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Urinary levels of the tobacco-specific carcinogen *N′*-nitrosonornicotine and its glucuronide are strongly associated with esophageal cancer risk in smokers

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***N′*-Nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) are tobacco-specific nitrosamines. NNN and NNK can induce cancers of the esophagus and lung, respectively, in laboratory animals, but data on human esophageal cancer are lacking. The association between levels of NNN and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), an NNK metabolite, in urine samples collected before diagnosis and risk of esophageal cancer was examined in 77 patients with esophageal cancer and 223 individually matched controls, all current smokers, from a cohort of 18244 Chinese men in Shanghai, China, followed from 1986 to 2008. Urinary total NNN (free NNN plus NNN-*N*-glucuronide) was significantly higher, whereas the percentage of its detoxification product NNN-*N*-glucuronide was significantly lower in cases than controls. Odds ratios (95% confidence intervals) of esophageal cancer for the second and third tertiles of total NNN were 3.99 (1.25–12.7) and 17.0 (3.99–72.8), respectively, compared with the first tertile after adjustment for urinary total NNAL and total cotinine and smoking intensity and duration ($P_{trend} < 0.001$). The corresponding figures for the percentage of NNN-*N*-glucuronides were 0.37 (0.17–0.80) and 0.27 (0.11–0.62) ($P_{trend} = 0.001$). Urinary total NNN and the percentage of NNN-*N*-glucuronides almost completely accounted for the observed association for urinary total NNAL (free NNAL plus its glucuronides), urinary total cotinine and smoking intensity with esophageal cancer risk. These findings along with results of previous studies in laboratory animals support a significant and unique role of NNN in esophageal carcinogenesis in humans.**

Introduction

Although esophageal cancer is rare in most Western countries, the incidence varies greatly worldwide and is relatively high in Asia (1). Tobacco smoking and alcohol use are established major risk factors for esophageal cancer (2–4). However, the association between cigarette smoking and esophageal cancer risk is not as strong as that for lung cancer. The relative risk for smokers of more than one pack a day relative to never-smokers ranges approximately from 2 to 5 for esophageal cancer (4–6), whereas it ranges from 15 to 30 for lung cancer (7,8). Furthermore, most smokers do not develop esophageal cancer over their lifetime. The variation underlying the susceptibility to smoking-related cancer may be determined by the amounts of carcinogens present in tobacco smoke, their uptake and metabolism and by genetic and environmental factors. There are >70 established carcinogens in cigarette smoke (3). Among these, the tobacco-specific nitrosamine *N′*-nitrosonornicotine (NNN) is present in greater concentrations than any other esophageal carcinogens (9,10). The carcinogenic potential of NNN has been clearly demonstrated in a number of animal models (reviewed in ref. 11). In addition to inducing esophageal tumors in rats, NNN causes lung tumors in mice, respiratory tract tumors in hamsters and nasal cavity tumors in rats and mink (11). A mixture of NNN and the related tobacco carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)-induced tumors in the oral cavity of rats (12). On its own, NNK is a potent systemic lung carcinogen in laboratory animals. NNK also can induce tumors of the liver and pancreas in rats, but it is not known to induce esophageal tumors (13). Based on laboratory animal data and related mechanistic studies, NNN and NNK are classified as human carcinogens (Group I) by the International Agency for Research on Cancer (13).

Human exposure to NNN can be measured via quantitation of unchanged NNN (also called free NNN) and its detoxification product NNN-pyridine-*N*-glucuronide (NNN-*N*-Gluc) in urine (14). The sum of free NNN and NNN-*N*-Gluc is referred to as total NNN. There have been no epidemiological studies on NNN in relation to risk of any cancer in humans. Based on the findings of experimental studies in laboratory animals, further investigation of the relationship between urinary total NNN and esophageal cancer risk could provide important information on the role of this laboratory animal carcinogen in the development of tobacco-induced esophageal cancer in humans. Moreover, while the measurement of urinary total NNN is informative in terms of overall exposure to NNN, separate analysis of free NNN and NNN-*N*-Gluc may also provide useful information on the evaluation of the relative efficiency of NNN detoxification in individual smokers. An increased understanding of esophageal cancer etiology in smokers can point the way to rational methods of preventing this generally fatal disease.

