BIOMARKERS, 2001, VOL. 6, NO. 3, 236–249

Importance of genetic polymorphisms of drug-metabolizing enzymes for the interpretation of biomarkers of exposure to styrene

VINCENT HAUFROID[1]*, JEAN-PIERRE BUCHET[1], SOPHIE GARDINAL[1], SERGIO GHITTORI[2], MARCELLO IMBRIANI[3], ARI HIRVONEN[4] and DOMINIQUE LISON[1]

[1]Industrial Toxicology and Occupational Medicine Unit, Catholic University of Louvain, Brussels, Belgium. e-mail: haufroid@toxi.ucl.ac.be

[2]Fondazione Salvatore Maugeri, IRCCS, Centro Medico di Pavia, Italy

[3]Dipartimento di Medicina Preventiva, Occupazionale e di Comunité, II Sezione di Medicina del Lavoro, Pavia, Italy

[4]Department of Industrial Hygiene and Toxicology, Finnish Institute of Occupational Health, Helsinki, Finland.

Received 11 August 2000, revised form accepted 11 October 2000

The objective of this study was to test the infiuence of genetic polymorphisms for metabolic enzymes (CYP2E1, mEH, GSTM1 and GSTT1) implicated in the biotransformation of styrene in humans on the interpretation of urinary biomarkers of exposure. Thirty workers from a fibreglass-reinforced plastics factory took part in the study. Ambient styrene concentration was determined during the whole workshift by passive sampling. Urine was collected at the end of the shift for the determination of mandelic acid (MA) and phenylglyoxylic acid (PGA) (major biotransformation pathway), *N*-acetyl-*S*-(1-phenyl-2-hydroxy)ethyl-L-cysteine (M1) and *N*-acetyl-*S*-(2-phenyl-2-hydroxy)ethyl-L-cysteine (M2) (minor metabolic pathway) and creatinine. The average airborne styrene concentration of 18.2 ppm (range: 0.9–68.9 ppm) was very close to the current threshold limit value (TLV-TWA) recently adjusted by ACGIH from 50 to 20 ppm. There was a better correlation between external and internal exposure as estimated by urinary MA + PGA ($r = 0.92$; $p < 0.0001$) compared with urinary M1 + M2 ($r = 0.74$; $p < 0.0001$). To investigate to what extent genetic polymorphisms in metabolic enzymes could explain interindividual variations observed in the concentration of urinary biomarkers related to a given external exposure, two 'metabolic indexes' (derived from the ratio between the sum of urinary metabolites for a specific pathway and ambient styrene concentration) were calculated for each worker and compared for different allelic combinations. Monovariate analyses showed that *GSTM1* polymorphism was clearly the most significant parameter infiuencing urinary concentrations of mercapturic acids. Based on *GSTM1* allelic status, two different biological exposure indexes (BEIs) for M1 + M2 in post-shift urinary samples corresponding to a 20 ppm styrene concentration are proposed (*GSTM1*null: 1330 μg g^{-1} creatinine, *GSTM1*+: 2878 μg g^{-1} creatinine). Multivariate regression analyses were also performed and revealed that the presence of the rare *CYP2E1*1B* allele linked to TaqI polymorphism (A1/A2) was associated with increased urinary concentrations of metabolites from both pathways. Two previously described polymorphisms for the *EPHX* gene were also tested but seemed not really relevant for interpretation of biomarkers. In conclusion, while *CYP2E1* genotyping, particularly assessment of the *CYP2E1*1B* allelic status, is useful for a more accurate interpretation of the concentration of urinary biomarkers, *GSTM1* genotyping is absolutely necessary when considering a biological monitoring programme based on determination of urinary mercapturic acids.

Keywords: styrene, biological monitoring, genetic polymorphisms, cytochrome P450 2E1, glutathione transferase, epoxide hydrolase, mercapturic acids.

* Corresponding author: Vincent Haufroid, Industrial Toxicology and Occupational Medicine Unit, Catholic University of Louvain, Clos Chapelle-aux-Champs 30/54, 1200 Brussels, Belgium.

Biomarkers ISSN 1354-750X print/ISSN 1366-5804 online
http://www.tandf.co.uk/journals
DOI: 10.1080/13547500010014540

Introduction

Styrene monomer (CAS: 100-42-5) is a very important chemical in the manufacture of polymers and reinforced plastics. Though there is a potential of environmental exposure from ambient air, food or drinking water, the most substantial human exposure to styrene occurs in occupational settings. Both environmental and occupational exposures to styrene occur predominantly via inhalation.

A small fraction (less than 5 %) of the absorbed styrene is eliminated unchanged with the expired air (Fernandez and Caperos 1977) and an even smaller amount (< 2 %) is eliminated unchanged in urine. The major biotransformation pathway of styrene is via oxidation to styrene 7,8-oxide (SO) which is present in two enantiomeric forms (*R*-SO and *S*-SO) (figure 1). Many *in vitro* studies have been conducted to examine the enzymatic processes involved in this oxidation. The conversion of styrene to SO is catalysed *in vitro* by cytochrome P450 2E1 (CYP2E1) (Guengerich *et al.* 1991, Kim *et al.* 1997) but other isoforms, particularly CYP2B6, could also be involved at higher concentrations of styrene (Nakajima *et al.* 1993, Kim *et al.* 1997) and a contribution of CYP2F1 has also been mentioned (Nakajima *et al.* 1994). In humans, to the best of our knowledge, the exact *in vivo* contribution of CYP2E1 in the formation of SO remains to be determined. The subsequent detoxification of SO involves two distinct metabolic pathways: it can be hydrolysed to styrene glycol (SG) or conjugated with glutathione (GSH). The major pathway in humans consists of hydrolysis by microsomal epoxide hydrolase (mEH) to form SG. This metabolite is then oxidized by alcohol and aldehyde dehydrogenases to mandelic acid (MA) that can be either excreted as such in urine or further oxidized to phenylglyoxylic acid (PGA), which is also excreted by the kidneys. MA and PGA represent more than 95 % of urinary metabolites of styrene. Conjugation represents a minor biotransformation pathway and following the reaction between GSH and SO (*R*- and *S*- enantiomers), each mercapturic acid excreted in urine [*N*-acetyl-*S*-(1-phenyl-2-hydroxy)ethyl-L-cysteine (M1) and *N*-acetyl-*S*-(2-phenyl-2-hydroxy)ethyl-L-cysteine (M2)] consists of two diastereoisomeric forms: M1-S, M1-R and M2-S, M2-R, respectively (figure 1). The latter metabolites have been measured in low concentrations in urine of workers occupationally exposed to styrene (Ghittori *et al.* 1997).

