Urinary biomarkers of exposure and of oxidative damage in children exposed to low airborne concentrations of benzene

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Abstract

The aim of this work was to evaluate the oxidative damage to nucleic acids in children (5–11 years) associated with exposure to environmental pollutants and tobacco smoke (ETS). For each subject, urinary sampling was done twice (evening and next morning) to measure by tandem LC–MS–MS such oxidated products of nucleic acids as 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodGuo), 8-oxo-7,8-dihydroguanosine (8-oxoGuo), and 8-oxo-7,8-dihydroguanine (8-oxoGua). Methyl *tert*-butyl ether (U-MTBE), benzene (U-Benz), and its metabolites (*t,t*-muconic and *S*-phenylmercapturic acids, *t,t*-MA and *S*-PMA, respectively) were determined as biomarkers of exposure to air pollution, and cotinine as a biomarker of exposure to ETS.

Biomarkers of exposure (*S*-PMA and U-MTBE) and of DNA oxidation (8-oxodGuo) were dependent on the urbanization and industrialization levels and increased in the evening sample as compared to next morning ($p<0.05$). In both evening and next morning samples, 8-oxodGuo and 8-oxoGuo correlated with each other ($r=0.596$ and $r=0.537$, respectively, $p<0.01$) and with biomarkers of benzene exposure, particularly *S*-PMA ($r=0.59$ and $r=0.45$ for 8-oxodGuo and $r=0.411$ and $r=0.383$ for 8-oxoGuo, $p<0.01$). No such correlations were observed for U-MTBE and cotinine. Multiple linear regression analyses showed that 8-oxodGuo was positively associated with *S*-PMA at both sampling times ($\beta=0.18$ and $\beta=0.14$ for evening and next morning sampling, respectively; $p<0.02$) and weakly with U-MTBE ($\beta=0.07$, $p=0.020$) only in the evening urines. These results suggest that the selected biomarkers of

exposure to benzene, particularly *S*-PMA, are good tracers of exposure to complex mixtures of oxidative pollutants and that the associated oxidative damage to nucleic acids is detectable even at very low levels of exposure.

Keywords: Children; Benzene exposure; *S*-phenylmercapturic acid; Nucleic acid oxidation; Oil refinery

1 Introduction

Exposure to environmental air pollution, a complex mixture of hazardous chemicals, gases and suspended particulate matter, is associated with inflammation and oxidative stress, and has been considered a hazard to human health (Risom et al., 2005; Møller et al., 2014; Valavanidis et al., 2013; Levy et al., 2012; Henschel et al., 2012). The nature of air pollutant may present various physical, chemical, mutagenic and toxicological properties, according to geographic area and human socio-economic activities (Bono et al., 2014; Götschi et al., 2005, Ghio et al.,2012; Kampa and Castanas, 2008). Apart from the industrial emissions, in urban area the prevalent contribution to air pollution arises from motor vehicle exhausts and environmental tobacco smoke (ETS).

Benzene, one of the most studied air pollutant component, is a known human carcinogen (International Agency for Research on Cancer, 1982), practically ubiquitous both in industrial and urban areas worldwide. It is still used as intermediate in chemical industries, is present as an impurity in the aromatic hydrocarbon mixture added to gasoline as an antiknock agent, and is a component of the tobacco smoke (Darrall et al., 1998). Due to its human carcinogenicity, agencies like the World Health Organization, the European Commission provided air quality guidelines for ~~benzene.~~benzene (World Health Organization (WHO), 2000; European Commission, 2008). Moreover, several recent studies demonstrated that at even low-level occupational exposure, benzene can induce ~~haematological~~hematological and genotoxic effects in humans (Lan et al., 2004; Smith, 2010). The ubiquitous presence of benzene in the environment and its adverse effects on the general population cannot be neglected and to protect the health of the general population, also guidelines for air quality have been set (European Commission, 2008). In Europe a mean calendar year limit of 5 $\mu g/m^3$ is being enforced as of 1 January 2010 (European Commission, 2008). The different sources that contribute to increased air benzene levels should be identified in order to better regulate their emissions.

In this contest of public health and hence lifetime exposure, children are considered a high-risk group because they are much more susceptible than adults (van Leeuwen et al., 2008). Even when exposed at the same concentrations of environmental pollutants, they adsorb much more than adults, and hence the biologically effective dose that reach target organs is proportionally higher. Moreover, if the exposure to environmental toxics starts in the childhood, the risk of adverse effects with long latency increases (Wild and Kleinjans, 2003).

Where validated, biomarkers are available to assess low exposure levels, biological monitoring can an efficient instrument to characterize environmental exposures, not only for occupationally exposed subjects but also for the general population, in particular children. In the case of benzene, it is difficult to identify a single biomarker suitable for characterizing environmental exposure levels due to the very low concentrations and the different emission sources, including, among others, urban traffic and tobacco smoke. Several studies conducted on adults from the general population have investigated three different compounds, the urinary unmetabolized solvent and two metabolites, *trans,trans*-muconic acid (*t,t*-MA) and *S*-phenylmercapturic acid (*S*-PMA). As for the *t,t*-MA, all the researchers agree on its low specificity, since it is influenced by dietary factors (Cocco et al., 2003; Hoet et al., 2009); urinary unmodified benzene (U-Benz) and *S*-PMA are surely more specific but their usefulness in discriminating different degrees of low levels of exposure are currently under evaluation (Protano et al., 2010; Campagna et al., 2014; Fustinoni et al., 2010; Manini,et al., 2008; Weisel, 2010).

