La Medicina del Lavoro Med Lav 2012; *103*, 5: 338-346

Biological monitoring of low-level exposure to benzene

M. Campagna, Giannina Satta, Laura Campo*, Valeria Flore, A. Ibba, M. Meloni, Maria Giuseppina Tocco, G. Avataneo, C. Flore, Silvia Fustinoni*, P. Cocco

Department of Public Health, Clinical and Molecular Medicine, Occupational Health Section, University of Cagliari, Asse Didattico della Facoltà di Medicina, Monserrato (Cagliari), Italy

* Department of Occupational and Environmental Health, University of Milan and Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy

Key words

Benzene; biomarkers; low-level environmental exposure; biological monitoring

Parole chiave

Benzene; indicatori biologici; esposizione ambientale a basse dosi; monitoraggio biologico

Summary

Introduction: *Conflicting opinions exist about the reliability of biomarkers of low-level exposure to benzene. We compared the ability of the urinary excretion of trans,trans-muconic acid (t,t-MA), s-phenilmercapturic acid (s-PMA) and urinary benzene (U-Benz) to detect low level occupational and environmental exposure to benzene.* ***Methods:*** *We monitored airborne benzene by personal air sampling, and U-Benz, s-PMA, t,t-MA and cotinine (U-Cotinine) in spot urine samples, collected at 8 am and 8 pm, in 32 oil refinery workers and 65 subjects, randomly selected among the general population of urban and suburban Cagliari, Italy. Information on personal characteristics, diet and events during the sampling day was acquired through in person interviews.* ***Results:*** *The median concentration of airborne benzene was 25.2 $\mu g/m^3$ in oil refinery workers, and 8.5 $\mu g/m^3$ in the general population subgroup. U-Benz in morning and evening samples was significantly more elevated among oil refinery workers than the general population subgroup ($p=0.012$, and $p=7.4x10^{-7}$, respectively) and among current smokers compared to non-smokers ($p=5.2x10^{-8}$, and $p=5.2x10^{-5}$ respectively). Benzene biomarkers and their readings in the two sampling phases were well correlated to each other. The Spearman's correlation coefficient with airborne benzene was significant for U-Benz in the evening sample, while no correlation was seen with t,t-MA and s-PMA readings in either samplings. The two benzene metabolites were frequently below limit of detection (LOD), particularly among the general population study subjects (17-9% and 39%, for t,t-MA and s-PMA respectively). Morning U-Cotinine excretion showed a good correlation with U-Benz in the morning and in the evening sampling ($p<0.001$), and with s-PMA in the evening sample ($p<0.001$), but not with t,t-MA in either samplings. t,t-MA in the evening sample was the only biomarker showing a moderate inverse correlation with BMI ($p<0.05$). The multiple regression analysis adjusting by BMI and number of cigarettes smoked during the day confirmed the results of the univariate analysis.* ***Discussion:*** *Our results suggest that unmetabolized U-Benz would allow a more reliable biomonitoring of low-level exposure to benzene than s-PMA and t,t-MA.*

Pervenuto il 11.4.2012 - Accettato il 27.6.2012

Corrispondenza: Marcello Campagna, Department of Public Health, Clinical and Molecular Medicine, Occupational Health Section, University of Cagliari, Asse Didattico della Facoltà di Medicina, SS 554, km 4,500, 09042 Monserrato (Cagliari), Italy - Tel. +39 070 6753104 - Fax: +39 070 6754728 - E-mail: makamp@libero.it

