

Exposure Markers of Nitrated Aromatic Compounds and the Association with Nitrative Stress

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Cite This: *Environ. Sci. Technol. Lett.* 2023, 10, 728–734



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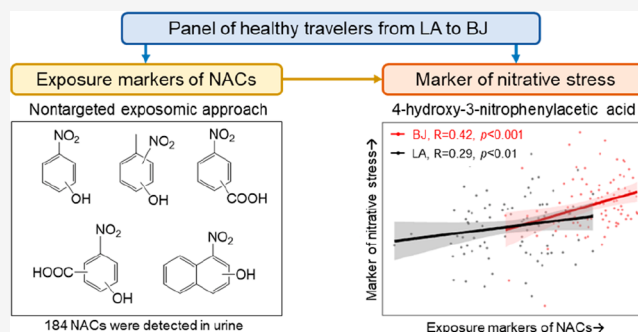
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Supporting Information

ABSTRACT: Nitrated aromatic compounds (NACs) belong to a group of ubiquitous environmental contaminants, and lacking exposure markers has restricted the accurate assessment of their exposure and subsequent health effects. Hence, based on gas chromatography and tandem mass spectrometry coupled with an electron capture ion source, we established a novel nontargeted exposomic approach to achieve highly sensitive and standard-independent detection for urinary NACs. Applying this technique to urine samples from a panel of 26 international travelers, we detected 184 NACs, of which 151 were inferred molecular formulas by accurate mass, and 25 were verified by reference standards. The isomeric profiles of the identified NACs suggested their direct excretion without functionalization metabolism after environmental absorption, which was corroborated by *in vitro* metabolism assays. Levels of 121 NACs, including nitrophenols, hydroxy-nitrobenzoic acids, and nitronaphthols, were increased from 1.3 to 21.1-fold after traveling from Los Angeles to Beijing, in association with ambient NO_2 levels. Most NACs were significantly associated with the marker of nitrative stress, with stronger associations observed in Beijing. This study provided an advanced analytical approach for exposure markers of NACs and preliminary evidence of their potential to trigger nitrative stress, an important but poorly understood response that may relate to adverse health effects.

KEYWORDS: *Exposomic approach, Exposure markers, Nitrated aromatic compounds (NACs), Nitrative stress, Urine*



INTRODUCTION

Nitrated aromatic compounds (NACs), with the structure of a nitro group(s) ($-\text{NO}_2$) covalently bonded to the aromatic ring, have received considerable attention in terms of their environmental ubiquity and potential impact. These pollutants can originate directly from incomplete combustion or be synthesized via nitration reactions involving aromatic precursors and nitrogen oxides (NO_x) in the atmosphere.¹ Such reactions can significantly increase the impact of these precursors, for instance, up to 5 orders of magnitude higher mutagenicity for nitrated polycyclic aromatic hydrocarbons (NPAHs).² As for the much higher levels of polar NACs (pNACs), typically containing hydroxyl and/or carboxyl groups, they have been acknowledged as brown carbon chromophores in ambient particles.³ However, little information was available on their health effects, such as the potential of inducing oxidative and nitrative stress.⁴ Notably, numerous NACs, including 1047 NPAHs and 1764 pNACs, have been identified in urban particles through a standard-independent approach in our previous studies,^{5,6} which significantly expanded our knowledge of NACs and highlighted a matched technique for exposure assessment to investigate their health effects.

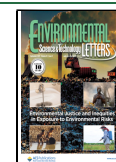
Accurate exposure assessment is the basis for a health effect evaluation. The practicability of external exposure assessment is severely limited by the complicated exposure routes of inhalation, ingestion, and even dermal contact.⁷ In comparison, internal exposure integrates the actual dose of total exposure, irrespective of temporal or spatial behavioral activity.⁸ Thus, based on exposure markers formed through nitro-reduction metabolism, five species of NPAHs were found to be associated with increased oxidative stress and endothelial dysfunction in healthy adults.⁹ However, the applicability of such exposure markers was constrained by interactions between nitro-reductase and bacteria in the anaerobic intestinal tract.¹⁰ Alternatively, NACs with hydroxyl and/or carboxyl groups may either be oxidized metabolites of NACs^{11,12} or directly excreted pNACs,^{13,14} providing potentially novel markers for NACs exposure.¹⁵

