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## Tobacco Smoke Biomarkers and Cancer Risk Among Male Smokers in the Shanghai Cohort Study

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### Abstract

Metabolites of tobacco smoke constituents can be quantified in urine and other body fluids providing a realistic measure of carcinogen and toxicant dose in a smoker. Many previous studies have demonstrated that these metabolites – referred to as biomarkers in this paper – are related to tobacco smoke exposure. The studies reviewed here were designed to answer another question: are these substances also biomarkers of cancer risk? Using a prospective study design comparing biomarker levels in cancer cases and controls, all of whom were smokers, the results demonstrate that several of these biomarkers – total cotinine, total 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), *r*-1-*t*-2,3,4-tetrahydroxy-1,2,3,4-tetrahydrophenanthrene (PheT), and total *N*'-nitrosonornicotine (NNN) – are biomarkers of cancer risk. Therefore, these biomarkers have the potential to become part of a cancer risk prediction algorithm for smokers.

### Keywords

tobacco smoke; biomarkers; cotinine; NNAL; PheT; NNN

## 1. Introduction

Tobacco products are responsible for 22% of all cancer death worldwide and 30% of cancer mortality in the United States <http://www.who.int/mediacentre/factsheets/fs297/en/index.html> [1]. At least eighteen different types of cancer are caused to varying extents by

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### Conflict of Interest Statement.

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

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tobacco products [2]. This paper focuses on lung and esophageal cancer. Approximately 90% of lung cancer mortality in populations with prolonged use is attributed to cigarette smoking [3]. More than 3000 people in the world succumb to this deadly and relatively incurable disease daily [4]. Cigarette smoking is also an important cause of esophageal cancer, both squamous cell carcinoma and adenocarcinoma [3].

In addition to the addictive but non-carcinogenic compound nicotine, cigarette smoke contains over 70 established carcinogens, including tobacco-specific nitrosamines, polycyclic aromatic hydrocarbons (PAH), and volatile carcinogens such as 1,3-butadiene and benzene [5]. There is no doubt that daily exposure to these compounds is a major cause of cancer in smokers. Professor Ramesh Gupta has made important contributions to our understanding of the relevant DNA damage mechanisms [6-9]. However, we have little ability to predict which of the 1.3 billion smokers in the world will actually get cancer. It is axiomatic in toxicology that “the dose makes the poison” and therefore it is logical that susceptibility to cancer in smokers is a function of carcinogen dose. Tobacco carcinogen and toxicant biomarkers – quantitative measures of tobacco constituents or their metabolites in body fluids – can inform us about dose much more accurately than counting numbers of cigarettes smoked, even if that information could be reliably obtained [10]. This is because there are wide differences in carcinogen and toxicant delivery among different tobacco products depending on their characteristics and the ways in which they are smoked. In the studies reviewed here, we focused on a panel of tobacco carcinogen and toxicant biomarkers representing some of the most important compounds in cigarette smoke.

The structures of the tobacco carcinogen and toxicant biomarkers considered here are shown in Figure 1 [10]. Cotinine and cotinine-*N*-glucuronide are metabolites of nicotine; their sum is “total cotinine.” Total cotinine is a reliable measure of uptake of the addictive compound nicotine. 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) and its glucuronides are metabolites of the potent tobacco-specific lung carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK); their sum is “total NNAL.” Total NNAL is a well established biomarker of NNK uptake. NNN-*N*-glucuronide is a metabolite of the tobacco-specific esophageal and oral cavity carcinogen *N*'-nitrosonornicotine (NNN); values for each of these are reported. *r*-1,*t*-2,3,*c*-4-Tetrahydroxy-1,2,3,4-tetrahydrophenanthrene (PheT) is a metabolite of phenanthrene, structurally related to carcinogenic PAH [11]. This metabolite is formed by the diol epoxide metabolism pathway, believed to be important in the metabolic activation to DNA binding intermediates of carcinogenic PAH. The mercapturic acids shown in Figure 1 are metabolites of the tobacco smoke volatiles 1,3-butadiene, ethylene oxide, benzene, acrolein, and crotonaldehyde, respectively [12]. 1,3-Butadiene, ethylene oxide, and benzene are considered carcinogenic to humans by the International Agency for Research on Cancer, based mainly on occupational studies and mechanistic data related to hematopoietic malignancies [13;14]. Acrolein is a highly toxic but marginally carcinogenic compound while crotonaldehyde is a relatively weak hepatocarcinogen [15]. Many studies have shown that concentrations of all of the biomarkers discussed here are higher in the urine and other body fluids of smokers than non-smokers and that in most cases their levels significantly decrease upon cessation of smoking [10]. Therefore, these substances are clearly biomarkers of tobacco smoke exposure and dose. But are they also biomarkers of

