Benzene Metabolism in Humans: Dose-dependent Metabolism and Interindividual Variability

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ABSTRACT

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Benzene Metabolism in Humans: Dose-dependent Metabolism
and Interindividual Variability
(Under the direction of Prof. Stephen M. Rappaport)

In order to gain a better understanding of dose-dependant metabolism of benzene and interindividual variation, we analyzed air benzene, urinary metabolites and personal information, including single nucleotide polymorphisms of key metabolic enzymes, from 389 subjects exposed to benzene occupationally/environmentally. The apparent levels of benzene metabolites increased with exposure, and levels of benzene metabolites were unambiguously different from background levels at: ~ 0.2 ppm for *E,E*-muconic acid (MA) and *S*-phenylmercapturic acid (SPMA), ~ 0.5 ppm for phenol (PH) and hydroquinone (HQ), and ~2 ppm for catechol (CA). After adjustment for the background levels, MA, PH, CA and HQ showed significant (p<0.001) downward trends of dose-related production between 0.027 and 15.4 ppm. The transitions were particularly accentuated at lower exposure (0.027 and 0.274 ppm) for all metabolites. These were confirmed with generalized linear models with natural splines (GLM+NS). Based on analysis using the molar fraction, CYP-mediated metabolic pathways favored MA and HQ below 20 ppm and favored PH and CA above 20 ppm. Noticeably, ~90% of the reductions in dose-specific levels occurred below ~3 ppm for each major metabolite. Metabolite levels were about 20% higher in females and decreased

between one and 2% per year of life. Also, levels of HQ and CA were greater in smokers. After adjustment for age, gender, BMI and smoking status, the following SNPs showed significant effect on various metabolites, either as main effects or as interactions with benzene exposure and/or smoking: *NQO1*2* for all metabolites, *CYP2E1* for all metabolites except CA, *GSTT1* and *GSTM1* for SPMA, *EPHX1* (Ex4+52A>G) for SPMA and CA, and *EPHX1* (Ex3-28T>C) for CA. Interestingly, variant alleles of all genes [except *EPHX1* (Ex4+52A>G)] appeared to be associated with lower levels of benzene metabolites relative to homozygous wild alleles. In conclusion, our results indicate that benzene metabolism is highly nonlinear with increasing benzene exposure above 0.03 ppm, and that metabolism shifts away from CA and PH at low doses in favor of MA and HQ. Also, metabolism of benzene is modulated by exposure, gender, age, smoking, and genetic polymorphisms.

To my family, my father, mothers, Myoung-Won, Ghun-Hee, Jae-Hee, and mother-in-law. With them, I could make it.

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LIST OF ABBREVIATIONS

ACGIH: American Conference Of Governmental Industrial Hygienists

AICc: bias corrected Akaike's Information Criterion

AML: acute myelocytic leukemia

ANLL: acute nonlymphocytic leukemia

BMI: bogy mass index [unit: kg/m^2]

BO: benzene oxide

BQ: benzoquinone

CA: catechol

CNS: central nervous system

CV: coefficient of variation

CYP: cytochrome P450

EI: electron ionization

EPHX1: epoxide hydrolase

GC-MS: gas chromatography-mass spectrometry

GLM: generalized linear model

GLM+NS: generalized linear model with natural splines

GST: glutathione *S*-transferase

GSTM1: glutathione *S*-transferase mu1

GSTP1: glutathione *S*-transferase pi1

GSTT1: glutathione S-transferase theta1

HQ: hydroquinone

HS-SPME: headspace solid phase microextraction

IQ range: interquartile range

LOD: limit of detection

m/z: mass-to-charge ratio

MA: *E,E*-muconic acid

MCV: mean corpuscular volume

MDS: myelodysplastic syndrome

min: minute

ml: milliliter

mM: millimolar

MPO: myeloperoxidase

MUC: *E,E*-Muconaldehyde

nM: nanomolar

NQO1: NAD(P)H: Quinone Oxidoreductase

NS: natural splines (restricted cubic splines)

OSHA: Occupational Safety and Health Administration

PEL: permissible exposure limit

PH: phenol

ppb: parts per billion

ppm: parts per million

PTFE: polytetrafluoroethylene

ROS: reactive oxygen species

SNP: single nucleotide polymorphism

SPMA: *S*-phenylmercapturic acid

TMS: trimethylsilyl

UBz: urinary benzene

µl: microliter

µM: micromolar

CHAPTER 1.

GENERAL INTRODUCTION

Benzene is a useful industrial chemical and is ubiquitous in the environment. It is an established human carcinogen (IARC, 1982; Wallace, 1996; ATSDR, 1997; NICNAS, 2001). Since early reports on benzene-induced hematotoxicities (Vigliani and Saita, 1964; Snyder, 2002), many studies have been carried out in the fields of epidemiology and exposure assessment. However, the mechanisms of hematotoxic effects and the shape of the exposure-response relationships [in particular below 1 ppm] are still controversial (Rothman *et al.*, 1996a; Savitz and Andrews, 1997; Ross, 2000; Snyder, 2000a; Snyder, 2002; Glass *et al.*, 2003; Lan *et al.*, 2004; Schnatter *et al.*, 2005). The present study is focused on these issues to provide a better understanding of human benzene metabolism, specifically, dose-dependant metabolic profiles of benzene and possible genetic predispositions involving benzene metabolism.

1.1. Exposure to Benzene

Benzene exposure occurs both in the workplace and the general environment. In the workplace, benzene is used primarily as an intermediate in production of Styrofoam, other plastics, synthetics and fibers. It is also an important raw material and excellent solvent for synthetic rubbers, lubricants, dyes, pharmaceuticals and pesticides (ATSDR, 2000). Strict

regulation of benzene exposure has been increasingly required due to the observed risks of exposure to benzene in occupational studies (ATSDR, 2000; ACGIH, 2004). The current permissible exposure limit (PEL) set by US OSHA is 1.0 ppm (OSHA, 1987) and the ACGIH threshold limit value (TLV) is 0.5 ppm (ACGIH, 2004). Environmental sources of benzene exposure are industrial pollution, automobile fuel and combustion products, and cigarette smoking. Ambient outdoor air levels of benzene globally are around 2 parts per billion (ppb) on average (range 0.6–3 ppb) (ATSDR, 1997). Smokers consuming a pack of cigarettes per day inhale a daily dose of approximately 1 mg of benzene, about 3 to 4% of the amount inhaled daily by a worker exposed at 1 ppm. Passive smokers typically have 50% greater exposure to benzene than nonsmokers (Wallace, 1996). Potential leakage from underground storage tanks and landfills and improper disposal of wastes can lead to benzene exposure via drinking water. Drinking water typically contains less than 0.1 ppb benzene. There have been reports that benzene has been detected at trace levels in bottled water, liquor, and food (ATSDR, 1997).

1.2. Health Effects of Benzene in Humans

While acute exposure to very high levels of benzene, occurring accidentally, can lead to death, depression of the central nervous system (CNS) or irritation, such situations are rare. Long-term chronic exposures are much more common, and can induce irreversibly fatal effects including leukemia (ATSDR, 1997; EPA, 2002). Hence, we focus on the chronic exposures to benzene.

1.2.1. Hematopoietic Toxicity

Exposure to excessive concentrations of benzene can induce bone marrow depression, which leads to reduction in blood cells and aplastic anemia (Aksoy, 1989; Rothman *et al.*,

1996a; Rothman *et al.*, 1996b; Hayes *et al.*, 1997; Snyder, 2002). Several mechanisms have been suggested to explain benzene-induced hematotoxicity, such as direct toxicity to hematopoietic progenitor cells by reactive benzene metabolites (Irons and Neptun, 1980; Chen *et al.*, 1994; Smith *et al.*, 2000; Zhang *et al.*, 2002), impairment of the bone marrow stromal microenvironment (Longacre *et al.*, 1981; Garnett *et al.*, 1983; Gaido and Wierda, 1985; Thomas *et al.*, 1990), and inhibition or alteration of regulatory cytokines produced by marrow cells (Miller *et al.*, 1994; Kalf *et al.*, 1996; Ross *et al.*, 1996).

Repeated benzene exposure at higher levels has been associated with benzene poisoning (Aksoy *et al.*, 1971; Midzenski *et al.*, 1992; Rothman *et al.*, 1996a; Rothman *et al.*, 1996b). Symptoms of benzene poisoning include decreased numbers of lymphocytes, white blood cells, red blood cells, platelets, hemoglobin and hematocrit, and an increase in the mean corpuscular volume (MCV) (Rothman *et al.*, 1996a). Dosemeci *et al.* showed a clear dose-response relationship between benzene exposure and benzene poisoning (Dosemeci *et al.*, 1997), and Rothman *et al.* reported that benzene poisoning was strongly associated with myelodysplastic syndrome (MDS) and/or acute myelocytic leukemia (AML) with a 71-fold higher risk (95% CI, 11–439) relative to controls without benzene poisoning (Rothman *et al.*, 1997).

1.2.2. Genotoxicity and Leukemia

Benzene metabolites can give rise to DNA damage, subsequent translocations, or other insults associated with inhibition of topoisomerase II. 1,4-benzoquinone (BQ), a metabolite of benzene, was suggested to bind to guanine or adenine residues, and inhibit mRNA(Rushmore *et al.*, 1984). It has also been suggested that *E,E*-Muconaldehyde (MUC), the ring opening product of benzene, could bind to DNA by forming DNA-protein cross links

(Schoenfeld and Witz, 1999). Interestingly, DNA adducts appear to be minor products of benzene metabolism with uncertain health implications (Subrahmanyam *et al.*, 1991) (Creek *et al.*, 1997; Snyder, 2002). Benzene exposure unequivocally produces chromosome aberrations (Tough and Brown, 1965; Tough *et al.*, 1970; Forni, 1996). High exposure to benzene (+31 ppm) can cause aneuploidy, long-arm deletions and translocations in chromosomes 1, 5, 7, 8 9 and 21 (Rothman *et al.*, 1996b; Zhang *et al.*, 1996; Smith *et al.*, 1998; Zhang *et al.*, 1998) and gene duplication in nucleated erythrocyte stem cells at the glycophorin A locus on chromosome 4 (Rothman *et al.*, 1995). It appears that benzene-induced chromosome aberrations are important to benzene-induced leukemia (Zhang *et al.*, 2002). Using CD34+, a surface antigen found in pluripotent stem cells or early progenitor cells in bone marrow, the benzene metabolite, hydroquinone (HQ) induced more aneusomy in CD34+ carrying cells on chromosomes 7 and 8, which are consistent with findings in myeloid leukemia (Smith *et al.*, 2000).

Since early reports on benzene-induced leukemia (Vigliani and Forni, 1969; Aksoy *et al.*, 1971; Vigliani and Forni, 1976), numerous epidemiologic studies have shown associations between benzene exposure and leukemia (Snyder, 2002). In the Pliofilm cohort studies, the standardized mortality ratio (SMR) was 560 for the exposed group at large, and projected SMR for excess of leukemia was 109-6637 at 40-400 ppm-year (Rinsky *et al.*, 1981; Rinsky *et al.*, 1987). However, there were controversies regarding the exposure-estimates used in those studies (Kipen *et al.*, 1988; Kipen *et al.*, 1989; Paustenbach *et al.*, 1992; Cody *et al.*, 1993; Paxton *et al.*, 1994; Utterback and Rinsky, 1995; Crump, 1996). The Chinese cohort studies, which recruited much larger number of subjects, showed clear evidence of significantly increased risks for AML, MDS, and aplastic anemia (Yin *et al.*,

1996). In the extended Chinese cohort study, acute nonlymphocytic leukemia (ANLL [or AML]) or MDS was found to be associated with recent exposure (<10 year prior to diagnosis) (Hayes *et al.*, 1997). A dose-response relationships was demonstrated between cumulative exposures to benzene and a spectrum of blood cancers and blood disorders, with increased risks at <40 ppm-yr. Relative risks for ANLL/MDS or other blood cancers were more than doubled with average exposures of about 10 ppm. Although there are further controversies regarding exposure estimates from the Chinese study (Dosemeci *et al.*, 1997; Schnatter *et al.*, 2005), these results suggest the possibility of an increased risk of leukemia below the levels of exposure suggested by the earlier Pliofilm cohort. As such, benzene is widely accepted as a leukemogen (primarily for AML) (IARC, 1982; ATSDR, 1997; EPA, 1998). Furthermore, we cannot exclude the linkage of benzene exposure to other leukemias (Savitz and Andrews, 1997) such as acute and chronic lymphocytic and myeloid leukemia (Hayes *et al.*, 1997; O'Connor *et al.*, 1999; Wong and Raabe, 2000; Hayes *et al.*, 2001; Schnatter *et al.*, 2005). It has recently been shown that cumulative exposures as low as 8 ppm-years have resulted in significantly increased leukemia risks (Glass *et al.*, 2003).

The mechanisms of leukemogenesis and benzene induced toxicities are not yet fully understood. Given that benzene metabolites can cause DNA damage, aneusomy or other insults (*e.g.* associated with inhibition of topoisomerase II), benzene metabolites can be termed as initiators. Also they can induce expansion of a less differentiated pool of myeloid cells, which is a property of a promoter (Snyder, 2000b; Snyder, 2000a). One suggested mechanism for benzene-induced leukemia is depicted in Fig. 1.1.

