Genetic polymorphisms and benzene metabolism in humans exposed to a wide Range of air concentrations

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Using generalized linear models with natural-spline smoothing functions, we detected effects of specific xenobiotic metabolizing genes and gene–environment interactions on levels of benzene metabolites in 250 benzene-exposed and 136 control workers in Tianjin, China (for all individuals, the median exposure was 0.512 p.p.m. and the 10th and 90th percentiles were 0.002 and 6.40 p.p.m., respectively). We investigated five urinary metabolites (*E,E*-muconic acid, *S*-phenylmercapturic acid, phenol, catechol, and hydroquinone) and nine polymorphisms in seven genes coding for key enzymes in benzene metabolism in humans {cytochrome P450 2E1 [*CYP2E1*, rs2031920], NAD(P)H: quinone oxidoreductase [*NQO1*, rs1800566 and rs4986998], microsomal epoxide hydrolase [*EPHX1*, rs1051740 and rs2234922], glutathione-*S*-transferases [*GSTT1*, *GSTM1* and *GSTP1*(rs947894)] and myeloperoxidase [*MPO*, rs2333227]}. After adjusting for covariates, including sex, age, and smoking status, *NQO1*2* (rs1800566) affected all five metabolites, *CYP2E1* (rs2031920) affected most metabolites but not catechol, *EPHX1* (rs1051740 or rs2234922) affected catechol and *S*-phenylmercapturic acid, and *GSTT1* and *GSTM1* affected *S*-phenylmercapturic acid. Significant interactions were also detected between benzene exposure and all four genes and between smoking status and *NQO1*2* and *EPHX1* (rs1051740). No significant effects were detected for *GSTP1* or *MPO*. Results generally support prior associations between benzene hematotoxicity and specific gene mutations, confirm earlier evidence that *GSTT1* affects production of *S*-phenylmercapturic acid, and provide additional evidence that genetic polymorphisms in *NQO1*2*, *CYP2E1*, and *EPHX1* (rs1051740 or rs2234922) affect metabolism of benzene in the human liver. *Pharmacogenetics and Genomics* 17:789–801 © 2007 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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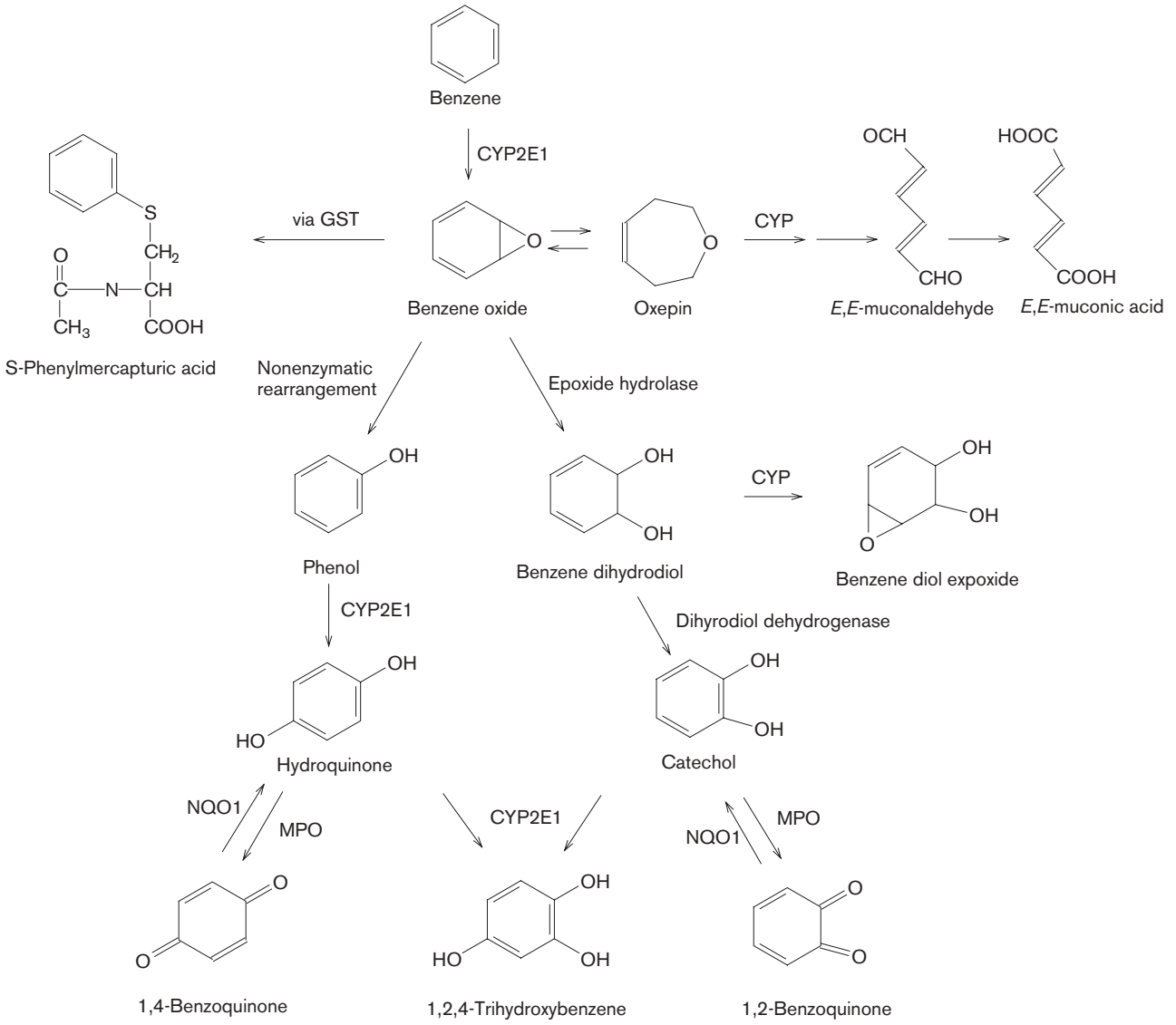
Introduction

Benzene is an important industrial chemical that is ubiquitous in the environment owing to vaporization from petroleum products and combustion of hydrocarbons [1–3]. Occupational exposures to benzene can cause blood disorders, including aplastic anemia, myelodysplastic syndrome, and acute myelogenous leukemia [4–7]. Significant decreases in the numbers of white blood cells and platelets have been reported in workers exposed to less than one p.p.m. of benzene in air [8]. Although these toxic effects are related to metabolism of benzene in the liver, the particular metabolite(s) that damage bone marrow cells and the mode of toxic action are subjects of debate [9–11].

