

# Metabolites of a Tobacco-specific Lung Carcinogen in the Urine of Elementary School-aged Children<sup>1</sup>

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## Abstract

**Limited data are available in the literature on carcinogen uptake by children exposed to environmental tobacco smoke (ETS). In this study, we quantified metabolites of the tobacco-specific lung carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in the urine of elementary school-aged children participating in the School Health Initiative: Environment, Learning, Disease study, a school-based investigation of the environmental health of children. The metabolites of NNK are 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) and its glucuronide (NNAL-Gluc). We also measured cotinine and its glucuronide (total cotinine). Urine samples were collected from 204 children. Seventy (34.3%) of these had total cotinine  $\geq 5$  ng/ml. NNAL or NNAL-Gluc was detected in 52 of 54 samples with total cotinine  $\geq 5$  ng/ml and in 10 of 20 samples with total cotinine  $< 5$  ng/ml. Levels of NNAL plus NNAL-Gluc and total cotinine were significantly higher when exposure to ETS was reported than when no exposure was reported. However, even when no exposure to ETS was reported, levels of NNAL, NNAL-Gluc, and NNAL plus NNAL-Gluc were higher than in children with documented low exposure to ETS, as determined by cotinine levels  $< 5$  ng/ml. Levels of NNAL, NNAL-Gluc, and cotinine were not significantly different in samples collected twice from the same children at 3-month intervals. Levels of NNAL plus NNAL-Gluc in this study were comparable with those observed in our previous field studies of adults exposed to ETS. There was a 93-fold range of NNAL plus NNAL-Gluc values in the exposed children. The results of this study demonstrate widespread and considerable uptake of the tobacco-specific lung carcinogen NNK in this group of elementary school-**

**aged children, raising important questions about potential health risks. Our data indicate that objective biomarkers of carcinogen uptake are important in studies of childhood exposure to ETS and cancer later in life.**

## Introduction

Consistently, epidemiological studies demonstrate a low-level increased risk for lung cancer in adult nonsmokers exposed to ETS<sup>3</sup> compared with nonexposed controls (1, 2). On the basis of these data, several agencies including the United States Department of Health and Human Services, the National Research Council, the United States Environmental Protection Agency, and the California Environmental Protection Agency have concluded that ETS causes lung cancer (3–7). The epidemiological evidence indicates that there is an  $\sim 20\%$  increase in risk for lung cancer in ETS-exposed adults (2). This is considerably less than the 1000–2000% increase in risk for lung cancer seen in smokers, but the uptake of tobacco smoke constituents in nonsmokers is also far less than in smokers.

Epidemiological investigations of childhood exposure to ETS and adult lung cancer have not produced consistent results. In a recent meta-analysis, the authors concluded that there was no increased risk but cautioned that the presence of some positive studies argues against concluding that there is no relationship (8). It is biologically plausible that children exposed to carcinogens in ETS could be at risk for cancer later in life. However, the extent of tobacco constituent uptake by children has not been quantified in any epidemiological study of childhood exposure and lung cancer risk.

Our goal in this study was to investigate the uptake by elementary school-aged children (grades 2–5) of the lung carcinogen NNK (Fig. 1). NNK is a potent pulmonary carcinogen in rodents and may play a significant role as a cause of lung cancer in smokers (9–11). NNK uptake can be quantified by analysis of two metabolites, NNAL and NNAL-Gluc, in urine (Fig. 1; Ref. 12). Because NNK is found only in tobacco products, the presence of NNAL and NNAL-Gluc in urine is a specific biomarker of tobacco carcinogen exposure (9). We have demonstrated previously the presence of these NNK metabolites in the urine of adult nonsmokers exposed to ETS, but there have been no reports of NNK uptake by ETS-exposed children (13–15). Although numerous studies have measured levels of the nicotine metabolite cotinine in blood, urine, saliva,

Received 4/20/01; revised 7/27/01; accepted 9/11/01.

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<sup>1</sup> This study was supported by National Cancer Institute Grant CA-81301 and Science to Achieve Results Grants R825813 and R826789 from the United States Environmental Protection Agency. The Shared Resources are supported in part by National Cancer Institute Cancer Center Support Grant CA-77598.

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<sup>3</sup> The abbreviations used are: ETS, environmental tobacco smoke; GC-MS/MS, gas chromatography-tandem mass spectrometry; GC-TEA, gas chromatography-nitrosamine selective detection; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; NNAL-Gluc, 4-(methylnitrosamino)-1-(3-pyridyl)-1-(*O*- $\beta$ -D-glucopyranuronosyl)butane [some of the pyridine-*N*-glucuronide, 4-(methylnitrosamino)-1-(3-pyridyl)-*N*- $\beta$ -D-glucopyranuronosyl)-1-butanolium inner salt, may also be present]; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; SHIELD, School Health Initiative: Environment, Learning, Disease; HPLC, high-performance liquid chromatography; ID, inner diameter.

