Monitoring Low Benzene Exposure: Comparative Evaluation of Urinary Biomarkers, Influence of Cigarette Smoking, and Genetic Polymorphisms

Silvia Fustinoni,[1] Dario Consonni,[1] Laura Campo,[1] Marina Buratti,[1] Antonio Colombi,[1] Angela C. Pesatori,[1] Matteo Bonzini,[1] Pier A. Bertazzi,[1] Vito Foà,[1] Seymour Garte,[2] Peter B. Farmer,[3] Leonard S. Levy,[4] Mauro Pala,[5] Federico Valerio,[5] Vincenzo Fontana,[5] Arianna Desideri,[5] and Domenico F. Merlo[5]

[1]Department of Occupational and Environmental Health, University of Milan and Fondazione Ospedale Maggiore Policlinico, Mangiagalli e Regina Elena, Milan, Italy; [2]School of Public Health, University of Medicine and Dentistry of New Jersey, Piscataway, New Jersey; [3]Cancer Biomarkers and Prevention Group, Biocentre and [4]Medical Research Council, Institute for Environmental and Health, University of Leicester, Leicester, United Kingdom; and [5]Environmental Epidemiology and Biostatistics, Department of Cancer Etiology and Epidemiology, National Cancer Research Institute, Genoa, Italy

Abstract

Benzene is a human carcinogen and an ubiquitous environmental pollutant. Identification of specific and sensitive biological markers is critical for the definition of exposure to low benzene level and the evaluation of the health risk posed by this exposure. This investigation compared urinary *trans,trans*-muconic acid (*t,t*-MA), *S*-phenylmercapturic acid, and benzene (U-benzene) as biomarkers to assess benzene exposure and evaluated the influence of smoking and the genetic polymorphisms CYP2E1 (*Rsa*I and *Dra*I) and NADPH quinone oxidoreductase-1 on these indices. Gas station attendants, urban policemen, bus drivers, and two groups of controls were studied (415 subjects). Median benzene exposure was 61, 22, 21, 9 and 6 μg/m^3, respectively, with higher levels in workers than in controls. U-benzene, but not *t,t*-MA and *S*-phenylmercapturic acid, showed an exposure-related increase. All the biomarkers were strongly influenced by cigarette smoking, with values up to 8-fold higher in smokers compared with nonsmokers. Significant correlations of the biomarkers with each other and with urinary cotinine were found. A possible influence of genetic polymorphism of CYP2E1 (*Rsa*I and/or *Dra*I) on *t,t*-MA and U-benzene in subjects with a variant allele was found. Multiple linear regression analysis correlated the urinary markers with exposure, smoking status, and CYP2E1 (*Rsa*I; R^2 up to 0.55 for U-benzene). In conclusion, in the range of investigated benzene levels (<478 μg/m^3 or <0.15 ppm), smoking may be regarded as the major source of benzene intake; among the study indices, U-benzene is the marker of choice for biomonitoring low-level occupational and environmental benzene exposure. (Cancer Epidemiol Biomarkers Prev 2005;14(9):2237–44)

Introduction

Benzene, a known human carcinogen (group 1 IARC; ref. 1), is a major chemical widely used all over the world in the production of monomers for the polymer industry and in the synthesis of intermediates. In some countries, benzene is still used as a solvent (2). As a consequence of these production activities, it is found as an airborne pollutant in industrial environments. Benzene is also distributed in the general environment by vehicles due to both fuel evaporation and gas-fueled engine emissions (3). Benzene is originally present in fuel (<1% v/v in the United States and European Union) and is additionally produced by engines because of incomplete combustion of the organic components of gas. Moreover, benzene is a component of mainstream and side-stream cigarette smoke (4, 5). Due to its multiple sources, benzene occupational or environmental exposure may concern the major part of the population.

In the last decades in developed countries, airborne benzene in traditionally polluted working settings has progressively decreased as a consequence of preventive actions. On the other hand, benzene concentration in living environments, particularly in large cities, has reached levels that cause concern for public health. Consequently, benzene exposure in working and living environments is often very similar, with concentrations in the 1 to 1,000 μg/m^3 range (<0.001-0.312 ppm).

For the definition of exposure to low levels of benzene as well as for the evaluation of health risks posed by this exposure, the identification of suitable, specific, and sensitive biological markers is needed. This need is further supported by recent evidence that indicates that benzene causes hematotoxicity at occupational levels below 1 ppm (6).

Inhaled benzene is readily absorbed into the blood at 40% to 70% of airborne doses by passive diffusion through alveolar capillary membranes. Major elimination occurs via the same mechanism with exhaled air. Once in the body, benzene is oxidized by cytochrome *P*450 enzymes (CYP2E1) to a reactive intermediate benzene oxide-oxepine. This highly reactive species is further metabolized through three different pathways. The predominant one leads to phenol via a nonenzymatic rearrangement. Phenol may be excreted in urine as sulfate or glucuronate derivatives (80-90% of the absorbed dose) or may be oxidized, via cytochrome *P*450, to 1,4- and 1,2-benzenediol, and this may be further oxidized to *p*- and *o*-benzoquinone. The quinones may be back-reduced to the original diols via NADPH quinone oxidoreductase-1 (NQO1). The second pathway leads to benzene 1,2-dihydrodiol via

