Interest of genotyping and phenotyping of drug-metabolizing enzymes for the interpretation of biological monitoring of exposure to styrene

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In the field of occupational and/or environmental toxicology, the measurement of specific metabolites in urine may serve to assess exposure to the parent compounds (biological monitoring of exposure). Styrene is one of the chemicals for which biological monitoring programs have been validated and implemented in environmental and occupational medicine. However, inter-individual differences in the urinary excretion exist both for the main end-products (mandelic acid and phenylglyoxylic acid) and for its specific mercapturic acids (phenylhydroxyethylmercapturic acids, PHEMA). This limits to a certain extent the use of these metabolites for an accurate assessment of styrene exposure. In a group of 26 volunteers selected with relevant genotypes, and exposed to styrene vapours (50 mg/m^3, 8 h) in an inhalation chamber, we evaluated whether genotyping or phenotyping relevant drug-metabolizing enzymes (CYP2E1, EPHX1, GSTM1, GSTT1 and GSTP1) may help to explain the observed inter-individual variability in the urinary metabolite excretion. Peripheral blood lymphocytes were used for genotyping and as reporter cells for the phenotyping of CYP2E1 and EPHX1. The *GSTM1* genotype was clearly the most significant parameter explaining the variance in urinary PHEMA excretion (6-fold lower in *GSTM1* null subjects; $P < 0.0001$) so that systematic *GSTM1* genotyping should be recommended routinely for a correct interpretation of PHEMA urinary levels. Variant alleles *CYP2E16* (7632T>A) and *His^{113}EPHX1* were associated with a significant reduction of, respectively, the expression ($P = 0.047$) and activity ($P = 0.022$) of the enzyme in peripheral blood lymphocytes. In combination with *GSTM1* genotyping, the phenotyping approach also contributed to improve the interpretation of urinary results, as illustrated by the combined effect of CYP2E1 expression and *GSTM1* allelic status that explained 77% of the variance in PHEMA excretion and allows the recommendation of mercapturates as specific and reliable biomarkers of exposure to styrene. *Pharmacogenetics* 12:691–702 © 2002 Lippincott Williams & Wilkins**

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Introduction

Styrene monomer remains an important chemical used worldwide in many applications, including the manufacture of polymers and reinforced plastics. Although percutaneous absorption is a potential exposure pathway in industrial settings, occupational and environmental exposures to styrene occur predominantly via inhalation [1]. To protect workers exposed to styrene from its toxic effects (neurotoxicity, irritation and central nervous system depression), the American Conference of Governmental Industrial Hygienists (ACGIH) reduced in 1997 the threshold limit value in air for a conventional 8-h workday and a 40-h workweek from 213 to 85 mg/m^3 (50–20 p.p.m.) [2]. Styrene has also been classified as possibly carcinogenic to humans (group 2B) by the International Agency for Research on Cancer (IARC) [3]. In humans, the metabolism of styrene is well characterized [4]; its biotransformation pathways are summarized in Fig. 1. The first step of styrene metabolism is via oxidation by cytochrome P450 enzymes (CYP) to styrene 7,8-oxide (SO). This oxidation leads to chiral SO (*R*-SO and *S*-SO), which is mainly hydrolyzed to styrene glycol (SG) by the microsomal epoxide hydrolase (EPHX1), and subsequently oxidized by alcohol and aldehyde dehydrogenases to the main urinary metabolites, mandelic acid and phe-

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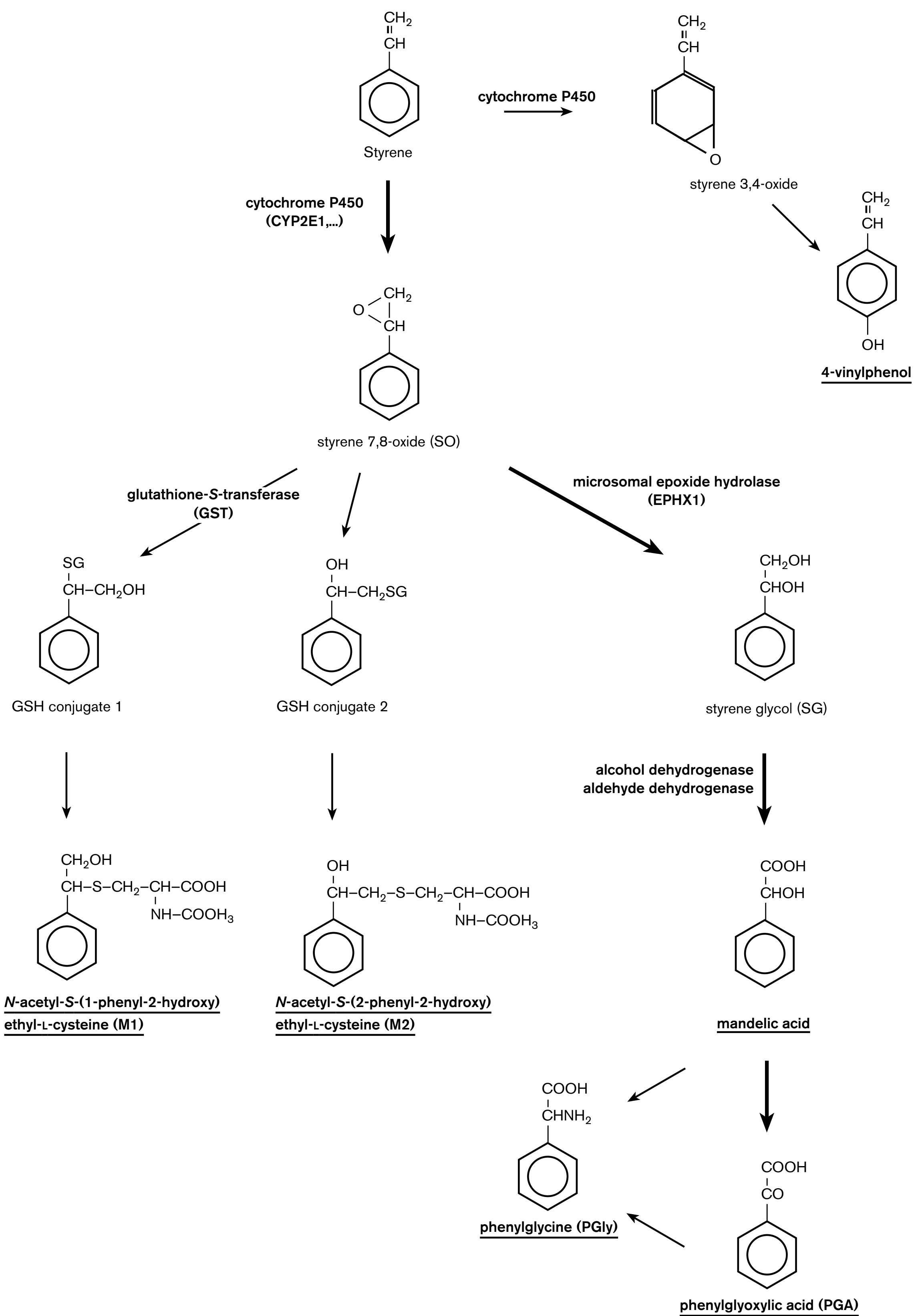
Fig. 1



