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Urinary Polycyclic Aromatic Hydrocarbon Metabolites as Biomarkers of Exposure to Traffic-Emitted Pollutants

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Abstract

1-nitro-pyrene has been considered a compound specific to diesel combustion emission, while 1- and 2-nitro-naphthalene are mainly produced through photochemical conversion of naphthalene released to the atmosphere. Metabolites of these compounds may serve as biomarkers of exposure to traffic related pollutants. We collected urine samples from 111 healthy and nonsmoking subjects within (i.e., during the Beijing Olympics) and outside (i.e., before and after the Olympics) a traffic control regime to improve Beijing's air quality. Urines were analyzed for the sum of 1&2-amino-naphthalene (metabolites of 1- and 2-nitro-naphthalene) and 1-amino-pyrene (a metabolite of 1-nitro-pyrene), using an HPLC-fluorescence method. Within the same time periods, PM_{2.5} mass and constituents were measured, including elemental carbon, sulfate, nitrate, PAHs, carbon monoxide, nitrogen dioxide, sulfur dioxide, ozone, and particle number concentrations. The associations between the urinary metabolites and air pollutants were analyzed using linear mixed-effects models. From the pre- to during-Olympic period, 1&2-amino-naphthalene and 1-hydroxy-pyrene decreased by 23% (p=0.066) and 16% (p=0.049), respectively, while there was no change

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

in 1-*amino*-pyrene (2% increase, $p=0.892$). From during- to post-Olympic period, 1&2-*amino*-naphthalene, 1-*amino*-pyrene and 1-*hydroxy*-pyrene concentrations increased by 26% ($p=0.441$), 37% ($p=0.355$), and 3% ($p=0.868$), respectively. Furthermore, 1&2-*amino*-naphthalene and 1-*hydroxy*-pyrene were associated with traffic related pollutants in a similar lag pattern. 1-*amino*-pyrene was associated more strongly with diesel combustion products (e.g. PN and elemental carbon) and not affected by season. Time-lag analyses indicate strongest/largest associations occurred 24–72 hours following exposure. 1&2-*amino*-naphthalene and 1-*hydroxy*-pyrene can be used as a biomarker of exposure to general vehicle-emitted pollutants. More data are needed to confirm 1-*amino*-pyrene as a biomarker of exposure to diesel combustion emissions. Controlling creatinine as an independent variable in the models will provide a moderate adjusting effect on the biomarker analysis.

Keywords

PAH metabolites; biomarkers; diesel exhaust particles; exposure assessment; traffic-emitted pollutants

1. Introduction

Nitrated polycyclic aromatic hydrocarbons (*nitro*-PAHs) are known for their mutagenic and carcinogenic toxicities (Talaska et al., 1996). The major sources of atmospheric *nitro*-PAHs include direct emissions from the incomplete combustion of fossil fuels, especially diesel (Feilberg et al., 2001; Bamford and Baker 2003), and the formation through photochemical reactions between parent PAHs and the hydroxyl radical during daytime and the nitrate radical during nighttime (Atkinson and Arey 1994; Arey and Arkinson 2003). Particle-bound PAHs can also be formed through the heterogeneous reactions between PAHs and $N_2O_5/NO_3/NO_2$ on the surface of particles (Zimmermann et al., 2013). A wide variety of *nitro*-PAHs have been detected in diesel exhaust particles and airborne particles from biomass burning in urban environments (Dimashki et al., 2000; Marino et al., 2000; Bamford and Baker 2003; Wang et al., 2011). Therefore, people can be exposed to *nitro*-PAHs through the inhalation of airborne particles from diesel and biomass combustion.

Post inhalation, *nitro*-PAHs can be metabolized to *amino*-PAHs that are ultimately excreted through urine (Poirier and Weisburger 1974; Nachtman and Wei 1982; van Bakkum et al., 1998). Therefore, it is biologically plausible to use urinary *amino*-PAHs as biomarkers of *nitro*-PAH exposure. A study by Laumbach et al. (2009) observed higher urinary 1-*amino*-pyrene concentrations among human volunteers following a one-hour exposure to a diesel exhaust mixture at $300 \mu\text{g}/\text{m}^3$ PM_{10} (particulate matter with an aerodynamic diameter smaller than $10 \mu\text{m}$) in an exposure chamber, compared to those exposed to clean air (Laumbach et al., 2009). This study supports earlier publications that demonstrated 1-*nitro*-pyrene as one of the *nitro*-PAH isomers to diesel combustion (Schuetzle et al., 1982; Paputapeck et al., 1983; Zwirner-Baier and Neumann 1999; Bamford and Baker 2003). In a more recent study, Neophytou et al. (2014) found associations between urinary *amino*-PAHs with vehicle exhaust-related $PM_{2.5}$ (particulate matter with an aerodynamic diameter smaller than $2.5 \mu\text{m}$) (Neophytou et al., 2014). Existing studies have also reported increases in

urinary 1-*amino*-pyrene in underground miners following a work shift using diesel-powered machinery (Seidel et al., 2002). However, few studies have examined whether urinary *amino*-PAHs are associated with diesel traffic exposure in urban residents.

