La Medicina del Lavoro

Med Lav 2012; *103*, 5: 324-337

Association between environmental exposure to benzene and oxidative damage to nucleic acids in children

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Key words

Benzene exposure; *S*-phenylmercapturic acid; nucleic acid oxidation

Parole chiave

Esposizione a benzene; acido *S*-fenilmercapturico; danno ossidativo agli acidi nucleici

Summary

Objectives: *To evaluate the association between environmental exposure to benzene and oxidative damage to nucleic acids in children, also considering the role of Environmental Tobacco Smoke (ETS).* **Methods:** *396 children living in central Italy were recruited in districts with different urbanization and air pollution. All biomarkers were determined in spot urine samples by mass spectrometric techniques to assess exposure [benzene (U-Benz), and its metabolites (t,t-muconic and S-phenylmercapturic acids, t,t-MA and S-PMA, respectively), cotinine] and nucleic acid oxidation [8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodGuo), 8-oxo-7,8-dihydroguanosine (8-oxoGuo), and 8-oxo-7,8-dihydroguanine (8-oxoGua)].* **Results:** *Biomarkers of exposure and nucleic acid oxidation increased with urbanization and were correlated with each other ($r>0.18$, $p<0.005$). In a multiple linear regression model, benzene exposure, assessed by S-PMA and t,t-MA, was associated ($p<0.0001$) with both 8-oxodGuo ($R^2=0.392$) and 8-oxoGuo ($R^2=0.193$) in all areas of residence, with similar slopes.* **Conclusions:** *(i) Biomarkers of exposure to benzene increased as a function of environmental air pollution and urbanization level; (ii) U-Benz clearly distinguished both exposure to ETS and areas of residence, whereas benzene metabolites were associated only with the latter; (iii) the variance of 8-oxodGuo and 8-oxoGuo was accounted for by environmental benzene exposure, thus suggesting that benzene is a good tracer of other components of complex mixtures of pollutants causing oxidative damage to nucleic acids.*

Riassunto

«Associazione tra esposizione ambientale a benzene e danno ossidativo al DNA nei bambini». **Obiettivi:** *Valutare l'associazione tra esposizione a benzene ambientale e danno ossidativo agli acidi nucleici in un gruppo di bam-*

Pervenuto il 11.4.2012 - Accettato il 27.6.2012

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bini, considerando anche il ruolo dell'esposizione passiva a fumo di tabacco (ETS). **Metodi:** *Sono stati reclutati 396 bambini, residenti in tre diverse zone dell'Italia centrale, distinte per grado di urbanizzazione ed inquinamento ambientale. Tutti gli indicatori di esposizione [Benzene urinario (U-Benz), acido t,t-muconico (t,t-MA), acido S-fenilmercapturico (S-PMA) e cotinina] e di danno ossidativo [8-idrossi-2'-deossiguanosina (8-oxodGuo), 8-idrossiguanosina (8-oxoGuo) e 8-idrossiguanina (8-oxoGua)] sono stati determinati mediante l'utilizzo di tecniche cromatografiche accoppiate alla spettrometria di massa, in campioni estemporanei di urina.* **Risultati:** *Gli indicatori di esposizione a benzene e di danno agli acidi nucleici sono fra loro correlati (r>0,18, p<0,005) e le loro concentrazioni aumentano in funzione del livello di urbanizzazione. In un modello di regressione lineare multipla, l'esposizione a benzene, valutata attraverso i metaboliti t,t-MA e S-PMA, è associata al danno ossidativo (p<0,0001), in particolare con 8-oxodGuo (R^2=0,392) e con 8-oxoGuo (R^2=0,193).* **Conclusioni:** *(i) Le concentrazioni degli indicatori di esposizione sono positivamente correlate con i livelli di inquinamento e di urbanizzazione; (ii) le concentrazioni di U-Benz sono significativamente influenzate sia da ETS che dal livello di urbanizzazione, mentre i metaboliti del benzene sono correlati solo a quest'ultimo; (iii) il danno ossidativo, in particolare 8-oxodGuo e 8-oxoGuo, è associato all'esposizione a benzene ambientale, suggerendo che quest'ultimo è un buon tracciante dell'inquinamento ambientale.*

Introduction

Benzene is a human carcinogen (25) causing haematotoxicity at occupational levels below 1 ppm (27, 43). As a component of fuel, benzene is associated with traffic emission and it is found in urban air. In Italy, the average benzene content in 95 Research Octane Number (RON) petrol is 0.7% (min-max, 0.29-0.96%) (17). Moreover, benzene is a major component of tobacco smoke (12). In Italy, the objective for air quality, as an average annual limit of benzene, was 5 $\mu g/m^3$ (18), and environmental benzene values obtained both from fixed monitoring stations (ARPA, data not shown) and from personal passive samplers on unexposed subjects (30) were found to be slightly lower than this limit. In the case of occupationally exposed subjects, like policemen, bus or taxi drivers and petrochemical workers, environmental background benzene concentrations were found to be in the 6-25 $\mu g/m^3$ range during the work shift, obtained from passive personal sampling devices (10, 22, 31, 32). Because the air benzene levels in occupational exposure are slightly higher than the environmental levels, the identification of suitable specific and sensitive exposure surrogates, i.e. biomarkers of exposure, is important (8, 23) for the characterization of exposure to low levels of benzene as well as for the assessment of health risks posed by this exposure.

After absorption, benzene is oxidized by cytochrome *P*-450 enzymes (*CYP2E1*) to a reactive intermediate benzene oxide-oxepin. This highly reactive species is further metabolized to various metabolites, including *trans,trans*-muconic (*t,t*-MA) and *S*-phenyl mercapturic acid (*S*-PMA) that are currently used as biomarkers of benzene exposure in occupational settings. Recent Italian studies on workers exposed to low benzene levels (such as traffic policemen, bus and taxi drivers, petrochemical plant workers) (32, 33) pointed out that an effective biomonitoring requires the use of more than one of the most sensitive and reliable benzene biomarkers, i.e. urinary benzene itself (U-Benz), *t,t*-MA and *S*-PMA (42).