So far, biological monitoring of exposure to styrene is routinely achieved by the measurement of MA and PGA in urine collected at the end of the shift and/or prior to the next shift (i.e. 16 h after exposure). Biological exposure indexes (BEIs) have been recommended for this purpose by the American Conference of Governmental Industrial Hygienists (ACGIH). More recently, Ghittori *et al.* (1997) proposed the measurement of specific mercapturic acids in post-shift urine for the biological monitoring of workers exposed to styrene but these authors reported a wide variation in the relationship between air concentration and urinary metabolites.

To date, biological monitoring methods and biological limit values applied in occupational medicine have been developed on the assumption that individuals do not differ significantly in their biotransformation capacities. In recent years, however, it has become clear that this is not the case and genetically determined variations in biotransformation enzyme activities have been reported or at least suggested. As to the *CYP2E1* gene, three different polymorphisms detectable with TaqI, DraI, RsaI and PstI restriction enzymes (Rannug *et al.* 1995) as well as a

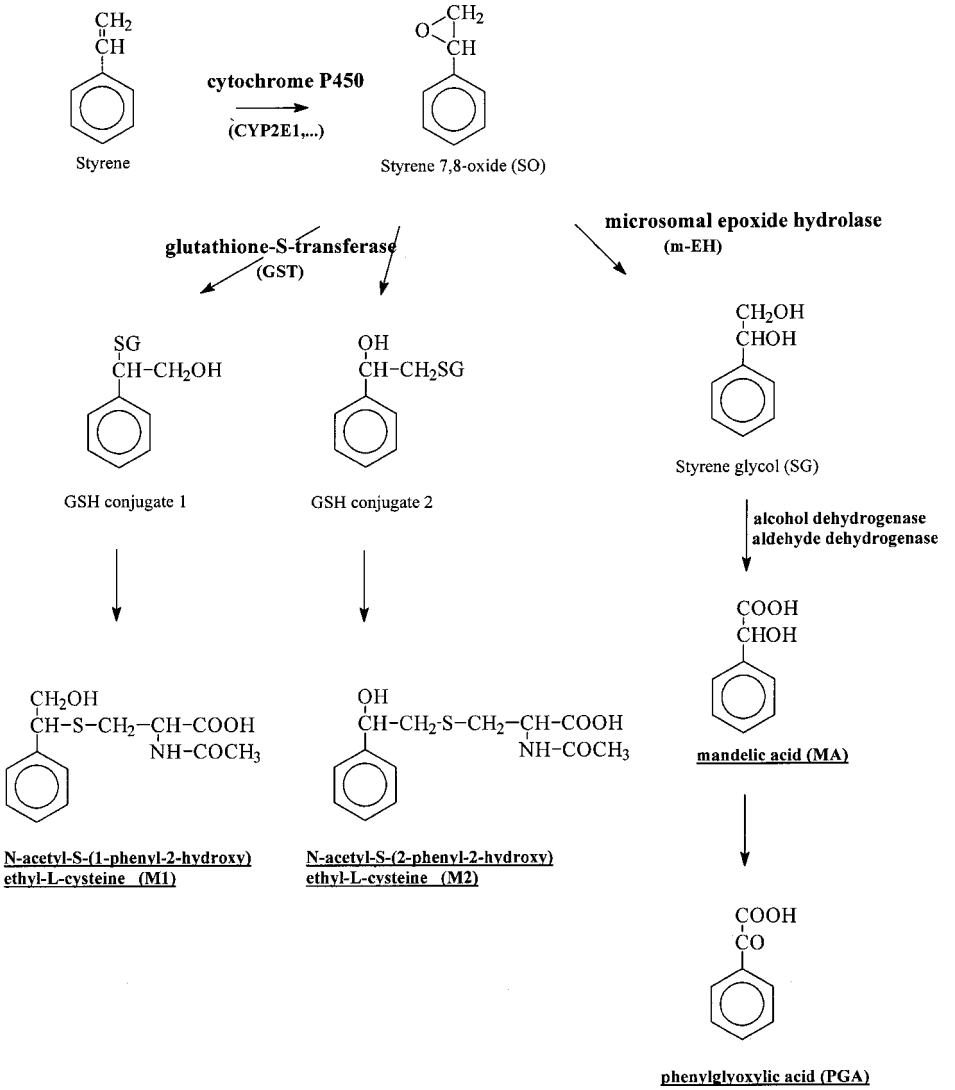

Figure 1. Biotransformation pathways of styrene.

96 bp-insertion polymorphism (McCarver *et al.* 1998, Fritsche *et al.* 2000) have been described (see also table 3). While reduced CYP2E1 activity in the presence of the rare C allele (*CYP2E1*5A*) has been suggested (Kim *et al.* 1995, Haufroid *et al.* 2000), the *in vivo* significance of most of these polymorphisms is, as yet, far from clear. In the *EPHX* gene, two polymorphic sites have been observed in exon 3 (Tyr113/His113) and exon 4 (His139/Arg139). Based on *in vitro* studies, the variant allele of one of these sites (exon 3) correlated with reduced mEH activity whereas the variant in the other site (exon 4) resulted in increased mEH activity (Hassett *et al.* 1994). Finally *GSTM1* and *GSTT1* are also polymorphic so that about 50% and 15% of Caucasians lack the respective genes.

The purposes of this study are therefore (1) to test the infiuence of *CYP2E1*, *EPHX*, *GSTM1* and *GSTT1* genetic polymorphisms on styrene metabolite

excretion rate and thus on the interpretation of biological monitoring data and (2) to reconsider the value of determining specific mercapturic acids for the biological monitoring of styrene exposure after consideration of genetic polymorphisms.

Materials and methods

After approval of the protocol by the local Ethical Committee, 30 workers from a fibreglass-reinforced plastics factory agreed to take part in the study and gave their informed consent. Workers did not wear protective devices (mask or gloves) so that the study population provides a relevant model to analyse the relationship between external and internal parameters. The main characteristics of the study population are summarized in table 1.