Biotransformation pathways of styrene in humans.

nylglyoxylic acid (PGA) (major pathway). Mandelic acid and PGA represent more than 95% of the urinary metabolites of styrene. Further transformation of PGA and mandelic acid, via transamination of α-keto- and α-hydroxyacids into the corresponding aminoacid, leads to phenylglycine (PGly). In humans, a minor metabolic pathway (about 1% of absorbed styrene) is the conjugation of SO with GSH via glutathione *S*-transferases (GSTs). This reaction takes place on one of the two carbons in the side chain giving rise, after the classic mercapturic acid pathway, to phenylhydroxyethyl mercapturic acids (PHEMA), namely *N*-acetyl-*S*-(1-phenyl-2-hydroxy)ethyl-L-cysteine (M1) and *N*-acetyl-*S*-(2-phenyl-2-hydroxy)ethyl-L-cysteine (M2), which are regioisomers, each existing in two diastereoisomeric forms ([*R*,*R*] and [*R*,*S*]). One additional minor pathway of styrene metabolism results from 3,4-ring epoxidation giving rise to 4-vinylphenol excreted in urine [4]. So far, biological monitoring of exposure to styrene is routinely achieved by the measurement of mandelic acid and PGA in urine collected at the end of the shift and/or prior to the next shift [2]. As an alternative, the measurement of PHEMA in post-shift urine has also been explored [5]. With both approaches, inter-individual variability in urinary metabolite excretion has been observed. As an example, after exposure to a low range of styrene concentrations (20–200 mg/m^3), the inter-individual variation of mandelic acid or mandelic acid + PGA excretion in urine amounted to 31–33% for a similar absorbed dose of styrene [6]. Although potentially interesting as biomarkers of exposure because of their high specificity, the determination of PHEMA in post-shift urine is subject to even higher interindividual variability [5]. These variabilities could be explained, at least partly, by differences in biotransformation capacity.

Although CYP2E1 has been identified as the main isoform responsible for styrene metabolism in humans [7–9], other isoforms, particularly CYP2B6, could also be involved at high styrene concentrations [9,10]. Recent in-vitro data confirmed that, in human liver tissue, CYP2E1 was the primary enzyme involved in the high-affinity component of styrene oxidation and that CYP-mediated styrene oxidation was subject to considerable inter-individual variation (eight-fold variation in V_{max} values) [11]. Previous studies had already reported large variations in CYP2E1 activity in human populations [12]. Among possible factors accounting for the observed inter-individual variability, the polymorphisms of the *CYP2E1* gene have been largely investigated; at least three different *CYP2E1* variant alleles detectable with *Taq*I (*CYP2E1*1B*; 9896C>G), *Dra*I (*CYP2E1*6*; 7632T>A), *Rsa*I and *Pst*I (*CYP2E1*5A*; −1293G>C and −1053C>T) restriction enzymes have been described [13]. While reduced CYP2E1 activity in the presence of the rare *CYP2E1*6* allele has been suggested [14–16], the in-vivo significance of most of these polymorphisms remains far from clear, and is therefore thought to be of limited importance. Another source of variability in CYP2E1 activity is its high degree of regulation by environmental factors like xenobiotics (alcohol, drugs, etc.), nutritional status (dietary fat, fasting, body mass index, etc.) and health status (diabetes, etc.) [17]. The classical test to assess CYP2E1 activity *in vivo* relies on the hydroxylation of chlorzoxazone (CZX) by this enzyme [18]. This method has, however, some limitations including practical aspects (administration of a drug, multiple blood sampling and urine collection) and difficulties in the interpretation when CZX is administered with other xenobiotics [19]. Based on experimental observations, expression of *CYP2E1* in the lymphocyte fraction of white blood cells may be influenced by the same factors that regulate the expression of the hepatic enzyme [20,21]. Consequently, we have developed a method for CYP2E1 mRNA quantification in human peripheral blood lymphocytes (PBLs). This assay has been validated for human field and controlled studies [22]. It is very sensitive, specific and it offers the possibility to address a mode of induction of the enzyme, especially when genetic polymorphisms of *CYP2E1* are located in non-coding regions.

For *EPHX1*, two polymorphic sites have been observed in exons 3 ($Tyr^{113}His$) and 4 ($His^{139}Arg$). Based on in-vitro studies, the *His[113]* variant allele correlated with reduced EPHX1 activity, whereas the *Arg[139]* variant allele resulted in increased EPHX1 activity [23] but such effects were not reproduced in an in-vitro study on SO hydrolysis [24]. Similarly to CYP2E1, a phenotyping approach has also been proposed through the measurement of EPHX1 activity in human PBLs; preliminary results showed that, on an individual basis, there appeared to be a strong correlation between lymphocyte and liver EPHX1 activity [25]. As EPHX1 polymorphisms seem to impact on protein stabilization [26] measuring enzyme activity is the most appropriate phenotyping assay for this DME.

Finally, among the cytosolic GSTs, which to date have been found to include at least eight subfamilies (A, K, M, P, S, T, Z and O), two major polymorphic genes (i.e. *GSTM1* and *GSTT1*) have been found, and largely investigated during recent years. About 50 and 15% of Caucasians lack the *GSTM1* and *T1* genes, respectively, and therefore are also deficient for the respective enzyme activities. Two recent publications have clearly demonstrated the importance of *GSTM1* status in PHEMA urinary excretion [27–28]. A third, relatively widely studied, *GST* gene polymorphism is in the exon 5 ($Ile^{105}Val$) of *GSTP1*; compared to the wild-type enzyme, isoforms encoded by the *GSTP1 Val[105]* allele have been shown to have a higher efficiency on diol

epoxides of polycyclic aromatic hydrocarbons (PAH) [29] while, in contrast, they present a reduced activity towards chlorodinitrobenzene (CDNB) when expressed in *Escherichia coli* [30,31].

The purpose of this study, involving human volunteers exposed to styrene, was: (1) to clarify the usefulness of genotyping and phenotyping of relevant drug-metabolizing enzymes (DMEs) in the interpretation of biomarkers of exposure to styrene; and, meanwhile, (2) to explore the relationship between genotypes and phenotypes for CYP2E1 and EPHX1 in PBLs.

Materials and methods

Selection of volunteers and genotyping analysis

For the initial genotype screening, 100 male subjects, between 20 and 40 years of age, were recruited from a population of students from Lodz University (Lodz, Poland). They were asked to provide a blood sample for genotyping analysis of DMEs. Two *CYP2E1* variant alleles, i.e. *CYP2E1*6* [32] and *CYP2E1*1B* [33] were determined by restriction fragment-length polymorphism (RFLP) analysis, as described elsewhere [34]. The *GSTM1* and *GSTT1* genotypes were determined by a previously described multiplex PCR method. Briefly, albumin specific signal was amplified as an internal control, while the *GSTM1* and *GSTT1* specific signals revealed the presence of these genes, respectively [35]. Based on previous results from a field study, which pointed to *GSTM1* genotype as the main determinant of PHEMA excretion upon styrene exposure [27], volunteers were first classified according to their *GSTM1* status. Then *CYP2E1*1B*, *GSTT1* and *CYP2E1*6* status were taken into account. Results of the initial genotype screening are summarized in Table 1.