Received: July 3, 2023

Revised: July 28, 2023

Accepted: July 31, 2023

Published: August 3, 2023



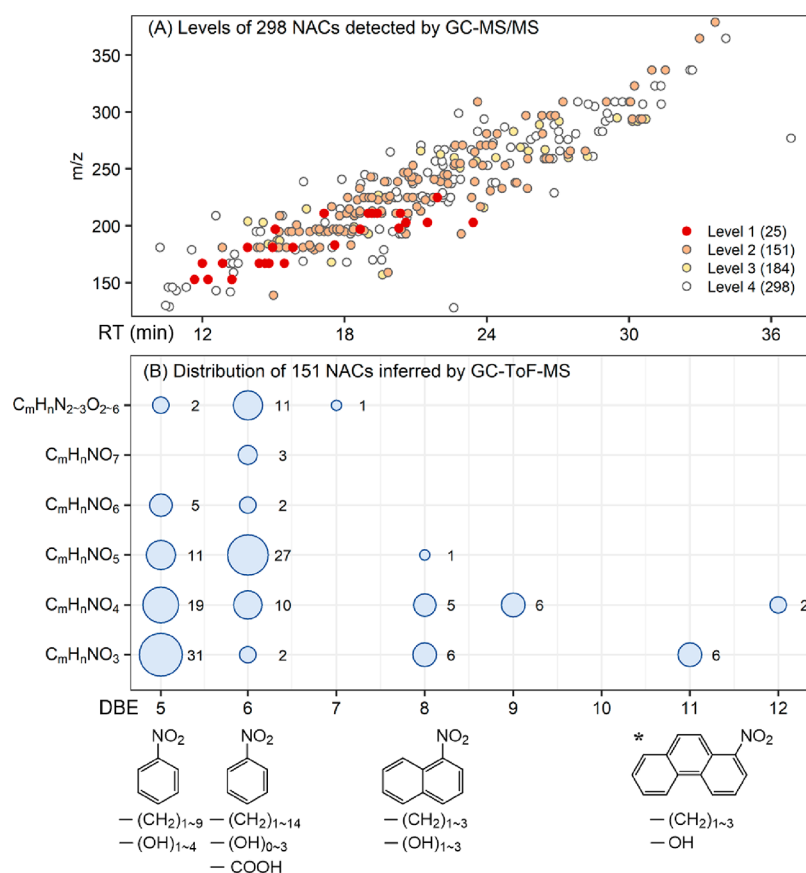


Figure 1. Distribution of detected urinary NACs by confidence levels (A) and that of identified NACs by DBE (B). Size of the circle (marked with number) represents the number of NACs species. Structure with asterisk was inferred by GC-ToF-MS.

Herein, to systematically assess NACs exposure, we developed a nontargeted exposomic approach for the discovery of potential markers of NACs in human urine and applied this technique to a panel of healthy travelers from Los Angeles (LA), USA, to Beijing (BJ), China. We further explored the reliability of urinary NACs as exposure markers and investigated their associations with oxidative and nitrate stress, the fundamental mechanism of adverse outcomes and diseases induced by environmental exposure, including cardiovascular and neurological diseases.¹⁶ As a preliminary exploration, this study intends to bridge the knowledge gap in the internal exposome of NACs and opens up possibilities to explore their potential health effects.

METHODS

Study Design and Sample Collection. As described previously,¹⁷ 26 healthy students from the University of California-Los Angeles (UCLA) were recruited in 2014 and 2015 to participate in a 70-day summer exchange program at Peking University (PKU). Seven to nine follow-ups were conducted before, during, and after the travel, yielding a total of 179 follow-ups. Demographic information was collected at the first visit. At each follow-up, the first-voided urine samples were collected after participants had fasted for >8 h, and questionnaires were conducted to acquire behavioral activities over the previous 3 days. Ambient pollutants were sourced from air monitoring stations located within a 30 km radius of each university campus. This study was approved by the Institutional Review Boards of UCLA and PKU, and informed consent was obtained from all participants.