To assess external exposure, air samples were collected during the whole workshift by a passive sampling device (3M 3500 organic vapour monitor ; St Paul, Minn, USA) attached to the clothing within the breathing zone of each worker. The sampler was eluted with carbon disulphide and analysed by gas chromatography (GC) with fiame ionization detection (FID) on a capillary column CP WAX 52 (length 25 m, internal diameter 0.32 mm, film thickness 1.2 μm). Under these conditions the detection limit of styrene was 0.1 ppm.

Urine was collected at the end of the shift for the measurement of MA, PGA, M1, M2 and creatinine. All urinary samples were transported at +4 ¡C to the laboratory within 6 h and aliquots of urine were frozen at −20 ¡C until analysis.

MA and PGA were determined by GC-FID. Briefiy, urine samples were acidified and extracted with diethyl ether; the organic layer was dried and the residue derivatized with BSTFA/chloroform before injection. The laboratory regularly participates in the inter-comparison programme organized by the Finnish Institute of Occupational Health (FIOH) for MA determination. During the study period, the accuracy of MA analysis was between 88 and 100% (mean: 96%) as compared with target values (LOD: 5 mg l^{-1}).

M1 and M2 were determined by an HPLC method with fiuorescence detection, as published previously (Ghittori *et al*. 1997) (LOD: 7 μg l^{-1}). Creatinine concentration in urine was measured by the method of Jaffe.

A blood sample (EDTA tube, 5 ml) was obtained from each worker in order to perform genotyping analyses.

Three *CYP2E1* polymorphisms, respectively c1→ c2 (Watanabe *et al*. 1990), D→ C (Uematsu *et al*. 1991), and A1→ A2 (McBride *et al*. 1987) were determined by restriction fragment-length polymorphism (RFLP) analysis as described elsewhere (Haufroid *et al*. 1998). Another *CYP2E1* insertion polymorphism initially described by McCarver *et al*. (1998) was determined using a simple PCR method developed in our laboratory and similar to another recently published (Hu *et al*. 1999).

GSTM1 and *GSTT1* polymorphisms were analysed by the method described by Arand *et al*. (1996) which allows differentiation between individuals possessing at least one undeleted allele (*GSTM1*+ or *GSTT1*+) and those possessing the gene homozygously deleted (*GSTM1*null or *GSTT1*null).

For *EPHX* genotyping, after two separate PCR reactions, the variant allele coding for an arginine residue in exon 4 (Arg139) was identified through the presence of the RsaI restriction site (Hassett *et al*. 1994) and the allele coding for an histidine residue in exon 3 (His113) through the presence of the AspI site (Lancaster *et al*. 1996).

All statistical analyses were performed with the SAS package (SAS Institute Inc., SAS/STAT version 6.12, Cary, NC, USA (1996)). When the concentration of a parameter was below the LOD, it was assigned a value equal to half the LOD. The strength of the relationship between two parameters was evaluated by calculating simple linear correlations. In order to evaluate the infiuence of multiple

Table 1. Characteristics of the study population.

	Mean ($\bar{x}$)	Standard deviation (SD)	Range
Age (years)	39.1	8.5	23–54
Body mass index (BMI)	26.6	4.3	19.3–34.6
Smokers (≥ 1 cig. per day)	60%		
Alcohol consumers (≥ 5 glasses per week)	63%		

All workers are males.

factors on styrene metabolism, two 'metabolic indexes' (defined as the ratio between urinary metabolites, expressed either as mg g^{-1} creatinine for the sum MA + PGA or μg g^{-1} creatinine for the sum M1 + M2, and ambient styrene expressed as ppm) were calculated for each worker. A logarithmic transformation was applied before statistical analysis to normalize the distribution of both variables and metabolic indexes 1 and 2 (I1 and I2) were respectively defined as log ((MA + PGA)/creatinine/styrene air) and log ((M1 + M2)/creatinine/styrene air). In a first analysis, subgroups, stratified according to single allelic status, were compared with respect to log transformed metabolic indexes by one-way analysis of variance (ANOVA) followed when appropriate by a Tukey–Kramer multiple comparison test. Where only two genotypic groups were represented, a double sided *t*-test was used. The level of statistical significance was set at $p < 0.05$. Potential determinants affecting the metabolic indexes were then identified in different models of stepwise regression analysis. For these multivariate regression analyses, each genotype was coded with a distinct dummy variable set at 0 (absence of mutant allele (*CYP2E1* and *EPHX*) or null genotype (*GST*)) or at 1 (presence of at least one mutant allele (*CYP2E1* and *EPHX*) or + genotype (*GST*)). Significant covariates of I1 and I2 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

The results of the environmental and biological monitoring are summarized in table 2. The mean time-weighted average (TWA) exposure (18.2 ppm) was very close to the current threshold limit value (TLV-TWA) recently adjusted by ACGIH from 50 to 20 ppm and the range of the measured individual values varied from 1 to 70 ppm. Results obtained for the metabolites of styrene in post-shift urinary samples after adjustment for creatinine concentration are presented in table 2.

As expected, a good relationship was observed between airborne styrene concentration and the concentration of MA and PGA measured in post-shift urinary samples. The relationship was not different when considering the sum of MA and PGA ($r = 0.92$, $p < 0.0001$, figure 2(a)), MA ($r = 0.90$, $p < 0.0001$, not shown) or PGA ($r = 0.93$, $p < 0.0001$, not shown) alone. Calculated urinary post-shift concentrations corresponding to a styrene airborne concentration of 50 ppm amounted to 1050, 768 and 282 mg g^{-1} creatinine, respectively and were in good agreement with proposed biological exposure indexes (BEIs) in end of shift urinary samples (1040, 800 and 240 mg g^{-1} creatinine for MA + PGA, MA and PGA, respectively). Concentrations corresponding to the newly proposed TLV-TWA value (20 ppm) were 420, 307 and 113 mg g^{-1} creatinine, respectively.

Table 2. Results of the environmental and biological monitoring.

