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RESEARCH ARTICLE

Biomarkers of exposure to aromatic hydrocarbons and methyl *tert*-butyl ether in petrol station workers

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Abstract

This cross-sectional study was aimed at reconstructing the exposure to gasoline in 102 petrol station attendants by environmental and biological monitoring of benzene, toluene, ethylbenzene and xylene (BTEX) and biomonitoring of methyl *tert*-butyl ether (MTBE). Airborne BTEX were higher for *manual refuelers* than *self-service assistants* and were highly correlated with each other. Significant relationships were found between airborne BTX and the corresponding urinary solvents (U-BTX) and beween airborne B and urinary MTBE (U-MTBE). Smokers eliminated higher values of U-B, *trans,trans*-muconic (*t,t*-MA) and *S*-phenylmercapturic (*S*-PMA) acids but not U-MTBE. All these biomarkers were, however, significantly raised during the shift, independently from smoking. Linear regression confirmed that occupational exposure was a main predictor of U-MTBE, U-B and *S*-PMA values, both the latter confounded by smoking habits. The study supports the usefulness of biomonitoring even at low exposure levels.

Keywords: Biological monitoring, exposure reconstruction, gasoline, benzene, methyl tert-butyl ether

Introduction

Petrol station attendants may be exposed to high levels of gasoline vapors emitted during the loading and unloading of fuels, and during the refueling of motor vehicles. Hazardous constituents of unleaded fuel include monoaromatic hydrocarbons, i.e. benzene, toluene, ethylbenzene and xylenes (BTEX). Benzene has been repeatedly classified by the International Agency for Research on Cancer (IARC) as a Group I carcinogenic chemical, known to cause leukemia after long-term exposure to high concentrations (IARC, 1982, 1987 and 2011), whereas toluene, ethylbenzene and xylenes are known to be neurotoxic. Due to the presence of carcinogenic and possibly carcinogenic compounds, like benzene and 1,3-butadiene (Group 2B), the IARC classified chronic exposure to gasoline as possibly carcinogenic to humans (Group 2B), with inadequate evidence for carcinogenicity in humans and limited evidence for carcinogenicity in experimental animals (IARC, 1989).

Owing to the toxicological properties of BTEX, their maximum limit level in 95 RON (Research Octane Number) gasoline sold in the European Union (EU) has been lowered from 42% to 35% by volume (vol./vol.), the value for benzene being 1.0% vol./vol. (European Directives 98/70/EC and 2009/30/EC). However, exposure to benzene is associated to early effects even at levels lower than 1 ppm (Lan et al. 2004; McHale et al. 2011).

In Europe, methyl *tert*-butyl ether (MTBE) and similar oxygenated compounds, have been growingly added to gasoline to enhance octane rating and to improve the combustion process after the abolition of lead since 1990 and further after the reduction of benzene content below 5% since 1998 (European Directive 98/70/EC). Thus, the maximum limit level of ethers containing five

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or more carbon atoms in 95 RON petrol sold in the EU has passed from 15% (European Directive 98/70/EC) to 22% (European Directive 2009/30/EC). According to IARC (IARC, 1999), MTBE is not classifiable as to its carcinogenicity to humans (group 3 of carcinogens) but it is toxic for liver, kidney and the central nervous system. Short-term effects, such as nausea, headache and eye irritation have been associated with occupational exposure to fuel containing oxygenated compounds (European Union, 2002).

In the past two decades, preventive measures supplementary to the reduction of benzene content in unleaded fuel, such as the installation of systems to recover gasoline vapors during delivery from road tanker only (Stage I) and also during refueling (Stage II), and the increasing number of *self-service* stations have considerably lowered benzene exposure in petrol station attendants as compared to the recent past (Carrieri et al. 2006, Periago and Prado, 2005). In particular, following the introduction of Stage II devices, a gradual decrease of benzene concentrations in petrol stations from 736 to 241 μg/m^3 has been documented in the period 1995–2003 (Periago and Prado, 2005). Similarly, a reduction in MTBE airborne concentrations from 15.3 to 3.4 mg/m^3 has been reported in a study comparing the exposure of customers to gasoline vapors during refueling in service stations with Stage I and Stage II vapor recovery systems (Hakkola et al. 2000). Occupational exposure to benzene in petrol station attendants may be reconstructed by biomonitoring activities, using validated biomarkers of exposure to benzene as the urinary concentrations of the unmetabolized solvent (U-B) or of the metabolites *trans,trans*-muconic (*t,t*-MA) and *S*-phenylmercapturic (*S*-PMA) acids (Fustinoni et al. 2005; Carrieri et al. 2006; Lovreglio et al. 2010; Manini et al. 2010; Rekhadevi et al. 2011). Particularly at the low current levels, all these biomarkers suffer a main confounding by tobacco smoking, as benzene is a main constituent of tobacco smoke (Darrall et al. 1998). Alternatively, MTBE levels in blood and urine have been evaluated as biomarkers of exposure to gasoline vapors in station attendants (Ghittori et al. 2005) or other gasoline exposed workers (Vainiotalo et al. 2006; Saarinen et al. 1998; Hakkola et al. 2001). In particular for benzene and MTBE, the reconstruction of occupational exposure through biomonitoring would allow the estimation of the whole absorbed dose by both inhalation and the dermal route (Petty et al., 2011; Prah et al., 2004). In service station workers, the uptake of MTBE via dermal exposure has been estimated to be around 8 % (European Union, 2002).