Human exposure to NNK can be assessed by urinary levels of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) and its glucuronides (sum of which is denoted as total NNAL). We and others recently demonstrated a strong association between total NNAL in serum or urine and risk of lung cancer incidence in smokers (15,16). It is not known if NNK can induce esophageal cancer in laboratory animals or humans. Epidemiological studies on NNAL in relation to risk of esophageal cancer in humans are lacking. Since both NNK and NNN are present in unburned tobacco and tobacco smoke (13), the simultaneous examination of both NNAL and NNN would provide more specific information for understanding their role in the development of esophageal cancer.

We conducted a prospective case–control study of esophageal cancer nested within the Shanghai Cohort Study to examine if smokers who developed esophageal cancer had higher levels of total NNN and total NNAL in their urine samples collected before cancer diagnosis as compared with their smoking counterparts who remained free of cancer. Since glucuronidation is a detoxification pathway, we also examined the relationship between the percentage of NNN-*N*-Gluc and risk of esophageal cancer.

Materials and methods

Subjects

Details of the Shanghai Cohort Study have been previously published (17,18). In brief, the cohort consisted of 18 244 men (constituting 80% of eligible subjects) enrolled from January 1 1986 through September 30 1989 who were between 45 and 64 years of age and resided in one of four small geographically defined communities in Shanghai, China. In addition to in-person interviews eliciting information on use of tobacco and alcohol, usual diet and medical history, we collected a 10 ml blood sample and one single spot urine sample from each participant at baseline. The collection of biospecimens from study subjects usually took place between 5 pm and 9 pm. Following collection, the urine sample was immediately put in an icebox and transported on the same day to the processing laboratory to be stored at 4°C. On the next morning, two aliquots of untreated urine samples (10 ml each) per subject were prepared and stored at −20°C until 2001 when they were moved to −70°C freezers until

Abbreviations: 5-MeNNN, 5-methyl-N′-nitrosonornicotine; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, N′-nitrosonornicotine.

analysis. Written informed consent was obtained from each subject. The Shanghai Cohort Study has been approved by the Institutional Review Boards at the University of Minnesota and the Shanghai Cancer Institute.

Identification of incident esophageal cancer cases and deaths was accomplished through annual in-person re-interviews to all surviving cohort members and routine review of reports from the population-based Shanghai Cancer Registry and from the Shanghai Municipal Vital Statistics Office. As of December 31 2008, losses to follow-up totaled 985 individuals (5.4%) after 18–22 years of study.

As of December 31 2008, 115 cohort participants developed esophageal cancer. Among them, 82 were smokers, 4 were former smokers and 29 were never-smokers at baseline. The primary objectives of the present study required all study subjects to be smokers at the time of urine collection at enrollment. For each case who smoked cigarettes at baseline, we selected three control subjects randomly from the Shanghai cohort members who were smokers at enrollment, free of any cancer and alive at the time of cancer diagnosis of the index case. Controls were matched to the index case by age at enrollment (±2 years), the year and month of biospecimen collection (±1 month) and neighborhood of residence at recruitment.

Laboratory measurements

Urine samples of all study subjects were retrieved from the biospecimen bank and shipped in boxes packed with dry-ice to the Hecht laboratory at the University of Minnesota where all urinary biomarkers were quantified. Four urine sample aliquots within a given matched case–control set (the case plus three controls) were arranged in random order, were identified only by unique codes and were assayed in the same batch for each urinary biomarker by laboratory personnel who had no knowledge of the case/control status of the test samples.

To separately analyze free NNN and NNN-*N*-Gluc in urine samples, we modified our previously published method for total NNN (19–21). Briefly, 2 ml urine samples were extracted with ethyl acetate after the addition of 5-methyl-*N*′′-nitrosonornicotine (5-MeNNN) as internal standard. The organic layer containing free NNN was concentrated to dryness and further purified by mixed mode cation exchange and normal phase extraction as described previously (20). To the aqueous layer containing NNN-*N*-Gluc, [$^{13}C_6$]NNN internal standard was added, and the mixture was treated with 10 N NaOH to convert NNN-*N*-Gluc to NNN (19). After the hydrolysis, the samples were purified as described previously for total NNN (20). The samples were analyzed by liquid chromatography–tandem mass spectrometry with selected reaction monitoring for *m/z* 178 → *m/z* 148 for NNN, *m/z* 192 → *m/z* 162 for 5-MeNNN and *m/z* 184 → *m/z* 154 for [$^{13}C_6$]NNN. The intra-day precision measures of the assays for free NNN and NNN-*N*-Gluc were 7.7% relative standard deviation (RSD) and 8.4% RSD, respectively. The corresponding inter-day precision measures were 10.6 and 12.8% RSD.

