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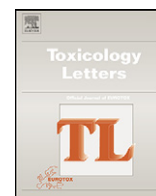
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Biomarkers of nucleic acid oxidation, polymorphism in, and expression of, *hOGG1* gene in styrene-exposed workers

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ABSTRACT

This study investigated nucleic acid oxidation associated with styrene exposure, mRNA expression levels of *hOGG1* gene and the role of the genetic polymorphism *Ser326Cys* of human 8-oxoguanine DNA N-glycosylase 1 (*hOGG1*) in 60 styrene-exposed workers and 50 unexposed clerks. Biomarkers of exposure (styrene in blood, mandelic and phenylglyoxylic acids and 4-vinylphenol in urine) and urinary biomarkers of nucleic acid oxidation, namely 8-oxo-7,8-dihydro-2'-deoxyguanosine (U-8-oxodGuo), 8-oxo-7,8-dihydroguanosine (U-8-oxoGuo) and 8-oxo-7,8-dihydroguanine (U-8-oxoGua) were determined by liquid chromatography–tandem mass spectrometry. The levels of 8-oxodGuo adduct and 2'-deoxyguanosine (dGuo) were measured by HPLC in DNA from white blood cells (WBC). Genomic DNA and RNA from blood samples were used to characterize the *Ser326Cys* polymorphism and the mRNA expression levels of the *hOGG1* gene, respectively, by PCR-based methods. Exposed workers showed lower values of 8-oxodGuo/10⁵ dGuo ratio in WBC-DNA but higher concentrations of U-8-oxoGuo compared to controls ($p = 0.002$ and $p = 0.008$, respectively, *t*-test for independent samples). In the whole group, all urinary biomarkers of nucleic acid oxidation correlated with both the sum of mandelic and phenylglyoxylic acids ($\rho > 0.33$, $p < 0.0001$) and 4-vinylphenol ($\rho > 0.29$, $p < 0.001$), whereas 8-oxodGuo/10⁵ dGuo in WBC showed a negative correlation with exposure parameters ($\rho < -0.24$, $p < 0.02$). Subjects bearing the *hOGG1 Ser/Ser* genotype showed lower values of 8-oxodGuo/10⁵ dGuo in WBC than those with at least one variant Cys allele (0.34 ± 0.16 vs 0.45 ± 0.21 , $p = 0.008$). In the subgroup of *hOGG1 Ser/Ser* subjects, laminators showed lower levels of WBC 8-oxodGuo/10⁵ dGuo ratio and significantly higher concentrations of U-8-oxoGua than controls ($p = 0.07$ and $p = 0.01$, respectively, *t*-test for independent samples). Interestingly, workers showed higher levels of *hOGG1* expression compared to controls ($p < 0.0005$). Styrene exposure seems to be associated with oxidation damage to nucleic acids, particularly to RNA and with an induction of the BER system.

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Abbreviations: *hOGG1*, human 8-oxoguanine DNA N-glycosylase 1; 8-oxodGuo, 8-oxo-7,8-dihydro-2'-deoxyguanosine; 8-oxoGuo, 8-oxo-7,8-dihydroguanosine; 8-oxoGua, 8-oxo-7,8-dihydroguanine; dGuo, 2'-deoxyguanosine; WBC, white blood cells; MTH1, mutT homolog-1; BER, base excision repair; 7,8-SO, styrene-(7,8)-oxide; MA, mandelic acid; PGA, phenylglyoxylic acid; 4-VP, 4-vinylphenol; LC-MS-MS, liquid chromatography–tandem mass spectrometry.

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1. Introduction

Styrene is a monomer widely used in the production of polymers, plastics and synthetic rubber. In humans, it is converted to styrene-(7,8)-oxide (7,8-SO) via the cytochrome P450 monooxygenase system (Nakajima et al., 1994). It is generally thought that most of styrene-induced genotoxicity is due to its electrophilic metabolite 7,8-SO, a highly reactive epoxide. Although styrene and 7,8-SO are known to induce both DNA adducts and DNA strand breaks in exposed workers, a recent re-evaluation of various genotoxic endpoints highlighted several inconsistencies in the overall current knowledge (Henderson and Speit, 2005; Vodicka et al., 2006). In

addition, the outcomes from cancer epidemiological studies were also inconclusive (Kogevinas et al., 1993). However, the International Agency for Research on Cancer (IARC) classified styrene as possibly carcinogenic with limited evidence for carcinogenicity in humans and experimental animals, whereas 7,8-SO as probably carcinogenic to humans (IARC, 1994).

A hypothesis has been postulated that oxidative stress arising as an imbalance between oxidant and antioxidant molecules may also contribute to the genotoxic effects of styrene (Marczynski et al., 2000). *In vitro* studies have demonstrated that exposure to styrene or 7,8-SO induces increased lipid peroxidation and DNA oxidation as well as glutathione depletion (Chakrabarti et al., 1993; Vettori et al., 2005). The guanine moiety of nucleotides represents one of the main targets for hydroxyl radicals and, depending on the molecular context (2'-deoxyribonucleotides, ribonucleotides, DNA, RNA), oxidized guanine may undergo different repair pathways resulting in different extracellular reaction products (Lunec et al., 2002; Cooke et al., 2008). 8-Oxo-7,8-dihydroguanine (8-oxoGua) in DNA is selectively cleaved by specific glycosylases of the base excision repair (BER) system, including the polymorphic 8-oxoguanine DNA N-glycosylase 1 (hOGG1) (Cooke et al., 2003). Alternatively, oxidized guanine may be released from DNA as 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodGuo) by an endonuclease-nucleotidase-based DNA repair system (Bessho et al., 1993). The same molecule is generated by the enzyme MTH1 (Tsuzuki et al., 2001) as product of repair of oxidized 2'-deoxyguanosine triphosphate in the cellular 2'-deoxyribonucleotide pool and by the nucleotide excision repair (NER) system, which releases oligonucleotides containing 8-oxodGuo (Patel et al., 2007). 8-Oxo-7,8-dihydroguanosine (8-oxoGuo) may originate from oxidized guanine in RNA, probably as a result of the turnover of the molecule, rather than as product of RNA repair mechanisms, that have not yet been well characterized (Nunomura et al., 2006). The turnover or repair of RNA may be responsible also for the generation of extracellular 8-oxoGua (Evans and Cooke, 2004).

