Title: Clinical relevance of uric tobacco-specific nitrosamine and severe abdominal aortic calcification in a national survey of the United States

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Clinical relevance of uric tobacco-specific nitrosamine and severe abdominal aortic calcification in a national survey of the United States

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Abbreviations and acronyms

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AA, arachidonic acid

AAC, abdominal aortic calcification

BMI, Body mass index

CDC, Centers for Disease Control and Prevention

CI, confidence interval

CS, Cigarette smoking

CVDs, cardiovascular diseases

DM, diabetes mellitus

DXA, dual-energy X-ray absorptiometry

ERB, Ethics Review Board

ID HPLC-ESI MS/MS, isotope-dilution high-performance liquid chromatography/electrospray ionization tandem mass spectrometry

IQR, interquartile range

IRB, Institutional Review Board

LC-MS/MS, liquid chromatography-tandem mass spectrometry

MEC, mobile examination center

NCHS, National Center for Health Statistics

NHANES, National Health and Nutrition Examination Survey

NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone

NNN, N'-nitrosonornicotine

OR, odds ratios

SD, standard deviation

SP, serum phosphorus

STC, serum total calcium

STROBE, STrengthening the Reporting of OBservational studies in Epidemiology

SUA, serum uric acid

TC, total cholesterol

TG, triglycerides

TSNA, tobacco-specific nitrosamine

US, United States

Abstract

Background: This cross-sectional study investigated the relationship between uric tobacco-specific nitrosamine (TSNA), N′-nitrosonornicotine (NNN), and abdominal aortic

calcification (AAC) in the United States (US) adults for the first time.

Methods: The final sample (2,713 participants aged 40 years and older) was obtained from the National Health and Nutrition Examination Survey (NHANES) 2013–2014. The risk of severe AAC according to uric NNN, dose–response relationship, and threshold effect were analyzed using the multivariate logistic regression models, cubic spline model, and a two-piecewise linear regression model, respectively.

Results: In the fully adjusted model, the odds ratios (OR) (95% confidence interval, CI) of severe AAC for participants in the high uric NNN group was 2.39 (1.59–3.61) compared with that in the low uric NNN group ($P < 0.001$). After adjusting for multiple covariates, the risk of severe AAC increased 1.515-fold for every 1 ng/dL increase in uric NNN when the concentration of uric NNN was less than 1.354 ng/dL. The association between uric NNN and severe AAC was stable among different subgroups.

***Conclusion*:** In a sample of US civilians, uric NNN levels positively correlated with the risk of severe AAC.

Key words: Cigarette smoking; Tobacco-specific nitrosamine; N′-nitrosonornicotine; Abdominal aortic calcification; Cross-sectional study; National Health and Nutrition Examination Survey

Significance statement

- The present study is the first to discuss the risk of severe AAC according to uric NNN, dose–response relationship, and threshold effect.
- Our findings may promote the public to realize the harm of cigarette smoking and the

benefit of cigarette smoking cessation, as smoking cigarettes releases at least 4000 constituents.

- Uric NNN may also be a potential biomarker to quantitatively evaluate the tobacco exposure and the effect of cigarette smoking cessation.
- Based on the basic, observational, and interventional studies in the future, it is of significance for CVD prevention and the finding of new therapeutic targets.

Introduction

Epidemiological studies strongly suggest that cigarette smoking (CS) is a major modifiable risk factor for the incidence and mortality of cardiovascular disease (CVD) [1-3]. The potential mechanism involves aggravated inflammation, oxidative stress, and thrombosis [4-6]. The dose-dependent correlation between CS and CVD risk remains unclear because it is difficult to measure the extent of tobacco use and exposure to environmental tobacco smoke [7-9]. Cigarettes release at least 4000 constituents, including tobacco-specific nitrosamines (TSNAs), which are well recognized as a leading class of carcinogens [10]. Owing to their specificity for tobacco, TSNAs may serve as biomarkers for tobacco carcinogen uptake. Among different types of TSNAs, N′-nitrosonornicotine (NNN) is particularly noteworthy, which has been proved to be associated with lung, oral cavity, and esophageal cancers in tobacco users [11-13]. In addition, the abundance of NNN is remarkably higher than that of other TSNAs [14]. However, the relationship between NNN and CVD has not yet been investigated.