Urban traffic and tobacco smoke (either active or passive) are main sources of benzene emission for the general population. Urinary methyl *tert*-buthyl ether (MTBE) has been studied as a biomarker of exposure to gasoline (De Palma et al., 2012) and urban traffic (~~Wiesel~~Weisel, 2010; Campo et al., 2011) and urinary cotinine is a validated biomarker of tobacco smoke (Protano et al., 2012).

Bio-activation of benzene via cytochrome *P*-450 enzymes (*CYP2E1*) leads to the formation of hazardous metabolites, like *trans,trans*-muconaldheyde and catechol, that could play an important role in benzene genotoxicity (Whysner et al., 2004) and in the generation of reactive oxygen species, which can directly induce deleterious changes in cellular macromolecules, including nucleic acids. Recently urinary 8-hydroxy-2'-deoxyguanosine (8-oxodGuo), 8-oxo-7,8-dihydroguanosine (8-oxoGuo), and 8-oxo-7,8-dihydroguanine (8-oxoGua) have been proposed as markers of oxidative damage to DNA, RNA and both, respectively. The measurement of such biomarkers is a snapshot at the time of sampling and represents the balance between formation of adducts and their repair (Poulsen et al., 2014). Several studies have reported association between 8-oxodGuo and exposure to air pollutants either in adults (Avogbe et al., 2005; Fracasso et al., 2010; Sørensen et al., 2003; Manini et al., 2010) and in children (Buthbumrung et al., 2008; Staessen et al., 2001, Andreoli et al., 2012).

In a recent study carried out on 396 children attending primary schools and living in Lazio (central Italy), we observed that (i) U-Benz, *t,t*-MA and *S*-PMA were sensitive enough to characterize the living areas with different urbanization and air pollutant levels (Protano et al., 2010); (ii) U-Benz significantly correlated with urinary cotinine, as marker of recent exposure to passive smoke, at evening sampling time (Protano et al., 2012); and (iii) *S*-PMA positively correlated with both 8-oxodGuo and 8-oxoGuo and was the best predictor of their variance, 20% in the case of 8-oxodGuo (Andreoli et al., 2012).

The present study was performed on children attending primary schools and residing in two different areas characterized by different urbanization and industrialization levels, in order to characterize their urinary oxidative damage to nucleic acids and to evaluate whether such oxidative damage levels were associated with exposure to pollutants, such as benzene and MTBE, which can be released from the oil refinery (Cottica and Grignani, 2013) located in the urban area, but also from urban traffic (Campo et al., 2011) and ETS (Avila-Tang et al., 2013). Finally, we aimed at investigating the influence of the sampling times (evening and next morning) on the relationship between biomarkers of exposure and of

oxidative damage.

2 Subjects and methods

2.1 Study design

The present study was conducted in Sicily, in the South of Italy (Milazzo and Nizza) (Fig. 1). We recruited 155 children living in Milazzo at a distance less than 15 ~~Km~~km from the oil refinery (Near), and 58 children resident in Nizza, a town 70 ~~Km~~km far from refinery and without important industrial hub (Far). The characteristics of both areas are reported in Table 1 (Sources by Sicily region, 2011). The subjects were recruited on a voluntary basis. All of the students attending each primary school of Milazzo and Nizza and their parents received information about the goals and plans for the research, and were invited to take part in the cross-sectional study. Once obtained parents' informed consent, sampling procedures (in particular on sampling storage) were provided together with a self-administered questionnaire to collected detailed information on socio-demographic characteristics, daily activities, living conditions and cohabitant smoking habits (Table 1). The study protocol fulfilled the Helsinki declaration and was approved by the local Ethical Committee of the University Hospital of Messina.

Fig. 1 The two sampling sites: Milazzo, Near oil refinery and Nizza, 70 ~~Km~~km Far away the industrial hub.

Table 1 Summary information on relevant urbanization indicators and air pollutant levels of the investigated areas in the study period (data from ARPA Sicily); main characteristics of the study group and geometric means [geometric standard deviations] of urinary cotinine levels (μg/g creat) classified by the number of smoking cohabitants.

Environmental variables		Far refinery	Near refinery
Resident population (*n*)		3674	31,860
Population density (persons per km^2)		279	1315
Motorization rate	No. of autovehicles per 100 inhabitants	58	62
	No. of two-wheeled vehicles per 100 inhabitants	12	15
	~~Means of air pollutants~~NO_2 ($\mu g/m^3$)	n.a.	10.9
Means of air pollutants	NO_x ($\mu g/m^3$)	n.a.	13.3
	Benzene ($\mu g/m^3$)	n.a.	0.45
Subjects' characteristics			

No.		55	150
Age (years, mean±SD)		9.2 ±1.1	9.7 ±1.0
Gender (male/female)		23/32	87/63
No. of smoking cohabitants (No. smoked cigarettes, mean±SD)	0	36 (0)	95 (0)
	1	14 (9.6±7.2)	44 (13.7±8.4)
	2	4 (31.7±10.4)	11 (21.5±10.6)
Cotinine (evening)	0	0.87 [2.14]¥,£	1.34 [1.67]*,§
	1	1.61 [2.65]*,§	2.49 [1.80]*,§,#
	2	14.4 [2.84]*,§	5.22 [2.40]*,§
Cotinine (next morning)	0	0.92 [1.99]§,¥	1.20 [1.60]*,§,†
	1	1.70 [1.92]*,§	2.23 [1.95]*,§,#
	2	12.5 [2.12]*,§	4.36 [2.47]*,§

Legend: n.a., not available; 0, absence of smoking cohabitants; 1, only one smoking cohabitant; 2, two or more smoking cohabitants.