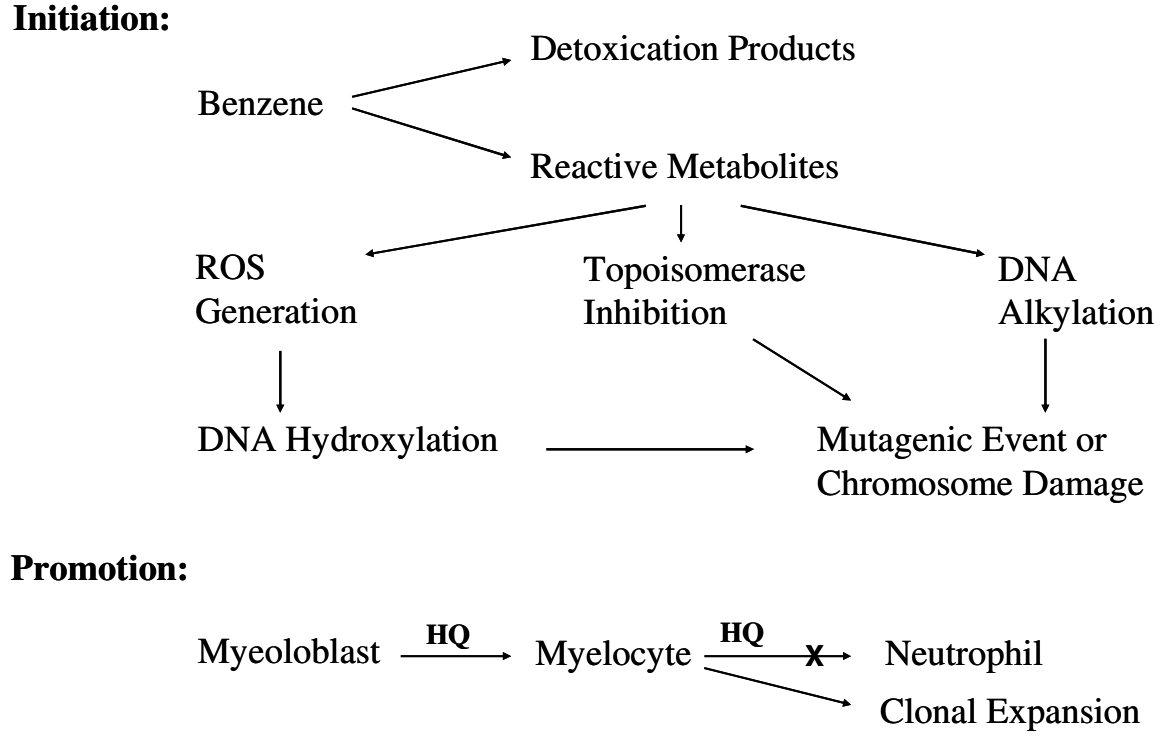

Figure 1. 1. Postulated processes of benzene-induced leukemia (modified from (Snyder, 2000b)). Legends: ROS, reactive oxygen species; HQ, hydroquinone.

1.2.3. Other Effects

Several reports have linked benzene exposure to a host of other effects (ATSDR, 1997) including menstruation disorders (Dahl *et al.*, 1999), spontaneous abortions (Xu *et al.*, 1998), melanoma, breast cancer (Petralia *et al.*, 1999) and childhood leukemia (Pearson *et al.*, 2000; Zeise and McDonald, 2000). However, causal relationships between exposure and these effects have not been established due to co-exposures to other xenobiotics and inconsistent results among studies (NICNAS, 2001).

1.3. Metabolism of Benzene

1.3.1. General Pathways of Benzene Metabolism

While it is generally accepted that benzene causes toxic effects via metabolism, the particular toxic metabolite(s) remains elusive (Snyder and Hedli, 1996; Snyder, 2000b). The major metabolic pathways are shown in Figure 1.2. Benzene is metabolized by cytochrome P450 (CYP) enzymes (primarily CYP2E1) to benzene oxide (BO, which is in equilibrium with its tautomer, oxepin), an electrophile that binds to macromolecules (Lindstrom *et al.*, 1998; Lindstrom *et al.*, 1999; Yeowell-O'Connell *et al.*, 2001; Rappaport *et al.*, 2002) and is the source of all other metabolites. Spontaneous rearrangement of BO produces phenol (PH) (Jerina and Daly, 1974), which can undergo another CYP oxidation to give hydroquinone (HQ)(Koop *et al.*, 1989; Valentine *et al.*, 1996). Hydrolysis of BO via epoxide hydrolase (EPHX1) produces benzene dihydrodiol which can be converted to catechol (CA), via dihydrodiol dehydrogenases, or to benzene diolepoxides via CYP oxidation(Bolcsak and Nerland, 1983). Hydroquinone and CA can be oxidized to 1,4-BQ and 1,2-BQ, respectively, which also bind to macromolecules (McDonald *et al.*, 1993; Waidyanatha *et al.*, 1998; Yeowell-O'Connell *et al.*, 2001). A second CYP oxidation of oxepin, followed by ring opening, produces the muconaldehydes (MUC) (Witz *et al.*, 1996). These reactive species are also capable of binding to macromolecules (Golding and Watson, 1999; Amin and Witz, 2001; Oshiro *et al.*, 2001) and are ultimately converted to *E,E*-muconic acid (MA) (Snyder and Hedli, 1996; Scherer *et al.*, 1998). The major phenolic metabolites, *i.e.*, PH, HQ, and CA, are easily conjugated and excreted in urine (Sabourin *et al.*, 1989; Seaton *et al.*, 1995; Snyder, 2004). Minor pathways of benzene metabolism include *S*-phenylmercapturic acid (SPMA) following reaction of BO with glutathione, supposedly via glutathione *S*-transferase (GST) (Boogaard and van Sittert, 1996; Henderson *et al.*, 2005a), and 1,2,4-trihydroxybenzene via CYP oxidation of HQ(Rushmore *et al.*, 1984; Ross, 2000). Of all the

benzene metabolites, 1,4-benzoquinone (derived from HQ) has most often been linked to the spectrum of toxic effects observed in humans and animals (Smith, 1999; Snyder, 2000a; Snyder, 2000b; Inayat-Hussain and Ross, 2005).

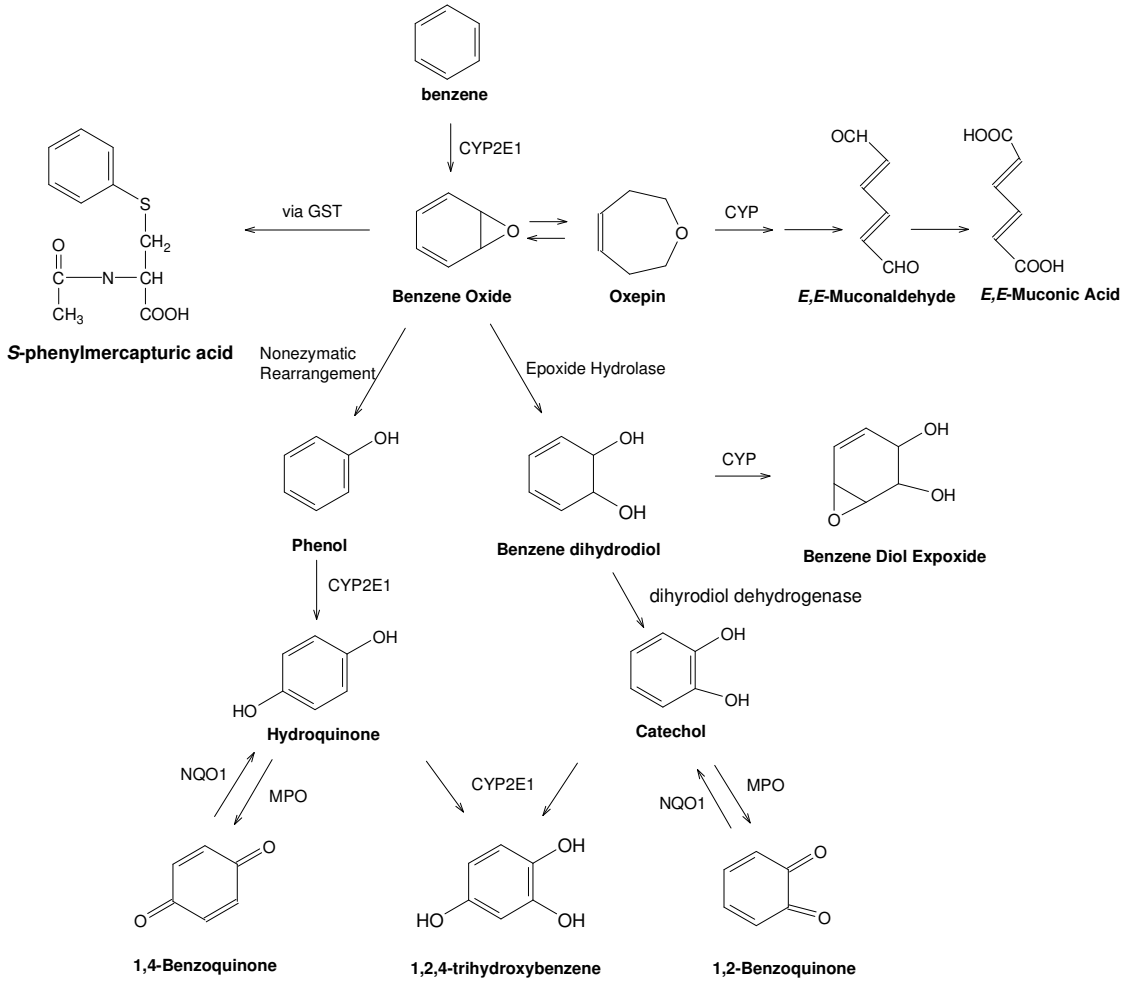

Figure 1. 2. Simplified metabolic scheme for benzene showing major pathways and metabolizing genes.

1.3.2. Toxicological Overview of Benzene Metabolites

As described earlier, there is no doubt that benzene must be metabolized in the liver before causing adverse effects on bone marrow depression. However, it has not been clearly determined (Snyder, 2002) whether the benzene metabolites from hepatic metabolism induce bone marrow depression, and if so, what the toxicological mechanism would be. Also, the role(s) that might be played by polymorphisms of metabolizing enzymes on susceptibility to benzene-induced toxicity (and/or metabolism) has not been established. Here, we provide a brief overview of benzene metabolism from a toxicological point of view.

It has been reported that a combination of benzene metabolites is needed to induce benzene toxicity in animal and human cells *in vitro* (Eastmond *et al.*, 1987; Snyder *et al.*, 1989; Subrahmanyam *et al.*, 1989; Subrahmanyam *et al.*, 1990; Levay and Bodell, 1992; Marrazzini *et al.*, 1994; Ross, 2000; Snyder, 2004). Specifically, Snyder *et al.* reported that the potency of metabolites (or their binary combinations) as potential inhibitors of erythropoesis in mice increased in the following order: HQ-sulfate < HQ < CA < 6-OH-*tt*-2,4-hexadienal < HQ + PH < PH < CA+ PH < 1,4-BQ < MUC < MUC + HQ (Snyder *et al.*, 1989; Snyder, 2004).

Numerous studies have showed that HQ and CA are further metabolized by myeloperoxidase (MPO) or prostaglandin H synthase to form semiquinones or benzoquinones, which can bind to macromolecules in bone marrow or blood (Post *et al.*, 1986; Bhat *et al.*, 1988; Schlosser and Kalf, 1989; Subrahmanyam *et al.*, 1989; Subrahmanyam *et al.*, 1990). Given more abundant MPO in bone marrow than in liver, it has

been speculated that activation of HQ and CA to toxic quinones is more likely in bone marrow than human liver (Snyder, 2002).

In addition to further metabolism of HQ, the oxygen stress from redox cycling or autooxidation can convert molecular oxygen into the superoxide anion, which is then converted to hydrogen peroxide, and the very reactive hydroxyl radical. These reactive oxygen species (ROS) damage proteins, DNA bases, structural chromosomes, lipid membranes, etc. (NICNAS, 2001). While redox cycling among HQ, 1,4-semiquinone, and 1,4-BQ can also generate ROS, it is unlikely at physiological pH owing to protonation of the semiquinone, thus limiting superoxide production (Boersma *et al.*, 1994). Alternatively, Brunmark *et al.* suggested autooxidation as an alternative source of ROS (Brunmark and Cadenas, 1988). The proposed mechanism proceeds as follows: 1,4-BQ is converted to an epoxide (2,3-epoxy-1,4-BQ), and subsequently, via reaction with glutathione, to glutathinoyl-2,3,5-trihydroxybenzene, a species which is predisposed to autooxidize and participate in redox cycling by generating superoxide anions.

Despite the lack of an animal model for benzene-induced leukemia (Snyder, 2004), the metabolism of benzene in mammals has been accepted to be qualitatively similar to that in humans (Henderson, 1996). Among mammalian species, mice are more susceptibile to benzene-induced toxicity than rats, and mice have more HQ in urine than rats at a same benzene exposure (Sabourin *et al.*, 1987; Sabourin *et al.*, 1988; Sabourin *et al.*, 1992). Also, mice produce proportionally much more MA at low doses than at high doses (Witz *et al.*, 1990). Sabourin *et al.* demonstrated that proportionally more MA and HQ were generated at lower doses in several rodent species, particularly mice. The dose-dependant shift of metabolites, which favors MA and HQ at lower exposure to benzene, has also been suggested

in humans based upon studies of occupational exposures in China (Rothman *et al.*, 1998; Waidyanatha *et al.*, 2001; Melikian *et al.*, 2002; Waidyanatha *et al.*, 2004).

1.3.3. Genetic Polymorphisms of Metabolic Enzymes

The dose of a benzene metabolites at the target organ should depend on the level of metabolic enzyme in the liver (Snyder, 2002). Therefore, considering benzene-induced toxicity, we need to verify the role of key metabolic enzymes such as CYP (in particular CYP2E1), GSTs, EPHX1, dihydrodiol dehydrogenase, NAD(P)H: quinone Oxidoreductase (NQO1), peroxidases (notably myeloperoxidase (MPO)) (Fig. 1.2) (Note: Sulfotransferase and glucuronyl transferase are also important detoxifying enzymes for PH, CA, and HQ. Since we do not focus on the potential roles of those conjugates in this study, we report levels of total phenolic metabolites (combined amounts with the free and conjugated forms).