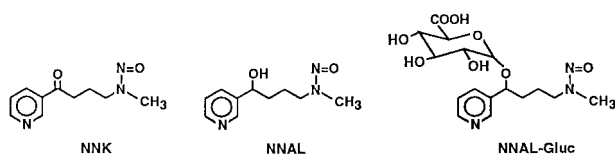


Fig. 1. Structures of NNK, NNAL, and NNAL-Gluc.

and hair of ETS-exposed children, there are scant data on tobacco carcinogen uptake by children (16). Higher levels of polycyclic aromatic hydrocarbon-albumin adducts and 4-aminobiphenyl-hemoglobin adducts have been observed in ETS-exposed *versus* unexposed children (17, 18). Comparisons of NNAL and NNAL-Gluc levels in children and adults exposed to ETS could provide an index of their relative uptake of NNK, perhaps resulting in additional insights concerning lung cancer risk. The children in this study were part of the SHIELD study, a novel school-based investigation of the environmental health of children in economically disadvantaged neighborhoods of Minneapolis (19).

## Materials and Methods

**Subjects and Data Collection.** This study was approved by the University of Minnesota Research Subjects' Protection Program Institutional Review Board: Human Subjects Committee. Details of the study population and objectives of the SHIELD study have been described (19). Briefly, children and their families eligible to participate in SHIELD were identified and contacted based on enrollment information obtained from the Minneapolis Public Schools. Students at either the Lyndale or Whittier elementary school were selected by stratified random sampling. All of the siblings of each selected student who were also students at those schools were also included in the study. All of the families received an initial contact letter from the principal of their child's school describing the study, encouraging participation, and explaining that a recruiter would contact them. Recruiters met with children and their families in their homes to explain the study and answer any questions. For those who agreed to volunteer, recruiters obtained verbal and written consent/assent and administered a questionnaire to the caregiver asking, among other things, about ETS exposure in the residence. The caregiver was asked the following questions: (a) in the past month, how many packs of cigarettes did you smoke inside the home in the presence of the child; and (b) how many smokers who live with the child smoke inside the child's house. If the answer was  $\geq 1$  to either of these questions, the child was considered exposed to ETS. We also asked about other exposures, as follows: (a) on average for the past month, what was the number of minutes or hours per week the child spent in a car, bus, van, or other enclosed vehicle with anyone who was smoking tobacco; and (b) on average for the past month, what was the number of minutes or hours per week the child spent in any other indoor or enclosed location with anyone who was smoking tobacco. Positive answers to these questions were taken to indicate exposure to ETS.

Urine samples were obtained at school under the supervision of the school nurse. Organic fruit juices were provided to the children at the start of the school day to increase the volume of the urine samples. Urine was collected in 4-oz polypropylene containers.

**Laboratory Analyses.** Total cotinine [cotinine plus pyridyl-*N*- $\beta$ -D-glucopyranuronosyl(-S)-(-)-cotininium inner salt] was

measured by gas chromatography-mass spectrometry, as described (20). All of the usable samples with total cotinine  $\geq 10$  ng/ml as well as a selection of those with total cotinine  $< 10$  ng/ml were analyzed for NNAL and NNAL-Gluc. This approach was taken to conserve time and resources, because the NNAL and NNAL-Gluc assay is more labor intensive than the total cotinine assay. The 10 ng/ml cotinine value was chosen to indicate potentially high exposure to ETS. NNAL and NNAL-Gluc were quantified by GC-TEA, using modifications of methods described previously (13, 14). Urine (20 ml) in a 50-ml disposable glass centrifuge tube (Kimble, Vineland, NJ) was adjusted to pH  $7.0 \pm 0.5$ . The sample was partitioned three times with equal volumes of ethyl acetate. The samples were shaken gently on a bench top shaker (Glas-Col, Terre Haute, IN) for 5–10 min, and any resulting emulsions were reduced by low speed centrifugation. The combined ethyl acetate extracts were placed in a 4-oz amber bottle, and 1 ng of *iso*-NNAL internal standard, dissolved in acetonitrile, was added along with  $\sim 5$  g of sodium sulfate. The mixture was briefly shaken. After  $\sim 0.5$  h, the ethyl acetate extract containing the free NNAL was transferred to a new 50-ml centrifuge tube and concentrated to dryness in portions on a model SVT200H SpeedVac centrifugal concentrator (Savant Instruments, Farmingdale, NY) and stored at  $-20^\circ\text{C}$  until HPLC cleanup. The extracted urine containing NNAL-Gluc was reduced to approximately two-thirds of its original volume in the SpeedVac to remove residual ethyl acetate, which may inhibit  $\beta$ -glucuronidase activity. This concentrated urine was treated with 25,000 units of  $\beta$ -glucuronidase type IX-A from *Escherichia coli* (Sigma Chemical Co., St. Louis, MO), and the solution was incubated overnight with gentle shaking at  $37^\circ\text{C}$ . The pH was adjusted to  $7.0 \pm 0.5$ , 1 ng of *iso*-NNAL was added, and the urine was extracted three times with equal volumes of methylene chloride. The methylene chloride extracts containing NNAL released from NNAL-Gluc were dried with sodium sulfate and concentrated to dryness in the same manner used for the ethyl acetate extracts.