In humans, 8-oxodGuo has been extensively studied either in DNA isolated from white blood cells (WBC) or as free urinary deoxynucleoside, although some inconclusive results have been reported in the field of occupational end environmental exposures (Pilger and Rudiger, 2006). More recently, the advent of liquid chromatography–tandem mass spectrometry (LC–MS–MS) has enabled the determination of oxidatively modified guanine derivatives in urine samples (U–), such as U-8-oxodGuo, U-8-oxoGuo, and U-8-oxoGua (Weimann et al., 2002). Such an approach limits the risk of artifactual oxidation during pre-analytical phases, owing to the minimal sample manipulation prior to injection. The combined evaluation of oxidized guanine derivatives both in DNA from white blood cells (WBC-DNA) and in urine may allow a better understanding of genotoxic mechanisms at the molecular level. DNA oxidation may be modulated by germ line variants in DNA repair genes, the most prominent being hOGG1. For this enzyme, a functional genetic polymorphism is known (Ser326Cys) and the variant allele distributes in Caucasian with a prevalence of about 20% (Marchand et al., 2002). Although the association between hOGG1 genotype and the enzyme activity of OGG1 has not been definitely proven so far (Weiss et al., 2005), experimental investigations demonstrate that the hOGG1 Cys326 isozyme has impaired function (by about 2-fold) compared to the Ser326 isoform (Luna et al., 2005; Bravard et al., 2009). In agreement with these data, a recent epidemiological study (Vodicka et al., 2007) has shown that subjects with the hOGG1 Cys/Cys genotype exhibit a 50% lower DNA repair capacity when compared to hOGG1 Ser/Ser subjects.

The present study was carried out to investigate the levels of oxidized guanine derivatives in a group of styrene-exposed workers with accurately characterized internal dose levels, and an

Table 1

Characteristics of the studied population and characterization of styrene exposure. Exposure biomarkers are reported as median and range.

| | Controls (n = 50) | Exposed (n = 60) |
|--------------------------------|---------------------|---------------------|
| Sex (male/female) | 39/11 | 42/18 |
| No. of current/never-smokers | 13/37 | 26/34 |
| Age (years) | 40.0 ± 12.1 (26–62) | 37.6 ± 11.1 (21–64) |
| Years of employment | – | 4.0 ± 3.4 (1–14) |
| Styrene air, mg/m ³ | n.d. | 107.4 ± 66.7 |
| Blood styrene, mg/L | 0.20 (n.d. to 0.43) | 1.20 (n.d. to 3.94) |
| MA + PGA, mg/g creatinine | 0.47 (0.17–3.24) | 286.7 (4.16–2022) |
| 4-VP, mg/g creatinine | 0.19 (0.01–4.08) | 3.39 (0.19–22.6) |

Note: 1 ppm of styrene is equal to 4.25 mg/m³. MA + PGA: mandelic acid + phenylglyoxylic acid; 4-VP: 4-vinylphenol; n.d.: not detectable.

unexposed control group. As an additional aim, we evaluated the modulating role of both gene expression and genetic polymorphism of the hOGG1 gene on oxidatively generated DNA damage associated with styrene exposure. In particular, we evaluated the relationships between the levels of the oxidized guanine in WBC-DNA (determined as 8-oxodGuo/10⁵ dGuo) that is the relevant substrate for hOGG1 activity and the urinary concentrations of the reaction product 8-oxoGua in subjects classified by the hOGG1 Ser326Cys polymorphism.

2. Materials and methods

2.1. Subjects and sampling

Sixty styrene-exposed workers employed in two plastics lamination plants in the same geographical area and 50 unexposed clerks volunteered to participate in the study. Confounding factors, like X-rays, medical drug treatment, dietary and lifestyle were carefully controlled by detailed questionnaire. The study was conducted on healthy individuals and exclusion criteria comprised a recent exposure to X-rays, current drug use or viral infections experienced in the last 3 months. The main characteristics of the studied population are reported in Table 1. The local Ethical Committee approved the study protocol and the participating subjects provided their written informed consent. The sampling of biological material was carried out according to the Helsinki Declaration (WHO, 1964).

Spot urine samples (50 mL) were collected at the end of the shift, divided into two aliquots and frozen at –20 °C until analysis. Blood samples (40 mL) were collected from all subjects in the middle of the work shift (ensuring saturation) on the same day as collection of urine samples.