Based on this initial screening, 26 students were selected to obtain a balanced distribution between the variant alleles of *CYP2E1*, *GSTM1* and *GSTT1* (the latter among GSTM1 *null* volunteers). At this stage, their *GSTP1* and *EPHX1* genotypes were also determined. In *GSTP1* genotyping analysis, after PCR *Sna*BI restriction enzyme digestions were performed for aliquots of the amplification product; the presence of the *Sna*BI restriction site identified the *GSTP1 Val105* allele [36]. Similarly, in *EPHX1* genotyping analysis, after two separate PCRs *Asp*I and *Rsa*I restriction enzyme digestions were performed for aliquots of the amplification products; presence of the *Asp*I and *Rsa*I restriction sites identified the His^{113} [37] and Arg^{139} [23] alleles, respectively. Because the presence of another polymorphism located in codon 119 of *EPHX1* has been shown to interfere with the detection of Tyr^{113}/His^{113} and His^{113}/His^{113} genotypes using the RFLP method [38], these results were confirmed by a real-time PCR method, which is not affected by the codon 119 polymorphism [39]. The main characteristics of the final study population are summarized in Table 2.

Exposure of volunteers

The 26 volunteers were exposed to styrene at a stable concentration of 50 mg/m^3 $\pm$ 0.42% (about 12 p.p.m.) for 8 h in a chamber of 12 m^3 with an air turnover rate of 350 m^3/h. The generation of styrene vapors was based on continuous injection of liquid compound into the pressed air stream, with controlled speed. The

Table 1 **Initial genotype screening on 100 male volunteers**

GSTM1 positive ($n = 42$)	*CYP2E1* C^{9896}/C^{9896} ($n = 34$)	*GSTT1* positive ($n = 27$)	*CYP2E1* T^{7632}/T^{7632} ($n = 21$)	**5**[c]
			CYP2E1 T^{7632}/A^{7632} ($n = 6$)[b]	**2**
		GSTT1 null ($n = 7$)	*CYP2E1* T^{7632}/T^{7632} ($n = 6$)	
			CYP2E1 T^{7632}/A^{7632} ($n = 1$)	
	CYP2E1 C^{9896}/G^{9896} ($n = 8$)[a]	*GSTT1* positive ($n = 7$)	*CYP2E1* T^{7632}/T^{7632} ($n = 7$)	**4**
			CYP2E1 T^{7632}/A^{7632} ($n = 0$)[d]	
		GSTT1 null ($n = 1$)	*CYP2E1* T^{7632}/T^{7632} ($n = 1$)	
			CYP2E1 T^{7632}/A^{7632} ($n = 0$)[d]	
GSTM1 null ($n = 58$)	*CYP2E1* C^{9896}/C^{9896} ($n = 47$)	*GSTT1* positive ($n = 37$)	*CYP2E1* T^{7632}/T^{7632} ($n = 31$)	**3**
			CYP2E1 T^{7632}/A^{7632} ($n = 6$)	**2**
		GSTT1 null ($n = 10$)	*CYP2E1* T^{7632}/T^{7632} ($n = 5$)	**3**
			CYP2E1 T^{7632}/A^{7632} ($n = 5$)[e]	**3**
	CYP2E1 C^{9896}/G^{9896} ($n = 11$)	*GSTT1* positive ($n = 7$)	*CYP2E1* T^{7632}/T^{7632} ($n = 7$)	**4**
			CYP2E1 T^{7632}/A^{7632} ($n = 0$)[d]	
		GSTT1 null ($n = 4$)	*CYP2E1* T^{7632}/T^{7632} ($n = 4$)	
			CYP2E1 T^{7632}/A^{7632} ($n = 0$)[d]	

[a] *CYP2E1*1B* alleles.
[b] *CYP2E1*6* alleles.
[c] Number of volunteers selected in each group.
[d] *CYP2E1*1B* and *CYP2E1*6* alleles were never present simultaneously.
[e] One volunteer was homozygous for *CYP2E1*6* (not included in the 26 final volunteers).
CYP2E1 allele frequencies: **1B* (0.095) and **6* (0.095)
GSTM1-null (58%) and *GSTT1*-null (22%)

Table 2 Distribution of *CYP2E1*, *GST* and *EPHX1* genotypes in the group of 26 selected volunteers exposed to styrene

Genotypes		Number (%)	Allele frequencies
*CYP2E1*6*	T^{7632}/T^{7632}	19 (73)	Selected subjects
	T^{7632}/A^{7632}	7 (27)	
*CYP2E1*1B*	C^{9896}/C^{9896}	18 (69)	Selected subjects
	C^{9896}/G^{9896}	8 (31)	
GSTM1	Positive	11 (42)	Selected subjects
	Null	15 (58)	
GSTT1	Positive	20 (77)	Selected subjects
	Null	6 (23)	
GSTM1-GSTT1[a]	Null-positive	9 (60)	Selected subjects
	Null-null	6 (40)	
GSTP1 exon5	Ile^{105}/Ile^{105}	14 (54)	Ile^{105}: 0.75
	Ile^{105}/Val^{105}	11 (42)	Val^{105}: 0.25
	Val^{105}/Val^{105}	1 (4)	
EPHX1 exon3	Tyr^{113}/Tyr^{113}	13 (50)	Tyr^{113}: 0.71
	Tyr^{113}/His^{113}	11 (42)	His^{113}: 0.29
	His^{113}/His^{113}	2 (8)	
EPHX1 exon4	His^{139}/His^{139}	14 (54)	His^{139}: 0.71
	His^{139}/Arg^{139}	9 (35)	Arg^{139}: 0.29
	Arg^{139}/Arg^{139}	3 (11)	

[a]Combination designed to test the effect of *GSTT1*.

concentration of styrene in the chamber was monitored with a gas chromatograph every 4 min. Since the volunteers did not use respiratory protection, ambient concentration constituted a reliable index of exposure. The experimental protocol was approved by the local Ethical Committee and all volunteers gave their informed consent to participate in the study. They also passed medical examination before exposure and were insured.

Sample collection and analysis

Blood samples were collected before exposure for the measurement of CYP2E1 mRNA content and EPHX1 activity both in peripheral blood lymphocytes, as well as for the measurement of the background amount of ethanol and styrene in whole blood. CYP2E1 mRNA in PBLs was determined by real-time reverse transcription–polymerase chain reaction (RT–PCR) as previously described [22], the results being expressed as a ratio with housekeeping expression (cyclophilin). The only modification compared to the published method was a 10-fold dilution of the reverse transcription product before real-time PCR. Lymphocyte EPHX1 activity was determined by a fluorescence based assay using a HPLC separation system and monitoring the epoxide hydrolase mediated conversion of (±)-benzo[a]pyrene-4,5-epoxide to (±)-trans-benzo[a]pyrene-4,5-dihydrodiol [25]. The results were expressed as pmol diol formed per mg lymphocyte protein per min.