The residue of the ethyl acetate extract was transferred with 400  $\mu\text{l}$  of potassium phosphate buffer (pH 7.0) 0.1 M (prepared from 0.1 M  $\text{KH}_2\text{PO}_4$  adjusted to pH 7 with  $\text{H}_3\text{PO}_4$ ) and 500  $\mu\text{l}$  of  $\text{H}_2\text{O}$  to an autosampler vial. The residue of the methylene chloride layer was similarly transferred with 50  $\mu\text{l}$  of methanol, 400  $\mu\text{l}$  potassium phosphate buffer 0.1 M (pH 7.0) and 450  $\mu\text{l}$  of  $\text{H}_2\text{O}$ . Ten  $\mu\text{l}$  of an aqueous solution of the collection markers consisting of 50  $\mu\text{g}$  2-pyridylcarbinol acetate and 50  $\mu\text{g}$  of 3-acetylpyridine were added to each vial. The HPLC eluant was monitored at 254 nm, and the fraction between the apices of the two marker compounds was collected. The column used was a 150 mm  $\times$  4.6 mm Bondclone C18 (Phenomenex, Torrance, CA) with a flow rate of 1 ml/min. Solvent "A" was  $\text{H}_2\text{O}$  and solvent "B" was methanol. The solvent program was as follows: a linear gradient from 90% to 55% A over 20 min, then to the initial composition over 2 min, then re-equilibration for 15 min. The collection markers eluted at  $\sim 11$  and  $\sim 16$  min. The fraction containing NNAL and *iso*-NNAL was collected in a 15-ml glass centrifuge tube (Kimble) and was concentrated to dryness on the SpeedVac.

For normal phase cleanup, the residue from the reverse-phase HPLC collection was dissolved in 200  $\mu\text{l}$  of ethyl acetate containing 5% isopropyl alcohol. 3-Pyridylpropanol (0.5  $\mu\text{g}$ ) was added as a collection marker. The column was a 250 mm  $\times$  4.0 mm Luna 5  $\mu$  silica column (Phenomenex), which was used at a flow rate of 1 ml/min. The solvent system was isocratic: 82% chloroform and 18% isopropyl alcohol. The NNAL/*iso*-NNAL fraction was collected starting just after the marker peak

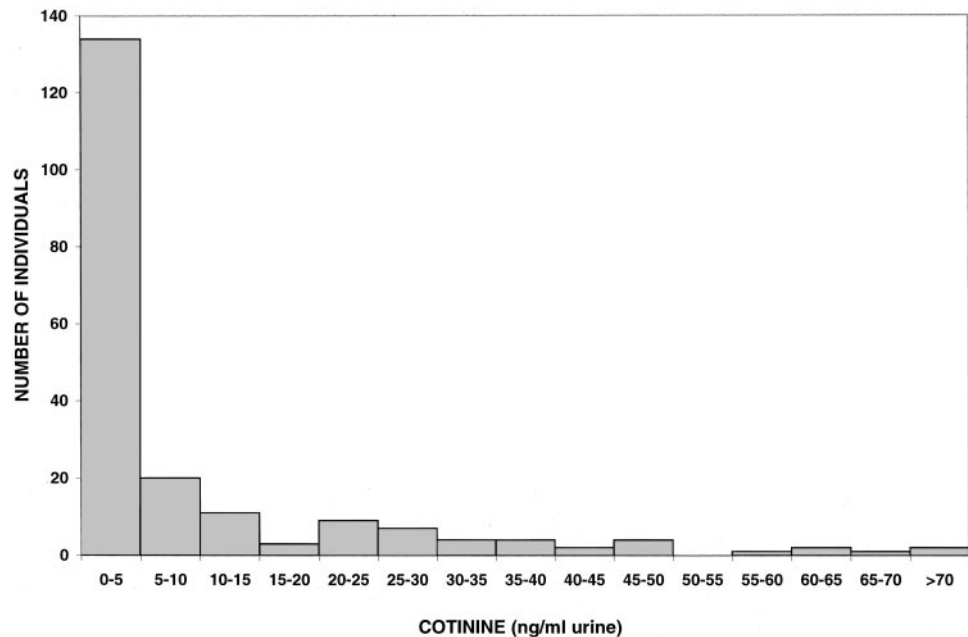


Fig. 2. Distribution of total cotinine levels in the urine of 207 elementary school-aged children.

eluted. The collection ended 8 min later. The collected material was concentrated to dryness using the SpeedVac.

The residue was transferred with methanol to a microvial (Kimble) and was brought to dryness using the SpeedVac. Five  $\mu\text{l}$  of 99% bis-trimethylsilyltrifluoroacetamide containing 1% trimethylchlorosilane (Regis Technologies, Morton Grove, IL) was added. *N*-Nitrosopentyl-3-picolylamine (TRC, North York, Ontario) was added to each vial as an injection standard at a final concentration of 0.40 ng/ $\mu\text{l}$ . The vials were capped, heated at 50°C for 60 min, and mixed intermittently. Four  $\mu\text{l}$  were injected in the pulse splitless mode on the GC-TEA from a cooled autosampler tray. The GC-TEA consisted of a HP 6890 gas chromatograph (Agilent Technologies, Wilmington, DE) interfaced to a model 543 Thermal Energy Analyzer (Orion Research, Beverly, MA). The pyrolyzer of the TEA and interface temperatures were 500 and 275°C, respectively. The separation was performed on a 30 m  $\times$  0.32 mm ID, 0.25- $\mu\text{m}$  film thickness, DB-1701 column (Agilent Technologies) attached to a 2 m  $\times$  0.32 mm ID deactivated retention gap. The injection port temperature was 225°C. A pressure program was used to keep the flow rate of helium at a constant 2.6 ml/min. The oven temperature was initially held at 80°C for 2 min and was then ramped to 180°C at 20°C/min. It was then ramped to 210°C at 2°C/min. From 210°C it was increased to 250°C at 20°C/min and held for 5 min.

GC-MS/MS was carried out as described (14).