2.2. Chemicals

Styrene (purity 99%), DL-mandelic acid (MA, 98%), phenylglyoxylic acid (PGA, 98%), 2'-deoxyguanosine (dGuo, 99–100%), and 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodGuo, 98%) were purchased by Sigma–Aldrich (Taufkirchen, Germany and Milan, Italy). 8-Oxo-7,8-dihydroguanosine (8-oxoGuo, 98%) and 8-oxo-7,8-dihydroguanine (8-oxoGua, 90%) were from Cayman (MI, USA). Isotopically labeled compounds used as either internal standards (ISs), i.e. [¹³C₁, ¹⁵N₂]8-oxoGua (¹³C₁, 98%, ¹⁵N₂, 98%) and [¹⁵N₅]Guo (96–98%, used as IS for 8-oxoGuo), or for the synthesis of ISs, i.e. [¹⁵N₅]dGuo (96–98%), were obtained from Cambridge Isotope Laboratories Inc. (MA, USA). [¹⁵N₅]8-oxodGuo had been synthesized from [¹⁵N₅]dGuo according to Hu et al. (2004), with minor modifications. All standards were used without further purification.

2.3. Styrene exposure at the workplace

The concentration of airborne styrene at the workplace was determined by personal dosimeters on the day of the sampling, as previously described (Vodicka et al., 1995). Results are summarized in Table 1.

2.4. Exposure biomarkers

Styrene in the blood was determined as previously described (Vodicka et al., 1995, 2001). Styrene metabolites, namely MA, PGA, and 4-VP were determined by LC–MS–MS as previously described (Manini et al., 2002). Concentrations of urinary metabolites were expressed as a function of creatinine concentration (mg/g creat.), measured by the method of Jaffe (Kroll et al., 1986). Sample with creatinine concentrations lower than 0.3 g/L or higher than 3.0 g/L were excluded from statistical analysis according to the American Conference of Governmental Industrial Hygienists recommendation (ACGIH, 2004). For quantitative analyses, calibrations were performed in a matrix by spiking pooled urine samples from non-exposed subjects with appropriate standard mixtures. The concentrations of styrene in blood and

styrene metabolites in styrene-exposed workers and unexposed clerks are reported in Table 1.

2.5. Urinary biomarkers of nucleic acid oxidation

Urinary levels of 8-oxodGuo, 8-oxoGuo and 8-oxoGua, were determined by isotopic dilution LC–MS–MS, by adapting the method proposed by Weimann et al. (2002). Filtered samples (0.2 mL) were added with an equal volume of IS mixture containing 2.5 µg/L of [¹⁵N₅]8-oxodGuo, 7.5 µg/L of [¹⁵N₅]Guo and 75 µg/L of [¹³C₁,¹⁵N₂]8-oxoGua dissolved in 100 mM lithium acetate (pH 6.4). Then, samples were kept at 37 °C for 10 min, vortexed, centrifuged (10 min at 10,000 × g) and injected (30 µL). Separation of urinary 8-oxodGuo, 8-oxoGuo and 8-oxoGua was performed on an Atlantis[®]dC₁₈ column (100 mm × 2.0 mm i.d., 3 µm; Waters, Milford, MA, USA) using variable proportions of 10 mM aqueous formic acid (pH 3.75) and methanol at a flow-rate of 0.2 mL/min. After the column, a flow of 0.07 mL/min of methanol was added to the chromatographic flow to improve the ionization efficiency. The analytes and the ISs were ionized in positive ion mode and the detection was obtained in selected-reaction monitoring mode by following the transitions: *m/z* 284 → 168 and *m/z* 289 → 173 for 8-oxodGuo and its IS [¹⁵N₅]8-oxodGuo; *m/z* 300 → 168 and *m/z* 289 → 157 for 8-oxoGuo and its IS [¹⁵N₅]Guo; and *m/z* 168 → 140 and *m/z* 171 → 142 for 8-oxoGua and its IS [¹³C₁,¹⁵N₂]8-oxoGua. For quantitative analysis, working calibrations were obtained by spiking pooled urines with standard solutions in the concentrations ranges 0.25–25 µg/L for 8-oxodGuo, 0.75–75 µg/L for 8-oxoGuo and 7.5–750 µg/L for 8-oxoGua. For each analyte, calibration curves were constructed by linear regression analysis of the analyte-to-IS area ratio vs the known concentration of analytes injected (*r*² > 0.998). The limits of quantification (LoQs) were 0.2, 0.3 and 1.0 µg/L for 8-oxodGuo, 8-oxoGuo and 8-oxoGua, respectively. Intra- and inter-day precision ranged between 2.5% and 6.8% for all analytes. Results were expressed as a function of creatinine concentration (µg/g creat.). All analytical determinations of urinary biomarkers were performed on a PE-Sciex API 365 triple-quadrupole mass spectrometer (Applied Biosystems, Thornhill, Canada) equipped with a TurbolonSpray[™] interface.

2.6. 8-OxodGuo adduct in WBC-DNA

Blood samples (9 mL) were collected in EDTA-treated tubes, immediately frozen at –20 °C and used for 8-oxodGuo adduct determination. White blood cells (WBC) were collected by centrifugation with 35 mL of 0.9% NH₄Cl, 10 mM Na₂EDTA similar to the procedure of Fichtinger-Schepman et al. (1987). DNA was extracted with chloroform following operation of Dahlhaus and Appel (1993) and recommendations of ESCODD (2002) to avoid artefacts during DNA preparation. 8-OxodGuo adduct isolation was carried out by previously published procedure (Marczynski et al., 2002). For the analysis of nucleosides in WBC-DNA, a Shimadzu HPLC/UV apparatus, connected to a Coulochem II (model 5200) electrochemical detector (ESA, Chelmsford, MA, USA), was used.