During the 2008 Beijing Olympics, aggressive air pollution control measures were implemented to reduce traffic emission and improve Beijing's air quality (Wang et al., 2009a). The air pollution control measures included the introduction of new vehicular emission standards, restrictions on diesel-powered vehicles in Beijing's urban areas, limited operation of local industrial and commercial combustion facilities and enforcement of alternate day driving that removed approximately half of the vehicles (~1.5 million) from the local roads each day (Wang et al., 2009a; Rich et al., 2012). Various studies found substantial reductions in traffic-emitted air pollutants, including *nitro*-PAHs, during the Olympic period compared to before and after the Olympics (Wang and Xie 2009; Wang et al., 2009a; Wang et al., 2011; Rich et al., 2012). Taking advantage of this unique opportunity, we conducted a study to measure urinary 1&2-*amino*-naphthalene and 1-*amino*-pyrene in a panel of Beijing residents (Zhang et al., 2013). By assessing the association between the urinary *amino*-PAHs and exposure to traffic-emitted air pollutants, we aim to evaluate the validity of using 1&2-*amino*-naphthalene and 1-*amino*-pyrene as internal markers of exposure to *nitro*-PAHs in traffic-emitted pollutants. Because 1-*hydroxy*-pyrene as a major metabolite of pyrene has been often used as a biomarker of PAH exposure, we also aim to examine the association between urinary 1-*hydroxy*-pyrene and traffic-emitted pollutants (Strickland and Kang 1999; Hu et al., 2006).

2. Methods

2.1. Study Design and Subjects

Centered around the air pollution control measures described above, three sampling periods were defined in our study as: the pre-Olympic period (2 June 2008–19 July 2008) where some relatively mild controls were implemented, the during-Olympic period (July 20, 2008–September 19, 2008) where the full-scale control measures were implemented, and the post-Olympic period (September 20, 2008–October 30, 2008) where the majority of the control measures were relaxed. In previous studies, drastic reductions in the concentrations of air pollutants were observed during the Olympic period compared to the pre- and post-Olympic periods (Wang and Xie 2009; Wang et al., 2009a; Wang et al., 2010; Rich et al., 2012). Therefore, our three-period study design (the pre-during-post Olympics) followed a 'high-low-high' air pollution changing pattern.

In the current analysis, study subjects included 111 (55 male and 56 female) nonsmoking individuals, 22–27 years of age. The subjects were recruited from the pool of medical residents at the Peking University First Hospital (hereinafter referred to as 'the hospital'). All study participants worked on the campus of the hospital and resided in dormitories at either the hospital or Peking University Health Sciences Center located within 5 km from the hospital. In each of the three Olympic periods, a spot urine sample was collected from each subject in the morning of the visit (between 8–10am). Each subject was scheduled to visit the clinic the same day of the week for all the three visits unless adverse events such as severe respiratory infectious diseases kept the subjects from timely clinical visits (Zhang et

al., 2013). The sample collection in each of the three Olympic period lasted for four weeks. The study protocol was approved by the Institutional Review Board of the University of Medicine and Dentistry of New Jersey as well as the Ethics Committee of the Peking University Health Sciences Center and Peking University First Hospital.

2.2. Air Pollution Monitoring

The air pollution measurements were conducted as described previously (Rich et al., 2012; Zhang et al., 2013). In brief, air samplers and monitors were placed on top of a seven-story building located in the center of the Peking University First Hospital campus. Carbon monoxide (CO), nitrogen dioxide (NO₂), ozone (O₃), PM_{2.5}, elemental carbon (EC), sulfur dioxide (SO₂), sulfate (SO₄²⁻), nitrate (NO₃⁻), and particle number concentrations (PN) in the size range of 13.0–764.8 nm were monitored throughout the three study periods, beginning one week before the start of urine sample collection. Ambient temperature and relative humidity (RH) were monitored continuously and concurrently at the same site. PM_{2.5} were collected on a 37 mm Teflon filter using a Quad Channel Ambient Particulate Sampler equipped with an impactor that has an aerodynamic cut-off of 2.5 µm (TH-16A, Tianhong Inc. China) at a flow rate of 16.7 L/min. Polycyclic aromatic hydrocarbon concentrations in PM_{2.5} collected during the pre- and the during-Olympic periods were analyzed from fine particles using a method employing a gas chromatography–mass spectroscopy system (He et al., 2006; Huang et al., 2006). The method details can be found in the Supplementary Materials (Appendix I).