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Requests for reprints: Silvia Fustinoni, Department of Occupational and Environmental Health, University of Milan and Instituti Clinici di Perfezionamento, Via S. Barnaba, 8, 20122 Milan, Italy. Phone: 39-2-503-20116; Fax: 39-2-503-20110. E-mail: silvia.fustinoni@unimi.it

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epoxide hydrolase, which may be oxidized to *trans,trans*-muconic aldehyde and to *trans,trans*-muconic acid (*t,t*-MA), which is excreted in urine (3-18% of the adsorbed dose). The minor pathway leads to pre-phenyl mercapturic acid, after conjugation with glutathione via glutathione *S*-transferase, and finally to *S*-phenylmercapturic acid (S-PMA), which is excreted in urine (<1% of the absorbed dose; reviewed in ref. 7). Nonmetabolized benzene may be also excreted in urine (U-benzene) after passive diffusion from blood to urine through the kidney glomerular tuft (<0.1% of the absorbed dose; ref. 8).

For prevention of risk arising from exposure to benzene, an occupational limit value of 3,200 $\mu g/m^3$ (1 ppm) has been adopted by the European Union (9), whereas a threshold limit value of 1,600 $\mu g/m^3$ (0.5 ppm) as maximal time-weighted average during an 8-hour shift is recommended by the American Conference of Governmental Industrial Hygienists (10). As biological exposure indices, the use of *t,t*-MA and S-PMA in urine samples collected at the end of work shift are indicated. Blood benzene is also suggested as a nonquantitative, but specific, index. In addition to these indices, some authors suggested U-benzene as a sensitive and specific marker for the biological monitoring of benzene exposure (8, 11-15). Although the cited biomarkers have proven to be suitable for assessment of exposure to airborne benzene concentrations close or above the occupational limit values, their validity to discriminate lower exposures is currently under evaluation.

The aim of this work was to compare the ability of *t,t*-MA, S-PMA, and U-benzene to detect occupational and environmental low benzene exposure and to determine how smoking habits and genetic polymorphism of metabolic enzymes (i.e., CYP2E1 and NQO1) may influence such indices. With this aim, gas station attendants, urban policemen, bus drivers, and controls working in two large Italian cities, Milan and Genoa, and the suburban areas surrounding them were investigated.

Materials and Methods

Study Population. The study was carried out from October 1999 to June 2000 on subjects exposed to benzene as a pollutant of the general and/or occupational environment of urban areas within or near two large cities in northern Italy, Milan, and Genoa. In the Milan area, the investigated subjects were recruited among gas station attendants (n = 78), urban traffic policemen (n = 77), and subjects working in offices and hospital facilities located downtown, designated as Milan referents (n = 58). In the Genoa area, subjects were recruited among municipal bus drivers (n = 153) and subjects working as clerks and researchers at the National Institute for Cancer Research in the urban environment and designated as Genoa referents (n = 49). Referent (nonexposed) subjects were matched by gender, age, and smoking habits. Workers received information about the aim of the research, and written informed consent was obtained. Detailed information about smoking habits, lifestyle, medical history, and occupational activity of the investigated subjects was obtained by a self-administered questionnaire.

Personal Benzene Exposure

Airborne Benzene. Airborne benzene was collected by a passive sampler (stainless steel tube, 9 mm internal diameter × 90 mm length) containing Chromosorb 106 and equipped with a diffusion chamber that was worn by the investigated subjects near the breathing zone during part of the work shift (typically from 8:00 a.m. to 2:00 p.m.; ref. 16). At the end of the monitored period, the passive sampler was closed with a brass cap and nut, equipped with a polyperfluoroethylene ferule, and kept at −20°C until analysis. Benzene in samplers was determined by thermal desorption followed by gas chromatography/flame ionization detector analysis. The limit of detection for airborne benzene was 6 $\mu g/m^3$ (0.002 ppm).

Biological Monitoring

Specimen Collection. Urine spot samples were collected twice for each subject, once at the beginning and the other at the end of the monitored period. Specimens were partitioned in plastic tubes for cotinine (pre-monitoring sample, T_0), *t,t*-MA (pre-monitoring and post-monitoring samples, T_0 and T_1), and S-PMA [post-monitoring sample, T_1; 6 mol/L HCl/1 mL urine (12.5 μL) was added as a preserving agent]. For U-benzene, a 7-mL aliquot was poured into a precleaned 8-mL glass vial, promptly closed with a rubber lid with a polyperfluoroethylene lining, and crimped with an aluminum seal (pre-monitoring and post-monitoring samples, T_0 and T_1, collected only for the Milan subjects). All samples were coded, frozen at −20°C, and delivered to the various laboratories, where analyses were done without knowledge of their origin.

Urinary t,t-MA. Determination of urinary *t,t*-MA was carried out by pre-purification of urine with solid-phase extraction using a strong anion exchange column (300 mg, Supelco, Milan, Italy) followed by high-performance liquid chromatography and UV detection according to a published procedure (17). The detection limit of the procedure was 10 μg/L.

Urinary S-PMA. Determination of urinary S-PMA was based on an immunoassay technique according to a previously published procedure (18). The immunoassay plate-based kit and reagents were from AB Biomonitoring Ltd. (Cardiff, Wales, United Kingdom). The detection limit of the procedure was 0.2 μg/L.