Analysis of total NNAL was performed as described previously (22). Briefly, the method involved treatment of urine with β-glucuronidase to convert any NNAL-glucuronides to free NNAL, which was followed by solid-phase extraction and analysis by liquid chromatography – tandem mass spectrometry. The intra-day and inter-day precision values of the assay were 7.1 and 7.4% RSD, respectively.

Quantification of total cotinine was carried out by gas chromatography–mass spectrometry as described previously (23,24). The intra-day precision of the assay for total cotinine was 1.8% RSD and the inter-day precision was 2.8% RSD. Urinary creatinine (Cr) was assayed by Fairview-University Medical Center Diagnostic Laboratories (Minneapolis) with a Kodak Ektachem 500 chemistry analyzer.

Of the 82 cases and 246 matched controls, 5 cases and 23 controls were excluded due to insufficient urine samples after previous measurements. We tested urine samples of 77 cases and 223 controls for all urinary biomarkers described above. Both free NNN and NNN-*N*-Gluc were detected in all samples (limit of detection, 0.5 fmol/ml urine). The assay for urinary NNAL failed on eight subjects (three cases and five controls). Urinary total NNAL was detectable (limit of detection, 0.01 pmol/ml) in all but two urine samples. We included these two subjects in the data analysis after the half value of the limit of detection was assigned. Thus, the present study included 77 esophageal cancer cases and their 223 individually matched control subjects for the analysis of NNN. For the analysis of urinary total NNAL, we included 74 cases and 218 controls.

Statistical analysis

Urinary concentrations of free NNN, NNN-*N*-Gluc, total NNN (the sum of free NNN and NNN-*N*-Gluc) and total NNAL were expressed as fmol/mg creatinine (Cr) and cotinine as nmol/mg Cr to correct for varying water contents of individual spot urine samples. The percentage of NNN-*N*-Gluc was calculated as the concentration of NNN-*N*-Gluc divided by total NNN multiplied by 100. The distributions of all urinary biomarkers measured except the percentage of NNN-*N*-Gluc were markedly skewed toward high values, which were corrected to a large extent by transformation to logarithmic values. Therefore, formal statistical tests were performed on logarithmically transformed values and geometric means are presented.

The χ^2 test and the *t*-test were used to compare the distributions of selected demographics, cigarette smoking and alcohol consumption between esophageal cancer cases and controls. The analysis of covariance (ANCOVA) method (25) was used to examine the relationship of concentrations of total NNN and total NNAL with number of cigarettes per day and levels of urinary total cotinine. We used the same ANCOVA method to assess the statistical differences in concentrations of urinary biomarkers between cases and controls.

We used standard statistical methods to analyze data from matched case–control sets (26). Conditional logistic regression models were used to calculate odds ratios (ORs) and their corresponding 95% confidence intervals (CIs) and *P* values. Study subjects were grouped into tertiles according to the distribution of each biomarker measured among all control subjects. The linear trend test for the association between levels of biomarkers and esophageal cancer risk was based on ordinal values. Multivariate logistic regression models were used to assess the independent effect of one factor while simultaneously adjusting for other variables in the same model. To examine the interaction effect, a product term of the two variables of interest was created and included in the multivariate logistic regression model that also included the two main term variables. The result of the product term determined whether the two had a synergistic effect on risk of the disease.

All statistical analyses were carried out using SAS software version 9.1 (SAS Institute, Cary, NC). All *P* values reported are two sided and those that were <0.05 were considered as statistically significant.

Results

Of the 77 cases of esophageal cancer, 58 were confirmed histologically; there were 49 (84% of confirmed cases) squamous cell cancers, 4 (7%) adenocarcinomas and 5 (9%) other histological types. The remaining 19 cases were based on clinical diagnosis. The mean age (± standard deviation) of all case patients at cancer diagnosis was 68.3 (±8.0) years. The corresponding figure for matched control subjects at the time of cancer diagnosis of index cases was 67.9 (±7.5) years ($P = 0.74$). The average time interval between baseline biospecimen collection and cancer diagnosis was 11.5 (±5.6) years, ranging from 2 months to 20.7 years. Patients with esophageal cancer had slightly lower body mass index (21.3 ± 2.4 kg/m^2) than control subjects (21.9 ± 2.7 kg/m^2) ($P = 0.055$). Case patients attained higher level of education than controls ($P = 0.006$).