2.3. Urinary amino-PAH measurements

The current study used a modified method to analyze urinary *amino*-PAHs as described in a previous publication (Laumbach et al., 2009). In brief, 2 ml of urine sample were incubated with 20 µl of β-glucuronidase from *Helix pomatia* Type H-2 (Sigma-Aldrich, St. Louis, MO) in 2 ml 0.1 M sodium acetate buffer (pH 5.0) at 37°C overnight. The hydrolyzed urine samples were adjusted to pH>10 with the addition of 25 µl of 10 M sodium hydroxide and extracted with 4 ml of ethyl acetate. After mixing on a shaker for 10 min, the samples were centrifuged at 3500 rpm for 10 min. The supernatant was evaporated to dryness under nitrogen in a TurboVap LV evaporator operated at 35°C. The residue was reconstituted in 200 µl of methanol and 20 µl was injected into an HPLC-fluorescence system for the detection of 1&2-*amino*-naphthalene (as one single peak without resolving 1-AN and 2-AN), 1-*amino*-pyrene, and 1-*hydroxy*-pyrene. The chromatographic separation was achieved on a Supelco-Ascentis RP-Amide column (250 × 4.6 mm, 5 µm, Sigma-Aldrich, St. Louis, MO). The mobile phase was 50% acetonitrile (A) and 100% acetonitrile (B), with a linear gradient from 0% B at 0 min to 70% B at 30 min. The fluorescence detector was set up at 254/425 nm (Ex/Em).

The limits of detection were estimated as 3 times the standard deviation of 8 injections of a lower concentration calibration standard (0.25 ng/ml). The recovery of the assay was expressed as the ratio (%) of the concentration measured to the concentration spiked into a real urine sample (two concentrations were spiked including 1 ng and 10 ng). The precision of the assay was expressed as the coefficient of variation (%) of 8 repeated injections. In summary, the limit of detection of the three PAH metabolites were 0.04, 0.02, and 0.04

ng/ml, the recoveries were 84.3%, 88.3%, and 74.1%, and the precision were 6.0%, 10.1%, and 6.0% for 1&2-*amino*-naphthalene, 1-*amino*-pyrene, and 1-*hydroxy*-pyrene, respectively. The representative chromatograms of unspiked and spiked urine samples were provided in the Supplementary Materials (Figure S8).

2.4. Statistical analysis

Linear mixed-effects models were used to estimate the changes in urinary *amino*-PAH levels across the three sampling periods (pre-, during-, and post-Olympic period). Since the concentrations of the three urinary metabolites of PAHs followed a log-normal distribution, we used their log-transformed concentrations as the dependent variables in these mixed-effects models. Period was set as a categorical fixed effect, and individual subjects were treated as a random effect. A compound symmetry covariance structure was applied in the models as it provided the best fit (with the smallest values of Akaike Information Criterion) to the repeated observation data (Zhang et al., 2013). We also controlled for ambient temperature, relative humidity, gender, and the day of week as covariates in the models. To control for the family-wise type I error rate at a 0.05 level, a Bonferroni correction was applied. With 6 between-period comparisons (3 biomarkers by 2 between-period changes), each individual 2-sided test was considered statistically significant relative to a 0.008 significance level.

Furthermore, we performed analyses to examine associations between the three urinary biomarkers and the traffic pollutants using linear mixed-effects models with a single pollutant. In these models, the period effect was replaced by the measured concentrations of individual air pollutants. The other covariates, random-effect variables, and the covariance structure were the same as described earlier in this section. Concentrations of the traffic pollutants were averaged over various time periods before the time when urine samples were collected, categorized as lag 0 (0–23 h), lag 1 (24–47 h), lag 2 (48–71 h), and lag 3 (72–95 h). Each model estimated the association of each PAH metabolite with one pollutant at one lag day. Spearman correlation analysis was performed among the traffic pollutants to detect potential collinearity.

2.5. Different creatinine adjustment methods

Considering the diluting effect on urine samples, the PAH metabolites were adjusted for creatinine in the period effect analysis and their associations with the air pollutants. We used three creatinine adjustments on the PAH metabolites, including (1) creatinine-unadjusted: no adjustment for creatinine was made on the PAH metabolite data, (2) creatinine-standardized: the concentrations of the PAH metabolites were standardized by dividing the levels of creatinine, and (3) creatinine-independent: creatinine was controlled in the mix-effect models as an independent variable. These two ways of the creatinine adjustment were performed since we observed in a previous study that creatinine-standardized biomarker data showed less significant results than creatinine-unadjusted outcome, which may indicate a potential over adjustment for creatinine in a study designed to examine the within-subject effects (Gong et al., 2013).

3. Results

3.1. Changes in Air pollutant Concentrations by Period

The changes in air pollutant concentrations across the three Olympic periods have been reported in previous publications (Huang et al., 2012; Rich et al., 2012; Gong et al., 2013). In summary, we observed a 13–60% reduction in the mean concentration of major traffic-related pollutants (Table 1) during the extensive traffic control period. Furthermore, ozone concentrations showed a non-significant increasing trend (24%), which may be due to the reduction in NO emission from the traffic sources since NO is a major scavenger of ozone in the atmosphere. Similar to the other traffic-emitted pollutants, pyrene and the total PAHs decreased from the pre- to during-Olympic period by 37% ($p<0.01$) and 38% ($p<0.01$), respectively.

