg daily)	0.719	0.728	0.503	0.730	0.816	0.690	
Cigarettes daily	0.528	0.563	0.445	0.606	0.674	0.574	0.724

[a]Three outliers removed from raw data. MLE, mouth level exposure.

the biomarkers, while it was ten-fold for the nominal tar yields.

There were three urine samples of smokers with NNN concentrations > 200 pg ml^{-1} (1331.9, 430.3 and 203.8 pg ml^{-1}), which differed greatly from the rest of the accumulated NNN data. These values (one in each smoking group) were treated as outliers, and eliminated from the evaluation, as it was believed that including them would have raised the mean for total NNN unrealistically. Median urinary NNN levels were similar in smokers of cigarettes with 10 and 4 mg tar, and about twofold lower in smokers of 1 mg cigarettes.

There were significant correlations ($p < 0.001$) between NNAL, NNN, NAB and NAT (Table 2). The strongest correlation was found between total NAB and total NAT in urine ($r = 0.98$). NNAL, NAB, and NAT also correlated significantly ($r > 0.5$) with markers of smoking dose, such as daily cigarette consumption, salivary cotinine and urinary Tneq. It should be noted that the correlations with NNN were weaker ($r = 0.4$–0.5) and became

significant only after removal of NNN concentrations > 200 pg ml^{-1}.

Discussion

Biomarkers of exposure are widely regarded in the scientific community as the best indicators of the level of internal or absorbed dose of a toxicant in exposed subjects. Commonly, these biomarkers are measured in urine, although blood, saliva and breath samples can also be used. An appropriate urinary biomarker for a specific chemical may be the parent chemical itself or one or more of its metabolites.

A number of key criteria must be met to ensure that candidate biomarkers are 'fit for purpose'. These are highlighted in the IOM report 'Clearing the smoke' (Institute of Medicine 2001) which indicates they should demonstrate adequate specificity, sensitivity, reproducibility and a dose–response relationship that is understood on a mechanistic basis.

With these criteria in mind, samples of 24-h urine collections (n = 108) were analysed from smokers and non-smokers, using a newly developed and validated LC-MS/MS method for determining total NNAL, NNN, NAB and NAT.

Comparing the urinary TSNA levels of smokers reported by Stepanov and Hecht (2005) with smokers of 10 mg tar cigarettes in this study, the average concentrations of total NNN (8.9 vs 47.2 pg ml^{-1}) and total NNAL (215.1 vs 473.8 pg ml^{-1}) were lower in this study, whereas the concentrations of total NAT (344.6 vs 53.2 pg ml^{-1}) and total NAB (82.6 vs 11.3 pg ml^{-1}) were lower in the Stepanov study. It is important to note that the NNN data in this study were corrected for putative artifact formation; in particular, a concentration of 1331.9 pg ml^{-1} was removed from this group. Including this value would have raised the mean for total NNN to 61.8 pg ml^{-1}. Since artifactual formation of NNN was not observed during sample analysis, we suggest that the artifactual formation might have occurred during sampling or storage of the samples. NNN levels might also potentially be confounded by diet, through the exposure to myosmine, which is readily nitrosated to produce NNN (Tyroller et al. 2002). Bacterial reduction of NNN-*N*-oxide to NNN, similar to approved reductions of the *N*-oxides of NNK and NNAL (Atawodi & Richter 1996, Carmella et al. 1997), is another possibility of artifactual NNN formation. Furthermore, polymorphisms in the CYP2A6 enzyme may lead to high variations in the urinary NNN excretion rates, given the fact that only about 1% of the absorbed NNN dose is excreted into urine (Urban et al. 2009).

The mean total urinary cotinine levels of smokers in this study (2.95 μg ml^{-1}) were comparable with levels generated from smokers in the Stepanov study (2.68 μg ml^{-1}), which indicates similar levels of smoke exposure. Similarities were also observed in the ratio of total NAT/total NAB in urine between the Stepanov study (4.7) and this study (4.2), with both approximately half as much as the NAT/NAB ratio reported in mainstream cigarette smoke (8.8) (IARC 2004). Assuming similar TSNA content in the cigarettes of the two studies, the lower urinary excretion of NNAL and NNN, and 6–7-fold higher excretion of NAB and NAT compared with the Stepanov study, requires further explanation. However, one explanation may be inherent differences in the cigarettes used in both studies.