2.7. hOGG1 expression levels

Peripheral blood lymphocytes were isolated from 10 mL of peripheral blood and mRNA was immediately extracted by TRIzol[®], according to the producer's procedure (Invitrogen, Paisley, UK). RNA quality and quantity was checked by UV VIS spectrophotometry on Carry 300 (Varian, Palo Alto, CA, USA) and horizontal agarose gel electrophoresis. cDNA was synthesized from 0.5–1 µg of total mRNA using First strand cDNA synthesis kit (MBI Fermentas, Vilnius, Lithuania). As a negative control, we used the same sample, but reverse transcriptase was omitted from the mixture. Quality of cDNA was confirmed by PCR amplification of fragment from the control gene ubiquitin C (Soucek et al., 2005). The PCR product (190 bp) in the negative sample indicates whether cDNA was contaminated by genomic DNA (1009 bp).

Expression of hOGG1 gene was determined with a 7500 Real Time PCR System (Applied Biosystems, Foster City, USA) as absolute quantification, using TaqMan Gene Expression Assays 4331182 (Hs00213454.m1/hOGG1/, Applied Biosystems). As standards for absolute quantification of gene expression, we used bacterial plasmids containing coding sequences of hOGG1 and GAPDH, prepared by the Gateway[™] cloning technology (Invitrogen, Paisley, UK) as previously described (Baranová et al., 2005; Suzuki et al., 2005). The expression of hOGG1 was normalized to the expression of GAPDH (TaqMan Endogenous control Human GAPDH 4333764; Diodovich et al., 2004) and expressed in arbitrary units.

2.8. hOGG1 genotype analysis

Genomic DNA was extracted from 3 mL of peripheral whole blood by a commercial kit (PureGene, GENTRA SYSTEMS, Minneapolis, MN). hOGG1 Ser326Cys polymorphism (rs 1052133) was characterized according to a previously published PCR-RFLP method (Wikman et al., 2000). In the whole study group, the following figures were observed: hOGG1 Ser/Ser genotype was present in 69 individuals (65%), Ser/Cys in 30 (28%), and Cys/Cys in 7 (7%). Similar genotype frequencies were observed among workers (*n* = 59), i.e. 39 (66%) individuals with Ser/Ser, 18 (31%) with Ser/Cys, and 2 (3%) with Cys/Cys genotypes, and controls (*n* = 47), i.e. 30 (64%) with the Ser/Ser, 12 (26%) with the Ser/Cys and 5 (10%) with the Cys/Cys genotypes. These frequencies were consistent with the expectations for the Hardy–Weinberg equilibrium. Deter-

mination of hOGG1 genotypes was confirmed by random re-genotyping, using the TaqMan allelic discrimination assay (Applied Biosystems, Foster City, USA).

2.9. Statistical analyses

Statistical analyses were carried out by the SPSS software (version 14.0 for Windows[®], Chicago, IL). Although all analytical determinations were above the corresponding limit of detections, valid samples (having urinary creatinine between 0.3 and 3.0 g/L) amounted to 104 and complete information including urinary exposure indices, biomarkers of nucleic acid oxidation, and hOGG1 genotypes was available for 96 subjects. The normality of the distribution was assessed by the one-sample Kolmogorov–Smirnov test. Parametric statistical tests were applied to log-transformed values ensuring a normal distribution of variables. Biomarkers of nucleic acid oxidation were normally distributed, 4-VP followed a log-normal distribution, whereas MA+PGA values were not distributed normally or log-normally. Differences between the exposed and control groups were assessed using the *t*-test for independent samples. Pearson's *r* was used to assess the correlation between variables. Non-parametric tests were also applied, when necessary (Mann–Whitney *U*-test and Spearman's *rho*). The significance level for all tests was *p* ≤ 0.05 (two-tailed). Multiple linear regression analysis models were used to assess the contribution of sex, age, smoking habits, urinary creatinine concentration and exposure to styrene (as MA+PGA concentrations) to the variability of biomarkers of nucleic acid oxidation. Stepwise regression analyses were run using a significance level 0.05 for entry and 0.10 for variables' removal from the model. Due to the limited sample size, in statistical analyses the hOGG1 genotypes were collapsed into two groups (according to a recessive model of inheritance), one represented by the homozygous wild-type (Ser/Ser) subjects and the other including people bearing at least one Cys allele (Ser/Cys and Cys/Cys).

3. Results

3.1. Internal dose markers

The concentrations of internal dose markers, i.e. styrene in blood and urinary metabolites (MA, PGA, and 4-VP) in subjects classified according to exposure status (workers vs controls) are summarized in Table 1. As biomarkers were not normally distributed in the whole study group, their concentrations are reported as medians and ranges. MA+PGA concentrations in controls were lower than the 95th percentile of the frequency distribution of the biomarker in the general unexposed population, i.e. 3.5 mg/g creat. (Manini et al., 2004). Conversely, all workers showed MA+PGA concentrations higher than such value. The distributions of internal dose biomarkers in controls and workers did not overlap.

In the whole study group, markers of exposure significantly correlated to each other (*p* < 0.0005), the Spearman's correlation coefficients being *rho* = 0.64 between styrene in blood and MA+PGA, *rho* = 0.53 between styrene in blood and 4-VP, and *rho* = 0.90 between MA+PGA and 4-VP. All these correlations were observed in the worker group (*rho* = 0.49, 0.38 and 0.94, respectively, *p* ≤ 0.0005 for all) whereas among controls only urinary biomarkers were significantly correlated (*rho* = 0.65, *p* < 0.0005).