3.2. Changes in Urinary Biomarker Concentrations by Period

Geometric means (GM) of the three PAH metabolites by period are shown in Table 2. For the creatinine-unadjusted values, from the pre- to during-Olympic period, the concentrations of urinary 1&2-*amino*-naphthalene, 1-*amino*-pyrene and 1-*hydroxy*-pyrene decreased by 29% ($p=0.016$), 10% ($p=0.497$) and 30% ($p=0.003$). From the during- to post-Olympic period, 1&2-*amino*-naphthalene and 1-*amino*-pyrene increased by 11% ($p=0.737$) and 10% ($p=0.789$) without reaching statistical significance. While the concentration of 1-*hydroxy*-pyrene continued to decrease by 23% ($p=0.328$) from the during- to the post-Olympic period without reaching statistical significance. With adjustment for creatinine using both methods, the reductions in 1&2-*amino*-naphthalene and 1-*hydroxy*-pyrene from the pre- to the during-Olympic period were in lower levels compared to the result using the method unadjusted for creatinine. Furthermore, we observed an increase in 1-*amino*-pyrene from pre- to during-Olympic period with adjustment for creatinine. The Spearman correlation coefficients among the three PAHs metabolites were 0.402 ($p<0.0001$) between 1&2-*amino*-naphthalene and 1-*amino*-pyrene, 0.425 ($p<0.0001$) between 1-*amino*-pyrene and 1-*hydroxy*-pyrene, and 0.639 ($p<0.0001$) between 1&2-*amino*-naphthalene and 1-*hydroxy*-pyrene, indicating a moderate correlation between each two of the three urinary PAH metabolites.

3.3. Associations between Urinary Biomarkers and Traffic-related Air Pollutants

Percent changes in the PAH metabolites associated with interquartile range increases in all pollutants are shown in Figure 1A–C. The results were obtained through mixed-effects models with creatinine controlled as an independent variable. A consistent pattern was found for the changes in the associations between 1&2-*amino*-naphthalene and the pollutants (except CO and ozone), showing that the percent changes increased from lag 0 to lag 2 and then started to decrease at lag 3, and the largest percent changes at lag 2 were statistically significant (Figure 1A). Associations between 1&2-*amino*-naphthalene and CO were significant across lag 0 to 4, with the largest percent changes appearing at lag 1.

Figure 1B shows that the percent changes in 1-*amino*-pyrene was associated with the changes in air pollutants from lag 0 to 3 in a similar pattern similar comparing to 1&2-*amino*-naphthalene. The percent changes in 1-*amino*-pyrene associated with NO₂, PN, and EC increased from lag 0 to lag 2 and decreased from lag 2 to 3, while the percent changes

associated with CO, PM_{2.5}, sulfate, and nitrate increased from lag 0 to lag 1 and then decreased from lag 1 to lag 3. The percent changes in 1-*amino*-pyrene associated with changes in SO₂, NO₂, PM_{2.5}, PN, EC, sulfate, and nitrate were statistically significant at lag 1 and lag 2, while none of the changes were statistically significant in the associations between 1-*amino*-pyrene and CO, SO₂, pyrene, or total PAHs.

Figure 1C shows that the percent changes in 1-*hydroxy*-pyrene associated with NO₂, EC, pyrene and the total PAHs increased from lag 0 to lag 2 and then decreased at lag 3, while the changes associated with CO, PM_{2.5}, sulfate, and nitrate showed the largest values at lag 1. We observed significant percent changes in 1-*hydroxy*-pyrene associated with changes in all the measured air pollutants except ozone.

Due to negative correlations of ozone with the other pollutants (Table 3), ozone expectedly showed negative or non-significant associations with 1&2-*amino*-naphthalene, 1-*amino*-pyrene, and 1-*hydroxy*-pyrene at various lags. The creatinine-standardized and creatinine-unadjusted results of percent changes in the PAH metabolites associated with the changes in air pollutants were showed in the Supplementary Materials (Figure S1–S6).

4. Discussion

The current study reported comparable levels of urinary 1&2-*amino*-naphthalene and 1-*amino*-pyrene with other studies. Grimmer et al. (2000) reported the urinary levels of 1&2-*amino*-naphthalene in humans, and found that the sum of the two urinary metabolites was 189.7 and 591.2 ng/day in non-smokers and smokers. In the current study, assuming a typical daily urine volume of 1 liter, the daily excretion doses of 1&2-*amino*-naphthalene observed in healthy adults (non-smokers) were approximate 340, 240, and 270 ng/day in the pre-, during-, and post-Olympic periods, respectively. The doses observed in the current study were between the levels of non-smokers and smokers observed in Grimmer et al (2000) study. Laumbach et al. (2009) measured urinary 1-*amino*-pyrene levels in subjects after 1-hr exposure to ~300 µg/m³ diesel exhaust particles, and found that the concentrations of urinary 1-*amino*-pyrene in subjects with exposure to diesel exhaust particles and to the clean air were 324.0±442.4 and 234.4±852.9 pg/mg creatinine (Laumbach et al., 2009). These results were comparable to the creatinine-standardized data of the current study (Table 2). In another study, Scheepers et al. (1995) used ELISA method to measure urinary 1-*amino*-pyrene from railroad workers and found that the 1-*amino*-pyrene levels approximately ranged from 3 –13 µmol/mol creatinine (5.8–25.0 pg/µg creatinine), which was about one to two orders of magnitude higher than what we reported in our study (Scheepers et al., 1995).