Measurement of Urine Samples. Briefly, a 2 mL aliquot of each urine sample was spiked with surrogate standards, then hydrolyzed at pH 5.2 and 37 °C by β -glucuronidase and sulfatase overnight. After acidification to pH < 1, analytes were extracted three times with hexane/methyl *tert*-butyl ether mixture (1:1, v/v). Afterward hydroxyl and carboxyl groups were methylated with fresh diazomethane for 5 h and purified by silica gel chromatography. Finally, deuterium-labeled 1-nitronaphthalene and 9-nitroanthracene were spiked as internal standards for instrumental analysis. Meanwhile, 13 pooled samples mixed from all urine and 12 blank samples of ultrapure water were prepared.

NACs were detected by an 8890–7010B gas chromatography and tandem mass spectrometry (GC-MS/MS; Agilent Technologies, Santa Clara, CA, USA) coupled with an electron capture negative ionization (ECNI) source.⁶ A multiple reaction monitoring (MRM) mode, with prescreened precursor ions and a fixed product ion at mass-to-charge (m/z) of 46 (representing NO_2^-), was applied for all samples (Table S1 in the Supporting Information). Meanwhile, pooled samples were analyzed by an Agilent 7890B-7200 GC and time-of-flight MS (GC-ToF-MS) coupled with an ECNI source in the same chromatographic method for qualification.

Previously detected exposure markers of PAHs (i.e., OH-PAHs) and oxidative stress markers (i.e., malonaldehyde [MDA]) were adjusted by urinary creatinine.¹⁷ To verify potential sources of urinary NACs, we conducted an *in vitro* metabolism using human liver microsomes with nitrotoluene, methyl-nitrophenol, nitrobenzoic acid, and 1-nitronaphthalene as precursors. Detailed methods for urinary NACs detection

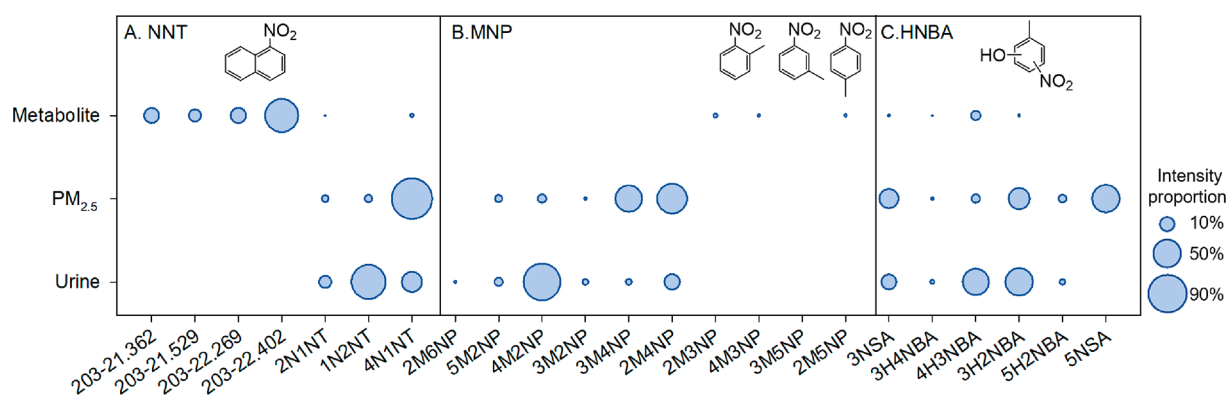


Figure 2. Isomeric profile of NACs formed through metabolism and that in $PM_{2.5}$ and urine samples. Precursors for the metabolic assays: 1-nitronaphthlene for panel A; three nitrotoluene isomers for panel B, six methyl-nitrophenol isomers for panel C. Size of the circle represents the intensity proportion of metabolites, isomers in $PM_{2.5}$, or urine samples. NNT, nitronaphthol; MNP, methyl-nitrophenol; HNBA, hydroxyl-nitrobenzoic acid.

and *in vitro* metabolism are described in the [Supporting Information](#).

