BIOMARKERS, 2000, VOL. 5, NO. 1, 33–45

Biomonitoring of environmental tobacco smoke (ETS)-related exposure to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)

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Received 12 February 1999, revised form accepted 8 July 1999

The exposure of non-smokers to the tobacco-specific *N*-nitrosamine 4-(*N*-methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), a rodent lung carcinogen, was determined in the air of various indoor environments as well as by biomonitoring of non-smokers exposed to environmental tobacco smoke (ETS) under real-life conditions using the urinary NNK metabolites 4-(*N*-methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) and [4-(*N*-methylnitrosamino)-1-(3-pyridyl)but-1-yl]-β-*O*-D-glucosiduronic acid (NNAL-Gluc). NNK was not detectable (< 0.5 ng m^{-3}) in 11 rooms in which smoking did not occur. The mean NNK concentration in 19 rooms in which smoking took place was 17.5 (2.4–50.0) ng m^{-3}. The NNK levels significantly correlated with the nicotine levels ($r = 0.856$; $p < 0.0001$). Of the 29 non-smokers investigated, 12 exhibited no detectable NNAL and NNAL-Gluc excretion (< 3 pmol day) in their urine. The mean urinary excretion of NNAL and NNAL-Gluc of the 17 remaining non-smokers was 20.3 (< 3–63.2) and 22.9 (< 3–90.0) pmol day^{-1}, respectively. Total NNAL excretion (NNAL + NNAL-Gluc) in all non-smokers investigated significantly correlated with the amount of nicotine on personal samplers worn during the week prior to urine collection ($r = 0.88$; < 0.0001) and with the urinary cotinine levels ($r = 0.40$; $p = 0.038$). No correlation was found between NNAL excretion and the reported extent of ETS exposure. Average total NNAL excretion in the non-smokers with detectable NNAL levels was 74 times less than in 20 smokers who were also investigated. The cotinine/total NNAL ratios in urine of smokers (9900) and non-smokers (9300) were similar. This appears to be at variance with the ratios of the corresponding precursors (nicotine/NNK) in mainstream smoke (16 400) and ETS (1000). Possible reasons for this discrepancy are discussed. The possible role of NNK as a lung carcinogen in non-smokers is unclear, especially since NNK exposure in non-smokers is several orders of magnitude lower than the ordinary exposure to exogenous and endogenous *N*-nitrosamines and the role of NNK as a human lung carcinogen is not fully understood.

Keywords: tobacco-specific nitrosamines, environmental tobacco smoke, passive smoking, urinary metabolites.

Abbreviations: ATNC, apparent total *N*-nitroso compounds; BSA, bis-(trimethylsilyl)acetamide; CV, coefficient of variation; ETS, environmental tobacco smoke; GC-NPD, gas chromatograph equipped with a nitrogen phosphorous detector; GC-TEA, gas chromatograph equipped with a thermal energy analyser; HPB, 4-hydroxy-1-(3-pyridyl)-1-butanone; *iso*-NNAL, 4-(*N*-methylnitrosamino)-4-(3-pyridyl)-1-butanol; LOD, limit of detection; NNAL, 4-(*N*-methylnitrosamino)-1-(3-pyridyl)-1-butanol; NNAL-Gluc, [4-(*N*-methylnitrosamino)-1-(3-pyridyl)but-1-yl]-b-*O*-D-glucosiduronic acid; [pyridyl-5-^{3}H] NNAL-Gluc, [pyridyl-5-^{3}H] [4-(*N*-methylnitrosamino)-1-(3-pyridyl)but-1-yl]-b-*O*-D-glucosiduronic acid; NNK, 4-(*N*-methylnitrosamino)-1-(3-pyridyl)-1-butanone; [pyridyl-5-^{3}H] NNK, [pyridyl-5-^{3}H] 4-(*N*-methylnitrosamino)-1-(3-pyridyl)-1-butanone; PS, personal sampler for nicotine measurement; RIA, radio immunoassay; THF, tetrahydrofuran; TMCS, trimethylchloro silane; TMS, trimethylsilyl ether.

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Biomarkers ISSN 1354-750X print/ISSN 1366-5804 online © 2000 Taylor & Francis Ltd
http://www.tandf.co.uk/journals/tf/1354750X.html

Introduction

NNK is a lung carcinogen in rodents and occurs in cured tobacco as well as in mainstream and sidestream smoke of cigarettes, and in ETS (Hoffmann and Hoffmann 1997). NNK is mainly metabolized by keto reduction resulting in the urinary metabolites 4-(*N*-methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) and its *O*-glucuronide 4-(*N*-methylnitrosamino)-1-(3-pyridyl)but-1-yl]-b-*O*-D-glucosiduronic acid (NNAL-Gluc), and by α-hydroxylation leading to methylating and pyridyloxobutylating reactive intermediates (figure 1) (Hecht 1996). Pyridine-*N*-oxidation is only a minor metabolic pathway of NNAL in man (Carmella *et al.* 1997). NNK has been suggested to be a causative agent for adenocarcinoma of the lung in smokers (Hoffmann *et al.* 1996). NNK uptake by non-smokers is of particular interest, since epidemiological studies report an association between ETS exposure and lung cancer in non-smokers (for a recent review see Hackshaw *et al.* (1997)) and adenocarcinoma is the most frequent type of lung tumour found in non-smokers (Hoffmann *et al.* 1996).