Since the pioneering work of Parke and Williams' [12,13], the metabolism of benzene has been extensively investigated (reviewed in [9,10]). The major metabolic pathways, shown in Fig. 1, begin with cytochrome P450 CYP2E1-mediated oxidation of benzene to benzene oxide (BO), which is in equilibrium with its tautomer, oxepin. BO–oxepin is the source of all other major metabolites, namely, phenol (PH), *E,E*-muconic acid (MA), hydroquinone (HQ), and catechol (CA), and the minor product, *S*-phenylmercapturic acid (SPMA). All of these metabolites are excreted in urine, either free or in conjugated form. Additional metabolism of the primary metabolites produces additional electrophilic species, including the muconaldehydes (from CYP oxidation of

Fig. 1



Simplified metabolic scheme for benzene showing major pathways and metabolizing genes.

oxepin followed by ring opening), and 1,2-benzoquinone and 1,4-benzoquinone (BQ) (from spontaneous or peroxidase-mediated oxidation of CA and HQ, respectively).

As shown in Fig. 1, numerous enzyme systems are involved in the metabolism of benzene and its metabolites. In addition to the CYP oxidations of benzene (to BO), oxepin (to the muconaldehydes and ultimately MA) and PH (to HQ) [14–16], microsomal epoxide hydrolase (*EPHX*) catalyzes the hydrolysis of BO to initiate the CA pathway [14,17], various glutathione-*S*-transferases (GSTs) catalyze production of SPMA [18], and NAD(P)H: quinone oxidoreductase (*NQO1*) and peroxidases [notably myeloperoxidase (*MPO*)] are thought to catalyze transformations between CA and HQ and the corresponding quinones (1,2-BQ and 1,4-BQ, respectively) [10,14,19,20].

It has been speculated that polymorphic genes of the above enzymes predispose some individuals to benzene toxicity through metabolism [21–23]. In particular, individuals with wild-type *MPO* (more active) and a variant of *NQO1* (less active) were found to be at greater risk of reduced numbers of white blood cells at low levels of benzene exposure [8]. Yet, although *GSTT1* polymorphisms have been shown to affect production of the minor metabolite SPMA [24–26], there is only sketchy evidence that the major metabolites (PH, MA, HQ,

and CA) are affected by polymorphic forms of *CYP2E1*, *EPHX*, *NQO1*, or *MPO*.

A major difficulty in elucidating the connections between genotypes of metabolizing genes and the corresponding in-vivo phenotypes has been the inability to control for the effects of benzene exposure, and important physiological and lifestyle factors, in observational studies. Indeed, the relationship between metabolite levels and benzene exposure is highly nonlinear and is significantly affected by sex, age, smoking status, and body mass index (BMI) [27,28]. Using generalized linear models (GLM) with natural spline (NS) smoothing functions, we were able to elucidate the effects of sex, age, smoking status and BMI, after adjustment for benzene exposure (between 0.003 and 88.9 p.p.m.) in a sample of 326 individuals, who were exposed to benzene occupationally and environmentally in Tianjin, China [28]. In the current paper, we extend our application of GLM + NS models to investigate the effects of polymorphic forms of *CYP2E1*, *EPHX*, *NQO1*, *MPO*, and *GST*s on urinary levels of PH, MA, HQ, CA, and SPMA in the same population.

Materials and methods

Study population, and air and biological sample collection

Exposed and control study participants were recruited with informed consent from two shoe-making factories and three clothes-manufacturing factories, respectively, in Tianjin, China as described by Lan *et al.* [8]. Characteristics of the workplaces and levels of benzene exposure have been described previously in detail [8,27,29]. After excluding four controls, who were missing measurements of metabolites and/or exposures, the samples included 250 exposed individuals and 136 controls. Exposed and control participants were frequency-matched with respect to sex. Table 1 shows summary statistics for the sex, age and smoking status of participants. Methods of sampling air and urine were also described previously [8,27,29]. Briefly, personal full-shift air measurements were matched with urine samples after shift from exposed and control workers. Of the 386 participants in this analysis, 139 had repeated measurements of air and urine, making a total of 617 matched air/urine samples. Among participants with repeated measurements, the median number of paired air and urine samples was 3 (range 2–4). Information about height, weight, smoking status and other relevant factors were obtained by questionnaire [8].

This study was approved by the Institutional Review Boards of the University of North Carolina, the University of California, Berkeley, the US National Cancer Institute and the Chinese Academy of Preventive Medicine.

Measurements of air and urinary analytes

Measurements of analytes in air and urine were described previously [27–29]. Briefly, benzene and toluene were measured in air using passive personal monitors (Organic Vapor Monitors, 3M, St Paul, Minnesota, USA) followed by solvent desorption and gas chromatography [29]. Air measurements of benzene and toluene were below limits of detection [(LOD), nominally 0.2 p.p.m. for benzene and 0.3 p.p.m. for toluene] or were missing for all controls and for some exposed participants (missing values, $n = 23$; measurements below the LOD, $n = 70$ for benzene and $n = 67$ for toluene). Air levels were predicted in these censored and missing air samples from the corresponding urinary levels of benzene and toluene, as described previously for benzene [28]. As summarized in Table 1, the median air level of benzene was 0.512 p.p.m., the 10th percentile level was 0.002 p.p.m. and the 90th percentile was 6.40 p.p.m. The median air level of toluene was 1.77 p.p.m.

Urinary benzene was determined by gas chromatography–mass spectrometry (GC–MS) using head–space solid-phase microextraction according to Waidyanatha *et al.*

Table 1 **Demographics, benzene exposure and other characteristics of the study population ($n = 386$)**

	Women	Men	All
Occupational exposure[a]			
Controls (%)	84 (61.8)	52 (38.2)	136 (100.0)
Exposed (%)	164 (65.6)	86 (34.4)	250 (100.0)
Benzene exposure[b]			
Air benzene (p.p.m.)	0.517 (0.002–6.63)	0.487 (0.002–3.78)	0.512 (0.002–6.40)
Age (years)[b]	31 (21–44)	24 (20–39)	28 (21–43)
BMI (kg/m^2)[b]	21.9 (18.7–27.0)	22.0 (18.4–26.5)	21.9 (18.7–26.9)
Current smoking status[a]			
Nonsmoker (%)	240 (81.4)	55 (18.6)	295 (100.0)
Smoker (%)	8 (8.8)	83 (91.2)	91 (100.0)
Toluene exposure[a]			
Low [$\leq$ 1.77 p.p.m. (%)]	130 (67.4)	63 (32.6)	193 (100.0)
High [$>$1.77 p.p.m. (%)]	118 (61.1)	75 (38.9)	193 (100.0)

BMI, body mass index.
[a]Number (percent).
[b]Median (10–90th percentiles).