Statistical Analyses. The intensities of all NACs were adjusted by the internal standard and creatinine after undetected NACs were replaced with half of the minimum detected intensity. Skewed variables were log-transformed to achieve approximately normal distribution and then analyzed by the Kolmogorov–Smirnov test. The fold change of NACs between cities was conducted by a linear mixed-effect (LME) model with participants as a random intercept to control the individual variation. The influence of behavioral activities and ambient pollutants on NACs was analyzed separately for each city, using a LME model. Associations between oxidative/nitrative stress and NACs were explored by the LME model with city (LA and BJ) as interaction term, to confirm the consistency of the associations in significantly different exposure scenarios.¹⁸ In the sensitivity analysis, phase (before, during, and after the travel) was applied as the interaction term to adjust for possible variations within LA. All statistical analyses were performed by R software, and two-tailed *p*-value < 0.05 was regarded as significant. The NTA Study Reporting Tool (SRT) was used in the preparation of this paper ([Table S2](#)).^{19,20}

RESULTS AND DISCUSSION

Detection of Urinary NACs. In this study, based on the high electrophilicity of $-NO_2$ and its resonance structure on the aromatic ring, we established a nontargeted exposomic approach on urinary NACs (postmethylation) to enable a highly sensitive and crucially standard-independent detection through GC-MS/MS coupled with an ECNI source.^{5,6} Through monitoring the negative product ion fixed at *m/z* 46 in MRM mode of tandem MS, all candidates of NACs with unit molecular weight and retention properties could be captured from a small urine volume of 2 mL, allowing the detection of a large number of samples.

As proposed previously,²¹ all candidate NACs were classified into four confidence levels ([Figure S1](#) and [Table S3](#)). Briefly, 298 species of NACs were detected in urine (Level 4; [Figure 1A](#)), with the chromatogram of a pooled sample shown in [Figure S2](#). Here, 184 species were classified as Level 3, which met the quality assurance and quality control measures of detection rate > 80%, blank-to-urine ratio < 30%, and coefficient of variation in pooled samples < 50%. Assisted by

GC-ToF-MS, molecular formulas of 151 species were inferred through the following criteria: retention time variation < 0.10 min, *m/z* accuracy < 20 ppm, at least one $-NO_2$ group, and an integer double bond equivalent (DBE) ≥ 5 (Level 2). Finally, 25 NACs, including three nitrophenols (NP), six methyl-NP, one dimethyl-NP, one nitrocatechol (NC), two methyl-NC, two nitrobenzoic acids (NBA), five hydroxy-NBA, one di-NP, three nitronaphthols (NNT), and 4-hydroxy-3-nitrophenyl-acetic acid (NHPA), were confirmed and quantified by reference standards (Level 1; [Table S4](#)).

Categorized by DBE, the distribution of NACs at Level 2 is depicted in [Figure 1B](#). The numbers of nitrogen and oxygen atoms indicated the presence of functional groups of $-NO_2$, $-OH$, and $-COOH$ in the metabolites of NACs, with up to four fused aromatic rings. Specifically, the congeners of NP (DBE = 5, $C_mH_nNO_3$) and hydroxy-NBA (DBE = 6, $C_mH_nNO_5$) were the most abundant species.