**Statistical Analysis and Related Considerations.** Children were sampled with probabilities specific to strata defined by school, grade, ethnicity, and sex, so all of the analyses were weighted to account for these probabilities and for nonresponse. Furthermore, if a child with siblings in the school was selected, all of the siblings were selected as well. Because children in the same family had correlated exposures, all of the analyses accounted for this correlation. Because only a fraction of urine samples with cotinine level <10 ng/ml were analyzed for NNAL and NNAL-Gluc levels, there were different selection probabilities depending on cotinine level. Thus, analyses were additionally weighted for these different sampling rates. Anal-

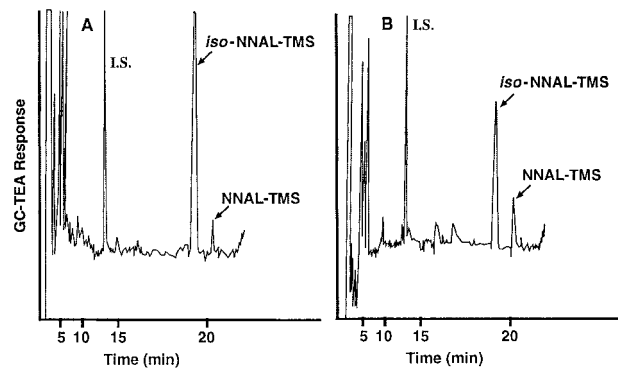


Fig. 3. Chromatograms obtained on GC-TEA analysis of the (A) NNAL fraction and (B) NNAL-Gluc fraction of a child's urine. I. S., injection standard; TMS, trimethylsilyl ether.

yses were performed on log-transformed laboratory values to correct for skewness in the distributions, and transformed means were exponentiated to obtain geometric means. Confidence intervals were calculated in the transformed scale and back-transformed by taking logs.

A randomization test using the weighted means and preserving the family grouping was used to compare total cotinine and NNK metabolite levels in the urine of children with reported ETS exposure to those of children with no reported ETS exposure. In the one instance where siblings reported different exposures, the index child's exposure was used for the family. To compare February and May values, a randomization test using weighted means that preserved pairing and family grouping was used. Correlations between repeated laboratory measurements were estimated and tested by weighted linear models.

On the basis of previous data in the literature, subjects with <5 ng/ml total cotinine in their urine were not aware of any ETS exposure (21, 22). Thus, for analyses relating NNAL and NNAL-Gluc levels to total cotinine levels, a cutoff of 5

Table 1 NNAL, NNAL-Gluc, and total cotinine<sup>a</sup> in the urine of elementary school-aged children<sup>b</sup>

Group	No. of families/children	No. of children							
		NNAL or NNAL-Gluc detected	NNAL and NNAL-Gluc not detected <sup>c</sup>	Total cotinine $\geq 5$ ng/ml	Total cotinine $< 5$ ng/ml	Total cotinine $\geq 5$ ng/ml		Total cotinine $< 5$ ng/ml	
						NNAL or NNAL-Gluc detected	NNAL and NNAL-Gluc not detected	NNAL or NNAL-Gluc detected	NNAL and NNAL-Gluc not detected
All	66/74	62	12	54	20	52	2	10	10
Exposure reported <sup>d,e</sup>	33/38	35	3	34	4	33	1	2	2
No exposure reported <sup>d,e</sup>	32/35	26	9	20	15	19	1	7	8

<sup>a</sup> Cotinine plus cotinine-*N*-Gluc.

<sup>b</sup> Samples analyzed for total cotinine, NNAL, and NNAL-Gluc.

<sup>c</sup> Detection limit, 0.003 pmol/ml.

<sup>d</sup> Based on questionnaire data.

<sup>e</sup> Questionnaire data not available on 1 subject.

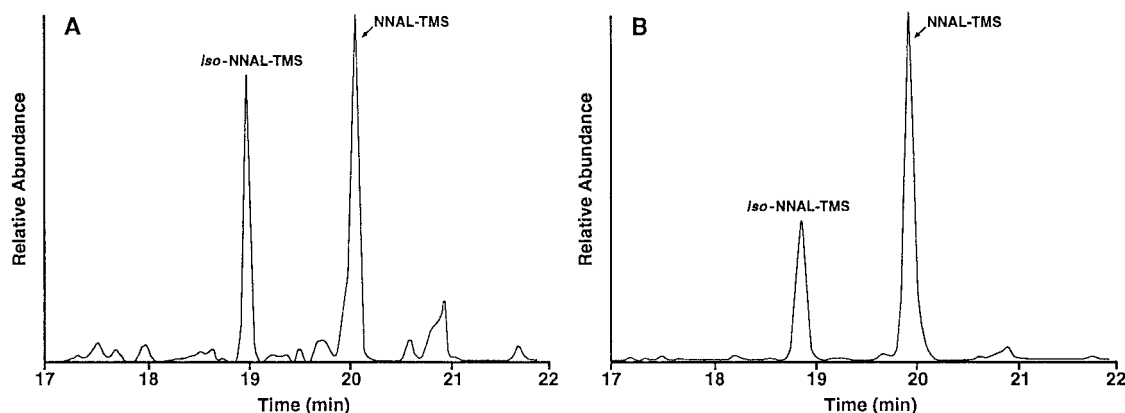


Fig. 4. Chromatograms obtained on GC-MS/MS analysis ( $m/z$  282 $\rightarrow$  $m/z$  162) of the (A) NNAL fraction and (B) NNAL-Gluc fraction of a child's urine. TMS, trimethylsilyl ether

ng/ml total cotinine was used to indicate potential ETS exposure. In agreement with these data, a recent study found mean total cotinine levels in nonsmoking men and their spouses, who reported no ETS exposure, to be  $2.2 \pm 3.1$  ng/ml urine (15). Tests of NNAL detection fraction by these cotinine strata were performed using weighted log-linear models.