U-benzene. Determination of U-benzene was done by headspace solid-phase microextraction followed by gas chromatography/mass spectrometry analysis according to a published method (19) with some modifications. Briefly, 0.5 mL urine was poured in a 2-mL autosampler vial containing 300 mg NaCl. The internal standard solution of benzene-d_6 (1 μL) in methanol (0.475 μg/L) was added, and the vial was immediately closed with a screw-holed cap equipped with a silicone-polyperfluoroethylene gasket. Benzene was sampled from the urine headspace by the solid-phase microextraction technique using a PDMS fiber. Sampling was operated at room temperature with a Varian CX8200 autosampler. Analyte separation was done by gas chromatography using a BD1 column (60 m length, 0.25 mm internal diameter, 1 μm film thickness). Quantification was done using a mass spectrometry detector operating in the electron impact mode. The detection limit of the procedure was 15 ng/L.

U-cotinine. Determination of urinary cotinine was carried out by high-performance liquid chromatography and UV detection according to a published procedure (20). The detection limit of the procedure was 50 μg/L.

Genetic Polymorphisms. For analysis of CYP2E1, samples were amplified using the following primers: 5′-CCAGTCGAGTCTACATTGTCA and 3′-TTCATTCTGTCTTCTAACTGG. PCR was done for 30 cycles with denaturing at 95°C for 1 minute, annealing at 55°C for 1 minute, and extension at 72°C for 1 minute. PCR products are digested with excess *Rsa*I or *Dra*I.

For analysis of the NQO1 RFLP, DNA samples were amplified in a total reaction volume of 50 mL containing 1.2 mmol/L deoxynucleotide triphosphate, 1.2 mmol/L oligonucleotide primers, and 2.5 units Taq polymerase (Amplitaq, Perkin-Elmer, Boston, MA) using the following primers: 5′-GAGACGCTAGCTCTGAACTGAT and 5′-ATTTGAATTCGGGCGTCTGCTG. PCR was done for 35 cycles with denaturing at

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Table 1. Selected characteristics of investigated subjects divided according to city and job title

Location	Jobs	No. subjects	No. males	No. females	Age (mean ± SD)	% Smokers	No. cigarettes daily (mean ± SD)
Genoa	Referents	49	47	2	41.5 ± 6.5	27	15.6 ± 6.9
	Bus drivers	153	150	3	38.9 ± 7.9	31	17.0 ± 9.3
Milan	Referents	58	39	19	38.6 ± 10.7	41	14.7 ± 9.8
	Traffic policemen	77	47	30	31.7 ± 5.5	36	17.0 ± 7.6
	Gas station attendants	78	69	9	42.3 ± 12.7	41	17.0 ± 7.6
Both cities	All jobs	415	352	63	38.5 ± 9.6	35	16.0 ± 8.4

93°C for 1 minute, annealing at 65°C for 1 minute, and extension at 72°C for 1 minute. PCR products were digested with excess *Hin*fI for 3 hours and then electrophoresed through 1.8% agarose and visualized by ethidium bromide staining.

Statistical Analyses. We assessed the relationship of benzene exposure and the urinary biomarkers by making comparisons among the occupational groups and between pre-shift and post-shift samples and by using correlation/regression techniques. A value corresponding to one-half of the detection limit was assigned to measurements below analytic detection. Because variables were highly positively skewed, we conducted parallel analyses with parametric methods on $\log_{10}$-transformed variables (t test for independent or paired samples, ANOVA, and Pearson's correlation coefficient) and with corresponding nonparametric techniques (Mann-Whitney, Wilcoxon, Kruskal-Wallis test, and Spearman's ρ). Multiple linear regression analyses were done on $\log_{10}$-transformed variables. In every model we included, as dependent variable, the different urinary biomarkers and, as predictors, the exposure variables (airborne benzene and current exposure to cigarette smoke, measured as U-cotinine) and the CYP2E1 (*Rsa*I) polymorphism (0 = wild-type, 1 = at least one mutant allele), which had been found to be associated with the biomarkers in some of the univariate analyses. We then tested the two-way interactions between airborne benzene, U-cotinine, and CYP2E1 (*Rsa*I) polymorphism by introducing product terms in the model; because none of these terms was statistically significant, they were omitted from the final model which had the following form: log(U-Biomarker) = constant + log(BenzeneAir) × β_1 + log(U-cotinine) × β_2 + CYP2E1 (*Rsa*I) × β_3. A two-sided P of 0.05 was considered significant. The statistical analyses were carried out using SPSS 12.0 for Windows.

Results

Study Population. Selected characteristics of study subjects are reported in Table 1. Comparing Genoa and Milan subjects, we noticed that the former were almost entirely males, whereas the latter had a higher prevalence of females (overall, ~30%), mainly distributed in referents and traffic policemen. Self-reported smoking prevalence was similar across job titles and cities, with an overall 35% of cigarette smokers and a mean daily consumption of 16 cigarettes. The good correlation coefficient found between self-reported daily cigarette consumption and the level of urinary cotinine (r = 0.791) proves the reliability of smoking level collected thought questionnaire.