	Mean ($\bar{x}$)	Range
Airborne styrene (ppm)	18.2	0.9–68.9
MA (mg g^{-1} creatinine)	314.6	15.5–1410.2
PGA (mg g^{-1} creatinine)	120.3	4.8–347.7
MA + PGA (mg g^{-1} creatinine)	434.9	20.3–1757.8
M1 (μg g^{-1} creatinine)	893.1	56.6–4292.3
M2 (μg g^{-1} creatinine)	1625.2	57.4–5027.9
M1 + M2 (μg g^{-1} creatinine)	2518.3	129.3–9320.2

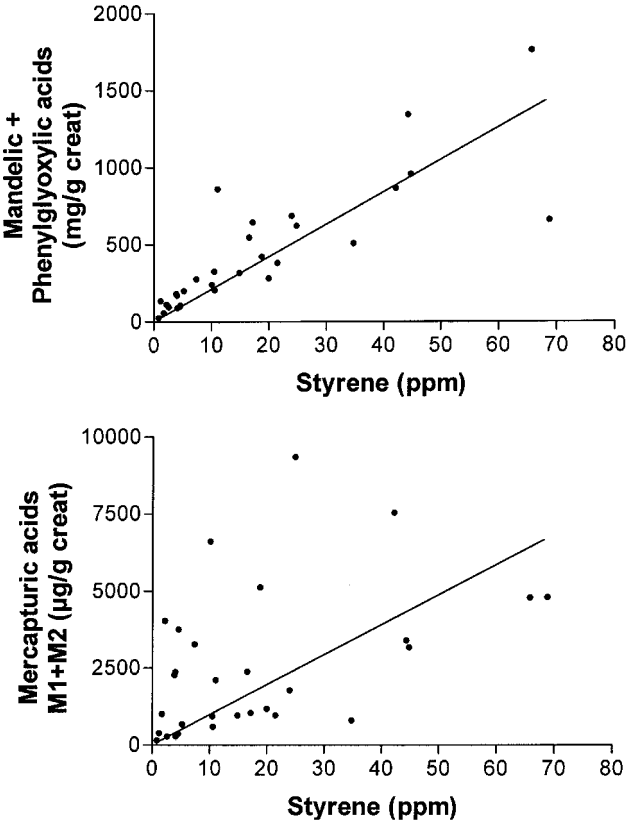

Figure 2. Relationship between airborne styrene and (a) the sum of mandelic and phenylglyoxylic acid or (b) the sum of mercapturic acids in end of shift urinary samples.

The excretion of mercapturic acids also exhibited a significant correlation with airborne styrene concentration. The M2 mercapturic acid showed a better correlation with airborne styrene ($r = 0.80$, $p < 0.0001$, not shown) than M1 ($r = 0.59$, $p = 0.0005$, not shown). Based on the existing correlations, urinary post-shift concentrations of 3366 and 1515 µg g^{-1} creatinine (or 1347 and 606 µg g^{-1} creatinine) corresponded to an airborne concentration of 50 ppm (or 20 ppm) of styrene for M2 and M1 mercapturic acids, respectively. Figure 2(b) illustrates the relationship between airborne styrene concentration and total mercapturic acids (Mtot = M1 + M2) in post-shift urinary samples ($r = 0.74$, $p < 0.0001$) for which a total concentration of 4881 (or 1952) µg g^{-1} creatinine corresponds to a 50 (or 20) ppm concentration of styrene.

The distribution of *CYP2E1*, *EPHX* and *GST* genotypes in the population study are presented in table 3. Concerning *CYP2E1* insertion polymorphism, all individuals were wild homozygous (i.e. without the 96 bp-insertion) except for one which was heterozygous. This particular polymorphism was therefore excluded for further statistical analysis. As previously observed for other *CYP2E1* polymorphisms (Haufroid *et al*. 2000), the presence of the rare c2 allele was always associated with the rare C allele (*CYP2E1*5A* allele) but in two cases, the rare C allele was present alone (*CYP2E1*6* allele).

As shown in table 4, for D/C polymorphism a lower mean I1 value was observed in individuals homozygous for the mutant allele C as compared with

Table 3. Distribution of *CYP2E1*, *EPHX* and *GST* genotypes in the study population.

	Homozygous wild type	Heterozygous	Homozygous mutant	Allele frequencies
CYP2E1 [a]				
5′-flanking region polymorphism	c1c1	c1/c2	c2c2	c1: 0.88
cl/c2	25 (83.3)	3 (10.0)	2 (6.7)	c2: 0.12
Intron 6 polymorphism	DD	DC	CC	D: 0.85
D/C	23 (76.6)	5 (16.7)	2 (6.7)	C: 0.15
Intron 7 polymorphism	A2A2	A1A2	A1A1	A2: 0.80
A1/A2	19 (63.3)	10 (33.3)	1 (3.3)	A1: 0.20
EPHX				
Exon 3 polymorphism	YY	YH	HH	Y: 0.75
Tyr113/His113 (Y/H)	19 (63.3)	7 (23.3)	4 (13.3)	H: 0.25
Exon 4 polymorphism	HH	HR	RR	H: 0.87
His139/Arg139 (H/R)	22 (73.3)	8 (26.7)	0 (0)	R: 0.13
GST				
GSTM1 polymorphism	GSTM1+		GSTM1null	
+/null	12 (40)		18 (60)	
GSTT1 polymorphism	GSTT1+		GSTT1null	
+/null	25 (83)		5 (17)	

Values are the number of individuals (%).

CYP2E1 insertion polymorphism; all individuals were homozygous wild type (without insertion) except one who was heterozygous.

[a] CYP2E1 proposed nomenclature (http://www.imm.ki.se/CYPalleles/cyp2e1.htm)

*CYP2E1*1A*	wild allele
*CYP2E1*1B*	A2 allele
*CYP2E1*5A*	association c2 and C allele
*CYP2E1*6*	C allele only

individuals homozygous for the wild allele D. An identical pattern was observed for mean I2 values. However ANOVA analysis did not reveal statistically significant differences between each combination of alleles. An opposite trend was observed for A1/A2 polymorphism (I1 and I2 values). As only one individual was homozygous for the mutant allele (A1A1), a double sided *t*-test was applied to compare individuals homozygous for the wild allele and individuals possessing at least one mutant allele. A statistically significant difference was observed for I2 values while this difference was nearly significant when considering I1.

A statistically significant difference was observed for I2 values between individuals *GSTM1*+ and those lacking this gene. A similar difference was not observed for *GSTT1* polymorphism applied to I2 values or for both *GST* polymorphisms applied to I1 values. Figure 3 illustrates the relationship between airborne styrene and the sum of mercapturic acids (Mtot) in end of shift urinary samples after taking into account the *GSTM1* allelic status. Better correlations were observed ($r = 0.95$, $p < 0.0001$ for *GSTM1*null and $r = 0.75$, $p = 0.0029$ for *GSTM1*+ individuals) as compared with the whole population (figure 2(b), $r = 0.74$). Two distinct biological exposure indexes corresponding to a 20 ppm level of styrene could be proposed for Mtot based on *GSTM1* allelic status (*GSTM1*null: 1330 μg g^{-1} creatinine, *GSTM1*+: 2878 μg g^{-1} creatinine). Similar indexes corresponding to a 50 ppm TLV-TWA value were 3325 and 7196 μg g^{-1} creatinine, respectively.