cancer risk? We addressed this question in the studies reviewed here by quantifying tobacco carcinogen and toxicant biomarkers in pre-diagnostic urine samples from the Shanghai Cohort Study [16-20].

## 2. Approach

The Shanghai Cohort Study enrolled 18,244 men between January 1, 1986 and September 30, 1989 [20;21]. They were between 45 and 64 years old and lived in one of four geographically defined communities in Shanghai, China. In-person interviews were conducted and a urine sample was obtained from each subject upon enrollment. Cases of lung cancer or esophageal cancer were identified annually by in-person re-interviews of all surviving cohort members and review of reports from the Shanghai Cancer Registry and the Shanghai Municipal Vital Statistics Office. For the lung cancer studies, we identified cases who smoked cigarettes at baseline and then randomly selected one control subject for each case from all cohort members who were current smokers at enrollment, free of cancer, and alive at the time of cancer diagnosis of the index case. Controls were matched to the index case by age at enrollment, date of urine collection, and neighborhood of residence at recruitment. The selection of controls was the same for the esophageal cancer study, except that 3 control subjects were randomly selected for each case in order to increase statistical power due to the smaller number of esophageal cancer than lung cancer cases.

Urine samples were retrieved from the biospecimen bank and analyzed for the following tobacco smoke carcinogen and toxicant biomarkers (Figure 1): total cotinine (the sum of cotinine and cotinine-*N*-glucuronide); total NNAL (the sum of NNAL, NNAL-*O*-glucuronide and NNAL-*N*-glucuronide); PheT; free NNN and total NNN (the sum of free NNN and NNN-*N*-glucuronide); 1-hydroxy-2-(*N*-acetylcysteiny)-3-butene and 1-(*N*-acetylcysteiny)-2-hydroxy-3-butene (collectively called MHBMA for monohydroxybutyl mercapturic acid, metabolites of 1,3-butadiene); 2-hydroxyethyl mercapturic acid (HEMA), a metabolite of ethylene oxide; *S*-phenyl mercapturic acid (SPMA), a metabolite of benzene; 3-hydroxypropyl mercapturic acid (HPMA), a metabolite of acrolein; and 4-hydroxybut-2-yl mercapturic acid (HBMA), a metabolite of crotonaldehyde. The analyses were carried out as previously described [10;16-19].

For total cotinine, total NNAL, and PheT there were urine samples from 476 lung cancer cases and 476 controls. Due to sample depletion, there were 392 urine samples from lung cancer cases and 343 from controls for measurement of mercapturic acids. For total NNN, there were urine samples from 74 esophageal cancer cases and 218 from controls.

## 3. Results

Urinary levels of all biomarkers increased significantly with numbers of cigarettes smoked per day. Furthermore, levels of all urinary biomarkers were significantly higher in cancer cases than in controls.

The results of the lung cancer study are summarized in Table 1. Total cotinine, total NNAL, and PheT were all independently and significantly associated with lung cancer risk, even after adjustment for number of cigarettes smoked per day, number of years of smoking, and

the other two biomarkers (e.g., results for total cotinine adjusted for total NNAL and PheT). The odds ratios in the highest versus the lowest tertile in the adjusted models were 2.97 for total cotinine, 1.67 for total NNAL, and 1.69 for PheT. The trend for increasing odds ratios with increasing tertile of biomarker were statistically significant in all cases,  $P < 0.0001$  for total cotinine,  $P = 0.0436$  for total NNAL, and  $P = 0.0157$  for PheT.

All of the mercapturic acids were significantly associated with lung cancer risk after correction for number of cigarettes smoked per day and number of years of smoking. The odds ratios in the highest versus the lowest tertile were 1.44 for MHBMA, 1.81 for HEMA, 1.59 for SPMA, 1.67 for HPMA, and 1.87 for HBMA. However, adjustment for cotinine resulted in a null association for all of the mercapturic acids with lung cancer risk.