Personal Benzene Exposure and Biological Monitoring. In Table 2, data on personal exposure to airborne benzene, as time-weighted average value, excretion of *t,t*-MA (T_0 and T_1), S-PMA, and U-benzene (T_0 and T_1), in subjects divided according to city, job title, and smoking habits are reported. Using parametric statistics, higher personal exposures were found in gas station attendants, followed by traffic policemen and bus drivers, with comparable levels, and finally by the two

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Table 2. Summary of biological markers of benzene exposure in the investigated subjects divided according to city, job titles, and smoking habits

Location	Jobs	Air benzene (μg/m^3), median (min-max) $N_{<lod}/N$*		*t,t*-MA T_0 (μg/L), median (min-max) $N_{<lod}/N$	*t,t*-MA T_1 (μg/L), median (min-max) $N_{<lod}/N$	S-PMA T_1 (μg/L), median (min-max) $N_{<lod}/N$	U-benzene T_0 (ng/L), median (min-max) $N_{<lod}/N$	U-benzene T_1 (ng/L), median (min-max) $N_{<lod}/N$
Genoa	Referents	9† (<6-46) 16/49	Nonsmokers	72 (<10-398) 3/34	51 (<10-181) 3/35	9.0 (0.2-182.2) 0/36	Not determined	Not determined
			Smokers‡	190 (519-1,850) 0/13	195 (<10-444) 1/13	13.7 (3.0-19.9) 0/13	Not determined	Not determined
	Bus drivers	21† (<6-92) 5/152	Nonsmokers	85 (<10-2,014) 16/106	57 (<10-536) 13/106	5.6 (0.2-13.3) 0/86	Not determined	Not determined
			Smokers‡	136 (<10-600) 4/47	174 (<10-695) 1/47	9.3 (0.2-65.9) 0/38	Not determined	Not determined
Milan	Referents	6† (<6-115) 25/58	Nonsmokers	35 (<10-576) 9/34	33 (<10-1,089) 7/34	4.1 (0.2-12.5) 0/34	155 (72-1,770) 0/34	133 (<15-409) 1/33
			Smokers‡	51 (<10-246) 4/23	71 (<10-270) 3/23	8.0 (0.2-13.9) 0/22	560 (160-3,414) 0/24	331 (64-4,615) 0/22
	Traffic policemen	22† (9-316) 0/77	Nonsmokers	86 (<10-1,400) 8/49	82 (<10-416) 10/49	5.3 (0.2-13.8) 0/49	256 (98-846) 0/47	151 (25-943) 0/49
			Smokers‡	222 (<10-1,400) 0/28	213 (52-909) 0/28	9.1 (2.4-13.8) 0/28	2055 (313-6,430) 0/27	753 (54-4,246) 0/28
	Gas station attendants	61† (11-478) 0/78	Nonsmokers	35 (<10-672) 6/46	49 (<10-581) 5/46	5.8 (0.2-10.9) 0/38	459 (147-2,708) 0/44	342 (42-2,836) 0/46
			Smokers‡	81 (17-305) 0/32	144 (15-321) 0/32	7.5 (0.2-24.8) 0/27	2467 (233-5,616) 0/28	1168 (55-5,111) 0/32

*Observations below the detection limit versus total observation.
†Gas station attendants higher than bus drivers and traffic policemen, higher than controls (P < 0.01).
‡Smokers higher than nonsmokers for all the biomarkers (P < 0.001); statistical analysis was done with t test for independent samples on $\log_{10}$-transformed variables.

referent groups. A similar trend was observed in the excretion of U-benzene in Milan (both T_0 and T_1) but not of *t,t*-MA and S-PMA. The comparison between pre-monitoring and post-monitoring samples resulted in no difference in *t,t*-MA and unexpectedly higher levels of U-benzene in T_0 than in T_1 specimens. No differences in airborne benzene exposure between smokers and nonsmokers were found within the same job title. On the contrary, following cigarette consumption, the levels of the urinary biomarkers (*t,t*-MA, S-PMA, and U-benzene) were systematically higher (up to ~8-fold). The application of nonparametric statistics confirms these results with the exception of *t,t*-MA in Milan referents, which did not differ in smokers compared with nonsmokers, in both T_0 and T_1 samples. Figure 1 shows box plots of post-monitoring *t,t*-MA, S-PMA, and U-benzene in Milan subjects divided according to smoking and job titles.

Table 3 reports Pearson's correlations coefficient between environmental and biological markers in all the investigated subjects and in subjects divided according to smoking habits. Significant correlations were found between both U-benzene T_0 and T_1 but not with *t,t*-MA, S-PMA, and personal exposure, with the highest *r*s in nonsmokers and the lowest in smokers (for U-benzene T_0: r = 0.56 versus 0.30). Benzene exposure biomarkers were mostly correlated to each other, with the highest Pearson's r for *t,t*-MA or U-benzene in pre-monitoring versus post-monitoring urine samples. Good correlations with U-cotinine were found in all subjects and in smokers; as expected, such correlations were not found in nonsmokers. Spearman's correlation analysis between environmental and biological markers fully confirms these results.

Genetic Polymorphisms. The observed genotypes agree with allele frequencies reported previously for other European populations (21). No differences in the frequencies of genotypes among the groups with different job titles were found (χ^2 test was used for comparison). Based on this observation, the total study group (n = 415) was used for analyses of the effects of polymorphic genotypes on exposure biomarkers. The results of t test comparison, reported in Table 4, showed higher median levels of *t,t*-MA T_0, but not of *t,t*-MA T_1, in subjects with at least one variant allele in CYP2E1 (*Rsa*I) or CYP2E1 (*Dra*I). Furthermore, a lower median level of U-benzene T_1 in subjects with one variant allele in CYP2E1 (*Rsa*I) was found. A similar, but not significant, difference was found for U-benzene T_0. No influence of genetic polymorphism of NQO1 or of the combination of CYP2E1 (*Rsa*I) and CYP2E1 (*Dra*I) polymorphisms on urinary biomarkers was observed. When the Kruskal-Wallis test was applied to untransformed data for group comparison, slight differences with the previous analysis were noticed. In particular, higher median levels of *t,t*-MA T_0, but not of *t,t*-MA T_1, in subjects with at least one variant allele in CYP2E1 (*Dra*I; P = 0.03) but not in CYP2E1 (*Rsa*I; P = 0.07) was observed. Furthermore, a lower median level of both U-benzene T_0 and U-benzene T_1 in subjects with one variant allele in CYP2E1 (*Rsa*I; P < 0.01 and 0.03) was found.