NNK levels in various indoor environments where smoking takes place have been found to range from 0.2 to 23.8 ng m^{-3} with mean levels at about 5–10 ng m^{-3} (Brunnemann *et al.* 1992, Klus *et al.* 1992, Tricker *et al.* 1994). A possible biomarker for measuring chronic exposure to NNK is based on the hydrolytic release of 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB) from haemoglobin adducted with tobacco-specific nitrosamines. However, a large overlap in the HPB levels between smokers and non-smokers has been reported (Hecht *et al.* 1993a, Falter *et al.* 1994). It is, therefore, not surprising that no relation exists between ETS exposure and HPB–haemoglobin adduct levels in non-smokers (Branner *et al.* 1998). The reason for the limited suitability of HPB–haemoglobin adducts as a biomarker for NNK exposure is unclear (Scherer and Richter 1997). The urinary NNK metabolites NNAL and NNAL-Gluc appear to be more suitable biomarkers for NNK exposure. In smokers, a strong correlation between these metabolites and cotinine, the main metabolite of nicotine, was observed (Carmella *et al.* 1993, Meger *et al.* 1996). In non-smokers, urinary NNAL and NNAL-Gluc excretion increases after experimental exposure to high concentrations of cigarette sidestream smoke in an experimental chamber (Hecht *et al.* 1993b). Recently, Parsons *et al.* (1998) demonstrated in a small field study the presence of NNAL-Gluc in the urine of non-smokers exposed to ETS. In both studies (Hecht *et al.* 1993b, Parsons *et al.* 1998), the urinary NNAL levels significantly correlated with those of cotinine.

The aim of the present study was to quantify the urinary excretion of NNAL and NNAL-Gluc in non-smokers in relation to their self-reported and objectively measured exposure to ETS. For comparison, the urinary NNAL and NNAL-Gluc excretion of smokers was also investigated. In addition, the NNK and nicotine levels in different smoking and non-smoking indoor environments were determined.

Materials and methods

Design of biomonitoring study

Twenty self-reported smokers (male, age 20–40 years) and 30 self-reported non-smokers (age 20–60 years; 17 males, 13 females) collected 24 h urine samples and completed a questionnaire on their daily tobacco smoke exposure over 1 week (smokers: cigarette consumption; non-smokers: duration and intensity of ETS exposure). Non-smokers wore a passive diffusion sampler for nicotine for 1 week prior

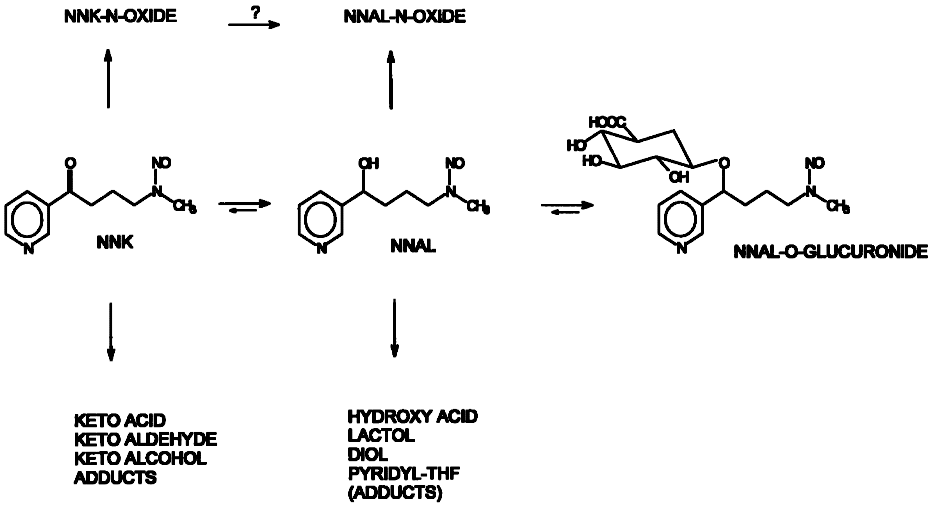

Figure 1. Simplified scheme of metabolic pathways of NNK and NNAL (Hecht 1996).