Riassunto

«Monitoraggio biologico dell'esposizione a basse dosi di benzene». **Introduzione:** *La validilità dei biomarcatori di bassi livelli d'esposizione a benzene è oggetto di opinioni contrastanti. Abbiamo confrontato la validità dell'acido trans,trans muconico (t,t-MA), dell'acido s-fenilmercapturico (SPMA) e del benzene urinario (U-Benz) nel monitoraggio dell'esposizione a basse dosi occupazionali ed ambientali a benzene.* **Metodi:** *Sono stati dosati i livelli di esposizione ambientale (AB) ed analizzati i livelli di U-benz, SPMA e t,t-MA in due campioni estemporanei di urina (alle ore 8 e alle ore 20) di 32 operai di una raffineria di petrolio e 65 soggetti non esposti estratti a caso tra la popolazione generale residente nell'area metropolitana di Cagliari. È stata inoltre dosata la cotinina (U-Cotinina) nel campione del mattino e calcolato l'indice di massa corporea (BMI). Informazioni sull'esposizione nel giorno del monitoraggio sono state raccolte mediante questionario.* **Risultati:** *I valori ambientali mediani di benzene sono risultati pari a 25.2 μg/m³ negli operai di raffineria e 8.5 μg/m³ nella popolazione generale. U-benz è risultato significativamente più elevato tra gli operai di raffineria in entrambi i campioni ($p=0.012$, e $p=7.4x10^{-7}$ rispettivamente) e tra i fumatori rispetto ai non fumatori ($p=5.2x10^{-8}$ e $p=5.2x10^{-5}$ rispettivamente). I vari marcatori si sono mostrati ben correlati tra di loro e tra i due campionamenti. U-benz nel campione serale ha mostrato una buona correlazione con l'esposizione ambientale ($p<0.001$), mentre s-PMA e t,t-MA non hanno mostrato una correlazione in nessuno dei due campionamenti. U-Cotinina ha mostrato una buona correlazione con U-benz al mattino e alla sera ($p<0.001$) e s-PMA nel campione serale ($p<0.001$), ma non con t,t-MA in nessuno dei due campioni. t,t-MA è risultato l'unico biomarcatore a mostrare una moderata correlazione inversa con il BMI ($p<0.05$). L'analisi della regressione multipla, corretta per BMI e numero di sigarette fumate nel corso della giornata ha confermato i risultati dell'analisi univariata.* **Discussione:** *I nostri risultati suggeriscono che la determinazione del benzene urinario possa costituire un biomarcatore piu' affidabile rispetto a s-PMA e t,t-MA nel monitoraggio dell'esposizione occupazionale ed ambientale a bassi livelli di benzene.*

Introduction

Long after its first regulation (10), benzene is still a reason for concern as an environmental contaminant. Following preventive action in the last decades (12, 30), automobile exhausts and industrial emissions share with cigarette smoking the role of major environmental sources of outdoor and indoor exposure to benzene (20). Benzene is a known human carcinogen (20-22) and it is labeled H350 (may cause cancer to humans) by the European regulation No. 1272/2008 (13), because it causes non lymphatic leukemia. Recent epidemiological findings suggest it might be implicated in the etiology of some lymphoma subtypes as well (5, 7, 36).

In consideration of its genotoxic potential even at low-level environmental exposure (39), and its possible contribution to childhood cancer (37), biomonitoring environmental exposure to benzene at the general population level has been recognized as an important public health issue (12). Unmetabolized urinary benzene (U-Benz) has been recently suggested as a biomarker in alternative to its metabolites *trans-trans*-muconic (*t,t*-MA) and *s*-phenil mercapturic acid (*s*-PMA), due to its sensitivity, lesser influence of confounding factors and good correlation with low airborne benzene levels (14, 28). In this paper, we compared the reliability of *t,t*-MA, *s*-PMA and U-Benz in detecting low benzene airborne levels from occupational and environmental origin and explored other covariates, including time of sampling, smoking habits and body mass index (BMI), that might affect such biomarkers.

Methods

Within a 2006-2007 multicentre Italian project on biomonitoring exposure to polycyclic aromatic hydrocarbons (PAH) and benzene, 182 adult subjects (age range 25-74) agreed to participate in the metropolitan area of Cagliari, the major urban area

in Sardinia, Italy. Study subjects were randomly selected among three population groups: oil refinery workers, whose exposure to benzene was regularly monitored within the occupational health and safety surveillance program; residents within 5 km from the oil refinery; and residents in the urban area, about 20 km apart. For the purposes of this analysis, we selected 97 male subjects, including 32 oil refinery workers and 65 male subjects from the general population subgroups. Female subjects were not included, as there were none among oil refinery workers. Subjects with definite or probable exposure to benzene, such as gasoline station attendants, cabdrivers, and policemen, were also excluded. No age matching was conducted. For each participating subject, two urine spot samples were collected at 8:00 a.m. (m) and 8:00 p.m. (e), for the analysis of *t,t*-MA, *s*-PMA, U-Benz and urinary cotinine (U-cotinine), as a biomarker of exposure to personal or environmental tobacco smoke. Personal exposure to benzene was monitored with passive samplers (Radiello®) worn by the subjects in between the two samplings. The analysis of airborne benzene was performed by gas chromatography/ mass spectrometry (GC/MS); the limit of quantification (LOQ) was 1 $\mu g/m^3$ (0.3 ppb).