Recently, many studies have reported an association between dietary data, laboratory

indices, and abdominal aortic calcification (AAC), using data from the National Health and Nutrition Examination Survey (NHANES) 2013–2014 [15-22]. AAC has been widely discussed because it is an important predictor of CVD mortality [23-27], and can be noninvasively and precisely examined in hospital [28-31]. However, the relationship between TSNAs and AAC remains unclear. This is the first cross-sectional study to explore the dose–response relationship between uric NNN levels and severe AAC in a noninstitutionalized US civilian population. We aimed to identify a reliable biomarker reflecting exposure to both mainstream and environmental tobacco smoke, and its correlation with CVD risk.

Methods

Ethics

The study protocol was approved by the Ethics Review Board (ERB) of the National Center for Health Statistics. All participants signed an informed consent form before participating in the study. All data in the present study are available to the public at https://wwwn.cdc.gov/nchs/nhanes/continuousnhanes/default.aspx?BeginYear=2013. The ethics committee approval number of NHANES 2013-2014 is the National Center for Health Statistics (NCHS) Institutional Review Board (IRB) / ERB Protocol Number: Protocol #2011-17, which can be found at https://www.cdc.gov/nchs/nhanes/irba98.html. The entire manuscript was organized following the STROBE (Strengthening the Reporting of Observational Studies in Epidemiology) statement.

Study population and design

The present study was performed using data from the NHANES of 2013–2014. NHANES is a program of studies designed to assess the health and nutritional status of adults and children in the United States. It is a major program of the National Center for Health Statistics, part of the Centers for Disease Control and Prevention. In NHANES 2013–2014, 10,175 individuals were involved in the interviews, in which AAC was evaluated in people aged 40 years and older. We first included 7,910 participants with uric NNN information. We further excluded 5,038 participants with missing AAC scores and 159 participants who did not consume alcohol. Finally, a total of 2,713 participants were enrolled in this study. A detailed flowchart of the participant recruitment process is shown in **Figure 1**.

Data collection and measurements

As previously documented [17], demographics, health conditions, and lifestyle data were derived from household interview questionnaires and mobile examination center questionnaires administered by trained interviewers. The demographic data included age, sex, and race/ethnicity. Race/ethnicity included Mexican American, other Hispanic, non-Hispanic white, non-Hispanic black, and other ethnicities. Due to this facilitation, we merged Mexican Americans and other Hispanics into one group (Hispanic) and non-Hispanic white, non-Hispanic black, and other races into another (non-Hispanic) in the subgroup analysis (**Figure 3**), as previously reported [17]. The health condition data composed of hypertension and diabetes mellitus (DM) history, which was examined through the item “Doctor or other health professional told you have DM and/or high blood pressure.” The alcohol intake was

assessed by “Had at least 12 alcohol drinks per year.” Body mass index (BMI) was expressed as weight in kilograms divided by height in meter squared (kg/m^2) and obtained from the NHANES body measurement data.

NHANES 2013–2014 used the Beckman UniCel DxC800 Synchron System (Beckman, Fullerton, CA, USA) to measure the serum total cholesterol (TC), triglycerides (TG), and serum uric acid (SUA) through a timed-endpoint method; the serum total calcium (STC) through indirect (or diluted) ion selective electrode methodology; the serum phosphorus (SP) through a timed-rate method; and the serum creatinine through the Jaffe rate method (kinetic alkaline picrate). The 25-hydroxyvitamin D levels were measured using standardized liquid chromatography-tandem mass spectrometry (LC-MS/MS). Detailed information on the laboratory tests is available at https://wwwn.cdc.gov/nchs/nhanes/search/datapage.aspx?Component=Laboratory&CycleBeginYear=2013.

Uric NNN examination

Urine TSNAs were measured using isotope-diluted high-performance liquid chromatography/electrospray ionization tandem mass spectrometry (ID HPLC-ESI MS/MS). More details are available at the official website: https://wwwn.cdc.gov/Nchs/Nhanes/2013-2014/TSNA_H.htm.

