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Title: Styrene-oxide N-terminal valine haemoglobin adducts in reinforced plastic workers: possible influence of genetic polymorphism of drug-metabolising enzymes

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Keywords: Styrene; occupational exposure; haemoglobin adducts; biological monitoring; genetic polymorphisms

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Abstract: Styrene is one of the most important organic chemicals used worldwide. In humans, styrene metabolism involves oxidation by cytochrome P450 monooxygenases (CYPs) to styrene-7,8-oxide, an epoxide thought to be responsible for the genotoxic effects of styrene exposure, and detoxification by means of epoxide hydrolase (mEH) and glutathione S-transferases (GSTs). The objective of this study was to investigate if genetic polymorphisms of metabolic enzymes modulate the level of urinary styrene metabolites and styrene oxide adducts with N-terminal valine of human globin (SO-Hb) in 75 workers occupationally exposed to styrene and 77 unexposed controls. The mean air concentration of styrene in the breathing zone of workers (30.4 ppm) was higher than the threshold limit value of 20 ppm recommended by the American Conference of Governmental Industrial Hygienists (ACGIH), and the biological exposure index adopted by the ACGIH for exposure to styrene prior to the next shift (MA+PGA= 400mg/g creatinine) was exceeded, indicating that styrene exposure for this group of workers was higher than recommended. A highly significant correlation was observed between styrene concentration in the breathing zone and the MA+PGA in urine of

workers ($r=0.85$, $P<0.001$). The levels of SO-Hb adducts in exposed workers were significantly increased as compared with controls, although no difference was observed between subjects stratified as high and medium exposure categories based on MA+PGA excretion. Regarding the effect of the genetic polymorphisms we found that the level of SO-Hb adducts might be modulated by the predicted mEH enzymatic activity in the exposed workers. From our data we conclude that SO-Hb adduct measurement is a complementary method to MA+PG measurement for assessing exposure to styrene at occupational and environmental levels, which reflects a more extensive exposure period.

Styrene is widely used in the production of various plastics, synthetic rubber and resins. Occupational exposure occurs mainly via inhalation and relatively high exposure occurs due to its use in manual application techniques.

The aim of this study was to evaluate if genetic polymorphisms of metabolic enzymes modulate the level of urinary styrene metabolites and styrene oxide adducts with N-terminal valine of human globin (SO-Hb) in 75 workers occupationally exposed to styrene and 77 unexposed controls.

The mean air concentration of styrene in the breathing zone of workers (30.4 ppm) was higher than the threshold limit value of 20 ppm recommended by the American Conference of Governmental Industrial Hygienists (ACGIH), and the biological exposure index adopted by the ACGIH for exposure to styrene prior to the next shift (MA+PGA= 400mg/g creatinine) was exceeded, indicating that styrene exposure for this group of workers was higher than recommended

The levels of SO-Hb adducts were significantly higher ($P<0.01$) in the exposed subjects (5.98 ± 0.41 pmol/g globin) when compared with controls (2.59 ± 0.25 pmol/g globin) and a significant difference ($P<0.02$) was found in levels of SO-Hb adducts between non-smokers (2.19 ± 0.27 pmol/g globin) and smokers (3.55 ± 0.51 pmol/g globin) among the control group. From our data we conclude that SO-Hb adduct measurement is a sensitive and specific means of assessing exposure to styrene at occupational and environmental level.

Regarding the effect of the genetic polymorphisms, we found that the level of SO-Hb adducts might be modulated by the predicted mEH enzymatic activity in the exposed workers. In exposed individuals, *EPHX1* genotypes associated with low hydroxylation reaction yielded an increase of SO-Hb adducts levels compared to high activity *EPHX1* genotypes. Since we know that mEH rapidly converts SO to phenyl

ethylene glycol (which is considered as rather non-toxic), in the case of excessive styrene exposure we can hypothesise that individuals with low enzymatic activity have higher levels of free SO that can rapidly react with the N-terminal valine in haemoglobin. However, this result should be regarded as preliminary, since the number of individuals in each category of mEH expected enzymatic activity is small.