The phase-I enzyme CYP2E1 has been established as a primary metabolic enzyme for benzene (Seaton *et al.*, 1994; Valentine *et al.*, 1996; Nedelcheva *et al.*, 1999) forming BO and subsequently PH and HQ (see Fig. 1.2). According to Seaton *et al.*, the interindividual difference in CYP2E1 activity (determined by hydroxylation of *p*-nitrophenol) varied by 13-fold (Seaton *et al.*, 1994) in human liver specimens. The distribution of genetic polymorphisms was different by races (Kato *et al.*, 1992). While *in vitro* studies showed a reduction in transcriptional activity of the gene (Hayashi *et al.*, 1991), the effect of functionality and genotypes have not be established (Bolt *et al.*, 2003). Although results among studies have been observed regarding the functionality of CYP2E1 (Lucas *et al.*, 1995; Carriere *et al.*, 1996; Kim *et al.*, 1996; Rothman *et al.*, 1997; Nedelcheva *et al.*, 1999; Wang *et al.*, 1999; Wormhoudt *et al.*, 1999; Carere *et al.*, 2002) (Ingelman-Sundberg *et al.*, 2006) (Seaton *et al.*,

1994; Seaton *et al.*, 1995), a recent *in vivo* pharmacokinetic study demonstrated that variant *CYP2E1* (*RsaI*) caused less activity than wild-type *CYP2E1* (Le Marchand *et al.*, 2002).

GSTs promote the reaction of glutathione with electrophilic benzene metabolites (in particular, BO, MUC, and the benzoquinones) (Jerina and Daly, 1974; Goon *et al.*, 1993). Several studies showed that variant forms of GSTs caused benzene-induced toxicities (Hsieh *et al.*, 1999; Morgan and Smith, 2002; Dirksen *et al.*, 2004; Kim *et al.*, 2004). There have also been suggestions that GST polymorphisms are associated with increased risks of oxidative stress (Hayes *et al.*, 2005).

The EPHX1 gene catalyzes hydrolysis of arene epoxides or other reactive aliphatic metabolites (usually produced by CYP) into dihydrodiol derivatives (Morisseau and Hammock, 2005). According to a recent study using EPHX1-deficient mice, EPHX1 seems to be an important detoxifying enzyme in male mice (Bauer *et al.*, 2003a). There have been reports of an 8-fold difference in the enzymatic activity (determined by levels of a benzo[a]pyrene metabolite in liver biopsy specimens) among 40 human subjects (Hassett *et al.*, 1997).

NQO1 catalyzes various substrates such as quinones via the two- or four-election reduction (Nebert *et al.*, 2002), and thereby can retain quinones in their reduced and nontoxic forms. Among the polymorphisms of NQO1 (Nebert *et al.*, 2002), *NQO1*2* was investigated extensively (Traver *et al.*, 1997; Siegel *et al.*, 1999; Ross *et al.*, 2000). Recent data suggest that active forms of NQO1 protect against benzene-induced toxicities (Rothman *et al.*, 1997; Long *et al.*, 2002; Bauer *et al.*, 2003b), and a high ratio of MPO to NQO1 in bone marrow has been suggested to confer more susceptibility to cellular damage (Ganousis *et al.*, 1992; Ross *et al.*, 1996; Ross, 2005). NQO1 can be highly inducible by electrophiles and oxidative

stress (De Long *et al.*, 1987; Moran *et al.*, 1999; Ross, 2005), and functions as a superoxide scavenger (Thor *et al.*, 1982; Ross, 2005).

1.4. Research Aims of this Project

Since the risks of hematotoxicity and leukemia are thought to arise from human metabolism of benzene, it is important that the dose-related production of benzene metabolites be understood. According to several recent studies (Rothman *et al.*, 1998; Waidyanatha *et al.*, 2001; Melikian *et al.*, 2002), benzene metabolism appears to be dose-dependant. These studies had several limitations including the followings: insufficient sample size, no adjustment of background metabolites of benzene, no measurement of air exposure for control subjects, incomplete coverage of all key metabolites of benzene, limited consideration of factors influencing inter-individual variability (*e.g.* life style, metabolic polymorphisms, etc).

In chapter 2 we discuss dose-related patterns of human benzene metabolism using urinary biomarkers. Here, we group subjects by the levels of benzene exposure and aggregate metabolite levels in these groups using empirical (non-parametric) analysis after adjusting for background levels of benzene metabolites. Then, we determine the uncertainty from the estimates of environmental exposure using a boot-strapping technique.

In chapter 3, we construct a parametric model for metabolism of benzene in humans exposed to benzene both occupationally and environmentally. The model explains the dose-specific productions of metabolites more extensively while addressing nonlinear relationship between benzene exposure and urinary metabolites of benzene. Effects of covariates (other

than metabolic polymorphisms) on benzene metabolites are also tested. Then, we use boot-strapping to determine the uncertainties in estimated dose-metabolite models.

In chapter 4, we extend the models used in chapter 3 to evaluate effects of genetic polymorphisms of metabolic enzymes. Thus, the enhanced models include nonlinear effects of benzene exposure, life-style factors, single nucleotide polymorphisms (SNP) of the key enzymes for benzene metabolism, and two-way interactions between SNPs and exposure or smoking.

CHAPTER 2.

USING URINARY BIOMARKERS TO ELUCIDATE DOSE-RELATED PATTERNS OF HUMAN BENZENE METABOLISM

[Kim S, Vermeulen R, Waidyanatha S, Johnson AB, Lan Q, Rothman N, et al. Carcinogenesis 2006;27:772-781.]

2.1. Abstract

Although the toxicity of benzene has been linked to its metabolism, the dose-related production of metabolites is not well understood in humans, particularly at low levels of exposure. We investigated unmetabolized benzene in urine (UBz) and all major urinary metabolites [phenol (PH), *E,E*-muconic acid (MA), hydroquinone (HQ), and catechol (CA)] as well as the minor metabolite, *S*-phenylmercapturic acid (SPMA), in 250 benzene-exposed workers and 139 control workers in Tianjin, China. Median levels of benzene exposure were about 1.2 ppm for exposed workers (interquartile range: 0.53–3.34 ppm) and 0.005 ppm for control workers (interquartile range: 0.002 – 0.007 ppm). (Exposures of control workers to benzene were predicted from levels of benzene in their urine). Metabolite production was

investigated among groups of 30 workers aggregated by their benzene exposures. We found that the urine concentration of each metabolite was consistently elevated when the group's median benzene exposure was at or above the following air concentrations: 0.2 ppm for MA and SPMA, 0.5 ppm for PH and HQ, and 2 ppm for CA. Dose-related production of the 4 major metabolites and total metabolites (μmol/l per ppm benzene) declined between 2.5 and 26 fold as group median benzene exposures increased between 0.027 and 15.4 ppm. Reductions in metabolite production were most pronounced for CA and PH below one ppm, indicating that metabolism favored production of the toxic metabolites, HQ and MA, at low exposures.

2.2. Introduction

Benzene is an important industrial chemical that is also emitted into the air from gasoline, engine exhausts, and combustion of organic materials (including cigarette smoke) (IARC, 1987; Wallace, 1996). Occupational exposures to benzene at air levels greater than about 10 ppm, have long been linked to hematotoxicity and to acute myelogenous leukemia (IARC, 1982; Hayes *et al.*, 1997; Savitz and Andrews, 1997). A recent report of hematotoxic effects in workers exposed to benzene below one ppm (Lan *et al.*, 2004) has raised additional concerns regarding the health consequences of low exposures to this contaminant.

While it is generally accepted that benzene causes toxic effects via metabolism, the particular toxic metabolite(s) remains elusive(Snyder, 2000a). The major metabolic pathways are shown in Figure 2.1. Benzene is metabolized by CYP enzymes (primarily

CYP2E1) to benzene oxide (BO, which is in equilibrium with its tautomer, oxepin), an electrophile that binds to macromolecules (Lindstrom *et al.*, 1998; Lindstrom *et al.*, 1999; Yeowell-O'Connell *et al.*, 2001; Rappaport *et al.*, 2002) and is the source of all other metabolites. Spontaneous rearrangement of BO produces phenol (PH), which can undergo

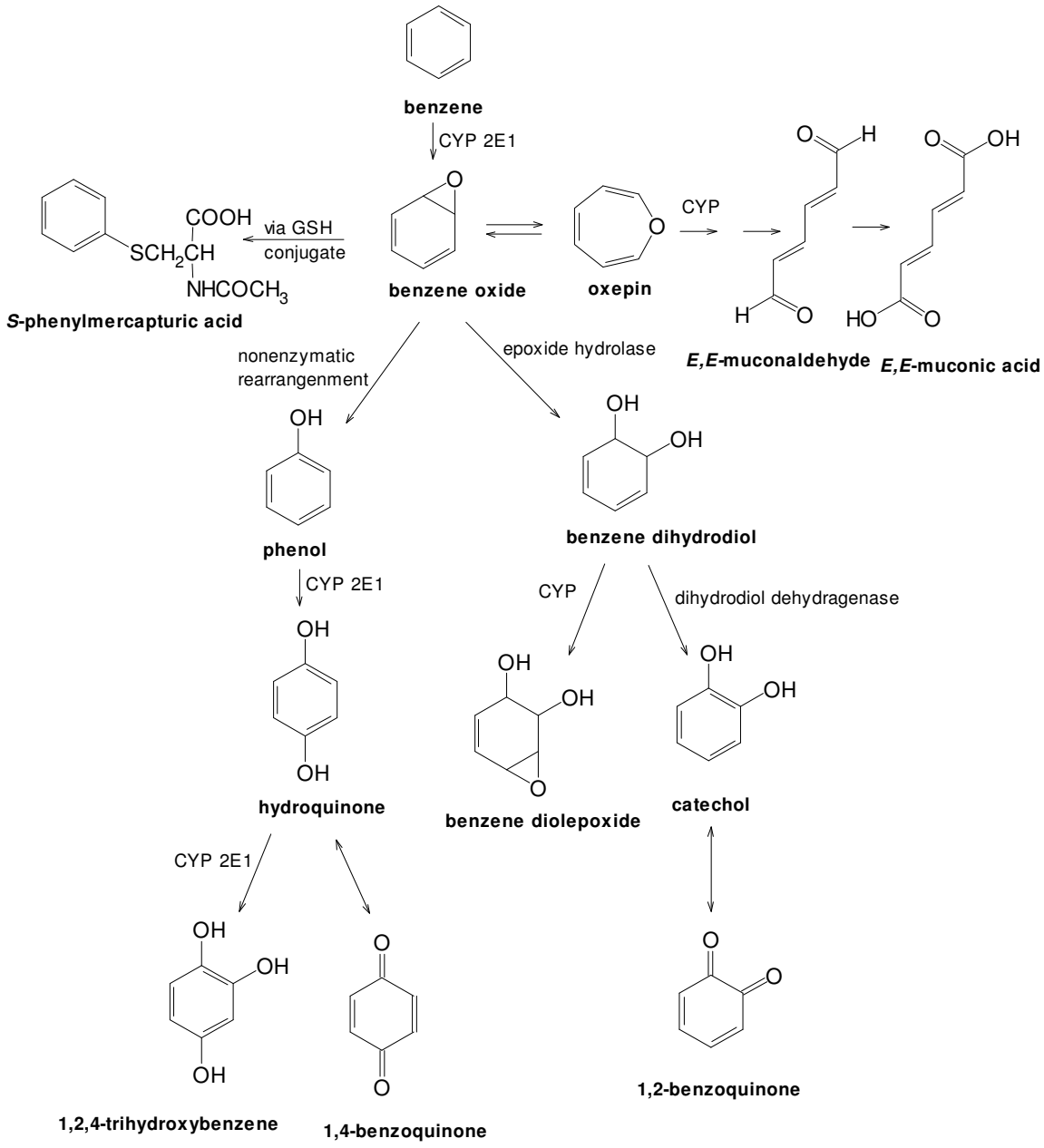

Figure 2.1. Simplified metabolic scheme for benzene showing major pathways.

another CYP oxidation to give hydroquinone (HQ). Hydrolysis of BO via epoxide hydrolase produces benzene dihydrodiol which can be converted to catechol (CA), via dihydrodiol dehydrogenases, or to benzene diolepoxides via CYP oxidation. Hydroquinone and CA can be oxidized to 1,4-benzoquinone and 1,2-benzoquinone, respectively, which also bind to macromolecules (McDonald *et al.*, 1993; Waidyanatha *et al.*, 1998; Yeowell-O'Connell *et al.*, 2001). A second CYP oxidation of oxepin, followed by ring opening, produces the muconaldehydes. These reactive species are also capable of binding to macromolecules (Golding and Watson, 1999; Amin and Witz, 2001; Oshiro *et al.*, 2001; Henderson *et al.*, 2005b) and are ultimately converted to *E,E*-muconic acid (MA) (Snyder and Hedli, 1996). The major phenolic metabolites, i.e., PH, HQ, and CA are easily conjugated and excreted in urine (Sabourin *et al.*, 1989; Kenyon *et al.*, 1995; Seaton *et al.*, 1995). Minor pathways of benzene metabolism include *S*-phenylmercapturic acid (SPMA) following reaction of BO with glutathione (Henderson *et al.*, 2005a), and 1,2,4-trihydroxybenzene via CYP oxidation of HQ. Of all the benzene metabolites, 1,4-benzoquinone (derived from HQ) has most often been linked to the spectrum of toxic effects observed in humans and animals (Smith, 1996; Snyder, 2000a; Snyder, 2000b; Inayat-Hussain and Ross, 2005).

Since risks of hematotoxicity and leukemia are thought to arise from benzene metabolism, it is important that the dose-related production of benzene metabolites be understood. Two recent studies have reported levels of urinary metabolites in both benzene-exposed workers and control workers in China (Rothman *et al.*, 1998; Qu *et al.*, 2000; Waidyanatha *et al.*, 2001; Melikian *et al.*, 2002). Metabolite profiles from those studies suggested shifts among the competing metabolic pathways at increasing levels of benzene

exposure. The Chinese studies also pointed to relatively high levels of the phenolic metabolites, PH, CA and HQ in control workers, presumably from dietary and endogenous sources of these compounds. These background levels of metabolites tend to obscure the amounts of PH, CA and HQ derived from ambient benzene exposure and from cigarette consumption. Also, because benzene air concentrations levels were not reported for control subjects in previous Chinese studies, they could not be used to determine the utility of the various metabolites as biomarkers of exposure to benzene at environmental levels.