* $p<0.0001$, 0 ~~*vs*~~ *vs.* 1, 0 ~~*vs*~~ *vs.* 2; and 1 ~~*vs*~~ *vs.* 2 for Near Refinery; 0 *vs.* 2; and 1 ~~*vs*~~ *vs.* 2 for Far Refinery;

§ $p<0.05$, 0 ~~*vs*~~ *vs.* 1 for Far Refinery. One-way ANOVA followed by Bonferroni post-hoc test for urinary cotinine levels.

¥ $p<0.01$,

# $p<0.05$ Far Refinery ~~*vs*~~ *vs.* Near Refinery;

£ $p<0.01$;

† $p<0.05$ evening *vs.* next morning; t-test for independent data.

2.2 Sampling procedure

Two urine samples for each participant were collected: the first in the Wednesday evening (just before bedtime) and the second in the Thursday next morning (before going to school), during the ~~April–June~~April–June 2013 period, in weeks without school vacancy. During the night, the evening samples were stored in the refrigerator at 4 °C. The next morning, both refrigerated urine samples were delivered to schools where the research team collected and transported all samples to the laboratory. Spot urine samples were divided into several aliquots and frozen at –20 °C within two hours from the last sample collection, until analyses.

2.3 Biological monitoring

All determinations were performed by chromatographic techniques coupled with mass spectrometry and analytical procedures have been described in details in other studies (De Palma et al., 2012; Manini et al., 2008; Andreoli et al., 2011).

Briefly, U-MTBE and U-Benz was determined by headspace solid-phase microextraction followed by gas chromatography-mass spectrometry (GC–MS) according to procedures outlined in De Palma et al. (2012). The limit of detection (LOD), calculated as the signal to noise ratio (*S*/*N*)>3, was 10 ng/L and 20 ng/L for U-Benz and U-MTBE, respectively; and the coefficient of variation of the method (%CV) was below 7% for all intra- and inter-day determinations. All other urinary determinations were performed by isotopic dilution liquid chromatography ~~tandem~~tandem mass spectrometry (LC–MS–MS) using a AB SCIEX API4000 triple-quadrupole mass spectrometer (AB SCIEX, Framingham, MA, USA) equipped with a Turbolonspray interface for pneumatically assisted electrospray as previously described (Andreoli et al., 2011; Manini et al., 2008). The LOQs of the benzene metabolites were 0.05 µg/L for *S*-PMA and 2.5 µg/L for *t,t*-MA. The LOQs 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 was below 6.8% for all analytes and for all intra- and inter-day determinations. The laboratory in which the determinations were performed is involving in an inter-laboratory project which include quality control to assess urinary concentration of 8-oxodGuo, organized by European Standards Committee on Urinary (DNA) Lesion Analysis (Barregard et al., 2013). Concentrations of urinary analytes, but not U-MTBE and U-Benz, were expressed as a function of creatinine concentration (µg/g creat), which was measured by the method of Jaffe (Kroll et al., 1986). We excluded very diluted (creatinine concentrations lower than 0.3 g/L) or very concentrated (creatinine concentration higher than 3.0 g/L) urine samples (World Health

Organization (WHO), 1996). For this reason, we excluded 8 children (five living in Milazzo and three in Nizza) who had at least one urinary creatinine value out of the above creatinine range. Excluded urine samples were not replaced and therefore statistical analyses were done on 205 subjects.

2.4 Determination of MTBE and benzene stability in urine samples

In order to study MTBE and Benzene kinetic evaporation, 6 pools of 10 urinary specimens were collected and stored at 4 °C for several time (0, 12, 24, 100 and 240 h). Three of the 6 urine pools were added with 2 µg/L of MTBE and Benzene. At each time, 2 ml from the 6 pool urine samples were immediately transferred in SPME vials and stored at –20 °C. Samples were collected in duplicate in order to have 12 replicate. Reproducibility calculated as RSD% was always within 5% for both solvents showing that there were no differences between MTBE and Benzene time-depletion in unmodified and spiked urine. Time course evaporation study showed that MTBE and Benzene urinary concentration follows the first order kinetic. Table 2 shows the linear regressions parameters (log% solvent concentration *vs.* storage time at 4 °C) for both analytes and their half-life ($t_{1/2}$), a value independent of the starting concentration. MTBE half-life, was higher than Benzene due to its greater urine solubility (logK_{OW} MTBE=1.04 *vs.* LogK_{OW} Benzene=2.13 at 25 °C) and despite of its higher boiling point (T_B MTBE=55.3 °C *vs.* T_B Benzene=80.15 °C). These data demonstrate that the solvent solubility affects more the evaporation rate with respect to the boiling point. After 12 h MTBE and Benzene% concentrations were 96.7±4.6% and 95.7±4.3%, respectively, confirming that the sampling procedure applied in this study was appropriate.

Table 2 First order kinetic linear regression: log% solvent~~log % solvent= k hours+log % at 0~~=–*k* hours+log% at 0 ~~hours~~h, where *K* is the first order rate constant (1/hours).

	~~K~~K	~~R~~R^2	$t_{1/2}$(hours)
U-MTBE	0.001	0.92	693
U-Benz	0.0035	0.99	198

Legend: U-MTBE, urinary methyl *tert*-butyl ether; U-Benz, urinary benzene.