3.2. Biomarkers of nucleic acid oxidation

Urinary levels of oxidatively modified guanine derivatives significantly correlated to each other. The Pearson's correlation coefficients were *r* = 0.86 between U-8-oxodGuo and U-8-oxoGuo,

Table 2

Biomarkers of nucleic acid oxidation in controls (*n* = 50) and workers (*n* = 60). Urinary biomarkers have been expressed as a function of creatinine concentration. Values are expressed as mean ± S.D.

| Biomarker | Controls (<i>n</i> = 50) | Exposed (<i>n</i> = 60) | <i>p</i> |
|------------------------------------|---------------------------|--------------------------|----------|
| WBC 8-oxodGuo/10 ⁵ dGuo | 0.46 ± 0.23 | 0.33 ± 0.13 | 0.002 |
| U-8-oxoGua (µg/g creat.) | 16.92 ± 5.90 | 18.73 ± 8.35 | 0.739 |
| U-8-oxodGuo (µg/g creat.) | 3.81 ± 1.49 | 3.91 ± 1.38 | 0.243 |
| U-8-oxoGuo (µg/g creat.) | 4.55 ± 1.53 | 5.58 ± 2.03 | 0.008 |

U-: urinary; 8-oxodGuo: 8-oxo-7,8-dihydro-2'-deoxyguanosine; dGuo: 2'-deoxyguanosine; 8-oxoGua: 8-oxo-7,8-dihydroguanine; 8-oxoGuo: 8-oxo-7,8-dihydroguanosine.

Table 3

Predictors of the urinary excretion of biomarkers of nucleic acid oxidation (set as dependent variable) according to a stepwise multiple linear regression model: (U-biomarker) = constant + (U-creatinine) $\times \beta_1$ + (U-MA + PGA) $\times \beta_2$ + (Age) $\times \beta_3$. Values of constant and β coefficient, with S.E., partial r^2 (r_p^2) and significance (p) for each term are given. The adjusted r^2 (r_{adj}^2) 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.

| | U-8-oxodGuo | | | U-8-oxoGuo | | | U-8-oxoGua | | |
|----------------------------|-----------------|---------|---------|-----------------|---------|---------|----------------|---------|---------|
| | β (S.E.) | r_p^2 | p | β (S.E.) | r_p^2 | p | β (S.E.) | r_p^2 | p |
| Constant | 0.32 (0.24) | – | 0.179 | –1.19 (0.62) | – | 0.057 | 3.81 (1.12) | – | 0.001 |
| U-creatinine | 3.04 (0.23) | 0.642 | <0.0005 | 4.35 (0.30) | 0.674 | <0.0005 | 12.63 (1.08) | 0.578 | <0.0005 |
| U-MA + PGA | 0.0012 (0.0004) | 0.026 | 0.006 | 0.0019 (0.0006) | 0.029 | 0.001 | – | – | – |
| Age | – | – | – | 0.037 (0.014) | 0.020 | 0.01 | – | – | – |
| Whole model r_{adj}^2, p | | 0.662 | <0.0005 | | 0.714 | <0.0005 | | 0.573 | <0.0005 |

U-: urinary; 8-oxodGuo: 8-oxo-7,8-dihydro-2'-deoxyguanosine; 8-oxoGuo: 8-oxo-7,8-dihydroguanosine; 8-oxoGua: 8-oxo-7,8-dihydroguanine; MA + PGA: mandelic acid + phenylglyoxylic acid.

$r=0.65$ between U-8-oxodGuo and U-8-oxoGua, and $r=0.75$ between U-8-oxoGuo and U-8-oxoGua ($p<0.0005$). These correlations were also observed within the subgroups of workers and controls (data not shown). Urinary biomarkers of nucleic acid oxidation were strongly correlated with urine creatinine, the Pearson's correlation coefficient ranging between 0.76 and 0.82 ($p<0.0005$).

No correlation was detected between WBC 8-oxodGuo/ 10^5 dGuo and urinary oxidized guanine derivatives. Moderate, but significant, correlations were observed among biomarkers of exposure and biomarkers of nucleic acid oxidation in the whole study group. WBC 8-oxodG/ 10^5 dGuo was inversely correlated with both MA + PGA ($\rho=-0.24$, $p=0.019$) and 4-VP ($\rho=-0.25$, $p=0.016$), whereas U-8-oxodGuo, U-8-oxoGuo and U-8-oxoGua were positively correlated with both MA + PGA ($\rho=0.33$, 0.41 and 0.34, respectively, $p<0.0001$) and 4-VP ($\rho=0.32$, 0.40 and 0.29, respectively, $p<0.001$). Age did not modulate significantly any of the above markers (data not shown).