In the present study we report levels of benzene in air and urine, as well as urinary levels of PH, CA, HQ, MA and SPMA, among 389 subjects (250 exposed and 139 controls) in a Chinese population that has shown evidence of hematotoxic effects (Lan *et al.*, 2004). Because some personal measurements of airborne benzene were below the limits of detection (LOD) or were missing, we predict benzene exposures in these (mostly control) subjects from the levels of unmetabolized benzene in urine (UBz). Then, we use the data to characterize relationships between metabolite levels and benzene exposures in groups of subjects stratified by exposure level. This analysis identifies useful ranges of the various metabolites as biomarkers of benzene exposure. Finally, we estimate the dose-related production of each metabolite (μM urine per ppm benzene) after adjusting for background levels.

2.3. Materials and Methods

2.3.1. Chemicals

Benzene, CA (99%) and HQ (>99%) were obtained from Fluka Chemical Co. (Switzerland). NaCl was purchased from Fisher Scientific (Pittsburg, PA) and was heated at

120ºC overnight prior to use. PH (99%+, redistilled), MA (98%), and [$^{2}H_6$]benzene (99.86%) were obtained from Aldrich Chemical (Milwaukee, WI). [$^{13}C_6$]PH (99%) and [$^{2}H_6$]CA (98%) were purchased from Cambridge Isotope Laboratories (Woburn, MA). Phenylmercapturic acid, Tri-Sil reagent, and concentrated hydrochloric acid (Optima grade) were purchased from TCI America (Portland, OR), Pierce (Rockford, IL), and Fisher Scientific (Pittsburgh, PA), respectively. Ethyl acetate (analytical reagent grade) and hexane (nanograde) were obtained from Mallinckrodt Baker (Paris, KT). Anhydrous Na_2SO_4 was purchased from J.T. Baker (Phillipsburg, NJ). 2,5-[$^{13}C_2$]MA, [$^{2}H_5$]SPMA and [$^{13}C_6$]HQ were kindly provided by Drs. Avram Gold, Ramiah Sangaiah and Alistair Henderson from the University of North Carolina at Chapel Hill. All headspace solid phase microextraction (HS-SPME) supplies were obtained from Supelco (Bellefonte, PA).

2.3.2. Subject Recruitment and Sample Collection

Subjects (n = 390) were recruited with informed consent from five factories in Tianjin, China. Exposed subjects (n = 250) worked in two shoe-making factories, and control subjects (n = 140) worked in three clothes-manufacturing factories as previously described (Lan *et al.*, 2004; Vermeulen *et al.*, 2004). The eligibility and exclusion criteria for participation were the same as in previous reports, and one control subject who did not provide a urine sample was removed. Exposed and control subjects were frequency matched by gender. As shown in Table 2.1, demographic characteristics were similar among exposed and control workers in terms of gender, age and smoking status. Smoking status was highly correlated with gender. Personal protective equipment was not used.

Table 2.1. Demographic characteristics of the study population.

Exposure Status	Gender	*n* (%)	Age median (range)	Current smokers *n* (%)	Smoking intensity[a] median (range)
Control	Male	52 (37.4)	28 (18–51)	36 (69.2)	10 (1–40)
	Female	87 (62.5)	28 (18–51)	3 (3.45)	NR[b]
	All	139 (100)	28 (18–51)	39 (28.1)	10 (1–40)
Exposed	Male	86 (34.4)	23 (18–44)	47 (54.7)	10 (1–30)
	Female	164 (65.6)	33 (18–52)	5 (3.05)	4.5 (2–10)
	All	250 (100)	29 (18–52)	52 (20.8)	7 (1–30)

[a] Average number of cigarettes/day.

[b] Not reported.

Full-shift exposures to benzene were monitored with passive samplers (Organic Vapor Monitors, 3M Corp., St. Paul, MN), and urine was collected from each participant at the end of each working shift for which air had been monitored. Air samples were analyzed for benzene by gas chromatography with flame-ionization detection as described previously (Vermeulen *et al.*, 2004). The nominal LOD was 0.20 ppm of benzene in air.

Urine samples were collected repeatedly (up to 4 times) in 139 subjects at intervals of about 42 days (median value) from June 2000 to June 2001. The other 250 subjects provided a single urine specimen. Urine samples were aliquoted immediately after collection, and stored at -80^{o}C for 1.6 – 4.0 y (for UBz) and 2.2 – 3.8 y (for urinary metabolites) prior to analysis. Samples were identified by randomly assigned numbers. Information about exposure levels and demographic factors were released after all assays had been completed and results reported to collaborators. A total of 620 urine specimens were analyzed from 389 subjects. Urinary creatinine was quantified with a commercial kit (Monarch Instrument, Amherst, NH) as described previously (Rothman *et al.*, 1998).

For quality control purposes, positive controls were assayed with each batch of urine samples. They were prepared from a single urine specimen obtained from an unexposed volunteer to which had been added sufficient quantities of standard analytes to achieve the following concentrations: UBz – 24.5 nM, MA – 32.3 μM, SPMA – 2.65 μM, and PH – 92.8 μM, CA – 94.1 μM and HQ – 69.7 μM. Batches of positive controls were aliquoted into 2-ml vials and stored at -80^{o}C prior to analysis.

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

2.3.3. Measurement of Urinary Benzene

Levels of UBz were determined according to the method of Waidyanatha *et al.* (Waidyanatha *et al.*, 2001), with minor modifications. Briefly, to 0.5 ml of urine in a 2-ml vial containing ca. 0.5 g of NaCl was added one μl of 59.4 μM [$^{2}H_{6}$]benzene in methanol (internal standard). The vial was immediately sealed with a PTFE/silicone septum and benzene was extracted from the headspace with a 30-μm polydimethylsiloxane fiber (Supelco, PA) at 40^{o}C for 15 min. Samples were analyzed by gas chromatography-mass spectrometry (GC-MS) in electron ionization (EI) mode using a HP 5980 Series II gas chromatograph coupled to a HP 5971-A mass selective detector. Sample injections were performed with a Varian Model 8200 autosampler (Walnut Creek, CA) equipped with a carousel (Strumenti Scientific, Padova, Italy). The injector, MS-transfer line and ion source temperatures were 200, 280, and 168^{o}C, respectively. A DB-1 fused silica capillary column (60 m, 0.25-mm i.d., 0.25-μm film thickness) was used with He as the carrier gas at a flow rate of 1 ml/min. The GC oven was held at 45^{o}C for 9 min, and was then increased at 10

^{o}C/min to 100 ^{o}C. Late eluting compounds were removed by rapidly raising the oven temperature to 250^{o}C. The mass spectrometer was operated with an electron energy of 70 eV, and ions were monitored at m/z 78 and 84 for benzene and [$^{2}H_6$]benzene, respectively. The retention times were 8.56 and 8.50 min for benzene and [$^{2}H_6$]benzene, respectively. Quantification was based on peak areas relative to the isotopically labeled internal standard.

2.3.4. Measurement of Benzene Metabolites

Levels of CA, HQ, MA, PH and SPMA were determined according to the method of Waidyanatha *et al.*, with minor modifications (Waidyanatha *et al.*, 2001). Briefly, to 0.5 ml of urine in a 4-ml vial was added 10 µl of a mixture (in methanol) of 1.73 mM [$^{13}C_2$]MA, 0.205 mM [$^{2}H_5$]SPMA, 6.24 mM [$^{13}C_6$]PH, 2.19 mM [$^{2}H_4$]CA, and 2.15 mM [$^{13}C_6$]HQ (internal standards). After adding 50 µl of concentrated HCl, the mixture was extracted with 1.5 ml of ethyl acetate and the organic phase, containing MA, SPMA, and free PH, CA, and HQ, was transferred to another 4-ml vial. Then, a 10-µl aliquot of a mixture containing the above quantities of internal standards of PH, CA, and HQ was added to the aqueous phase, which was heated at 100^{o}C for one h to hydrolyze the conjugates. After cooling to room temperature, the aqueous phase was extracted with an additional 1.5 ml of ethyl acetate. The two organic extracts were combined, dried with anhydrous Na_2SO_4, reduced under N_2, transferred to a 500-µl flat-bottomed insert, and brought to dryness under N_2. The residue was dissolved in 100 µl hexane and derivatized with 100 µl Tri-Sil reagent at 70^{o}C for one h.

Samples were analyzed by GC-MS in EI mode with the same instrumentation described above using automated liquid injection. A 2-µl injection was analyzed in splitless mode for SPMA; here, the GC oven was maintained at 75^{o}C for 4 min, and was then increased at 8^{o}C/min to 245^{o}C, where it was held for 10 min. A 1-µl portion was analyzed in (1:20) split

mode for PH, CA, HQ, and MA; here, the GC oven was maintained at 75 ^{o}C for 4 min, and was then increased at 10 ^{o}C/min to 230^{o}C and held for 4 min. In both cases, late eluting compounds were removed by rapidly raising the oven temperature to 270^{o}C and holding this temperature for 15 min. The following ions were monitored for trimethylsilyl (TMS)-derivatives of the analytes: TMS-PH [m/z 166, M^+], TMS-[$^{13}C_6$]PH [m/z 172, M^+], TMS-CA [m/z 254, M^+], [$^{2}H_4$]CA [m/z 258, M^+], TMS-HQ [m/z 254, M^+], TMS-[$^{13}C_6$]HQ [m/z 260, M^+], TMS-MA [m/z 271, $(M-15)^+$], TMS-[$^{13}C_2$]MA [m/z 273, $(M-15)^+$], TMS-SPMA [m/z 252, $(M-NH_2COCH_3)^+$], and TMS-[$^{2}H_5$]SPMA [m/z 257, $M-NH_2COCH_3)^+$]. The respective retention times were 12.66, 12.66, 17.65, 17.62, 18.83, 18.83, 21.91, and 21.91 min for TMS-PH, TMS-[$^{13}C_6$]PH, TMS-CA, TMS-[$^{2}H_4$]CA, TMS-HQ, TMS-[$^{13}C_6$]HQ, TMS-MA, and TMS-[$^{13}C_2$]MA in split mode, and 32.65 and 32.59 for TMS-SPMA and TMS-[$^{2}H_5$]SPMA in splitless mode. Quantification was based on peak areas relative to the corresponding isotopically labeled internal standards.

2.3.5. Creatinine Adjustment for Benzene Metabolites

It is customary to adjust urinary metabolite levels for urinary creatinine to control for urine volume at the time of collection. However, such adjustments can introduce biases and reduce precision, due to variation of creatinine levels with gender, age, body mass index, and other physiological factors (Zacur *et al.*, 1997; Hall Moran *et al.*, 2001; Ikeda *et al.*, 2003). In preliminary regression models, we observed lower adjusted-R^2 values using creatinine-adjusted metabolite levels compared to unadjusted values. We also found significant effects of age, gender and body mass index upon creatinine levels in our samples of workers. Thus, for all statistical analyses we used metabolite levels without adjustment for urinary creatinine. The following median levels of urinary creatinine were observed in our samples

of workers: all subjects – 11.6 mM; female controls – 10.4 mM; male controls – 11.5 mM; female exposed – 11.4 mM; and male exposed – 13.9 mM.

2.3.6. Predicting Low-level Exposures from Urinary Benzene

All air samples from control subjects were below the LOD (n = 160) and some measurements from exposed subjects were either below the LOD (n = 70) or were missing (n = 23). In order to predict the levels of benzene exposure in these samples, we used the log-scale linear regression of UBz on air benzene (228 data pairs) shown in Figure 2.2.A. For subjects having multiple air and urine samples, the mean values of the natural logarithms of urine and air levels were used. The following calibration curve was derived from the parameters of the simple linear regression: $\text{benzene (ppm)} = \exp\left(\frac{\ln(\text{UBz, nmol/l}) - 5.42}{0.886}\right)$.

Note that these predicted values of benzene exposure include contributions from smoking. Uncertainties in this relationship were evaluated via a bootstrapping technique as described below.

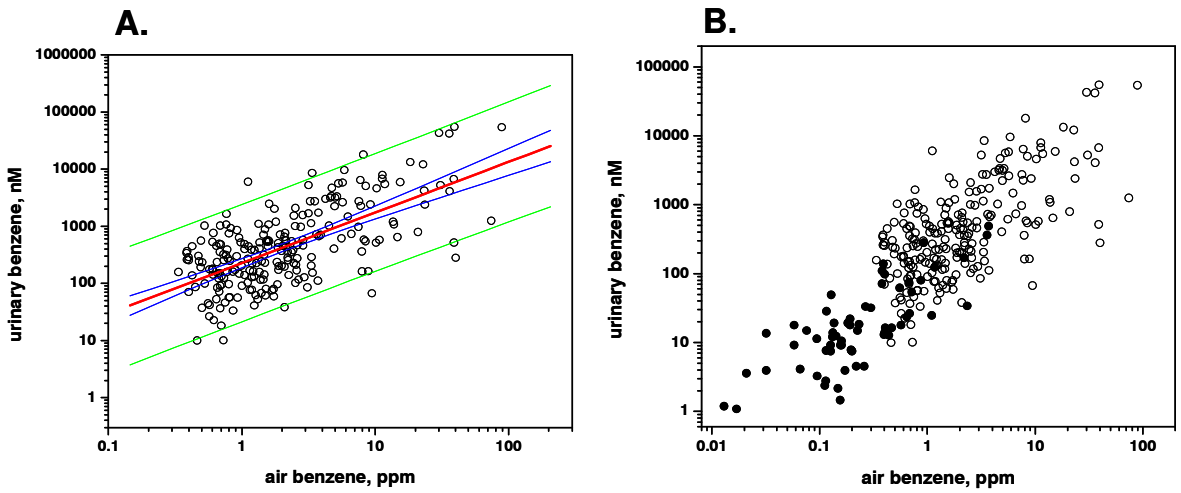

Figure 2.2. Calibration curve for predicting air concentrations of benzene from the corresponding urinary levels. (A) Points represent observed air and urine concentrations and the line represents the least-squares regression equation: ln(urinary benzene, nM) = 5.42

+ 0.886×ln(air benzene, ppm) (n = 228, adjusted R^2 = 0.428; 95 confidence of intercept, 5.24 – 5.61; 95 confidence interval of slope, 0.752 - 1.02). The curves represent 95% confidence intervals and 95% prediction intervals. (B) Scatter plot of urinary benzene versus air benzene from the current study (open circles) and data from Ghittori *et al.* (Ghittori *et al.*, 1993) obtained from measurements from 63 nonsmoking subjects exposed to benzene (closed circles).