Concerning *EPHX* genetic polymorphisms, no statistically significant difference was observed neither between the three allele combinations for exon 3 polymorphism (Y/H) or for both allele combinations observed for exon 4 polymorphism (H/R) (table 4).

Table 4. Influence of genetic polymorphisms on metabolic indexes.

(n=30)	Whole population	Homozygous Wild type	Heterozygous	Homozygous mutant	p value[a]
c1/c2 polymorphism		c1c1 (n=25)	c1c2 (n=3)	c2c2 (n=2)	
I1	1.43 (0.98–1.99)	1.44 (0.98–1.99)	1.51 (1.43–1.61)	1.22 (1.14–1.31)	0.30
I2	2.17 (1.35–3.23)	2.18 (1.35–3.23)	2.18 (1.86–2.76)	2.00 (1.76–2.25)	0.88
D/C polymorphism		DD (n=23)	DC (n=5)	CC (n=2)	
I1	1.43 (0.98–1.99)	1.45 (0.98–1.99)	1.45 (1.27–1.61)	1.22 (1.14–1.31)	0.34
I2	2.17 (1.35–3.23)	2.21 (1.35–3.23)	2.03 (1.73–2.76)	2.00 (1.76–2.25)	0.64
A1/A2 polymorphism		A2A2 (n=19)	A1A2 (n=10)	A1A1 (n=1)	
I1	1.43 (0.98–1.99)	1.38 (0.98–1.65)	1.51 (1.24–1.99)	1.66	0.10[c] (0.06[d])
I2	2.17 (1.35–3.23)	2.04 (1.35–2.76)	2.31 (1.64–2.90)	3.23	0.09[c] (0.03[d])
Y/H polymorphism		YY (n=19)	YH (n=7)	HH (n=4)	
I1	1.43 (0.98–1.99)	1.41 (0.98–1.99)	1.48 (1.16–1.66)	1.45 (1.14–1.61)	0.75
I2	2.17 (1.35–3.23)	2.11 (1.64–2.90)	2.27 (1.35–3.23)	2.26 (1.76–2.76)	0.71
H/R polymorphism		HH (n=22)	HR (n=8)	RR (n=0)	
I1	1.43 (0.98–1.99)	1.41 (0.98–1.99)	1.50 (1.33–1.66)		0.28[b]
I2	2.17 (1.35–3.23)	2.12 (1.35–2.90)	2.31 (1.85–3.23)		0.33[b]
GSTM1 polymorphism		GSTM1+ (n=12)		GSTM1null (n=18)	
I1	1.43 (0.98–1.99)	1.43 (0.98–1.66)		1.43 (1.14–1.99)	0.97[b]
I2	2.17 (1.35–3.23)	2.59 (1.84–3.23)		1.89 (1.35–2.44)	<0.0001[b]
GSTT1 polymorphism		GSTT1+ (n=25)		GSTT1null (n=5)	
I1	1.43 (0.98–1.99)	1.41 (0.98–1.99)		1.53 (1.24–1.88)	0.27[b]
I2	2.17 (1.35–3.23)	2.15 (1.35–3.23)		2.24 (1.64–2.64)	0.71[b]

Values are given as mean with range.
[a] Differences between genotype categories were assessed by ANOVA
[b] Double sided t-test.
[c] Double sided t-test between homozygous wild type and heterozygous (ANOVA not possible).
[d] Double sided t-test between homozygous wild type and individuals possessing at least one mutant allele.

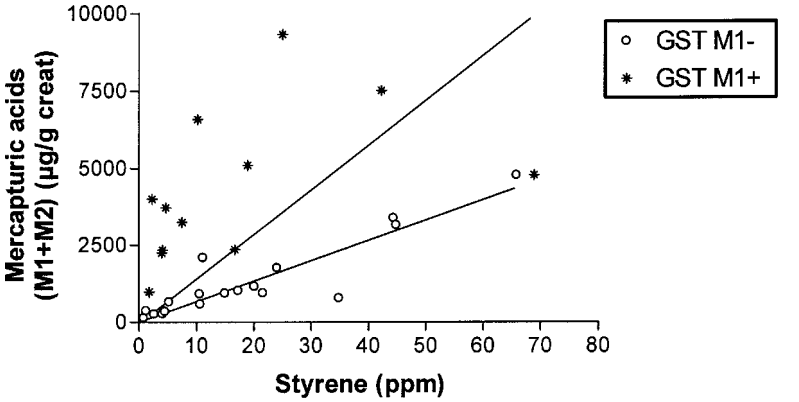

Figure 3. Relationship between airborne styrene and the sum of mercapturic acids (M1 + M2) in end of shift urinary samples taking into account the GSTM1 allelic status.

The infiuence on I1 and I2 values of some other parameters not directly related to genomic status (body mass index, alcohol and tobacco consumption) is presented in table 5. No differences on metabolic parameters were observed between alcohol or tobacco consumers. Only individuals with higher BMI values (>25 kg m^{-2}) showed a statistically significant lower MA + PGA urinary excretion.

Multivariate regression analyses were also performed in order to test interaction terms between genetic polymorphisms involving enzymes acting at different levels in the biotransformation pathway of styrene. Statistical models were constructed using either I1 or I2 as dependent variable and six unrelated single polymorphisms (D/C, A1/A2, Y/H, H/R, GSTM1, GSTT1), five first order interaction terms (GSTM1 combined with each other polymorphisms), BMI, age, tobacco and alcohol consumption as independent variables. Based on the results of single polymorphism analysis (table 4), only GSTM1 was taken into account for interaction terms. Results of stepwise multiple regression analyses are presented in table 6 which shows that A1/A2 polymorphism for *CYP2E1* gene explained a substantial part of the variance observed for I1 and I2 values (17 and 6%, respectively). However BMI was the most important determinant of I1 values with a slightly negative infiuence. The presence of Arg139 residue (R) in mEH also

Table 5. Infiuence of BMI, alcohol and tobacco consumption on metabolic indexes.

			p value[a]
Body mass index (BMI)	BMI≤25 (n = 13)	BMI>25 (n = 17)	
I1	1.55 (1.24–1.99)	1.34 (0.98–1.66)	0.004
I2	2.23 (1.64–2.76)	2.12 (1.35–3.23)	0.51
Alcohol consumption	no (n = 11)	yes (n = 19)	
I1	1.38 (0.98–1.89)	1.46 (1.16–1.99)	0.29
I2	2.11 (1.64–2.90)	2.20 (1.35–3.23)	0.63
Tobacco consumption	no (n = 12)	yes (n = 18)	
I1	1.39 (1.27–1.66)	1.46 (0.98–1.99)	0.33
I2	2.18 (1.73–3.23)	2.16 (1.35–2.90)	0.88

Values are given as mean with range.
[a] Double sided *t*-test.