The goal of the present cross-sectional study was to reconstruct the occupational exposure to gasoline vapors in a group of petrol station attendants by a combined approach including environmental and biological monitoring of BTEX and biomonitoring of MTBE. Since information about the actual concentrations of MTBE in fuel sold in Italy was not available, we relied on a recent report by the DG Environment of the European Commission on fuel quality in the EU for year 2006, which reported a mean content of MTBE in 95 RON of about 9.1% (vol./vol.), about 11 times more than benzene (0.8% vol./vol.) (European Commission, 2006).

Methods

Subjects

One hundred and two petrol station attendants (69 males, 37 smokers, mean age 41.1 ± 13.4 years) participated in this study that was conducted in the period April–May 2008 in the city of Parma, Italy. Twenty-two petrol stations were located in the town and surroundings, nine in the province and one along the highway passing in the proximity of Parma. According to the method of fuel delivering, the stations could be classified into three groups: *served*, i.e. relying on manual refueling of cars by attendants, *self-service*, in which attendants gave assistance to customers refueling the cars by themselves and *mixed*, in which the workers could be involved in both the tasks. Thus, we classified petrol station attendants according to both the kind of station and the number of cars personally fuelled with gasoline in the sampling day as *manual refuellers* (n = 58, employed in *served* and *mixed* stations and refueling at least 10 vehicles with gasoline) or *self-service* assistants (n = 44, employed in *self-service* and *mixed* stations, in the latter case refueling less than 10 vehicles with gasoline). During the sampling period, the following atmospheric parameters (expressed as medians and ranges of values) were monitored: temperature, 13.8 (9.9–22.6)C; air humidity, 64 (41–85) %; atmospheric pressure, 1013 (1001–1019) hPa; wind speed, 4.1 (2.6–12.3) m/s (data available from the website: www.ilmeteo.it). Information concerning both the job and the smoking habits was collected by a questionnaire administered by a physician specialized in occupational health. This information is summarized in Table 1. The study protocol was approved by the local

Table 1. Demographic characteristics of the study group and summary of information collected by questionnaire.

	All	*Self-service*	*Manual refueling*
Subjects (No.)	102	44	58
Gender (male/female, No.)	69/33	26/18	43/15
Age (years, mean ± SD)	41.1 ± 13.4	37.5 ± 12.3	43.9 ± 13.6*
Current smokers/nonsmokers (No.)	38/64	15/29	23/35
Body mass index (kg/m^2), mean ± SD	25.1 ± 3.9	24.7 ± 3.9	25.7 ± 3.8
[a]Gasoline cars, medians (min–max)	10 (0–320)	3 (0–8)	20 (10–320)

[a]Number of cars refueled with gasoline by petrol station attendants in the sampling day; *$p < 0.05$, Student's t-test.

Ethical Committee and the subjects participated after giving written, informed consent.

Chemicals

Benzene, toluene, ethylbenzene, *o*-, *m*-, *p*-xylenes (BTEX, purity 99%), the corresponding deuterated standards (TEX-d_6), and $^{13}C_6$-benzene ($^{13}C_6$-B) used as internal standards (ISs), ethyl *tert*-butyl ether (ETBE) used as MTBE IS, *trans,trans*-muconic acid (*t,t*-MA, 98%), cotinine (Cot, 98%), cotinine-d_3 (99%), carbon disulphide (99.9+%), analytical grade formic acid and ammonium hydroxide were purchased by Sigma Aldrich (Milan, Italy). DL-Phenylmercapturic acid (*S*-PMA, purity 98%) was supplied by TCI America (Portland, OR, USA). All standards were used without further purification. *S*-PMA-d_5 and *t,t*-MA-$d_4$were obtained biosynthetically from rat urine and purified by solid-phase extraction (SPE) and HPLC (Melikian et al. 1999). HPLC-grade water and methanol were from Carlo Erba (Milan, Italy). Stock solutions containing about 1 g/l of BTEX, ETBE and *S*-PMA were prepared in methanol; cotinine (1 g/l) was dissolved in water. The solubilization of *t,t*-MA (about 0.5 mg/l) in 0.1 N aqueous sodium hydroxide was achieved by heating and stirring.

Air monitoring

Personal exposure to ambient concentrations of BTEX was assessed by using Radiello® passive-diffusive samplers (Fondazione S. Maugeri, Padua, Italy) in all workers. The sampler was worn by petrol station attendants for the whole work-shift (on average, 9.7 ± 2.0 h). Twenty µl of methanolic solution (500 µg/l) of $^{13}C_6$-B, E-d_6, T-d_6 and *p*-X-d_6 were added at each Radiello® as I.Ss. The samplers were desorbed according to the instructions given by the manufacturer, using low-benzene carbon disulfide. Analytical determinations were performed on a HP 5890 gas chromatograph coupled with a HP 5973A mass spectrometer (GC-MS) as previously described (Andreoli et al. 1999).