2.3.7. Investigating Exposure-Metabolite Relationships

The relationships between levels of metabolites and exposure were explored using median values and interquartile ranges. Based upon 13 groups of 30 subjects stratified by exposure the following median air concentrations (ppm) were observed: 0.001, 0.002, 0.004, 0.007, 0.027, 0.273, 0.508, 0.689, 1.02, 1.57, 2.41, 5.45, and 15.4. Multiple comparison tests were utilized to generate step-functions showing statistically significant increases in geometric mean (GM) metabolite levels from group to group. These step functions were used to define levels of benzene exposure at which metabolite concentrations were statistically distinguishable from background values.

A similar grouping scheme (30 subjects stratified by exposure level) was used to investigate the dose-related production of each benzene metabolite, after adjustment for background metabolite levels. Background levels were estimated as median metabolite concentrations in 60 subjects with the lowest benzene exposures (median predicted benzene concentration = 0.002 ppm). Adjustment involved subtracting background metabolite levels from the corresponding urine concentrations observed in each subject. After grouping by benzene exposure (n = 30 per group) the median metabolite level in each group was divided by the median air concentration of benzene to estimate dose-related production of each metabolite (μM per ppm benzene) for the 9 groups with estimated median benzene exposures between 0.027 and 15.4 ppm. Uncertainties in estimated median values of benzene exposure

and background-adjusted metabolite levels were evaluated via a bootstrapping technique as described below.

2.3.8. Statistical Analyses

All statistical analyses were performed using SAS software for Windows v. 9.12 (SAS Institute, Cary, NC). Between one and four concurrent air and urine samples from each subject were available for statistical analysis (median = 2/subject). For subjects with multiple samples, estimated individual GM levels were used for all analyses. Non-detectable levels of urinary analytes were replaced by LOD/$\sqrt{2}$ [UBz: n = 8 (1.29%); SPMA: n = 30 (4.84%)] (Hornung and Reed, 1990). The precision of the assay for each urinary analyte was estimated as the coefficient of variation (*CV*) from 84 pairs of duplicate urine samples (from exposed and control workers), which had been aliquoted and assigned random identification numbers prior to shipment to the laboratory. The *CV* was estimated as $CV = \sqrt{\exp(s_\varepsilon^2) - 1}$, where s_ε^2 is the estimated error variance obtained from a one-way analysis of variance of the log-transformed levels of each analyte (Proc NESTED of SAS).

To evaluate possible effects of sample storage at -80^{o}C upon the levels of UBz and the benzene metabolites, the logged level of each urinary analyte was regressed upon the corresponding logged air concentration of benzene and the time of storage using a mixed-effects model, which included a random-subject effect to account for multiple urine specimens being collected from some persons (Proc MIXED of SAS).

Median levels of benzene exposure and those of the urinary metabolites were compared by exposure status, gender, and smoking status using the Wilcoxon two-sample test (two-sided tests with a significance level of 0.05, Proc NPAR1WAY of SAS). Trends of (median) metabolite levels versus benzene exposure were tested with Spearman correlation

coefficients, (Proc CORR of SAS). Differences in mean values of logged metabolite levels among groups of 30 subjects aggregated by benzene exposure were tested using Tukey's multiple comparisons test (Proc GLM of SAS). These tests were used to determine levels of benzene exposure where metabolite levels could be differentiated from background values.

We predicted air exposures of control subjects from their levels of urinary benzene and used these predicted exposures to group subjects for aggregated analyses. Thus, to estimate dose-related profiles of metabolite production, and the associated uncertainties, bootstrapping was performed based upon median values of background-adjusted metabolite levels and the corresponding levels of benzene exposure. First, resampling was conducted (with replacement) 1000 times to predict nondetected air exposures from urinary benzene levels, using the 228 data pairs shown in Figure 2.2. For each of the 1000 samples, predicted air concentrations were combined with observed air concentrations, median background levels from the lowest 60 exposed persons were subtracted from individual metabolite levels, and data were aggregated for groups of 30 subjects. Then, 5^{th}, 50^{th} and 95^{th} percentiles of the distributions of estimated median values of air and metabolite concentrations were obtained. Differences in dose-related production of each metabolite between adjacent exposure groups were evaluated with Kruskal-Wallis tests for overall group effects and trends (Proc FREQ of SAS).

2.4. Results

2.4.1. Precision, Sensitivity and Storage Stability

Estimates of *CV*s for the urinary analytes were as follows: UBz – 21.1%, MA – 6.15%, SPMA – 46.2%, PH – 4.97%, CA – 6.50%, and HQ – 4.81%. Based on a volume of 0.5 ml urine and a signal-to-noise ratio of 3:1, the following LODs were estimated: UBz, 0.128 nM,

MA, 5.66 nM (one µl injected with 1:20 split), and SPMA, 0.836 nM (2 µl with splitless injection). Values of LOD were not estimated for PH, CA, and HQ due to the presence of background levels of these compounds in all urine specimens. The LOD for measurements of airborne benzene had previously been assigned a nominal value of 0.2 ppm (Vermeulen *et al.*, 2004). When the period of storage was included as a fixed effect in mixed-effects models of the logged levels of UBz and the benzene metabolites on the logged benzene air concentrations, the observed regression coefficients were very small relative to the intercepts and showed no evidence of statistical significance ($p > 0.2$). This indicates that all analytes were stable during long periods of storage at -80^{o}C in this study.

2.4.2. Summary Statistics

Estimates of median levels, ranges and interquartile ranges of air exposures to benzene and all urinary analytes are summarized in Table 2.2, after stratification by exposure status, gender, and smoking status. Among occupationally exposed subjects, air levels of benzene ranged from 0.017 to 88.9 ppm (interquartile range: 0.529 – 3.34 ppm), with median values of about 1.2 ppm in all categories of subjects. Among control subjects, median exposures (predicted from UBz levels) were between 3 and 6 parts per billion (ppb) depending upon gender and smoking status. Although air and urinary concentrations of benzene were comparable in all categories of exposed workers, females had significantly higher median levels of SPMA ($p = 0.048$) and PH ($p = 0.038$) than males. No significant differences were observed between smokers and nonsmokers in the exposed group. Control subjects who were smokers had higher median levels of all urinary analytes than nonsmokers, notably UBz ($p = 0.003$), CA ($p = 0.034$) and HQ ($p = 0.001$), indicating the likely presence of these analytes in cigarette smoke. Although there were no significant effects of gender on

metabolite levels in control subjects, when nonsmokers were further stratified by gender, females (n = 84) had significantly higher median levels of PH (63.3 vs. 46.4 μM, p = 0.037) and CA (13.2 vs. 7.99 μM, p = 0.010) than males (n = 16).

Table 2.2. Summary statistics for exposure to benzene and urinary analytes.

Variable	Control[§]				Exposed[§§]			
	Female (n = 87)	**Male (n = 52)**	**Nonsmoker (n = 100)**	**Smoker (n = 39)**	**Female (n = 164)**	**Male (n = 86)**	**Nonsmoker (n = 198)**	**Smoker (n = 52)**
Air benzene[†]								
Median	3.4	3.71	3.09	6.07	1.28	1.05	1.18	1.18
Range	0.146-21.2	0.146-533	0.146 -21.2	0.146 -533	0.017-88.9	0.122-50.2	0.017-88.9	0.122-40.1
IQ range	1.70-6.80	2.13-9.71	1.70 -5.91	2.56 -17.6	0.523-4.21	0.572-2.36	0.520-3.34	0.588-3.02
p-value	0.273		0.003		0.588		0.889	
UBz (nM)								
Median	1.48	1.59	1.36	2.47	283	216	267	197
Range	0.091-7.47	0.091-130	0.091-7.47	0.091-130	6.21-53900	19.4-42600	6.21-53900	19.4-41600
IQ range	0.799-2.73	0.973-3.74	0.797-2.41	1.15-6.32	87.3-1020	96.3-643	96.3 -810	85.3-654
p-value	0.273		0.003		0.635		0.702	
MA (μM)								
Median	1.06	1.09	1.06	1.11	13.5	10.3	12.3	12.4
Range	0.152-6.17	0.132-5.78	0.152-6.17	0.132-4.96	0.644-426	1.50-370	0.644-426	2.15-347
IQ range	0.549-1.75	0.774-1.72	0.543-1.70	0.803-1.74	6.33-34.5	5.39-22.4	6.13-28.5	6.22-28.4
p-value	0.565		0.384		0.272		0.473	
SPMA (nM)								
Median	1.94	3.24	1.96	3.17	262	137	228	171
Range	0.591-86.4	0.591-68.1	0.591-86.4	0.591-68.1	1.50-29400	3.68-33000	1.50-29400	4.06-33000
IQ range	1.17-3.62	1.04-6.93	1.13-4.25	1.09-7.37	50.6-788	48.9-353	50.1-631	47.9 -412
p-value	0.479		0.493		0.048		0.638	
PH (μM)								
Median	61.4	53.2	61.3	56.3	171	134	158	135
Range	9.48-208	7.58-336	9.48-208	7.58-336	15.9-3740	41.8-4140	15.9-4140	41.8-3380
IQ range	45.0-103	28.8-82.0	43.7-89.6	29.0-98.5	102-310	86.6-190	100-261	84.6-313
p-value	0.117		0.415		0.038		0.391	
CA (μM)								
Median	13.3	12.7	12.1	15.7	21.8	21	20.5	23.7
Range	2.30-61.1	2.45-46.6	2.30-61.1	2.45-46.6	3.50-420	4.20-438	3.50-438	7.08-348
IQ range	8.06-17.0	7.07-18.9	7.50-15.8	9.90-19.8	13.3-33.0	12.6-29.4	12.6-32.1	13.8-35.5
p-value	0.786		0.034		0.411		0.153	
HQ (μM)								
Median	5.95	7.35	5.93	8.52	17.7	18.5	17.1	19.6
Range	1.54-39.1	1.32-41.7	1.40-39.1	1.32-41.7	2.29-427	3.20-341	2.29-427	5.76-375
IQ range	4.44-8.88	4.48-11.7	4.11-8.86	5.65-13.9	11.2-36.8	11.5-27.8	10.4-29.1	14.9-35.9
p-value	0.106		0.001		0.925		0.084	

Legend: IQ range - interquartile range, benzene - benzene in air, UBz - urinary benzene, MA - *E,E*- muconic acid, SPMA - *S*-phenylmercapturic acid, PH - phenol, CA - catechol, HQ - hydroquinone. [†] Exposure to benzene for control subjects was predicted from the relationship between UBz and air exposure. § unit of air benzene, ppb. §§ unit of air benzene, ppm.

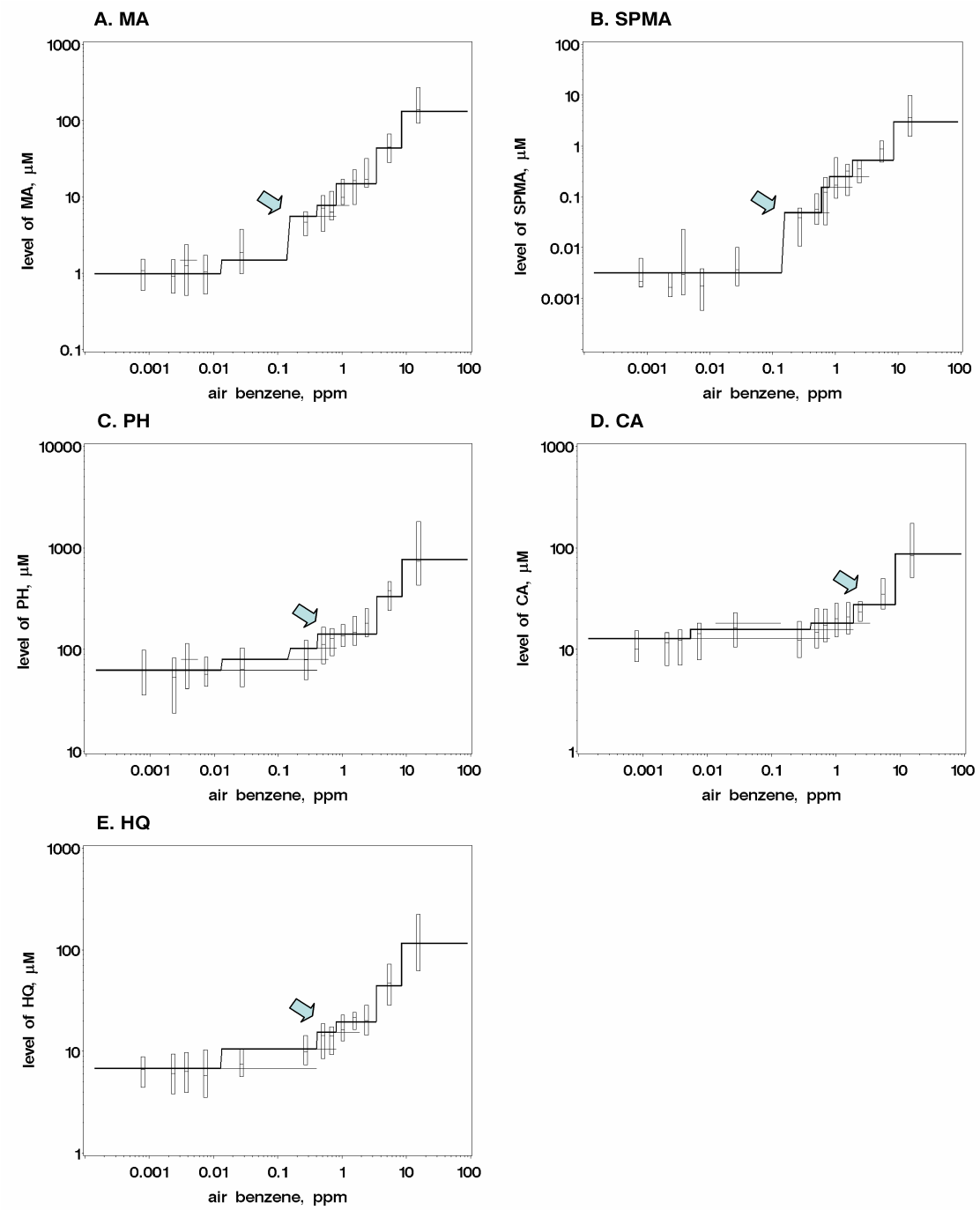

Figure 2.3. Levels of urinary metabolites versus air exposures to benzene. Each vertical box depicts median, 25th and 75th percentiles of a given metabolite at the median level of exposure for data grouped by exposure levels (30 subjects per group). Horizontal lines represent groups with equivalent metabolite levels (Tukey's multiple comparison tests). Arrows represent air exposure levels where it is possible to distinguish benzene metabolites from background sources. Legend: MA - *E,E*-muconic acid, SPMA - *S*-phenylmercapturic acid, PH – phenol, CA - catechol, and HQ– hydroquinone.