Table 6. Determinants of metabolic indexes I1 and I2.

Dependent variables	Independent variables	Partial r^2	Slope	p value[a]
I1	BMI	0.30	-0.03	0.002
MA + PGA pathway	A1/A2	0.17	0.25	0.007
	H/R	0.12	0.17	0.009
	age	0.08	0.007	0.023
		Model r^2: 0.67	Intercept: 1.79	
I2	GSTM1	0.58	0.46	0.0001
Mercapturic acids pathway	Interaction 3 (GSTM1*Y/H)	0.08	0.44	0.020
	A1/A2	0.06	0.24	0.021
		Model r^2: 0.72	Intercept: 1.82	

[a] Partial r^2 p value.

Tested independent variables were six single unrelated polymorphisms (D/C, A1/A2, Y/H, H/R, GSTM1 and GSTT1), five first order interaction terms including GSTM1 (interaction 1: GSTM1* D/C, interaction 2: GSTM1* A1/A2, interaction 3: GSTM1* Y/H, interaction 4: GSTM1* H/R and interaction 5: GSTM1* GSTT1), BMI, alcohol consumption, tobacco consumption and age.

c1/c2 polymorphism was not included because it is linked with D/C polymorphism.

explained 12% of the variance observed with I1 values. In accordance with previous statistical analyses, *GSTM1* allelic status remained the most important predicting factor when considering the mercapturic acids pathway. Alone it explained 58% of the I2 variance and 8% more when combined with the presence of the H allele of *EPHX*.

Discussion

Biological monitoring of exposure to styrene is currently performed by the measurement of the urinary excretion of MA and PGA, which originate from the conversion of SO, an electrophilic compound considered responsible for most toxic effects of styrene. Several national or international organizations have proposed biological limit values for these metabolites in urine collected at the end of the shift and/or prior to the next shift (i.e. 16 h after exposure). Biological exposure indexes (BEIs) recommended by the American Conference of Governmental Industrial Hygienists (ACGIH) for MA and PGA are 800 and 240 mg g^{-1} creatinine or 300 and 100 mg g^{-1} creatinine, in end of shift or prior to the next shift urinary samples which corresponds to a TLV-TWA of 50 ppm. More recently, Ghittori *et al.* (1997) proposed the measurement of specific mercapturic acids in post-shift urine for the biological monitoring of workers exposed to styrene but these authors observed large inter-individual variations in urinary excretion of these metabolites.

In the present study, without taking genotype into consideration, a better correlation was observed between the well validated biomarkers of styrene exposure (MA and PGA) in post-shift urinary samples and ambient styrene than that observed with the sum of mercapturic acids (Mtot). Similar experimental data have already been published by Ghittori *et al.* (1997). These authors had then arbitrarily divided their study population into two groups on the basis of the median value of the conversion percentage (0.173%) from absorbed styrene to mercapturic acid (Mtot) in post-shift urine. Their approach was conducted to

simulate the existence of two different groups, respectively rapid and poor metabolizers of SO by GST but was not based on objective data on metabolizing status. In view of the results in table 4 suggesting a significant infiuence of *GSTM1* genotype on urinary excretion of mercapturic acids (I2), we have followed a similar approach by dividing our study population into two groups (*GSTM1*null and *GSTM1*+) which are clearly distinct on the graph and for which specific biological exposure indexes corresponding to a 50 or 20 ppm concentration of styrene in ambient air are proposed (3325 and 7196 μg g^{-1} creatinine or 1330 and 2878 μg g^{-1} creatinine for *GSTM1*null and *GSTM1*+, respectively).

Our results indicate that the knowledge of the *GSTM1* allelic status is important for a correct interpretation of urinary mercapturic acid determination. This observation is somewhat discordant with published *in vitro* results obtained in human lymphocyte cultures. Based on the *in vitro* induction of sister chromatid exchanges (SCEs) by SO in human whole-blood lymphocytes cultures, Uuskula *et al.* (1995) concluded that GSTM1-mediated GSH conjugation was not an important detoxification pathway for SO while thereafter the same group suggested that GSTT1 was much probably involved in this detoxification process (Ollikainen *et al.* 1998). In an *in vivo* study, Lambert *et al.* (1995) found however that styrene-exposed workers with GSTM1 deficiency showed significantly higher frequency of hypoxanthine phosphoribosyl transferase (hprt)-mutant T-cell than *GSTM1*+ workers (*GSTT1* genotype not determined). Different *GST* activity profiles between blood and other organs involved in xenobiotic metabolism (liver, kidney, lung, etc.) could explain the discordance observed between *in vitro* and *in vivo* studies. The high GSTT activity in erythrocytes (Pemble *et al.* 1994) may be important in lymphocyte cultures and might also affect the level of genotoxic damage observed in peripheral lymphocytes of styrene-exposed reinforced plastics workers. However, when considering the urinary excretion of SO-specific mercapturic acids formed systemically, the infiuence of the *GSTT1* polymorphism seems of less importance than that observed with *GSTM1* polymorphism. The low frequency of *GSTT1* null workers could be another explanation of the fact that *GSTT1* genotype does not appear important for mercapturic acids excretion in the present population.