Biological monitoring

Urine samples collected at the beginning (BS) and at the end (ES) of the work-shift were divided into three aliquots and frozen at –20°C until analyses of (i) unchanged BTEX and MTBE, (ii) benzene metabolites *S*-PMA and *t,t*-MA, and (iii) cotinine, respectively. In particular, urine samples (2 ml) for analysis of BTEX and MTBE were immediately transferred into 4.0-ml SPME glass vials containing NaCl (1.0 g), added with 2 µl of a IS mixture [$^{13}C_6$-B (0.5 µg/l), E-d_6 (1 µg/l), T-d_6 and *p*-X-d_6 (2 µg/l), ETBE (1 µg/l)] and, after vigorous shaking, were stored at –20°C until analysis.

Urinary BTEX and MTBE were determined by solid phase microextraction gas chromatography-mass spectrometry (SPME GC-MS), as previously described (Andreoli et al. 1999) with some modifications to determine urinary MTBE in the same chromatographic run. A 75 µm Carboxen PDMS fiber (Supelco, Bellefonte, PA, USA) mounted on a Combi/Pal System autosampler (CTC Analytics, Zwingen, Switzerland) was used for headspace SPME sampling, performed at 45°C for 30 min under stirring conditions. Analyses were carried out on HP 6890 GC coupled with a HP 5973 mass spectrometer. The limits of detection (LODs) were, respectively, 0.005 µg/l for both U-B and U-T, 0.010 µg/l for U-E and U-Xy and 0.020 µg/l for U-MTBE. The coefficient of variation of the method (% CV) was within 5 and 7 % for all intra- and inter-day determinations.

Benzene metabolites, *t,t*-MA and *S*-PMA, and urinary free cotinine were determined as previously described (Manini et al. 2006 and 2008) by isotopic dilution liquid chromatography *tandem* mass spectrometry (LC-MS-MS) using a PE-Sciex API 365 triple-quadrupole mass spectrometer (Applied Biosystems, Thornhill, Canada) equipped with a Ionspray interface for pneumatically assisted electrospray (ESI). The LODs calculated as the ratio signal/noise >3 were respectively 2.5 µg/l for *t,t*-MA, 0.1 µg/l for *S*-PMA and 0.2 µg/l for cotinine. The % CV was within 1.3% and 2.6% for all intra- and inter-day determinations.

Concentrations of urinary metabolites were expressed as a function of creatinine (creat.) concentration (µg/g creat.), that was measured by the method of Jaffe (Kroll et al. 1986). We adopted the exclusion criteria of the American Conference of Governmental Industrial Hygienists recommendation (ACGIH, 2011) for very diluted (creatinine concentrations lower than 0.3 g/L) or very concentrated (creatinine concentration higher than 3.0 g/L) urine samples (WHO, 1996).

Statistics

Statistical analyses were carried out using the PASW Statistics 18.0 for Windows™ statistical package (IBM SPSS Inc., Chicago, IL, USA). Parametric or non-parametric statistical tests were applied to (log) normally or not normally distributed variables, after evaluation of distributions by the one-sample Kolmogorov–Smirnov test. Airborne BTEX did not follow a normal distribution, also after logarithmic transformation of data, whereas all biomarkers followed a log-normal distribution. In subgroups of subjects classified according to the smoking habits, cotinine also followed a log-normal distribution. Parametric tests on log-transformed biomarkers included the Student's *t*-test for independent and paired samples and the Pearson's *r* correlation analysis, to evaluate correlations between biomarkers. Non parametric tests included the Mann–Whitney U-test, to assess differences of airborne BTEX levels between groups, and the Spearman's ρ, to assess correlations between airborne BTEX and biomarkers. Multivariate linear regression analysis models were then run to assess the contribution of exposure (as airborne BTEX), type of gasoline refuelling (*manual refuellers vs. self-service assistants*) and personal characteristics (age, gender, ES creatinine and cotinine) to the variability of ES values of biomarkers, set as dependent variables. The significance

level for all tests was $p \leq 0.05$ (two-tailed). Stepwise regression analyses were run using a significance level of 0.05 for entry and 0.10 for removal from the model.

Results

Air monitoring

All analytical determinations were above the corresponding LODs. Table 2 summarizes the distributions of airborne concentrations of BTEX in the whole sample and in subgroups of workers classified according to the job task. Workers employed in the *manual refueling* of gasoline showed significantly higher concentrations of BTEX than *self-service assistants* ($p < 0.005$ for toluene, $p < 0.0001$ for the others), whereas airborne levels of aromatic compounds were not influenced by the smoking habits (data not shown). The airborne BTEX concentrations were highly correlated with each other (Spearman's ρ coefficients ranging from 0.650 to 0.943, in every case $p < 0.0001$).