Table 2 Distributions of genetic polymorphisms among participants in the study

Gene name, SNP region, SNP ID (notes)	Genotype	No. participants	(%)	Presumed phenotype	Reference
CYP2E1, −1054C→T, Rs2031920, (ascribed to *RsaI*)	C/C	239	(62.1)	Active	[33]
	C/T	127	(33)	Less active	
	T/T	19	(4.9)	Least active	
NQO1, Ex6+40C→T, Rs1800566, (*NQO1*2*)	C/C	105	(27.3)	Active	[34]
	C/T	173	(44.9)	Less active	
	T/T	107	(27.8)	Inactive	
NQO1, Ex4−3C→T, Rs4986998, (*NQO1*3*)	C/C	359	(93.7)	Active	[35]
	C/T	24	(6.3)	Less active	[36]
	T/T	0	(0.0)	Least active	
MPO, −642G→A, Rs2333227	G/G	297	(77.3)	Active	[37]
	G/A	76	(19.8)	Less active	
	A/A	11	(2.9)	Least active	
GSTM1, del{GSTM1}, n/a	+/+	28	(7.4)	Conjugator	[38]
	+/−	141	(37.2)		
	−/−	210	(55.4)	Null	
GSTT1, del{GSTT1}, n/a	+/+	33	(8.6)	Conjugator	[38]
	+/−	187	(48.8)		
	−/−	163	(42.6)	Null	
GSTP1, Ex5−24A→G, Rs947894	A/A	224	(58.6)	Conjugator	[39]
	A/G	142	(37.2)		
	G/G	16	(4.2)	Less active	
EPHX1, Ex3−28T→C, Rs1051740, (Y113H)	T/T	143	(38)	Normal	[40]
	T/C	176	(46.8)		[41]
	C/C	57	(15.2)	Slow metabolizer	
EPHX1, Ex4+52A→G, Rs2234922, (H139R)	A/A	302	(79.9)	Normal	[40]
	A/G	71	(18.8)		[41]
	G/G	5	(1.3)	Rapid metabolizer	

SNP, single nucleotide polymorphism.

[30]. Urinary PH, CA, HQ, MA, and SPMA were measured as trimethylsilylether derivatives by GC–MS, after digestion of urine to release conjugates, according to Waidyanatha *et al.* [31]. Quantification of all urinary analytes was based on peak areas relative to the corresponding isotopically labeled internal standards. The minor metabolite, SPMA, was not detected in 30 urine specimens; a value of $\mathrm{LOD}/\sqrt{2} = 0.591\mathrm{nmol/l}$ was imputed to these samples [32].

Genotyping

We selected nine polymorphisms in seven genes coding for key enzymes in benzene metabolism, on the basis of the evidence of functionality in experimental or human studies (described in the discussion section). As summarized in Table 2 [33–41], the following genetic polymorphisms were chosen: *CYP2E1* (rs2031920: C→T), two alleles of *NQO1* [*NQO1*2* (rs1800566: C→T) and *NQO1*3* (rs4986998: C→T)], *MPO* (rs2333227: G→A), *GSTM1*, *GSTT1*, and *GSTP1* (rs947894: A→G), and two alleles of *EPHX1* [(rs1051740: T→C) and (rs2234922: A→G), respectively] [42]. Genotyping was performed with an ABI 7900HT detection system using TaqMan end points as described on the website, http://snp500cancer.nci.nih.gov [43]. The numbers of participants with each polymorphism of the various metabolizing genes are summarized in Table 2. Quality control procedures have been described previously [44]. In brief, blind replicate samples were randomly interspersed throughout the study sample plates and showed intrasubject agreement >99% for all genotype assays.

Statistical analyses

Relationships between levels of the urinary metabolites and the corresponding air concentrations of benzene were examined using GLM + NS models, as described previously [28]. The smoothing functions were based upon 5-knot models for all metabolites, after comparing NS models with 5–7 knots by visual inspection and corrected Akaike's Information Criteria (AICc) [45]. (The candidate knots in the 5-knot model were 0.001, 0.009, 0.512, 1.54, and 11.3 p.p.m. of benzene in air, corresponding to the 5th, 27.5th, 50th, 72.5th, and 95th percentiles [46], respectively). To avoid overparameterization, nonsignificant knots for each exposure–metabolite relationship were removed by stepwise elimination using a value of $P < 0.10$ for retention (PROC REG of SAS; SAS Institute, Cary, North Carolina, USA) [28]. For participants with repeated measurements of air and urine, the estimated geometric mean air and metabolite concentrations were used in all statistical analyses.

After establishing NS smoothing functions for each metabolite, we used GLM to investigate effects of genetic polymorphisms and their interactions with benzene exposure and smoking status, after adjusting for the following covariates: sex (0, women; 1, man), age (centered around the estimated mean value of 29.8 years, $n = 386$), smoking status (0, nonsmoker; 1, smoker), BMI (centered around the estimated mean value of 22.5 $\mathrm{kg/m^2}$, $n = 384$). We also investigated effects of toluene exposure (0, low exposure; 1, high exposure; median as a cutoff point, 1.77 p.p.m.) on levels of each metabolite.

Potential effects of genetic polymorphisms were screened in two stages to explore exposure-related interactions and smoking-related interactions, respectively. In both stages, the number of effects was restricted to less than 10% of observations to avoid overfitting. Then, important main effects and interactions were pooled from the two stages to build final models. Every candidate model for a given benzene metabolite was sorted by AICc, and the final model was selected from the best and second best candidates, using the following criteria: ΔAICc, evidence ratio, and the significance and biological plausibility of explanatory variables [45]. Modeling was performed using PROC GLMSELECT of SAS/STAT with the selection/stop option of AICc [45,47].

In coding each genetic polymorphism, the homozygous wild-type was defined as the reference group. For *EPHX1* (rs2234922: A→G), variant homozygotes and heterozygotes were combined in the analysis because the former contained only five participants (1.3%) [44]. Tests for Hardy–Weinberg equilibrium (HWE) among participants were conducted on the basis of observed genotype frequencies using PROC ALLELE of SAS/GENETICS (using a Pearson's χ^2 test with one degree of freedom). All genotypes were in HWE except *MPO* (rs2333227: G→A) ($P = 0.04$). The quality control data were rechecked and the precision of genotyping for this genetic polymorphism in blind replicates was confirmed; so this slight departure from HWE is likely due to chance. Tukey–Cramer adjustment was carried out for multiple comparisons of least-squares means among genetic polymorphisms in the final models.

All statistical analyses were performed using SAS software for Windows ver. 9.13 (SAS Institute).