"Response to Reviews"

- In order to respond to the first comment of the referee, I agree that the last sentence of the abstract is a little bit strong, as we show that MA+PGA correlated with styrene in air but Hb adducts did not. (However the Hb adducts are measuring dose over a much longer period that MA+PGA). I have suggested an alternative end to the abstract and on page 15.

- The reviewer's second comment relates to background levels and the effect of smoking. I have added a bit more about background levels on page 14 (add new reference Toxicology Letters, 1999, vol 108, 117-126). I presume that any effects of smoking in the exposed population may be overwhelmed by the variation in occupational exposure over the lifetime of the adduct formation. It is also conceivable that there is saturation in the metabolite production or adduct formation! This would explain why high doses do not increase the adduct and why smoking does not increase the adduct in the exposed population. I have modified the text a little on page 14 .

- Concerning to normality of the variables only the styrene in air departed significantly from the normality.

MA+PGA (exposed): Median=381and Mean=443mg/g cr;

Hb Adducts (control): Median=1.95 and Mean=2.59pmol/g globin

and exposed median=5.78 and mean=5.98pmol/g globin

- As said the reviewer the limit of detection is expressed in mg/L instead of mg/g creatinine, in the paper we put in mg/g creatinine by distraction!

- Finally, as suggested by the reviewer we changed the Table 5 and the text in page 11 concerning to this table.

LIST OF CHANGES

- Abstract, line 19"…, although no difference was observed between subjects stratified as high and medium exposure categories based on MA+PG excretion."

- Abstract, line 22 "From our data we conclude that SO-Hb adduct measurement is a complementary method to MA+PG measurement for assessing exposure to styrene at occupational and environmental levels, which reflects a more extensive exposure period."

- Statistical analysis, page 8, line 16, "The level of styrene in air was the only parameter that departed significantly from normality (median=19.0 ppm vs mean=30.4 ppm in exposed group)."

- Results, page 9, line 8 "…limit of detection (14 mg/L)."

- Since all the individuals in control group had values of MA and PGA below the LOD we did not use in the statistical analysis.

- Discussion, page 14, line 3 "…and exposure data a significant difference…"

- Discussion, page 14, line 7 "An effect in the exposed group may be undetectable because it is overwhelmed by the variability in the adduct levels induced by occupational exposure, or conceivably because of saturation in the metabolic activation process of styrene and/or adduct formation."

- Discussion, page 14, line 15 "Rappaport and Yeowell O'Connell calculate that the background levels of the SO-albumin adduct are too great to have arisen from non-occupational exposures to styrene or from cigarette smoking (Rappaport and Yeowell O'Connell, 1999). Possibilities were suggested that these adducts represent unknown exposures or endogenous production of reactive species which react with proteins to produce the same analyte in the assay."

- Discussion, page 14, line 22 “but despite the fact that a significant correlation was found between MA+PGA and SO-Hb adducts, this difference was not statistically significant. It should be appreciated also however that the exposure classification was based on urinary metabolites which reflect a different exposure period than Hb adducts.

Styrene-oxide *N*-terminal valine haemoglobin adducts in reinforced plastic workers: possible influence of genetic polymorphism of drug-metabolising enzymes

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Abstract

Styrene is one of the most important organic chemicals used worldwide. In humans, styrene metabolism involves oxidation by cytochrome P450 monooxygenases (CYPs) to styrene-7,8-oxide, an epoxide thought to be responsible for the genotoxic effects of styrene exposure, and detoxification by means of epoxide hydrolase (mEH) and glutathione S-transferases (GSTs). The objective of this study was to investigate if genetic polymorphisms of metabolic enzymes modulate the level of urinary styrene metabolites and styrene oxide adducts with N-terminal valine of human globin (SO-Hb) in 75 workers occupationally exposed to styrene and 77 unexposed controls. The mean air concentration of styrene in the breathing zone of workers (30.4 ppm) was higher than the threshold limit value of 20 ppm recommended by the American Conference of Governmental Industrial Hygienists (ACGIH), and the biological exposure index adopted by the ACGIH for exposure to styrene prior to the next shift (MA+PGA= 400mg/g creatinine) was exceeded, indicating that styrene exposure for this group of workers was higher than recommended. A highly significant correlation was observed between styrene concentration in the breathing zone and the MA+PGA in urine of workers ($r=0.85$, $P<0.001$). The levels of SO-Hb adducts in exposed workers were significantly increased as compared with controls, although no difference was observed between subjects stratified as high and medium exposure categories based on MA+PGA excretion. Regarding the effect of the genetic polymorphisms we found that the level of SO-Hb adducts might be modulated by the predicted mEH enzymatic activity in the exposed workers. From our data we conclude that SO-Hb adduct measurement is a complementary method to MA+PG measurement for assessing exposure to styrene at