Multiple Linear Regression Analysis. The adjusted multiple regression analyses (Table 5) substantially confirmed the findings of univariate analyses. We observed significant relationships between each of the urinary biomarkers and smoking (measured as U-cotinine); instead, after adjustment for smoking and CYP2E1 (*Rsa*I) polymorphism, only U-benzene was significantly positively related to airborne benzene; this variable alone explained ~20% of the variability in U-benzene. Accordingly, the adjusted R^2 for the models containing U-benzene as the dependent variable were 0.55 and 0.41 for pre-shift and post-shift measurements, respectively, much higher than those obtained when the other biomarkers were analyzed (0.10-0.20). We found a significant increase of *t,t*-MA

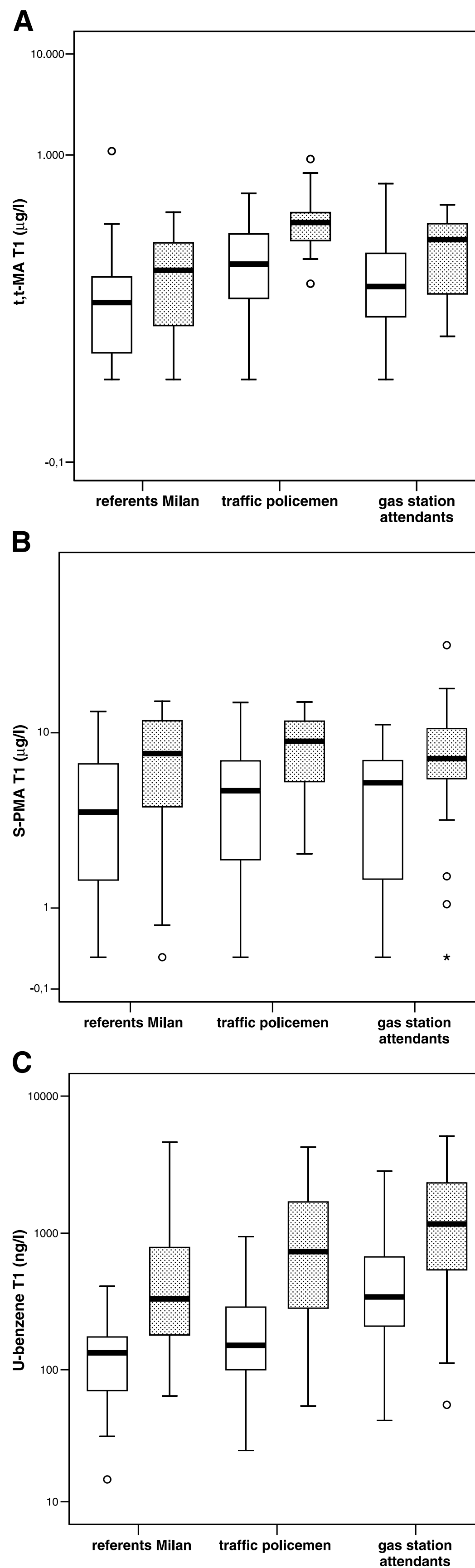

Figure 1. Box plots of *t,t*-MA (**A**), S-PMA (**B**), and U-benzene (**C**) in Milan subjects divided according to smoking habit and job title. *Plain boxes,* nonsmokers; *dashed boxes,* smokers (○, outliers; *, extremes).

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Table 3. Pearson's correlations between environmental and biological markers and between biomarkers themselves in all investigated subjects, nonsmokers, and smokers

		t,t-MA T_0	*t,t*-MA T_1	S-PMA	U-benzene T_0	U-benzene T_1	U-cotinine
Air benzene	All subjects	0.05 (411)	0.10* (412)	0.04 (370)	0.41† (204)	0.38† (210)	0.05 (412)
	Nonsmokers	0.00 (268)	0.09 (269)	0.04 (242)	0.56† (125)	0.53† (128)	0.04 (269)
	Smokers	0.09 (143)	0.06 (143)	−0.03 (128)	0.30† (79)	0.23* (82)	−0.07 (143)
t,t-MA T_0	All subjects		0.55† (412)	0.27† (369)	0.31† (203)	0.20† (210)	0.30† (412)
	Nonsmokers		0.47† (269)	0.24† (241)	0.10 (125)	0.03 (128)	−0.12 (269)
	Smokers		0.60† (143)	0.17 (128)	0.32† (78)	0.13 (82)	0.48† (143)
t,t-MA T_1	All subjects			0.43† (370)	0.35† (203)	0.27† (210)	0.44† (413)
	Nonsmokers			0.34† (242)	0.08 (125)	0.04 (128)	0.02 (270)
	Smokers			0.47† (128)	0.40† (78)	0.28* (82)	0.60† (143)
S-PMA	All subjects				0.39† (189)	0.40† (197)	0.33† (370)
	Nonsmokers				0.25† (117)	0.17 (120)	0.13 (242)
	Smokers				0.30* (72)	0.54† (77)	0.38† (128)
U-benzene T_0	All subjects					0.71† (201)	0.64† (203)
	Nonsmokers					0.62† (124)	0.00 (125)
	Smokers					0.54† (77)	0.47† (78)
U-benzene T_1	All subjects						0.53† (210)
	Nonsmokers						0.07 (128)
	Smokers						0.33† (82)

*$P < 0.05$.
†$P < 0.01$, the number of pairs in parentheses; statistical analysis was done on $\log_{10}$-transformed variables.