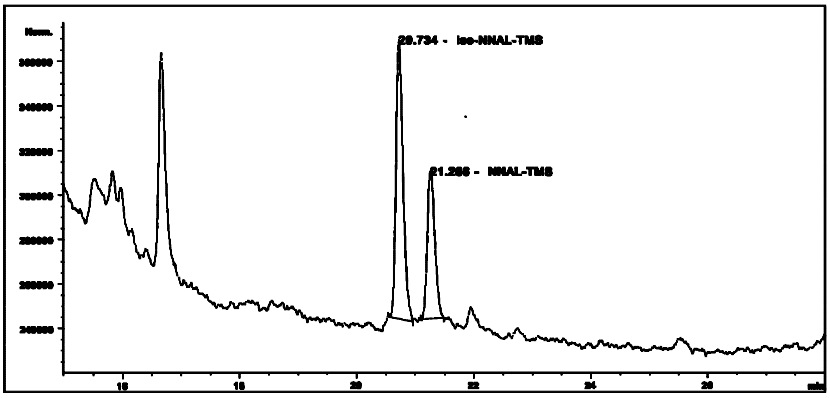

Figure 2. Chromatogram obtained by GC-TEA of a non-smoker's urine sample. *iso*-NNAL-TMS is the trimethylsilyl derivative of the internal standard *iso*-NNAL. NNAL-TMS is the trimethylsilyl derivative of the analyte NNAL.

to urine collection (Ogden and Maiolo 1992). Urine samples were collected in 2l plastic bottles containing 10 g sodium hydroxide to prevent bacterial growth and artifactual *N*-nitrosamine formation. Samples were split into 250 ml aliquots and stored at –30 °C prior to analysis. The stability under alkaline conditions was examined in a frozen pool urine sample, aliquots of which were thawed once a month over 9 months and analysed for NNAL and NNAL-Gluc. The levels did not systematically change. Identical NNAL and NNAL-Gluc concentrations were measured in urine samples stabilized with NaOH or sulphamic acid. Similar results were reported by another laboratory (Carmella *et al.* 1995).

Design of air monitoring study

Indoor air was sampled for about 3 h at 30 different locations. NNK was absorbed on acidified (0.1 M sodium hydrogen sulphate) Cambridge filters (44 mm, Borgwaldt, Hamburg, Germany), spiked with 5 ng *N*-nitrosopentylpicolylamine (Toronto Research Chemicals, Ontario, Canada). Nicotine was sampled on XAD 4 resin (SKC Inc., Eighty Four, PA, USA).

Analytical methods

NNK and nicotine levels in ETS were determined by GC-TEA and GC-NPD, respectively, according to published methods (Begutter *et al.* 1985, Ogden and Maiolo 1992). Nicotine on the personal samplers was determined by GC-NPD (Ogden and Maiolo 1992). Urinary cotinine was determined by RIA (Haley *et al.* 1983).

Analysis of NNAL. To 20–100 ml (smokers) or 100–500 ml (non-smokers) of urine, 4 ng *iso*-NNAL (internal standard, generous gift from Dr Dietrich Hoffmann, Valhalla, NY, USA) were added. Samples from non-smoker were concentrated on a rotary evaporator at 40 °C and 3 kPa to 100 ml. The sample was adjusted to pH 5.0 with hydrochloric acid. The aqueous solution was transfered to a glass column (450 mm × 40 mm, G3 frit) filled with Extrelut® (Merck, Darmstadt, Germany) and allowed to soak for 30 min. The column was eluted with 250 ml ethyl acetate (Code 3427, Promochem, Wesel, Germany), and the eluate was evaporated to 5 ml at 40 °C and 20 kPa. The concentrate was added to 5 ml water, adjusted to pH 2.0 with hydrochloric acid, and transfered to a separation funnel. After washing the aqueous layer three times with ethyl acetate, the pH was adjusted to 5.0 with aqueous sodium hydroxide and absorbed on a column containing Extrelut®. The column was eluted with 80 ml ethyl acetate, the eluate dried over anhydrous sodium sulphate, and concentrated to 2 ml *in vacuo*. The final extract was applied to 8 g aluminium oxide (activity II-III, ICN Biomedicals, Eschwege, Germany), equilibrated with 20 ml ethyl acetate (glass column, 150 mm × 15 mm, G2 frit). The column was washed with 10 ml ethyl acetate. Unconjugated NNAL was eluted with 20 ml ethyl acetate/methanol (10:1 v/v) (methanol, Code 9835, Promochem, Wesel, Germany) and evaporated to 1 ml. The purified fraction was transfered to a reaction vial, the solvent was removed under nitrogen, and the residue was derivatized by adding 48 µl bis-(trimethylsilyl)acetamide (BSA) and 2 µl trimethylchloro silane (TMCS) (Aldrich, Steinheim, Germany) at 50 °C for 30 min.

Analysis for NNAL-Gluc. *Iso*-NNAL (4 ng) was added as an internal standard to 20–100 ml urine (smokers) or 100–500 ml urine (non-smokers), the sample was adjusted to pH 6.9 and incubated overnight at 37 °C with 50.000–100.000 units of b-glucuronidase (type IX A, Sigma, Deisenhofen, Germany). After adjusting to pH 5.0, the sample was worked up and analysed as described above. The amount of NNAL-Gluc was obtained by subtracting the previously determined amount of free NNAL.