Urine samples were collected before exposure (spot sample), every 2 h until the 16th hour from the beginning of exposure and until the 24th hour (10 urinary samples in total for each volunteer). Mandelic acid and PGA in urine were determined by gas chromatography (Hewlett Packard 5890) according to a method previously published [6] in a single laboratory participating in the Quality Assurance program for organic solvent metabolites run by the Finnish Institute of Occupational Health (FIOH, Helsinki, Finland). M1, M2, 4-vinylphenol (sulfate, 4-vinylphenol-S and glucuronide, 4-vinylphenol-G) and PGlyc were determined by LC-MS-MS on a PE-Sciex API 365 triple-quadruple mass spectrometer (Sciex, Thornhill, Canada) according to a method previously published [40]. For all urinary metabolites, results were expressed as the total amount excreted during the 24 h following the beginning of exposure.

Statistical analysis

Statistical analysis was carried out by using the SAS package (SAS Institute Inc., SAS/STAT version 6.12, Cary, NC, USA; 1996). The parameters were assumed normally distributed; no transformation was applied and parametric tests were used. Differences between group means were assessed using the double-sided Student's *t*-test for independent samples. Simple linear regression analyses were carried out using the least-square method (Pearson's correlation). Analysis of variance (ANOVA) models were first applied and multiple regression analysis models were then used to assess the contribution of genotypes or phenotypes of DMEs and other covariates (age, smoking, drinking, body mass index; BMI) to the inter-individual variability of urinary biomarkers. For these multivariate regression analyses, each genotype was coded with a distinct dummy variable set at 0 (absence of mutant allele (*CYP2E1*, *EPHX1* and *GSTP1*) or null genotype (*GSTM1* and *GSTT1*)) or at 1 (presence of at least one mutant allele (*CYP2E1*, *EPHX1* and *GSTP1*) or positive genotype (*GSTM1* and *GSTT1*)). Based on in-vitro functional expression of variant alleles at respective residues 113 and 139, three predicted EPHX1 enzymatic activity levels were assigned as follows [41]: low activity for individuals $His^{113}/His^{113}-His^{139}/His^{139}$, $His^{113}/His^{113}-His^{139}/Arg^{139}$, $Tyr^{113}/His^{113}-His^{139}/His^{139}$, or $His^{113}/His^{113}-Arg^{139}/Arg^{139}$; intermediate activity for individuals $Tyr^{113}/Tyr^{113}-His^{139}/His^{139}$, $Tyr^{113}/His^{113}-His^{139}/Arg^{139}$, or $Tyr^{113}/His^{113}-Arg^{139}/Arg^{139}$; high activity for individuals $Tyr^{113}/Tyr^{113}-Arg^{139}/Arg^{139}$, or $Tyr^{113}/Tyr^{113}-His^{139}/Arg^{139}$. When appropriate, significant covariates of urinary biomarkers were traced by a stepwise regression procedure using a significance level of 0.25 for entry and 0.05 for staying in the model.

Results

Allelic frequencies in a Polish population

Initial genotype screening on a sample of Polish young male students ($n = 100$) revealed frequencies of 58 and 22% for *GSTM1* and *GSTT1* null genotypes, respectively. For both *CYP2E1* variant alleles (*CYP2E1*1B* and *CYP2E1*6*), the frequencies were 0.095. Within the subgroup of selected volunteers ($n = 26$), frequencies of the variant alleles were 0.29, 0.29 and 0.25 for

EPHX1 His[113], *EPHX1 Arg*[139] and *GSTP1 Val*[105], respectively. It is important to note that four *EPHX1* exon 3 genotyping results were discordant between classic PCR–RFLP and real-time PCR analysis (67% of the heterozygous were initially falsely classified as homozygous for the variant allele using the classic PCR–RFLP method (four out of six)).

Influence of biotransformation enzyme genotype

As shown in Table 3, among individuals lacking *GSTM1*, significantly lower urinary excretions were observed for all PHEMA and, in particular, for three of them, namely diastereoisomers [*R*,*R*]-M1, [*S*,*R*]-M1 and [*S*,*R*]-M2. While the greatest difference (16-fold) was observed for the [*R*,*R*]-M1 diastereoisomer, an overall 6-fold difference was found in *GSTM1* deficient individuals for the excretion of total PHEMA (Mt). Significant effects of the *GSTT1* genotype were observed mainly on [*R*,*R*]-M2 excretion but also on [*R*,*R*]-M1 and total PHEMA. However, when restricting this analysis to *GSTM1* null volunteers (9 *GSST1* positive vs. 6 *GSTT1* null), only the effect on [*R*,*R*]-M2 excretion remained significant ([*R*,*R*]-M1, 44.1 vs. 36.7, $P = 0.32$; [*R*,*R*]-M2, 31.8 vs. 18.0, $P < 0.01$; total PHEMA, 176 vs. 153 μg/24 h, $P = 0.45$, for mean values respectively). No effect of *GSTP1* genotype was noted on urinary excretion of styrene specific metabolites. As for *CYP2E1*, a lower mean value was observed in the excretion of both mandelic acid + PGA and the two main PHEMA ([*R*,*R*]-M1 and [*S*,*R*]-M2) for individuals carrying at least one rare *CYP2E1*6* allele. However, the observed differences did not reach statistical significance. A similar tendency was observed for individuals carrying the rare *CYP2E1*1B* allele. Concerning *EPHX1* genotypes, no statistically significant association was observed between styrene specific metabolite excretion and exon 3 or exon 4 polymorphisms. The influence of the above genetic polymorphisms on the urinary excretion of 4-vinylphenol and PGlyc was also tested (Table 4). None of the variations were statistically significant and the most notable was a decrease (1.6-fold; $P = 0.07$) in 4-vinylphenol total (G + S) excretion in individuals carrying at least one *CYP2E1*6* allele.

The influence on metabolite excretion of some other parameters not directly related to genetic factors (body mass index, alcohol and tobacco consumption) is presented in Table 5. Although not statistically significant, an overall decrease in metabolite excretion was observed for individuals with higher BMI values (> 25 kg/m^2), and, with the exception of PGly, the opposite tendency was noted for individuals reporting occasional alcohol consumption. Alcohol consumption on the day of exposure was excluded as verified by negative ethanol blood determination for all volunteers. A statistically significant increase in excretion of 4-vinylphenol-S (1.6-fold; $p = 0.04$) was observed in smokers.