The effect of exposure and potentially confounding factors, such as sex and smoking habits, on biomarkers of nucleic acid oxidation was initially assessed by univariate analyses. The WBC 8-oxodGuo/ 10^5 dGuo ratio was significantly lower in the exposed group than in the controls ($p=0.002$, t -test for independent samples, Table 2). As urinary creatinine was not significantly different in workers and controls (0.96 ± 0.61 g/L vs 0.79 ± 0.48 g/L, $n.s.$) and according to our yet unpublished results showing that urinary biomarkers of nucleic acid oxidation and urinary creatinine

showed similar kinetics of excretion, we normalized oxidation markers by creatinine concentration. Workers showed significantly higher U-8-oxoGuo levels compared to controls ($p=0.008$), whereas no significant difference was observed for U-8-oxoGua and U-8-oxodGuo (Table 2). No differences either in the levels of oxidation damage biomarkers or in the concentration of urinary creatinine were found in the group stratified by sex (data not shown). Conversely, in this sample, smokers showed higher levels of urinary creatinine than non-smokers (1.04 ± 0.54 g/L vs 0.79 ± 0.54 g/L, $p=0.028$).

Multiple regression models run to assess the role of styrene exposure (as MA + PGA) and other predictors (sex, age, smoking habits and urinary creatinine) on urinary biomarkers of nucleic acid oxidation substantially confirmed the findings of univariate analyses. The results of stepwise models are summarized in Table 3, where partial r^2 values are reported to evaluate the individual contribution of each predictor to the overall variance. Creatinine alone accounted for more than 58% of variance of all biomarkers (with partial r^2 ranging between 0.58 and 0.67, $p<0.0001$ for all). Significant relationships between urinary biomarkers of nucleic acid oxidation and exposure biomarkers were observed for U-8-oxodGuo ($p=0.006$) and U-8-oxoGuo ($p=0.001$), exposure accounting for 2.6 and 2.9% of variance, respectively. In any case, the smoking status and gender did not significantly affect the concentration of urinary biomarkers. Age significantly influenced the levels of U-8-oxoGuo only ($p=0.01$).

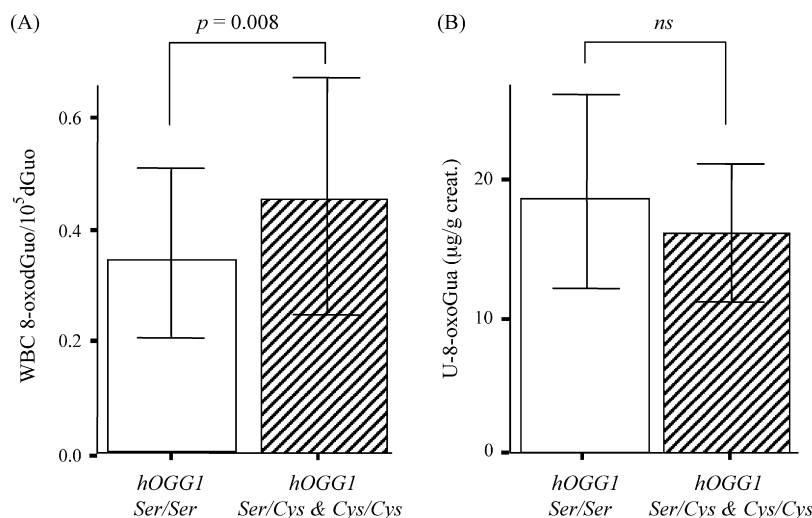


Fig. 1. Comparison between (A) the levels of the WBC 8-oxodGuo/ 10^5 dGuo ratio and (B) the concentrations of U-8-oxoGua in subjects stratified according to the *hOGG1* polymorphism (t -test for independent samples). Data are expressed as mean \pm S.D. *hOGG1* genotypes were collapsed into two groups (recessive model): the homozygous wild-type (*Ser/Ser*) genotype ($n=64$) and genotypes including at least one Cys allele (*Ser/Cys* and *Cys/Cys*, $n=35$).

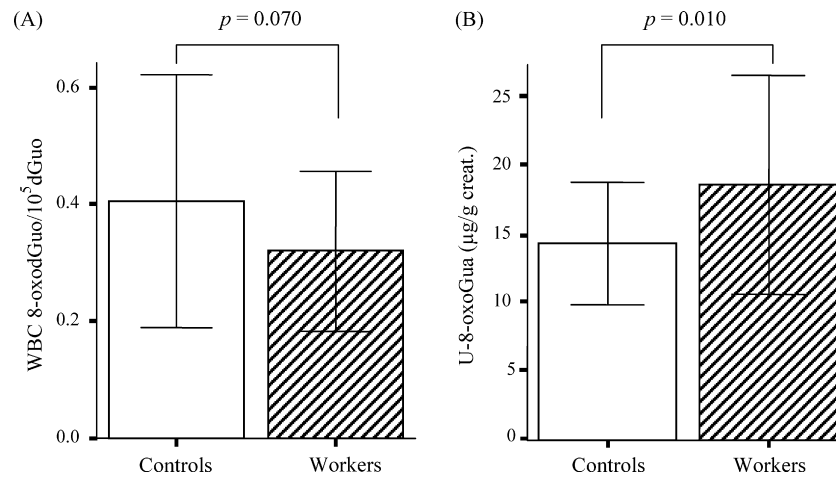


Fig. 2. Comparison between (A) WBC 8-oxodGuo/10⁵ dGuo and (B) U-8-oxoGua in controls and styrene-exposed workers. Only subjects bearing the *hOGG1 Ser/Ser* genotype were considered (*t*-test for independent samples). Data are expressed as mean ± S.D.