2.4.3. Distinguishing Benzene Metabolites from Background Sources

Figure 2.3 shows median levels and interquartile ranges for concentrations of each metabolite among groups of 30 subjects aggregated by benzene exposure. As exposure increased, the levels of metabolites also increased (estimated Spearman coefficients of median values for PH: 0.973, HQ: 0.945, CA: 0.934, MA: 0.973, and SPMA: 0.973, all with $p < 0.001$). The step functions signify nominal exposure concentrations at which statistically significant increases in metabolite levels were observed. As exposure increased, the contribution of benzene to each metabolite could consistently be discerned at some point (Tukey's multiple comparisons test), as indicated by an arrow in Figure 2.3. Although there were marginal increases in median levels of MA, PH, CA, and HQ at median benzene exposures as low as 0.02 ppm, elevations were not unambiguous below air concentrations of 0.2 ppm for MA and SPMA, 0.5 ppm for PH and HQ and 2 ppm for CA. From these results, it appears that none of the metabolites would be useful for biomonitoring of benzene at environmental levels (generally below 0.01 ppm).

2.4.4. Dose-Related Production of Benzene Metabolites

Median urine concentrations of all metabolites per ppm of benzene exposure are presented in Figure 2.4 for the 9 groups of workers having exposures above 0.01 ppm (after adjustment for background values). Each point in the figure and the corresponding error bars represent median values and 5^{th} and 95^{th} percentiles of distributions of median values, as determined by bootstrapping. These curves point to the dose-related production of benzene metabolites according to the pathways shown in Figure 2.1. Over the range of exposures investigated (median benzene concentrations between 0.027 and 15.4 ppm) significant downward trends in metabolite production were observed ($p < 0.001$, Kruskal-Wallis tests)

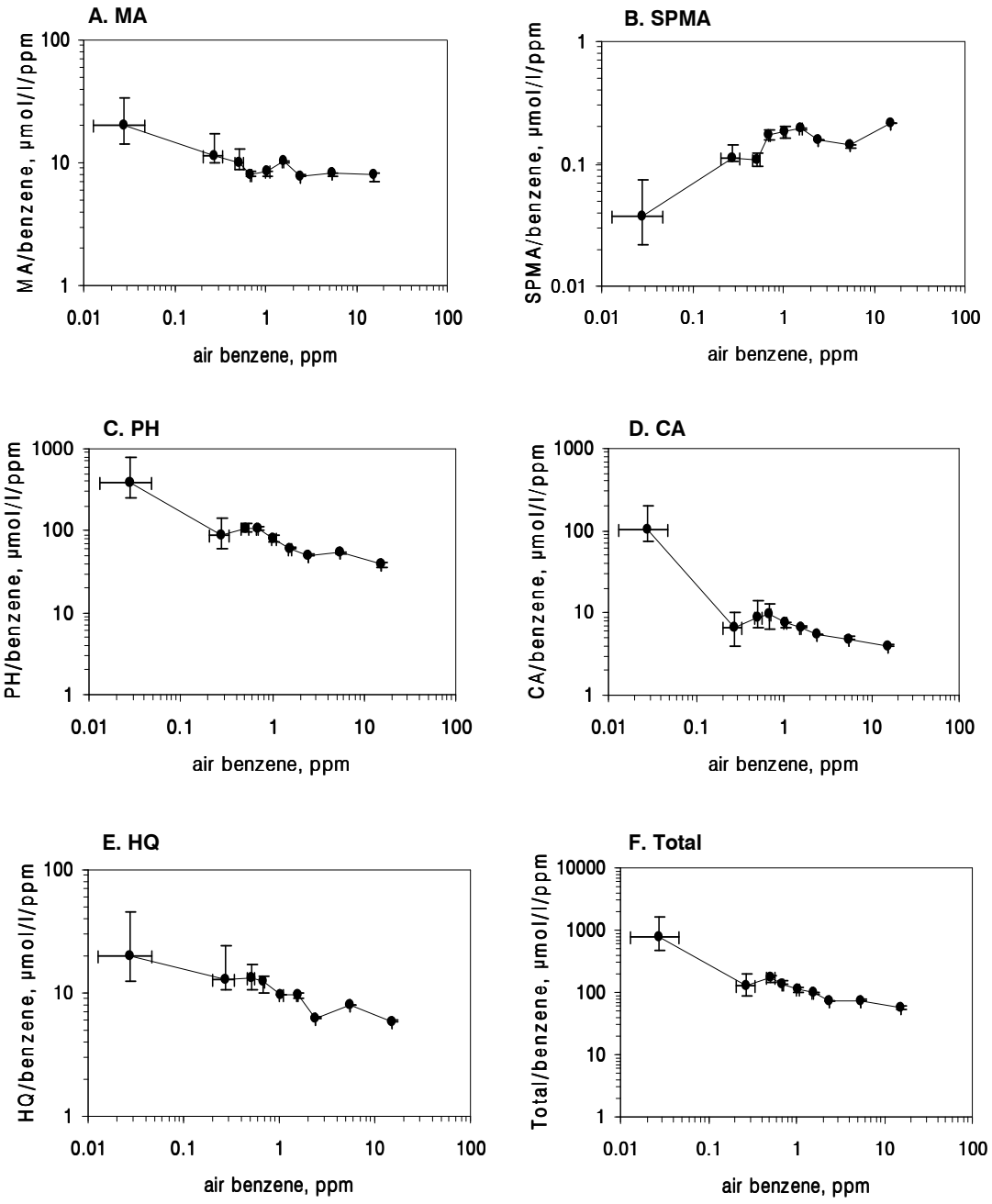

Figure 2.4. Dose-related production of urinary metabolites versus air exposure to benzene. Each point and the corresponding error bars represent median values and 5th and 95th percentiles of distributions of estimated median values of air benzene and dose-related metabolite levels (background-adjusted metabolite concentration/air benzene concentration) for groups of 30 subjects, determined by bootstrapping (1000 iterations). Legend: MA - *E,E*-muconic acid, SPMA - *S*-phenylmercapturic acid, PH – phenol, CA - catechol, and HQ– hydroquinone Total – total metabolites.

for all major metabolites (PH, CA, HQ and MA) and for total metabolites ($p < 0.001$), while a significant upward trend was observed for SPMA ($p < 0.001$). The downward trend of total benzene metabolites is consistent with partial saturation of CYP metabolism of benzene between 0.027 and 15.4 ppm. The transitions were particularly apparent between the first and second exposure groups (median benzene concentrations between 0.027 and 0.274 ppm for all metabolites.

2.5. Discussion

The connections between benzene metabolism and toxicity have been extensively debated (Smith, 1996; Golding and Watson, 1999; Snyder, 2000b; Lovern *et al.*, 2001; Snyder, 2004). To gain a better understanding of human benzene metabolism, we measured the key metabolites (PH, CA, HQ, MA and SPMA) and UBz in 250 benzene exposed workers and 139 control workers from a Chinese population. Of the studies devoted to urinary biomarkers of benzene in Asian populations, none compared levels of all key metabolites with air levels representing both environmental and occupational exposures (Ong *et al.*, 1995; Ong *et al.*, 1996; Rothman *et al.*, 1998; Qu *et al.*, 2000; Melikian *et al.*, 2002; Waidyanatha *et al.*, 2004). Our goals were to determine the usefulness of the urinary metabolites as biomarkers of benzene exposure in exposed and control subjects and to elucidate the dose-related patterns of benzene metabolism along the prominent pathways shown in Figure 2.1.

Since control workers in our study were exposed to benzene in environmental air and tobacco smoke, we predicted their exposures from the corresponding UBz concentrations,

using a calibration curve obtained from exposed subjects (Figure 2.2). Such predictions should be reasonably unbiased, given previous findings of strong linear trends between levels of benzene in urine and environmental air (Ghittori *et al.*, 1993; Ghittori *et al.*, 1995; Fustinoni *et al.*, 1999; Fustinoni *et al.*, 2005a; Fustinoni *et al.*, 2005b). This conjecture is reinforced by Figure 2.2B which plots levels of UBz versus air benzene from the current study (open circles) as well as those reported by Ghittori *et al.* (Ghittori *et al.*, 1993) for 63 nonsmoking workers exposed to measured benzene concentrations between 0.01 and 4 ppm (closed circles). The two sets of measurements point to a linear log-scale relationship down to the lowest measured air level of about 0.01 ppm. Also, the median benzene air concentration predicted from UBz levels for our control subjects (3.55 ppb, $n = 139$) is similar to air concentrations estimated from personal monitoring of adult populations in the U.K. (3.82 ppb daytime mean value, $n = 50$) (Leung and Harrison, 1998), in Germany (3.44 ppb median value, $n = 113$) (Hoffmann *et al.*, 2000), in the U.S. (2.29 – 7.01 ppb range of median values for 5 cities, $n = 421$) (Rappaport and Kupper, 2004), and Italy (2.82 and 1.88 ppb for control subjects in two cities, $n = 107$) (Fustinoni *et al.*, 2005b).

Comparing levels of urinary analytes between smoking and nonsmoking controls, the median values of UBz, CA, HQ, MA and SPMA were all higher in smokers, while the median level of PH was marginally lower in smokers (Table 2.1). The apparent anomaly for PH is probably related to gender because male controls had lower PH levels than females and 92% of the smokers were male. [Among male controls, smokers had a higher median level of PH (58.7 μM, $n = 36$) than male nonsmokers (46.4 μM, $n = 16$)]. Other investigators have reported that non-occupationally exposed smokers had significantly higher urinary levels of UBz (Ghittori *et al.*, 1993; Ghittori *et al.*, 1995; Fustinoni *et al.*, 1999; Fustinoni *et al.*,

2005a), MA (Melikian *et al.*, 1994; Ghittori *et al.*, 1995; Scherer *et al.*, 1998; Melikian *et al.*, 1999; Taniguchi *et al.*, 1999; Waidyanatha *et al.*, 2004), and SPMA (Ghittori *et al.*, 1995; Maestri *et al.*, 1997; Waidyanatha *et al.*, 2004) than nonsmokers.

To estimate the equivalent air level of benzene from cigarette consumption, we regressed the (logged) predicted air concentration of benzene on the number of cigarettes smoked per day among control subjects. This led to the following relationship:

ln(benzene, ppm) = -5.81 + 0.050(cigarettes/d) (n = 134, p-value for slope = 0.003),

from which we predict that smoking 20 cigarettes/d would be equivalent to an occupational benzene exposure of 8.2 ppb (26 $\mu g/m^3$). This estimated benzene exposure of 8.2 ppb is the same as that predicted from British data (Duarte-Davidson *et al.*, 2001) for an urban smoker consuming 20 cigarettes/d.

In order to distinguish benzene-derived metabolites from background levels of the same compounds, we investigated median metabolite levels for groups of 30 subjects, aggregated by benzene exposure (Figure 2.3). Using statistical significance to discern exposure-related increases in urinary levels, we detected marginal elevations at benzene exposures between 0.02 and 0.1 ppm for all metabolites except SPMA. However, when we considered exposure levels which produced unambiguous increases in metabolite concentrations, MA and SPMA were the most sensitive biomarkers of exposure to benzene (about 0.2 ppm), followed by PH and HQ (about 0.5 ppm) and CA (about 2 ppm).

Using aggregated data, we also observed changes in dose-related metabolism of benzene (Figure 2.4) for median benzene exposures between 0.027 and 15.4 ppm. We anticipated that saturable metabolism would be detected in this range, given results from other Chinese studies (Rothman *et al.*, 1998; Waidyanatha *et al.*, 2001; Melikian *et al.*, 2002; Waidyanatha

et al., 2004), and because benzene, oxepin and PH are all thought to compete for the same enzymes (CYP2E1) (Gilmour *et al.*, 1986; Snyder *et al.*, 1993; ATSDR, 1997). And, in fact, downward trends were apparent for dose-related production of PH (9.9-fold), CA (26-fold), HQ (3.4-fold), MA (2.5-fold), and total metabolites (14-fold) (Figure 2.4). The 2.5-fold decrease observed for MA is comparable to a 3.3-fold reduction that we estimated from grouped data reported by Melikian *et al*. (Melikian *et al.*, 2002) (using differences between post-work and pre-work measurements of MA) for Chinese workers exposed to benzene concentrations between 0.34 and 22.6 ppm (estimated mean values).