Significant correlations between the total NNN, NNAL, NAB and NAT and the various indicators of smoke exposure, including daily cigarette consumption, Tneq in urine and salivary cotinine, were observed (Table 2). This is in good agreement with correlations reported by Stepanov and Hecht (2005). Similarly, there was clear agreement with the strong correlations found in the Stepanov study, between the total TSNA in urine, and for the correlation between NAT and NAB (r = 0.98). It should be noted that the correlation between total NNN and the smoking dose variables, or other TSNA, become significant only after elimination of the outliers.

From the levels of TSNA measured in the urine of smokers and non-smokers in the present study, it is possible to make power calculations. This allows the opportunity to estimate the number of subjects required to satisfy a minimum 80% discrimination between the ISO tar yield groups and non-smokers for each TSNA biomarker as shown in Table 3. These results indicate that in most cases a larger population than used in

Table 3. Pairwise power calculations of NAB, NAT, NNAL and NNN in urine of smokers and non-smokers (NS)

Variable	Pack tar (mg)	n	Pairwise power calculations	Power (%)	Sample number required to achieve 80% study power
NAB ng 24 h^{-1}	NS	25			
	1	25	NS vs 1	99	7
	4	33	1 vs 4	42–52	63
	10	25	4 vs 10	25–31	115
NAT ng 24 h^{-1}	NS	25			
	1	25	NS vs 1	99	6
	4	33	1 vs 4	69–81	33
	10	25	4 vs 10	9–11	517
NNN ng 24 h^{-1} (outliers removed)	NS	25			
	1	24	NS vs 1	94–95	16
	4	32	1 vs 4	38–50	67
	10	24	4 vs 10	31–41	86
NNN ng 24 h^{-1} (outliers included)	NS	25			
	1	25	NS vs 1	28	99
	4	33	1 vs 4	1	1381
	10	25	4 vs 10	21	165

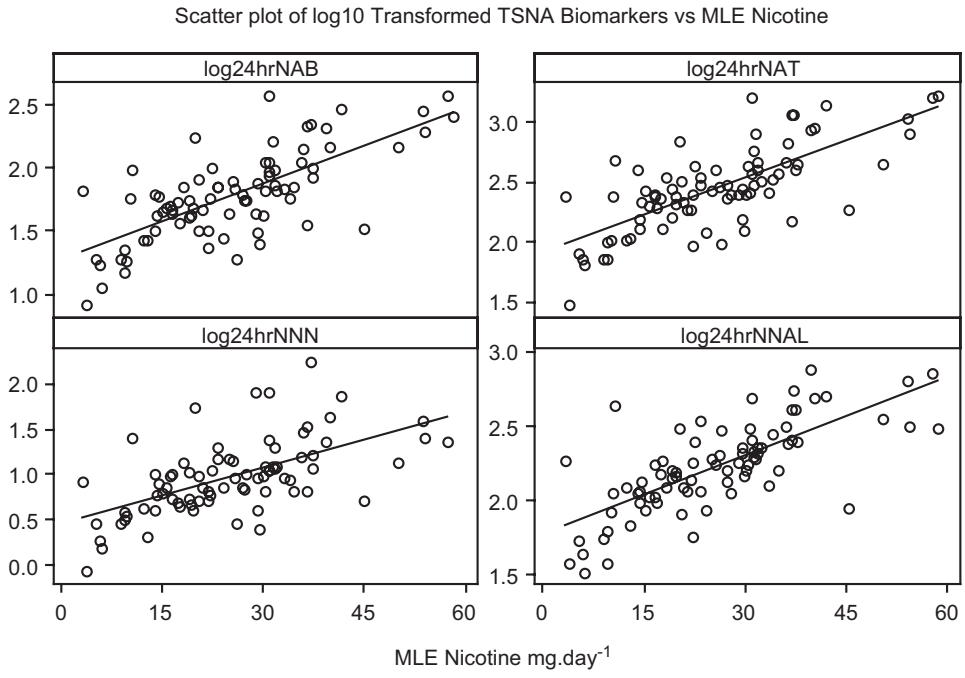

Figure 2. Scatterplot of $\log_{10}$ transformed tobacco-specific nitrosamine (TSNA) biomarkers plotted against mouth level exposure (MLE) to nicotine (mg daily). NNN outliers have been removed.

this study is required to show a significant difference between the different groups. This is especially true for the smoker groups exposed to higher tar products (smokers of ISO 4 mg products and ISO 10 mg products). Including the NNN outliers led to unrealistically high number of subjects required. Therefore, in future clinical studies these data can advise on the numbers of subjects required to investigate adequately potential changes in exposure to the TSNA.