3.3. *hOGG1 Ser326Cys* polymorphism and expression levels of *hOGG1*

In the whole sample, subjects bearing the *Ser/Ser* genotype showed significantly lower levels of WBC 8-oxodGuo/10⁵ dGuo (about 26%, on average) than those carrying the variant *Cys* allele (0.34 ± 0.16 vs 0.46 ± 0.21 , $p = 0.008$, Fig. 1A). A similar trend was observed among workers (0.31 ± 0.11 vs 0.38 ± 0.17 , $p = 0.09$), but the difference was not significant. Fig. 1B shows that in the whole sample, individuals with the wild-type *Ser/Ser* genotype tended to excrete higher concentration of U-8-oxoGua than those carrying variant *Cys* allele (18.18 ± 7.91 µg/g creat. vs 16.76 ± 5.50 µg/g creat., *n.s.*). Among workers with homozygous wild-type genotype (*hOGG1 Ser/Ser*), significantly lower 8-oxodGuo/10⁵ dGuo levels (0.31 ± 0.11 vs 0.40 ± 0.22 , $p = 0.05$) and significantly higher concentrations of U-8-oxoGua than in controls (18.73 ± 8.01 µg/g creat. vs 14.4 ± 4.48 µg/g creat., $p = 0.01$) were observed (Fig. 2).

Styrene-exposed workers also showed increased levels of *hOGG1* expression compared to controls (0.0032 ± 0.0015 a.u. vs 0.0020 ± 0.0009 a.u., $p < 0.0005$, *t*-test for independent samples). Moreover, females showed higher levels of *hOGG1* expression compared to males (0.0032 ± 0.0013 a.u. vs 0.0025 ± 0.0014 a.u., $p = 0.022$).

The *hOGG1 Ser326Cys* polymorphism did not significantly affect the levels of *hOGG1* expression. No differences were observed between subjects bearing the *Ser/Ser* genotype and those carrying variant *Cys* allele, either in the whole sample (0.0027 ± 0.0015 a.u. vs 0.0026 ± 0.0013 a.u.), or in subgroups of styrene-exposed workers (0.0033 ± 0.0017 a.u. vs 0.0032 ± 0.0014 a.u.) and controls (0.0020 ± 0.0010 a.u. vs 0.0018 ± 0.0007 a.u.).

4. Discussion

This is the first communication reporting the application of a panel of blood DNA and urinary biomarkers of nucleic acid oxidation to characterize the extent oxidative stress in workers exposed to styrene. In the present study, urinary biomarkers of oxidation damage have been evaluated by a method based on isotopic dilution LC–MS–MS, which limits sample manipulation prior to analysis and further reduces the risk of guanine oxidation during preparative steps. It should be noted that the sources and the meaning of urinary nucleobases, 2'-deoxyribonucleosides and ribonucleosides reflecting oxidation damage to nucleic acids have not been fully characterized (Cooke et al., 2008). Whereas oxidation of 8-position of guanine in DNA leads to a unique product, determined

as 8-oxodGuo adduct in WBC–DNA, several urinary biomarkers may be generated, depending on the localization of the guanine residue (DNA, RNA or the nucleotide pool) and the specificity and efficiency of the involved repair systems.

As many recent studies pointed out, the net oxidative damage to DNA results from the three-way balance between oxidizing species, antioxidants and DNA repair (Friedberg, 2001). Thus, the actual levels of 8-oxodGuo/10⁵ dGuo in DNA from WBC are indicative of not yet repaired oxidation damage to DNA. The present study shows that styrene-exposed workers have significantly lower amount of oxidation damage in WBC–DNA compared to controls. An opposite result was previously reported in a small study, where higher levels of WBC 8-oxodGuo/10⁵ dGuo were found in styrene-exposed boat builders (Marczynski et al., 1997). Several aspects may account for the discrepancy, e.g. different exposure conditions, co-exposure to other oxidant compounds, like diisocyanates, and the limited group of 17 workers, precluding any robust statistical evaluation. In our study, significantly higher *hOGG1* transcript levels were observed among workers, suggesting an induction of *hOGG1* gene by styrene exposure, that would be fully consistent with the observed levels of WBC 8-oxodGuo/10⁵ dGuo.

Despite the debate about the measurement and the meaning of urinary oxidized guanine derivative species in urine (Cooke et al., 2008), we know that U-8-oxoGua originates, at least in part, from the glycosylase activity (BER) on oxidized guanine residues of DNA, but a major source is likely to be RNA, too. On the other hand, urinary concentrations of 8-oxodGuo may reflect either the repair (by MTH1 or NUDT1) of oxidized guanine triphosphate in the nucleotide pool or the repair of 8-oxodGuo from DNA (by an endonuclease/nucleotidase system) or even repair by NER (Patel et al., 2007). It is reasonable to assume that U-8-oxoGua originates from oxidation of RNA (Evans and Cooke, 2004). In our study, the urinary levels of U-8-oxoGua were significantly increased in workers compared to controls and significantly associated with styrene exposure, as a consequence of cytoplasmic oxidative stress. RNA is single-stranded and its bases are protected neither by hydrogen bonds nor by structural proteins and may be more susceptible to oxidative insults than DNA (Nunomura et al., 2006). Moreover, the localization of styrene metabolizing CYP in the endoplasmic reticulum that are an important source of ROS, is compatible with secondary oxidation of RNA molecules that are located in the neighbouring cytoplasm. Despite urinary concentrations of 8-oxodGuo were comparable in controls and exposed, they were significantly associated with styrene exposure in multivariate models. In the case of U-8-oxoGua, the product of *hOGG1* activity, we did not

observe any difference between exposed workers and controls and any association with styrene exposure, probably due to the individual genetic background, represented here by the *hOGG1* Ser326Cys polymorphism. In addition, RNA could be an additional source of extracellular 8-oxoGua.