Surprisingly, SPMA displayed an upward trend of production, with a 5.8-fold increase between 0.027 and 15.4 ppm (Figure 2.4). This might reflect the action of glutathione-*S*-transferase or be the result of mechanisms (Henderson *et al.*, 2005a) or sites of formation of SPMA which differ fundamentally from those of the other metabolites.

It is noteworthy that the most dramatic reductions in dose-related metabolism occurred between the first two groups in Figure 2.4, with benzene exposures of 0.027 and 0.274 ppm, respectively. In this range, production of CA dropped by 16-fold and that of PH by 4.4-fold. Yet, only marginal reductions were observed in this exposure range for production of MA (44% reduction) and HQ (36% reduction). Thus, it appears that metabolism shifted away from CA and PH at low doses in favor of MA and HQ, the only major benzene metabolites requiring two CYP oxidations (Figure 2.1). When exposures exceeded about one ppm, the curves in Figure 2.4, representing major metabolites (PH, MA, HQ, and CA), became quasi-parallel, suggesting that metabolism of benzene to BO had become rate-limiting.

If our conjecture is correct that exposures to benzene below one ppm favor production of HQ and MA, there could be important implications for risk assessment. Certainly, HQ is the precursor of 1,4-benzoquinone, which is generally regarded as most hematotoxic of the benzene metabolites (Smith, 1996; Snyder and Hedli, 1996; Smith *et al.*, 2004; Snyder, 2004; Inayat-Hussain and Ross, 2005), and MA is derived from the extremely reactive and toxic muconaldehydes (Witz *et al.*, 1990; Witz *et al.*, 1996; Golding and Watson, 1999; Amin and Witz, 2001; Oshiro *et al.*, 2001). We are currently applying various nonlinear models to these data to more fully examine the dose-related metabolism of benzene and to estimate effects of physiological and genetic factors upon benzene metabolites.

CHAPTER 3.

MODELING HUMAN METABOLISM OF BENZENE FOLLOWING OCCUPATIONAL AND ENVIRONMENTAL EXPOSURES

[Kim S, Vermeulen R, Waidyanatha S, Johnson AB, Lan Q, Rothman N, et al. Cancer Epidemiol Biomarkers Prev 2006, in press.]

3.1. Abstract

We used natural spline (NS) models to investigate nonlinear relationships between levels of benzene metabolites [*E,E*-muconic acid (MA), *S*-phenylmercapturic acid (SPMA), phenol (PH), hydroquinone (HQ), and catechol (CA)] and benzene exposure among 386 exposed and control workers in Tianjin, China. After adjusting for background levels (estimated from the 60 control subjects with the lowest benzene exposures), expected mean trends of all metabolite levels increased with benzene air concentrations over 0.03 – 88.9 ppm. Molar fractions for PH, HQ and MA changed continuously with increasing air concentrations, suggesting that competing CYP-mediated metabolic pathways favored MA and HQ below 20 ppm and favored PH above 20 ppm. Mean trends of dose-specific levels (μM/ppm benzene) of MA, PH, HQ, and CA all decreased with increasing benzene exposure, with an overall 9-fold reduction of total metabolites. Surprisingly, about 90% of the

reductions in dose-specific levels occurred below about 3 ppm for each major metabolite. Using general linear models (GLM) with NS-smoothing functions we detected significant effects upon metabolite levels of gender, age and smoking status. Metabolite levels were about 20% higher in females and decreased between one and two percent per year of life. Also, levels of HQ and CA were greater in smoking subjects. Overall, our results indicate that benzene metabolism is highly nonlinear with increasing benzene exposure above 0.03 ppm and that current human toxicokinetic models do not accurately predict benzene metabolism below 3 ppm. Our results also suggest that GLM+NS models are ideal for evaluating nonlinear relationships between environmental exposures and levels of human biomarkers.

3.2. Introduction

Benzene is an important industrial chemical that is also ubiquitous in the environment due to emissions from gasoline and combustion of hydrocarbons and tobacco (Wallace, 1996; ATSDR, 1997). Occupational exposure to benzene can cause blood disorders, including aplastic anemia, myelodisplastic syndrome, and acute myelogenous leukemia (Hayes *et al.*, 1997; Savitz and Andrews, 1997). Significant decreases in the numbers of white blood cells and platelets have recently been reported in workers exposed to less than one ppm of benzene (Lan *et al.*, 2004). These toxic effects are thought to arise from metabolism of benzene, which proceeds along several lines, as illustrated in Figure 3.1. Of the various metabolites, 1,4-benzoquinone and the muconaldehydes are regarded as the most toxic species. However, the mechanism by which benzene causes toxicity and the shape of the exposure-response relationship are not well understood (Ross, 2000; Snyder, 2004).

We recently reported dose-specific urine concentrations of the major urinary metabolites of benzene, namely, phenol (PH), catechol (CA), hydroquinone (HQ), and *E,E*-muconic acid (MA), and a minor metabolite, *S*-phenylmercapturic acid (SPMA), in 250 benzene-exposed and 139 control workers from Tianjin, China (Kim *et al.*, 2006b). After grouping subjects according to their benzene exposures (30 subjects per group), median metabolite levels increased non-linearly with increasing median benzene concentrations between 0.03 and 20 ppm while median dose-specific levels of total metabolites (μM/ppm benzene) decreased about 10 fold.

We sought a parsimonious statistical model with which to elaborate on our previous grouped analyses (Kim *et al.*, 2006b) and to determine effects of significant covariates, such as gender, age and smoking status, on the levels of benzene metabolites. Given the nonlinear relationships involved, we selected natural splines (NS) as basis functions for these models because they use standard (least-squares or maximum-likelihood) methods for estimating parameters and for conducting formal tests; they can be used to represent predictors in final models; and they can easily be added to generalized linear models (GLM) for considering covariate effects (Durrleman and Simon, 1989; Heuer, 1997; Harrell, 2001; Samoli *et al.*, 2005). Although generalized linear models with natural splines (GLM+NS) have been used in time-series studies of health effects associated with community air pollution (Dominici *et al.*, 2002), we could find no reports of their applications to characterize exposure-biomarker relationships.

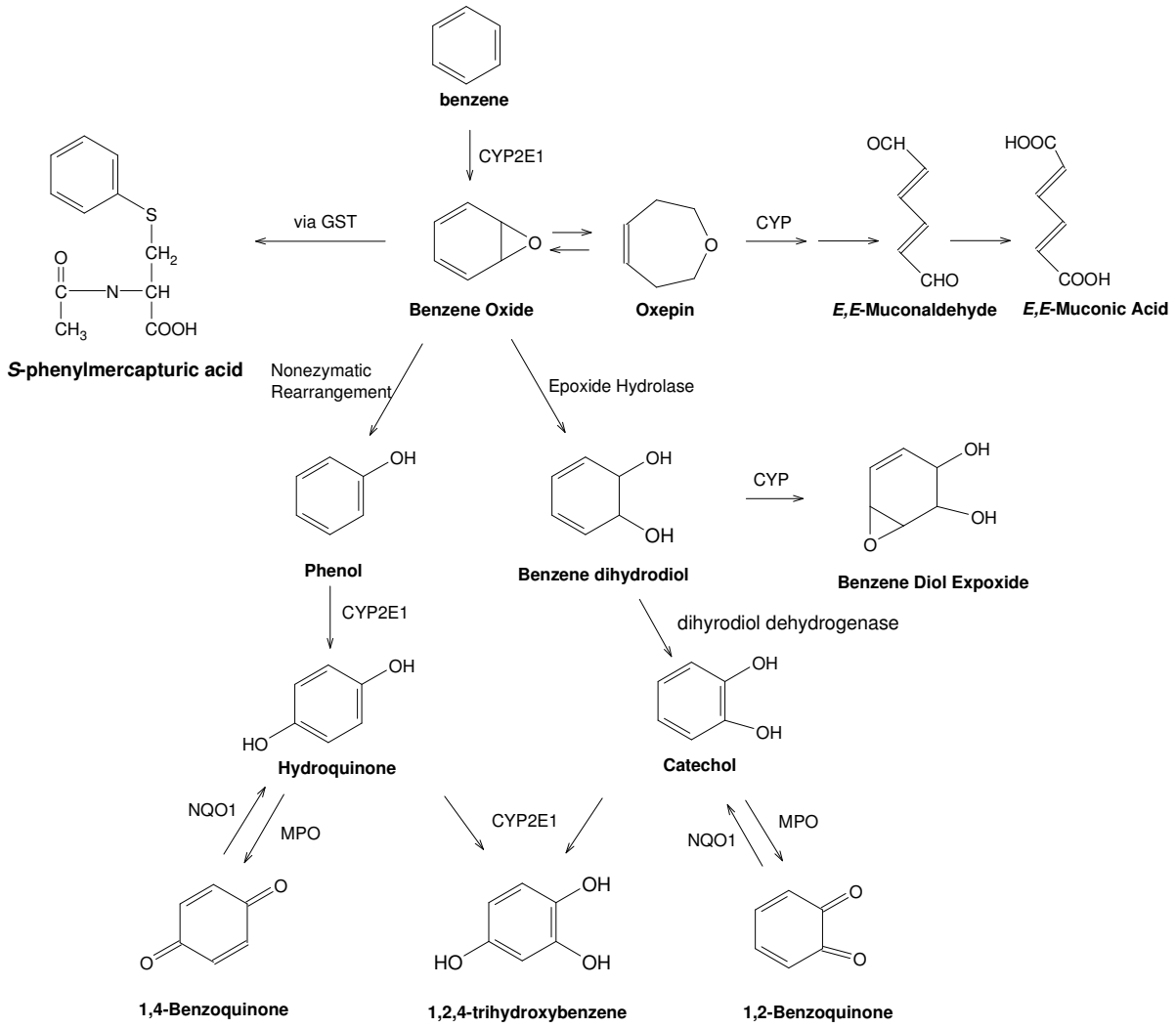

Figure 3.1. Simplified metabolic scheme for benzene showing major pathways.

3.3. Materials and Methods

3.3.1. Subject Recruitment and Sample Collection

Exposed and control subjects, from two shoe-making factories and three clothes-manufacturing factories, respectively, in Tianjin, China, were recruited with informed consent as described previously (Lan *et al.*, 2004; Vermeulen *et al.*, 2004; Kim *et al.*, 2006b). After excluding three control subjects, who had missing values of at least one metabolite, the

samples included 250 exposed subjects and 136 control subjects. Exposed and control subjects were frequency matched by region and gender. Table 3.1 shows summary statistics for the gender, age and smoking status of participants. Demographic data were obtained by questionnaires at the time of recruitment.

Table 3.1. Demographic characteristics of the study population.

Exposure Status	Gender	*n* (%)	Air Benzene	Age	Current smokers	Smoking intensity[a]
			Median (range)	Median (range)	*n* (%)	Median (range)
Control	**Male**	52 (38.2)	3.71 (0.146 – 533) ppb	28 (18–51)	36 (69.2)	10 (1–40)
	Female	84 (61.8)	3.39 (0.146 – 21.2) ppb	28 (18–51)	3 (3.57)	NR[b]
	All	136 (100)	3.48 (0.146 – 533) ppb	28 (18–51)	39 (28.71)	10 (1–40)
Exposed	**Male**	86 (34.4)	1.05 (0.122 – 50.2) ppm	23 (18–44)	47 (54.7)	10 (1–30)
	Female	164 (65.6)	1.28 (0.017 – 88.9) ppm	33 (18–52)	5 (3.05)	4.5 (2–10)
	All	250 (100)	1.18 (0.017 – 88.9) ppm	29 (18–52)	52 (20.8)	7 (1–30)

[a] Average number of cigarettes/day.

[b] Not reported.

Methods of sampling air and urine were also previously described (Lan *et al.*, 2004; Vermeulen *et al.*, 2004; Kim *et al.*, 2006b). Briefly, personal full-shift air measurements were matched with post-shift urine samples from exposed and control workers. Of the 386 subjects in this analysis, 139 had repeated measurements of air and urine, making a total of 617 matched air/urine samples. Among subjects with repeated measurements, the median number of paired air and urine samples was three (range: 2 – 4).

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

3.3.2. Measurements of Air and Urinary Analytes

The methods of measuring analytes in air and urine were described previously (Vermeulen *et al.*, 2004; Kim *et al.*, 2006b). Briefly, benzene and toluene were measured in air using passive personal monitors (Organic Vapor Monitors, 3M, St. Paul, MN) followed by solvent desorption and gas chromatography (Vermeulen *et al.*, 2004). Urinary benzene was determined by gas chromatography-mass spectrometry (GC-MS) using head-space solid-phase microextraction according to Waidyanatha *et al.* (Waidyanatha *et al.*, 2001). Urinary PH, CA, HQ, MA and SPMA were measured as trimethylsilylether derivatives by GC-MS according to Waidyanatha *et al.* (Waidyanatha *et al.*, 2004). Quantification of all urinary analytes was based on peak areas relative to the corresponding isotopically labeled internal standards.

All air samples from control subjects were below the nominal limits of detection (LOD) of 0.2 ppm for benzene and 0.3 ppm for toluene. In addition, some air measurements from exposed subjects were below the LOD (n = 70 for benzene and 67 for toluene) or were missing (n = 23). Air concentrations for these samples were predicted from the simple linear regression of levels of urinary benzene or toluene on the corresponding air levels (in log scale) as described previously for benzene(Kim *et al.*, 2006b). The minor metabolite, SPMA, was not detected in 30 urine specimens; a value of LOD/$\sqrt{2}$ = 0.591 nM was imputed to these samples (Hornung and Reed, 1990).