As the parent compound of 1-*amino*-pyrene, 1-*nitro*-pyrene comes primarily from direct emissions of diesel combustion, and it is one of the most abundant *nitro*-PAHs in diesel exhaust (Bamford and Baker 2003; Reisen and Arey 2005). During the Olympics, heavy-duty vehicles, which typically consume diesel, had been strictly forbidden to enter the urban area (within the 5th ring road) of the city, which led to significant reductions in the diesel exhaust emissions (Wang et al., 2009a). Since diesel truck traffic has been associated with the black carbon concentrations in Beijing (Westerdahl et al., 2009), the 73% reduction in

black carbon during the Olympic period observed by Wang et al. (2009b), indicates a reduction in diesel exhaust emissions during the Olympic period.(Wang et al., 2009b).

However, we did not consistently observe lower concentrations of urinary 1-*amino*-pyrene during the Olympic period through the creatinine-unadjusted and adjusted methods (Table 2). Even though a 10% reduction in 1-*amino*-pyrene was obtained by using creatinine-unadjusted method, it was not statistically significant. After the adjustment for creatinine, the reduction in 1-*amino*-pyrene disappeared from the pre- to the during-Olympic period. Therefore, our data were not sufficient to associate the period changes in the urinary 1-*amino*-pyrene to the period changes in exposure to 1-*nitro*-pyrene. Similar result was reported by Neophytou et al. (2014) that there was no significant associations of urinary 1-*amino*-pyrene and any of the microenvironment exposure measures, including PM_{2.5}, elemental and organic carbons, in their study (Neophytou et al., 2014). More data are needed to confirm the eligibility of using urinary 1-*amino*-pyrene as a biomarker of exposure to 1-*nitro*-pyrene.

Different from 1-*nitro*-pyrene, 1-*nitro*-naphthalene and 2-*nitro*-naphthalene are two major products of gas-phase photochemical reactions between naphthalene and the hydroxyl radical (daytime) or the nitrate radical (night time) in the atmosphere (Arey et al., 1987; IARC 1989; Atkinson and Arey 1994; Reisen and Arey 2005). Therefore, ambient concentrations of 1-*nitro*-naphthalene and 2-*nitro*-naphthalene are dependent on atmospheric concentrations of naphthalene and photochemical reaction activity. As a major source of ambient naphthalene, the vehicle emissions were reduced from the pre-Olympics to the during-Olympics, followed by a 31% reduction in ambient naphthalene (Wang et al., 2011). Furthermore, He et al. (2010) found that hydrogen peroxide, an important precursor of the hydroxyl radical, was significantly decreased from the 29th of July (before the Olympics) to the 15th of August (during the Olympic full-scale control period) (He et al., 2010). Both of the above findings were favorable to a low level of ambient naphthalene and free radical gas-phase reaction activities. In fact, Wang et al. (2011) reported a 28% ($p=0.002$) reduction in 2-*nitro*-naphthalene concentration during the Olympic period compared to the non-Olympic period (Wang et al., 2011). In the current study, we observed reductions in urinary 1&2-*amino*-naphthalene (regardless adjustments for creatinine) from the pre- to the during-Olympic period, consistent with reduced concentrations of *nitro*-naphthalene from which 1&2-*amino*-naphthalene were derived inside the human body. After the Olympics when all the traffic control measures were relaxed, the concentration of 1&2-*amino*-naphthalene went up to the level in the pre-Olympic period (Table 2). This finding suggests that 1&2-*amino*-naphthalene might be a biomarker of exposure to both general traffic-emitted pollutants and secondary sources. A similar conclusion was obtained by Neophytou et al. (2014) as well, whose findings suggested associations of 1&2-*amino*-naphthalene with more general measures of traffic-related air pollution (Neophytou et al., 2014).

As the most abundant metabolite of pyrene, 1-*hydroxy*-pyrene may be used as a biomarker of exposure to airborne pyrene or the total PAHs when dietary and other sources of PAHs are not important (Strickland and Kang 1999). In the current study, we observed significant reductions in urinary 1-*hydroxy*-pyrene (using the creatinine-independent method) from the

pre- to the during-Olympic period, following the changes in air pollutants (Table 1). It may suggest urinary 1-*hydroxy*-pyrene as a biomarker of exposure to general traffic-emitted pollutants. After the Olympics, the level of 1-*hydroxy*-pyrene remained the same as in the during-Olympic period. It is notable that in the post-Olympic period when the traffic control measures were relaxed, the traffic emissions increased as reflected by increased atmospheric concentrations of CO, NO₂, PN, etc. (Table 1). Meanwhile, the concentration of PM_{2.5}-bound pyrene continued to decrease by -59% from the during- to the post-Olympics. Therefore, it may also suggest that urinary 1-*hydroxy*-pyrene may serve as a biomarker of exposure to ambient pyrene.