Results

GLM + NS models and covariate effects

The following NS smoothing functions were used for the five benzene metabolites:

$$\begin{aligned}\text{MA}:&\mathrm{E}[\ln(Y_{MA,j})\,|\,\ln(X_j)]\\ &= 0.754 + 0.127[\ln(X_j)]\\ &\quad + 0.005[\ln(X_j) - \xi_1]_+^3\\ &\quad - 0.019[\ln(X_j) - \xi_3]_+^3\end{aligned}$$

$$\begin{aligned}\text{SPMA}:&\mathrm{E}[\ln(Y_{SPMA,j})\,|\,\ln(X_j)]\\ &= -6.65 - 0.119[\ln(X_j)]\\ &\quad + 0.030[\ln(X_j) - \xi_1]_+^3\\ &\quad - 0.040[\ln(X_j) - \xi_2]_+^3\end{aligned}$$

$$\begin{aligned}\text{PH}:&\mathrm{E}[\ln(Y_{PH,j})\,|\,\ln(X_j)]\\ &= 4.38 + 0.053[\ln(X_j)]\\ &\quad + 0.005[\ln(X_j) - \xi_2]_+^3\end{aligned}$$

$$\begin{aligned}\text{CA}:&\mathrm{E}[\ln(Y_{CA,j})\,|\,\ln(X_j)]\\ &= 3.02 + 0.098[\ln(X_j)]\\ &\quad - 0.005[\ln(X_j) - \xi_1]_+^3\\ &\quad + 0.011[\ln(X_j) - \xi_2]_+^3\end{aligned}$$

and

$$\begin{aligned}\text{HQ}:&\mathrm{E}[\ln(Y_{HQ,j})\,|\,\ln(X_j)]\\ &= 1.72 - 0.016[\ln(X_j)]\\ &\quad + 0.004[\ln(X_j) - \xi_1]_+^3\\ &\quad - 0.123[\ln(X_j) - \xi_5]_+^3\end{aligned}$$

where $\mathrm{E}[\ln(Y_{m,j})\,|\,\ln(X_j)]$ is the conditional mean value of $\ln(Y_{m,j})$ representing the natural log transform of the level of the mth metabolite level in the jth individual exposed at $\ln(X_j)$, the corresponding (logged) air concentration of benzene (p.p.m.), and ξ_i is the location of the ith knot (in log-scale of benzene exposure): $\xi_1 = \ln(0.001\text{ p.p.m.})$, $\xi_2 = \ln(0.009\text{ p.p.m.})$, $\xi_3 = \ln(0.512\text{ p.p.m.})$, $\xi_4 = \ln(1.54\text{ p.p.m.})$, $\xi_5 = \ln(11.3\text{ p.p.m.})$. The

Table 3 Parameter estimates for the final model of MA. [The dependent variable was the natural logarithm of the MA concentration (μmol/l); $n = 382$, $R^2 = 85.0\%$]

Independent variable	Description	Parameter estimates	Standard error	*P*-value	Cumulative ΔR^2 (%)
Intercept		**0.937**	0.197	<0.0001	
Age (years)	Centered at mean (29.8 years)	**−0.017**	0.005	<0.001	
Sex	Male	**−0.276**	0.099	0.005	
Smoking	Smoker	**0.177**	0.107	0.099	
BMI	Centered at mean (22.5 kg/m^2)	**0.019**	0.011	0.076	
$\ln(X_j) \times$ *NQO1*2*	$\ln(X_j) \times$ **1/*2*, less active	**−0.076**	0.027	0.005	
	$\ln(X_j) \times$ **2/*2*, least active	**−0.070**	0.029	0.016	0.37
*NQO1*2* (rs1800566: C→T)	**1/*2*, less active	**−0.174**	0.099	0.081	
	**2/*2*, least active	**−0.184**	0.110	0.094	0.38
$\ln(X_j) \times$ *CYP2E1*	$\ln(X_j) \times$ C/T, less active	0.001	0.024	0.955	
	$\ln(X_j) \times$ T/T, least active	**−0.150**	0.042	<0.001	1.00
CYP2E1 (rs2031920: C→T)	C/T, less active	0.037	0.087	0.667	
	T/T, least active	**−0.406**	0.191	0.034	1.01

BMI, body mass index; MA, *E,E*-muconic acid.
$\ln(X_j)$ represents the natural logarithm of the air benzene concentration (p.p.m.) in the jth participant.

Table 4 Parameter estimates for the final model of SPMA. [The dependent variable was the natural logarithm of the SPMA concentration, (μmol/l); $n = 365$, $R^2 = 82.0\%$]

Independent variable	Description	Parameter estimate	Standard error	*P*-value	Cumulative ΔR^2 (%)
Intercept		**−4.811**	0.685	<0.0001	
Age (years)	Centered at mean (29.8 years)	−0.012	0.009	0.161	
Sex	Male	−0.280	0.181	0.123	
Smoking	Smoker	**−0.690**	0.337	0.042	
BMI	Centered at mean (22.5 kg/m^2)	−0.025	0.019	0.189	
GSTM1	+/−, less active	−0.458	0.254	0.072	
	−/−, null	**−0.590**	0.248	0.018	0.31
$\ln(X_j) \times$ *EPHX1*	$\ln(X_j) \times$ (A/G or G/G, faster)	**0.101**	0.051	0.049	0.55
EPHX1 (rs2234922: A→G)	A/G or G/G, faster	0.234	0.187	0.213	0.56
$\ln(X_j) \times$ *NQO1*2* (rs1800566: C→T)	$\ln(X_j) \times$ **1/*2*, less active	**−0.149**	0.049	0.003	
	$\ln(X_j) \times$ **2/*2*, least active	−0.069	0.053	0.199	0.98
$\ln(X_j) \times$ *CYP2E1*	$\ln(X_j) \times$ C/T, less active	**0.114**	0.045	0.011	1.44
	$\ln(X_j) \times$ T/T, least active	−0.038	0.076	0.617	
CYP2E1 (rs2031920: C→T)	C/T, less active	0.146	0.159	0.357	1.50
	T/T, least active	−0.480	0.345	0.164	
Smoking × *NQO1*2*	Smoker × **1/*2*, less active	**1.108**	0.385	0.004	
	Smoker × **2/*2*, least active	**1.041**	0.413	0.012	1.94
*NQO1*2* (rs1800566: C→T)	**1/*2*, less active	**−0.654**	0.203	0.001	
	**2/*2*, least active	**−0.646**	0.227	0.005	2.01
$\ln(X_j) \times$ *GSTT1*	$\ln(X_j) \times$ +/−, less active	−0.035	0.069	0.614	
	$\ln(X_j) \times$ −/−, null	**−0.143**	0.070	0.041	2.63
GSTT1	+/−, less active	**−0.592**	0.251	0.019	
	−/−, null	**−1.444**	0.258	<0.0001	4.86

SPMA, *S*-phenylmercapturic acid; BMI, body mass index.
$\ln(X_j)$ represents the natural logarithm of the air benzene concentration (p.p.m.) in the jth participant.