## Results

Urine was collected from most of the children participating in the SHIELD study (19). Forty-eight percent of these children were female. The mean ( $\pm$ SD) age of the children was  $8.91 \pm 1.21$  years. In 95 children, urine samples were collected in both February and May, 2000. In another 77 children, urine was collected in February only, whereas in 32 children, samples were obtained in May only. The total number of urine samples was 299, collected from 204 children.

Seventy of the 204 children (34.3%) had total cotinine levels  $\geq 5$  ng/ml urine. The distribution of total cotinine values is illustrated in Fig. 2. The mean level of total cotinine in the initial samples with amounts  $\geq 5$  ng/ml urine was  $25.5 \pm 22.6$  (SD) ng/ml. Among the 95 children for whom urine samples were available in both February and May, 38 (40.0%) had total cotinine levels  $\geq 5$  ng/ml in February. Of these, 24 also had total cotinine  $\geq 5$  ng/ml in May. There was no significant difference between levels of total cotinine in the urine of these children in February *versus* May (paired *t* test). The correlation

of total cotinine levels in individual children in February and May was 0.703 ( $P < 0.0001$ ).

There were a total of 90 samples for which NNAL and NNAL-Gluc were measured. Fifty-seven urine samples (from 43 children in 39 families) of the 71 urine samples in which total cotinine levels were  $>10$  ng/ml and 33 samples (from 31 children in 27 families) of the 228 with  $<10$  ng/ml total cotinine were analyzed for NNAL and NNAL-Gluc. Of these samples, 50 were from children who gave urine in February, 8 in May, and 32 were from children who gave urine in both February and May.

Typical GC-TEA traces from samples of urine positive for NNAL and NNAL-Gluc in children are illustrated in Fig. 3. The identities of NNAL and NNAL-Gluc were confirmed by GC-MS/MS of selected samples (Fig. 4). Both types of analysis showed clear peaks for NNAL-trimethylsilyl ether.

The relationship between total cotinine and NNAL or NNAL-Gluc in urine is summarized in Table 1. NNAL or NNAL-Gluc was detected in 52 of 54 samples in which total cotinine was  $\geq 5$  ng/ml and in 10 of 20 samples in which total cotinine was  $< 5$  ng/ml. Thus, detection of NNAL or NNAL-Gluc was significantly more likely when total cotinine was  $\geq 5$  ng/ml than when it was  $< 5$  ng/ml ( $P < 0.0001$ ). When exposure to ETS was reported by questionnaire, NNAL or NNAL-Gluc was detected in 35 of 38 samples, whereas it was detected in 26 of 35 samples in which no exposure was



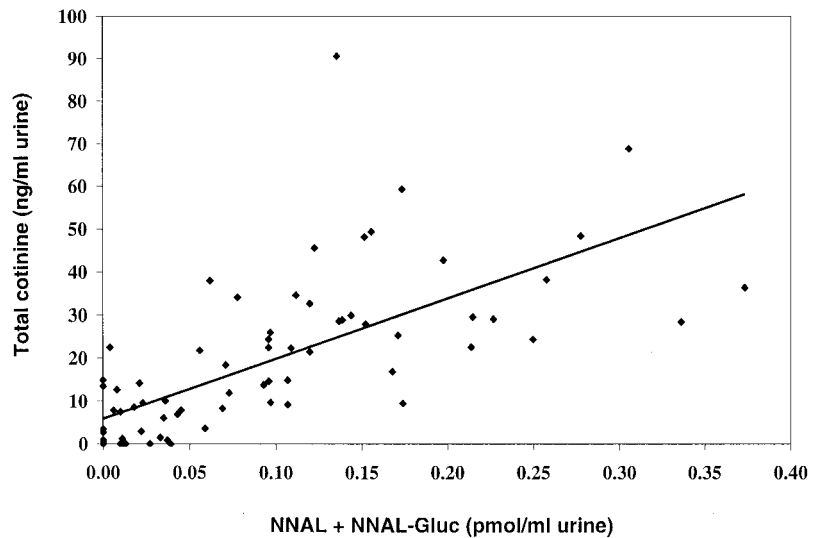


Fig. 5. Relationship between levels of total cotinine and NNAL plus NNAL-Gluc in the urine of 74 children.  $r = 0.71$ ;  $P < 0.0001$ .