occupational and environmental levels, which reflects a more extensive exposure period.

Keywords: Styrene; occupational exposure; haemoglobin adducts; biological monitoring; genetic polymorphisms

1. Introduction

Styrene is a monomer widely used in the production of plastics, synthetic rubber and polyester resins. The highest human exposure to styrene occurs during the production of fiberglass-reinforced polyester products (Miller *et al.,* 1994), where unsaturated resins containing about 40% styrene as a reactive diluent are commonly used, and as much as 10% of it can evaporate into the workroom air during lamination by hand procedures.

In humans, styrene metabolism has been well characterised (Sumner and Fennell, 1994). The first step of styrene metabolism is oxidation by cytochrome P450 enzymes (CYP) including CYP2E1, CYP2B6, CYP1A2 and other isozymes (Nakajima *et al.,* 1994) to styrene-7,8-oxide (SO), which is a highly reactive epoxide and has been classified as a probable human carcinogen (Group 2A) (IARC, 1994). Styrene-7,8-oxide is subsequently hydrolysed by microsomal epoxide hydrolase (mEH), producing phenylethylene glycol, which is further oxidized by alcohol dehydrogenase and aldehyde dehydrogenase to the main urinary metabolites, mandelic acid (MA) and phenylglyoxylic acid (PGA). A minor metabolic pathway is the conjugation of SO with glutathione via glutathione-S-transferases (GSTs), resulting in subsequent formation of phenyl hydroxyethyl mercapturic acids (PHEMAs) (Sumner and Fennell, 1994). Biological monitoring of styrene exposure is achieved by measuring urinary metabolites MA and PGA at the end of the work shift and/or prior to the next shift (ACGIH, 2003). Numerous investigations have focused on the relationship between these two metabolites with airborne styrene concentrations (Apostoli *et al.,* 1983; Brenner *et al.,* 1991); however, individual differences in urinary metabolites were observed (Hallier *et al.,* 1995; Wenker *et al.,* 2001). Hallier *et al.* (1995) point to marked individual

differences in metabolism of styrene, hypothesized to be related to enzyme polymorphisms.

Blood protein adducts are generally stable and, unlike most DNA adducts, are not enzymatically repaired. They are therefore very suitable for use as exposure biomarkers. Proteins most commonly used in biomonitoring studies are haemoglobin (Hb) and albumin (Alb), which are easily isolated from blood and purified in large quantities. The relatively long and well-controlled life span of Hb (approximately 4 months in humans) is one important reason for choosing this protein as a dose monitor for electrophilically reactive compounds. Styrene-7,8-oxide is an electrophilic compound which forms covalently bound adducts with nucleophilic centres in proteins (e.g. Hb and albumin) (Christakopoulos *et al.,* 1993; Fustoni *et al.,* 1998; Johanson *et al.,* 2000), and its quantification may be used as an exposure biomarker. The sites of interaction of SO within Hb include the amino groups on the N-terminal amino acid (valine in both α and β chains of the protein), cysteine and histidine. Several studies have reported a styrene-related increase of N-terminal valine adducts in haemoglobin in reinforced plastic workers (Brenner *et al.,* 1991; Christakopoulos *et al.,* 1993; Vodicka *et al.,* 1999).

The aim of the present study was to evaluate the possible influence of individual genetic polymorphisms of styrene-metabolizing enzymes on the level of urinary styrene metabolites and styrene oxide adducts with N-terminal valine of human globin (SO-Hb) following occupational exposure to styrene.