Biological monitoring

All analytical determinations were above the corresponding LODs. Table 3 shows the distributions of BS and ES biomarker concentrations in the whole sample and in workers classified by smoking habits. Smokers excreted significantly higher concentrations of benzene and its metabolites ($p < 0.0001$ for all comparisons, both for BS and ES samples) than nonsmokers, at both the sampling times. The same held true for cotinine, whereas an interference of smoking habits on U-T levels was apparent only in ES samples ($p < 0.05$). Conversely, U-EB, U-Xy and U-MTBE levels were not affected by smoking habits at any sampling time. The paired sample analysis of BS *vs.* ES biomarker levels, showed a significant increase of ES concentrations for U-B ($p < 0.005$), U-MTBE ($p < 0.0001$), *S*-PMA ($p < 0.0001$) and *t,t*-MA ($p < 0.005$) in the whole sample, as well as in workers stratified by the smoking habits (with lower *p* values among nonsmokers). No significant increase of cotinine concentration was apparent in ES samples.

After sample stratification by job task, we found that workers engaged in the *manual refueling* of gasoline excreted significantly higher U-B and U-MTBE concentrations than *self-service* assistants both in BS (GM [GSD] of 546 [3.85] *vs.* 292 [3.24] ng/l and 1290 [2.91] *vs.* 805 [2.52] ng/l, respectively, $p < 0.05$ for both) and ES samples (GM [GSD] of 811 [4.63] *vs.* 418 [4.02] ng/l, $p < 0.05$ and 2841 [2.27] *vs.* 11056 [2.39] ng/l, $p < 0.0001$, respectively). No significant difference was apparent for other biomarkers.

Table 2. Distributions of BTEX airborne concentrations (μg/m³), as medians (5th–95th percentiles) in the whole group of petrol station attendants and in subgroups of workers classified according to the working task, i.e. employed in the manual refueling of gasoline ($n = 58$) or attending self-service stations ($n = 44$).

Xenobiotics	All workers ($n = 102$)	*Self-service* ($n = 44$)	*Manual refueling* ($n = 58$)
Benzene	38.3 (12.1–107.5)	30.2 (5.5–71.4)	47.9 (14.7–118.2)***
Toluene	169.3 (20.5–447.1)	140.4 (18.8–291.4)	195.0 (45.7–495.8)**
Ethylbenzene	11.0 (2.8–62.8)	7.2 (2.6–28.54)	13.0 (4.6–68.0)***
Xylenes	38.4 (8.5–259.8)	20.2 (7.9–120.7)	50.0 (15.9–309.0)***

**$p < 0.05$, ***$p < 0.0001$, Mann–Whitney *U* test.

Table 3. Distributions of urinary biomarkers (U-B, U-T, U-E, U-Xy: benzene, toluene, ethylbenzene, o-, m-, p-xylenes; U-MTBE: methyl tert-butyl ether; *S*-PMA: *S*-phenylmercapturic acid; *t,t*-MA: *trans*,*trans* muconic acid) in the whole group of petrol station attendants and in subgroups classified by smoking habits. Values are expressed as geometric means [geometric standard deviations] of determinations on urine samples collected at the beginning (BS) and at the end of the shift (ES).

Biomarkers	*All workers* ($n = 102$)	*Nonsmokers* ($n = 64$)	*Smokers* ($n = 38$)	$p^{\#}$
BS U-B, ng/l	417.77 [3.70]	237.53 [2.83]	1104.72 [3.17]	<0.0001
ES U-B, ng/l	604.50 [4.55]**	323.46 [3.23]*	1774.65 [4.25]*	<0.0001
BS U-T, ng/l	332.84 [1.57]	312.15 [1.56]	371.74 [1.57]	0.068
ES U-T, ng/l	362.40 [1.61]	332.81 [1.58]	419.66 [1.61]	0.015
BS U-EB, ng/l	355.87 [1.73]	373.23 [1.72]	327.85 [1.75]	0.264
ES U-EB, ng/l	373.57 [1.75]	397.37 [1.83]	335.89 [1.60]	0.238
BS U-Xy, ng/l	366.63 [1.57]	361.06 [1.62]	376.43 [1.49]	0.517
ES U-Xy, ng/l	388.78 [1.39]	372.00 [1.40]	419.48 [1.36]	0.061
BS U-MTBE, ng/l	1063.34 [2.81]	974.21 [2.82]	1236.38 [2.80]	0.309
ES U-MTBE, ng/l	1861.01 [2.63]***	1788.60 [2.53]***	1992.65 [2.83]*	0.582
BS *S*-PMA, μg/g creat.	0.67 [2.51]	0.49 [2.33]	1.20 [2.12]	<0.0001
ES *S*-PMA, μg/g creat.	0.98 [2.33]***	0.78 [2.33]***	1.48 [1.99]*	<0.0001
BS *t,t*-MA, μg/g creat.	42.79 [2.09]	34.46 [1.90]	62.88 [2.10]	<0.0001
ES *t,t*-MA, μg/g creat.	57.97 [2.02]**	46.88 [1.85]**	84.56 [2.02]*	<0.0001
BS COT, μg/g creat.	15.71 [20.01]	1.88 [2.61]	682.99 [2.55]	<0.0001
ES COT, μg/g creat.	16.13 [19.52]	1.96 [2.10]	685.44 [3.25]	<0.0001

*$p < 0.05$, **$p < 0.005$, ***$p < 0.0001$, Student's *t*-test for paired samples, ES *vs.* BS.
#Student's *t*-test for independent samples, smokers *vs.* nonsmokers.