Table 5 Parameter estimates for the final model of PH. [The dependent variable was the natural logarithm of the PH concentration, (μmol/l); $n = 382$, $R^2 = 64.8\%$]

Independent variable	Description	Parameter estimate	Standard error	*P*-value	Cumulative ΔR^2 (%)
Intercept		**4.645**	0.100	<0.0001	
Age (years)	Centered at mean (29.8 years)	**−0.010**	0.004	0.014	
Sex	Male	**−0.313**	0.090	0.001	
Smoking	Smoker	−0.204	0.167	0.222	
BMI	Centered at mean (22.5 kg/m^2)	0.007	0.010	0.477	
Smoking × *NQO1*2*	Smoker × **1/*2*, less active	0.311	0.192	0.106	0.67
	Smoker × **2/*2*, least active	**0.538**	0.204	0.009	
*NQO1*2* (rs1800566: C→T)	**1/*2*, less active	**−0.196**	0.085	0.022	1.31
	**2/*2*, least active	**−0.337**	0.097	0.001	
$\ln(X_j) \times$ *CYP2E1*	$\ln(X_j) \times$ C/T, less active	0.026	0.022	0.230	2.75
	$\ln(X_j) \times$ T/T, least active	**−0.141**	0.038	<0.001	
CYP2E1 (rs2031920: C→T)	C/T, less active	0.037	0.079	0.640	3.08
	T/T, least active	**−0.613**	0.174	0.001	

BMI, body mass index; PH, phenol.
$\ln(X_j)$ represents the natural logarithm of the air benzene concentration (p.p.m.) in the jth participant.

function $[\ln(X_j) - \xi_i]_+^3$ equals $[\ln(X_j) - \xi_i]^3$ for positive values and equals zero otherwise.

Final GLM + NS models are summarized in Tables 3–7 for the five benzene metabolites. Referring to the nongenetic effects, results are similar to those reported previously without adjustment for genetic polymorphisms [28]. Women participants had higher levels of MA, PH, CA, and HQ than men ($P < 0.05$) and younger individuals (below 30 years) had higher levels of MA, PH, and HQ than older individuals. Smokers generally had higher levels of benzene metabolites than nonsmokers, but the relationships were complicated by gene–smoking interactions for SPMA, PH, and CA. No significant effects were observed on any of the benzene metabolites for either BMI or coexposure to toluene.

Effects of genetic polymorphisms

After adjusting for exposure and covariates, the following genetic polymorphisms were found to significantly affect levels of the various metabolites, either as main effects or as interactions with benzene exposure and/or smoking: *NQO1*2* (rs1800566: C→T) for all metabolites, *CYP2E1* (rs2031920: C→T) for all metabolites except CA, *GSTT1* and *GSTM1* for SPMA, *EPHX1* (rs2234922: A→G) for SPMA and CA, and *EPHX1* (rs1051740: T→C) for CA. (Note that negative values of estimated parameters indicate lower metabolite levels and vice versa). The interaction between *CYP2E1* and benzene exposure accentuated the effects of polymorphic forms of this gene on levels of MA, PH, and HQ among individuals exposed to higher benzene concentrations (Tables 3, 5 and 7). For each of these metabolites, individuals having

Table 6 Parameter estimates for the final model of CA. [The dependent variable was the natural logarithm of the CA concentration, (μmol/l); $n = 370$, $R^2 = 57.2\%$]

Independent variable	Description	Parameter estimate	Standard error	*P*-value	Cumulative ΔR^2 (%)
Intercept		**3.072**	0.285	<0.0001	
Age (years)	Centered at mean (29.8 years)	<0.001	0.004	0.997	
Sex	Male	**−0.272**	0.087	0.002	
Smoking	Smoker	**0.567**	0.127	<0.0001	
BMI	Centered at mean (22.5 kg/m^2)	−0.007	0.009	0.448	
$\ln(X_j) \times$ *NQO1*2*	$\ln(X_j) \times$ **1/*2*, less active	−0.037	0.023	0.113	0.67
	$\ln(X_j) \times$ **2/*2*, least active	**−0.058**	0.025	0.021	
*NQO1*2* (rs1800566: C→T)	**1/*2*, less active	**−0.186**	0.089	0.036	0.96
	**2/*2*, least active	**−0.237**	0.097	0.015	
EPHX1 (rs2234922: A→G)	A/G or G/G, faster	**0.143**	0.076	0.061	1.43
$\ln(X_j) \times$ *EPHX1* (rs1051740: T→C)	$\ln(X_j) \times$ (T/C, slow)	**−0.039**	0.021	0.063	2.22
	$\ln(X_j) \times$ (C/C, slower)	**−0.069**	0.028	0.014	
Smoking × *EPHX1*	Smoker × (T/C, slow)	−0.163	0.156	0.299	3.32
	Smoker × (C/C, slower)	**−0.633**	0.206	0.002	
EPHX1 (rs1051740: T→C)	T/C, slow	−0.019	0.086	0.827	3.36
	C/C, slower	0.096	0.118	0.420	

BMI, body mass index; CA, catechol.
$\ln(X_j)$ represents the natural logarithm of the air benzene concentration (p.p.m.) in the jth participant.

Table 7 Parameter estimates for the final model of HQ. [The dependent variable was the natural logarithm of the HQ concentration, (μmol/l); $n = 382$, $R^2 = 72.6\%$]

Independent variable	Description	Parm. est.	Standard error	*P*-value	Cumulative ΔR^2 (%)
Intercept		**1.758**	0.118	<0.0001	
Age	Centered at mean (29.8 years)	**−0.011**	0.004	0.006	
Sex	Male	**−0.302**	0.082	<0.001	
Smoking	Smoker	**0.473**	0.089	<0.0001	
BMI	Centered at mean (22.5 kg/m^2)	−0.010	0.009	0.248	
*NQO1*2* (rs1800566: C→T)	× *1/*2*, less active	−0.087	0.070	0.215	0.34
	**2/*2*, least active	**−0.164**	0.078	0.036	
$\ln(X_j) \times$ *CYP2E1*	$\ln(X_j) \times$ C/T, less active	−0.008	0.020	0.676	1.25
	$\ln(X_j) \times$ T/T, least active	**−0.125**	0.035	<0.001	
CYP2E1 (rs2031920: C→T)	C→T, C/T, less active	−0.036	0.072	0.617	1.77
	C→T, T/T, least active	**−0.657**	0.160	<0.0001	