2.5 Statistics

Statistical analyses were carried out by the SPSS software (version 20.0 for Windows, Chicago, IL). All the measured variables followed a log-normal distribution, as assessed by the Kolmogorov–Smirnov test, and further statistical analyses were conducted on decimal log-transformed values. We applied the Student *t* for independent samples to assess differences between Near *vs.* Far groups and the Student *t* for dependent samples for evening *vs.* next morning; the ANOVA test followed by the Bonferroni *post-hoc* test was used for multiple comparisons. Pearson's *r* was used to assess correlation between variables on log-scale, expressed not as a function of creatinine. Multiple linear regression analysis models were applied to predict U-Benz, *S*-PMA and *t,t*-MA as singly dependent variables, as a function of gender, age, urinary concentration of creatinine, MTBE and cotinine for each sampling time. Parameters like gender, daily activities (physical activity, indoor or outdoor) and living conditions like presence of petrol station or a parking near the residences were removed from the models after verifying that they did not affect the biomarkers excretion profile. After evaluating the effect of markers of urban traffic and ETS exposure on benzene exposure biomarkers, new multiple linear regression analysis models were used adding the exposure to benzene (as *S*-PMA, *t,t*-MA and U-Benz concentrations) to other independent variables (age, creatinine, U-MTBE, cotinine) to assess the variability of biomarkers of nucleic acid oxidation (8-oxodGuo/8-oxoGuo/8-oxoGua). All the biomarkers were insert expressed as concentration (µg/l) and not as a function of creatinine. The final model was as follows: Log(U-biomarker)=constant+β_{1}×(Age)+β_{2}×Log(U-creatinine)+β_{3}×Log(U-MTBE)+β_{4}×Log(U-cotinine)+β_{5}×Log(U-Benz)+β_{6}×Log(*S*-PMA)+β_{7}×Log (*t,t*-MA). Values of constant, regression coefficient (β), its 95% Confidence Interval (C.I. 95%), its standard error (SE) and significance (*p*) for each predictive variable were given. The adjusted r^2 (r^2_{adj}) and significance (*p*) for the whole model are also reported in the last row.

3 Results

3.1 Biological monitoring

All analytical determinations were above the corresponding LODs. Table 3 summarizes the distribution of biomarkers of exposure to benzene (U-Benz; *S*-PMA, *t,t*-MA), MTBE (U-MTBE) and ETS (cotinine) and of nucleic acid oxidation in children classified by the distance of the schools from the oil refinery (school location: “Near” and “Far”) and also stratified by time of sampling (evening and morning). Biomarker levels are expressed as geometric means [and geometric standard deviation] (GM [GSD]) due to their log-normal distribution. At the evening, the urinary levels of U-MTBE, *S*-PMA, cotinine and 8-oxodGuo were significantly higher in the “Near” as compared to the “Far” group (*t*-test for independent data, p=0.031, p=0.011, p=0.033 and p<0.0001 for U-MTBE, *S*-PMA, cotinine and 8-oxodGuo, respectively), and 8-oxoGuo that showed an opposite trend (p=0.001). Similar differences were observed also in the morning samples for U-MTBE (p=0.041), 8-oxodGuo and 8-oxoGuo (p<0.0001). Children living near the oil refinery excreted significantly higher concentrations of *S*-PMA and *t,t*-MA, cotinine and 8-oxodGuo in the evening than in the next morning samples (*S*-PMA 0.20 ~~vs~~*vs.* 0.15 µg/g creat and *t,t*-MA 48.1 ~~vs~~*vs.* 38.9 µg/g creat, p<0.0001, cotinine 1.78 ~~vs~~*vs.* 1.58 µg/g creat and 8-oxodGuo 3.95 ~~vs~~*vs.* µg/g creat, p<0.001, respectively). Similar differences were observed in children living Far from oil refinery only for *S*-PMA and 8-oxodGuo concentrations (0.17 ~~vs~~*vs.* 0.14 µg/g creat and 3.05 ~~vs~~*vs.* 2.63 µg/g creat, respectively, p<0.0001 for both).

Table 3 Distribution of biomarkers of exposure, nucleic acid oxidation and passive smoking in children stratified by living area and sampling time. Values are expressed as geometric mean and geometric standard deviation. Concentrations are expressed as µg/g creatinine, but not U-MTBE and U-Benz (µg/L).

Biomarker	Near refinery[a]		Far refinery[a]		Near ~~vs~~ *vs.* Far[b], *P*	
	Evening	Morning	Evening	Morning	Evening	Morning
U-MTBE	0.79 [2.73]	0.82 [2.89]	0.56 [2.27]	0.59 [2.56]	0.031	0.041
U-Benz	0.20 [1.41]	0.21 [1.50]	0.22 [1.30]	0.23 [1.35]	n.s.	n.s.
S-PMA	0.20 [1.63]	0.15 [1.69]**	0.17 [1.50]	0.14 [1.60]**	0.011	n.s.
t.t-MA	48.1 [2.47]	38.9 [2.26]**	53.9 [2.85]	40.4 [3.87]	n.s.	n.s.
Cotinine	1.78 [2.02]	1.58 [1.93]*	1.28 [3.04]	1.31 [2.66]	0.033	n.s.
8oxodGuo	3.95 [1.47]	3.65 [1.42]*	3.05 [1.50]	2.63 [1.53]**	<0.0001	<0.0001
8oxoGuo	9.43 [1.83]	9.09 [1.68]	12.5 [1.50]	12.1 [1.56]	0.001	<0.0001
8oxoGua	17.8 [2.66]	15.7 [2.91]	15.9 [2.94]	16.4 [2.86]	n.s.	n.s.

[a] Student's *t*-test for paired samples.

** $p<0.0001$.

* $p<0.01$.

[b] Student's *t*-test for independent samples; U-MTBE, urinary methyl *tert*-butyl ether; U-Benz, urinary 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.