Table 3 **Influence of genetic polymorphisms on urinary excretion of styrene specific metabolites**

Genotypes		*n*	MA (mg/24 h)	MA+PGA (mg/24 h)	[*R*,*R*]-M1 (μg/24 h)	[*S*,*R*]-M1 (μg/24 h)	[*R*,*R*]-M2 (μg/24 h)	[*S*,*R*]-M2 (μg/24 h)	Mt (M1+M2) (μg/24 h)
*CYP2E1*6*	T^{7632}/T^{7632}	19	50.0 ± 14.9	78.9 ± 23.4	290 ± 313	19.6 ± 17.4	31.7 ± 14.7	217 ± 183	558 ± 514
	T^{7632}/A^{7632}	7	43.0 ± 10.0	63.7 ± 17.5	188 ± 293	19.7 ± 12.0	31.0 ± 15.3	193 ± 191	431 ± 506
*CYP2E1*1B*	C^{9896}/C^{9896}	18	50.4 ± 12.1	77.7 ± 20.3	283 ± 332	22.1 ± 18.0	33.3 ± 16.1	233 ± 199	572 ± 553
	C^{9896}/G^{9896}	8	42.9 ± 17.3	68.3 ± 27.7	215 ± 247	14.0 ± 8.2	27.6 ± 9.9	161 ± 131	418 ± 387
GSTM1	Positive	11	48.2 ± 15.3	74.7 ± 24.3	564 ± 243***	33.1 ± 16.2***	38.6 ± 16.5*	376 ± 168***	1012 ± 425***
	Null	15	48.0 ± 13.4	74.9 ± 22.2	41.2 ± 13.6	9.7 ± 4.1	26.3 ± 10.6	89.7 ± 38.1	167 ± 57.9
GSTT1	Positive	20	48.8 ± 14.4	76.9 ± 23.8	330 ± 319*	22.2 ± 17.2	35.5 ± 14.0**	248 ± 191	636 ± 528*
	Null	6	45.6 ± 13.4	67.9 ± 18.6	36.7 ± 8.7	11.1 ± 5.1	18.0 ± 5.6	86.6 ± 32.0	153 ± 38.6
GSTP1 exon 5	Ile^{105}/Ile^{105}	14	48.0 ± 13.5	74.9 ± 20.7	212 ± 320	16.7 ± 16.4	31.4 ± 15.4	181 ± 185	441 ± 530
	Other	12	48.2 ± 15.1	74.7 ± 25.7	320 ± 289	23.0 ± 15.4	31.7 ± 14.1	246 ± 178	621 ± 478
EPHX1 exon 3	Tyr^{113}/Tyr^{113}	13	46.9 ± 13.3	72.0 ± 22.1	223 ± 260	19.5 ± 15.9	32.4 ± 11.4	184 ± 144	459 ± 421
	Other	13	49.2 ± 15.0	77.7 ± 23.8	301 ± 351	19.8 ± 16.6	30.6 ± 17.6	238 ± 215	589 ± 588
EPHX1 exon 4	His^{139}/His^{139}	14	47.1 ± 11.8	75.2 ± 20.3	259 ± 293	16.9 ± 11.0	30.6 ± 13.9	204 ± 182	510 ± 486
	Other	12	49.2 ± 16.6	74.3 ± 26.1	266 ± 332	22.8 ± 20.3	32.6 ± 15.8	219 ± 189	540 ± 548

Values are given as arithmetic mean ± standard deviation, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.0001$.
MA, mandelic acid.

Table 4 **Influence of genetic polymorphisms on urinary excretion of styrene metabolites**

Genotypes		*n*	4-VP-G (mg/24 h)	4-VP-S (mg/24 h)	4-VP-T (mg/24 h)	PGlyc (mg/24 h)
*CYP2E1*6*	T^{7632}/T^{7632}	19	0.49 ± 0.28	0.29 ± 0.18	0.79 ± 0.40	2.64 ± 1.95
	T^{7632}/A^{7632}	7	0.33 ± 0.15	0.17 ± 0.07	0.49 ± 0.20	1.84 ± 0.56
*CYP2E1*1B*	C^{9896}/C^{9896}	18	0.46 ± 0.29	0.22 ± 0.13	0.68 ± 0.39	2.07 ± 0.25
	C^{9896}/G^{9896}	8	0.43 ± 0.19	0.35 ± 0.20	0.78 ± 0.35	3.23 ± 0.92
GSTM1	Positive	11	0.52 ± 0.17	0.31 ± 0.22	0.83 ± 0.37	2.22 ± 1.95
	Null	15	0.40 ± 0.30	0.22 ± 0.10	0.62 ± 0.37	2.58 ± 1.57
GSTT1	Positive	20	0.47 ± 0.27	0.27 ± 0.18	0.74 ± 0.40	2.67 ± 1.88
	Null	6	0.37 ± 0.20	0.22 ± 0.10	0.59 ± 0.29	1.62 ± 0.49
GSTP1	Ile^{105}/Ile^{105}	14	0.48 ± 0.29	0.25 ± 0.10	0.73 ± 0.35	2.70 ± 1.90
exon 5	Other	12	0.42 ± 0.22	0.27 ± 0.22	0.69 ± 0.42	2.11 ± 1.49
EPHX1	Tyr^{113}/Tyr^{113}	13	0.39 ± 0.18	0.26 ± 0.18	0.64 ± 0.32	2.33 ± 1.57
exon 3	Other	13	0.51 ± 0.31	0.26 ± 0.15	0.78 ± 0.42	2.52 ± 1.91
EPHX1	His^{139}/His^{139}	14	0.45 ± 0.21	0.28 ± 0.21	0.73 ± 0.39	2.01 ± 0.79
exon 4	Other	12	0.45 ± 0.31	0.24 ± 0.09	0.69 ± 0.37	2.91 ± 2.34

Values are given as arithmetic mean ± standard deviation.
4-VP, vinylphenol (G, glucuronide, S, sulfate and T, total (G + S)); PGly, phenylglycine.

Table 5 **Influence of BMI, alcohol and tobacco consumption on urinary excretion of styrene specific metabolites**

		n	MA (mg/24 h)	MA+PGA (mg/24 h)	Mt (M1+M2) (μg/24 h)	4-VP-G (mg/24 h)	4-VP-S (mg/24 h)	4-VP-T (mg/24 h)	PGlyc (mg/24 h)
BMI	⩽ 25	14	49.5 ± 14.8	76.0 ± 23.8	668 ± 592	0.49 ± 0.20	0.32 ± 0.20	0.81 ± 0.36	2.76 ± 2.01
(kg/m^2)	> 25	12	46.4 ± 13.4	73.5 ± 22.3	357 ± 330	0.40 ± 0.32	0.19 ± 0.09	0.60 ± 0.38	2.04 ± 1.26
Alcohol[a]	No	8	45.5 ± 13.0	69.6 ± 22.1	266 ± 205	0.32 ± 0.15	0.19 ± 0.08	0.51 ± 0.21	2.71 ± 1.84
	Yes	18	49.2 ± 14.6	77.1 ± 23.1	639 ± 559	0.51 ± 0.28	0.29 ± 0.19	0.80 ± 0.40	2.30 ± 1.70
Tobacco[b]	No	15	49.8 ± 13.2	78.1 ± 18.8	557 ± 581	0.39 ± 0.19	0.20 ± 0.09*	0.60 ± 0.25	2.76 ± 1.98
	Yes	11	45.8 ± 15.3	70.4 ± 27.4	480 ± 401	0.53 ± 0.32	0.34 ± 0.21	0.86 ± 0.47	1.98 ± 1.22

[a]Occasional alcohol consumption defined as ⩾ 1 unit (equivalent of 50 ml of 40% alcohol)/week.
[b]Tobacco consumption defined as ⩾ 1 cigarette/day.
Values are given as arithmetic mean ± standard deviation, $*P < 0.05$.
MA, mandelic acid; VP, vinylphenol.