Separation and quantitation of NNAL was performed using gas chromatography (5890 Series II gas chromatograph (GC), Hewlett Packard, Waldbronn, Germany) with *N*-nitrosamine specific detection (Thermal Energy Analyzer, TEA 502, Waltham, USA). Sample aliquots of 2–10 µl were injected in splitless mode (HP 7673 A automatic sampler). Separation was performed on a DB 1301 (30 m × 0.53 mm × 1.0 µm) fused silica capillary column (J&W, Folsom, California, USA). The oven temperature was programmed (initial, 80 °C; 1 min hold; 15 °C min^{-1} to 180 °C; 5 °C min^{-1} to 210 °C, hold for 5 min; 10 °C min^{-1} to 250 °C, 20 min hold). The retention times for *iso*-NNAL and NNAL were 20.7 and 21.3 min, respectively. The analytical method was calibrated by spiking a pooled urine sample from non-smokers, previously determined to be free of NNAL and NNAL-Gluc with known amounts of NNAL (generous gift from Dr Dietrich Hoffmann, Valhalla, NY, USA). Deconjugation of NNAL-Gluc was shown to be complete under these conditions by addition of authentic [pyridyl-5-^{3}H]NNAL-Gluc (isolated from bile of [pyridyl-5-^{3}H]NNK-treated rats (Schulze *et al.* 1992)) and high performance liquid chromatography with solid phase radioactivity monitoring. Recovery rates of *iso*-NNAL ($n = 23$) and NNAL ($n = 8$), spiked at a concentration of 4 ng/100 ml urine, averaged 80.5 ± 10 % and 82.4 ± 8 %, respectively. Recoveries were not affected by preconcentration or treatment with β-glucuronidase. Multiple analysis ($n = 4$) of urine from a smoker and a non-smoker gave an average of 4656 ± 241 pmol day^{-1} (CV = 5 %) and 71 ± 6 pmol day^{-1} (CV = 8 %). The limit of detection for NNAL was 3 pmol l^{-1} after inclusion of the preconcentration step (urine: 500 ml, final volume: 50 µl; injection volume: 10 µl; limit of detection, signal/noise ratio 3:1; 40 pg per injection; recovery: 80 %).

To confirm identity, aliquots of most samples were reanalysed on a different coated capillary column (DB 1701, 30 m × 0.32 mm × 0.25 µm). Positive results were confirmed by UV photolysis at 366 nm prior to reinjection (Krull *et al.* 1979).

Results

NNK and nicotine levels in indoor environments

Concentrations of NNK and nicotine were measured in 30 indoor environments. Table 1 shows the results from stationary monitoring in homes, offices, bars, and restaurants. NNK concentrations were below the LOD of 0.5 ng m^{-3} in non-smoking homes and offices. At sites where smoking took place, NNK concentrations ranged from 2.4 to 50.0 ng m^{-3} (mean: 17.5 ± 13.4 ng m^{-3}) with nicotine levels ranging from 2.2 to 33.5 µg m^{-3} (12.4 ± 9.6 µg m^{-3}). NNK and nicotine concentrations significantly correlated ($r = 0.856$; $p < 0.0001$; figure 3).

Table 1. Ranges and means (± standard deviation) of NNK and nicotine levels in different indoor environments.

Location	NNK (ng m^{-3})	Nicotine (mg m^{-3})
Non-smoker homes ($n = 6$)	< LOD[a]	< LOD–0.1
Non-smoker offices ($n = 5$)	< LOD	< LOD–0.5 (0.3 ± 0.2)
Bar and restaurants (smoking permitted) ($n = 10$)	7.0–50.0 (22.7 ± 15.7)	2.2–33.5 (12.3 ± 10.7)
Smoker homes ($n = 5$)	4.1–23.0 (13.6 ± 8.8)	3.6–28.5 (13.6 ± 10.3)
Smoker offices ($n = 4$)	2.4–15.2 (9.3 ± 6.0)	4.0–20.0 (11.1 ± 7.6)

[a] LOD: Limit of detection, 0.5 ng m^{-3} for NNK and 0.1 µg m^{-3} for nicotine.

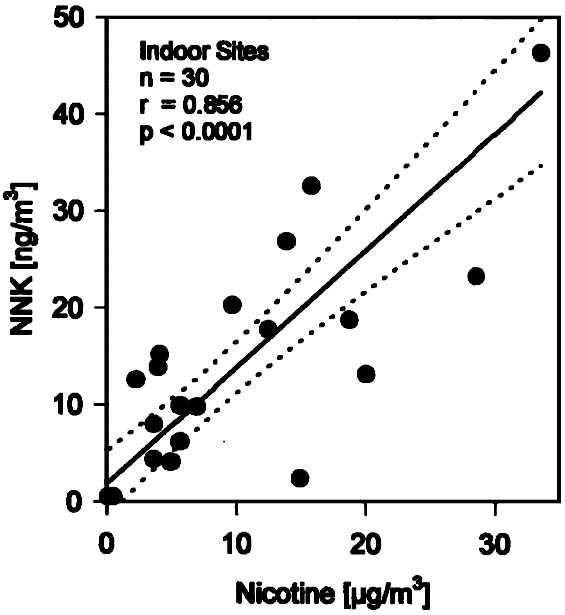

Figure 3. Linear regression between nicotine and NNK levels in indoor air at 30 different sites. The equation of the regression line (solid line) is $y = 1.85 \times 10^{-3} + x\ 1.21 \times 10^{-3}$; the dashed lines represent the 95% confidence intervals.