It has been shown that metabolism of benzene is associated with the generation of reactive oxygen species that induce oxidative DNA damage (5). Oxidative damage to DNA may be important in benzene-induced carcinogenesis since DNA base lesions such as 8-hydroxy-2'-deoxyguanosine (8-oxodGuo) are highly mutagenic (28). 8-OxodGuo, one of the major oxidative DNA base products, is not a specific marker for benzene exposure but a general biomarker for oxidative damage to DNA. Most 8-oxodGuo is removed by the DNA repairing enzyme system (41). Recently, it has been demonstrated that other extracellular oxidized guanine derivatives, such as 8-oxo-7,8-dihydroguano-

sine (8-oxoGuo), and 8-oxo-7,8-dihydroguanine (8-oxoGua) together with 8-oxodGuo itself, can be detected in urine samples (2, 50). These biomarkers arise from different repair pathways and/or turnover of nucleic acids, e.g., 8-oxodGuo may reflect either the repair of oxidized 2'-deoxyguanosine triphosphate in the cellular 2'-deoxyribonucleotide pool by MTH1 (47) or the repair of 8-oxodGuo from DNA by an endonuclease/nucleotidase based DNA repair system (7) or even repair by a nucleotide excision repair (NER) system; 8-oxoGuo may originate from oxidized guanine in RNA, as a result of its turnover or repair, even though RNA repair mechanisms have not yet been well characterized (34); 8-oxoGua originates, at least in part, from the activity of specific glycosylases of the base excision repair (BER) system on oxidized guanine residues of DNA (11) or from turnover or repair of RNA (19).

It has been reported that exposure to urban pollutants in young children and adolescents is associated with increased urinary levels of 8-oxodGuo (9, 45). Children are particularly sensitive to the effects of air pollution, for various reasons, including: incomplete metabolic systems, immature host defences, high rates of infection by respiratory pathogens, and activity patterns (15). Thus, it is of relevant concern to investigate the association between environmental exposure to benzene and oxidative damage to nucleic acids, however there is still a gap in this research area, also in Italy.

We recently characterized benzene exposure in children living in two different urbanized areas of central Italy (Rural Area and Town) by applying analytical techniques based on mass spectrometry (40). We observed that U-Benz, *t,t*-MA and *S*-PMA were sensitive enough to characterize the areas of residence with different urbanization and air pollutant levels. Furthermore, a significant correlation between U-Benz and urinary cotinine, as markers of recent exposure to active and passive smoking, was found (38). The present study, performed on children exposed to different environmental benzene levels, was planned with the following aims: (i) to evaluate the relationships between biomarkers of benzene exposure and of nucleic acid oxidation; (ii) to assess the role of Environmental Tobacco Smoke (ETS) on the excretion profile of the same biomarkers.

SUBJECTS AND METHODS

Study design and Subjects

The research was conducted in three primary-school districts located respectively in the northern, central, and southern areas of the Lazio Region (central Italy), comprising a total of 665 children and whose urbanization characteristics allowed us to classify them as Rural, Town or City (metropolitan area). The classification of the three areas is reported in Table 1 and is mainly based on urbanization characteristics (i.e., population density, green area density and motorization rate) obtained from national databases (i.e., National Institute of Statistics, Italian Automobile Club).

We collected detailed information by a self-administered questionnaire filled in by parents of participants. The following topics were investigated: socio-demographic characteristics, daily activities, living conditions and cohabitant smoking habits. The study protocol was approved by the local Ethical Committee and all subjects participated after written, informed consent from the parents: 501 out of 665 children participated in the study, which a response rate of 75%. However, 46 urine samples were rejected because of unsatisfactory sealing of sample containers; therefore, analytical determinations of benzene metabolites, cotinine and markers of nucleic acid oxidation were performed on 455 urinary samples. In addition, we excluded 59 children who had at least one parent who was not Italian from the data analysis to avoid interference due to well-known language comprehension problems, and ethnic differences in the metabolism and excretion of benzene and cotinine (6, 36, 48), hence the analysis was performed on 396 children.

Biological monitoring

One urine sample for each participant was collected in the evening (just before bedtime) and stored in the refrigerator at 4°C; the next morning, the sample was placed into a polystyrene cooler

Table 1 - Distributions of relevant urbanization indicators of the three study areas in the monitoring campaign years (2007-2009) and demographic characteristics of the study group, summary of information collected by questionnaires and geometric means [geometric standard deviations] of urinary cotinine divided according to smoking cohabitants. Concentrations are expressed as μg/g creatinine ($\mu g/g_{creat}$)

Environmental variables	Rural Area	Town	City
Resident population (No.)	3308	32,886	2,743,796
Population density (persons per km^2)	120	395	2,098
Green area density (% of total municipal territory)	>85	<85	<85
Motorization rate			
Number of motorvehicles per 100 inhabitants	66	76	69
Number of two- wheeled vehicles per 100 inhabitants	8	10	15
Characteristics of subjects			
No. of subjects	97	87	212
Age (years, mean ± SD)	8.1±1.5	8.1±1.3	8.0±1.4
Gender (male/female)	53/44	44/43	109/103
No. of smoking cohabitants (%)			
0	43 (44%)	66 (76%)	105 (50%)
1	34 (35%)	15 (17%)	68 (32%)
2 or more	20 (21%)	6 (7%)	39 (18%)
Cotinine ($\mu g/g_{creat}$)			
0	2.15 [1.84]	2.07 [1.99]	1.62 [1.65]§,†
1	3.89 [2.16]*	4.13 [1.99]*	3.36 [2.59]#
2 or more	5.41 [2.19]#	7.19 [2.72]#	5.07 [2.52]#