AAC evaluation

As previously reported [17, 19, 21, 22], AAC was accurately assessed through a lateral scan

of the lumbar spine (vertebrae L1–L4) using dual-energy X-ray absorptiometry. The Kauppila score system was applied to quantify the extent of AAC [19, 21, 22, 30]. More details are available at the official website: https://wwwn.cdc.gov/Nchs/Nhanes/2013-2014/DXXAAC_H.htm. The AAC score ranged from 0 to 24, and more than 6 score (＞6) was defined as severe AAC in light of previous studies [17, 19, 29, 31].

Statistical analysis

Data are presented as mean ± standard deviation or median (interquartile range, IQR) for continuous variables and as frequency or percentage (n, %) for categorical variables. The normality of the distribution was confirmed using the Shapiro–Wilk test. Uric NNN levels were analyzed as categorical variables and the low NNN group was defined as the reference. In the analysis of baseline characteristics, the statistical differences among groups of uric NNN were tested using one-way analysis of variance for continuous variables and chi-square test for categorical variables. The odds ratios (ORs) and 95% confidence intervals (CIs) for severe AAC in the uric NNN category were determined using multivariate logistic regression models. Both the crude and adjusted models (Models I–III) are shown. The potential covariates included age, sex, race, BMI, alcohol intake, hypertension, DM, TC, TG, SUA, STC, SP, total 25-hydroxyvitamin D, and serum creatinine levels. Trends were estimated using linear regression by entering the median value of each uric NNN group as a continuous variable in the models.

A generalized additive model was used to assess the nonlinear relationship between uric

NNN levels and risk of severe AAC. The nonlinearity was tested using a cubic spline term. Considering the smoothing curve, a two-piecewise linear regression model was designed to determine the threshold effect with adjustment for potential confounders. The threshold level of the uric NNN was determined using a recurrence method that included selecting the turning point along a predefined interval and choosing the turning point that yielded the maximum likelihood model. A likelihood ratio test was used to compare the two-piecewise linear regression model with the one-line linear model. Subgroup analyses were performed using stratified logistic regression models. Interactions across the subgroups were tested using a likelihood ratio test.

All statistical analyses were performed using the statistical package R (http://www.R-project.org, The R Foundation) and Free Statistics software version 1.8. The packages of tableone, rms, ggplot2 and forestplot were used for statistical analyses. Statistical significance was defined as a two-sided *P* value <0.05.

Results

The database is presented in **Supplementary Table 1**. The demographic characteristics of the 2,713 participants (1,311 men and 1,402 women), stratified by uric NNN levels are presented in **Table 1**. The mean age was 59.0 ± 11.9 years and the median (IQR) uric NNN level was 0.2 (0.2, 0.2) ng/dL. Due to the lack of a well-established standard, the uric NNN level was categorized as low or high by means of the median concentration (0.2 ng/dL) in the present study. Overall, individuals with high uric NNN levels were younger and more likely to be men, have a lower BMI, have a habit of alcohol intake, and have lower levels of serum total

25-hydroxyvitamin D (all $P < 0.05$).

The associations between uric NNN levels and severe AAC are shown in **Table 2**. The prevalence of severe AAC was 8.7% (n = 237). In all adjusted models (Models I–III), but not in the crude model, the risk of severe AAC increased in the high uric NNN group compared with the low uric NNN group. In the fully adjusted model (Model III, adjustment for age, gender, race, BMI, alcohol intake, hypertension, DM, TC, TG, SUA, STC, SP, total 25-hydroxyvitamin D and serum creatinine), the adjusted OR for participants in high uric NNN group was 2.39 (95% CI: 1.59, 3.61), compared with that in the low uric NNN group ($P < 0.001$).