In person interviews were conducted by trained interviewers for all subjects. The questionnaire recorded information on demographics, occupation, activities during the day (type of transportation, duration of commuting, jobs performed, intake of food and beverages) and smoking habits. Subjects who reported current active smoking were defined as smokers.

To assess *t,t* -MA, *s*-PMA and U-Benz urinary excretion, samples were transferred in vials upon deliverance by the study subject, promptly sealed with screw open-top closure with silicone-polyperfluoro-ethylene gaskets, crimped with an aluminum nut in order to prevent loss of benzene from urinary samples, and stored at -20°C up to analysis. U-Benz was assessed by headspace solid-phase microextraction (HS-SPME) followed by GC/MS analysis (18). Readings were expressed by volume (ng/L), with 15 ng/L as the limit of detection (LOD). *t,t*-MA concentration was assessed by high performance liquid chromatography (HPLC) equipped with an UV-VIS detector following solid phase extraction (SPE) using a SAX column (9); values were expressed by volume (μg/L), with 5μg/L as LOD. *s*-PMA was measured by liquid chromatography followed by MS analysis (LC/MS) and expressed by volume (μg/L). LOD was 0.1μg/L (14). U-cotinine was assessed in the morning samples by liquid chromatography/triple quadrupole mass detector (LC-MS-MS), in the presence of cotinine-d_3 as internal standard. The LOQ was 10 μg/L. Urinary creatinine excretion was assessed by Jaffe's colorimetric method (23) in a limited number of samples to normalize measurements of *t,t*-MA, *s*-PMA and U-cotinine and expressed by volume (g/L). As no substantial variation in the statistical analysis occurred after using values normalized for creatinine, results using measurements by volume are presented throughout the paper. Values below LOD or LOQ were attributed half the limit value.

The assays were performed in the laboratory of the Occupational Health Section of the Department of Public Health, Clinical and Molecular Medicine of the University of Cagliari, Italy (*t,t*-MA and urinary creatinine) and in the laboratory of Occupational and Environmental Toxicology of the Department of Occupational and Environmental Medicine, University of Milan and Fondazione IRCCS Ospedale Maggiore Policlinico, Milan (U-benz, *s*-PMA and U-cotinine).

Descriptive statistics, parametric and non-parametric tests were performed as appropriate, using SPSS®. Multivariate regression analysis was conducted, considering all subjects included in the study, in order to explore the correlation between exposure covariates (airborne benzene and smoking) and the outcomes (benzene biomarkers) adjusting for BMI and number of cigarettes smoked during the sampling day (only for the evening samplings).

Results

Overall, the mean age of the study population was 46.6 years (SD 13.7), and it was significantly lower in oil refinery workers (mean: 39.2, SD

12.4), than the general population subgroups combined (mean: 50.4, SD 12.8) (t=7.93; p=5.53x10^{-12}).

Smokers from the general population (mean number of cigarettes=15, *SD* 8.9) and the oil refinery subgroups (mean number of cigarettes=13, *SD* 8.6) roughly smoked a similar number of cigarettes per day (t=0.23; p=0.82). U-cotinine median values were 25 μg/L (IQ range <10-25) in the general population (non-smokers: median 25 μg/L, IQ range <10-25; smokers: median 1707 μg/L, IQ range 1294-1784) and <10 μg/L (IQ range <10-651) in the oil refinery subgroup (non-smokers: median <10 μg/L, IQ range <10 - <10; smokers: median 779 μg/L, IQ range 488-1442). U-creatinine median values were 1 g/L (IQ range 1-1.77) in general population (non-smokers: median 1.3 g/L, IQ range 1-1.7; smokers: median 1.7 g/L, IQ range 1.4-2.1) and 2 g/L (IQ range 1.4-2.3) in the oil refinery subgroup (non-smokers: median 2 g/L, IQ 1.4-2.3; smokers: median 1.9g/L, IQ range 1.1-2.3).

Urinary benzene biomarkers were not related to age (Spearman's correlation coefficients: U-Benz-m=-0.128, p=0.26; U-Benz-e=-0.116; p=0.31; *s*-PMA=-0.056 p=0.69; *t,t* –MA-m=-0.173 p=0.14; *t,t*-MA-e =-0.230 p=0.06) (data not shown in the tables).