Table I shows the urinary levels of total NNAL, total NNN, free NNN, NNN-*N*-Gluc and the percentage of NNN-*N*-Gluc by the number of cigarettes smoked per day or urinary levels of total cotinine among control subjects only. Urinary levels of total NNAL, total NNN, free NNN and NNN-*N*-Gluc all increased with increasing number of cigarettes per day and urinary levels of total cotinine (all Ps < 0.001). The percentage of NNN-*N*-Gluc, however, was not associated with the number of cigarettes per day or urinary levels of total cotinine.

Urinary levels of total NNN, free NNN and NNN-*N*-Gluc were highly correlated with each other. Among control subjects only, the Spearman's correlation coefficient was 0.93 between total NNN and free NNN, 0.95 between total NNN and NNN-*N*-Gluc and 0.80 between free NNN and NNN-*N*-Gluc (all Ps < 0.001). Urinary total NNN also was correlated with urinary total NNAL ($r = 0.48$, $P < 0.001$) and total cotinine ($r = 0.67$, $P < 0.001$), although the correlation coefficients were not as high as those with free NNN and NNN-*N*-Gluc. The percentage of NNN-*N*-Gluc, however, was not associated with either total NNN ($r = -0.07$, $P = 0.35$) or total NNAL ($r = -0.06$, $P = 0.35$) but was inversely correlated with free NNN ($r = -0.39$, $P < 0.001$).

Subjects who developed esophageal cancer had significantly higher levels of free NNN, NNN-*N*-Gluc and total NNN in urine at baseline than control subjects (all Ps < 0.001). In contrast, the percentage of NNN-*N*-Gluc was significantly lower in cases than in controls (Table II).

Urinary level of total NNN was significantly associated with increased risk of developing esophageal cancer (Table III). Compared with the lowest tertile, ORs (95% CIs) of esophageal cancer for the second and third tertiles of total NNN were 3.99 (1.25–12.7) and 17.0 (3.99–72.8), respectively, after adjustment for urinary total NNAL,

Table I. Urinary levels of total NNAL, total NNN, free NNN, NNN-*N*-Gluc and the percentage of NNN-*N*-Gluc by number of cigarettes per day and urinary total cotinine among current smokers of control subjects only, The Shanghai Cohort Study 1986–2008

	No. of subjects	Geometric mean (95% CI), fmol/mg creatinine				Mean percentage of NNN-*N*-Gluc (95% CI)
		Total NNAL[a]	Total NNN	Free NNN	NNN-*N*-Gluc	
No. of cigarettes/day						
<10	36	210 (158–280)	23.0 (15.8–33.4)	9.7 (6.5–14.7)	12.3 (8.4–17.9)	55.0 (50.0–60.1)
10 to <20	79	372 (308–449)	43.3 (33.7–55.6)	18.0 (13.6–23.7)	23.1 (17.9–29.8)	55.6 (52.2–59.0)
20+	108	475 (404–559)	58.1 (46.9–72.0)	22.7 (18.0–28.8)	31.9 (25.6–39.6)	57.4 (54.5–60.3)
P for trend		<0.001	<0.001	<0.001	<0.001	0.34
Urinary total cotinine (nmol/mg creatinine)						
First quartile (<5.22)	56	197 (161–241)	15.0 (11.8–19.0)	6.1 (6.6–8.0)	8.1 (6.3–10.3)	56.1 (52.1–60.1)
Second quartile (5.22–11.52)	56	326 (267–398)	37.2 (29.4–47.2)	16.0 (12.1–21.0)	19.3 (15.1–24.5)	54.4 (50.3–58.4)
Third quartile (11.53–18.89)	56	454 (373–553)	63.3 (50.0–80.3)	24.0 (18.3–31.6)	38.4 (28.6–46.3)	59.1 (55.1–63.1)
Fourth quartile (>18.90)	55	747 (609–916)	119.1 (93.6–151.5)	48.3 (36.6–63.8)	63.4 (49.7–80.9)	55.9 (51.9–60.0)
P for trend		<0.001	<0.001	<0.001	<0.001	0.65

[a]Based on 218 subjects in total after five controls with missing values for total NNAL were excluded from this analysis.