Multivariate regression analysis highlights some other interesting points. First, A1/A2 polymorphism explains a substantial part of the variance observed for I1 and I2 values (17 and 6%, respectively). In both models, the slope value is of similar sign, which is consistent with the fact that CYP2E1 contributes to both biotransformation pathways. These results suggest that the presence of mutant A1 allele (*CYP2E1*1B*) is associated with increased activity of CYP2E1 *in vivo* and that genotypic status should be considered for interpreting exposure biomonitoring. The mechanistic basis of the infiuence of this polymorphism, which is located in a non-coding region of the *CYP2E1* gene, remains however unclear. After an oral administration of chlorzoxazone to 70 healthy volunteers, Kim *et al.* (1995) have already shown that the presence of mutant C allele (*CYP2E1*5A*) is associated with a decreased fractional clearance by 6-hydroxylation, a parameter considered until now as the best for CYP2E1 phenotyping purposes (Streetman *et al.* 2000). On the other hand, we have reported a 'reverse linkage' between D/C and A1/A2 polymorphisms so that the presence of at least one mutant allele for one polymorphic site always excluded the presence of the mutant allele in the other (Haufroid *et al.* 2000). The present observation is therefore in complete accordance with previously cited studies.

As already discussed, *GSTM1* genotype is important for interpreting SO-specific mercapturic acids excretion and it remains the principal parameter to take into account to explain the observed variability (58% of the observed variance can be explained by *GSTM1* allelic status). However, a particular association of *GSTM1* genotype with *EPHX* genetic polymorphism seems also of particular interest. In fact *GSTM1+* individuals possessing at least one mutant allele in exon 3 (His113, H) would produce more mercapturic acids than other workers. This association is consistent with the *in vitro* observation of Hassett *et al.* (1994) showing, with an expression assays using cloned mEH DNA templates transiently transfected into COS-1 cells, that His113 subtitution for Tyr113 was associated with a 39% loss in mEH activity. With regard to styrene metabolism, such a decresead mEH activity could explain a preferential detoxification of SO by the GSH pathway. The other *EPHX* polymorphism (exon 4, H/R) is more important to explain part of the variation observed in MA and PGA production. Because of the positive slope value, individuals possessing at least one mutant allele in exon 4 (Arg139, R) produce more MA and PGA than individuals homozygous for the wild allele. Once more this observation is consistent with *in vitro* experimental data of Hassett *et al.* (1994) suggesting 125% increased mEH activity when His139 is substituted by Arg139.

Besides genetic polymorphisms for enzymes involved in styrene biotransformation, the infiuence of some 'environmental' factors on urinary metabolites excretion was also tested. Alcohol consumption, tobacco consumption and age seemed to have negligible effect on both metabolic indexes (MA + PGA and mercapturic acids). Since ethanol is a well known CYP2E1 inducer after chronic intake (Lieber *et al.* 1997), one could have expected that urinary excretion of styrene biomarkers would be increased for both pathways in the subgroup of alcohol consumers (≥ 5 glasses per week). This trend was in fact verified in the monovariate analysis for variables I1 and I2 but the differences observed remained not statistically significant. The infiuence of BMI on the MA + PGA pathway deserves further comments. Obesity is in fact a known factor of CYP2E1 induction (Lieber *et al.* 1997). Using chlorzoxazone as a metabolic probe of *in vivo* CYP2E1 activity, Lucas *et al.* (1999) recently confirmed that chlorzoxazone 6-hydroxylation activity was higher in obese individuals (defined as BMI > 30 kg m^{-2}). We also showed previously that biotransformation of sevofiurane, an anaesthetic agent predominantly oxidized by CYP2E1, was significantly higher in individuals with BMI > 25 kg m^{-2}compared with those presenting low BMI (< 25 kg m^{-2}) (Haufroid *et al.* 2000). Therefore the negative infiuence of BMI on styrene biotransformation is in apparent contradiction with these observations. Two hypotheses could be proposed to explain this discrepancy: (1) a greater deposition of styrene in a peripheral fatty compartment resulting in a delayed biotransformation or (2) an effect of BMI on the activity of other enzymes (e.g. alcohol and/or aldehyde dehydrogenase) involved in the further biotransformation of SO.

In conclusion, although based on a limited number of subjects, this study shows that the interpretation of biological monitoring data for styrene exposure is improved by taking into account the frequent genetic polymorphisms for metabolic enzymes. *CYP2E1* genotyping, particularly assessment of A1/A2 allelic status, is useful because of the presence of the rare *CYP2E1*1B* allele which enhances MA and PGA urinary excretion in post-shift samples. The study also confirms the value of specific mercapturic acids for the biological monitoring of styrene exposure and

highlights the need of a *GSTM1* genotyping for a correct interpretation of M1 and M2 concentrations. Different biological exposure indexes (BEIs) corresponding to a 20 ppm styrene concentration are therefore proposed according to *GSTM1* status: 1330 and 2878 μg g^{-1} creatinine for *GSTM1*null and *GSTM1*+, respectively. The equations reported in table 6 also allow individualized BEIs to be derived according to the genotypic and phenotypic characteristics of a subject. For instance, one can calculate that a male individual 25 years old, with a BMI of 22 kg m^{-2}, heterozygous for the A2 allele of *CYP2E1* and homozygous HH for *EPHX* in exon 4 would excrete 718 mg of MA + PGA g^{-1} creatinine at the end of the shift when exposed to 20 ppm styrene (as compared with 420 mg g^{-1} creatinine when considering that all individuals respond similarly, figure 2(a)).

Acknowledgements

This study was supported by the National Fund for Scientific Research, Belgium and by the European Commission (Environment and Health, 5th framework programme, contract QLRT-1999-01368). The technical assistance of F. Toubeau, D. Boesmans, C. Nicolas and J. Casters is gratefully acknowledged.

References

Arand, M., Mühlbauer, R., Hengstler, J., Jäger, E., Fuchs, J., Winkler, L. and Oesch, F. 1996, A multiplex polymerase chain reaction protocol for the simultaneous analysis of the glutathione S-transferase GSTM1 and GSTT1 polymorphisms. *Analytical Biochemistry*, **236**, 184–186.

Fernandez, J. G. and Caperos, J. R. 1977, Styrene exposure. An experimental study of pulmonary absorption and excretion. *International Archives of Occupational and Environmental Health*, **40**, 1–12.

Fritsche, E., Pittman, G. S. and Bell, D. A. 2000, Localization, sequence analysis, and ethnic distribution of a 96-bp insertion in the promoter of the human CYP2E1 gene. *Mutation Research Genomics*, **432**, 1–5.

Ghittori, S., Maestri, L., Imbriani, M., Capodaglio, E. and Cavalleri, A. 1997, Urinary excretion of specific mercapturic acids in workers exposed to styrene. *American Journal of Industrial Medicine,* **31**, 636–644.