2. Materials and methods

2.1. Study subjects

The study population consisted of 75 individuals, who have been occupationally exposed to styrene for 9 years (SD 10 years) in factories manufacturing glass-fibre reinforced plastics in Portugal. The workers were mainly involved in hand-spraying lamination processes using unsaturated polyester. The control group included 77 healthy volunteers of comparable sex and age distribution, with no history of styrene exposure and living in the same areas as the exposed individuals. Demographic characteristics of the study population are shown in Table 1.

Prior to the study, a questionnaire was supplied concerning health status, smoking habits, alcohol consumption, medication and occupational history. This study was conducted with the informed consent of all subjects and approval by the institutional ethical board of the Portuguese National Institute of Health.

2.2. Environmental monitoring

Personal air sampling was performed in the breathing zone for representative working periods (minimum 90 min each sample). Air samples were collected in activated charcoal tubes connected to battery-powered personal air sampling pumps operating at a flow rate of 100–200 ml/min. Charcoal tubes were analyzed using gas chromatography after desorption with 2 ml carbon disulfide according to the NIOSH method (NIOSH,

1994). Analysis of the samples allowed the calculation of the 8-h time-weighted average (TWA) level of exposure to styrene for each subject.

2.3. Analysis of MA and PGA in urine samples

Urine samples were collected before the start of the work shift on the following morning. The urinary metabolites of styrene, MA and PGA were measured in all subjects. Urinalysis was performed using a high-pressure liquid chromatography method (Kivisto *et al.,* 1993). In all urine samples, creatinine (Cr) was also measured according to Jaffe's method and the values of MA and PGA were expressed as mg/g Cr. All samples were coded and analyzed under blind conditions.

2.4. Determination of SO N-terminal valine (SO-Hb) adducts in human globin

SO-N-terminal valine adducts were analysed by gas chromatograph-mass spectrometry (VG 70-SEQ (Micromass; Manchester, UK) tandem instrument, linked with a Hewlett Packard Model 5890A gas chromatograph) using a modification of the protocol described by Tavares *et al.* (1996) and an octadeuterated internal standard (manuscript in preparation). The limit of detection was 1 pmol adducts/g globin.

2.5. Genotyping

DNA was isolated from lymphocytes using a commercially available kit according to the manufacturer's instructions (QIAamp DNA extraction kit; Qiagen, Hilden, Germany). *CYP2E1, GSTT1, GSTP1, GSTM1* and *EPHX1* polymorphisms were all determined as described by Teixeira *et al.* (2004). All genotype determinations were carried out twice in independent experiments and inconclusive samples were reanalysed. Regarding, the *EPHX1* genotypes, once the individuals were classified for codon 113 and 139 polymorphisms they were included in one of the following groups according to their expected enzymatic activity (Sarmanova *et al.,* 2000):

Low activity: His/His–His/His; His/His–His/Arg; Tyr/His–His/His; His/ His–Arg/Arg

Medium activity: Tyr/Tyr–His/His; Tyr/His–His/Arg; Tyr/His–Arg/Arg

High activity: Tyr/Tyr–Arg/Arg; Tyr/Tyr–His/Arg.

2.6. Statistical analysis

The normality of variables was evaluated by Kolmogorov–Smirnov test. The level of styrene in air was the only parameter that departed significantly from normality (median=19.0 ppm vs mean=30.4 ppm in exposed group). Statistical analysis of differences in SO-Hb adducts among the different groups was carried out using Student's t-test. Analysis of variance (ANOVA) was employed to test an association between biomarkers and the particular genotype. Bonferroni's correction was used for multiple comparisons among groups. The associations between two variables were analysed by Pearson's correlation. Statistical evaluation was conducted using SPSS for Windows statistical package, version 11.0 (Illinois, USA).

3. Results

The mean values, standard errors (SE) and ranges recorded for styrene in air, styrene metabolites in urine and for SO-Hb adducts are reported in Table 2. During sample preparation for the SO-Hb adducts, 6 control and 18 exposed samples were lost, due to technical reasons.