Stepwise multiple regression analyses were also performed in order to trace the contribution of genotypes and other covariates (BMI, alcohol consumption, tobacco consumption and age) to the inter-individual variability of styrene specific urinary biomarkers (Table 6). No statistically significant independent variable was found to explain variability of mandelic acid + PGA or 4-vinylphenol-T urinary excretion. The *GSTM1* genotype remained the most significant independent variable when considering PHEMA excretion; it explained 71% of the mercapturate variance while tobacco consumption explained an additional 7% variance. It is interesting to note that 'presence of *His*^{113} allele' in EPHX1 was the next independent variable selected by the model but finally rejected because the *P*-value was greater than 0.05 (positive slope, $P = 0.062$).

Comparison of phenotype and genotype for CYP2E1 and EPHX1

Since data on CYP2E1 mRNA quantification in PBLs and on *CYP2E1* genotype was available for each volunteer, we could evaluate the correlation between

Table 6 **Determinants of the urinary excretion of styrene metabolites (including *CYP2E1* genotyping)**

Dependent variables	Independent variables	Partial r^2	Slope	*P* value[a]
Mandelic acid + PGA excretion	*CYP2E1*6*	0.09	Negative	0.131[b]
Total 4-vinylphenol excretion	BMI	0.13	Negative	0.066[b]
Total mercapturic acids	*GSTM1*	0.71	Positive	0.0001
	Tobacco consumption	0.07	Negative	0.014
		Model r^2: 0.78		

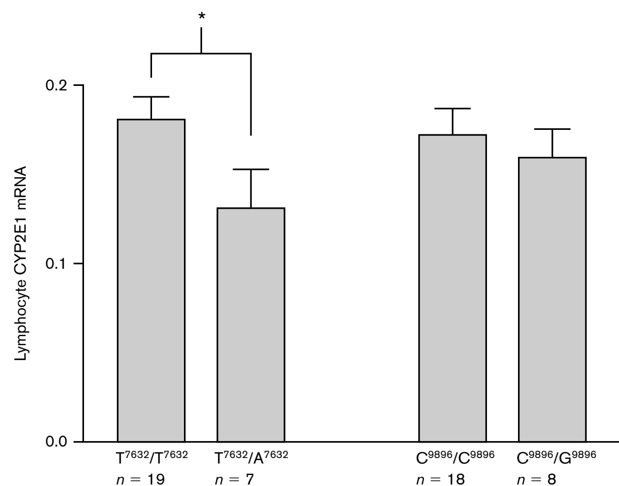
[a]Partial r^2 *P* value.
Tested independent variables were seven unrelated polymorphisms (*CYP2E1*6*, *CYP2E1*1B*, *GSTM1*, *GSTT1*, *GSTP1*, *EPHX1* (exons 3 and 4)), BMI, alcohol consumption, tobacco consumption and age.
[b]First independent variable selected by the model but excluded in the first step because $P > 0.05$.

these parameters. As illustrated in Fig. 2, a statistically significant reduction in CYP2E1 mRNA expression was observed in PBLs of individuals carrying at least one rare *CYP2E1*6* allele ($P = 0.047$), whereas *CYP2E1*1B* status had no effect in this context. A similar approach was conducted for the 21 cases for which results of both lymphocyte EPHX1 activity and *EPHX1* genotype (exon 3 and 4) were available. Figure 3 shows a significant reduction in EPHX1 activity in PBLs of individuals bearing at least one His^{113} variant allele (−25%, $P = 0.02$), while a nearly significant higher activity (24%, $P = 0.09$) was noted for individuals possessing at least one Arg^{139} variant allele. No difference was observed between heterozygous and homozygous volunteers bearing a polymorphism on exon 3 or 4. After classification according to the three predicted EPHX1 enzymatic activity levels (low–intermediate–high), ANOVA revealed statistically significant differences between group means ($P = 0.013$), and a statistically significant linear trend was evidenced ($r = 0.58$, $P = 0.006$; Fig. 3c).

Influence of phenotype

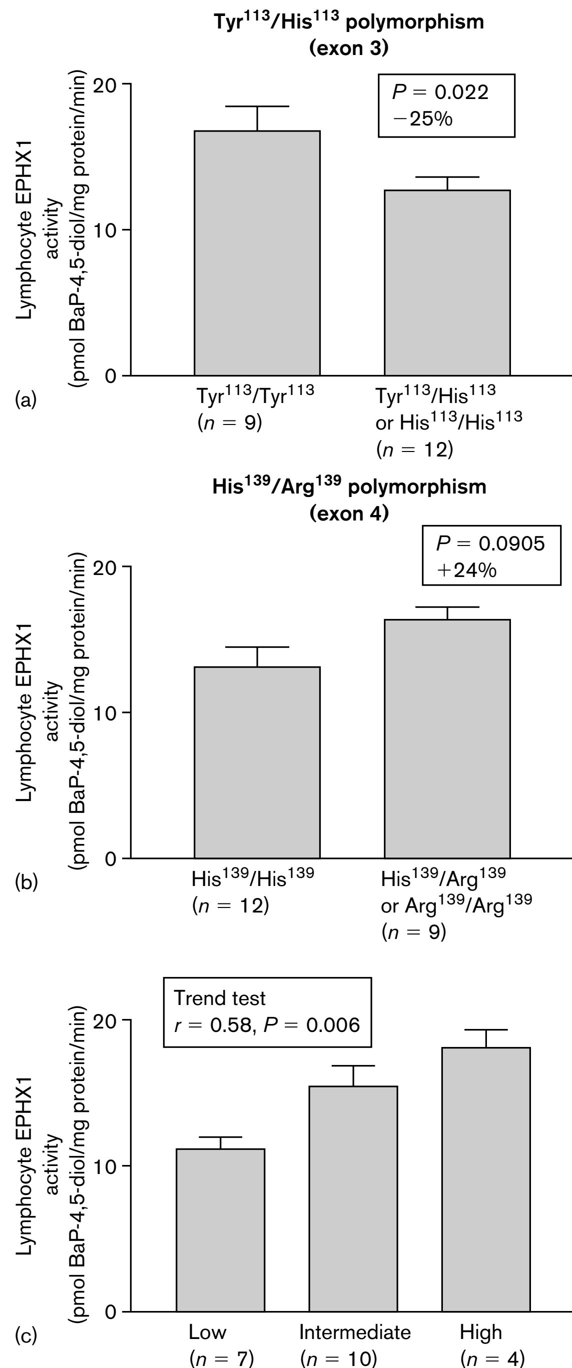
Stepwise multiple regression analyses were reprocessed in models taking into account PBLs CYP2E1 mRNA content (phenotyping approach) instead of *CYP2E1*1B* and *CYP2E1*6* genotypes as independent variable. This phenotyping parameter alone explained 22% of the variance in mandelic acid excretion (Table 7), as also illustrated in Fig. 4. Using the same independent variables, the *GSTM1* genotype remained the most significant parameter that explained PHEMA excretion. However, as shown in Fig. 5, a combined effect of lymphocyte CYP2E1 expression and *GSTM1* status was apparent. Therefore, after exclusion of one 'outlier' and the consideration of three first order interaction terms (see Table 7), the *GSTM1**CYP2E1 mRNA interaction remained the only independent variable retained by

Fig. 2



CYP2E1 mRNA expression in PBLs (expressed as the ratio with cyclophilin mRNA) according to *CYP2E1*6* (7632T>A) and *CYP2E1*1B* (9896C>G) allelic status.