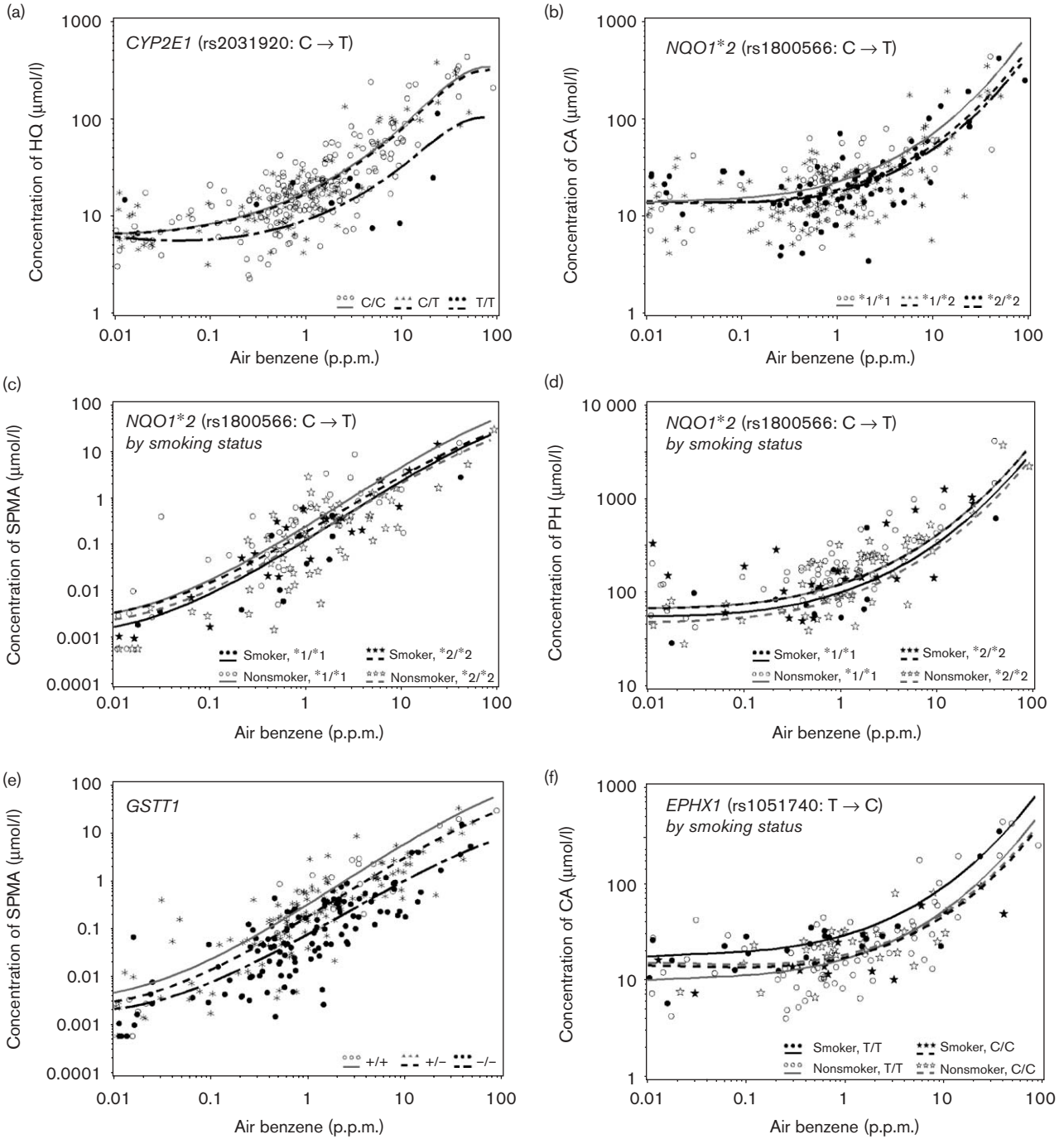
BMI, body mass index; HQ, hydraquinone.
$\ln(X_j)$ represents the natural logarithm of the air benzene concentration (p.p.m.) in the jth participant.

both variant alleles of *CYP2E1* had lower metabolite levels than those with at least one wild-type allele. For example, the relationships for HQ, shown in Fig. 2a, indicate that homozygous variants produced appreciably less metabolite than heterozygotes or homozygous wild-types at air concentrations greater than 0.1 p.p.m. (Tukey's test, $P < 0.05$). Similar behaviors were observed for PH, where significant departure was observed above 0.2 p.p.m., and for MA above 2 p.p.m. For SPMA, the effect of *CYP2E1* was unclear. Individuals with both variant alleles of *CYP2E1* had the lowest metabolite levels at benzene concentrations between 0.02 and 88.9 p.p.m.; however, the difference was not statistically significant ($P > 0.05$).

Participants with at least one variant allele of *NQO1*2* (rs1800566: C→T) had lower levels of all metabolites than homozygous wild-types or heterozygotes (Tables 3–7). This gene was also found to interact strongly with benzene exposure (for MA, SPMA, and CA) and/or smoking status (for PH and SPMA). These interactions with benzene exposure resulted in participants with at least one variant allele of *NQO1*2* (rs1800566: C→T) having lower levels of CA above 0.8 p.p.m. ($P < 0.05$, Fig. 2b), lower levels of MA above 6 p.p.m. ($P < 0.05$), and lower levels of HQ over the entire range of exposure (significance, $P < 0.1$). Among nonsmokers, participants with *NQO1*1/*1* produced more SPMA above 0.5 p.p.m. (Fig. 2c, $P < 0.05$) and more PH over the entire range of exposure (Fig. 2d, $P < 0.01$) than participants with *NQO1*2/*2*. Among smokers, however, these effects were diminished (Figs. 2d and e). No effects were observed for *NQO1*3* (rs4986998: C→T) polymorphisms.

Strong effects of *GSTT1* and *GSTM1* were observed on levels of SPMA, with homozygous variants producing the highest levels, followed by heterozygotes and homozygous null individuals (Table 4). A significant interaction was also observed between benzene exposure and *GSTT1* null individuals, such that these workers produced increasingly less SPMA at higher air concentrations (see Fig. 2e). No effect of polymorphic forms of *GSTP1* (rs947894: A→G) was observed on SPMA levels.

Fig. 2



Effects of genetic polymorphisms on urinary metabolites of benzene in humans (representative plots). Each panel depicts (log scale) effects of a particular genetic polymorphism on levels of a given metabolite versus the air concentration of benzene.

Participants with variant alleles of *EPHX1* (rs1051740: T→C) had lower levels of CA, particularly among smokers, than homozygous wild-types (see Table 6). Owing to the interaction of *EPHX1* (rs1051740: T→C) with benzene exposure, this effect was accentuated among smokers at air concentrations above 0.8 p.p.m. ($P < 0.05$) (Fig. 2f). Participants with at least one variant allele of *EPHX1* (rs2234922: A→G) had higher CA levels than homozygous wild-types ($P = 0.06$). Interestingly, participants with variant *EPHX1* (rs2234922: A→G) also had higher levels of SPMA at benzene concentrations above 6 p.p.m. ($P < 0.1$).