Our results showed that MA+PGA levels in the control group were below the limit of detection (14 mg/L). The study of workplace air revealed the presence of toluene and acetone, although air levels of these two chemicals were less than 1% of the styrene concentration. The styrene exposures, expressed as TWA for each subject, were between 0.5 and 114 ppm, mean 30.4 ppm (Table 2). Considering the current threshold limit value time-weighted average (TLV-TWA) for styrene (20 ppm) adopted by the American Conference of Governmental Industrial Hygienists (ACGIH, 2003), this level was exceeded for 36 (48%) workers. Biological exposure index (BEI) adopted by the ACGIH (2003) for exposure to styrene prior to the next shift (MA+PGA= 400 mg/g Cr) was exceeded in 36 (48%) workers; among this workers only 5 did not exceeded the TLV-TWA of styrene in the air (one worker 7 ppm and four workers with exposure levels 15-20 ppm).

Levels of SO-Hb adducts were significantly higher ($P<0.01$) in the exposed subjects (5.98 ± 0.41 pmol/g globin) when compared with controls (2.59 ± 0.25 pmol/g globin) (Table 2).

The effect of cigarette smoking on the levels of SO-Hb adducts was further investigated (Table 3). Among the control subjects the smokers had a significant increase of SO-Hb adducts when compared with the non-smokers ($P<0.02$). In the

exposed group, no significant difference was observed between smokers and non-smokers.

To further investigate the effect of styrene exposure on the levels of SO-Hb adducts, the subjects were classified into three exposure categories on the basis of urinary levels of MA+PGA [zero (controls); MA+PGA ≤ 400 mg/g Cr (medium–level exposure, mean 151 ± 16 mg/g Cr, range 23-390 mg/g Cr); MA+PGA > 400 mg/g Cr (high-level exposure, mean 813 ± 63 mg/g Cr, range 423-1770 mg/g Cr)]. The cut-off value, 400 mg/g Cr, was based on the BEI value for MA+PGA currently proposed by the ACGIH (2003) for exposure to styrene prior to the next shift. Figure 1 summarizes the mean levels of SO-Hb adducts recorded for the subjects stratified by styrene exposure. A significant difference was found between medium–level exposure group (5.71 ± 0.45 pmol/g globin) and control group (2.59 ± 0.25 pmol/g globin; $P<0.01$), and the high-level exposure group (6.25 ± 0.68 pmol/g globin) when compared with the control group ($P<0.01$). Levels of SO-Hb adducts in the high-level exposure group were higher than in the medium–level exposure group; however, the increase was not statistically significant.

A strong and significant correlation was found between the concentration of styrene in air and concentration of MA+PGA in the urine of the workers ($r=0.85$, $P<0.001$, $N=75$). A significant correlation was also found between the individual levels of MA+PGA and the SO-Hb adducts among exposed individuals ($r=0.30$, $P<0.02$, $N=57$). However, no correlation was obtained between SO-Hb adducts and concentration of styrene in the air, age and years of exposure.

The results of genotype analysis for sets of exposed workers, controls, and the whole set are presented in Table 4. The distribution of genotypes in each group was in Hardy–Weinberg equilibrium.

Table 5 shows results obtained for the effect of EPHX1 genetic polymorphisms studied on the levels of protein adducts. Concerning the *EPHX1* genotypes in codons 113 and 139, individuals were classified according to the expected enzymatic activity (Sarmanová *et al.,* 2000). We could not find any difference in the urinary metabolites for each genotype of styrene metabolizing enzymes. However, the levels of SO-N-terminal valine adducts in haemoglobin suggest that *EPHX1* genotypes might modulate the levels of this endpoint (Table5). A decrease in the levels of this biomarker is observed with an increase in EPHX1 activity in the exposed groups (altogether and by the level of exposure). No effect was observed for the other polymorphisms evaluated in this study on SO-Hb adducts.