NNK exposure of non-smokers

Based on a cut-off point of 1000 nmol day^{-1} urinary cotinine, one self-reported non-smoker proved to be a smoker and was therefore excluded from the analysis. In table 2, the individual exposure data of 29 non-smokers are shown. Arithmetic means of all non-smokers, non-smokers with detectable and undetectable NNAL or NNAL-Gluc levels in urine as well as smokers are shown in table 3. The non-smoking subjects reported ETS exposure ranging from 0 to 100 (12.8 ± 22.3) hours per week. Of the 29 non-smoking subjects, 20 had worn a personal sampler for nicotine over the week prior to urine sampling. Nicotine levels on the samplers ranged from 0.05 to 4.07 (0.74 ± 1.09) µg m^{-3}. Urinary cotinine excretion varied from 20.5 to 821.3 (180.9 ± 205.5, $n = 27$) nmol day^{-1} .

Table 2. ETS exposure markers and urinary excretion of NNAL and NNAL-Gluc of 29 non-smokers.

Subject No.	ETS exposure duration (h week^{-1})	Nicotine on PS[a] (μg m^{-3})	Urinary cotinine (nmol day^{-1})	Urinary NNAL-Gluc (pmol day^{-1})	Urinary free NNAL (pmol day^{-1})	Urinary total NNAL[b] (pmol day^{-1})
1	100	0.81	179.7	2.3	6.2	8.5
2	5	0.77	762.5	< LOD[c]	34.9	34.9
3	0	–[d]	87.5	< LOD	< LOD	< LOD
4	1	–	–	< LOD	19.1	19.1
5	0	–	50.9	< LOD	< LOD	< LOD
6	3	–	148.3	57.1	< LOD	57.1
7	16	0.06	121.4	< LOD	< LOD	< LOD
8	2	0.11	57.3	< LOD	3.8	3.8
9	0	0.11	198.9	< LOD	< LOD	< LOD
10	6	0.43	112.5	< LOD	26.8	26.8
11	45	1.00	821.3	< LOD	19.1	19.1
12	0	–	68.2	< LOD	< LOD	< LOD
13	–	3.42	422.7	74.5	63.2	137.7
14	2	0.11	81.8	< LOD	11.5	11.5
15	1	0.21	34.8	8.6	< LOD	8.6
16	0	–	–	< LOD	< LOD	< LOD
17	6	0.14	55.2	7.3	< LOD	7.3
18	4	0.52	78.4	< LOD	< LOD	< LOD
19	34	0.67	107.4	27.0	< LOD	27.0
20	2	–	221.6	90.9	57.4	148.3
21	1	0.06	40.9	< LOD	< LOD	< LOD
22	18	0.05	36.4	< LOD	< LOD	< LOD
23	48	0.54	158.9	9.3	3.4	12.7
24	0	0.09	20.5	< LOD	< LOD	< LOD
25	12	1.08	337.8	20.5	54.6	75.1
26	0	–	25.0	< LOD	< LOD	< LOD
27	39	4.07	387.5	49.9	38.3	88.2
28	10	0.47	125.0	33.5	< LOD	33.5
29	4	–	142.6	< LOD	< LOD	< LOD

[a] PS: Personal sampler for nicotine, worn over 1 week.
[b] Total NNAL: sum of NNAL-Gluc and free (unconjugated) NNAL.
[c] LOD: limit of detection for NNAL, 3 pmol l^{-1} corresponding to about 3 pmol day^{-1}.
[d] – : No data available.

Of the 29 non-smokers, 17 had detectable amounts of NNAL or NNAL-Gluc in their urine (LOD = 3 pmol l^{-1}, corresponding to about 3 pmol day^{-1}). The non-smokers with detectable NNAL levels in their urine (subgroup 1) reported a 5.5-fold longer ETS exposure (19.8 versus 3.6 hours per week), exhibited 6.6 times more nicotine on their personal samplers (0.99 versus 0.15 μg m^{-3}), and excreted 2.8 times more cotinine (250.9 versus 89.1 nmol day^{-1}) than the non-smokers with non-detectable NNAL (subgroup 2, $n = 12$). The differences between the two subgroups are statistically significant ($p<0.05$).

The amounts of NNAL-Gluc, unconjugated NNAL, and total NNAL (sum of NNAL + NNAL-Gluc) in all non-smokers ranged from < LOD to 90.9 (14.1 ± 24.2) pmol day^{-1}, < LOD to 63.2 (12.5 ± 19.0) pmol day^{-1}, and < LOD to 148.3 (26.6 ± 39.2) pmol day^{-1}, respectively.