Table 1 shows environmental monitoring data in the two study subgroups by smoking habit. As expected, petrochemical workers showed the highest median airbone benzene exposure level, which was significantly more elevated among non-smokers compared to the smoking workmates and to smokers and non-smokers of the general population subgroup (Kruskal-Wallis test=24.905; p=1.62 x10^{-5}). Table 1 also shows summary statistics of benzene biomarkers by sampling time, study subgroup and smoking habit. Numbers of subject with valid measurement data for each biomarker are shown in the table. It is also of interest to note that measurements below LOD were null for U-Benz-m and

Table 1 - Summary of airborne benzene exposure and benzene biomarkers by study subgroup and smoking habit. The number of valid readings and its percentage over the participating subjects in the respective study subgroup are shown along with median and IQ range values

Study subgroups	N. subjects	Airborne benzene (μg/m^3)*		U-benz-m** (ng/L)		U-benz-e** (ng/L)		t,t-MA-m (μg/L)		t,t-MA-e* (μg/L)		s- PMA* (μg/L)	
		N. (%)	Median (IQ range)	N. (%)	Median (IQ range)	N. (%)	Median (IQ range)	N. (%)	Median (IQ range)	N. (%)	Median (IQ range)	N. (%)	Median (IQ range)
General population	65	55 (85)	9 (6-11)	59 (91)	148 (74-251)	54 (83)	126 (69-226)	62 (95)	12 (<5-46)	45 (69)	25 (10-44)	59 (91)	0.2 (<0.1-0.3)
Non smokers	51	43 (84)	6 (1-9)	48 (94)	115 (71-179)	46 (90)	120 (67-176)	49 (96)	11 (<5-32)	36 (71)	25 (10-41)	48 (94)	0.1 (<0.1-0.2)
Smokers	14	12 (86)	10 (9-15)	11 (79)	941 (310-2729)	8 (57)	819 (355-2024)	13 (93)	47 (6-78)	9 (64)	23 (15-101)	11 (79)	0.5 (0.2-1.5)
Oil refinery workers	32	32 (100)	25 (12-64)	22 (69)	236 (122-506)	30 (94)	391 (211-662)	32 (100)	30 (21-66)	32 (100)	52 (30-105)	31 (97)	0.14 (<0.1-0.3)
Non smokers	19	19 (100)	30 (12-123)	12 (63)	149 (115-227)	12 (63)	267 (151-557)	19 (100)	28 (17-61)	19 (100)	35 (26-66)	18 (95)	<0.1 (<0.1-0.2)
Smokers	13	13 (100)	16 (13-31)	10 (77)	641 (477-1097)	11 (85)	431 (359-1217)	13 (100)	53 (24-70)	13 (100)	92 (34-130)	13 (100)	0.2 (0.1-0.8)
Total	97	87 (90)	9 (7-26)	81 (84)	149 (82-346)	84 (87)	168 (81-403)	94 (97)	23 (8-57)	77 (79)	33 (18-72)	90 (93)	0.1 (<0.1-0.3)

* Note: Kruskal-Wallis test: * <0.05; ** p<0.001

U-Benz-e; 16 (17%) for *t,t*-MA-m and 7 (9%) for *t,t*-MA-e, all but one belonging to the general population subgroup in both samplings; and 35 (39%) for *s*-PMA, which measurement was available only in the evening sample, approximately evenly distributed by study subgroup (22/65 in the general population subgroup, and 13/32 in the petrochemical workers subgroup). U-Benz-m, U-Benz-e, and *t,t*-MA-e were significantly more elevated among oil refinery workers than the general population subgroup. However, U-Benz-e showed the greatest discriminating power between the two study groups overall (Mann-Whitney test=4.815, p=7.4x10^{-7}). U-Benz-m was most effective in discriminating between smokers and non-smokers, overall (Mann-Whitney test=5.320, p=5.2x10^{-8}) and in the general population subgroup (Mann-Whitney test=4.094, p=2.1x10^{-5}). *t,t*-MA excretion also differed by study subgroup, particularly among non-smokers and in the evening sample (Mann-Whitney test=4.118, p=1.9x10^{-5}), and by smoking status in the morning sample in the overall study population (Mann-Whitney test=2.852, p=0.004), but not within the two study subgroups. *s*-PMA excretion was significantly more elevated among non-smoking oil refinery workers compared to non-smokers in the general population subgroup (Mann-Whitney test=2.189, p=0.03), and it differed between smokers and non-smokers within the general population subgroup (Mann-Whitney test=2.010, p=0.04). However, a higher proportion of the study population readings were below the LOD in both the study subgroups, and the p–values for the difference between study subgroups and smoking status were much less relevant than for U-Benz and *t,t*-MA in both sampling times.