The results of the esophageal cancer study are summarized in Table 2. Total NNN and free NNN were significantly associated with esophageal cancer risk, even after adjustment for number of cigarettes per day, number of years of smoking, number of alcoholic drinks per day, urinary total cotinine, and urinary total NNAL. The odds ratios in the upper tertile compared to the lowest tertile were 17.0 for total NNN and 15.8 for free NNN ( $P < 0.0001$ ). A higher percentage of NNN-*N*-glucuronide was also significantly associated with a lower risk of esophageal cancer, after adjustment for the same factors listed above.

#### 4. Discussion

The studies summarized here indicate that the tobacco smoke carcinogen and toxicant biomarkers total cotinine, total NNAL, PheT, NNN, and total NNN are risk biomarkers for cancer in addition to their well established roles as exposure biomarkers. These findings have considerable implications for future research directions, in which these biomarkers may ultimately contribute to prediction of individual cancer risk in smokers, an area of great importance in prevention and possibly early detection of cancer.

The results for total cotinine and total NNAL are consistent with previous work. Several studies including ours have noted a relationship between cotinine levels in serum or urine and lung cancer risk in smokers [16;22-24], although one previous report was negative in this respect [25]. Cotinine and cotinine glucuronide are metabolites of the non-carcinogen nicotine, so the relationship of their levels to lung cancer is an indirect one. It results from the fact that smokers regulate their use of cigarettes to achieve a certain level of nicotine, thus satisfying their addiction. When they smoke, they are simultaneously exposed to nicotine and multiple carcinogens.

Two previous smaller studies, nested in the Singapore Chinese Health Study and the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial, demonstrated a significant relationship between total NNAL and lung cancer, in agreement with the results summarized here [16;25]. The relationship between total NNAL levels and lung cancer is more direct than that of total cotinine because NNAL and its glucuronides are metabolites of the tobacco-specific lung carcinogen NNK which readily induces lung tumors in rats, mice, hamsters, and ferrets after systemic treatment with even low doses [26].

This was the first large study to demonstrate a relationship between a specific biomarker that integrates PAH exposure and metabolism – PheT - and lung cancer in smokers. PAH are classic carcinogens widely considered to be important causative factors for lung cancer in smokers based on their occurrence in cigarette smoke, strong carcinogenic activities in rodent models including lung cancer models, and metabolic, DNA binding, and genetic consequences in humans [27-30]. Thus, our results are consistent with a large body of experimental data on PAH. One previous smaller study did not detect a significant relationship between serum PheT and lung cancer [25].

While the relationships of total NNAL and PheT to lung cancer in the Shanghai Cohort Study were significant, they were not as strong as one might have expected based on various lines of evidence connecting NNK and PAH to lung cancer in smokers. With respect to total NNAL, it is important to note that NNK levels in Chinese cigarettes are relatively low compared to a typical American blend [31]. The mean levels of total NNAL in the urine of our subjects were 0.20 pmol/mg creatinine in controls and 0.28 pmol/mg creatinine in cases, 4-5 times less than the amounts of total NNAL typically observed in the urine of U.S. cigarette smokers [10;17]. We have also observed that levels of PheT are considerably higher in *non-smokers* from Shanghai than in non-smokers from the U.S. [32]. Exposure to higher levels of PAH among non-smokers in Shanghai could be due to air pollution, diet, or other factors. These exposures, particularly from diet, would tend to blunt the effects of PAH exposure specifically from cigarette smoke and consequently diminish the utility of PheT as a specific biomarker of lung cancer risk in smokers from Shanghai.