The associations between the PAH metabolites and the individual air pollutants further confirm the findings we obtained from the period-specific comparisons discussed above. As shown in Figure 1A, urinary 1&2-*amino*-naphthalene was significantly associated with the air pollutants from both primary sources, including CO, SO₂, NO₂, PM_{2.5}, PN, EC, pyrene, and the total PAHs, and secondary sources, such as PM_{2.5}, sulfate, and nitrate. Wang et al. (2011) also reported that the greater formation of *nitro*-PAHs after the Olympic compared to the Olympic period was the result of both meteorological conditions and increased vehicle emissions (Wang et al., 2011). Figure 1B showed that 1-*amino*-pyrene was significantly associated with primary pollutants, like NO₂, PM_{2.5}, EC, and PN, but not with CO (a good indicator of general vehicle emissions) (Reisen and Arey 2005). In Figure 1C, we found that 1-*hydroxy*-pyrene was significantly associated with all the measured pollutants except ozone, which was consistent with the findings in the period comparison, and both suggest that 1-*hydroxy*-pyrene is a good marker of exposure to traffic-emitted pollutants. Notably that the total PAHs showed significant associations with 1&2-*amino*-naphthalene and 1-*hydroxy*-pyrene at lag 2, but not with 1-*amino*-pyrene (Figure 1). Since the total PAH was correlated with both the primary and secondary pollutants (Table 2), these findings suggest that 1&2-*amino*-naphthalene and 1-*hydroxy*-pyrene were biomarkers of exposure to general traffic-emitted and secondary pollutants, while the sources contributing to urinary 1-*amino*-pyrene was more complicated which might be a mixture of vehicle exhaust in general and other medias that contain 1-*nitro*-pyrene.

In addition, the lag analysis on the associations between the *amino*-PAH metabolites and individual air pollutants can provide insights about the kinetics of *nitro*-PAHs from exposure to excretion in the urine. In Figure 1A and 1B, we consistently found an inverted 'U' shape for the changing patterns of the associations between the *amino*-PAHs and the air pollutants from lag 0 to 3, with the largest changes consistently appearing at lag 1 or lag 2. These observations indicate that the time of the maximum excretion of 1&2-*amino*-naphthalene and 1-*amino*-pyrene was likely to occur between 24 to 72 hours after *nitro*-PAH exposure. Huyck et al. (2010) estimated the time of maximum excretion of urinary 1-*amino*-pyrene, which was measured from healthy volunteers after a controlled diesel exhaust exposure; and they found that about one third of the subjects had maximum excretion times longer than 24 hours, while the rest had a median time of maximum excretion of 5.37 hours (Huyck et al., 2010).

Ozone was associated with the *amino*-PAHs in a nearly opposite pattern, in terms of statistical significance, to the associations between *amino*-PAHs and other traffic-related

pollutants (Figure 1A and 1B). Similar findings were also reported in other related studies, which were mainly attributed to the negative correlations between ozone and other traffic-related pollutants (Huang et al., 2012; Rich et al., 2012; Gong et al., 2013). In the current study, we observed that ozone was negatively correlated with NO₂ ($r=-0.58$, $p<0.01$), PN (-0.33 , $p<0.01$), EC ($r=-0.30$, $p<0.01$), and CO ($r=0.13$, $p>0.05$) (Table 3).

The current study suggests that creatinine adjustments are necessary in the analysis of urinary biomarkers, since we found that the two creatinine adjustments affected both the between-period comparison of the three metabolites and their associations with the air pollutants in different extents. By using the creatinine-standardized method, the period effects on the three metabolites and their associations with the air pollutants were largely weakened in terms of both the magnitude and the significance (Table 2 and Appendix III). Gong et al. (2013) reported similar findings on urinary malondialdehyde (a biomarker of lipid peroxidation), namely that the period specific changes and associations with air pollutants became non- or less significant after the adjustment for creatinine using creatinine-standardized method (Gong et al., 2013). Both studies suggest that the creatinine-standardized method might result in a decrease in the sensitivity of the statistical analysis by introducing additional errors in studies comparing within-subject effects. In comparison, when creatinine was controlled as an independent variable in the mixed-effects models, the associations between the three metabolites and the air pollutants showed more significant results. Therefore, controlling creatinine as an independent variable in the applied models showed a more moderate adjusting effect than standardizing the biomarker levels by creatinine.

Besides inhalation of PM_{2.5}, there are multiple other sources of *nitro*-PAH exposure. For example, inhalation of larger ambient particles (PM_{2.5-10}) that contains 1-*nitro*-pyrene inhalation of gas-phase 1-*nitro*-pyrene, dermal exposure to the particulate- and gas-phase 1-*nitro*-pyrene, and ingestion of diet (including water and food) that contains 1-*nitro*-pyrene. 1-*nitro*-pyrene in all the exposure routes will result in the formation of 1-*amino*-pyrene. Although there have been other sources contributing to the measured levels of *amino*-PAH, these sources appear to be relatively constant within the same individual. Our study design enabled us to observe significant within-individual temporal changes in *amino*-PAH concentrations driven by anticipated temporal changes in *nitro*-PAH concentrations as a result of source control during the Beijing Olympics. Though traffic-related *nitro*-PAH exposure was not measured simultaneously with the urinary *amino*-PAH measurements, by comparing the period-specific changes of the *amino*-PAHs and associating them with the primary and secondary pollutants, we found evidence to support the use of 1&2-*amino*-naphthalene and 1-*hydroxy*-pyrene as internal markers for exposure to general vehicle-emission sources, and more data are need to support the utilization of 1-*amino*-pyrene as a biomarker of exposure to diesel-combustion emission.