4. Discussion

Our group of workers had highly variable exposure to styrene (0.5-114 ppm) and in 48% of them, the recommended TLV-TWA (20 ppm) by the ACGIH (2003) was exceeded, indicating that styrene exposure for this group of workers was higher than recommended. This was also confirmed by analysis of the urinary metabolites of styrene (MA+PGA), which in 48% of the cases was higher than the biological exposure index (BEI) proposed by the ACGIH (2003). The present data on styrene exposure clearly showed a good correlation between styrene concentrations in inhaled air and its urinary metabolites. The two major urinary metabolites, MA and PGA, are routinely used as biomarkers of internal dose of styrene, and a clear correlation between their levels and styrene in the workplace air has been reported in a number of studies (Vodicka *et al.,* 1999; Migliore *et al.,* 2006).

However, SO is metabolised by two distinct metabolic pathways: microsomal epoxide hydrolase (mEH) and glutathione-S-transferases (GST). In the urine of styrene exposed factory workers, the impact of these metabolic pathways has been measured by correlating variation in urinary metabolite levels with polymorphism in key metabolic enzymes, including CYP2E1, mEH, glutathione-S-transferase M1 (GSTM1), GSTT1 and GSTP1 (De Palma *et al.,* 2001; Haufroid *et al.,* 2001).

In this study we evaluated the effect of these genetic polymorphisms on the main metabolites of styrene in urine, MA+PGA. None of the different genotypes studied had an effect on the levels of MA+PGA. These results are in accordance with previous studies (De Palma *et al.,* 2001; Haufroid *et al.,* 2001). However, the studies cited above suggest that *GSTM1* has a major influence on the level of GST-conjugated styrene metabolites in urine, phenylhydroxyethyl mercapturic acids (PHEMA). De Palma *et al.*

(2001) found that individuals bearing the present *GSTM1* genotype excreted five to six times more PHEMA compared to null *GSTM1*, and there was no effect of *EPHX1*, *GSTT1*, and *GSTP1* polymorphisms on PHEMA excretion. Concerning the effects of these polymorphisms on the levels of MA+PGA, De Palma *et al.* (2001) only found a slight interference of the *GSTT1* genotype. Individuals with *GSTT1* had higher levels of MA+PGA when compared with the *GSTT1* null genotype. Haufroid *et al.* (2001) found that the highest urinary levels of PHEMA were present in workers with active *GSTM1* but there was little correlation with the *EPHX1* genotype.

The level of N-terminal valine adducts in haemoglobin served as an indicator of both internal exposure to styrene and the ability of styrene intermediate SO to attack nucleophilic sites. In accordance with some previous studies (Fustinoni *et al.,* 1988; Christakopoulos *et al.,* 1993), we observed that SO-Hb adducts were significantly higher in exposed subjects as compared to controls and correlated with internal dose parameter (MA+PGA).

In a previous study by Christakopoulos *et al.* (1993) an average haemoglobin adduct level of 18 pmol/g globin was observed in reinforced plastic workers who had been exposed to an average styrene concentration of 74 ppm (0.24 adducts per ppm of styrene) . The adduct level observed in this study is about a 3-fold less. However, the styrene air concentration in Christakopoulos´ study was about 2.5 fold higher than that of this study. The adduct levels observed in these two studies are therefore similar if we expressed the amount of adducts per unit of exposure (0.24 versus 0.20 adducts/ppm styrene). Significant differences have also been observed by Fustinoni *et al.* (1988), who found significant differences in Hb adduct levels between workers (average exposure estimated at 24 ppm) and controls.

Subjects were divided according to smoking status to determine any role that cigarette smoking may have on SO-Hb adduct formation. By comparing the smoking status and exposure data a significant difference was observed in levels of SO-Hb adducts between non-smokers and smokers within the control group. In contrast, no effect was observed within the exposed group when smoking status was taken into account. From these results we conclude that the only significant effect of smoking status on SO-Hb adducts is within the control group. An effect in the exposed group may be undetectable because it is overwhelmed by the variability in the adduct levels induced by occupational exposure, or conceivably because of saturation in the metabolic activation process of styrene and/or adduct formation.