The univariate correlation matrix between airborne benzene concentration, benzene biomarkers, and BMI is shown on table 2. All study subjects, non-smokers and smokers are included. U-Benz-e was the only showing a significant correlation with airborne benzene concentration. U-cotinine, which was measured only in the morning sample, was well correlated to U-Benz-m values, and it also affected *s*-PMA concentration in the evening. BMI showed a moderate inverse correlation with *t,t*-MA-e, but not with the other benzene biomarkers. U-Benz-m and *t,t*-MA-m were good predictors of the respective values in the evening hours. Both U-Benz values showed a moderate correlation with *t,t*-MA-e, but not with *t,t*-MA-m. *s*-PMA was not correlated with airborne benzene, and it was moderately correlated with U-Benz-m, U-Benz-e, and *t,t*-MA-e.

The multiple regression analysis was designed to predict the benzene biomarkers in the morning as a function of log-transformed values of airborne benzene and U-cotinine adjusting by BMI and, for the evening samples, by the number of cigarettes smoked during the sampling day. The results of the univariate analysis were confirmed (table 3): U-cotinine in the morning hours, number of cigarettes smoked during the sampling day in the evening

Table 2 - Correlation matrix between benzene monitoring data, urinary cotinine, body mass index and benzene biomarkers (Spearman correlation coefficients

	Airborne benzene	U-cotinine	BMI	U-Benz-m	U-Benz-e	*s*-PMA	*t,t*-MA-m	*t,t*-MA-e
Airborne benzene	1.000	-0.169	-0.040	0.220	0.519[b]	-0.077	0.149	0.017
U-cotinine	-	1.000	0.007	0.566[b]	0.376[b]	0.458[b]	0.045	0.194
BMI	-	-	1.000	-0.047	0.005	-0.116	-0.159	-0.272[a]
U-Benz-m	-	-	-	1.000	0.661[b]	0.426[b]	0.262[a]	0.337[b]
U-Benz-e	-	-	-	-	1.000	0.371[b]	0.155	0.346[b]
s-PMA	-	-	-	-	-	1.000	0.222[a]	0.322[b]
t,t-MA-m	-	-	-	-	-	-	1.000	0.650[b]
t.t-MA-e	-	-	-	-	-	-	-	1.000

[a] =p<0.05; [b] =p<0.01

Table 3 - Multivariate regression predicting biomarkers of benzene as a function of airborne benzene level and smoking habit: slopes are adjusted by BMI and cigarettes smoked during the sampling day. Continuous values (airborne benzene, U-cotinine and benzene biomarkers) were log-transformed for analysis

	U-cotinine		Airborne benzene		BMI	
	β	$se_β$	β	$se_β$	β	$se_β$
U-benz-m	0.695	0.036**	0.220	0.062*	0.220	0.062*
U-benze-e	0.390	0.130**	0.307	0.054**	0.047	0.013*
t,t-MA-m	0.066	0.052	0.202	0.080	-0.163	0.018**
t,t-MA-e	0.308	0.181	-0.024	0.103	-0.180	0.019**
*s-P*MA	0.203	0.154	-0.212	0.067*	-0.057	0.014*

* $p<0.05$; ** $p<0.001$

hours, and airborne benzene in both samplings, were good predictors of unmetabolized benzene excretion, while *t,t*-MA and *s*-PMA values were unaffected. Following the reciprocal adjustments, the corrected slopes for BMI were significantly inverse for all log-transformed benzene biomarkers, although of low absolute value compared to airborne benzene and smoking covariates.