Guengerich, F. P., Kim, D. H. and Iwasaki, M. 1991, Role of human cytochrome P-450 IIE1 in the oxidation of many low molecular weight cancer suspects. *Chemical Research in Toxicology,* **4**, 168–179.

Hassett, C., Aicher, L., Sidhu, J. S. and Omiecinsky, C.J. 1994, Human microsomal epoxide hydrolase : genetic polymorphism and functional expression *in vitro* of amino acid variants. *Human Molecular Genetics*, **3**, 421–428.

Haufroid, V., Clippe, A., Knoops, B., Bernard, A. and Lison, D. 1998, Genotyping in urine: an interesting tool for epidemiological studies. *Clinical Chemistry*, **44**, 2210–2211.

Haufroid, V., Gardinal, S., Licot, C., Villalpando, M. L., Van Obbergh, L., Clippe, A. and Lison, D. 2000, Biological monitoring of exposure to sevofiurane in operating room personnel by the measurement of hexafiuoroisopropanol and fiuoride in urine. *Biomarkers*, **5**, 141–151.

Hu, Y., Hakkola, J., Oscarson, M. and Ingelman-Sundberg, M. 1999, Structural and functional characterization of the 5´-fianking region of the rat and human cytochrome P450 2E1 genes: identification of a polymorphic repeat in the human gene. *Biochemical and Biophysical Research Communications*, **263**, 286–293.

Kim, R. B., O'Shea, D. O. and Wilkinson, G. R. 1995, Interindividual variability of chlorzoxazone 6-hydroxylation in men and women and its relationship to CYP2E1 genetic polymorphisms. *Clinical Pharmacology and Therapeutics*, **57**, 645–654.

Kim, H., Wang, R. S., Elovaara, E., Raunio, H., Pelkonen, O., Aoyama, T., Vainio, H. and Nakajima, T. 1997, Cytochrome P450 isozymes responsible for the metabolism of toluene and styrene in human liver microsomes. *Xenobiotica,* **27**, 657–665.

Lambert, B., Bastlova, T., Osterholm, A. M. and Hou, S. M. 1995, Analysis of mutation at the hprt locus in human T lymphocytes. *Toxicology Letters*, **82–83**, 323–333.

Lancaster, J. M., Brownlee, H. A., Bell, D. A., Futreal, P. A., Marks, J. R., Berchuck, A., Wiseman, R. W. and Taylor, J. A. 1996, Microsomal epoxide hydrolase polymorphism as a risk factor for ovarian cancer. *Molecular Carcinogenesis,* **17,** 160–162.

LIEBER, C. S. 1997, Cytochrome P-4502E1: Its physiological and pathological role. *Physiological Reviews*, **77**, 517–544.

LUCAS, D., FERRARA, R., GONZALEZ, E., BODENEZ, P., ALBOREZ, A., MANNO, M. and BERTHOU, F. 1999, Chlorzoxazone, a selective probe for phenotyping CYP2E1 in humans. *Pharmacogenetics,* **9**, 377–388.

MCBRIDE, O. W., UMENO, M., GELBOIN, H. V. and GONZALEZ, F. J. 1987, A Taq I polymorphism in the human P450IIE1 gene on chromosome 10 (CYP2E). *Nucleic Acids Research*, **15**, 10071.

MCCARVER, D. G., BYUN, R., HINES, R. N., HICHME, M. and WEGENEK, W. 1998, A genetic polymorphism in the regulatory sequences of human *CYP2E1*: association with increased chlorzoxazone hydroxylation in the presence of obesity and ethanol intake. *Toxicology and Applied Pharmacology*, **152**, 276–281.

NAKAJIMA, T., ELOVAARA, E., GONZALEZ, F. J., GELBOIN, H. V., VAINIO, H. and AOYAMA, T. 1993, *Butadiene and Styrene: Assessment of Health Hazards*. IARC Scientific Publication, **127** (Lyon: IARC), pp. 101–108.

NAKAJIMA, T., ELOVAARA, E., GONZALEZ, F. J., GELBOIN, H. V., RAUNIO, H., PELKONEN, O., VAINIO, H. and AOYAMA, T. 1994, Styrene metabolism by cDNA-expressed human hepatic and pulmonary cytochromes P450. *Chemical Research in Toxicology,* **7**, 891–896.

OLLIKAINEN, T., HIRVONEN, A. and NORPPA, H. 1998, Infiuence of GSTT1 genotype on sister chromatid exchange induction by styrene-7,8-oxide in cultured human lymphocytes. *Environmental and Molecular Mutagenesis*, **31**, 311–315.

PEMBLE, S., SCHROEDER, K. R., SPENCER, S. R., MEYER, D. J., HALLIER, E., BOLT, H. M., KETTERER, B. and TAYLOR, J. B. 1994, Human glutathione S-transferase theta (GSTT1): cDNA cloning and the characterization of a genetic polymorphism. *Biochemical Journal*, **300**, 271–276.

RANNUG, A., ALEXANDRIE, A.-K., PERSSON, I. and INGELMAN-SUNDBERG, M. 1995, Genetic polymorphism of cytochromes P450 1A1, 2D6 and 2E1: regulation and toxicological significance. *Journal of Occupational and Environmental Medicine,* **37**, 25–36.

STREETMAN, D. S., BERTINO, JR J. S. and NAFZIGER, A.N. 2000, Phenotyping of drug-metabolizing enzymes in adults: a review of in-vivo cytochrome P450 phenotyping probes. *Pharmacogenetics,* **10**, 187–216.

UEMATSU, F., KIKUCHI, H., OHMACHI, T., SAGAMI, I., MOTOMIYA, M., KAMATAKI, T., KOMORI, M. and WATANABE, M. 1991, Two common RFLPs of the human CYP2E gene. *Nucleic Acids Research*, **19**, 2803.

UUSKULA, M., JARVENTAUS, H., HIRVONEN, A., SORSA, M. and NORPPA, H. 1995, Infiuence of GSTM1 genotype on sister chromatid exchange induction by styrene-7,8-oxide and 1,2-epoxy-3-butene in cultured human lymphocytes. *Carcinogenesis,* **16**, 947–950.

WATANABE, J., HAYASHI, S. I., NAKACHI, K., IMAI, K., SUDA, Y., SEKINE, T. and KAWAJIRI, K. 1990, PstI and RsaI RFLPs in complete linkage disequilibrium at the CYP2E gene. *Nucleic Acids Research*, **18**, 7194.