No effects were observed for polymorphic forms of *MPO* (rs2333227: G→A).

Discussion

Although the hematotoxicity of benzene was reported more than a century ago, the mechanism is not yet fully understood [9]. It has been speculated that genetic and lifestyle factors can influence the toxic effects of benzene, but current evidence is far from conclusive [9–11]. Rothman *et al.* [23] reported that heavily benzene-exposed workers who were rapid chlorzoxazone metabolizers (a measure of *CYP2E1* phenotype) and also possessed variant *NQO1*2* (rs1800566: C→T), were at elevated risk of benzene poisoning. More recently, Wan *et al.* [48] reported increased benzene poisoning in workers with variant *NQO1*2* (rs1800566: C→T) and in those with null-type *GSTT1* or *CYP2E1 DraI*, and Lan *et al.* [8] found lower white blood cell counts in workers with variants of *NQO1*3* (rs4986998: C→T) and *MPO* (rs2333227: G→A). Other studies of polymorphisms among benzene-exposed workers reported that individuals with variant *NQO1*2* (rs1800566: C→T) had increased DNA single-strand breaks [49] but decreased aneuploidy [50] in peripheral lymphocytes. The latter study also reported increased aneuploidy in benzene-exposed workers with null deletions of *GSTT1* and *GSTM1* and with either of two *CYP2E1* mutations (*Dra*I or *Rsa*I) [50].

In the present study, we focused upon the effects of metabolizing genes on production of five prominent metabolites (MA, SPMA, PH, CA and HQ). These metabolites are not 'biological effect markers' [51] *per se*, but rather reflect primary metabolism in the liver, which appears to be a necessary prelude to benzene-induced toxicity in target organs [9,10]. Previous attempts to link levels of benzene metabolites with polymorphic forms of metabolizing genes have been hampered by methodological and practical problems, including low benzene exposures (below 0.1 p.p.m.) [18,25,26,52,53], no measurements of air exposure [38,48,54,55], small numbers of participants [25,26,50,53], and difficulties in adjusting for covariates and nonlinear effects of exposure [24,49]. As benzene exposures were typically very low in previous studies, only SPMA and MA (metabolites with high specificity for benzene) tended to be measured, and the only consistent effect of any genetic polymorphism was that of lower SPMA levels in individuals with *GSTT1* (null deletion) [24–26].

We detected several effects of genetic polymorphisms on benzene metabolite patterns and interactions with benzene exposure that have not been reported previously (Tables 3–7). Our study had substantially more participants (386) and therefore, greater power to detect such effects. Further strengths of our study were the ability to examine effects over a wide range of benzene exposures, determined in both benzene-exposed workers and controls, broad exploration of genetic variants in key genes, evaluation of all major benzene urinary metabolites, and use of GLM + NS models to adjust for exposure and covariates. At the same time, it is possible that some findings could be false positives, and these findings need to be replicated in other large studies.

Individuals with *NQO1*2* (rs1800566: C→T) had lower levels of all five metabolites in our study. As NQO1 catalyzes two-election or four-election reductions of quinones [34,56–59], and the *NQO1*2* polymorphism is associated with a lack of *NQO1*2* protein [20], it is reasonable that the levels of CA and HQ would be lower in individuals with *NQO1*2* (rs1800566: C→T) (Tables 6 and 7). When combined with evidence that less active forms of *NQO1* are associated with benzene poisoning and DNA damage [8,23,48,49], this finding is also consistent with speculation that 1,4-BQ and/or 1,2-BQ (the oxidized forms of HQ and CA, respectively) play roles in benzene-induced toxicity [10,14,60–62]. The fact that levels of the other three metabolites (MA, SPMA, and PH) were also lower among participants with *NQO1*2* (rs1800566: C→T), suggests a more general antioxidant role for NQO1 [20]. Furthermore, the interaction effects between *NQO1*2* (rs1800566: C→T) and both benzene exposure and smoking status point to induction of *NQO1* by reactive benzene metabolites or other reactive species [20,63,64]. Such induction could come about via either the antioxidant or xenobiotic response element in the *NQO1* promoter region [19,65,66]. We found that *NQO1*2* (rs1800566: C→T) but not *NQO1*3* (rs4986998: C→T) was associated with lower levels of benzene metabolites and that single nucleotide polymorphisms (SNPs) of MPO appeared not to affect metabolism in liver. Considering our previous report of the presence of greater hematotoxicity in workers having the combination of variant *NQO1*3* (*C/T*, less active) and wild-type *MPO* (*A/A*, more active) [8], the present results are interesting. They probably point to differences in the balance between *NQO1* and *MPO* activities in liver (where metabolites are produced) and bone marrow (where metabolites are activated and deactivated in target hematopoietic cells) [60,67,68].

We observed significant effects of *CYP2E1* (rs2031920: C→T) variants on levels of MA, PH, and HQ (Tables 3, 5, and 7). Controversy has surrounded the relationship between the genotype and phenotype of *CYP2E1*, an important gene that metabolizes many small molecules of toxicological interest, including benzene [21,22,69–79]. There is some evidence that a variant type *CYP2E1* (rs2031920: C→T, also referred to as *Rsa*I−), is associated with decreased *CYP2E1* activity *in vivo* [33,52,71,73,80,81]. In the present study, we found that participants with variant *CYP2E1* (rs2031920: C→T)

produced lower levels of benzene metabolites at a given benzene exposure than homozygous wild-types, and that the effect was accentuated at higher benzene levels due to gene-environment interactions. The difference in metabolite levels between homozygous wild-types and homozygous variants was detected at benzene exposures in the range of 0.1–2 p.p.m. for HQ, PH and MA (Tables 3, 5 and 7 and Fig. 2a). This interaction effect could point to induction *CYP2E1* (rs2031920: C→T) by benzene exposure, or to more rapid saturation of metabolism among homozygous variant individuals [22]. Therefore, our results substantially support evidence from previous studies [33,52,71,73,80,81] that *CYP2E1* (rs2031920: C→T) mutations functionally reduce the metabolism of CYP2E1 substrates.