Fig. 3



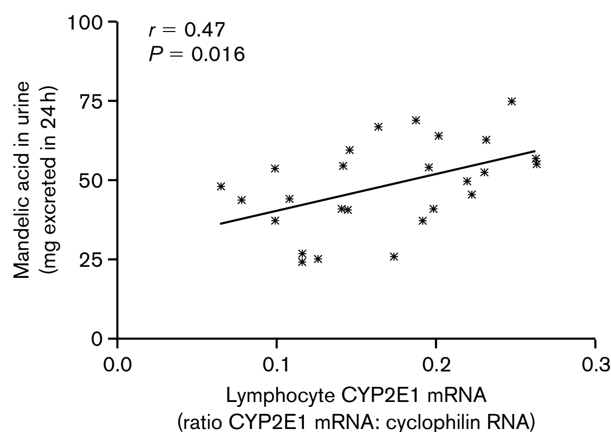
EPHX1 activity in PBLs according to (a) allelic status in exon 3 (*Tyr113*/*His113*), (b) allelic status in exon 4 (*His139*/*Arg139*) and (c) predicted low–intermediate–high EPHX1 activity based on genotypes in exon 3 and 4. See text for details.

Table 7 Determinants of the urinary excretion of styrene metabolites (including CYP2E1 phenotyping)

Dependent variables	Independent variables	Partial r^2	Slope	*P* value[a]
Mandelic acid excretion	CYP2E1 mRNA	0.22 Model r^2: 0.22	Positive	0.016
Total 4-vinylphenol excretion	BMI	0.13	Negative	0.066[c]
Total mercapturic acids	*GSTM1* Tobacco consumption	0.71 0.07 Model r^2: 0.78	Positive Negative	0.0001 0.014
Total mercapturic acids[b]	*GSTM1**CYP2E1 mRNA	0.77 Model r^2: 0.77	Positive	0.0001

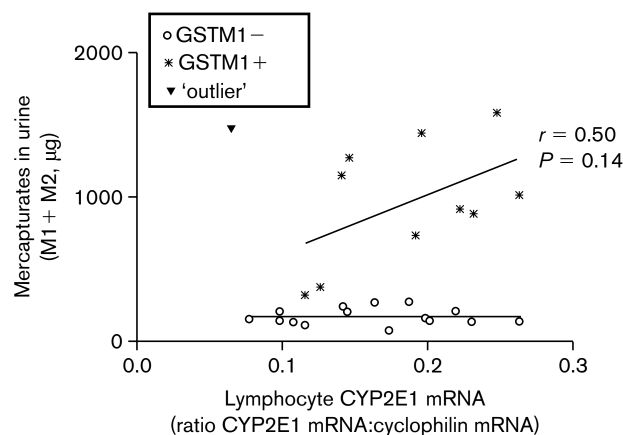
[a]Partial r^2 *P* value.
Tested independent variables were five unrelated polymorphisms (*GSTM1*, *GSTT1*, *GSTP1*, *EPHX1* (exons 3 and 4)), CYP2E1 mRNA expression in BPLs, BMI, alcohol consumption, tobacco consumption and age.
[b]The same independent variables were selected with three first order interaction term *GSTM1**CYP2E1 mRNA, *GSTM1**GSTT1 and *GSTM1***GSTP1* (after exclusion of one 'outlier').
[c]First independent variable selected by the model but excluded in the first step because $P > 0.05$.

Fig. 4



Relationship between the amount of CYP2E1 mRNA in PBLs (expressed as the ratio with cyclophilin mRNA) and the amount of mandelic acid excreted in urine during the 24 h following styrene exposure in the inhalation chamber.

Fig. 5



Relationship between the amount of CYP2E1 mRNA in PBLs (expressed as the ratio with cyclophilin mRNA) and the amount of total PHEMA excreted in urine (expressed in µg) during the 24 h following styrene exposure taking into account the *GSTM1* genotype. All *GSTM1* positive individuals are also positive for *GSTT1*.

the stepwise multiple regression analysis and explained by itself 77% of the variance in PHEMA excretion. This interaction parameter explained an even more important part of the variance for [*R*,*R*]-M1 excretion with a partial r^2 of 0.82 for the interaction term and additional partial r^2 of 0.03, 0.03 and 0.02 for Tyr^{113}/His^{113} (positive slope), tobacco consumption (negative slope) and BMI (negative slope), respectively (model r^2: 0.90; data not shown). It should be emphasized that, among *GSTM1* positive volunteers, the 'outlier' subject presented the lowest lymphocyte EPHX1 activity (9.21 pmol BaP-4,5-diol/mg protein/min vs. a mean of 15.45 for others). In all previously tested models, the only independent variable explaining a significant part of the variation of [*R*,*R*]-M2 was GSTT1 status. Finally, when considering the subgroup of the 21 volunteers for which both EPHX1 activity and CYP2E1 mRNA content in PBLs were available, the same models of stepwise multiple regression analysis yielded equations for mandelic acid + PGA or PHEMA identical to those presented in Table 7. In the PHEMA model, however, EPHX1 activity was the next independent variable selected but finally rejected by the stepwise procedure (negative slope, $P = 0.084$). When EPHX1 activity was forced in a PHEMA model taking into account only *GSTM1* status and both phenotyping results (EPHX1 and CYP2E1), the total variance explained by the model was 77% ($P < 0.0001$), with a respective contribution of dependent variables as follows: *GSTM1* status (positive slope, $P < 0.0001$), EPHX1 activity (negative slope, $P = 0.031$) and CYP2E1 mRNA content in PBLs (positive slope, $P = 0.35$, not significant).

Discussion

Allele frequencies observed in the Polish population for the different polymorphisms analyzed were similar to

those observed in European Caucasians [42,43] and were in accordance with previously published data on *GST* polymorphisms in another Polish cohort [44]. Moreover, as underlined in a recent paper [45], the present data confirm the absolute necessity to check all results obtained with the classic PCR–RFLP assay for the *EPHX1* codon 113 polymorphism by an appropriate method taking into account the silent polymorphism found in codon 119.

The lower expression of CYP2E1 mRNA in PBLs of individuals carrying at least one rare *CYP2E1*6* allele deserves some comments. There is now suggestive evidence that the variant *CYP2E1*6* allele is associated with reduced CYP2E1 activity, at least in Caucasians [14,46]. The lower excretion of both mandelic acid + PGA and PHEMA ([*R*,*R*]-M1 and [*R*,*S*]-M2) in individuals heterozygous for the *CYP2E1*6* allele is in line with these observations. It has also been shown that, although Caucasians heterozygous for the *CYP2E1*6* variant allele did not show a significant difference in basal CYP2E1 activity compared with the wild-type homozygotes, in alcoholics *in vivo* chlorzoxazone metabolism was slower in subjects with the heterozygous genotype than in those with the homozygous wild-type genotype [15]. This suggested that the *CYP2E1*6* allele was associated with a reduced inducibility of *CYP2E1*. By demonstrating a significant effect at the pretranslational level, possibly reflecting a reduced CYP2E1 mRNA expression in the presence of the *CYP2E1*6* allele, our results give further support to this hypothesis.