Table 2 NNAL, NNAL-Gluc, and total cotinine in the urine of elementary school-aged children

Group	No. of families	No. of children	pmol/ml			Total cotinine (ng/ml)
			NNAL	NNAL-Gluc	NNAL + NNAL-Gluc	
All	66 <sup>b</sup>	74				
Mean <sup>a</sup> ( $\pm$ SD)			0.018 <sup>c</sup> ( $\pm$ 0.030)	0.040 <sup>d</sup> ( $\pm$ 0.050)	0.056 <sup>e</sup> ( $\pm$ 0.076)	12.0 ( $\pm$ 17.8)
Geometric mean			0.005	0.012	0.015	2.5
95% CI			(0.003 to 0.009)	(0.006 to 0.022)	(0.008 to 0.028)	(1.2 to 5.0)
Exposure reported	33 <sup>b</sup>	38				
Mean <sup>a</sup> ( $\pm$ SD)			0.032 <sup>f</sup> ( $\pm$ 0.039)	0.064 <sup>g</sup> ( $\pm$ 0.056)	0.095 <sup>h</sup> ( $\pm$ 0.088)	24.5 <sup>i</sup> ( $\pm$ 22.4)
Geometric mean			0.011	0.028	0.04	12.6
95% CI			(0.006 to 0.021)	(0.015 to 0.05)	(0.022 to 0.074)	(7.4 to 21.3)
No exposure reported	32 <sup>b</sup>	35				
Mean <sup>a</sup> ( $\pm$ SD)			0.010 ( $\pm$ 0.020)	0.026 ( $\pm$ 0.040)	0.035 ( $\pm$ 0.058)	5.0 ( $\pm$ 8.7)
Geometric mean			0.003	0.007	0.008	1.0
95% CI			(0.001 to 0.007)	(0.003 to 0.019)	(0.003 to 0.023)	(0.4 to 2.9)
Total cotinine <5 ng/ml	19	20				
Mean <sup>a</sup> ( $\pm$ SD)			0.005 ( $\pm$ 0.010)	0.012 ( $\pm$ 0.020)	0.016 ( $\pm$ 0.030)	1.2 ( $\pm$ 1.6)
Geometric mean			0.002	0.004	0.004	0.45
95% CI			(0.001 to 0.003)	(0.002 to 0.01)	(0.002 to 0.012)	(0.2 to 1.0)

<sup>a</sup> Mean values are calculated from among family means, which in turn, are calculated from the student means within each family. The means are weighted to account for nonresponse and sample selection. For samples with levels below the detection limit, half of the minimum detectable level (0.0015 pmol/ml for NNAL and NNAL-Gluc and 0.2 ng/ml for cotinine) was added to calculate mean values.

<sup>b</sup> Of the 66 families that were tested for NNAL and NNAL-Gluc, only 1 family with 1 child did not have questionnaire data on exposure.

<sup>c</sup> Includes 28 not detected (ND), see footnote a.

<sup>d</sup> Includes 15 ND, see footnote a.

<sup>e</sup> Includes 12 ND, see footnote a.

<sup>f</sup> Significantly higher than "No exposure reported,"  $P = 0.002$ .

<sup>g</sup> Not significantly higher than "No exposure reported,"  $P = 0.053$ .

<sup>h</sup> Significantly higher than "No exposure reported,"  $P = 0.031$ .

<sup>i</sup> Significantly higher than "No exposure reported,"  $P < 0.001$ .

reported. Total cotinine was  $\geq 5$  ng/ml in 34 of 38 samples from children having reported exposure to ETS and in 20 of 35 samples with no reported exposure. Exposure data were not available for 1 child.

NNAL plus NNAL-Gluc and total cotinine correlated in the 74 children ( $r = 0.71$ ;  $P < 0.0001$ ; Fig. 5). Levels of NNAL, NNAL-Gluc, and total cotinine are summarized in Table 2. Mean levels ( $\pm$ SD) of NNAL, NNAL-Gluc, and NNAL plus NNAL-Gluc were  $0.018 \pm 0.030$ ,  $0.040 \pm 0.050$ , and  $0.056 \pm 0.076$  pmol/ml, respectively, in the 74 children. Levels of NNAL, NNAL-Gluc, NNAL plus NNAL-Gluc, and

total cotinine were higher when exposure was reported than when it was not, and the differences were mostly significant: NNAL ( $P = 0.002$ ); NNAL-Gluc ( $P = 0.053$ ); NNAL plus NNAL-Gluc ( $P = 0.031$ ); and total cotinine ( $P < 0.001$ ). However, NNAL ( $0.010 \pm 0.020$  pmol/ml), NNAL-Gluc ( $0.026 \pm 0.040$  pmol/ml), NNAL plus NNAL-Gluc ( $0.035 \pm 0.058$  pmol/ml), and total cotinine ( $5.0 \pm 8.7$  ng/ml) were detected even when no exposure was reported. These levels were higher than "background levels" of NNAL, NNAL-Gluc, NNAL plus NNAL-Gluc, and total cotinine determined in the urine of children with cotinine levels  $< 5$  ng/ml (Table 2).

Table 3 NNAL, NNAL-Gluc, and total cotinine in the urine of elementary school-aged children collected in two different seasons

Subject	pmol/ml			Total cotinine (ng/ml)
	NNAL	NNAL-Gluc	NNAL + NNAL-Gluc	
1 <sup>a</sup>	ND <sup>b</sup>	0.021	0.021	14.2
	0.017	0.11	0.13	53.8
2	0.11	0.20	0.31	68.8
	0.33	0.18	0.51	41.9
3	ND	0.013	0.013	ND
	ND	ND	ND	17.7
4	0.03	0.17	0.20	42.8
	ND	0.06	0.059	20.5
5	0.12	0.095	0.22	29.6
	0.014	0.026	0.04	54.8
6	ND	0.036	0.036	10.1
	0.013	0.043	0.056	26.8
7	0.046	0.076	0.12	21.5
	0.044	0.16	0.20	22.3
8	0.018	0.089	0.11	14.9
	0.043	0.13	0.17	32.1
9	0.081	0.087	0.17	16.9
	0.036	0.11	0.14	42.3
10	0.014	0.042	0.056	21.8
	0.016	0.081	0.097	26.0
11	0.065	0.27	0.34	28.4
	0.024	0.19	0.21	10.2
12	0.045	0.051	0.096	24.4
	0.049	0.12	0.17	91.7
13	ND	0.097	0.097	9.7
	0.074	0.026	0.10	ND
14	0.049	0.17	0.21	22.6
	0.19	0.27	0.45	22.9
15	0.033	0.079	0.11	34.6
	0.033	0.14	0.17	25.2
16	0.11	0.14	0.25	24.4
	0.016	0.024	0.040	20.1

<sup>a</sup> First line, urine collected in February 2000; second line, urine collected in May 2000.