Individuals with variant alleles of *EPHX1* (rs1051740: T→C) produced lower levels of CA in our study (Table 6 and Fig. 2f). *EPHX1* enzymes hydrolyze epoxides through the formation of hydroxyl alkyl-enzyme intermediates [82]. Although *EPHX1* should logically be involved in benzene metabolism, notably in catalyzing BO to the dihydrodiol, the functional role of this enzyme has been ambiguous in benzene-exposed participants [56,83]. Two polymorphisms have been identified; one, in exon 3, decreased enzymatic activity 50% *in vitro,* whereas the other, in exon 4, increased activity 25% [84]. Our findings that workers with variant allele(s) of *EPHX1* (rs1051740: T→C) had lower levels of CA than homozygous wild-types, whereas those with at least one variant of *EPHX1* (rs2234922: A→G) had marginally higher CA levels, support the in-vitro results. We also detected significant interactions between *EPHX1* (rs1051740: T→C) and both benzene exposure and smoking status. The gene-smoking interaction tended to accentuate differences in CA levels between smoking individuals who had different alleles of *EPHX1* (rs1051740: T→C) and to obscure effects among nonsmokers (Fig. 2f). The gene-exposure interaction produced differences in CA levels that could be distinguished between smokers having homozygous wild-types and variant-types of *EPHX1* (rs1051740: T→C) at benzene concentrations above 1 p.p.m. Our results are intriguing in light of recent epidemiologic studies indicating that, among smokers, fast *EPHX1* metabolizers had greater risks of colorectal adenomas [85,86] than slow metabolizers. Interestingly, fast metabolizers of *EPHX1* (rs2234922: A→G) also had marginally higher levels of SPMA at benzene concentrations above 6 p.p.m. ($P < 0.10$). Although the mechanism for production of SPMA is not yet established [87,88], the apparent effect of *EPHX1* on SPMA levels may offer clues regarding formation of this minor benzene metabolite [82].

Among the GST isozymes, both *GSTM1* and *GSTT1* affected the production of SPMA (Table 4, Fig. 2e), with individuals having variant forms of these enzymes producing lower levels. Of the two isozymes, *GSTT1* produced more substantial effects, based upon evidence ratios [45] (data not shown), and produced different profiles for each combination of alleles (Fig. 2e). This finding is consistent with previous studies [24–26]. No effect of *GSTP1* (rs947894: A→G) was detected.

Finally, it is worth commenting upon the amounts of variability in metabolite levels that were explained by the observed genetic effects and the magnitudes of interindividual differences in metabolism that can be attributed to particular genes. The GLM + NS models summarized in Tables 3–7 had R^2 (%) values of 85.0 for MA, 82.0 for SPMA, 64.8 for PH, 57.2 for CA, and 72.6 for HQ, among which benzene exposure and nongenetic covariates explained between 53 and 84% of the variability in metabolite levels. The corresponding percentages of variability explained collectively by all significant genes and gene-environment interactions were 1.0 for MA, 4.9 for SPMA, 3.1 for PH, 3.4 for CA, and 1.8 for HQ. Thus, although many significant genetic effects were detected, they collectively contributed rather little to the explained variation in benzene metabolism.

Regarding interindividual differences in metabolism that would be expected for a given genetic polymorphism, Table 8 lists the ratios of predicted metabolite levels for homozygote variants to homozygous wild-types, based upon least-squares means of the models summarized in Tables 3–7. These ratios represent the mean fold ranges for variant/referent that would be expected for each genetic polymorphism after adjusting for benzene exposure as well as covariates and other genetic effects. The values shown in Table 8 suggest that interindividual differences in metabolite production were generally rather modest, with most ratios lying between about 0.3 and 2.0. Indeed, differences as great as two-fold to 3.5-fold would only be anticipated for most metabolite–gene combinations when persons were exposed to very high benzene concentrations (100 p.p.m.). The exception to this rule is the large effect of *GSTT1* on SPMA production, where homozygous referents would typically have SPMA levels three-fold to eight-fold greater than those of homozygous variants. This large fold range undoubtedly contributed to the earlier reports of significant effects of *GSTT1* on SPMA levels [24–26].

In conclusion, we used GLM + NS regression to detect numerous effects of particular metabolizing genes and gene–environment interactions on levels of benzene metabolites in 386 Chinese workers. Of the nine genetic polymorphisms investigated, *NQO1*2* (rs1800566: C→T) affected all five metabolites, *CYP2E1* (rs2031920: C→T) affected all metabolites but CA, *EPHX1* (1051740: T→C or 2234922: A→G) affected CA and SPMA, and *GSTT1* and *GSTM1* affected SPMA. Significant interactions were detected between benzene exposure and all four genes [including *CYP2E1* (rs2031920: C→T), *NQO1*2*

Table 8 Effects of genetic polymorphisms on benzene metabolites at various levels of benzene exposure. (Least-squares-mean ratios of variant/variant to wild/wild)

Metabolite	SNP	Air concentration (p.p.m.)			
		0.1	1	10	100
E,E-muconic acid (MA)	*CYP2E1* (rs2031920: C→T)	0.941	0.666	0.472	0.333
	*NQO1*2* (rs1800566: C→T)	0.976	0.832	0.708	0.603
S-Phenylmercapturic acid (SPMA)	*CYP2E1* (rs2031920: C→T)	0.675	0.619	0.567	0.519
	EPHX1 (rs2234922: A→G)	1.00	1.26	1.59	2.01
	GSTT1	0.328	0.236	0.17	0.122
	GSTM1	0.554	0.554	0.554	0.554
	*NQO1*2* (rs1800566: C→T, nonsmokers)	0.614	0.524	0.448	0.382
	*NQO1*2* (rs1800566: C→T, smokers)	1.74	1.48	1.27	1.08
Phenol (PH)	*CYP2E1* (rs2031920: C→T)	0.750	0.542	0.392	0.283
	*NQO1*2* (rs1800566: C→T, nonsmokers)	0.714	0.714	0.714	0.714
	*NQO1*2* (rs1800566: C→T, smokers)	1.22	1.22	1.22	1.22
Catechol (CA)	*EPHX1* (rs2234922: A→G)	1.15	1.15	1.15	1.15
	*NQO1*2* (rs1800566: C→T)	0.902	0.789	0.690	0.603
	EPHX1 (rs1051740 : T→C, nonsmokers)	1.29	1.10	0.938	0.800
	EPHX1 (rs1051740 : T→C, smokers)	0.685	0.584	0.498	0.425
Hydroquinone (HQ)	*CYP2E1* (rs2031920: C→T)	0.692	0.518	0.389	0.291
	*NQO1*2* (rs1800566: C→T)	0.849	0.849	0.849	0.849

MA, *E,E*-muconic acid; PH, phenol; HQ, hydroquinone; CA, catechol; SPMA, *S*-phenylmercapturic acid; SNP, single nucleotide polymorphism.

(rs1800566: C→T), *EPHX1* (*2234922*: A→G), and *GSTT1*] and between smoking status and *NQO1*2* (rs1800566: C→T) and *EPHX1* (rs1051740: T→C). Results are generally consistent with previous reports of associations between benzene hematotoxicity and specific gene mutations and provide additional evidence regarding functionality of SNPs of *NQO1*, *CYP2E1*, and *EPHX1* in humans exposed to benzene.

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