Mice knocked out at the *OGG1* locus showed a 26% reduction in the levels of U-8-oxoGua compared to the wild-type strain (Rozalski et al., 2005). In agreement with above study, we found reduced WBC 8-oxodGuo/10⁵ dGuo levels in subjects bearing the *Ser/Ser* genotype, compared to those carrying the variant Cys allele. In the same subjects, we observed a complementary, though not significant, increase in mean U-8-oxoGua excretion in *Ser/Ser* subjects (Fig. 1). The effect of *hOGG1* polymorphism seems to be more specific for the substrate (8-oxodGuo/10⁵ dGuo in WBC-DNA) than for the reaction product (U-8-oxoGua), which may derive from alternative pathways. The *hOGG1* Ser326Cys polymorphism did not affect the levels of *hOGG1* mRNA expression and this is consistent with the functional nature of the polymorphism that affects the enzyme activity by the Ser326Cys change in the primary protein structure (Bravard et al., 2009).

To evaluate the interaction between *hOGG1* polymorphism and styrene exposure, we limited the analysis to individuals with the wild-type *Ser/Ser* genotype. In this subgroup of subjects, workers showed lower levels of the substrate 8-oxodGuo/10⁵ dGuo in WBC and significantly higher concentrations of reaction product U-8-oxoGua than controls (Fig. 2), suggesting that styrene exposure may induce BER enzymes. In agreement with this, the lower levels of WBC 8-oxodGuo/10⁵ dGuo in exposed subjects are accompanied with the higher *hOGG1* expression levels, providing pilot mechanistic data.

We observed a strong correlation between urinary concentrations of oxidatively modified guanine derivatives and creatinine (Pearson's $r > 0.76$, $p < 0.0005$). This result has never been reported before. It is recommended that, in future studies, possible differences in urinary creatinine should be checked in the investigated groups and results should be confirmed by multivariate models including urinary creatinine concentration as covariate or independent variable (Barr et al., 2005). Although urinary creatinine alone accounted for more than 58% of total biomarker variance, regression analysis revealed that about 2–3% of the variability of U-8-oxodGuo and U-8-oxoGua could be ascribed to styrene exposure (Table 3). However, our data should be considered cautiously, due to the limited sample size of the study population. Comparison of present results with those published earlier by Marczyński et al. (1997) and Vodicka et al. (2004) shows the importance of a well-matched control group without any exposure to styrene.

The present study seems to indicate that styrene exposure is associated with oxidation damage to nucleic acids, particularly to RNA, and with an induction of the BER system, as suggested by increased *hOGG1* expression levels in exposed workers and by the complementary distribution of DNA damage in blood and repaired DNA damage in urine in subjects bearing the *hOGG1* *Ser/Ser* genotype (Fig. 2). Within the exposure range explored in this study, styrene-induced oxidative damage appeared to be counterbalanced by DNA repair capacity. About 75% of workers showed urinary concentrations of MA + PGA lower than 600 mg/g creat., a value corresponding to the Biologischer Arbeitsstoff-Toleranz-Wert (BAT, biological tolerance values) proposed by the German Deutsche Forschungsgemeinschaft (DFG). This value corresponds to the maximum workplace concentration (MAK) of airborne styrene that do not have an impact on health even for long-term exposure, i.e. 20 ppm (DFG, 2007). For these workers, the BER mechanisms appeared to be effective in removing oxidation damage from WBC DNA. Conversely, Fracasso et al. (2009) recently reported a significant decrease of DNA repair activity in workers exposed to about 47 ppm styrene compared to controls. We speculate that

styrene exposure could result in the induction of DNA oxo-repair enzymes, though this mechanism could be overwhelmed by higher dose levels. Interestingly, the threshold for the onset of clastogenic genotoxic effects due to styrene exposure has been identified at 125 mg/m³ (or 30 ppm) (Nestmann et al., 2005). Below this threshold, the induction of DNA repair enzyme would be sufficient to balance the DNA damage, as shown by the results of our study. Above this threshold, a reduced DNA repair capacity (Fracasso et al., 2009) could lead to the persistence of reactive styrene metabolites responsible for clastogenic effects of styrene in humans.

The present data are consistent with our previous findings, indicating that both single strand break repair and removal of oxidized guanine are induced by styrene exposure, as assessed by methods based on the comet assay (Vodicka et al., 2004). In this study, we focused on accurate analyses of specific oxo-adducts in various biological matrices, both revisiting well-know biomarkers of nucleic acid oxidation, like 8-oxodGuo/10⁵ dGuo in WBC-DNA and U-8-oxodGuo, and investigating less frequently considered biomarkers, like U-8-oxoGua and U-8-oxoGua.

5. Conclusion

This study demonstrated a significant relationship between styrene exposure and biomarkers of nucleic acid oxidation. Among urinary biomarkers, 8-oxoGua (specific for RNA oxidation) and 8-oxodGuo (biomarker of DNA oxidation) were the most susceptible to oxidative damage secondary to styrene exposure, whereas 8-oxoGua (the main product of DNA oxidation) was increased only in exposed subjects bearing the *hOGG1* *Ser/Ser* genotype. The *hOGG1* Ser326Cys polymorphism could display some interference as an effect modifier *in vivo*. Finally, at the observed exposure levels, styrene was able to induce the expression and the activity of *hOGG1*.

Conflict of interest statement

Authors declare that they have no competing interests.

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