Table II. Urinary levels of total NNN, free NNN and NNN-*N*-Gluc and the percentage of NNN-*N*-Gluc by esophageal cancer status among current smokers, The Shanghai Cohort Study 1986–2008

	Cases (n = 77)	Controls (n = 223)	P[a]
Total NNN (fmol/mg creatinine)	118 (89.1–156)[b]	45.1 (38.3–53.1)[b]	<0.001
Free NNN (fmol/mg creatinine)	58.2 (43.0–78.7)[b]	18.2 (15.3–21.8)[b]	<0.001
NNN-*N*-Gluc (fmol/mg creatinine)	49.8 (37.5–66.1)[b]	24.4 (20.6–28.8)[b]	0.004
Percentage of NNN-*N*-Gluc (%)	46.7 (43.1–50.3)[c]	56.4 (54.2–58.5)[c]	<0.000

[a]Two-sided *P* values and means were derived from analysis of variance models retaining matched case–control sets, of which controls were matched with index cases on current smoking status, age, neighborhood and year and month of urine collection.
[b]Geometric mean (95% CI).
[c]Arithmetic mean (95% CI).

urinary total cotinine, smoking intensity and duration and alcohol consumption (*P* for trend <0.001). Similarly, a positive dose-dependent association between urinary free NNN levels and esophageal cancer risk was observed. In contrast, a higher percentage of NNN-*N*-Gluc was associated with lower risk of esophageal cancer. Compared with the lowest tertile, the multivariate-adjusted ORs (95% CIs) for the second and third tertiles of the percentage of NNN-*N*-Gluc were 0.37 (0.17–0.80) and 0.27 (0.11–0.62), respectively (*P* for trend = 0.001). Further adjustment for total NNN did not alter the associations between the percentage of NNN-*N*-Gluc and risk of esophageal cancer. Similarly, adjustment for the percentage of NNN-*N*-Gluc did not materially change the total NNN-esophageal cancer risk association (data not shown).

Urinary levels of total NNAL and total cotinine were higher in case patients than in control subjects. Compared with the lowest tertile, ORs (95% CIs) of esophageal cancer for the second and third tertiles of total NNAL were 1.69 (0.79–3.64) and 3.63 (1.60–8.21), respectively (*P* for trend = 0.002) (Table IV). After adjustment for urinary total NNN and the percentage of NNN-*N*-Gluc, the association between urinary total NNAL and esophageal cancer risk no longer existed (*P* for trend = 0.98). Similarly, positive associations for esophageal cancer with urinary total cotinine and number of cigarettes per day disappeared after adjustment for urinary total NNN and the percentage of NNN-*N*-Gluc. The number of years of smoking was significantly associated with risk of esophageal cancer even after adjustment for urinary total NNN and the percentage of NNN-*N*-Gluc.

There was a strong dose-dependent association between alcohol consumption and risk of esophageal cancer. Adjustment for urinary total NNN and the percentage of NNN-*N*-Gluc did not materially alter the alcohol–esophageal cancer risk association (Table IV). We further examined the potential modifying effect of alcohol intake on the association between urinary total NNN and risk of esophageal cancer. Among non-drinkers, ORs (95% CIs) of esophageal cancer for the second and third tertiles of total NNN were 0.98 (0.21–4.64) and 2.17 (0.49–9.63), respectively, compared with the lowest tertile after adjustment for smoking duration and the percentage of NNN-*N*-Gluc (*P* for trend = 0.25). The corresponding figures among alcohol drinkers were 13.4 (2.62–68.6) and 34.7 (6.82–176.5) (*P* for trend <0.001). A test for the interaction effect between alcohol intake and urinary total NNN on risk of esophageal cancer was statistically significant (*P* for interaction = 0.036).

Discussion

This study demonstrates for the first time that levels of NNN in urine samples collected years before cancer diagnosis are significantly associated with the risk of developing esophageal cancer in smokers. The present study also showed a strong inverse association between the percentage of NNN-*N*-Gluc, a detoxifying metabolite of NNN, and risk of esophageal cancer. Urinary total NNAL, a metabolite of NNK, however, was not independently associated with risk of esophageal cancer after adjustment for total NNN and the percentage of NNN-*N*-Gluc. These findings are entirely in line with the results of laboratory experiments that consistently demonstrate the induction by NNN of esophageal tumors in rats. NNK is a tobacco carcinogen that targets the lung and other organ sites but has not been shown to induce esophageal cancer in laboratory animals (13). Furthermore, urinary total NNN and the percentage of NNN-*N*-Gluc almost completely accounted for the observed associations of esophageal cancer risk with number of cigarettes per day and urinary total cotinine (an objective marker of uptake and metabolism of nicotine). These findings along with data from previous animal experiments strongly suggest that NNN in tobacco smoke may play a significant and unique role in esophageal carcinogenesis in smokers.