This supports the hypothesis of Rappaport *et al.* (1996) that cigarette smoking is a source of SO-background adducts among people not occupationally exposed to styrene. Because this adduct is also observed in the blood of non-smoking individuals this indicates that other factors such as diet or environmental pollution can also contribute to the formation of SO-Hb adducts. The presence of background levels of alkylated proteins among control subjects as well as in unexposed experimental animals has been reported for several chemicals (Welie *et al.*, 1992; Farmer *et al.*, 1993) including styrene (Brenner *et al.*, 1991; Christakopoulos *et al.,* 1993; Rappaport *et al.*; 1996; Yeowell-O'Connell *et al.*, 1996). Rappaport and Yeowell O'Connell calculate that the background levels of the SO-albumin adduct are too great to have arisen from non-occupational exposures to styrene or from cigarette smoking (Rappaport and Yeowell O'Connell, 1999). Possibilities were suggested that these adducts represent unknown exposures or endogenous production of reactive species which react with proteins to produce the same analyte in the assay.

To better evaluate the effect of styrene exposure on the levels of SO-Hb adducts we stratified the subjects by styrene exposure on the basis of urinary levels of metabolites (zero, medium-level, and high-level exposure). After stratification by these three exposure categories, differences were found in levels of SO-Hb adducts between the two exposed groups (medium-level and high-level exposure) and the controls. The high-level exposure group had more SO-Hb adducts than the medium-level exposure group, but despite the fact that a significant correlation was found between MA+PGA and SO-Hb adducts, this difference was not statistically significant. It should be appreciated also however that the exposure classification was based on urinary metabolites which reflect a different exposure period than Hb adducts.

Age and exposure time did not influence the SO-Hb adducts, which is not surprising because SO-Hb adducts persist with a half span of erythrocytes (120 days), and their level is closely related to the concentration of SO in circulation.

Regarding the effect of the genetic polymorphisms, we found that the level of SO-Hb adducts might be modulated by the predicted mEH enzymatic activity in the exposed workers. In exposed individuals, *EPHX1* genotypes associated with low hydroxylation reaction yielded an increase of SO-Hb adducts levels compared to high activity *EPHX1* genotypes. Since we know that mEH rapidly converts SO to phenyl ethylene glycol (which is considered as rather non-toxic), in the case of excessive styrene exposure we can hypothesise that individuals with low enzymatic activity have higher levels of free SO that can rapidly react with the N-terminal valine in haemoglobin. However, this result should be regarded as preliminary, since the number of individuals in each category of mEH expected enzymatic activity is small.

In conclusion, the results of this study based on a reasonable number of observations show a strong correlation between styrene concentration in the workplace

and styrene urinary metabolites and that SO-Hb adduct measurement is a sensitive and specific means of assessing exposure to styrene at occupational and environmental level. The present results suggest the importance of individual susceptibility factors in modulating genotoxicity, although cautious interpretations are required since the size of the studied population limits the power of many of the analysis. Because the effects of these polymorphisms are relatively subtle, and some important alleles are relatively rare, a much larger study population will be necessary to evaluate their effects on biomarkers, especially when gene-gene interactions are considered. Conducting adequate studies for addressing these questions represents a major challenge.

Acknowledgments

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Table 1. Characteristics of the study subjects

Study population	**Controls (n=77)**	**Exposed (n=75)**
Males/ Females	53/24	54/21
Age, mean ± SD (years)	37 ± 11	34 ± 12
Years of employment, mean ± SD (range)		9 ± 10 (0.5-35)
Smokers/ Nonsmokers	23/54	23/52
Cigarettes/day, mean ± SD	5 ± 7	6 ± 6

Table 2. Results of external exposure and biomarkers of exposure in the studied population

Study population	Controls	Exposed Workers
Styrene (TWA) (ppm)	___	30.4 ± 3.7 (0.5-114)
MA+PGA (mg/g Cr)	Not detected	443 ± 44 (23-1770)
SO-Hb adducts (pmol/g globin)	2.59 ± 0.25 (N=71)	5.98 ± 0.41 (N=57)*

*P<0.01, when compared the SO-Hb adducts between the controls and the exposed group

Table 3. Effect of smoking status on the SO-Hb adducts (pmol/g globin)

	Controls	**Exposed workers**
Non-smokers	2.19 ± 0.27 (N=50)*	6.35 ± 0.56 (N =35)
Smokers	3.55 ± 0.51 (N =21)	5.39 ± 0.56 (N =22)