Legend: 0, absence of smoking cohabitants; 1, only one smoking cohabitant; 2, two or more smoking cohabitants.
#p<0.0001, * p<0.005, 0 *vs* 1 and 0 *vs* 2; only for Group 0: §p<0.01, City *vs* Rural Area; †p<0.03 City *vs* Town;
One-way ANOVA followed by Bonferroni post-hoc test for urinary cotinine levels.

containing an ice pack and was delivered to the research team. Then, spot urine samples were divided into several aliquots and frozen at -20°C until analyses of benzene metabolites, urinary cotinine and biomarkers of nucleic acid oxidation. All determinations were performed by chromatographic techniques coupled with mass spectrometry and analytical procedures that have been described in detail in other studies (31, 49). Briefly, U-Benz was determined by headspace solid-phase microextraction followed by gas-chromatography-mass spectrometry (GC-MS) according to procedures outlined in Vitali et al. (49); the limit of detection (LOD), calculated as the signal to noise ratio (S/N) > 3, was 8 ng/l and the coefficient of variation of the method (%CV) was below 9.8% for all intra- and inter-day determinations. All other urinary determinations were performed by isotopic dilution liquid chromatography *tandem* mass spectrometry (LC-MS-MS) using a AB SCIEX API 4000 triple-quadrupole mass spectrometer (AB SCIEX, Framingham, MA, USA) equipped with a TurboIonspray interface for pneumatically assisted electrospray (ESI) as previously described (3, 31). The LODs of the benzene metabolites were 0.1 μg/L for S-PMA and 2.5 μg/L for t,t-MA. The LODs of other analytes were 0.03, 0.09, 0.2 and 0.5 μg/L for 8-oxodGuo, 8-oxoGuo, cotinine and 8-oxoGua, respectively. The %CV ranged between 1.3% and 6.8% for all analytes and for all intra- and inter-day determinations. Concerning the analysis of biomarkers of oxidative DNA damage, the laboratory in which the determinations were performed is participating in an inter-laboratory project which includes quality control to assess urinary concentration of 8-oxodGuo, organized by European Standards Committee on Urinary (DNA) Lesion Analysis (20). Concentrations of urinary metabo-

lites, but not U-Benz, were expressed as a function of creatinine concentration (μg/g creat.), which was measured by the Jaffe method (26).

Statistics

Statistical analyses were carried out by the SPSS software (version 18.0 for Windows, Chicago, IL). All analytical measurements were above the corresponding detection limit. The normality and log-normality of the distributions were assessed by the one-sample Kolmogorov-Smirnov test, and all the measured variables followed a log-normal distribution. Differences between groups were assessed using the Student *t* test for independent samples and ANOVA followed by the Bonferroni *post-hoc* test for multiple comparisons. A chi-square test was performed when categorical data were compared and, in the case of multiple comparisons, a Bonferroni correction was applied. Pearson's *r* was used to assess correlation between variables on either linear or log-scale depending on the normality of the residuals. Multiple linear regression analysis models were used to assess the contribution of age, urinary creatinine concentration and exposure to benzene (either as *S*-PMA, *t,t*-MA and U-Benz concentrations) to the variability of biomarkers of nucleic acid oxidation.

Results

Exposure to environmental tobacco smoke

All children were considered to be exposed to ETS if they lived in households where at least one person was a smoker. As reported in table 1, the number of children who lived without any smoker cohabitants was significantly higher in Town (76%) compared to Rural Area (44%) and City (50%) ($p<0.001$ in both cases). Urinary cotinine was dependent on the number of smoking cohabitants in all the areas (table 1).

Biological monitoring

Table 2 summarizes the distribution of biomarkers of exposure and nucleic acid oxidation in children classified according to residential area (Rural Area, Town and City) and further classified for ETS exposure (NETS, unexposed to Environmental Tobacco Smoke; EETS, exposed to Environmental Tobacco Smoke). Biomarker levels are expressed as geometric means [and geometric standard deviation] (GM [GSD]). Significantly different concentrations of all benzene biomarkers (U-Benz, *S*-PMA and *t,t*-MA) were detected in children living in areas characterized by different levels of urbanization with the exception of *t,t*-MA in City *vs* Town (one-way ANOVA followed by Bonferroni post-hoc test, $p \leq 0.002$). In particular, children living in the City excreted higher concentrations of benzene biomarkers than those living in Town (U-Benz 372 *vs* 264 ng/l, $p<0.0001$, and S-PMA 0.92 *vs* 0.27 μg/g $_{creat}$, respectively) and in the Rural Area (U-Benz 372 *vs* 185 ng/l, 0.92 *vs* 0.20 μg/g $_{creat}$ and *t,t*-MA 114 *vs* 60.5 μg/g $_{creat}$, respectively). Again, U-Benz, *S*-PMA and *t,t*-MA concentrations were higher in children living in Town than those living in a Rural Area (U-Benz 264 *vs* 185 ng/l, 0.27 *vs* 0.20 μg/g $_{creat}$ and *t,t*-MA 112 *vs* 60.5 μg/g $_{creat}$, respectively). After stratification for absence (NETS) or presence (EETS) of smoking cohabitants, the same differences were observed for *S*-PMA and *t,t*-MA, in both groups ($p<0.001$) but not for U-Benz, for which increasing level was seen only in NETS one (table 2).