Given the remarkably strong dose-dependent relationship of esophageal cancer risk with urinary NNN, but lack of association with urinary total NNAL, additional studies are required to identify factors that influence amounts of urinary NNN. Some of the variability is probably due to differing amounts of NNN in the smoke of various cigarette brands and differences in the ways in which these cigarettes were smoked. Virtually, all unburned commercial tobacco products contain NNN and NNK, and they always occur together (13). There is a great variation in levels of NNN and NNK in mainstream smoke of cigarettes. This is mainly due to differences in tobacco types used, agricultural practices, curing methods and manufacturing processes

Table III. Urinary levels of total NNN, free NNN and the percentage of NNN-*N*-Gluc in relation to risk of esophageal cancer among current smokers, The Shanghai Cohort Study 1986–2008

	Tertile levels			*P* for trend
	First	Second	Third	
Total NNN (fmol/mg creatinine)	<29.2	29.2–66.5	>66.5	
No. of cases/no. of controls	6/74	23/76	48/73	
Matched OR (95% CI)[a]	1.00 (referent)	4.51 (1.64–12.36)	13.4 (4.64–38.8)	<0.001
Adjusted OR (95% CI)[b]	1.00 (referent)	4.17 (1.32–13.17)	18.3 (4.40–76.3)	<0.001
NNAL-adjusted OR (95% CI)[c]	1.00 (referent)	3.99 (1.25–12.72)	17.0 (3.99–72.8)	<0.001
Free NNN (fmol/mg creatinine)	<10.4	10.4–28.5	>28.5	
No. of cases/no. of controls	7/74	15/76	55/73	
Matched OR (95% CI)[a]	1.00 (referent)	2.97 (1.02–8.65)	15.5 (5.18–46.6)	<0.001
Adjusted OR (95% CI)[b]	1.00 (referent)	3.30 (0.95–11.52)	16.6 (4.29–64.4)	<0.001
NNAL-adjusted OR (95% CI)[c]	1.00 (referent)	3.24 (0.92–11.36)	15.8 (4.02–62.3)	<0.001
Percentage of NNN-*N*-Gluc (%)	<50.5	50.5–62.2	>62.2	
No. of cases/no. of controls	45/74	18/76	14/73	
Matched OR (95% CI)[a]	1.00 (referent)	0.35 (0.18–0.69)	0.24 (0.11–0.52)	<0.001
Adjusted OR (95% CI)[b]	1.00 (referent)	0.36 (0.17–0.77)	0.25 (0.11–0.59)	<0.001
NNAL-adjusted OR (95% CI)[c]	1.00 (referent)	0.37 (0.17–0.80)	0.27 (0.11–0.62)	0.001

[a]ORs were derived from conditional logistic regression models that retained the case–control matched sets, of which controls were matched with index cases on current smoking status, age, neighborhood, and year and month of urine collection.
[b]In addition to matching factors, ORs were adjusted for number of cigarettes per day, number of years of smoking, number of alcoholic drinks per day and urinary total cotinine in tertile.
[c]Further adjusted for urinary total NNAL in tertile; an indicator variable for the missing values on total NNAL was created for three cases and five controls who were included in these analyses.

Table IV. Urinary total NNAL and total cotinine, cigarette smoking and alcohol consumption in relation to risk of esophageal cancer among current smokers, The Shanghai Cohort Study 1986–2008