Table 4 resumes the results of Pearson's correlations analysis among biomarkers in children classified by the sampling time. Metabolites of benzene were significantly correlated each other at both the sampling times (r=0.33 and 0.35 in the evening and next morning samples, respectively, $p<0.01$ for both), whereas U-Benz was significantly correlated with S-PMA only in the next morning samples (r=0.22, $p<0.05$). Among biomarkers of nucleic oxidation, only 8-oxodGuo and 8-oxoGuo were significantly correlated (r=0.54 and 0.60, in the evening and next morning samples, respectively, $p<0.01$ for both). Both the biomarkers were significantly correlated with benzene metabolites at both the sampling times ($0.29<r<0.59$). and, to a lesser extent, with U-Benz ($0.16<r<0.21$), with the exception of 8-oxoGuo in the evening sample. Cotinine was positively correlated with U-Benz (r=0.164, $p<0.05$), and *S*-PMA (r=0.190, $p<0.01$) in the morning samples only and showed significantly negative correlations with 8-oxoGua at both the sampling times (r=–0.165, $p<0.05$ and r=–0.145, $p<0.05$, respectively). U-MTBE was positively correlated with 8-oxodGuo (r=0.14, $p<0.05$) in the evening samples and negatively correlated with U-Benz (r=–0.18, $p<0.01$) and *t,t*-MA (r=–0.16, $p<0.05$) in the morning samples. All correlations between biomarkers (respectively between S-PMA and *t,t*-MA, S-PMA and 8-oxodGuo, S-PMA and 8-oxoGuo, and 8-oxodGuo and 8-oxoGuo) were also present in subgroups of children stratified according to school location (data not shown).

Table 4 Pearson's correlation coefficients between urinary biomarkers in children classified by sampling time (evening and morning).

	U-Benz	*S*-PMA	*t.t*-MA	U-Cot	8-oxodGuo	8-oxoGuo	8-oxoGua
Evening							
U-MTBE	−0.108	0.045	−0.021	0.050	0.140*	0.017	−0.037
U-Benz		0.136	0.108	0.104	0.160*	0.122	−0.088
S-PMA			0.328**	0.084	0.591**	0.411**	−0.080
t.t-MA				0.003	0.357**	0.366*	0.034

U-Cotinine					0.030	0.041	−0.165*
8-oxodGuo						0.596**	0.006
8-oxoGuo							0.046
Morning							
U-MTBE	−0.184**	−0.075	−0.156*	−0.056	0.045	−0.122	−0.119
U-Benz		0.218**	0.085	0.164*	0.156*	0.212**	−0.006
S-PMA			0.353**	0.190**	0.449**	0.383**	−0.033
t,t-MA				0.018	0.286**	0.331**	0.117
U-Cotinine					0.103	0.097	−0.145*
8-oxodGuo						0.537**	0.064
8-oxoGuo							0.125

U-MTBE, urinary methyl *tert*-butyl ether; U-Benz, urinary 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.

** $p<0.01$.

* $p<0.05$.

Multiple regression analysis models posing biomarkers of exposure to benzene as dependent variables and creatinine, U-MTBE, cotinine as independent variables showed that U-Benz, S-PMA and *t,t*-MA were not affected by U-MTBE and cotinine levels in the evening samples. On the other hand, in the next morning samples, a weakly negative effect of U-MTBE toward U-Benz (β=−0.061, p=0.028) and *t,t*-MA (β=−0.142, p=0.037) and a positive relationship between urinary cotinine and *S*-PMA (β=0.117, p=0.046) were apparent. In all cases the multicollinearity test was performed and the VIF values were always lower than 2.2 (data not shown).

Table 5 shows results of the multiple linear regression analysis with biomarkers of oxidative damage to nucleic acids as outcomes, in the whole study population at both the sampling times, and all biomarkers of exposure, together with age, and creatinine as independent variables. For 8-oxodGuo as outcome, the regression models explain about 59% and 38% of its variance in the evening and morning samples, respectively (p<0.0001 for both). Apart from creatinine, *S*-PMA significantly affected the 8-oxodGuo excretion, with similar effect at both the sampling times, (p<0.02), whereas U-MTBE showed a lower effect only in the evening samples (p=0.02).

Table 5 Predictors of the urinary excretion (evening and next morning samples) of biomarkers of nucleic acid oxidation (set as dependent variables) according to multiple linear regression model: Log(U-biomarker)=constant+(Age)×β_1+Log(U-creatinine)×β_2+log(U-MTBE)×β_3+log(U-cotinine)×β_4+log(U-Benz)×β_5+log(*S*-PMA)×β_6+log(*t,t*-MA)×β_7. For each independent variable, the values of constant β coefficient (β), with corresponding 95% confidence interval (C.I. 95%) and standard error (SE), and significance (p) 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 set at 0.05 for entry and 0.10 for removal from the model.

Model	Evening			Morning		
1. 8-oxodGuo	β (C.I. 95%)	SE	p	β (C.I. 95%)	SE	p
Constant	0.78 (0.48, 1.09)	0.16	<0.0001	0.62 (0.34, 0.90)	0.14	<0.0001
Age	−0.00 (−0.03, 0.02)	0.01	0.797	0.00 (−0.02, 0.03)	0.01	0.814
U-Creatinine	0.62 (0.47, 0.77)	0.08	<0.0001	0.70 (0.52, 0.88)	0.09	<0.0001
U-MTBE	0.07 (0.01, 0.12)	0.03	0.020	0.04 (−0.01, 0.09)	0.03	0.133
U-Cotinine	−0.03 (−0.11, 0.04)	0.04	0.387	0.02 (−0.06, 0.09)	0.04	0.690