A further comparison was made between each individual smoker's TSNA and their MLE to nicotine, as measured by filter analysis (nicotine data were obtained from a previous study) (Shepperd et al. 2009). MLE constitutes an estimate of the amount of smoke entering the smoker's mouth, and the filter methodology described has been shown previously to be a good indicator of the amount of nicotine exiting the cigarette filter (Shepperd et al. 2006, St Charles et al. 2006). Nicotine MLE data may function as a more reliable marker of smoke exposure than ISO tar yield due to its consideration of the individual smoking habit (Baker & Dixon 2006). The results from this comparison identify significant correlations between the $\log_{10}$ transformed excreted TSNA biomarker levels and the MLE nicotine. Furthermore, the data shown in Figure 2 support the use of these biomarkers in quantifying the exposure to TSNA from cigarette smoke. One may consider that the combination of these two approaches – filter analysis measurements of nicotine MLE and urinary biomarkers – could establish a potentially acceptable framework for the assessment of conventional cigarettes and future, potentially reduced exposure products.

In conclusion, an analytical method for the simultaneous determination of total NNAL, NNN, NAB and NAT in human urine has been applied to urine samples of 25 non-smokers and 83 smokers. All four TSNA were found to be significantly higher in the urine of smokers compared with non-smokers. Furthermore, total NNAL, NAB and NAT significantly correlated with markers of smoke exposure, and the MLE to nicotine. A weaker correlation was found for total NNN. Accordingly, the data also demonstrate a good dose–response relationship between the biomarkers and exposure to TSNA in tobacco smoke. It is anticipated that these TSNA biomarkers will complement the current suite of available exposure markers, with the aim that these can be monitored in future clinical studies of potentially reduced exposure tobacco products.

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References

Atawodi SE, Richter E. (1996). Bacterial reduction of N-oxides of tohbacco-specific nitrosamines (TSNA). *Hum Exp Toxicol* 15:329–34.

Baker RR. (1999). Smoke chemistry. In: Davis DL, Nielsen MT, editors. *Tobacco – Production, Chemistry and Technology*. Malden, MA: Blackwell Science. p. 398–439.

Baker RR, Dixon M. (2006). The retention of tobacco smoke constituents in the human respiratory tract. *Inhal Toxicol* 18:255–94.

Bernert JT, Jain RB, Pirkle JL, Wang L, Miller BB, Sampson EJ. (2005). Urinary tobacco-specific nitrosamines and 4-aminobiphenyl hemoglobin adducts measured in smokers of either regular or light cigarettes. *Nicotine Tobacco Res* 7:729–38.

Brunnemann KD, Hoffmann D. (1991). Analytical studies on tobacco-specific N-nitrosamines in tobacco and tobacco smoke. *Crit Rev Toxicol* 21:235–40.

Carmella SG, Borukhova A, Akerkar SA, Hecht SS. (1997). Analysis of human urine for pyridine-N-oxide metabolites of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, a tobacco-specific lung carcinogen. *Cancer Epidemiol Biomarkers Prev* 6:113–20.

Chamberlain WJ, Severson RF, Chortyk OT, Sisson VE. (1992). Effects of curing and fertilization on ntirosamine formation in Bright and Burley tobacco. *Beitr Tabakforsch Int* 15:87–92.

de Roton C, Wiernik A, Wahlberg I, Vidal B. (1992). Factors influencing the formation of tobacco-specific nitrosamines in French air-cured tobaccos in trials and at the farm level. *Beitr Tabakforsch Int* 216:305–20.

Fischer S, Spiegelhalder B, Preussmann R. (1989). Preformed tobacco-specific nitrosamines in tobacco-role of nitrate and influence of tobacco type. *Carcinogenesis* 10:1511–17.