Total NNAL excretion correlated significantly with the amount of nicotine on the personal sampler ($r = 0.88$, $p < 0.0001$, figure 4) and the urinary cotinine

Table 3. Ranges and means (± standard deviation) of tobacco smoke exposure and urinary NNAL excretion in non-smokers and smokers[a]

	Self-reported ETS exposure (h/week)	Nicotine on PS (μg/m³)	Urinary cotinine (nmol/d)	Urinary NNAL-Gluc (pmol/d)	Urinary free NNAL (pmol/d)	Urinary total NNAL (pmol/d)	Molar ratio (cotinine/total NNAL)	Molar ratio (NNAL-Gluc/free NNAL)
All non-smokers								
n	28	20	27	29	29	29		
Range	0–100	0.05–4.07	20.5–821.3	< LOD–90.9	< LOD–63.2	< LOD–148.3		
Mean ± SD	12.8 ± 22.3	0.74 ± 1.09	180.9 ± 205.5	14.1 ± 24.2	12.5 ± 19.0	26.6 ± 39.2		
Non-smokers (NNAL > LOD)								
n	16	12	16	17	17	17	16	12
Range	1–100	0.11–4.07	34.8–821.3	< LOD–90.9	< LOD–63.2	3.8–148.3	1494–39869	0–2.8
Mean ± SD	19.8 ± 27.2	0.99 ± 1.22	250.9 ± 241.4	22.9 ± 28.6	20.3 ± 21.8	43.3 ± 44.4	9287 ± 10198	0.7 ± 0.8
Non-smokers (NNAL < LOD)								
n	12	8	11	12	12	12		
Range	0–18	0.05–0.52	20.5–198.9	< LOD	< LOD	< LOD		
Mean ± SD	3.6 ± 6.5	0.15 ± 0.18	89.1 ± 63.2					
Smokers	Self-reported consumption (cigarettes/d)							
n	20	20	20	20	20	20	20	
Range	8–29	3400–49600	3–3548	225–4510	373–8058	4926–22535	0–3.7	
Mean ± SD	20.8 ± 4.6	27600 ± 13100	1724 ± 946	1494 ± 1090	3218 ± 1934	9861 ± 4380	1.5 ± 1.0	

[a] In cases where NNAL or NNAL-Gluc were below the detection limit (<LOD), half the LOD (1.5 pmol day^{-1}) was used for the calculation of statistical parameters.

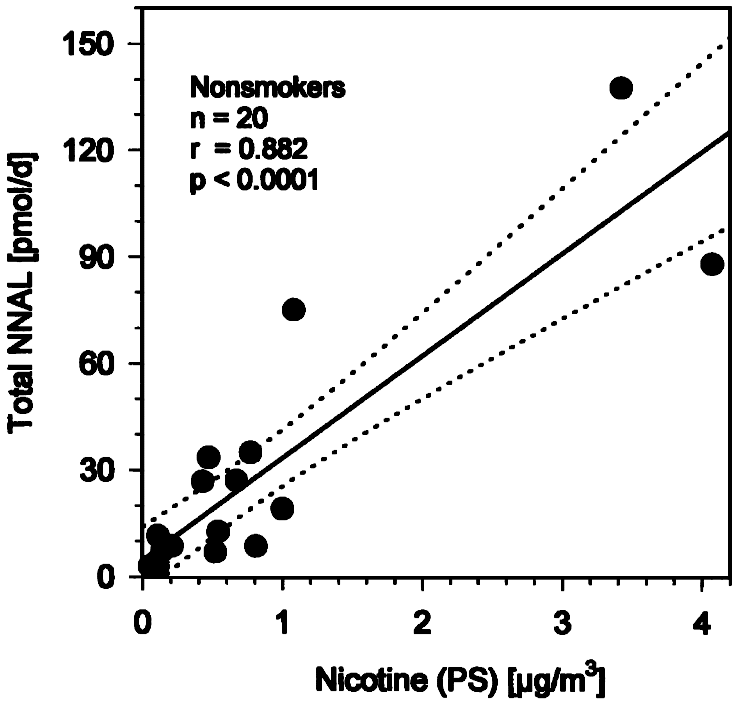

Figure 4. Linar regression between nicotine measured on a personal sampler (PS) and total urinary NNAL excretion of 20 nonsmokers (no personal sampler data were available from nine non-smokers). The equation of the regression line (solid line) is $y = 3.77 + x\ 29.1$; the dashed lines represent the 95% confidence intervals.

excretion ($r = 0.40$, $p = 0.038$). No correlation was found between total NNAL excretion and self-reported ETS exposure duration ($r = 0.05$, $p = 0.8$).

Comparison with NNK exposure of smokers

The means and ranges of the exposure data for 20 smokers are shown in table 3 together with those for the non-smoking groups. Self-reported numbers of cigarettes smoked per day ranged from 8 to 29 (20.8 ± 4.6). Urinary cotinine excretion varied from 3.4 to 49.6 (27.6 ± 13.1) µmol day^{-1}. This is 153 times higher than in all non-smokers and 110 times higher than in non-smokers with detectable NNAL level.