Higher concentrations of 8-oxodGuo and 8-oxoGua were detected in children living in the City as compared to those living in Town (8-oxodGuo 6.69 *vs* 4.27 μg/g_{creat} and 8-oxoGua 21.8 *vs* 14.5 μg/g_{creat}, respectively) and in Rural Area (8-oxodGuo 6.69 *vs* 3.55 μg/g_{creat} but not 8-oxoGua 21.8 *vs* 18.4 μg/g_{creat}, respectively), whereas higher levels of 8-oxoGuo were observed in children living in Town (9.85 μg/g_{creat}) and in the City (9.56 μg/g_{creat}) compared to those living in a Rural area (8.33 μg/g_{creat}). Considering exposure to passive smoking, the differences between areas of residence for benzene metabolites were confirmed in NETS group. For ETS, only 8-oxodGuo concentrations significantly increased, going from Rural Area (3.53 μg/g_{creat}) to Town (4.34 μg/g_{creat}) and to City (6.44 μg/g_{creat}). Considering only the different urbanization levels, urinary cotinine concentration was in-

Table 2 - Distribution of biomarkers of exposure, nucleic acid oxidation and cotinine in subgroups of children dividied according to residential area and exposure to passive smoking (NETS= unexposed to environmental tobacco smoke; EETS= exposed to environmental tobacco smoke). Values are expressed as geometric mean and geometric standard deviation. Concentrations are expressed as μg/g creat, but not U-Benz (ng/l)

	Rural			Town			City			*p*[a]		
Biomarker	Overall	NETS	EETS	Overall	NETS	EETS	Overall	NETS	EETS	Overall	NETS	EETS
U-Benz	185 [2.54]	**91.0** [1.86]	**380*** [1.91]	264 [2.35]	**216** [2.03]	**516*** [2.64]	372 [1.83]	**290** [1.49]	**468*** [1.96]	<0.001	<0.001	ns
S-PMA	0.20 [1.60]	**0.18** [1.67]	**0.23*** [1.54]	0.27 [1.68]	0.26 [1.66]	0.30 [1.75]	0.92 [1.69]	**0.98** [1.78]	**0.86*** [1.60]	<0.001	<0.001	<0.001
t,t-MA	60.5 [1.83]	63.1 [2.07]	59.5 [1.70]	112 [1.76]	109 [1.68]	126 [2.02]	114 [2.06]	112 [1.97]	113 [2.18]	<0.001	<0.001	<0.001
8-oxodGuo	3.55 [1.35]	3.57 [1.36]	3.53 [1.35]	4.27 [1.39]	4.26 [1.33]	4.34 [1.57]	6.69 [1.50]	6.84 [1.50]	6.44 [1.50]	<0.001	<0.001	<0.001
8-oxoGuo	8.33 [1.36]	8.14 [1.37]	8.49 [1.37]	9.85 [1.46]	9.88 [1.40]	9.83 [1.65]	9.56 [1.54]	9.96 [1.50]	9.14 [1.48]	0.002	0.007	ns
8-oxoGua	18.4 [2.16]	17.6 [2.21]	20.3 [2.11]	14.5 [2.14]	14.0 [1.99]	15.8 [2.70]	21.8 [2.21]	23.6 [2.27]	20.0 [2.16]	<0.001	<0.001	ns
Cotinine	3.20 [2.21]	**2.15** [1.84]	**4.30*** [2.20]	2.53 [2.22]	**2.07** [1.99]	**4.69*** [2.25]	2.53 [2.42]	**1.62** [1.65]	**3.81*** [2.59]	0.05	0.002	ns

Legend: U-Benz, Benzene; *S*-PMA, *S*-phenylmercapturic acid; *t,t*-MA, *trans,trans*-muconic acid; 8-oxodGuo, 8-oxo-7,8-dihydro-2'-deoxyguanosine; 8-oxoGuo, 8-oxo-7,8-dihydroguanosine; 8-oxoGua, 8-oxo-7,8-dihydroguanine.

[a] One-way ANOVA with Bonferroni post-hoc test. *$p < 0.05$, NETS *vs* EETS, *t*-student test for independent samples in subgroups of children divided according to their residential area.

dependent of the residential areas. After stratification for exposure to passive smoking, in NETS, higher concentrations of urinary cotinine were observed in children living in Rural Area (2.15 $\mu g/g_{creat}$) compared to those living in Town (2.07 $\mu g/g_{creat}$) or in a City (1.62 $\mu g/g_{creat}$). Considering the exposure to passive smoking, in the whole group EETS excreted significantly higher amounts of U-Benz and of cotinine than NETS (452 [2.03] *vs* 217 [1.90] ng/l for U-Benz and 4.00 [2.43] *vs* 1.88 [1.81] $\mu g/g_{creat}$ for cotinine, $p<0.0001$). The differences were confirmed in subgroups of children classified according to residential areas (Rural Area: U-Benz 380 *vs* 91.0 ng/l and cotinine 4.30 *vs* 2.26 $\mu g/g_{creat}$, Town 516 *vs* 216 ng/l and 4.69 *vs* 2.07 $\mu g/g_{creat}$, City 468 *vs* 290 ng/l and 3.81 *vs* 1.63 $\mu g/g_{creat}$).