Potential Source of Urinary NACs. Theoretically, urinary NACs may act as either effect or exposure markers based on the dominant endogenous or exogenous sources. We have identified several endogenous NACs in urine, such as NHPA, a major metabolite of 3-nitrotyrosine (3NT), which is a recognized biomarker of nitrative stress implicated in inflammatory responses as well as pulmonary, neurological, and breast diseases.^{16,22} Nevertheless, because most NACs were first reported in urine, their reliability as exposure markers is largely unknown. Urinary NACs can be generated via two pathways. One involves the hydroxylation or carboxylation of NACs by enzymes such as CYP450, and the other involves the direct excretion without functionalization metabolism of absorbed environmental pNACs such as those in fine particulate matter ($PM_{2.5}$).

To investigate potential sources of urinary NACs, we compared the isomeric profiles of three NACs, namely, NNTs (*n* = 7), methyl-NPs (*n* = 10), and hydroxy-NBAs (*n* = 6) in urine samples, $PM_{2.5}$ samples in Beijing,^{5,6} and human liver microsomes incubated with hypothetical precursors of NNTs (1-nitronaphthalene), methyl-NPs (three nitrotoluene isomers), and hydroxy-NBAs (10 methyl-nitrophenol and three nitrobenzoic acid isomers; [Figure 2](#)).

In the case of NNT, $-NO_2$ and $-OH$ are typically homocyclic in atmospheric NNT,²³ matching the structure of those observed in urine. Conversely, $-NO_2$ and $-OH$ are heterocyclic in the metabolites of 1-nitronaphthalene as

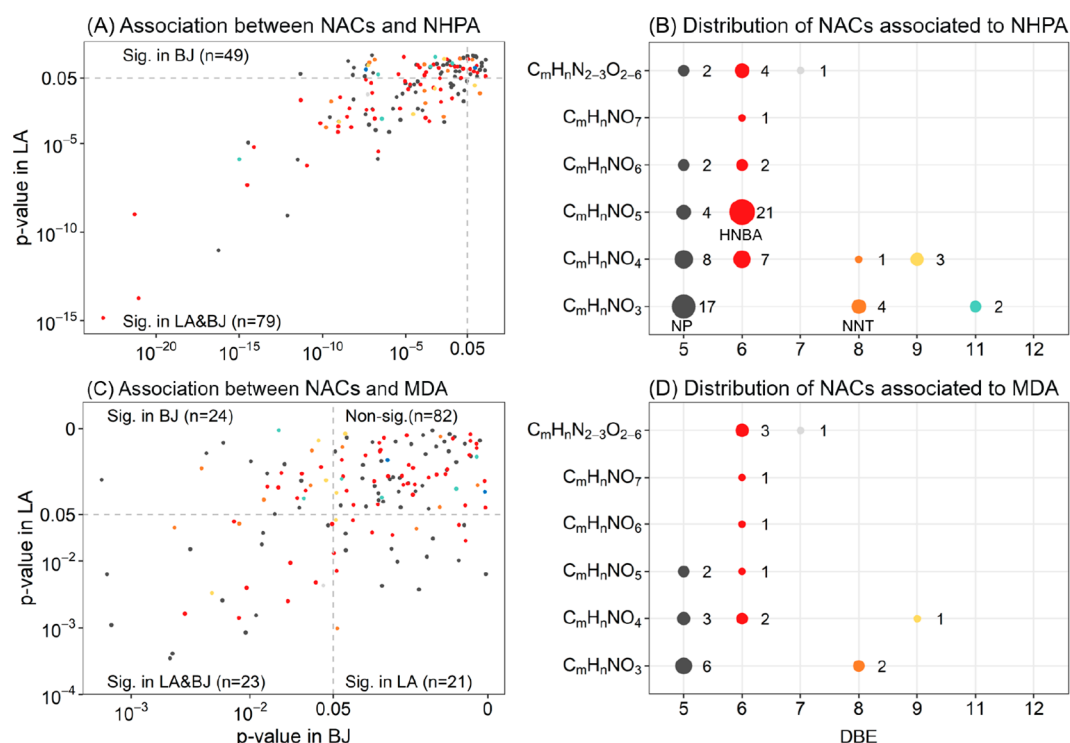


Figure 3. Associations between NACs and NHPA (A) and MDA (C) and distribution of NACs with significant association to NHPA (B) or MDA (D). Size of the circle (marked with number) represents the number of NAC species, and color of the circle corresponds to DBE.