<sup>b</sup> ND, not detected; detection limit 0.4 ng/ml.

NNAL and NNAL-Gluc were not detected in water blanks analyzed at the same time as the urine samples of the children.

Data from urine samples collected from 16 children in both February and May are summarized in Table 3. Some values increased between February and May, and others decreased or did not change. Overall, levels of total cotinine, NNAL, NNAL-Gluc, and NNAL plus NNAL-Gluc were not significantly different at the two collection intervals.

The distributions of NNAL-Gluc:NNAL ratios in the SHIELD children and in 231 adult smokers from previous studies are illustrated in Fig. 6. These distributions were not significantly different.

## Discussion

The results of this study demonstrate widespread and substantial uptake of nicotine and NNK, as measured by urinary levels of their metabolites total cotinine, NNAL, and NNAL-Gluc, in this group of economically disadvantaged elementary school-aged children. Our results confirm the uptake of these tobacco smoke components even in the absence of reported exposure to tobacco smoke at home or in vehicles, as determined by questionnaires. This is the first study to demonstrate the uptake of the tobacco-specific lung carcinogen NNK in children of this age group. Based both on questionnaire and biomarker data, it

is likely that uptake of nicotine and NNK by these children was attributable to ETS exposure, but we cannot exclude the possibility that some children may have smoked a cigarette.

More than 34% of the 204 children had urinary total cotinine  $\geq 5$  ng/ml. Among the samples with  $\geq 5$  ng/ml total cotinine, which were also analyzed for NNAL and NNAL-Gluc, 96.2% were positive for these carcinogen metabolites. But surprisingly, 10/20 (50%) of samples in which total cotinine levels were  $< 5$  ng/ml were also positive for NNAL or NNAL-Gluc. The mean level of NNAL plus NNAL-Gluc in these 20 samples was  $0.016 \pm 0.030$  pmol/ml urine. We did not analyze all of the samples for NNAL and NNAL-Gluc, but extrapolation of our results suggests that 134 of 204 (66%) of the children would have positive NNAL or NNAL-Gluc levels. The more frequent detection of NNAL and NNAL-Gluc than of total cotinine may relate to differences in the pharmacokinetics of these metabolites. We have shown previously that the decay of NNAL and NNAL-Gluc from urine is slow after cessation of smoking ( $t_{1/2\alpha}$ , 3–4 days;  $t_{1/2\beta}$ , 40–45 days) compared with rapid disappearance of total cotinine (20). Therefore, the probability of detecting NNAL or NNAL-Gluc after a given exposure to tobacco smoke may be greater than that of detecting cotinine. The widespread occurrence of NNAL and NNAL-Gluc in the urine of elementary school-aged children is a cause for concern.

There was consistency in our data, as indicated by comparisons of the NNAL and NNAL-Gluc levels in 16 children sampled in both February and May (Table 3). There were no significant differences in these biomarkers at the two different sampling times. There were also no significant differences between total cotinine levels in 95 children who were sampled at both times.

Levels of NNAL, NNAL plus NNAL-Gluc, and total cotinine were significantly higher in the urine of children classified as “exposed to ETS” than in the urine of children classified as “unexposed,” based on questionnaire data. However, substantial amounts of NNAL plus NNAL-Gluc were detected even in the urine of nominally unexposed children. The mean level of NNAL plus NNAL-Gluc in these children was  $0.035 \pm 0.058$  pmol/ml urine, which was substantially greater than in the urine of children with cotinine  $< 5$  ng/ml ( $0.016 \pm 0.030$  pmol/ml). The level of NNAL plus NNAL-Gluc in these supposedly unexposed children can be compared with a level of  $0.050 \pm 0.068$  in the urine of women who live with smokers (Table 4; Ref. 15). These amounts are about 2–3% of the levels typically found in smokers.

Levels and ranges of NNAL and NNAL-Gluc detected in this study are compared with those reported in our previous studies of adult nonsmokers in Table 4. The amounts found here were comparable with those in the urine of women exposed to ETS because of the smoking by their spouse (15) and in the urine of hospital workers exposed occupationally to ETS (14). They were lower than those in men exposed heavily to ETS in a chamber (13). The present amounts were higher than in the amniotic fluid of mothers who smoke but lower than in the urine of newborns exposed transplacentally to NNK (23, 24). The range of detected values (93-fold) for all of the children in the present study was greater than in our previous studies. The range of NNAL plus NNAL-Gluc in children with reported exposure to ETS was 0.004–0.373 pmol/ml (93-fold), and in those with no reported exposure it was 0.008–0.258 pmol/ml (32-fold). These large ranges may have important implications for interpretation of epidemiological studies of childhood exposure to ETS and cancer later in life. All such studies have been based on questionnaire data, which may lead