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in pre-shift urine samples (but not in post-shift samples) for subjects carrying a mutant CYP2E1 (*Rsa*I) allele; these subjects also had a significant reduction in the excretion of U-benzene, both pre-shift and post-shift.

Discussion

In the last decade, several studies were carried out to evaluate personal exposure to benzene in different categories of workers exposed to petrol engine exhaust fumes and/or to gasoline vapors (22–35). In the present investigation, gas station attendants, traffic policemen, bus drivers, and the general population working within or near two large cities of northern Italy, Genoa, and Milan were investigated through environmental and biological monitoring.

Comparing personal exposure to benzene in the different job titles, we observed that gas station attendants showed the highest level (median, 61 μg/m^3 or 0.019 ppm) followed by traffic policemen (22 μg/m^3 or 0.007 ppm) and bus drivers (21 μg/m^3 or 0.007 ppm), with comparable exposure, and finally by the two groups of referents (9 and 6 μg/m^3 or 0.003 and 0.002 ppm) with the lowest concentrations (Table 2). This exposure rank is in line with what was expected based on previous experience, although the concentrations measured in this study reflect a trend toward lower levels in comparison with the past (25-27, 31, 32). Such improvement in air quality may be due to at least three major initiatives undertaken by the Italian government in the last years: (*a*) the lowering of the maximum permissible percentage of benzene in gasoline, (*b*) the economic incentive for purchasing new cars equipped with catalytic converters, and (*c*) the aspiration system applied to the nozzle of the gasoline pump for vapor recovery (36, 37).

Considering the workers with the highest exposure levels (i.e., gas station attendants), median and maximum benzene levels of 61 and 478 μg/m^3 (0.019 and 0.15 ppm) are, respectively, 52- and 7-fold lower than the European Union occupational limit value of 3,200 μg/m^3 (1 ppm; ref. 9) recently adopted in Italy but also lower than the limit value recommended or stated by industrial hygiene associations or governmental authorities (10). On the contrary, exposure to benzene in referents, with median and maximum levels up to 9 and 115 μg/m^3 (0.003 and 0.036 ppm), is about equal to and up to 12-fold higher than the European Union air quality standard of 10 μg/m^3 (0.003 ppm; ref. 38).

Considering urinary *t,t*-MA as a biomarker of occupational exposure to benzene, we found that in the different job titles there were no differences related to personal exposure or correlation with airborne benzene (Tables 2 and 3). This finding is consistent with previous studies, as reviewed by Scherer et al. (39) and Dor et al. (40). Moreover, recent publications on traffic policemen, gas station attendants, and

Table 4. Biomarkers of exposure in all investigated subjects divided according to different polymorphic genotypes

Polymorphic genotype/ genotype subgroups	*t,t*-MA T_0 μg/L, median (min-max), *n*	*t,t*-MA T_1 (μg/L), median (min-max), *n*	S-PMA T_1 (μg/L), median (min-max), *n*	U-benzene T_0 (ng/L), median (min-max), *n*	U-benzene T_1 (ng/L), median (min-max), *n*
CYP2E1 (*Rsa*I)					
Wild-type	76 (<10-1,850), 382	79 (<10-1,089), 382	6.7 (0.2-182.2), 340	458 (77-6,430), 189	283 (15-5,111), 194
ht variant	118 (<10-2,014), 27	58 (<10-286), 28	5.6 (0.2-16.0), 28	264 (72-6,235), 13	149 (54-1,170), 14
*P**	0.05	0.14	0.23	0.09	0.03
CYP2E1 (*Dra*I)					
Wild-type	74 (<10-1,850), 349	77 (<10-1,089), 349	6.6 (0.2-182.2), 308	450 (77-6,430), 173	273 (25-5,111), 177
ht + ho variant	115 (<10-2,014), 60	88 (<10-452), 61	6.7 (0.2-18.5), 60	347 (72-6,235), 29	251 (15-3,296), 31
*P**	0.02	0.69	0.75	0.63	0.46
NQO1					
Wild-type	87 (<10-2,014), 238	84 (<10-1,089), 239	6.6 (0.2-182.2), 212	421 (72-6,430), 115	248 (25-4,246), 121
ht + ho variant	67 (<10-672), 171	72 (<10-909), 171	6.5 (0.2-65.9), 156	466 (81-5,106), 87	283 (15-5,111), 87
*P**	0.40	0.40	0.78	0.69	0.96

NOTE: Comparison between genotype subgroups was done by *t* test for independent samples.
**P* for comparison with *t* test for independent samples on $\log_{10}$-transformed variables between the genotype subgroups.