demonstrated in rat lung microsomes and infant monkey airways.^{24,25} While for methyl-NP, $-NO_2$ and $-OH$ were at the ortho- or para-position for both atmospheric and urinary MNP.^{13,26} However, nitrotoluene isomers were metabolized to mainly carboxylated products, with a small portion of meta-hydroxylated MNP.²⁷ Furthermore, the structure of atmospheric hydroxy-NBA corresponds to that in urine, with $-NO_2$ and $-OH$ at the ortho- or para-position relative to $-COOH$ group.^{6,28} The metabolic source of HNBA could be further ruled out due to the low metabolic rates of NBA hydroxylation or MNP carboxylation. As evidenced by the abundant species, the structure of urinary NACs isomers more closely resembles that of atmospheric pNACs.

Additionally, comparing chromatograms of pNACs in both urine and $PM_{2.5}$ samples, we identified 96 NACs present in both urine and $PM_{2.5}$ samples with identical m/z and retention time.^{5,6} The median concentrations of these NACs in urine and $PM_{2.5}$ samples were significantly correlated ($R = 0.49$, $p < 0.001$; Figure S3), suggesting the exogenous sources of these NACs in the urine.

Leveraging the natural experiment with contrasted pollution levels between LA and BJ, we observed significant increases in 121 NACs, ranging from 1.3 to 21.1-fold in BJ compared with LA (Figure S4). Based on data from questionnaires and ambient monitoring stations, we investigated the factors that could influence urinary NACs (Table S5). Specifically, the dominant factors were NO_2 and driving duration in LA, which were associated with $\sim 20\%$ urinary NACs, while ambient pollutants were associated with $\sim 30\%$ urinary NACs in BJ. Notably, NO_2 was a critical factor on NACs in both LA and BJ, manifesting from 1.2 to 3.3-fold and 1.5 to 13.3-fold increases in urinary NACs as NO_2 levels doubled, respectively. As previously reported, $PM_{2.5}$ -bound pNACs could have originated from traffic emissions and/or atmospheric reactions,

among which NO_2 was the major promoting factor.²⁶ Therefore, urinary NACs, in general, could be applied as exposure markers for pNACs absorbed directly from environmental sources.

Association between Urinary NACs and Nitritative Stress. Oxidative stress, characterized by an overabundance of reactive oxygen species (ROS), is a rapid consequence of pollutants and is also considered as the initiation of various health effects. Similarly, excessive reactive nitrogen species (RNS) can initiate nitritative stress, yet little was known about their responding species.^{16,29} The presence of $-NO_2$ might imply the potential to generate a RNS from NACs. To test such hypothesis, we applied MDA and NHPA as markers of oxidative and nitritative stress, respectively, and examined their associations with 151 urinary NACs at Level 2.

As shown in Figure 3, more than a half of NACs ($n = 79$) were significantly associated with NHPA in both cities, with NP, HNBA, and NNT as the dominant species. Among them, 25 NACs showed statistically higher associations in BJ than in LA, such as 4-methyl-2-NP, 3-NBA, 3-hydroxy-4NBA, 3-nitrosalicylic acid (NSA), and 2-nitro-1-naphthols. MDA maintained stable associations with 23 NACs, mainly NP, in both cities but without intercity differences, suggesting that certain NACs could activate nitritative and oxidative stress (Table S6). Additionally, urinary OH-PAHs in both cities were positively correlated with MDA, but not with NHPA, hinting at the specificity of nitritative stress for NACs (Figure S5). These findings remained consistent in the sensitivity analysis with the phase as the interaction term.

Previous studies have assessed the effects of certain NACs, such as the vasodilatory, estrogenic, and antiandrogenic activities of 4-NP, 2-methyl-4NP, and 3-methyl-4NP by rat thoracic artery and *in vitro* assays.³⁰ Eleven NACs in $PM_{2.5}$ showed positive correlations with interleukin (IL)-6 and 8-

hydrox-2'-deoxyguanosine (8-OHdG) in urine and saliva of 18 homemakers.³¹ However, a comprehensive investigation of NACs based on exposure markers is largely absent from the literatures.