*P<0.02, when compared with smokers, in the same group

Table 4. Frequency of metabolic genotypes in the study population

Genotypes	All (%)	Controls (%)	Exposed (%)
CYP2E1 D/D	117 (79)	57 (74)	60 (83)
CYP2E1 D/C	32 (21)	20 (26)	12 (17)
CYP2E1 C/C	0	0	0
EPHX codon 113 Tyr/Tyr	97 (65)	49 (64)	48 (67)
EPHX codon 113 Tyr/His	37 (25)	21 (27)	16 (22)
EPHX codon 113 His/His	15 (10)	7 (9)	8 (11)
EPHX codon 139 His/His	109 (73)	52 (67)	57 (79)
EPHX codon 139 His/Arg	36 (24)	23 (30)	13 (18)
EPHX codon 139 Arg/Arg	4 (3)	2 (3)	2 (3)
GSTM1 present	77 (48)	40 (52)	37 (51)
GSTM1 null	72 (52)	37 (48)	35 (49)
GSTT1 present	125 (84)	67 (87)	58 (81)
GSTT1 null	24 (16)	10 (13)	14 (19)
GSTP1 Ile/Ile	75 (50)	41 (53)	34 (47)
GSTP1 Ile/Val	64 (43)	30 (39)	34 (47)
GSTP1 Val/Val	10 (7)	6 (8)	4 (6)

Table 5. Effect of *EPHX1* genetic polymorphisms on protein adducts[a]

Genotypes	Controls	Exposed		
		All	Medium exposure	High exposure
EPHX1-low actv	2.21±0.40 (20)	6.59±0.70 (17)	6.46±0.93 (9)	6.73±1.13 (8)
EPHX1-medium actv	2.70±0.38 (38)	6.26±0.61 (30)	5.47±0.60 (15)	7.04±1.05 (15)
EPHX1-high actv	2.87±0.52 (13)	4.27±0.51 (7)	4.89±0.70 (4)	3.45±0.48 (3)

[a]mean ± SE (nº)

Figure

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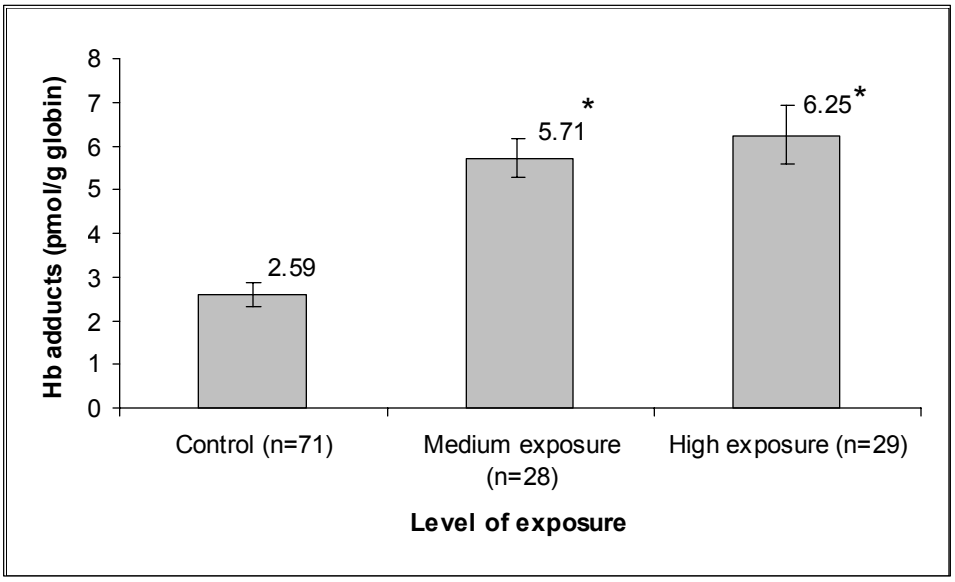

Figure 1. Levels of SO-Hb adducts by level of exposure

*P< 0.01, significant difference with regard to the control group