Perhaps the most remarkable finding in this study was the strong relationship of total NNN to esophageal cancer risk – 17-fold higher for the upper tertile of exposure after adjustment for cotinine and total NNAL, the latter showing no relationship to esophageal cancer. These results demonstrate *coherence* between epidemiologic data in smokers and rat carcinogenicity studies, in which NNN but not NNK, is a potent esophageal carcinogen [26]. The results therefore add considerable support to the role of NNN as a major cause of esophageal cancer in smokers, proposed previously based on its levels in cigarette smoke and on metabolism, DNA binding, and carcinogenicity studies mainly in rats [28;33]. These results underline the urgent need for reduction of NNN and NNK levels in tobacco products. The technology to do this exists and is well validated but has not been applied to most tobacco products [33]. The results also point to a possible previously unrecognized role of nornicotine in tobacco carcinogenesis. Nornicotine is easily nitrosated to produce NNN [34], and some studies indicate that this occurs in humans [35;36]. The strong relationship of total NNN to esophageal cancer in the Shanghai cohort study could result partially from endogenous formation of NNN from nornicotine and dietary nitrite, particularly since levels of tobacco-specific nitrosamines in the smoke of cigarettes used in Shanghai are relatively low, as noted above.

We did not observe a significant relationship between the volatile toxicants and carcinogens and lung cancer in smokers, after adjustment for cotinine. Our interpretation of these data is that the lung carcinogenicity of these compounds, if it exists in humans, is not strong enough to overcome the power of cotinine as a representative of all other smoke constituents [19]. Among these volatiles, only 1,3-butadiene, ethylene oxide, and benzene have been shown to

induce lung tumors in laboratory animals, and then only in mice [19]. Overall, their potency as lung carcinogens appears to be considerably weaker than those of PAH or NNK [19]. Although 1,3-butadiene, ethylene oxide, and benzene are recognized as human carcinogens, the data from occupational studies indicate that their effects are not mainly on lung cancer [13;14].

Levels of the biomarkers quantified in this study will depend on carcinogen or toxicant dose plus metabolism (with the exception of free NNN, which represents the parent compound in smoke). Thus, there are two variables which affect the final value: dose and metabolism. Since levels of all of these biomarkers are higher in smokers than non-smokers, and in most cases their levels decrease upon smoking cessation, dose is a more important factor than metabolism. But differences in metabolic pathways for each of these compounds could differentially affect cancer risk in individuals exposed to the same dose. A variety of enzymes are involved in the metabolism of the carcinogens and toxicants considered here, and further studies are required to determine their effects on cancer risk.

The studies reviewed here have certain limitations. The cohort consists only of males from Shanghai and the results may not be representative of female smokers, or smokers from other parts of the world where different types of cigarettes are smoked. There was only a single urine sample collected at baseline from all subjects. That urine sample may not be representative of the subjects' continuing exposure, although longitudinal studies of some of these biomarkers have shown only relatively minor variation over time [37].

In summary, the results reviewed here demonstrate that several tobacco smoke toxicant and carcinogen biomarkers – total cotinine, total NNAL, PheT, and total NNN – are not only biomarkers of exposure but also are biomarkers of cancer risk. Based on comparison of cases and controls who were all smokers, total cotinine, total NNAL, and PheT were risk biomarkers for lung cancer while total NNN was a risk biomarker for esophageal cancer. These results provide new directions for possible early identification of smokers at high risk for cancer.

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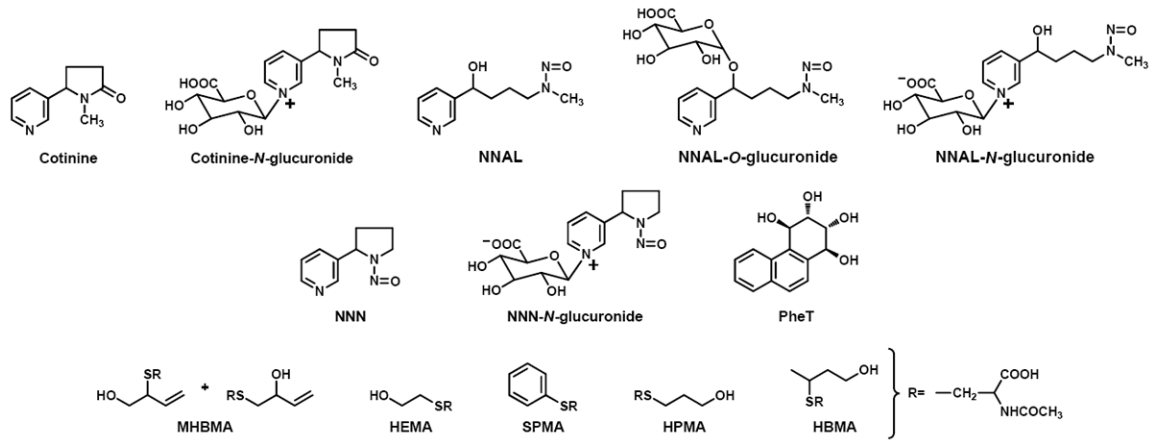
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**Fig. 1.**  
Chemical structures of the tobacco carcinogen and toxicant biomarkers discussed in the text.