The dose–response relationship between uric NNN and severe AAC is shown in **Figure 2** with a restricted cubic spline model (P for nonlinear=0.025) after adjusting for age, sex, race, BMI, alcohol intake, hypertension, DM, TC, TG, SUA, STC, SP, total 25-hydroxyvitamin D, and serum creatinine levels. To evaluate the threshold effect of uric NNN levels on severe AAC, after adjusting for the confounding factors mentioned above, a two-piecewise linear regression model was developed according to the smoothing curve shown in **Figure 2**. As shown in **Table 3**, the risk of severe AAC was positively correlated with uric NNN levels until it reached 1.354 ng/dL (95% CI: 1.201–1.507) and OR was 2.515 (95% CI: 1.100–5.748, P=0.029). When the uric NNN level exceeded 1.354 ng/dL, the OR was 0.798 (95% CI: 0.459–1.387, P=0.423), suggesting that the risk of severe AAC did not increase significantly with a further increase in uric NNN levels (P=0.017 for the likelihood ratio test) (**Table 3** and **Figure 2**).

Stratified and interactive analyses were performed to determine whether the association

between uric NNN and severe AAC was stable among the different subgroups. In general, the association in the stratified analysis was consistent with that in the multivariate logistic regression analysis (**Figure 3**). The data indicated that age played an interactive role in the relationship between uric NNN and severe AAC (P =0.033). In participants who were younger than 65 years, the association between uric NNN and severe AAC was stronger in high uric NNN group (OR 3.66 [95% CI: 2.02–6.64, P< 0.001]), compared with the low uric NNN group. No significant association was observed in participants older than 65 years (OR, 1.51 [95% CI: 0.83–2.75, P=0.176]). None of the other variables, including gender (female and male), BMI (＜ 25 kg/m^2 and ≥25 kg/m^2), and race (Hispanic and non-Hispanic), significantly influenced the relationship between uric NNN and severe AAC (all P for interaction ＞0.05) (**Figure 3**).

Discussion

The present study investigated the dose–response relationship between uric NNN and severe AAC in noninstitutionalized US civilians. In general, uric NNN levels were positively associated with the risk of severe AAC. In addition, for the first time, this study found a threshold effect of uric NNN on severe AAC, with a breakpoint of 1.354 ng/dL (95% CI: 1.201–1.507). Multiple potential covariates, including demographics, lifestyle, CVD risk factors, and laboratory measurements, were adjusted.

CS is a serious addiction and a leading modifiable risk factor for CVD [1, 12, 32]. The underlying mechanisms mainly include endothelial dysfunction, oxidative stress, platelet activation, sympathetic activation, and inflammation [3, 6]. The biomarkers involved in these

pathophysiological processes cannot accurately reflect cigarette consumption owing to their lack of specificity. To precisely assess the exposure to CS, especially in passive smokers, and the condition of CS cessation, it is necessary to find a reliable biomarker. The carcinogenic activities of nitrosamines have been well established over the past few decades [10]. TSNAs belong to the specific compounds known to be present in tobacco smoke [13, 14]. Compared with the well-documented carcinogenic activities [33], the association between TSNAs and CVD has rarely been discussed. To the best of our knowledge, this is the first study to systematically examine the dose–response relationship between TSNAs and CVD. The most widely studied TSNA-NNN [11-14], was focused on, and AAC was considered a predictor of CVD mortality, as previously documented [23-27]. It is worth noting that both NNN and AAC can be quantitatively assessed noninvasively in hospitals [28]. In this cross-sectional study, 2,713 noninstitutionalized US civilians were recruited from the NHANES 2013–2014 (**Figure 1**). The normal range of uric NNN is unknown because the relationship between NNN and CVD has not been previously investigated. In the present study, uric NNN levels were categorized as low or high, based on the median concentration (0.2 ng/dL). The overall AAC prevalence was 29.9%, and the prevalence of severe AAC was 8.7%, which is similar to that reported in other studies based on NHANES 2013–2014 [17, 22, 29] (**Tables 1** and **2**). Additionally, both prevalence rates were higher in the high-NNN group (**Tables 1** and **2**). Although the association between uric NNN and severe AAC was not statistically significant in the crude model, uric NNN levels were positively correlated with the risk of severe AAC in all adjusted models (**Table 2**). The relationship between uric NNN levels and severe AAC is shown in **Figure 2**. According to the characteristic of the smoothing curve shown in **Figure 2**

(*P* for nonlinear=0.025), we assumed that there was a threshold effect of uric NNN levels on severe AAC and the number of breakpoints was one. Therefore, a two-piecewise linear regression model was developed as shown in **Table 3**. In summary, the risk of severe AAC increases 1.515-fold for every 1 ng/dL increase in uric NNN when the concentration of uric NNN is less than 1.354 ng/dL (95% CI: 1.201–1.507) (**Table 3** and **Figure 2**). Subgroup analysis revealed that the association between uric NNN levels and the risk of severe AAC was stable between layers, except for age and race (**Figure 3**). A reasonable explanation for this may be the small sample size of the high-NNN group in each layer (**Figure 3**).