	No. of cases	No. of controls	Matched OR (95% CI)	Total NNN-adjusted OR (95% CI)[a]
Urinary total NNAL (fmol/mg creatinine)[b]				
First tertile (<278)	14	69	1.00 (referent)	1.00 (referent)
Second tertile (278–522)	23	71	1.69 (0.79–3.64)	1.00 (0.42–2.41)
Third tertile (>522)	37	69	3.63 (1.60–8.21)	1.01 (0.36–2.87)
P for trend			0.002	0.98
Urinary total cotinine (nmol/mg creatinine)				
First tertile (<7.13)	16	74	1.00 (referent)	1.00 (referent)
Second tertile (7.13–15.64)	25	76	1.61 (0.78–3.33)	0.53 (0.21–1.32)
Third tertile (>15.64)	36	73	2.46 (1.22–4.99)	0.41 (0.15–1.15)
P for trend			0.011	0.11
No. of cigarettes per day				
<10	8	36	1.00 (referent)	1.00 (referent)
10 to <20	21	79	1.14 (0.46–2.85)	0.73 (0.26–2.05)
20+	48	108	1.85 (0.80–4.29)	1.17 (0.45–3.09)
P for trend			0.058	0.34
No. of years of smoking				
<20	9	32	1.00 (referent)	1.00 (referent)
20 to <40	34	140	0.98 (0.39–2.46)	0.93 (0.31–2.75)
40+	34	51	4.48 (1.48–13.7)	6.06 (1.58–23.2)
P for trend			0.001	0.001
No. of drinks of alcoholic beverages per day				
Non-drinkers	19	91	1.00 (referent)	1.00 (referent)
<2	17	64	1.43 (0.65–3.12)	1.74 (0.68–4.45)
2 to <4	21	43	2.56 (1.17–5.61)	3.34 (1.28–8.74)
4+	20	25	4.24 (1.87–9.61)	5.15 (1.97–13.5)
P for trend			<0.001	<0.001

[a]In addition to matching factors, ORs were adjusted for urinary total NNN and the percentage of NNN-*N*-Gluc.
[b]Three cases and 14 controls (nine who were individually matched controls of these three cases and five additional controls with missing values on total NNAL) were excluded from this analysis using conditional logistic regression models.

(13). Levels of NNN range from 20 to 58 000 ng/cigarette and NNK from 19 to 10 745 ng/cigarette in tobacco from commercial cigarettes sold in different parts of the world. In mainstream smoke, the ranges of NNN and NNK were reported from 4 to 2830 ng/cigarette and 3–1749 ng/cigarette, respectively (13). In China, for example, the levels of total tobacco-specific nitrosamine (sum of NNN plus NNK) were 30 times higher in mainstream smoke of Marlboro cigarettes (264 ng/cigarette) than a domestic brand of cigarettes (8.7 ng/cigarette) (27). Based on the findings of the present study, decreasing or eliminating NNN from tobacco products could be a straightforward way to reduce the exposure to NNN and subsequently decrease esophageal cancer risk for a smoker. Methods to decrease levels of tobacco-specific

nitrosamines, including NNN, in tobacco are well established but have not been widely applied (3).

Another factor contributing to the variability in urinary total NNN could be the endogenous formation of NNN from the tobacco alkaloids nornicotine and nicotine via nitrosation in the acidic environment of the stomach where nornicotine and nicotine from any sources are present (19,28). Endogenous formation of *N*-nitrosamines in humans through the reaction of dietary precursors with nitrosating agents from diet has been demonstrated in multiple studies (29,30). We recently reported significantly elevated urinary total NNN in some former smokers who used nicotine replacement therapy products that were virtually free of NNN (21,31,32). In the present study, among 92 control subjects who smoked the same number of cigarettes (one pack) per day, urinary total NNN of 5th and 95th percentiles were 11.3 and 282.8 fmol/mg Cr, respectively, an ∼25-fold difference. Given that the participants of the cohort most likely smoked domestic brands of cigarettes with comparable NNN contents due to their limited access to imported cigarettes in the mid-1980's, this large variation in urinary total NNN could be, at least partly, due to the difference in endogenous formation of NNN from nornicotine and nicotine in tobacco smoke. Endogenous formation of *N*-nitrosamines can be greatly affected by dietary factors including ascorbic acid, vitamin E and phenolic compounds or by bacteria in the oral cavity and stomach (33). These data suggest that endogenous formation of NNN may contribute to the risk of developing esophageal cancer in individuals who are exposed to nicotine and/or nornicotine from various sources.

A strong inverse association between the percentage of NNN-*N*-Gluc and risk of esophageal cancer in the present study suggests that NNN glucuronidation may play an important role in reducing the carcinogenic effect of free NNN on the esophagus. Several genetic polymorphisms in the uridine diphosphate-glucuronosyltransferase gene including *UGT2B10* can influence the glucuronidation of NNN (34). Future studies are warranted to study the potential modifying effect of these genetic polymorphisms on NNN-related esophageal cancer risk.