In the whole study group, significant correlations were observed between biomarkers of nucleic oxidation ($0.23<r<0.63$, $p<0.05$) and between biomarkers of exposure and biomarkers of nucleic acid oxidation ($0.18<r<0.63$, $p<0.05$), as reported in table 3. Cotinine showed significant correlations with U-Benz (r=0.574, $p<0.01$), *t,t*-MA (r=0.125, $p<0.05$), 8-oxoGuo (r=0.132, $p<0.05$) and creatinine (r=0.148, $p<0.01$). These correlations were maintained when children were stratified according to exposure to either passive smoking or the residential areas (data not shown). In a multiple linear regression model, benzene exposure assessed by *t,t*-MA and *S*-PMA, was associated ($p<0.0001$) with both 8-oxodGuo ($R^2=0.392$) and 8-oxoGuo ($R^2=0.193$) in all residential areas, with similar slopes. In detail, figure 1 shows the correlations be-

Table 3 - Pearson's correlation coefficients between urinary biomarkers in children (No.=396)

	S-PMA	*t,t*-MA	Cotinine	8-oxodGuo	8-oxoGuo	8-oxoGua	Creatinine
U-Benz	0.345**	0.217**	0.574**	0.284**	0.203**	0.051	0.171**
S-PMA		0.414**	0.020	0.629**	0.373**	0.223**	0.310**
t,t-MA			0.125*	0.488**	0.504**	0.182*	0.499**
Cotinine				0.072	0.132*	-0.053	0.148**
8-oxodGuo					0.629**	0.228*	0.647**
8-oxoGuo						0.309**	0.713**
8-oxoGua							0.133**

Legend: U-Benz, Benzene; *S*-PMA, *S*-phenylmercapturic acid; *t,t*-MA, *trans,trans*-muconic acid; 8-oxodGuo, 8-oxo-7,8-dihydro-2'-deoxyguanosine; 8-oxoGuo, 8-oxo-7,8-dihydroguanosine; 8-oxoGua, 8-oxo-7,8-dihydroguanine.
** $p < 0.01$, * $p < 0.05$, Pearson's correlation, two-tailed.

tween: (A,C,E) *S*-PMA and 8-oxodGuo, and (B,D,F) *S*-PMA and 8-oxoGuo in samples of children according to residential areas (Rural Area A,B; Town, C,D; City E,F).

Multiple regression models were run to assess the role of benzene exposure (either as *S*-PMA, *t,t*-MA or U-Benz) and other predictors (age, and creatinine) on urinary biomarkers of nucleic acid oxidation. In the multiple regression models, all the biomarkers were insert expressed as concentration (μg/l for all but not U-Benz, ng/l) and not as a function of creatinine. Exposure to passive smoking, both as urinary cotinine and smoking cohabitant's habits, was removed from the models after verifying that it was not statistically significant. The results of stepwise models are summarized in table 4, where partial r^2 values are reported to evaluate the individual contribution of each predictor to the overall variance. The models were highly significant for 8-oxodGuo and 8-oxoGuo (adjusted r^2 ranging from 0.455 to 0.623 using either *S*-PMA (Model 1), *t,t*-MA (Model 2) or U-Benz (Model 3) as exposure index. Although significant, the adjusted r^2 of the models 1 and 2 for 8-oxoGua were lower, i.e. 0.015-0.047. Creatinine was the most important predictor, accounting for about 42% and 51% of the variance of 8-oxodGuo and 8-oxoGuo, respectively (p<0.0001 for both). Significant relationships between oxidatively modified guanine derivatives and three exposure biomarkers were observed for 8-oxodGuo and 8-oxoGuo (p<0.0001), exposure accounting for 3-20% and 1-3% of variance, respectively. For 8-oxoGua, a significant contribution of exposure was observed only for *S*-PMA and *t,t*-MA (0.5% and 0.3% of the variance, respectively, p<0.0001). We observed a negative significant correlation between age and oxidative nucleic damage for 8-oxodGuo (p<0.032) and 8-oxoGuo (p<0.028) in all the examined Models, accounting for 1% and 2-3% of variance, respectively. In the whole group, passive smoking, evaluated both as urinary cotinine and by questionnaire did not significantly affect the concentration of oxidatively modified guanine derivatives.

Discussion

There is a growing body of evidence linking serious health consequences to exposure to environmental air pollutants towards which young people display high vulnerability, therefore child protection is a Public Health challenge. To the best of our knowledge, this is the first study investigating the association between exposure to environmental benzene and oxidative damage to nucleic acids in Italian children. To fulfill this purpose, we used a biomonitoring approach, which is simple, non-invasive and therefore applicable in field studies involving children. Urinary biomarkers of exposure to benzene (U-Benz, *S*-PMA and *t,t*-MA) reflect the whole absorbed dose and take into account the uptake of benzene from different sources, including environmental pollution and ETS. These biomarkers are good exposure surrogates which allow exposure reconstruction and may thus replace airborne sampling (14) that would involve practical problems, considering the high number of subjects par-