The mechanisms underlying the role of TSNAs in the development of CVD were rarely documented. Tithof *et al*. suggested that 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), a type of TSNAs, induces endothelial cell apoptosis through beta1- and beta2-adrenergic receptor-mediated release of arachidonic acid (AA)[34]. In contrast, other studies show that NNK promotes angiogenesis in tumorigenesis [35, 36]. Penn and Snyder indicated that the inhalation of butadiene (a cigarette smoke component), other than NNK, accelerates arteriosclerosis *in vivo* [37]. Further studies are required to identify the mechanisms by which TSNAs affect the occurrence and development of CVD.

Limitations

This study had some limitations. First, the causal relationship between uric NNN and severe AAC could not be assessed due to the cross-sectional nature of this study. Second, the uric NNN tests were conducted using a single measurement, which might have underestimated the strength of the associations if a regression dilution bias existed. Third, the

subjective description of several covariates in the database from NHANES 2013-2014 such as the alcohol intake may influence the results. Fourth, as a potential biomarker, the excretion and uptake of NNN may be affected by other factors, such as drinking alcohol. Finally, AAC was only detected in people aged 40 years and older, which may influence external validity. These limitations should be addressed in future studies.

Future directions

In the future, more studies are needed to verify the association between TSNAs and CVDs. It is necessary to reveal the mechanism by which TSNAs influence the development of CVDs, and to conduct cohort and interventional studies to examine whether NNN could affect the prognosis of CVDs. We believe that uric NNN may be a potentially reliable biomarker for evaluating the contribution of CS to CVD development and the benefit of CS cessation in both active and passive smokers.

Conclusion

We found that in a sample of noninstitutionalized US civilians, uric NNN levels were positively associated with the risk of severe AAC when the concentration of uric NNN was less than 1.354 ng/dL. The dose–response relationship between uric NNN and severe AAC, and the threshold effect of uric NNN levels on severe AAC were determined for the first time. The relationship in the subgroup analysis was consistent with that in the multivariate logistic regression analysis.

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Author contributions

Jingang Zheng designed this study. Fang Wang collected and analyzed the data, and completed the organization and writing of this article. Both authors approved the final manuscript.

Conflicts of interest

Both authors declare that they have no conflicts of interest.

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Figure Legends

Figure 1 Flowchart of the present study. NHANES, National Health and Nutrition Examination Survey. AAC, abdominal aortic calcification. NNN, N'-nitrosonornicotine.

Figure 2 The relationship between uric NNN levels and severe AAC in the database from NHANES 2013-2014.

The relationship was shown through a cubic spline model after adjustment for potential confounding factors (*P* for nonlinear=0.025). Four knots were chosen. Adjusted for age, gender, race, body mass index, alcohol intake, hypertension, diabetes mellitus, total cholesterol, triglycerides, serum uric acid, serum total calcium, serum phosphorus, serum creatinine and total 25-hydroxyvitamin D. AAC, abdominal aortic calcification. NHANES, National Health and Nutrition Examination Survey. OR, Odds Ratio. CI, Confidence Interval.

NNN, N’-nitrosonornicotine.

Figure 3 Subgroup analyses of the association between uric NNN levels and severe AAC in the database from NHANES 2013-2014.

Adjusted for age, gender, race, body mass index, alcohol intake, hypertension, diabetes mellitus, total cholesterol, triglycerides, serum uric acid, serum total calcium, serum phosphorus, serum creatinine and total 25-hydroxyvitamin D. AAC, abdominal aortic calcification. NHANES, National Health and Nutrition Examination Survey. OR, Odds Ratio. CI, Confidence Interval. BMI, body mass index. NNN, N’-nitrosonornicotine.