Food and Drug Administration (FDA). (2001). Guidance for Industry – Bioanalytical Method Validation. Washington, DC: US Department of Health and Human Services 1. Available at: http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM070107.pdf (last accessed 17 April 2009).

Gregg E, Hill C, Hollywood M et al. (2004). The UK smoke constituents testing study. Summary of results and comparison with other studies. *Beitr Tabakforsch Int* 21:117–38.

Hecht SS. (1998). Biochemistry, biology, and carcinogenicity of tobacco-specific *N*-nitrosamines. *Chem Res Toxicol* 11:559–603.

Hecht SS. (2002). Human urinary carcinogen metabolites: biomarkers for investigating tobacco and cancer. *Carcinogenesis* 23:907–22.

Hecht SS, Chen CHB, Young R, Hoffmann D. 1981. Mass spectra of tobacco alkaloid-derived nitrosamines, their metabolites, and related compounds. *Beitr Tabakforsch Int* 11:57–66.

Hecht SS, Hoffmann D. (1989). The relevance of tobacco-specific nitrosamines to human cancer. *Cancer Surv* 8:273–94.

Institute of Medicine. (2001). Clearing the Smoke: Assessing the Science Base for Tobacco Harm Reduction. Washington, DC: National Academy Press.

International Agency for Research on Cancer (IARC). (2004). *Tobacco smoke and involuntary smoking*. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans 83:1–1438.

International Agency for Research on Cancer (IARC). (2007). *Smokeless Tobacco and Some Tobacco-specific N-Nitrosamines*. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans 89:1–626.

Kavvadias D, Scherer G, Urban M, Cheung F, Errington G, Shepperd J, McEwan M. (2009). Simultaneous determination of four tobacco-specific *N*-nitrosamines (TSNA) in human urine. *J Chromatogr B Analyt Technol Biomed Life Sci* 877:1185–92.

Parsons WD, Carmella SG, Akerkar S, Bonilla LE, Hecht SS. (1998). A metabolite of the tobacco-specific lung carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in the urine of hospital workers exposed to environmental tobacco smoke. *Cancer Epidemiology, Biomarkers & Prevention* 7:257–60.

Shepperd CJ, Eldridge AC, Mariner DC, McEwan M, Dixon M. (2009). A study to estimate and correlate cigarette smoke exposure in smokers in Germany as determined by filter analysis and biomarkers of exposure. *Regul Toxicol Pharmacol* June 16 [Epub ahead of print].

Shepperd CJ, St Charles FK, Lien M, Dixon M. (2006). Validation of methods for determining consumer smoked cigarette yields from cigarette filter analysis. *Beitr Tabakforsch Int* 22:176–84.

St Charles FK, Krautter GR, Dixon M, Mariner DC. (2006). A comparison of nicotine dose estimates in smokers between filter analysis, salivary cotinine, and urinary excretion of nicotine metabolites. *Psychopharmacology (Berl)* 189:345–54.

Stepanov I, Hecht SS. (2005). Tobacco-specific nitrosamines and their pyridine-*N*-glucuronides in the urine of smokers and smokeless tobacco users. *Cancer Epidemiol Biomarkers Prev* 14:885–91.

Tyroller S, Zwickenpflug W, Richter E. (2002). New sources of dietary myosmine uptake from cereals, fruits, vegetables, and milk. *J Agric Food Chem* 50:4909–15.

Urban M, Scherer G, Kavvadias D, Hagedorn HW, Feng S, Serafin R, Kapur S, Muhammad R, Jin Y, Mendes P, Roethig H. (2009). Quantitation of *N*'-Nitrosonornicotine (NNN) in smokers' urine by liquid chromatography– tandem mass spectrometry. *J Anal Toxicol* 33:260–5.

Wiernik A, Christakopoulos A, Johansson L, Wahlberg I. (1995). Effect of air-curing on the chemical composition of tobacco. *Recent Adv Tobacco Sci* 21:39–80.

Xia Y, McGuffey JE, Bhattacharyya S, Sellergren B, Yilmaz E, Wang L, Bernert JT. (2005). Analysis of the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol in urine by extraction on a molecularly imprinted polymer column and liquid chromatography/atmospheric pressure ionization tandem mass spectrometry. *Anal Chem* 77:7639–45.