Discussion

In our study of low-level occupational and environmental exposure to benzene, we found exposure levels comparable to similar surveys conducted in Italy (4, 14, 26, 28). The top median value of U-Benz-m among smokers from the general population subgroup might be related with the prohibition of smoking for refinery workers, who were monitored at the beginning of their morning work shift, while participants from the general population subgroup were monitored at their residence, and had more time available and no restrictions to smoke. It is known that active smoking is a major source of benzene uptake and can represents the main factor increasing U-Benz excretion (16). Our findings are consistent with previous reports showing the contribution of smoking in adding up to occupational exposure to increase benzene biomarkers' levels (17).

Benzene biomarkers were well correlated to each other, and U-Benz and *t,t*-MA readings in the evening hours were well correlated with the respective reading in the morning hours. However, U-Benz-e was the only one showing a good correlation with airborne benzene monitoring data during the sampling day. Besides, none of the U-Benz readings was below the LOD, compared to a substantial fraction of *t,t*-MA and particularly *s*-PMA readings. U-Benz-m, and *s*-PMA excretion in the evening hours, but neither *t,t*-MA readings, showed a good correlation with U-cotinine. *t,t*-MA in the evening hours was the only showing a moderately significant inverse correlation with BMI.

Despite some limitations related to limited sample size, and the loss of some aliquots of urine for the determination of specific biomarkers for technical problems, our study apparently supports a higher sensitivity of U-Benz as a biomarker of low-level exposure to benzene, including airborne benzene and benzene intake from active smoking, compared to its urinary metabolites. *t,t*-MA limitations in the biological monitoring of low-level exposure to benzene were confirmed in our study, its low specificity being possibly related to the dietary intake of sorbic acid, as previously described (2, 6). It has been estimated that diet might account for *t,t*.MA levels corresponding to 2.5 ppm of airborne benzene in 13% of the general population (31, 33).

s-PMA was also effective in discriminating smokers, but its lack of correlation with low-level airborne benzene levels and the low sensitivity of the method, with a high fraction of readings below LOD, in our opinion, make it less reliable than unmetabolized benzene in detecting low-level occupational and environmental exposure.

Due to a high fat/air and fat/blood partition coefficient, and adipose tissue being the major storage site for hydrocarbons (31), an inverse correlation between BMI and benzene biomarkers has been postulated (2, 25, 34). In our study, we found a moderate inverse correlation with *t,t*-MA excretion in the evening hours, but not with any the other benzene biomarkers.

In 2008, the US ACGIH proposed biological exposure indexes (BEIs) for *s*-PMA and *t,t*-MA, but not U-Benz (1). However, to date, methods for unmetabolized benzene monitoring have reportedly achieved acceptable and constant analytical performances, and greater sensitivity in the assessment of airborne benzene concentrations below 1 ppm (or 3.2 mg/m^3) in respect to the benzene metabolites (14). Our results apparently support such statements. Therefore, the conditions exist for the scientific community and governmental regulatory agencies to consider setting recommended and regulatory limits also for U-Benz. Thanks to its higher specificity and sensitivity in respect to the other available biomarkers, U-Benz would allow better monitoring of low-level occupational and environmental exposure to benzene (16). In fact, this is currently a relevant public health issue, as subclinical effects have been described following low-level exposure to benzene (4, 24, 32, 38, 39), and an increased risk of mature B-cell lymphomas, and particularly, chronic lymphocytic leukaemia and follicular lymphoma, has been associated with occupational exposure to benzene (5). These findings support the efforts of governmental regulatory agencies to achieve zero benzene content in fuels and solvents, and to further reduce its use in the chemical industry in the near future.

In conclusion, despite the above mentioned limitations and the conflicting opinions about the reliability of biomonitoring low-level environmental exposure to benzene (3, 8, 15, 19, 25-29), our results seem to support the use of urinary benzene as a sensitive and specific biomarker of low-level occupational and environmental exposure to benzene, as an alternative to *s*-PMA and *t,t*-MA.