U-Benz	−0.09 (−0.08, 0.26)	0.09	0.292	0.00 (−0.15, 0.15)	0.08	0.986
S-PMA	0.18 (0.07, 0.30)	0.06	0.002	0.14 (0.03, 0.26)	0.06	0.012
t,t-MA	0.01 (−0.05, 0.06)	0.03	0.896	0.00 (−0.06, 0.06)	0.03	0.934
Whole model r^2_{adj}, *p*	0.541		<0.0001	0.383		<0.0001
2. 8-oxoGuo						
Constant	1.26 (0.81, 1.71)	0.23	<0.0001	1.44 (1.08, 1.818)	0.18	<0.0001
Age	−0.03 (−0.07, 0.01)	0.02	0.097	−0.04 (−0.07, −0.01)	0.08	0.007
U-Creatinine	0.68 (0.46, 0.90)	0.11	<0.0001	0.77 (0.54, 1.01)	0.12	<0.0001
U-MTBE	0.01 (−0.07, 0.09)	0.04	0.842	−0.04 (−0.11, 0.03)	0.04	0.247
U-Cotinine	−0.00 (−0.11, 0.11)	0.06	0.970	0.02 (−0.08, 0.12)	0.05	0.706
U-Benz	−0.10 (−0.15, 0.34)	0.13	0.453	0.13 (−0.07, 0.12)	0.10	0.201
S-PMA	0.03 (−0.14, 0.20)	0.09	0.715	0.05 (−0.10, 0.19)	0.07	0.525
t,t-MA	0.06 (−0.02, 0.15)	0.04	0.135	0.04 (−0.03, 0.12)	0.04	0.256
Whole model r^2_{adj}, *p*	0.323		<0.0001	0.335		<0.0001
3. 8-oxoGua						
Constant	0.69 (−0.03, 1.40)	0.36	0.060	1.20 (0.58, 1.83)	0.32	<0.0001
Age	0.01 (−0.04, 0.07)	0.03	0.624	−0.03 (−0.8, 0.2)	0.03	0.251
U-Creatinine	0.24 (−0.11, 0.59)	0.18	0.183	0.53 (0.12, 0.95)	0.21	0.011
U-MTBE	−0.04 (−0.17, 0.09)	0.07	0.521	−0.11 (−0.23, 0.01)	0.06	0.082
U-Cotinine	−0.18 (−0.35, −0.01)	0.09	0.042	−0.18 (−0.35, 0.00)	0.09	0.050
U-Benz	−0.25 (−0.64, 0.15)	0.20	0.216	−0.05 (−0.39, 0.30)	0.17	0.794
S-PMA	−0.22 (−0.48, 0.05)	0.14	0.113	−0.26 (−0.51, −0.00)	0.13	0.047
t,t-MA	0.03 (−0.11, 0.16)	0.07	0.687	0.05 (−0.08, 0.17)	0.07	0.489
Whole model r^2_{adj}, *p*	0.017		0.164	0.053		0.013

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

The model explained about 30% of 8-oxoGuo levels at both the sampling times, with a strong effect of creatinine ($p<0.0001$) at both the sampling times and a significant negative effect of age in the next morning samples only ($p=0.007$). The model explained a very low fraction of 8-oxoGua's variance (about 5%), significantly only in the next morning sample ($p=0.013$), with a significant role only for creatinine and *S*-PMA, the latter playing a negative effect ($\beta=-0.26$, $p<0.05$). A similar effect was observed for cotinine but without reaching a full statistical significance ($p=0.05$).

3.2 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 the questionnaire. As shown in Fig. 2, average urinary cotinine levels were strongly dependent on the number of smoking cohabitants, at both the sampling times and the behavior was the same independently from living “Near” or “Far” from the oil refinery (data not shown). The same results were obtained when the number of smoked cigarettes was used instead of the number of smoker cohabitants (Table 1), confirming the validity of the questionnaire. A positive correlation was observed between number of smoked cigarettes and cotinine both in the evening urine samples ($r=0.469$, $p<0.0001$) and in the next morning ones

(r=0.540, p<0.0001). After stratification for the lack or presence of smoking cohabitants, in children not exposed to ETS, we observed significantly higher urinary levels of cotinine in the "Near" as compared to the "Far" group both in the evening (p=0.003) and in the next morning (p=0.040) samples. No difference was apparent in children who live with smoker cohabitants. At the evening time, U-benz and *S*-PMA showed an increasing trend with the number of smoker cohabitants but without statistically differences (data not shown).

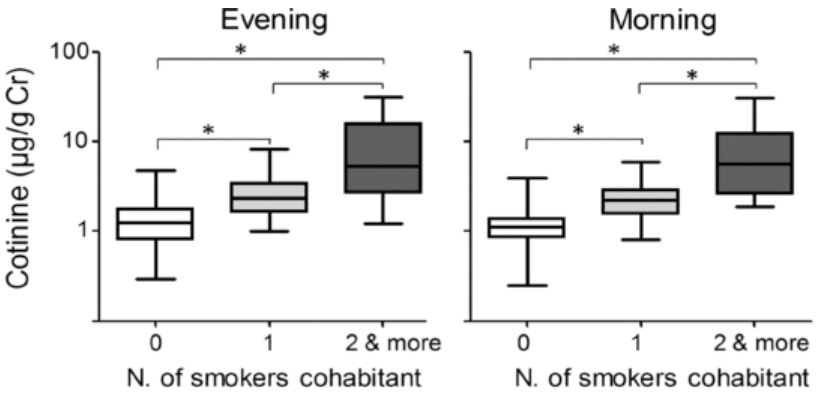

Fig. 2 Urinary cotinine concentration in children (n=205) classified according to the number of smoking cohabitants. * p<0.0001, ANOVA with Bonferroni *post-hoc* test.