Among *self-service* assistants, only benzene metabolites showed significantly higher ES as compared to BS values (GM [GSD] of 0.83 [2.40] *vs.* 0.59 [2.79], $p = 0.005$ and 62.57 [1.86] *vs.* 41.97 [2.06], $p < 0.0001$, for *S*-PMA and *t,t*-MA, respectively), whereas among *manual refuellers*, the same held true also for U-B and U-MTBE values (GM [GSD] of 811 [4.63] *vs.* 534 [3.85] ng/l, $p = 0.003$, 2841 [2.20] *vs.* 1280 [2.93], $p < 0.0001$, 1.11 [2.25] *vs.* 0.75 [2.28] μg/g creat, $p < 0.0001$ and 54.73 [2.14] *vs.* 43.42 [2.13] μg/g creat, for U-B, U-MTBE, *S*-PMA and *t,t*-MA, respectively)

In the range of airborne concentrations examined in this study, significant relationships were observed between airborne levels and ES urinary concentrations of unmetabolized solvents. As shown in Figure 1, both U-B and U-MTBE were significantly correlated with airborne concentrations of benzene ($\rho = 0.31$, $p < 0.005$ and $\rho = 0.50$, $p < 0.0001$, respectively), in the latter case with no interference by tobacco smoking. Significant correlations between airborne and urinary concentrations were observed for both toluene and xylene ($\rho = 0.22$ and 0.27, respectively, $p < 0.05$ for both), but not for ethylbenzene, that correlated with urinary xylene only ($\rho = 0.24$, $p < 0.05$). No significant relationship was apparent between airborne benzene levels and urinary ES concentrations of specific metabolites (*S*-PMA, *t,t*-MA).

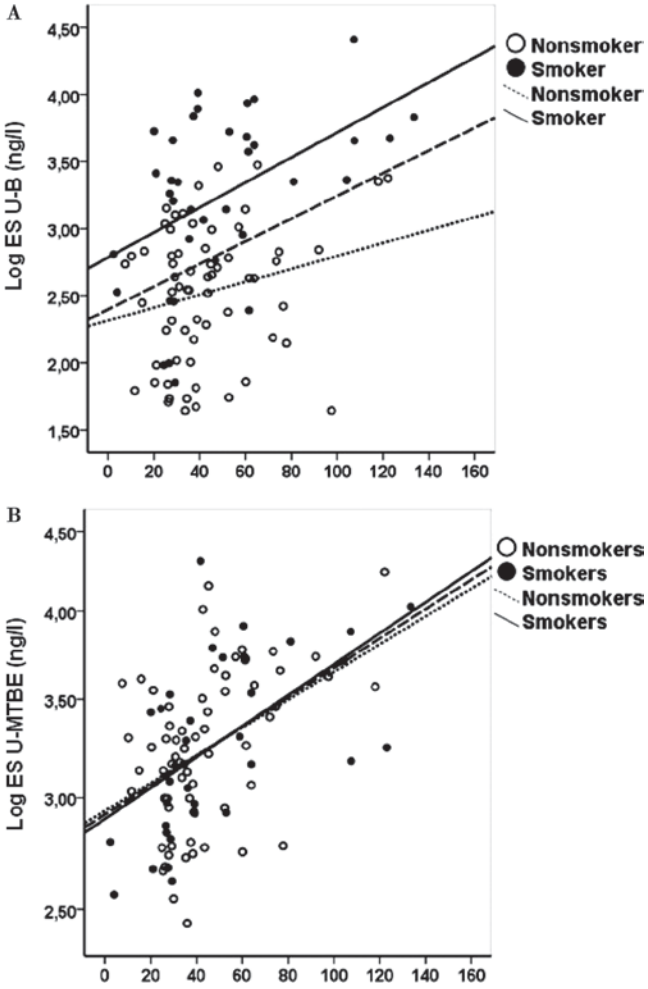

Figure 1. Relatioships between airborne benzene (A–B) concentrations and end-of-shift urinary levels of benzene (ES U-B) (1A) and methyl *tert*-butyl ether (ES U-MTBE) (1B) in workers stratified by smoking habits. The Spearman's correlation values between A–B and ES U-B were: $\rho = 0.31$ for the whole sample ($p = 0.002$); $\rho = 0.17$ for nonsmokers ($p = 0.169$); $\rho = 0.47$ for smokers ($p = 0.004$). The Spearman's correlation values between A and B and ES U-MTBE were: $\rho = 0.50$ for the whole sample ($p < 0.0001$); $\rho = 0.41$ for nonsmokers ($p = 0.001$); $\rho = 0.64$ for smokers ($p < 0.0001$).

Table 4 resumes the results of the Pearson's correlation analysis of ES values of biomarker values. U-EB and U-Xy were excluded from analysis, according to previous results. U-B levels were significantly correlated with those of U-T, U-MTBE (in particular among nonsmokers), benzene metabolites, cotinine and creatinine. U-T was correlated with both benzene metabolites, in particular *t,t*-MA, cotinine and creatinine. S-PMA was significantly correlated with U-MTBE among smokers, *t,t*-MA, and creatinine. Both *S*-PMA and *t,t*-MA were significantly correlated with cotinine in the whole group but not in workers stratified by smoking habits. Both the biomarkers, however, were significantly correlated with cotinine among smokers in BS samples ($r = 0.32$, $p < 0.05$ and $r = 0.44$, $p = 0.005$, respectively).