A previous study comparing both lymphocyte and liver EPHX1 activities in the same individuals showed that blood lymphocytes could be considered as a useful reporter for assessing epoxide hydrolase activity. These measurements were relatively stable over time and appeared to reflect activity levels in other organs, such as the lung [25]. Two common polymorphic amino acid loci (113 and 139) in the EPHX1 protein were then identified and in-vitro expression assays employing cloned EPHX1 DNA templates transiently transfected into COS-1 cells indicated that amino acid 113 and 139 polymorphisms altered EPHX1 expression, possibly through a protein stabilization mechanism. The $Tyr^{113}His$ substitution was associated to a 39% reduction in EPHX1 activity in comparison to the reference construct whereas replacing Arg^{139} for His resulted in a relative increase of 25% [23]. The functional significance of these polymorphisms was further tested by the same investigators on a panel of 40 liver samples but they did not appear to differentially affect the specific activity of EPHX1 [26]. To the best of our knowledge, the present study is the first that analyzes the relationship between the two common EPHX1 polymorphisms and EPHX1 activity in PBLs. The statistically significant reduction observed in EPHX1 activity in PBLs of volunteers carrying at least one His^{113} variant allele was of the same order of magnitude as the decrease observed by Hassett *et al. in vitro* (25 vs. 39%). Although not statistically significant, the mean increase observed for those carrying at least one Arg^{139} variant allele was identical to that observed *in vitro* (24 vs. 25%). Stratifying the genotyping data by the predicted EPHX1 activity [41] resulted in a statistically significant linear trend, giving further support towards a genotype-phenotype relationship. This observation was confirmed in an independent cohort of 33 workers occupationally exposed to styrene (personal unpublished data).

The measurement of PHEMA in post-shift urine for the biological monitoring of workers exposed to styrene was first proposed by Ghittori *et al.* [5] but these authors reported an 'unexplained', and probably genetically based, variation in the relationship between air concentration and urinary metabolites. PHEMA, and mercapturates in general, are potentially interesting biomarkers of exposure because of their high specificity. The determination of urinary PHEMA may also provide more toxicologically relevant parameters, as compared with mandelic acid and PGA, considering that they are a more direct reflection of the electrophilic intermediate SO. As recently elucidated in two field surveys [27,28], the present results confirm that the *GSTM1* genotype is clearly an important parameter modifying the rate of urinary excretion of PHEMA and that the mechanism by which GSTs catalyze GSH-conjugation of SO in humans is regio- and stereoselective. Consistent with the data of the Italian study, [*R*,*R*]-M1 is the main mercapturate affected by the *GSTM1* genotype and it accounts, in *GSTM1* positive subjects, for 56% of total PHEMA excreted during the 24 h following styrene exposure. For an identical level of exposure, workers carrying the *GSTM1* positive genotype showed a 6-fold increase in the 24-h excretion of PHEMA, which is totally in accordance with the 5- and 6-fold increases observed in the previous study for end-of-shift and next-morning samples, respectively [28]. Although less significant than that of GSTM1, an involvement of GSTT1 in the formation of PHEMAs is also possible. The influence of this genotype was specifically noted for the [*R*,*R*]-M2 metabolite, further illustrating the regio- and stereoselectivity of GST activity on SO. The differences noted for other metabolites (Table 3) were most likely due to the fact that all *GSTM1* positive volunteers were also *GSTT1* positive, and were not confirmed when the analysis was restricted to *GSTM1* null volunteers. A modifying role of GSTT1 has also been reported regarding the induction of sister chromatid exchanges by SO in cultured human lymphocytes [47]. This in-vitro observation can be explained especially if it is considered that whole blood lymphocyte cultures are mainly affected by the

high GSTT1 activity in erythrocytes [48]. Despite a good balance among *GSTP1* genotypes (12 out of 26 subjects possessing at least one *Val*[105]), we were unable to demonstrate an effect of this polymorphism on the urinary excretion of PHEMA. Our results are therefore different from those of a recent study indicating a significant effect of the *Val*[105] genotype on hypoxanthine phosphoribosyl transferase mutant frequency in PBLs of workers occupationally exposed to styrene [41].

The significant relationship observed between urinary excretion of mandelic acid during the 24 h following styrene exposure and CYP2E1 mRNA in PBLs demonstrates the importance of CYP2E1 in the metabolism of styrene in humans, confirming recent in-vitro data on human liver microsomes [11]. On the other hand, based on the biotransformation pathway of styrene in man, a combined effect of CYP2E1 phenotype and *GSTM1* genotype could be hypothesized to explain inter-individual variability in urinary PHEMA excretion. During a previous field study using the classic chlorzoxazone metabolic ratio (CMR) to assess CYP2E1 activity, we were unable to verify this hypothesis [46]. In contrast, the present results indicate such combined effect highlighting a potential value of CYP2E1 phenotyping, through the measurement of CYP2E1 mRNA in PBLs, in *GSTM1* positive subjects. The use of CMR to measure CYP2E1 activity is a first possible explanation for the discordance between both studies. While chlorzoxazone may be considered as an 'acceptable' probe substrate for CYP2E1 [49], the possibility exists that the CMR does not provide a measure accurate enough to finely reflect the CYP2E1 catalytic activity. Indeed, the correlation between the CMR and chlorzoxazone fractional clearance to 6-hydroxychlorzoxazone (considered as the most accurate kinetic parameter exploring 6-hydroxylation of CZX) has sometimes been reported as quite low [18]. Another explanation for the discordant findings might be that, unlike field studies, a volunteer study takes place under strictly controlled conditions. In a recent report involving twenty volunteers exposed to styrene in an inhalation chamber during which CMR was used to assess individual CYP2E1 metabolic capacity, no relationship was observed between CMR values and mandelic acid + PGA excretion but urinary PHEMA were not measured [50].

A minor pathway of styrene metabolism leads to the urinary excretion of 4-vinylphenol but CYP isoforms involved in this reaction have not been characterized in humans. The lower urinary excretion ($P = 0.07$) observed for this metabolite in individuals carrying at least one *CYP2E1*6* allele would indicate a possible involvement of CYP2E1, as recently suggested in rat and mouse liver [51]. Although positive, the relationship observed between 4-vinylphenol excretion and CYP2E1 mRNA in PBL was however not statistically significant.

In conclusion, although the present study shows that genotyping and/or phenotyping of relevant DMEs does not significantly improve the interpretation of urinary levels for the main metabolites of styrene in general, these analyses might greatly improve the interpretation of urinary concentrations of minor – but more specific – metabolites. In this respect, PHEMA determination can now be considered as a useful tool for biological monitoring of styrene exposure in occupational or environmental settings. The first practical recommendation of this study is to propose a systematic *GSTM1* genotyping to allow PHEMA data interpretation. Secondly, in *GSTM1* positive subjects excreting 'unexpected' amounts of PHEMA, the measurement of CYP2E1 mRNA in PBLs could be recommended to discriminate between individuals who are really exposed to different levels and those who are more likely to be subject to host-associated factors influencing *CYP2E1* expression, and hence, mercapturate production. This approach could be extended to other specific mercapturic acids (benzene, toluene, ethylene oxide, etc.) giving a new prospect for their use in the field of biological monitoring of exposure to chemical agents.

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