4 Discussion

The present study demonstrates that in children attending the primary school in a South region of Italy the urinary excretion of biomarkers of oxidative damage to nucleic acids, particularly 8-oxodGuo, is conditioned by environmental benzene exposure, measured as *S*-PMA. Children are vulnerable group of the general population. Moreover, they have lower mobility inside the area of residence and a simpler lifestyle than adults, thereby reducing the variability of exposure and possible interference by confounding factors (Bono et al., 2014).

To assess the exposure to environmental benzene, we used a biomonitoring approach because it is simpler, non-invasive and applicable in field studies involving children.

Urban traffic, industrial sites, such as oil refinery, and ETS increase the air borne benzene levels and need to be considered and evaluated when the relationship between benzene exposure and oxidative damage to nucleic acids is investigated. For these reasons together with urinary benzene and its metabolites, *t,t*-MA and *S*-PMA, we also quantified urinary MTBE, which share common sources with benzene, i.e. oil refinery and urban traffic (Weisel, 2010), and urinary cotinine as biomarkers of ETS.

The results of biomonitoring were able to distinguish benzene exposure of children according to their school location, though recorded values were within reference values for the Italian population (SIVR, 2015) and comparable to those previously measured in adults and children residing in urban and rural areas (Fustinoni et al., 2010; Lovreglio et al., 2011; Protano et al., 2010). Moreover, despite the lack of personal sampling, air monitoring data measured by a station located near the oil refinery were comparable with those reported by measurement systems situated in proximity of a similar plant in Sardinia, Italy (Fustinoni et al., 2012).

A main finding of this research is that the urinary concentrations of 8-oxodGuo, the most studied biomarker of oxidation to DNA (Loft et al., 2012), followed the same trend of exposure biomarkers, in particular U-MTBE and *S*-PMA, suggesting an influence of environmental pollution exposure on this biomarker of urinary oxidative damage. Average 8-oxodGuo concentrations were 1.3 fold higher in urine from children living near oil refinery and in the town with higher population density as compared to those living in area characterized by lower urbanization and absence of industrial hub (Table 3), when the samples were collected in the evening. The difference was even higher (1.4 fold, on average) in samples collected the next morning. This behavior, confirmed by correlation and regression analyses, would be consistent with results obtained in others studies investigating the relationships between environmental air pollutants and the urinary excretion of oxidative stress biomarkers (Bono et al., 2014; Svecova et al., 2009).

Both *S*-PMA and 8-oxodGuo showed significantly higher levels in the evening samples as compared to those collected in the next morning, independently from the living area ("Near" or "Far" from the refinery). The same behavior was observed also for *t,t*-MA and cotinine but only in children living near the refinery. In an our previous study on healthy adults, we could exclude a circadian rhythm for the excretion profiles of biomarkers of nucleic acid oxidation (Andreoli et al., 2010). In this case, the observed differences at different sampling times could be supported by differences of benzene intake (and nucleic acid damage repair) by external sources. Near the oil refinery, a fixed station registered hours by hours data related to air quality. In the sampling days, the means (medians) of air benzene measurements were 0.59 (0.45) µg/m^3 at 7.00–9.00 pm and 0.38 (0.38) µg/m^3 at 5.00–7.00 am range. Biomarkers of exposure to air pollutants and biomarkers of oxidative damage were sensitive enough to assess the reduction of pollution sources, such as urban traffic, industries and business activity and active smoke, that occurs during the night, mainly expected in the town with higher population

and industrial density.

8-OxodGuo was positively correlated with all benzene biomarkers (U-Benz-, *S*-PMA and *t,t*-MA) on both the sampling times. Even if the creatinine was the most important predictor, multiple regression analyses showed that *S*-PMA was significantly associated with oxidative damage to DNA both in the evening and in the next morning, suggesting this benzene metabolite as a better biomarker of oxidative damage to nucleic acids related to air pollutant exposure. Only in evening samples, a positive influence of U-MTBE was shown on the 8-oxodGuo excretion profile. Considering the half-life of the studied biomarkers of exposure (respectively 2 h for benzene, 4.8 h for U-MTBE, 5 h for *t,t*-MA, and 9.1 h for *S*-PMA), the differences in biomarkers kinetics may account for these results, that are in agreement with those of a previous study carried out on children living in the Center of Italy showing similar demographic and living characteristics (Andreoli et al., 2012). Pooling together these data results in a better characterization of the dose-effect relationship at the lower exposure levels (Fig. 3) and supports the role of benzene (namely urinary levels of *S*-PMA) as a reliable tracer of general oxidative environmental pollution, probably also of other oxidant substances that are typical of urban air (e.g., particulated matter, $PM_{2.5}$ and PM_{10}). As illustrated by Fig. 3, a threshold is not apparent, whereas a flexion point of the dose-effect curve is apparent at *S*-PMA concentrations around 0.6 μg/g creatinine, corresponding to airborne benzene concentrations of about 9 $\mu g/m^3$ (Carrieri et al., 2012), above which a change in slope can be appreciated. Such a change in slope can be interpreted as a reduction – if not saturation – in ROS detoxification.

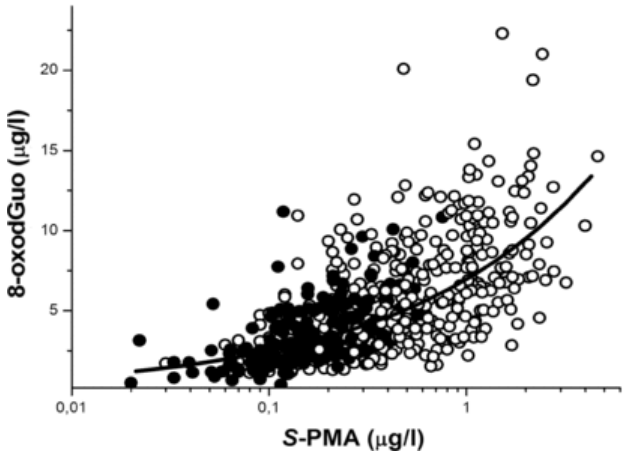

Fig. 3 Relationship between urinary concentrations of *S*-phenylmercapturic acid (*S*-PMA) and 8-oxo-2'-deoxy-7,8-dihydroguanosine (8-oxodGuo) at the evening sampling time, distinguished according to the results of this study (filled circle) and of previous one (empty circle, Andreoli et al., 2012).