Table 5 resumes the main results of the stepwise multiple regression analysis. About 56% of the variance of ES U-B levels was explained by the urinary concentrations of cotinine and creatinine, by airborne benzene levels and age. The best predictors of MTBE values (adjusted r^2 0.366, $p < 0.0001$) were airborne benzene levels and the job title as extent of gasoline refuelling. Creatinine and cotinine values and the extent of gasoline handling during the shift accounted for about 28% of the variance of *S*-PMA values. Both cotinine and creatinine values significantly affected the variance of *t,t*-MA levels (r^2 0.145 and 0.180, respectively, $p < 0.0001$ for both).

Discussion

The present study was aimed at evaluating biomarkers of exposure to BTEX and MTBE as tools to reconstruct occupational exposure to gasoline. As a main limitation of the study, airborne MTBE was not assessed by personal ambient monitoring. Several previous studies, however, have demonstrated good relationships between airborne MTBE and the unmetabolized compound in biological matrices, such as blood and urine (Vainiotalo et al. 1998; Saarinen et al. 1998; Hakkola et al. 2001; Ghittori et al. 2005; Fustinoni et al. 2010). As a significant correlation between airborne benzene and MTBE levels has been consistently reported in previous studies (Ghittori et al. 2005; Fustinoni et al. 2010), we used airborne benzene as a surrogate of airborne MTBE. Similarly, in a recent study investigating U-MTBE values in traffic policemen and lacking airborne MTBE measurement, airborne CO was used as a marker of vehicular traffic (Campo et al. 2011). Airborne BTEX concentrations determined in this study were on average 2- to 10-fold higher than those we observed in previous field studies on workers exposed to urban pollutants in the city of Parma (taxi drivers and traffic policemen, Manini et al. 2006 and 2008) but lower

Table 4. Pearson's correlation coefficients between end-of-shift urinary values of biomarkers (U-B, U-T: benzene, toluene; U-MTBE: methyl *tert*-butyl ether; *S*-PMA: *S*-phenylmercapturic acid; *t,t*-MA: *trans,trans* muconic acid; U-Cot: cotinine; U-creat: creatinine) in the whole worker sample ($n = 102$) and in subgroups of nonsmoker ($N = 64$) and smoker ($N = 38$).

		U-B	U-T	U-MTBE	*S*-PMA	*t,t*-MA	U-COT
U-T	*All*	0.593***					
	Smokers	0.691***					
	Non smokers	0.475***					
U-MTBE	*All*	0.329**	0.156				
	Smokers	0.301	0.299				
	Non smokers	0.396**	0.046				
S-PMA	*All*	0.512***	0.267*	0.174			
	Smokers	0.414*	0.242	0.385*			
	Non smokers	0.466***	0.201	0.079			
t,t-MA	*All*	0.486***	0.377***	0.047	0.329**		
	Smokers	0.398*	0.344*	0.074	0.318		
	Non smokers	0.377**	0.310*	–0.002	0.235		
U-COT	*All*	0.600***	0.335**	0.047	0.309**	0.382***	
	Smokers	0.490**	0.428*	–0.086	0.050	0.315	
	Non smokers	0.244	0.348**	0.030	0.057	0.170	
U-Creat	*All*	0.339**	0.366***	0.043	0.400***	0.424***	0.003
	Smokers	0.472**	0.429*	0.300	0.356*	0.414*	–0.037
	Non smokers	0.425***	0.376**	–0.093	0.468***	0.504***	0.383**

*$p < 0.05$, **$p < 0.005$, ***$p < 0.0001$, Pearson's correlation, two-tailed.

Table 5. Predictors of the end-of-shift urinary excretion of benzene (U-B), methyl *tert*-butyl ether (U-MTBE), *S*-phenylmercapturic acid (*S*-PMA) and trans,trans muconic acid (*t,t*-MA) according to a stepwise multiple linear regression model: Log (biomarker) = constant + (airborne levels) x β_1 + (creatinine) × β_2 + (age) × β_3 + (gender) × β_4 + (Log cotinine) × β_5 + (gasoline refueling). Values of constant and β coefficients, with SE, partial r^2 (r^2p) and significance (p) for each term are given. The adjusted r^2 (r^2adj) and significance (p) for the whole model are reported in the last row.