Through the between-period comparison of the three PAH metabolites and their associations with the traffic-emitted pollutants, the current study found evidence that 1&2-*amino*-naphthalene and 1-*hydroxy*-pyrene can be used as biomarkers of exposure to general vehicle-emitted pollutants, and more data are needed to confirm 1-*amino*-pyrene as a biomarker of exposure to diesel combustion emissions. We also found that controlling

creatinine as an independent variable in the applied models will provide a moderate adjusting effect on the biomarker analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

1. Urinary polycyclic aromatic hydrocarbon metabolites were analyzed as exposure markers.
2. Associations were found between polycyclic aromatic hydrocarbon metabolites and general traffic emissions.
3. More data are needed for associations between 1-amino-pyrene and diesel exhaust particles.
4. Adjustment for creatinine is essential in urinary biomarker analyses.

A

B

C.

Figure 1.

Percent changes in urinary concentrations of (A) 1&2-amino-naphthalene, (B) 1-amino-pyrene, and (C) 1-hydroxy-pyrene associated with one interquartile range changes in CO (0.65 ppm), SO₂ (5.4 ppb), NO₂ (18.7 ppb), O₃ (25.4 ppb), PM_{2.5} (76.8 µg/m³), PN (6572 cm⁻³), EC (1.4 µg/m³), sulfate (28.0 µg/m³), nitrate (18.7 µg/m³), PYR (pyrene, 1.9 ng/m³), and TPAH (41.1 ng/m³) from lag 0 to lag 4. The results were from the mixed-effects models controlling for temperature, relative humidity, gender, day of week, and creatinine. The label of x axis, lag day, means that the percent changes in the PAH metabolites were shown by pollutant from lag 0 to lag 4. Circles indicate the mean percent changes in the PAH metabolites and the vertical error bars indicate the 95% confident interval of the mean changes.

Table 1

Period-specific means and percent changes in the air pollutants among the three Olympic periods

	Pre-Olympics	During-Olympics		Post-Olympics		During-Pre Olympics		Post-During Olympics	
	GM ^a (95% CI)	GM (95% CI)	GM (95% CI)	GM (95% CI)	GM (95% CI)	% change	95% CI	% change	95% CI
PM _{2.5} , µg/m ³	98.9 (70.2, 127.7)	71.9 (42.3, 101.5)	85.3 (55.2, 115.3)			-27	(-64, 9)	19	(-66, 103)
EC, µg/m ³	2.2 (1.6, 2.8)	1.4 (0.8, 2.1)	3.4 (2.7, 4.0)			-36*	(-70, -2)	133**	(35, 232)
PN, cm ⁻³	16480 (13979, 18980)	12853 (10131, 15575)	19477 (16797, 22157)			-22*	(-42, -2)	52**	(15, 88)
CO, ppm	1.23 (0.97, 1.49)	0.64 (0.37, 0.91)	0.81 (0.54, 1.08)			-48**	(-73, -24)	27	(-114, 168)
NO ₂ , ppb	25.6 (18.4, 32.8)	14.6 (7.3, 22.0)	41.4 (33.9, 48.9)			-43*	(-76, -10)	183**	(49, 317)
ozone, ppb	31.8 (24.5, 39.2)	39.6 (32.0, 47.1)	15.1 (7.5, 22.8)			24	(-13, 62)	-62**	(-85, -39)
SO ₂ , ppb	7.5 (5.2, 9.7)	3.0 (0.4, 5.6)	6.8 (4.4, 9.2)			-60**	(-97, -23)	129	(-224, 482)
sulfate, µg/m ³	26.5 (15.0, 37.9)	23.0 (10.5, 35.5)	13.7 (1.5, 25.8)			-13	(-73, 47)	-41	(-133, 52)
nitrate, µg/m ³	18.3 (11.1, 25.4)	9.3 (1.5, 17.2)	17.6 (9.9, 25.2)			-49*	(-96, -1)	88	(-165, 342)
temperature, °C	25.2 (23.5, 27.0)	27.8 (26.0, 30.0)	16.7 (14.9, 18.5)			10	(-0.4, 20)	-40*	(-48, -31)
relative humidity, %	65.6 (57.8, 73.5)	64.9 (56.9, 73.0)	49.5 (41.4, 57.7)			-1	(-18, 16)	-24*	(-42, -6)
Pyrene, ng/m ³	3.2 (2.6, 3.7)	2.0 (1.4, 2.6)	0.8 (0.3, 1.4)			-37**	(-57, -16)	-59	(-138, 199)
Total PAH, ng/m ³	74.6 (62.9, 86.4)	46.4 (33.4, 59.3)	26.4 (14.0, 38.8)			-38**	(-58, -18)	-43	(-121, 34)