Table 5. Evaluation of airborne benzene exposure, smoking status (as urinary cotinine), and CYP2E1 (*Rsa*I) genotype on urinary biomarkers according to linear multiple regression analysis: $\log_{10}$(U-Biomarker) = constant + $\log_{10}$(BenzeneAir) $\times\beta_1$ + $\log_{10}$(U-cotinine) $\times\beta_2$ + CYP2E1 (*Rsa*I) $\times\beta_3$

	t,t-MA T_0		*t,t*-MA T_1		S-PMA		U-benzene T_0		U-benzene T_1	
	β (SE)	R^2_P, P	β (SE)	R^2_P, P	β (SE)	R^2_P, P	β (SE)	R^2_P, P	β (SE)	R^2_P, P
Constant	1.27 (0.11)	—, <0.01	1.07 (0.10)	—, <0.01	0.28 (0.09)	—, <0.01	1.48 (0.09)	—, <0.01	1.35 (0.11)	—, <0.01
Benzene Air	0.05 (0.06)	<0.01, 0.43	0.09 (0.05)	0.01, 0.09	0.01 (0.05)	<0.01, 0.77	0.34 (0.05)	0.22, <0.01	0.36 (0.05)	0.18, <0.01
U-cotinine	0.24 (0.04)	0.09, <0.01	0.32 (0.03)	0.19, <0.01	0.21 (0.03)	0.10, <0.01	0.41 (0.03)	0.45, <0.01	0.33 (0.04)	0.28, <0.01
CYP2E1 (*Rsa*I)	0.29 (0.11)	0.02, 0.01	−0.07 (0.10)	<0.01, 0.47	−0.07 (0.09)	<0.01, 0.47	−0.21 (0.10)	0.02, 0.03	−0.25 (0.11)	0.03, 0.02
Whole model R^2_{adj}, P	0.10, <0.01		0.20, <0.01		0.10, <0.01		0.55, <0.01		0.41, <0.01	

NOTE: Values of constant and β coefficient, with SE, partial R^2 (R^2_P), and significance (P) for each term of the equation, are given. Adjusted R^2 (R^2_{adj}) and the significance (P) for the whole model are reported in the last row. Statistical analysis was done on $\log_{10}$-transformed variables.

bus drivers confirmed the lack of differences between workers and the general population in *t,t*-MA excretion (13, 28, 30). Indeed, a benzene threshold, ranging from 800 to 3,200 μg/m³ (0.25-1 ppm), below which *t,t*-MA may not be a useful biomarker for exposure to benzene, was suggested (11, 41, 42), In the present study, both the median and the highest benzene exposure were well below these values.

In addition to smoking and genetic polymorphisms (discussed later), the preserving agent sorbic acid and its salts (sorbates), contained in a large variety of food and drinks, may have contributed to the observed level of urinary *t,t*-MA (43-45). According to Ruppert et al. (43), as much as 50% of *t,t*-MA excreted in nonsmoking subjects and up to 25% in smoking, nonoccupationally exposed subjects may be ascribed to the contribution of these molecules. Because we did not collect information on food and drink intake in the study subjects, the contribution of sorbic acid and its salts in the excretion of *t,t*-MA could not be properly evaluated.

S-PMA is generally considered a very specific biomarker of benzene, successfully applied to distinguish exposed and nonexposed subjects (35, 41, 42, 46-49) and correlated with personal exposure starting from low concentrations [reviewed by Dor et al. (40)]. In the present study, S-PMA was also a poor biomarker of occupational exposure to benzene. This is consistent with a recent field study on traffic policemen in which S-PMA failed to distinguish exposed from unexposed subjects (28). Overall, available evidence does not allow setting an unequivocal threshold above which this index may be applied: benzene concentrations ranging from 64 to 960 μg/m³ (0.02-0.3 ppm) were suggested (23, 35, 49). Again, the majority of our subjects experienced exposure below these thresholds. Interestingly, the levels of S-PMA determined in the present study were higher than expected, with values ranging from <0.2 and 182 μg/L, and the median value in nonsmoking referents up to 9.0 μg/L, whereas concentrations in the range from 1.5 to 2 μg/g creatinine were reported in other studies (46-48). At the moment, the reason for this is unclear, but differences in the analytic procedure used may be important; in fact, an immunoassay technique was applied in this investigation (18), whereas high-performance liquid chromatography/fluorescence, gas chromatography/mass spectrometry, and liquid chromatography/tandem mass spectrometry were used in all the other studies.

U-benzene was the only biomarker suitable for discriminating among the different jobs and positively correlated with personal exposure. After the initial interesting results in the mid-1990s (8, 11), following which U-benzene was suggested as the most promising biological index for monitoring benzene exposure (40), few applications of this marker have been done. U-benzene was applied with success (12, 14) in industrial settings characterized by heavily personal exposures (median airborne exposure of 3 and 31 ppm, respectively). In studies characterized by much lower exposure, the validity of U-benzene needs further evidence. A correlation between personal exposure and U-benzene was detected in 12 gas station attendants (median benzene exposure, 300 μg/m³ or 0.094 ppm), but U-benzene could not discriminate between workers and controls (13). A correlation was also found among petrochemical workers exposed to 90 μg/m³ (0.028 ppm) of benzene (50). U-benzene failed to differentiate two groups of volunteers, one cycling in urban and the other in rural routes (51), and showed no correlation with personal exposure in parking attendants and laboratory workers (median exposure, 14 μg/m³ or 0.004 ppm; ref. 30).

The results of the present investigation further support the use of U-benzene as a biomarker of exposure to benzene even at low levels: based on our data, U-benzene is useful starting from airborne concentrations as low as 6 μg/m³ (0.002 ppm).