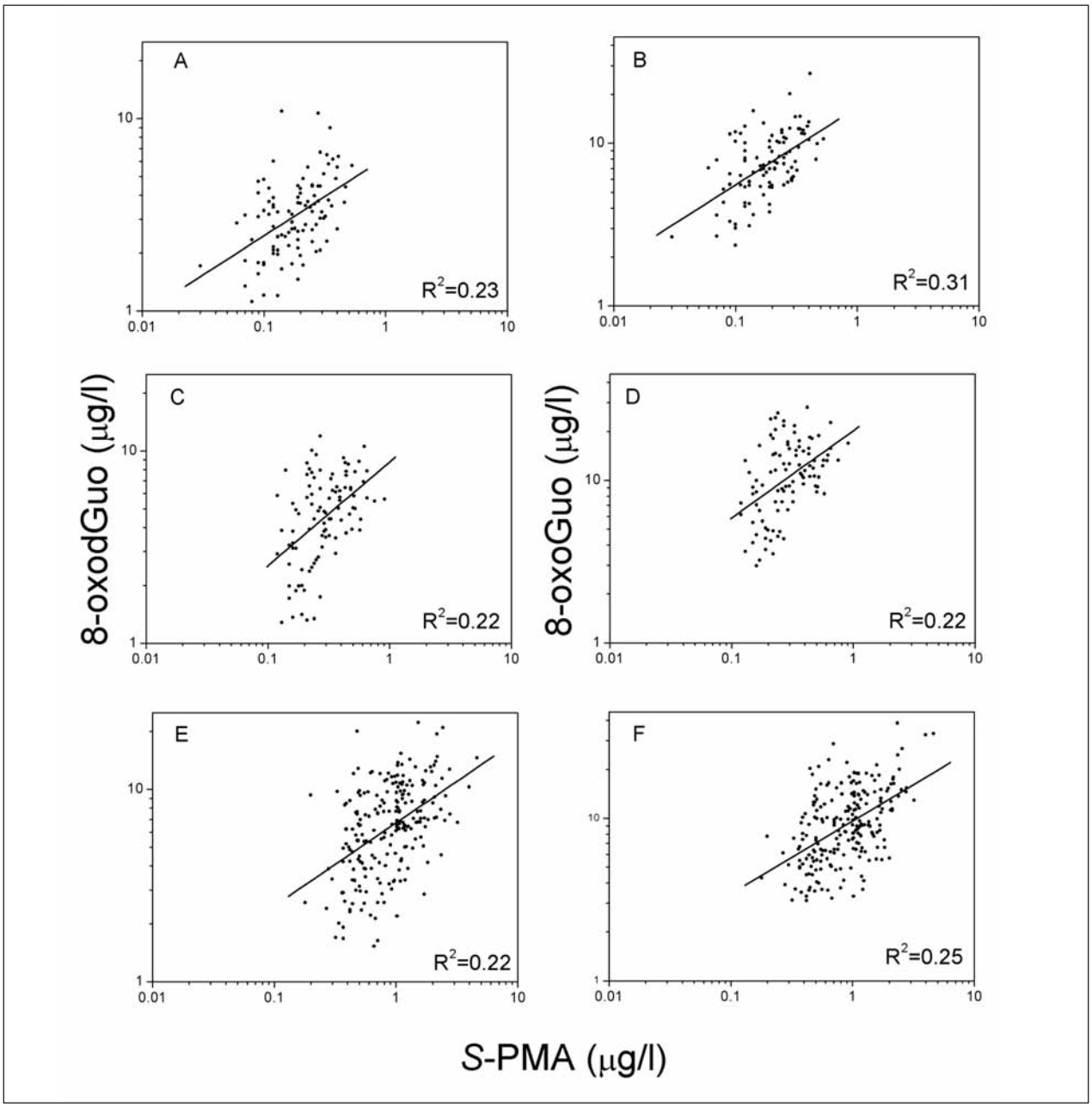

Figure 1 - Relationship between urinary concentrations of *S*-phenylmercapturic acid (*S*-PMA) and 8-oxo-7,8-dihydroguanosine [8-oxodGuo, (A, C, E)] and 8-oxoguanosine [8-oxoGuo, (B, D, F)] in children classified according to residential area [Rural Area (A, B), Town (C, D) and City (E, F), No.=396]

ticipating in the study, their young age and the difficulty in obtaining reliable samples. We developed LC-MS-MS methods allowing their determination with urinary cotinine (as biomarker of recent exposure to tobacco smoke) and urinary biomarkers of nucleic acid oxidation (8-oxodGuo, 8-oxoGuo and 8-oxoGua) in the same urine sample (2, 32).

In the case of primary school children, for whom smoking can be ruled out, ETS represents the most important confounding factor in the biological monitoring of exposure to benzene pollution in the

Table 4 - Predictors of urinary excretion of biomarkers of nucleic acid oxidation (taken as dependent variable) according to a stepwise multiple linear regression model: Log (U-biomarker) = constant + Log (U-creatinine) x β_1 + Log (*S*-PMA/*t,t*-MA/U-Benz) x β_3 + (Age) x β_4. Values of constant and standardized β coefficient (β_{stand}), partial r^2 (r^2_p) and significance (p) for each term are given. The adjusted r^2 (r^2_{adj}) and significance (p) for the whole model are reported in the last row. The significance level was 0.05 for entry and 0.10 for removal from the model

Model	8-oxodGuo			8-oxoGuo			8-oxoGua		
	β_{stand}	r^2_p	p	β_{stand}	r^2_p	p	β_{stand}	r^2_p	p
1. *S*-PMA									
Constant	0.859	-	<0.0001	1.078	-	<0.0001	1.319	-	<0.0001
Creatinine	0.516	0.420	<0.0001	0.690	0.508	<0.0001	-	-	-
S-PMA	0.467	0.200	<0.0001	0.160	0.023	<0.0001	0.223	0.050	<0.0001
Age	-0.074	0.005	0.018	-0.162	0.028	<0.0001	-	-	-
Whole model r^2_{adj}, p		0.623	<0.0001		0.555	<0.0001	-	0.047	<0.0001
2. *t,t*-MA									
Constant	0.458	-	<0.0001	0.814	-	<0.0001	0.945	-	<0.0001
Creatinine	0.557	0.420	<0.0001	0.647	0.508	<0.0001	-	-	-
tt-MA	0.209	0.035	<0.0001	0.183	0.029	<0.0001	0.182	0.033	<0.0001
Age	-0.081	0.006	0.032	-0.156	0.024	<0.0001	-	-	-
Whole model r^2_{adj}, p		0.456	<0.0001		0.557	<0.0001	-	0.031	<0.0001
3. U-Benz									
Constant	0.433		<0.0001	0.921		<0.0001	1.262	-	<0.0001
Creatinine	0.633	0.420	<0.0001	0.727	0.508	<0.0001	0.133	0.018	0.012
U-Benz	0.175	0.031	<0.0001	0.078	0.006	0.032	-	-	-
Age	-0.091	0.008	0.022	-0.167	0.028	<0.0001	-	-	-
Whole model r^2_{adj}, p		0.455	<0.0001		0.538	<0.0001	-	0.015	0.012

Legend: 8-oxodGuo, 8-oxo-7,8-dihydro-2'-deoxyguanosine; 8-oxoGuo, 8-oxo-7,8-dihydroguanosine; 8-oxoGua, 8-oxo-7,8-dihydroguanine; *S*-PMA, *S*-phenylmercapturic acid; *t,t*-MA, *trans,trans*-muconic acid; U-Benz, Benzene.