^aGM: Geometric mean

* p value < .05,

** p value < .01

Table 2
Period-specific means and percent changes in the PAH metabolites among the three Olympic periods

	Pre-Olympics	During-Olympics		Post-Olympics		During-Pre Olympics		Post-During Olympics	
	GM ^a (95% CI)	GM (95% CI)	GM (95% CI)	GM (95% CI)	% change	95% CI	% change	95% CI	% change
Creatinine-unadjusted									
1&2- <i>amino</i> -naphthalene, pg/μl	0.34 (0.26, 0.45)	0.24 (0.19, 0.32)	0.27 (0.17, 0.42)	0.27 (0.17, 0.42)	-29	(-46, -6)	11	(-39, 103)	
1- <i>amino</i> -pyrene, pg/μl	0.11 (0.076, 0.16)	0.099 (0.070, 0.14)	0.11 (0.063, 0.19)	0.11 (0.063, 0.19)	-10	(-34, 22)	10	(-46, 124)	
1- <i>hydroxy</i> -pyrene, pg/μl	0.37 (0.28, 0.48)	0.26 (0.20, 0.33)	0.20 (0.13, 0.30)	0.20 (0.13, 0.30)	-30*	(-44, -11)	-23	(-54, 30)	
Creatinine-standardized^b									
1&2- <i>amino</i> -naphthalene, pg/μg creatinine	0.48 (0.36, 0.65)	0.47 (0.36, 0.63)	0.61 (0.38, 0.97)	0.61 (0.38, 0.97)	-2	(-27, 31)	28	(-32, 142)	
1- <i>amino</i> -pyrene, pg/μg creatinine	0.16 (0.11, 0.22)	0.19 (0.14, 0.27)	0.25 (0.15, 0.42)	0.25 (0.15, 0.42)	22	(-9, 63)	31	(-33, 153)	
1- <i>hydroxy</i> -pyrene, pg/μg creatinine	0.53 (0.44, 0.63)	0.50 (0.42, 0.60)	0.46 (0.35, 0.60)	0.46 (0.35, 0.60)	-5	(-19, 12)	-9	(-37, 32)	
Creatinine-independent^c									
1&2- <i>amino</i> -naphthalene, pg/μl	0.31 (0.23, 0.41)	0.24 (0.19, 0.31)	0.30 (0.20, 0.47)	0.30 (0.20, 0.47)	-23	(-41, 2)	26	(-30, 127)	
1- <i>amino</i> -pyrene, pg/μl	0.096 (0.067, 0.14)	0.098 (0.071, 0.14)	0.13 (0.080, 0.22)	0.13 (0.080, 0.22)	2	(-24, 37)	37	(-30, 167)	
1- <i>hydroxy</i> -pyrene, pg/μl	0.30 (0.25, 0.37)	0.25 (0.21, 0.31)	0.26 (0.19, 0.36)	0.26 (0.19, 0.36)	-16	(-30, 0)	3	(-31, 54)	

^a GM: Geometric mean. Marker was log-transformed, and geometric means (median) and 95% confident intervals were provided.

^b Standardizing the PAH metabolites with creatinine by dividing the concentrations of the metabolites with that of creatinine.

^c Controlling creatinine as an independent variable in the mixed-effects models.

* p<.008

Table 3

Spearman correlation coefficients among air pollutants

	PM _{2.5}	EC	CO	NO ₂	PN	ozone	SO ₂	sulfate	nitrate	pyrene
PM _{2.5}	1									
EC	0.67 ^b	1								
CO	0.69 ^b	0.58 ^b	1							
NO ₂	0.42 ^b	0.80 ^b	0.53 ^b	1						
PN	0.11	0.56 ^b	0.27 ^b	0.63 ^b	1					
ozone	0.12	-0.30 ^b	-0.13	-0.58 ^b	-0.33 ^b	1				
SO ₂	0.74 ^b	0.71 ^b	0.58 ^b	0.64 ^b	0.40 ^b	0.02	1			
sulfate	0.90 ^b	0.40 ^b	0.57 ^b	0.12	-0.21	0.35 ^b	0.63 ^b	1		
nitrate	0.92 ^b	0.65 ^b	0.69 ^b	0.51 ^b	0.10	-0.12	0.64 ^b	0.78 ^b	1	
pyrene	0.76 ^b	0.75 ^b	0.69 ^b	0.61 ^b	0.41 ^b	-0.10	0.69 ^b	0.63 ^b	0.71 ^b	1
Total PAHs	0.81 ^b	0.82 ^b	0.66 ^b	0.59 ^b	0.41 ^b	-0.13	0.65 ^b	0.66 ^b	0.74 ^b	0.93 ^b

PN: particle number concentrations

^a p value < .05^b p value < .01