As previously reported, nitrate stress is triggered by RNS imbalance, such as peroxynitrite, formed by nitric oxide synthase (NOS)-synthesized NO with a superoxide anion. Peroxynitrite can nitrate tyrosine to form 3-nitrotyrosine (3NT), which can then be metabolized to NHPA and excreted in urine.³² Normally, NO is modestly generated by endothelial NOS (eNOS). External stimulation such as PM_{2.5} could induce oxidative stress and further upregulate inducible NOS (iNOS), which could in turn transiently release plentiful NO and inhibit eNOS through negative feedback.²⁹

As evidenced by our study, NACs could specifically trigger nitrate stress upon activating oxidative stress, especially in highly polluted scenarios in BJ, yet PAHs could not. Previous studies somewhat were consistent with our results. As a major source of NACs and PAHs, particulate exposure exerted a dose–response association with 3NT, along with iNOS upregulation and NO production.³³ Whereas carcinogenic PAHs, including benzo[a]pyrene, showed no association with 3NT in bus drivers.³⁴ NACs, including 1-NNAP and 9-NANT, reportedly reduced eNOS and subsequent NO production in both *in silico* and *in vitro* cellular assays, in contrast to their parent PAHs.³⁵ Although the underlying mechanisms remained unclear, it was plausible that the –NO₂ group in NACs could promote iNOS activity and suppress eNOS after intake, but PAHs could not.

MDA and NHPA levels in BJ were significantly increased by 1.18-fold ($p < 0.05$) and 1.66-fold ($p < 0.001$) compared to LA, suggesting that more NO could react with more superoxide anions with higher oxidative stress under high pollution scenarios, thereby amplifying the associations between NACs and nitrate stress.

In conclusion, this study pioneered a practical exposomic approach to discover exposure markers of NACs that were absorbed and directly excreted. Significantly, most NACs showed associations with nitrate stress, especially in the highly polluted urban region, suggesting that greater attention to NACs is warranted for their health effects. Nevertheless, it is noteworthy that the robust associations between urinary NACs and nitrate stress were confirmed by using different cities, but the underlying mechanism requires definitive toxicological studies. Despite illustrating potential sources of major NACs by comparing *in vitro* and PM_{2.5}, it is better to match internal and external exposure research.

■ ASSOCIATED CONTENT

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.estlett.3c00466>.

Detection method of urinary NACs; human liver microsomal incubation experiment; list of selected precursor ions in the MRM method of GC-MS/MS; demographic characteristics and environment exposure of participants and their influence on urinary NACs; identification criteria for NACs; representative chromatogram of pooled sample; corresponding levels of NACs in urine and PM_{2.5} samples; concentrations of urinary NACs and OH-PAHs in two cities; fold changes in

NHPA and MDA with a one-fold increase in urinary NACs and OH-PAHs in two cities (PDF)

Result of the NTA Study Reporting Tool (SRT); detailed results of NACs identification; concentrations of urinary NACs; association between urinary NACs and oxidative/nitrate stress (XLSX)

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Author Contributions

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This research was supported by the National Natural Science Foundation of China grants (NSFC, 42293324, 41961134034, and 21876002 to X.Q.), the National Institute of Environmental Health Sciences (NIEHS, 1R21ES024560 to Y.Z. and J.A.A.), the 111 Project of Urban Air Pollution and Health Effects (B20009), and the International Joint Laboratory for Regional Pollution Control, Ministry of Education. We acknowledge the extensive support from the Joint Research Institute in Science and Engineering by Peking University and UCLA. We also thank the volunteers who participated in our study; Erin Hakim and Larissa Harrison for assistance with recruitment; and Nu Yu, Yanwen Wang, and Hiromi Murata for assistance with sample collection.

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