general environment. Urinary cotinine showed a significant increasing trend along with the number of smoking cohabitants (0, 1 or 2) (table 1) independently of the residential area. Despite differences in the percentage of smoking cohabitants in the three areas, urinary cotinine excretion in the two subgroups of children exposed to ETS (1 and 2 or more, respectively) was comparable in the three areas (EETS 1: 3.89, 4.13, 3.36 $\mu g/g_{creat}$ and EETS 2 or more: 5.41, 7.19 and 5.07 $\mu g/g_{creat}$ for Rural area, Town and City, respectively), thus ruling out a role of environmental air pollutants on cotinine excretion. This result further confirms the validity of urinary cotinine as a specific biomarker to assess exposure to ETS, when measured with highly sensitive and selective analytical techniques (39). Unexpectedly, in NETS group, children living in Rural areas excreted significantly higher concentrations of urinary cotinine (2.15 $\mu g/g_{creat}$) than children who lived in Town or City (2.07 $\mu g/g_{creat}$ and 1.62 $\mu g/g_{creat}$, respectively). We can speculate that the sensitivity of the analytical method allowed us to identify a background exposure to nicotine that could be alternative to inhalation of tobacco smoke. Nicotine was used as an insecticide in Italy until 2010 and is also a natural constituent of some vegetables (potatoes, eggplant and tomatoes) (13).

The results of the present study demonstrate an increasing trend in the concentrations of all benzene biomarkers in subgroups of children living in Rural area < Town < City, confirming that urban children were exposed to higher levels of benzene (table 2). In the NETS subgroup, this gradient was observed for all the biomarkers, whereas in the EETS subgroup it was evident only for *S*-PMA,

the concentrations of which were 3.41- and 4.60-fold higher in samples from children living in a City than from those living in Town or Rural Area, respectively; moreover, *S*-PMA excretion in children living in Town was significantly higher than in children from Rural Area. These differences were also observed when children were stratified according to ETS (3.77-fold and 5.44-fold for NETS and 2.87-fold and 3.70-fold for EETS). A similar trend was observed in the concentrations of *t,t*-MA and U-Benz, even though the differences were less pronounced. Children living in Town or City excreted comparable amounts of *t,t*-MA (112 and 114 $\mu g/g_{creat,}$ respectively), which were 2-fold higher than those observed for children living in Rural area (60.5 $\mu g/g_{creat}$). A significant trend in the concentrations of U-Benz was observed among children living in areas with increasing urbanization (185, 264 and 372 ng/l for Rural Area, Town and City, respectively). When subjects were stratified according to ETS, the same trend was observed in the NETS group (91, 216 and 290 ng/l for Rural area, Town and City, respectively), whereas it was no longer evident in the EETS group (380, 516 and 468 ng/l for Rural area, Town and City, respectively). This behaviour could be ascribed, at least in part, to kinetic reasons related to the short half-life of U-Benz and its sampling time (24). Considering that urine samples were collected in the evening (just before bedtime), U-Benz concentrations reflect exposure to benzene in the late afternoon and evening. For children belonging to the EETS group we may hypothesize the occurrence of an acute exposure to ETS in the domestic environment, caused by the smoking habits of cohabitants in the hours prior to sampling. Conversely, in subjects belonging to NETS subgroup U-Benz may reflect the background exposure to airborne benzene during the day, including the evening, which is associated with urban air pollution and depends on the residential area.

In previous reports, where only children living in the area with the lowest levels of environmental pollutants were considered, i.e., Rural Area and Town, all three biomarkers of benzene exposure, U-Benz, *S*-PMA and *t,t*-MA, were useful to assess exposure to environmental benzene (40). However, only U-Benz was correlated with the passive smoking exposure (38, 40). In the present study, a third group of children living in the City of Rome, characterized by higher levels of urbanization and traffic was considered. The enlargement of the study group confirmed the correlation between U-benz and exposure to passive smoking expressed both as urinary cotinine (Pearson coefficient r=0.574, $p<0.001$) and as self-reported cohabitants' smoking habits (39). As highlighted by Protano et al (38), U-Benz levels of EETS children living in rural areas with low traffic density but exposed to ETS may be higher than those of children living in urban areas but unexposed to ETS. This fact may reduce the health benefits of living far from traffic pollution and highlights the need to promote educational initiatives among parents, with the aim of increasing their awareness of the negative impact of ETS exposure during childhood and instruct them about correct behaviour to protect their children's health.

Oxidative damage to DNA may indicate an increased risk of cancer and has been associated with exposure to polluted urban air (29). The main pathway of benzene toxicity is thought to involve redox cycling of quinones which induce oxidative damage in particular to DNA in the bone marrow (5). To the best of our knowledge, this is the first time that a set of urinary biomarkers of nucleic acid oxidation – evaluated by a method based on isotopic dilution LC–MS–MS – was applied to characterize the extent of oxidative stress in Italian children exposed to environmental benzene. Different biomarkers may be generated from oxidation of guanine at the C8-position, depending on the localization of the guanine residue (DNA, RNA or the nucleotide pool) and the efficiency of the repair systems involved during exposure to oxidizing agents (37).

The present study confirmed 8-oxoGua as the biomarker of nucleic acid oxidation with the highest inter-individual variability, as already observed in adults (2). In addition, its concentration in children is about 2 to 5 times higher than those of 8-oxoGuo and 8-oxodGuo. In agreement with other Authors (35, 46), the urinary concentrations of 8-oxodGuo and, to our knowledge for the first time,

also of 8-oxoGuo, were inversely correlated with pediatric age. In our study group a similar trend was observed for 8-oxoGuo and 8-oxo-dGuo, but not for 8-oxoGua, confirming a higher sensitivity of young children to benzene exposure and a role for growth in the development of defences against environmental pollutants and passive smoking. The small airways diameter in pediatric age is a susceptibility factor towards inflammation produced by air pollution. Children breathe more air per unit of body weight than adults, and thus receive proportionately higher doses of pollutants (15).