Urinary levels of tobacco carcinogen and toxicant biomarkers in relation to risk of lung cancer in current smokers, the Shanghai Cohort Study.

**Table 1**

	Levels of urinary biomarkers in tertiles			P for trend
	1 <sup>st</sup> tertile	2 <sup>nd</sup> tertile	3 <sup>rd</sup> tertile	
Total cotinine (nmol/mg creatinine)	<5.58	5.58 – 13.35	>13.35	
No. Controls/No. Cases	158/47	161/146	157/283	
Smoking-Adjusted OR (95% CI) <sup>1</sup>	1.00	2.33 (1.52 – 3.57)	3.70 (2.42 – 5.65)	<0.0001
Biomarkers-Adjusted OR (95% CI) <sup>2</sup>	1.00	2.05 (1.33 – 3.18)	2.97 (1.89 – 4.65)	<0.0001
Total NNAL (pmol/mg creatinine)	<0.14	0.14 – 0.28	>0.28	
No. Controls/No. Cases	158/76	161/166	157/234	
Smoking-Adjusted OR (95% CI) <sup>1</sup>	1.00	1.89 (1.24 – 2.87)	2.60 (1.68 – 4.04)	<0.0001
Biomarkers-Adjusted OR (95% CI) <sup>2</sup>	1.00	1.46 (0.94 – 2.27)	1.67 (1.04 – 2.69)	0.0436
Total PheT (pmol/mg creatinine)	<21.7	21.7 – 35.3	>35.3	
No. Controls/No. Cases	158/121	161/151	157/204	
Smoking-Adjusted OR (95% CI) <sup>1</sup>	1.00	1.37 (0.94 – 1.98)	2.01 (1.34 – 3.02)	0.0008
Biomarkers-Adjusted OR (95% CI) <sup>2</sup>	1.00	1.23 (0.83 – 1.82)	1.69 (1.10 – 2.60)	0.0157
MHBMA (pmol/mg creatinine)	<5.53	5.53 – 15.39	>15.39	
No. Controls/No. Cases	130/86	133/111	129/146	
Smoking-Adjusted OR (95% CI) <sup>3</sup>	1.00	1.14 (0.77 – 1.68)	1.44 (0.98 – 2.11)	0.0582
Cotinine-Adjusted OR (95% CI) <sup>4</sup>	1.00	0.87 (0.58 – 1.32)	0.97 (0.64 – 1.46)	0.9331
HEMA (pmol/mg creatinine)	<11.1	11.1 – 22.6	>22.6	
No. Controls/No. Cases	130/73	133/114	129/156	
Smoking-Adjusted OR (95% CI) <sup>3</sup>	1.00	1.32 (0.88 – 1.97)	1.81 (1.22 – 2.68)	0.0029
Cotinine-Adjusted OR (95% CI) <sup>4</sup>	1.00	1.01 (0.66 – 1.56)	1.05 (0.67 – 1.64)	0.8248
SPMA (pmol/mg creatinine)	<1.65	1.65 – 3.56	>3.56	
No. Controls/No. Cases	130/78	133/115	129/150	
Smoking-Adjusted OR (95% CI) <sup>3</sup>	1.00	1.23 (0.84 – 1.85)	1.59 (1.08 – 2.34)	0.0184
Cotinine-Adjusted OR (95% CI) <sup>4</sup>	1.00	1.01 (0.67 – 1.53)	1.11 (0.73 – 1.68)	0.6088
HPMA (pmol/mg creatinine)	<5946	5946 – 11852	>11852	