	U-B, ng/l β ± SE [β_{stand}]	r^2_p	p	U-MTBE, ng/l β ± SE [β_{stand}]	r^2_p	p
Constant	1.356 ± 0.196	—	0.021	2.826 ± 0.069		<0.0001
[a]Airborne benzene	0.007 ± 0.002 [0.271]	0.081	<0.0001	0.006 ± 0.001 [0.360]	0.115	<0.0001
Age	0.007 ± 0.003 [0.151]	0.022	0.017	—		—
Gender	—	—	—	—		—
ES Creatinine	0.317 ± 0.061 [0.365]	0.114	<0.0001	—	—	—
ES Cotinine,	0.301 ± 0.035 [0.587]	0.360	<0.0001	—	—	—
[b]Gasoline refueling	—	—	—	0.330 ± 0.071[0.394]	0.264	<0.0001
Whole model r^2_{adj}, p	0.558		<0.0001	0.366		<0.0001
	S-PMA, µg/l β ± SE [β_{stand}]	r^2_p	P	*t,t*-MA, µg/l β ± SE [β_{stand}]	r^2_p	p
Constant	–0.468 ± 0.090		<0.0001	1.403 ± 0.070		<0.0001
[a]Airborne benzene	—	—	—	—	—	—
Age	—	—	—	—	—	—
Gender	—	—	—	—	—	—
ES Creatinine	0.218 ± 0.050 [0.377]	0.160	<0.0001	0.208 ± 0.041 [0.423]	0.180	<0.0001
ES Cotinine,	0.098 ± 0.028 [0.297]	0.095	0.001	0.108 ± 0.024 [0.381]	0.145	<0.0001
[b]Gasoline refueling	0.175 ± 0.073 [0.207]	0.042	0.018	—	—	—
Whole model r^2_{adj}, p	0.275		<0.05	0.311		<0.0001

[a]Airborne benzene levels (µg/m^3); [b]*Manual refuellers vs. self-service* assistants.

(about 2–3 orders of magnitude) than the corresponding occupational exposure limits. The threshold limit values for 8-h time-weighted average concentrations (TLV-TWAs) recommended by the American Conference of Governmental Industrial Hygienists (ACGIH) for BTEX and MTBE are, respectively 0.5, 20, 20, 100 and 50 ppm (or 1.6, 75.4, 87, 434 and 180 mg/m^3) (ACGIH, 2011). In the EU, the corresponding occupational exposure limits (OELs) proposed by the Scientific Committee on Occupational Exposure Limits (SCOEL) for BTEX and MTBE are, respectively, 3.25, 192, 442, 221 and 183.5 mg/m^3 (or 1, 50, 100, 50 and 50 ppm) (SCOEL, 1992, 1995 and 2001; Directives 2004/37/EC and 2009/161/EU).

Airborne benzene concentrations fell in the range of values (medians between 21 and 61 µg/m^3) already reported by previous Italian studies on petrol station

attendants (Ghittori et al. 2005; Fustinoni et al. 2005; Carrieri et al. 2006; Lovreglio et al. 2010). Much higher benzene values (up to 1,500 μg/m^3) have been yet recently reported in service stations outside from Europe (de Oliveira et al. 2007; Rekhadevi et al. 2011). Notwithstanding the low exposure levels, benzene biomarkers could be successfully determined in all samples, their concentrations among nonsmokers being, on average, up to about 15 (for BS *t,t*-MA) and 51 folds (for BS *S*-PMA) lower than the corresponding biological exposure indices (BEI®: 500 and 25 μg/g creatinine, respectively) established by ACGIH (ACGIH, 2011). Airborne BTEX were highly correlated with each other and all, with the exception of ethylbenzene, with the respective urinary unmetabolized biomarker and, in any case, with U-B. The strong differences of airborne BTEX levels we observed in workers classified according to the extent of manual refueling of gasoline, confirmed the presence of all aromatic monohydrocarbons in gasoline vapors. Similar trends of exposure have been described for petrol station workers classified by the job task, with airborne concentrations of benzene and MTBE increasing in the order: cashiers *vs.* outdoor workers *vs.* fuel supply attendants (Ghittori et al. 2005). Personal ambient monitoring was not influenced by smoking habits, as already shown in our previous studies on taxi drivers and traffic policemen (Manini et al. 2006 and 2008) and as reported also by others (Fustinoni et al. 2005; Lovreglio et al. 2010).

Conversely, when biomarkers are considered, the smoking habits is a well-recognized main confounder in the biomonitoring of occupational exposure to benzene, especially at low exposure levels (Fustinoni et al. 2005; Maestri et al. 2005; Lovreglio et al. 2010). In our sample, the worker classification according to information about smoking habits was confirmed by urinary free cotinine levels, which showed a clear cut between smokers and nonsmokers. Cotinine, a biomarker of tobacco smoke with a long half-life (20–24 h), showed substantially unchanged values at both the sampling times, demonstrating that its concentration was at the steady state. A slight increase (about 15–20%) would, however, have been found if workers had substantially smoked during the work-shift, as previously observed in a taxi-driver cohort (Manini et al. 2006). Thus, our results confirmed that workers refrained from smoking during the work-shift as required by the smoking ban in petrol stations. Univariate analyses confirmed the interfering role of smoking habits on all biomarkers of exposure to benzene (i.e. U-B, *S*-PMA and *t,t*-MA), at both the sampling times but showed also a significant effect of occupational exposure, as demonstrated by the significant rising of ES as compared to BS values, both in smokers and nonsmokers. U-TEX, however, failed to show significant excretion rising at the end of the shift. These results let us to conclude that, among U-BTEX, only urinary benzene showed characteristics of sensitivity and selectivity adequate for the biological monitoring of petrol station workers, suggesting the use of this biomarker as a suitable tracer of exposure to aromatic monohydrocarbons in gasoline vapors.