The results of the present study show that the levels of urinary biomarkers of nucleic acid oxidation were higher in children living in urban polluted areas compared to those living in areas characterized by lower urbanization (table 2). Such differences were observed for all the three oxidized guanine derivatives in the whole group and, after stratification for exposure to passive smoking, in the NETS group. In the EETS subgroup a significant difference was evident only for 8-oxodGuo. Moreover, we observed a significant correlation between urinary benzene metabolites *S*-PMA and *t,t*- MA and biomarkers of DNA, RNA and nucleotide pool oxidation, 8-oxodGuo, 8-oxoGuo and 8-oxoGua. This is consistent with the results of several studies showing a positive association between exposure to environmental air pollutants and oxidative DNA damage in children (9, 46) and in adults (4, 21, 44). In particular, one of the studies conducted in the Czech Republic described significantly higher concentrations of 8-oxodGuo in children living in industrialized area compared to those living in agricultural areas (46), suggesting a higher health risk in these children.

The slopes of the regression line(s) between urinary *S*-PMA and the biomarker(s) of oxidative damage (figure 1) classified by residential areas, were similar and overlapped even if the range of benzene exposure, as S-PMA concentrations, was wide and significantly different in the three residential areas. This behaviour supports the role of benzene as a reliable marker for assessing general environmental pollution, particularly those oxidant substances that are typical of urban air (e.g. PM10).

Linear regression models applied to predictors of the variability of biomarkers of nucleic acid oxidation, e.g. urinary creatinine concentration, age, benzene exposure (as *S*-PMA in Model 1, *t,t*-MA in Model 2 and U-Benz in Model 3) confirmed that urinary creatinine is the most important predictor, accounting for about 42% and 51% of the variance of 8-oxodGuo, 8-oxoGuo, respectively, in all Models. Nevertheless, regression analysis showed that all three different Models of benzene exposure (Model 1: *S*-PMA, Model 2: *t,t*-MA and Model 3: U-Benz, respectively), in particular the one including *S*-PMA among independent variables, were significantly associated with oxidative damage to nucleic acids and explained a variable percentage of biomarker variance, in the range of 3-20% for 8-oxodGuo and 2-3% of 8-oxoGuo. A similar result was described in benzene-exposed workers, for whom exposure biomarkers accounted for about 3-10% of the variability of 8-oxodGuo and 8-oxoGuo (33). In addition, in children under investigation, we found a weak but significant association between *S*-PMA or *t,t*-MA and the most abundant urinary oxidized guanine derivative, 8-oxoGua, which was not observed in adults occupationally exposed to benzene (33). This was probably due to the absence of other confounders, including smoking habits and occupational exposure. Among oxidative damage biomarkers, both 8-oxodGuo and 8-oxoGuo were associated with unmodified U-Benz (Model 3).

In agreement with a previous study on a large number of subjects not occupationally exposed to benzene and other oxidizing agents (3), regression analysis in children did not suggest a marked effect of smoking habits (as either urinary cotinine or reported ETS) on biomarkers of nucleic acid oxidation. On the other hand, since cotinine concentrations detected in samples from ETS children were much lower (from 10 to 100 times) than those observed in smoking adults, this result was not unexpected. This means that the genetic damage is not directly related to benzene only, but rather that biomarkers of benzene exposure are surrogates of some other kind of "oxidative stress" possibly related to environmental pollution from road traffic. However, the lack of correlation between cotinine

and biomarkers of nucleic acid oxidation does not rule out the role of passive smoking. It should be noted that biomarkers of exposure integrate all sources of uptake, including ETS.

Conclusions

The present study shows that: (i) the concentration of both biomarkers of exposure to benzene and nucleic acid oxidation increases as environmental air pollution and urbanization level increase; (ii) exposure to passive smoking (in EETS subgroup) accounts for the similar concentration levels observed in U-Benz, 8-oxoGuo, 8-oxoGua and urinary cotinine in children from different residential areas; (iii) the percentage of variance of 8-oxodGuo and 8-oxoGuo associated with environmental benzene exposure is similar in all residential areas, suggesting that benzene is a good tracer of other oxidant substances that are typical of urban air. Among exposure biomarkers, S-PMA was the most sensitive in detecting differences between groups of children living in different areas, whereas U-Benz was more sensitive to exposure to passive smoking. In addition, when different models of exposure to benzene were compared, *S*-PMA was the best predictor of the variance of biomarkers of nucleic acid oxidation (20% in the case of 8-oxodGuo).

No potential conflict of interest relevant to this article was reported

Disclaimer

The author Paola Manini is employed with the European Food Safety Authority (EFSA) in its FEED Unit that provides scientific and administrative support to EFSA's scientific activities in the area of feed additives. At the time the author's gave her contribution to the research described in article the author was working at the University of Parma. The present article is published under the sole responsibility of the author and may not be considered as an EFSA scientific output. The positions and opinions presented in this article are those of the author alone and are not intended to represent the views or scientific works of EFSA. To know about the views or scientific outputs of EFSA, please consult its website http://www.efsa.europa.eu.

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ACKNOWLEDGEMENTS: *The study was supported by the Italian Ministry of Health as part of the project "Novel approaches to assess the carcinogenic potential of genotoxic pollutants" (PMS/42/2006), and by the National Institute for Occupational Safety and Prevention as parte of the project "Control methods and non-occupational exposure assessment to benzene in pediatric age" (B1/38/DML)*