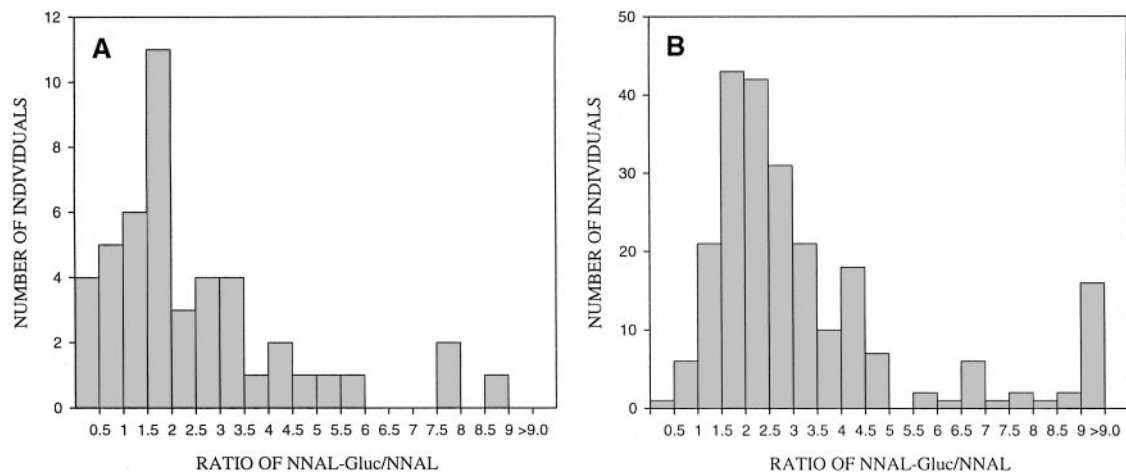


Fig. 6. Distribution of NNAL-Gluc:NNAL ratios in (A) 46 school children (NNAL or NNAL-Gluc were not detected in 28 samples) and (B) 231 adult smokers from previous work.

Table 4 NNK uptake by nonsmokers

Fluid	Analyte	Correlation with cotinine	Mean $\pm$ S.D. pmol/ml	Range <sup>a</sup> (fold)	Reference
Amniotic <sup>b</sup>	NNAL	No	0.025 $\pm$ 0.029 ( <i>n</i> = 21)	0.009–0.082 (9.1)	24
Urine of newborns <sup>b</sup>	NNAL + NNAL-Gluc	Yes	0.13 $\pm$ 0.15 ( <i>n</i> = 28)	0.044–0.4 (9.1)	23
Urine of women married to smokers	NNAL + NNAL-Gluc	No	0.050 $\pm$ 0.068 ( <i>n</i> = 23)	0.009–0.28 (31)	15
Urine of hospital workers	NNAL-Gluc	Yes	0.059 $\pm$ 0.028 ( <i>n</i> = 27)	0.005–0.11 (22)	14
Urine of men exposed to ETS in a chamber	NNAL + NNAL-Gluc	Yes	0.16 $\pm$ 0.10 <sup>c</sup> ( <i>n</i> = 7)	0.084–0.296 (4)	13
Urine of elementary school-aged children	NNAL + NNAL-Gluc	Yes	0.056 $\pm$ 0.076 ( <i>n</i> = 74)	0.004–0.373 (93)	This study

<sup>a</sup> Detected values only.

<sup>b</sup> Mothers were smokers.

<sup>c</sup> Approximate, based on excretion of 1 liter of urine/day.

to misclassification of exposed children and possibly to inaccurate conclusions about carcinogen uptake. It is clear that questionnaire data alone do not adequately characterize exposure by children to ETS.

NNAL plus NNAL-Gluc correlated with total cotinine in this study (Fig. 5). The correlation coefficient,  $r = 0.71$ , was similar to that in 223 smokers,  $r = 0.68$ , in which total cotinine and NNAL plus NNAL-Gluc were determined by essentially the same methods used here (10). We have not observed this correlation in all of our ETS studies (Table 4) but that may be attributable to the relatively small numbers of subjects in some of the studies. Collectively, the data strongly indicate that total cotinine and NNAL plus NNAL-Gluc are uptake biomarkers. Total cotinine is a biomarker of nicotine uptake, whereas NNAL plus NNAL-Gluc is a biomarker for uptake of NNK, a lung carcinogen. It is unclear whether total cotinine would be a good surrogate for NNAL plus NNAL-Gluc, because NNAL plus NNAL-Gluc are generally longer-lived biomarkers.

In summary, the results of this study demonstrate considerable uptake of the tobacco-specific lung carcinogen NNK in this group of elementary school-aged children. Although it is difficult to quantify the public health risk this uptake represents, it is potentially larger than acceptable. Our results indicate that biomarkers of carcinogen uptake should be incorporated into studies of childhood exposure to ETS and cancer development later in life.

## Acknowledgments

We thank Ky-Anh Le, London Losey, and Abigail Kritzer for their outstanding technical assistance; Peter Villalta, Analytical Chemistry and Biomarkers Facility, for MS data; and Robin Bliss, Biostatistics Facility, for statistical analyses. We thank the children and parents who volunteered to participate in this study, and appreciate the cooperation we received from the Minneapolis Public Schools and the principals and staff at both the Lyndale and Whittier schools.

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*Cancer Epidemiol Biomarkers Prev* 2001;10:1109-1116.

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