The main pathway of benzene toxicity is thought to involve redox cycling of quinones which induce oxidative damage to proteins (Spatari et al., 2012) and DNA (Sørensens et al., 2003), and several studies describe association between benzene exposure and oxidative stress (Uzma et al., 2010; Moro et al., 2013; Prasad et al., 2013). 8-OxodGuo, 8-oxoGuo and 8-oxoGua were excreted in a free form in urine and were used to evaluate the oxidative stress related to DNA (Poulsen et al., 2014), RNA (Poulsen et al., 2012) and both (Cooke et al., 2003, Evans and Cooke, 2004), respectively, and represent the actual balance between damage formation and repair rates. As just observed in other studies both in adults (Manini et al., 2010) and in children (Andreoli et al., 2012), we confirmed the urinary correlation between 8-oxodGuo and 8-oxoGuo, respectively raising mainly by oxidative damage to DNA and RNA, and the absence of the correlation between them and the other urinary oxidative damage biomarker, 8-oxoGua.

Different from our previously obtained results (Manini et al., 2010; Andreoli et al., 2012), in this cohort of children 8-oxoGuo is not influenced by benzene exposure biomarkers but only by creatinine, and age. Moreover the 8-oxoGuo concentration levels were 1.3 fold higher in children urines living far from the industrial hub respect to ones near the oil refinery at both sampling times. The interpretation of 8-oxoGuo level is global RNA oxidation but the knowledge about the repair mechanisms of oxidative damage to RNA and the role of it in the oxidative stress pathways are few as the interest for RNA and for its oxidative forms is a very young filed (Poulsen et al., 2012). The slight difference between the age of the two groups of children (9.2±1.1 *vs.* 9.7±1.0 years, respectively) together with the negative correlation between 8-oxoGuo and age could account for higher concentrations of urinary 8-oxoGuo levels in children living far from the refinery.

Even if the urinary cotinine is a sensitive biomarker of ETS, able to discriminate children according to the number of smoker cohabitants (Fig. 2) and correlating with S-PMA in the morning samples, no correlation between cotinine and 8-oxodGuo was observed. Conversely, an unexpected result was a negative correlation between cotinine and 8-oxoGua at both the sampling times. Tobacco smoke is a complex mixture of toxics that induces oxidative stress both in *in vitro* (Prabhulkar and Li, 2010) and in *in vivo* (Bono et al., 2014) and a positive correlation between cotinine and biomarkers of oxidative damage of nucleic acids is expected. 8-OxoGua, excreted in the free form in the urine, is the quote of the damage due to the oxidation of nucleic acids and mainly repaired by BER system by hOGG1 activity on the oxidized guanine residues of DNA (Cooke et al., 2003) or originates from turnover or repair of RNA (Evans and Cooke, 2004). On the contrary, we observed significantly higher 8-oxoGua concentrations in urines of children unexposed to passive smoke respect to exposed ones. Our results are consistent with those obtained by other groups (Fracasso et al., 2006) demonstrating that in

adults exposed to passive smoke, the DNA repair capacity had seriously decreased in non-smokers>smokers>ex-smokers, while the damage was repaired in a shorter time in never smokers. Urinary excretion of oxidized guanine derivatives represent a global measure of oxidative stress in an organism and the body average rate by which either DNA, RNA or nucleic acids pool is oxidized (Poulsen et al., 2014). A reduction of urinary levels of this biomarker not necessary represents a decrease in the suffered damage but may reflect a inhibition of the repair systems rate. Further studies are necessary to confirm this hypothesis and to clarify the involved repair mechanisms and the role of urinary 8-oxoGua.

5 Conclusions

Even though there are some limitations in this study due to the absence of environmental personal data, low range of benzene exposure as well as a relative small number of subjects, urban benzene exposure measured by its internal metabolites (U-Benz, *S*-PMA and *t,t*-MA), U-MTBE and cotinine may influence the levels of oxidative nucleic acids damage in urine of children, which suggests that even at low exposure levels, benzene may induce oxidative stress. *S*-PMA was a good tracer of exposure to complex mixtures of pollutants inducing oxidative damage to nucleic acids. Sampling time is important to better interpret correlations between different biomarkers, both of exposure and of oxidative damage, taking into account different kinetics and metabolic pathways.

Competing ~~Interests~~interests

Authors declare that they have no competing interests.

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Highlights

- Biomarkers of exposure and of DNA oxidation are dependent on the urbanization levels.
- Same biomarkers increased in the evening sample as compared to next morning one.
- In urine of children, 8-oxodGuo is positively correlated with all benzene biomarkers.
- A dose-effect curve between 8-OxodGuo and *S*-PMA is observed at low exposure levels.

Queries and Answers

Query:

Please confirm that given names and surnames have been identified correctly and are presented in the desired order.

Answer: Yes

Query:

“WHO, 2005” has been changed to “WHO, 2000” as per the reference list. Please confirm.

Answer: Yes

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The reference given here is cited in the text but is missing from the reference list – please make the list complete or remove the reference from the text: "Wiesel, 2010".

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