Urinary excretion of NNAL, NNAL-Gluc and total NNAL in smokers amounted to 225–4510 (1494 ± 1090) pmol day^{-1}, 3–3548 (1724 ± 946) pmol day^{-1}, and 373–8058 (3218 ± 1934) pmol day^{-1}, respectively. The total NNAL excretion in smokers is, on average, 121 times higher than in all non-smokers and 74-fold higher than in non-smokers with detectable NNAL levels in their urine. The molar ratio of NNAL-Gluc/free NNAL ranged from 0 to 3.7 (1.4 ± 1.0) in the smokers and from 0 to 2.8 (0.7 ± 0.8) in the non-smokers with detectable NNAL levels.

The molar ratio of urinary cotinine/total NNAL was found to be similar in smokers (9900 ± 4400) and non-smokers (9300 ± 10 00).

There was no significant correlation between daily cigarette consumption and total NNAL excretion ($r = 0.30$, $p = 0.197$), however, urinary cotinine levels significantly correlated with total NNAL excretion ($r = 0.69$, $p < 0.001$).

Discussion

NNK in indoor environments

The average NNK level of 17.5 ng m^{-3} observed in indoor environments with smoking taking place (table 1) is higher than in most studies reported earlier (Brunnemann *et al.* 1992, Klus *et al.* 1992, Tricker *et al.* 1994). The nicotine concentration measured in these rooms was, on average, 12.4 μg m^{-3}, which is above the nicotine range of 1–10 μg m^{-3} reported for smoky rooms under realistic conditions (Coultas *et al.* 1990, Oldaker III *et al.* 1990, Scherer *et al.* 1995, Phillips *et al.* 1998). This suggests that extensive smoking occurred in these environments. Under these conditions a strong correlation ($r = 0.856$) was found between NNK and nicotine (figure 3), with a molar ratio (nicotine/NNK) of 1000 ± 1300 (range: 200–6200). A similar ratio can be calculated from the reported nicotine and NNK concentrations in a chamber with sidestream smoke (Hecht *et al.* 1993b). The nicotine/NNK ratio in ETS is at least one order of magnitude lower than the corresponding ratio for mainstream smoke of cigarettes. On the basis of data reported by Tricker *et al.* (1991) the mean molar ratio of nicotine/NNK for mainstream smoke of 10 German filter cigarettes (0.3–1.0 mg/cig nicotine) is estimated to be 16 400 ± 5800 (8200-25 600). These ratios are of interest for our discussion of the excretion ratios of the corresponding biomarkers (cotinine and NNAL) in urine of smokers and non-smokers.

Urinary NNAL excretion of non-smokers and smokers

The developed GC-TEA method has a limit of detection of 3 pmol l^{-1} and is sensitive enough to determine NNAL and NNAL-Gluc levels in urine of ETS-exposed non-smokers. This sensitivity was achieved primarily by application of large urine volumes of about 500 ml and large GC-injection volumes of up to 10 μl.

We found that the urinary excretion of total NNAL (NNAL + NNAL-Gluc) in non-smokers is clearly related to their extent of exposure to ETS. The strongest correlation ($r = 0.88$) was observed between the total NNAL excretion and nicotine on the personal sampler worn during 1 week prior to urine collection (figure 4). A weaker, although still significant correlation, was found between NNAL and cotinine excretion ($r = 0.40$). No correlation existed when the self-reported ETS exposure duration was used as a measure for exposure. These results are in agreement with those obtained in two previous studies (Hecht *et al.* 1993b, Parsons *et al.* 1998). In an experimental study (Hecht *et al.* 1993b), elevated NNAL + NNAL-Gluc levels after exposure of five non-smokers for 3 h to high sidestream smoke levels in a small chamber were observed (127 versus 31 pmol day^{-1}). In a small field study with nine ETS-exposed workers (Parsons *et al.* 1998), elevated NNAL-Gluc levels were reported compared to five 'negative controls' (0.059 versus 0.012 pmol ml^{-1}). In both studies (Hecht *et al.* 1993b, Parsons *et al.* 1998), as in our study, a significant correlation between urinary NNAL and cotinine

excretion was found. The background levels of total NNAL in non-smokers of the experimental study (31 pmol day^{-1}) (Hecht *et al.* 1993b) is comparable to the levels found in our study (26.6 pmol day^{-1} for all non-smokers, table 3). The tobacco smoke exposure-related increase to 127 pmol day^{-1} (Hecht *et al.* 1993b) is certainly much lower than would have been expected, given the very high level of smoke exposure (38–230 μg m^{-3} nicotine over 3 h) compared with approximately 1 μg m^{-3} nicotine over 24 h in our study (table 2). However, it is clear that no steady-state NNAL excretion level was achieved after the sidestream smoke exposure (2 × 90 min), whereas NNAL excretion was probably in steady-state in our non-smokers. If we assume a urine volume of 1200 ml day^{-1}, NNAL-Gluc excretion in the small field study (Parsons *et al.* 1998) would amount to 70.8 pmol day^{-1} (ETS-exposed non-smokers) and 14.4 pmol day^{-1} (unexposed controls). The former level is higher than that observed in our subjects (14.1 pmol day^{-1} in all non-smokers, 22.9 pmol day^{-1} in non-smokers with detectable NNAL concentrations, table 3), whereas the level in the unexposed controls is comparable to our findings. Three reasons might be responsible for this difference:

(i) Parsons *et al.* (1998) have excluded all samples with low cotinine levels determined by RIA which shifts the extent of ETS exposure of the group to a higher level.
(ii) The ETS exposure of the hospital workers was indeed much higher than that of our non-smokers. No levels of ETS components were reported, but the authors (Hecht *et al.* 1993b) state that the results may be typical for higher ETS exposures.
(iii) Incomplete extraction of the unconjugated NNAL (extraction efficiency is reported to be about 75 %) (Carmella *et al.* 1995), which was performed prior to β-glucuronidase treatment and NNAL determination, might have increased the NNAL-Gluc levels by up to 25 %.

Average total NNAL excretion in our group of smokers (3.22 nmol day^{-1}) is lower than the average total NNAL excretion reported by Carmella *et al.* (1993) (11.41 nmol day^{-1}, mean of 11 smokers), but similar to that of another study of this group (Hecht *et al.* 1995) (3.28 nmol day^{-1}, mean of 11 smokers). In a larger study with 61 smokers (Carmella *et al.* 1995, Richie *et al.* 1997), creatinine-based NNAL excretion rates were reported. With an assumed daily creatinine excretion of 1.5 g, total NNAL elimination would amount 5.6 nmol day^{-1}, which is also consistent with our findings. In the study with 61 smokers (Carmella *et al.* 1995, Richie *et al.* 1997), a bimodal distribution of the urinary NNAL-Gluc/NNAL ratio was reported with means of 3.11 in 34 black smokers and 4.43 in 27 white smokers. In our data set of 20 Caucasian smokers, we found no indication of a bimodal distribution and the mean was 1.5 for smokers and 0.7 for non-smokers (table 3).

We found that non-smokers excrete 153 times less cotinine and 121 times less total NNAL than smokers. The corresponding factors for the group of non-smokers with detectable NNAL levels amount to 110 (cotinine) and 74 (total NNAL). Parsons *et al.* (1998) reported that levels of cotinine were 84 times and levels of NNAL-Gluc were 69 times less than in smokers. These ratios are somewhat lower than those for our group of non-smokers with detectable NNAL levels in urine, again suggesting a more intense ETS exposure of the exposed non-smokers in the study of Parsons (Parsons *et al.* 1998).

We observed similar molar ratios between cotinine and total NNAL in urine of

smokers (9900) and non-smokers (9300). This confirms findings by Hecht and coworkers who found cotinine/NNAL-Gluc ratios of 3800 in ETS exposed non-smokers (Parsons *et al.* 1998), 4600 in sidestream smoke exposed non-smokers (Hecht *et al.* 1993b) and 3600 in smokers (Carmella *et al.* 1995). These observations are somewhat surprising, since the average molar ratios between precursors in tobacco smoke (nicotine/NNK) differ by more than one order of magnitude in mainstream smoke of cigarettes (16 400) and ETS (1000). Possible reasons for this discrepancy are:

(i) Nicotine, a gas phase constituent of ETS, is taken up more effectively than the particle-bound NNK (Scherer *et al.* 1990).
(ii) Metabolic conversion of NNK to NNAL is more extensive in smokers than in non-smokers because of the inhibiting effects of nicotine and cotinine present in high enough concentrations only in smokers (Richter and Tricker 1994, Schulze *et al.* 1998).
(iii) The half-life of cotinine is longer in non-smokers than in smokers (Kyerematen *et al.* 1982, Haley *et al.* 1989). However, this is not supported by another author (Benowitz 1996).

In conclusion, a highly sensitive analytical method for the determination of NNAL and NNAL-Gluc in urine of non-smokers has been developed. For the first time, both NNK metabolites have been determined in the urine of non-smokers exposed to ETS under real-life conditions. Renal elimination of NNAL and NNAL-Gluc in non-smokers significantly correlates with nicotine measured on a personal sampler and with urinary cotinine excretion. ETS-exposed non-smokers excrete approximately 1% of the amount of total NNAL excreted by smokers. The cotinine/NNAL ratios in urine of smokers and non-smokers are similar, which is at variance with the corresponding ratios of the precursors (nicotine/NNK) in mainstream smoke and ETS. The biological role of NNK exposure of non-smokers in terms of a lung cancer risk remains to be determined.

Acknowledgements

NNAL and *iso*-NNAL were generous gifts from Dr D. Hoffmann, American Health Foundation, Valhalla, NY, USA. [pyridyl-5-^{3}H]NNAL-Gluc was a generous gift from Prof. E. Richter, Walther-Straub-Institute for Pharmacology and Toxicology, Ludwig-Maximilians-Universität München, Germany.

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