When levels of urinary biomarkers in T_0 and T_1 specimens were compared, no difference was found for *t,t*-MA, whereas a significant decrease in the levels of U-benzene at the end of the monitoring period was observed in all job tasks in both smokers and nonsmokers. This behavior of U-benzene is in contrast with the increase expected following occupational exposure. The drop in excretion is especially evident in smokers; a possible reason for this may be related to refraining from smoking during the work shift. Another explanation may be linked to the circadian rhythms in the biochemical activity of organs and tissues with a nocturnal slow down in the production of urine and metabolites (52). Due to the reduced transformation, at night, an increase in the concentration of circulating benzene released from storage tissues by passive diffusion is expected. Consequently, in morning samples, the concentration of U-benzene should reflect the amount present in blood. Later, the resumption of kidney and liver activities quickly subtracts benzene from circulation with a following decrease in its level. As, according to our knowledge, no evidence on daily rhythmic variation in benzene excretion in humans has been reported to date, this issue remains open and will be the object of further investigation.

Mainstream cigarette smoke contains relevant amounts of benzene and can lead to an average extra benzene inhalation of ~720 μg/d (16 cigarettes daily × 45 μg/cigarette; ref. 5) in smoking compared with nonsmoking subjects. Daily benzene exposure from inhalation of polluted air in working and living environments was calculated assuming that subjects were exposed for 8 hours daily to the benzene level measured during the investigated work shift and for the remaining 16 hours daily to the level measured in the referent subjects [work shift exposure μg/m³ (median value for the specific job title) × 0.9 m³/h (ventilation rate) × 8 hours daily (work shift duration) + 9 μg/m³ (median exposure for highest exposed referents) × 0.9 m³/h (ventilation × 16 hours daily, rest of the day)]. The estimated average intakes were 194, 288, and 526 μg/d for controls, bus drivers or traffic policemen, and gas station attendants. The ratio between daily intake computed in smokers (environmental benzene + smoke) and nonsmokers

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(only environmental benzene) was estimated to be 4.7 in controls, 3.5 in bus drivers or traffic policemen, and 2.4 in gas station attendants. These values are in good agreement with the ratio obtained in this study between urinary biomarkers detected in smokers and nonsmokers (1.5-3.8 for *t,t*-MA, 1.5-2.0 for S-PMA, and 2.5-8.0 for U-benzene; Table 2) as well as those reported in previous investigations (42, 43, 45, 51). Based on these calculations, we would predict a daily benzene intake higher in smoking referents (194 μg/d from inhalation of polluted air + 720 μg/d from cigarettes = 914 μg/d) than in nonsmoking gas station attendants (526 μg/d). This estimate is consistent with the levels of U-benzene measured in smoking referents (560 and 331 ng/L in T_0 and T_1 samples) compared with nonsmoking gas station attendants (459 and 342 ng/L in T_0 and T_1 samples).

U-cotinine significantly correlated with *t,t*-MA, S-PMA, and U-benzene in all subjects and in smokers. The correlations with personal exposure were significant only for U-benzene, not for *t,t*-MA and S-PMA. From the linear regression of U-benzene T_1 versus air benzene (all subjects), it was calculated that an exposure of 320 μg/m^3 (0.1 ppm) corresponds to a U-benzene excretion of 828 ng/L. This value agrees with those reported previously (13, 14). The correlations between biomarkers were generally higher in smokers compared with nonsmokers. These observations further support the relevance of additional benzene exposure due to cigarette smoking.

Rothman et al. have reported evidence of a genetic basis for human susceptibility to benzene-related disease (53). They found a higher probability of "benzene poisoning" (hematotoxicity) in heavily exposed Chinese workers having a rapid fractional excretion of chlorzoxazone (phenotype of CYP2E1) and two copies of the NQO1 ^{609}C→T mutation. Some evidences of the influence of genetic polymorphism in the production of benzene metabolites were also reported (23, 51, 54, 55). The result of our study suggests, for the first time, a role of the genetic polymorphisms of CYP2E1 *Rsa*I and *Dra*I in the metabolism of benzene (Table 4). The study population is large enough to allow for the detection of a small effect in a limited number of individuals as expected based on the mutation frequencies. These two polymorphisms in CYP2E1 have not been found to have a significant effect on benzene toxicity (53, 54) or CYP2E1 phenotype (56), although other studies have indicated a role for CYP2E1 genotype in benzene toxicity, gene expression, and disease risk (57-59). Our result on the effect of the CYP2E1 RFLP needs to be confirmed, as only *t,t*-MA T_0 and U-benzene T_1 were significantly modified, U-benzene T_0 showed a trend toward decrease, whereas *t,t*-MA T_1 was not influenced by the polymorphisms of CYP2E1.

Multiple linear regression analysis (Table 5) summarizes how the influence of genetic polymorphism on the variability of the investigated biomarkers seems to be limited in comparison with other factors: in fact, for U-benzene T_0, only 2% could be explained by genetic polymorphism of CYP2E1, whereas as much as 22% could be explained by personal exposure and 45% by smoking.

In conclusion, at the environmental levels of benzene exposure investigated, smoking plays a major role in individual benzene uptake. The comparison of *t,t*-MA, S-PMA, and U-benzene as biomarkers of benzene exposure shows that U-benzene is performing best and may be applied for biological monitoring purposes starting from airborne benzene levels as low as a 6 μg/m^3 (0.002 ppm).

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