	Levels of urinary biomarkers in tertiles			P for trend
	1 <sup>st</sup> tertile	2 <sup>nd</sup> tertile	3 <sup>rd</sup> tertile	
No. Controls/No. Cases	130/74	133/108	129/161	
Smoking-Adjusted OR (95% CI) <sup>3</sup>	1.00	1.18 (0.79 – 1.77)	1.67 (1.12 – 2.49)	0.0099
Cotinine-Adjusted OR (95% CI) <sup>4</sup>	1.00	0.83 (0.53 – 1.29)	0.96 (0.61 – 1.52)	0.9849
HBMA (pmol/mg creatinine)	<4340	4340 – 10380	>10380	
No. Controls/No. Cases	130/63	133/124	129/156	
Smoking-Adjusted OR (95% CI) <sup>3</sup>	1.00	1.60 (1.06 – 2.41)	1.87 (1.24 – 2.83)	0.0041
Cotinine-Adjusted OR (95% CI) <sup>4</sup>	1.00	1.22 (0.79 – 1.89)	1.12 (0.70 – 1.77)	0.7491

<sup>1</sup> Odds ratios (ORs) were derived from conditional logistic regression models that retained the case-control matched pairs, of which controls were matched to the index cases on current smoking status, age, neighborhood of residence, and year and month of urine collection, and adjusted for number of cigarettes/day and number of years of smoking; CI, confidence interval.

<sup>2</sup> Further adjusted for total NNAL and PheT in the case of total cotinine, PheT and total cotinine in the case of total NNAL, and total cotinine in the case of PheT.

<sup>3</sup> Odds ratios were derived from unconditional logistic regression models that included matching factors (age, neighborhood of residence, and duration of biospecimen storage before assays for urinary biomarkers), number of cigarettes smoked per day and number of years of smoking at baseline.

<sup>4</sup> In addition to matching factors, number of cigarettes smoked per day, number of years of smoking, odds ratios were adjusted for urinary total cotinine.

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

Table 2

	Levels of urinary biomarkers in tertiles			<i>P</i> for trend
	1 <sup>st</sup> tertile	2 <sup>nd</sup> tertile	3 <sup>rd</sup> tertile	
Total NNN (fmol/mg creatinine)	<29.2	29.2 – 66.5	>66.5	
No. Controls/No. Cases	74/6	76/23	73/48	
Matched OR (95% CI) <sup>1</sup>	1.00 (referent)	4.51 (1.64 – 12.36)	13.4 (4.64 – 38.76)	<0.0001
Adjusted OR (95% CI) <sup>2</sup>	1.00 (referent)	4.17 (1.32 – 13.17)	18.3 (4.40 – 76.32)	<0.0001
NNAL-adjusted OR (95% CI) <sup>3</sup>	1.00 (referent)	3.99 (1.25 – 12.72)	17.0 (3.99 – 72.80)	<0.0001
Free NNN (fmol/mg creatinine)	<10.4	10.4 – 28.5	>28.5	
No. Controls/No. Cases	74/7	76/15	73/55	
Matched OR (95% CI) <sup>1</sup>	1.00 (referent)	2.97 (1.02 – 8.65)	15.5 (5.18 – 46.62)	<0.0001
Adjusted OR (95% CI) <sup>2</sup>	1.00 (referent)	3.30 (0.95 – 11.52)	16.6 (4.29 – 64.43)	<0.0001
NNAL-adjusted OR (95% CI) <sup>3</sup>	1.00 (referent)	3.24 (0.92 – 11.36)	15.8 (4.02 – 62.28)	<0.0001
Percentage of NNN- <i>N</i> -Gluc (%)	<50.5	50.5 – 62.2	>62.2	
No. Controls/No. Cases	74/45	76/18	73/14	
Matched OR (95% CI) <sup>1</sup>	1.00 (referent)	0.35 (0.18 – 0.69)	0.24 (0.11 – 0.52)	<0.0001
Adjusted OR (95% CI) <sup>2</sup>	1.00 (referent)	0.36 (0.17 – 0.77)	0.25 (0.11 – 0.59)	0.0007
NNAL-adjusted OR (95% CI) <sup>3</sup>	1.00 (referent)	0.37 (0.17 – 0.80)	0.27 (0.11 – 0.62)	0.0013

<sup>1</sup> Odds ratios (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; CI, confidence interval.

<sup>2</sup> 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.

<sup>3</sup> Further adjusted for urinary total NNAL.