The levels of urinary NNN biomarkers observed in this study are in agreement with previous reports of the same biomarkers in smokers. We first reported that the arithmetic mean of urinary total NNN was 0.18 pmol/mg creatinine in smokers in the USA and that NNN-*N*-Gluc accounted for an average of 59.1% of total NNN in urine (14). The corresponding figures in this study population are comparable with those in the USA. The arithmetic mean of urinary total NNN was 0.22 pmol/mg creatinine and the percentage of NNN-*N*-Gluc was 56.4% in control subjects.

The lack of association between urinary total NNAL and risk of esophageal cancer in the present study is in agreement with the findings in experiments with rats. NNN, but not NNK, induces esophageal tumors in rats treated with the carcinogen in the drinking water (13). Furthermore, cultured rat esophagus readily catalyzes the metabolic activation of NNN, but not NNK, by α-hydroxylation. The cytochrome P450 enzyme involved in this process has not been identified (35,36). The positive association between urinary total NNAL and risk of esophageal cancer observed in univariate analysis disappeared after adjustment for urinary total NNN, suggesting that NNN plays a more important role than NNK in the development of esophageal cancer in humans, as in rats.

Alcohol consumption is an established risk factor for esophageal cancer in various populations (37–39). In this study, alcohol intake was a strong independent risk factor for esophageal cancer. The interaction between alcohol consumption and urinary total NNN for esophageal squamous cell cancer risk in the present study is consistent with results of previous reports of alcohol and cigarette smoking on esophageal cancer risk (38,39). The interaction between alcohol consumption and NNN suggests that they act independently of each other in the etiology of esophageal cancer. Studies in rats have not produced consistent results with respect to the effects of ethanol on esophageal tumor induction by NNN (11). Ethanol itself can cause local irritation of the upper gastrointestinal tract (40). It may act as a solvent to increase the physical contact with tobacco carcinogen NNN, thereby facilitating the entry of NNN into the esophageal mucosa, resulting in increased uptake of NNN in cigarette smoke (41). In studies with porcine oral mucosa, the permeability of the floor or the mouth to NNN was increased by ethanol (42). Given the relative small sample size of the present study, the modifying effect of alcohol intake on the NNN-esophageal cancer risk association needs to be confirmed.

The most important strength of this study is that the biomarkers were measured in urine samples collected years (11.5 years on average) before cancer diagnosis, thereby ruling out the possibility of a spurious association due to smoking behavior changes of patients close to their time of clinical diagnosis of esophageal cancer. The remarkably strong dose-dependent NNN-esophageal cancer association and consistency with animal studies further strengthen the findings of this study. The simultaneous measurement of urinary free and conjugated NNN and total NNAL allowed us to examine the independent effect of these biomarkers on the development of esophageal cancer risk and shed some light on esophageal carcinogenesis related to tobacco smoking.

The present study had several limitations. One limitation was the single-time assessment of exposure based on spot urine samples at baseline. Changes in exposure to tobacco-specific nitrosamines after baseline were not ascertained. However, these changes would occur equally in both case patients and control subjects and result in an underestimate of the true effect (i.e. ORs toward null). Timing of the urine collection also would have an impact on the concentration of urinary biomarkers measured. However, the spot urine samples were collected at a similar time of the day (5 pm–9 pm) from both cases and controls and processed in the same manner. Another concern is the stability of biomarkers in stored urines. Our data have shown that total NNN is stable for at least 2 years and total NNAL is stable for at least 4 years in urine samples stored at −20°C. Given that the duration of storage of controls was individually matched with that of index cases, the observed association between urinary NNN and risk of esophageal cancer would be less likely to be biased by the time of collection and/or handling of urine specimens or degradation of biomarkers in stored urines. Another limitation of the present study is its small sample size. Our findings need to be confirmed in a similar prospective study with a larger sample size. Future studies could also incorporate polymorphisms in genes that are possibly involved in the endogenous formation, activation and detoxification of NNN. The interaction between genetic polymorphisms in NNN metabolism and risk of esophageal cancer could further elucidate the mechanism of NNN in human esophageal carcinogenesis.

In summary, using prospectively collected urine samples from participants of a Chinese cohort, we demonstrated a remarkable dose-dependent association between urinary total NNN and increased risk of esophageal cancer in smokers. The study also showed that smokers with a higher percentage of glucuronidated NNN experienced significantly reduced risk of esophageal cancer. Urinary total NNAL was not independently associated with the risk of esophageal cancer after the effects of total NNN and the percentage of NNN-*N*-Gluc were taken into account. These data along with the results of previous studies in laboratory animals support a significant and unique role of NNN in esophageal carcinogenesis in humans.

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