No potential conflict of interest relevant to this article was reported

References

1. ACGIH: *Threshold limit values for chemical substances and physical agents and biological exposure indices for 2008*. American Conference of Governmental Industrial Hygienists. Cincinnati, OH: 2008
2. Barbieri A, Violante FS, Sabatini L, et al: Urinary biomarkers and low-level environmental benzene concentration: assessing occupational and general exposure. Chemosphere 2008; *74*: 64-69
3. Campo L, Cattaneo A, Consonni D, et al: Urinary methyl tert-butyl ether and benzene as biomarkers of exposure to urban traffic. Environ Int 2011; *37*: 404-411
4. Carugno M, Pesatori AC, Dioni L, et al: Increased mitochondrial DNA copy number in occupations associated with low-dose benzene exposure. Environ Health Perspect 2012; *120*: 210-215
5. Cocco P, 'T Mannetje A, Fadda D, et al: Occupational exposure to solvents and risk of lymphoma subtypes: results from the Epilymph case-control study. Occup Environ Med 2010; *67*: 341-347
6. Cocco P, Tocco MG, Ibba A, et al: Trans,trans-Muconic acid excretion in relation to environmental exposure to benzene. Int Arch Occup Environ Health 2003; *76*: 456-460
7. Cogliano VJ, Baan R, Straif K: Updating IARC's carcinogenicity assessment of benzene. Am J Ind Med 2011; *54*: 165-167
8. De Palma G, Poli D, Manini P, Andreoli R, Mozzoni P, Apostoli P, Mutti A. Biomarkers of exposure to aromatic hydrocarbons and methyl tert-butyl ether in petrol station workers. Biomarkers 2012; *17*: 343-351
9. Ducos P, Gaudin R, Robert A, et al: Improvement in HPLC analysis of urinary trans,trans-muconic acid, a promising substitute for phenol in the assessment of benzene exposure. Int Arch Occup Environ Health 1990; *62*: 529-534
10. European Commission. Council Directive of 27 July 1976 on the approximation of the laws, regulations and administrative provisions of the Member States relating to restrictions on the marketing and use of certain dangerous substances and preparations (76/769/EEC). Brussels, Belgium: The European Commission, 1976. http://www.dehp-facts.com/upload/documents/webpage/document37.pdf. (last accessed 11 November 2011)
11. European Commission: Directive 2000/69/EC of the European Parliament and of the Council of 16 November relating to limit values for benzene and carbon monoxide in ambient air. Brussels, Belgium: The European Commission, 2000. http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CELEX:32000L0069:en (last accessed 11 November 2011)