Despite a large number of human inhalation studies have been carried out on MTBE (Dekant et al. 2001; Prah et al. 2004; Vainiotalo et al. 2007; Pleil et al. 2007), there is not yet a general agreement about the more valid biomarker for biomonitoring purposes. Absorbed MTBE undergoes oxidative demethylation mainly by the cytochrome P450 2A6, which displays a 24-fold interindividual variability (Le Gal et al. 2003) and gives rise to tertiary butyl alcohol (TBA). This can be excreted in urine after conjugation with glucuronic acid or further oxidized to 2-methyl-1,3-propanediol and ultimately to 2-hydroxyisobutyrate, which is excreted in urine as the major metabolite of MTBE. According to some authors (Dekant et al. 2001), the analysis of the parent ether in biological matrices is to be preferred, owing to the lower specificity of both 2-hydroxyisobutyrate, formed endogenously at large concentrations (Liebich and Forst, 1984), and TBA, that may enter the body from sources other than gasoline (food processing and flavoring, CIREP, 1989). On the other hand, some authors (Pleil et al. 2007) have demonstrated that the biological damping effect of the metabolite production would give more stable estimates of previous exposure, thus allowing a more thorough exposure reconstruction in occupational settings. In a field study, higher correlation coefficients have been reported between MTBE concentration in the breathing zone and U-MTBE ($r = 0.830$) in samples collected after the shift as compared to the metabolite TBA ($r = 0.526$). An opposite behavior was shown for next morning samples (Hakkola et al. 2001). In the present study, we chose to measure U-MTBE for its higher specificity toward gasoline exposure as compared to benzene biomarkers and set-up a method allowing the determination of urinary BTEX and MTBE in a single chromatographic run.

In agreement with other studies in the field (Fustinoni et al. 2010, Campo et al. 2011), U-MTBE did not suffer any influence by the subjects' smoking status and reflected specifically the occupational exposure to gasoline vapors. In the present study, the average end-of-shift levels of U-MTBE (GMs of 1861.01 and 2841.19 ng/l for either the whole sample and workers who did the *manual refueling* of cars, respectively) were comparable to those already reported in a previous Italian study (medians of 1339 and 3043 ng/l for either the whole group and petrol station attendants, respectively; Ghittori et al. 2005). Higher values (medians ranging from 3000 to 7000 ng/l) were reported for workers involved in fuel transportation and loading (Saarinen et al. 1998; Hakkola et al. 2001; Vainiotalo et al. 2006). Assuming that the internal ratios in fuels are reflected by unmetabolized biomarkers, we may speculate that the MTBE concentration in the fuel handled by *manual refuellers* was, on average, similar to the expected value of 9.1% (European Commission, 2006) the ratio between ES U-MTBE and ES U-B (nonsmokers only) values (2841.19 and 401.14 ng/l, respectively) being about 7. Regression analyses confirmed the confounding effect of smoking (as cotinine in the models) on the end-of-shift concentrations

of urinary benzene and benzene metabolites. Only urinary benzene was significantly affected by airborne benzene levels, probably relying on the different kinetics of benzene biomarkers (half-life lengths in the order *S*-PMA > *t,t*-MA > urinary benzene) (Dor et al. 1999). Differences of kinetics may also underlie the lack of any relationship between benzene metabolites and cotinine levels at the end-of-shift among smokers, correlations being apparent in samples collected before the shift.

Actually, also *S*-PMA values were significantly influenced by occupational exposure, included in the regression analysis as the extent of manual refueling. Thus, both U-B and *S*-PMA, whose specificity for benzene is unique, allowed a sensitive biomonitoring of exposure to benzene deriving from different sources, including tobacco smoking and gasoline vapors. On the other hand, the assessment of low-level exposure relying on ES t,t-MA shows some limitations, probably due to the lower specificity of the biomarker, whose values suffer the confounding also by dietary sorbic acid (Negri et al. 2005).

In the regression model, we chose airborne benzene as a surrogate of airborne MTBE, relying on the significant correlation between airborne benzene and U-MTBE ($\rho = 0.502$, $p < 0.0001$), further supported by previous results in a cohort of gasoline pump attendants (Ghittori et al. 2005), exposed to airborne benzene concentrations (38 μg/m^3) and U-MTBE levels (see above) similar to those observed in the present study. About 37% of the variance of U-MTBE was explained by both the independent variables linked to occupational exposure (i.e. airborne benzene levels and the extent of gasoline exposure), and any interfering role of smoking habits was excluded.

Conclusions

The study demonstrates that biomonitoring is useful and reliable for exposure reconstruction even at so low exposure levels. U-MTBE is a sensitive and specific biomarker of exposure to gasoline vapors, that may help the interpretation of benzene biomonitoring, since it is not affected by smoking habits, as benzene is. Among benzene biomarkers, both U-B and *S*-PMA showed, apart the specificity, adequate sensitivity toward both smoking habits and occupational exposure, whereas *t,t*-MA gave rise to ambiguous results, probably relying on lower specificity.

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Declaration of interest

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