12. European Commission: Directive 2004/37/EC of the European Parliament and of the Council of 29 April 2004 on the protection of workers from the risks related to exposure to carcinogens or mutagens at work (Sixth individual Directive within the meaning of Article 16(1) of Council Directive 89/391/EEC). Brussels, Belgium: European Commission, 2004. Available at: http://eur-lex.europa.eu/LexUriServ/LexUri Serv.do?uri=OJ:L:2004:229: 0023: 0034:EN:PDF (last accessed 12 June 2010)
13. European Commission: Regulation (EC) No 1272/2008 of the European Parliament and the Council of 16 December 2008 on classification, labelling and packaging of substances and mixtures, amending and repealing Directives 67/548/EEC and 1999/45/EC, and amending Regulation (EC) No 1907/2006. Brussels, Belgium: The European Commission, 2008. http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2008:353:0001: 1355:EN:PDF (last accessed 11 November 2011)
14. Fustinoni S, Campo L, Mercadante R, et al: A quantitative approach to evaluate urinary benzene and S-phenilmercapturic acid as biomarkers of low benzene exposure. Biomarkers 2011; *16*: 334-345
15. Fustinoni S, Campo L, Mercadante R, Manini P: Methodological issues in the biological moniotring of urinary benzene and S-phenilmercapturic acid at low exposure levels. J Chromatography 2010; *878*: 2534-2540
16. Fustinoni S, Campo L, Satta G, et al: Environmental and lifestyle factors affect benzene uptake biomonitoring of residents near a petrochemical plant. Environ Int 2012; *39*: 2-7
17. Fustinoni S, Consonni D, Campo L, etal. Monitoring low benzene exposure: comparative evaluation of urinary biomarkers, influence of cigarette smoking, and genetic polymorphisms. Cancer Epidemiol Biomark Prev 2005; *14*: 2237-2244
18. Fustinoni S, Giampiccolo R, Pulvirenti S, et al: Headspace solid-phase microextraction for the determination of benzene, toluene, ethylbenzene and xylenes in urine. Journal of Chromatography B 1999; *723*: 105-115
19. Hopf NB, Kirkeleit J, Brâtveit M, et al. Evaluation of exposure biomarkers in offshore workers exposed to low benzene and toluene concentrations. Int Arch Occup Environ Health 2012; *85*: 261-271
20. International Agency for Research on Cancer: *A review of human carcinogens: chemical agents and related occupations.* Lyon: IARC, 2012 (IARC monographs on the evaluation of the carcinogenic risk of chemicals to humans no 100F)
21. International Agency for Research on Cancer: *Some anti-thyroid and related substances, nitrofurans and industrial chemicals.* Lyon: IARC, 1986 (IARC monographs on the evaluation of the carcinogenic risk of chemicals to humans no 7)
22. International Agency for Research on Cancer: *Some Industrial chemicals and dyestuffs.* Lyon: IARC, 1982 (IARC monographs on the evaluation of the carcinogenic risk of chemicals to humans no 29)
23. Kroll MH, Chesler R, Hagenbruger C, et al. Automated determination of urinary creatinine without sample dilution: theory and practice. ClinChem 1986; *32*: 446-452
24. Lan Q, Zhang L, Li G, et al: Hematotoxicity in workers exposed to low levels of benzene. Science 2004; *306*: 1774-1776
25. Lovreglio P, Barbieri A, Carrieri M, et al: Validity of new biomarkers of internal dose for use in the biological monitoring of occupational and environmental exposure to low concentrations of benzene and toluene. Int Arch Occup Environ Health 2010; *83*: 341-356
26. Lovreglio P, Barbieri A, Carrieri M, et al: Lesser validity of urinary benzene than S-phenylmercapturic acid for measuring occupational and environmental exposure to very low concentrations of benzene. G Ital Med Lav Ergon 2011; *33*: 117-124
27. Lovreglio P, Carrieri M, Barbieri A, et al: Applicability of urinary benzene to biological monitoring of occupational and environmental exposure to very low benzene concentrations. G Ital Med Lav Ergon 2011; *33*: 41-46
28. Lovreglio P, D'Errico MN, Fustinoni S, et al: Biomarkers of internal dose for the assessment of environmental exposure to benzene. J Environ Monit 2011; *13*: 2921-2928
29. Manini P, De Palma G, Andreoli R, et al: Biological monitoring of low benzene exposure in Italian traffic policemen. Toxicol Lett 2008; *181*: 25-30
30. Parlamento Italiano. Legge 4 novembre 1997, n. 413. Misure urgenti per la prevenzione dell'inquinamento atmosferico da benzene. Gazzetta Ufficiale N. 292. http://www.parlamento.it/parlam/ leggi/97413l.htm, 1997 (last accessed 11 November 2011)
31. Perry R, Gee IL: Vehicle emissions and effects on air quality: indoors and outdoors. Indoor Environ 1994; *3*: 224-236
32. Pesatori AC, Garte S, Popov T, et al: Early effects of low benzene exposure on blood cell counts in Bulgarian petrochemical workers. Med Lav 2009; *100*: 83-90
33. Pezzagno G, Maestri L, Fiorentino ML: Trans,trans-Muconic acid, a biological indicator to low levels of environmental benzene: some aspects of its specificity. Am J Ind Med 1999; *35*: 511-518
34. Satoa, Nakajima T, Fujiwara Y, Hirosawa K: Pharmacokinetics of benzene and toluene. Int Arch Arbeitsmed 1974; *33*: 169-182

35. Scibetta L, Campo L, Mercadante R, et al: Determination of low level methyl tert-butyl ether, ethyl tert-butyl ether and methyl tert-amyl ether in human urine by HS-SPME gas chromatography/mass spectrometry. Anal Chim Acta 2007; *581*: 53-62
36. Smith MT: Advances in understanding benzene health effects and susceptibility. Annu Rev Public Health 2010; *31*: 133-148
37. Steffen C, Auclerc MF, Auvrignon A, et al: Acute childhood leukaemia and environmental exposure to potential sources of benzene and other hydrocarbons; a case-control study. Occup Environ Med 2004; *61*: 773-778
38. Yang Jee Kim, Jun Yeol Choi, Yoon Hee Cho, et al: Micronucleus-centromere assay in workers occupationally exposed to low level of benzene. Hum Exp Toxicol 2010; *29*: 343-350
39. Zhang L, Rothman N, Li G, et al: Aberrations in chromosomes associated with lymphoma and therapy-related leukemia in benzene-exposed workers. Environ Mol Mutagen 2007; *48*: 467-474

ACKNOWLEDGMENTS: *The present work was partially funded by the PRIN COFIN project N 2003065175. The sponsor had no role in the study design, data analysis and preparation of the manuscript*