3.3.3. Statistical Analysis

For subjects with multiple measurements of air and urine, the estimated geometric mean air and urine concentrations were used in all statistical analyses. Relationships between levels of the urinary metabolites and the corresponding air concentrations of benzene were

examined using NS models with fixed knots. Since metabolite levels from the 60 subjects with the lowest benzene exposures were used to estimate background levels of these metabolites (described later), NS models were applied to the remaining 326 subjects. Each full NS model had the form,

$$E[\ln(Y_{m,j}) \mid \ln(X_j)] = \beta_{m,0} + \beta_{m,1}\ln(X_j) + \sum_{i=1}^{\kappa} \beta_{m,2i}[\ln(X_j) - \xi_i]_+^3 \quad (1)$$

where $E[\ln(Y_{m,j})|\ln(X_j)]$ is the conditional mean value of $\ln(Y_{m,j})$ representing the natural-log transform of the level of the m^{th} metabolite level in the j^{th} subject at $\ln(X_j)$, the corresponding (logged) air concentration of benzene (ppm), and ξ_i is the location of the i^{th} knot (in log-scale of benzene exposure) among total κ knots. The function $[\ln(X_j) - \xi_i]_+^3$ equals $[\ln(X_j) - \xi_i]^3$ for positive values and equals zero otherwise. Knots are joints of log-transformed air levels of benzene, showing different polynomial trends. They were assigned using equally spaced quantiles of the observations (Harrell, 2001). After comparing NS models with 3 to 7 knots, based upon 'corrected Akaike's Information Criteria' (AICc) (Burnham *et al.*, 2002), a 6-knot model was selected as a basis function for each metabolite. Then a final model was assigned to each metabolite after removing insignificant knots by stepwise elimination ($p = 0.10$ for retention) (Marsh, 1986), while retaining the linear term representing benzene exposure, $\beta_1\ln(X_j)$.

Effects of covariates on metabolite levels were determined using GLM+NS models having the form:

$$\mathrm{E}[\ln(Y_{m,j}) \mid \ln(X_j)] = \beta_{m,0} + \beta_{m,1}\ln(X_j) + \sum_{i^*=1}^{\kappa} \beta_{m,2i^*}[\ln(X_j) - \xi_{i^*}]_+^3 + \beta_{m,3k}\sum_{k=1}^{K} C_{kj} \qquad (2)$$

where i^* indicates the i^{th} knot in final NS model for the m^{th} metabolite (m = 1, …, 5, representing MA, SPMA, PH, CA and HQ, respectively), C_{kj} is the value of the k^{th} covariate (k = 1, …, K) in the j^{th} subject, and the remaining terms were the same as for Model (1). The following covariates were evaluated: gender (0, female; 1, male), age (centered around the estimated mean value of 30.1 y, n = 326), smoking status (0, nonsmoker; 1, smoker), body mass index (BMI, centered around the estimated mean value of 22.5 kg/m^2, n = 325), co-exposure to toluene (0, low exposure relative to the median concentration of 3.29 ppm (n = 326) ; 1, high exposure; antibiotics used within 30 days, (0, no; 1, yes), and current alcohol consumption status (0, no; 1, yes). Main effects and two-way interactions were evaluated using Proc GLMSELECT of SAS, with backward selection based upon the smallest AICc values (Burnham *et al.*, 2002), while retaining gender, age, BMI, and smoking status in all models. With these main effects in the model, no other covariate effects or interactions were retained in final models.

All statistical analyses were performed using SAS software for Windows v. 9.12 (SAS Institute, Cary, NC).

3.3.4. Molar Fractions and Dose-specific Metabolism

Let $\hat{Y}_{m,j|X_j}$ be the conditional mean value of $Y_{m,j}$, representing the m^{th} metabolite level in the j^{th} subject, given exposure level X_j under final Model (1). The molar fraction of the m^{th} metabolite, derived from benzene exposure of the j^{th} subject, was estimated as

$$\frac{(\hat{Y}_{m,j|X_j} - Y_{m,b})}{\sum_{m=1}^{5}(\hat{Y}_{m,j|X_j} - Y_{m,b})}$$, where $Y_{m,b}$ is the background level of the m^{th} metabolite and the

denominator term represents 'total' metabolites from benzene. We assigned values to $Y_{m,b}$ using the median levels of $Y_{m,j}$ observed in the 60 control subjects with the lowest benzene exposures (MA, 1.03 μM; SPMA, 0.002 μM; PH, 54.4 μM; CA, 11.7 μM; HQ, 6.43 μM) (Kim *et al.*, 2006b). Dose-specific production (μM/ppm benzene) of the m^{th} metabolite in the j^{th} subject was estimated as $(\hat{Y}_{m,j|X_j} - Y_{m,b}) / X_j$, where X_j is that subject's benzene exposure. Negative values of $(\hat{Y}_{m,j|X_j} - Y_{m,b})$ in the above computations were replaced by zeros. Because the proportions of negative values increased rapidly with decreasing exposure levels below 0.03 ppm, molar fractions and dose-specific metabolite levels were only evaluated for subjects exposed to benzene at or above 0.03 ppm (n = 267). The following percentages of negative values were observed between 0.03 and 88.9 ppm: MA - 0.29% and HQ - 14.6%.

Uncertainties in the model predictions of dose-specific metabolite levels were evaluated via bootstrap resampling with 500 iterations (implemented with the SAS macro, *%boot*). The pool of all observed benzene exposures (each representing a different subject, n = 386) was sampled, with replacement, to select a reference group (the 60 lowest observations) and an exposed group (the 326 remaining observations). Data from the exposed group (n = 326 observations) were then used to construct NS models for the various metabolites, as described above for the original data set.

3.4. Results

3.4.1. Natural Spline Models

After removal of non-significant terms from Model (1), the following reduced models were selected for the 5 metabolites:

MA: $E[\ln(Y_{MA,j}) \mid \ln(X_j)] = 1.11 + 0.188[\ln(X_j)] + 0.007[\ln(X_j)-\xi_1]_+^3 - 0.022[\ln(X_j)-\xi_3]_+^3$,

SPMA: $E[\ln(Y_{SPMA,j}) \mid \ln(X_j)] = -6.16 - 0.072[\ln(X_j)] + 0.040[\ln(X_j)-\xi_1]_+^3 - 0.077[\ln(X_j)-\xi_2]_+^3 + 0.110[\ln(X_j)-\xi_5]_+^3$

PH: $E[\ln(Y_{PH,j}) \mid \ln(X_j)] = 4.21 + 0.009[\ln(X_j)] + 0.004[\ln(X_j)-\xi_1]_+^3$,

CA: $E[\ln(Y_{CA,j}) \mid \ln(X_j)] = 2.64 + 0.034[\ln(X_j)] + 0.007[\ln(X_j)-\xi_2]_+^3$, and

HQ: $\ln(Y_{HQ,j}) = 2.01 + 0.036[\ln(X_j)] + 0.004[\ln(X_j)-\xi_1]_+^3 - 0.202[\ln(X_j)-\xi_6]_+^3$,

where ξ_1 = ln(0.004 ppm), ξ_2 = ln(0.040 ppm), ξ_3 = ln(0.513 ppm), ξ_4 = ln(1.05 ppm), ξ_5 = ln(2.37 ppm); ξ_6 = ln(13.8 ppm). These models are shown in Figures 3.2A-E along with the corresponding 95% confidence intervals and the individual observations for the 326 subjects.

3.4.2. Effects of Covariates

Effects of covariates, determined under Model (2) after adjustment for benzene exposure, are summarized in Table 3.2. Age and/or gender were important explanatory variables for all 5 metabolites, with males and older subjects typically having lower metabolite levels. Smokers had significantly higher levels of CA and HQ while lean subjects (lower BMI values) had significantly higher levels of CA. Co-exposure to toluene was not significant in any of the models. Likewise, alcohol consumption was not a significant predictor of any benzene metabolite, either all subjects (n = 326 including 95 current drinkers) or in male subjects (n = 118 including 88 current drinkers).

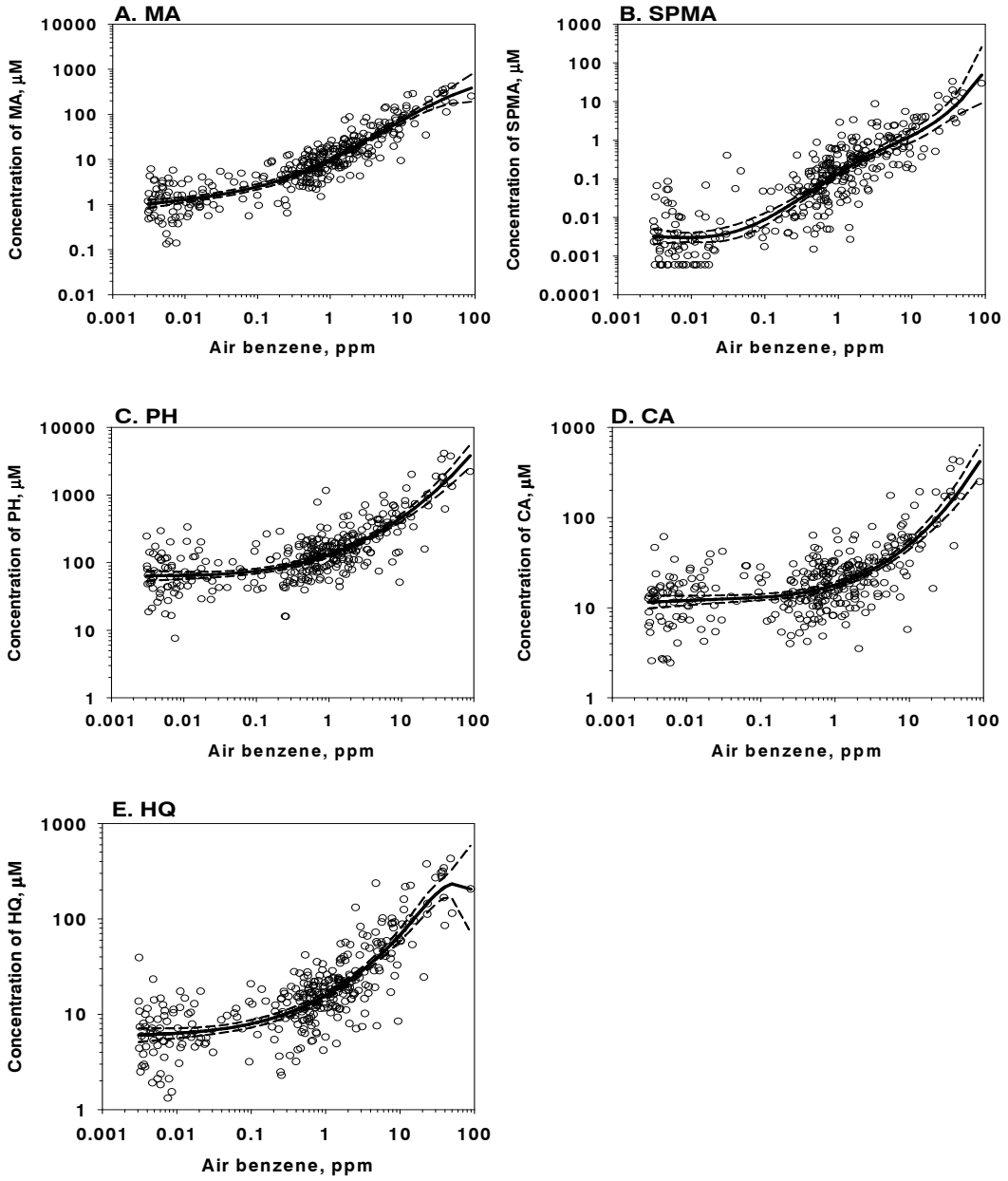

Figure 3.2. Basis functions of natural spline models fitting the 326 observations.
Legends: MA, *E,E*-muconic acid; SPMA, *S*-phenylmercapturic acid; PH, phenol; CA, catechol; HQ, hydroquinone; open circle, observation; solid line, expected mean trends from the model; short dash, upper or lower bound of 95% confidence limit for expected means of dependant variable

Table 3.2. Effects of significant covariates on metabolite levels (after adjustment for benzene exposure).

Metabolite	adj.R^2	Covariate	Parameter estimate	*p*-value
MA	0.814	Intercept	1.12	<.0001
		Age	-0.019	0.001
		Sex (male)	-0.273	0.013
		BMI	0.012	0.332
		Smoking	0.109	0.345
SPMA	0.741	Intercept	-6.19	<.0001
		Age	-0.015	0.136
		Sex (male)	-0.447	0.034
		BMI	-0.016	0.478
		Smoking	0.203	0.360
PH	0.605	Intercept	4.21	<.0001
		Age	-0.011	0.025
		Sex (male)	-0.209	0.032
		BMI	-0.003	0.773
		Smoking	0.042	0.684
CA	0.504	Intercept	2.65	<.0001
		Age	-0.002	0.716
		Sex (male)	-0.257	0.007
		BMI	-0.021	0.052
		Smoking	0.329	0.001
HQ	0.690	Intercept	1.95	<.0001
		Age	-0.011	0.015
		Sex (male)	-0.209	0.026
		BMI	-0.013	0.204
		Smoking	0.356	<0.001

Legend: MA, *E, E,*-Muconic acid; SPMA, *S*-phenylmercapturic acid; PH, phenol; CA, catechol; HQ, hydroquinone; adj.R^2, adjusted R^2 of final model; 95% CI 95% confidence interval of parameter; age, centered around the mean value of 30.2 y; gender, (female, reference); BMI, body mass index, centered around the mean value of 22.5 kg/m^2; smoking (nonsmoker, reference).

3.4.3. Molar Fractions and Dose-specific Metabolism

The background-adjusted urine concentration of m^{th} benzene metabolite in the j^{th} subject, i.e., $(\hat{Y}_{m,j|X_j} - Y_{m,b})$, increased monotonically with benzene exposure from 0.03 to 88.9 ppm, as shown in Figure 3.3A. The corresponding molar fractions, i.e., $\frac{(\hat{Y}_{m,j|X_j} - Y_{m,b})}{\sum_{m=1}^{5}(\hat{Y}_{m,j|X_j} - Y_{m,b})}$ are plotted versus benzene exposure in Figure 3.3B.

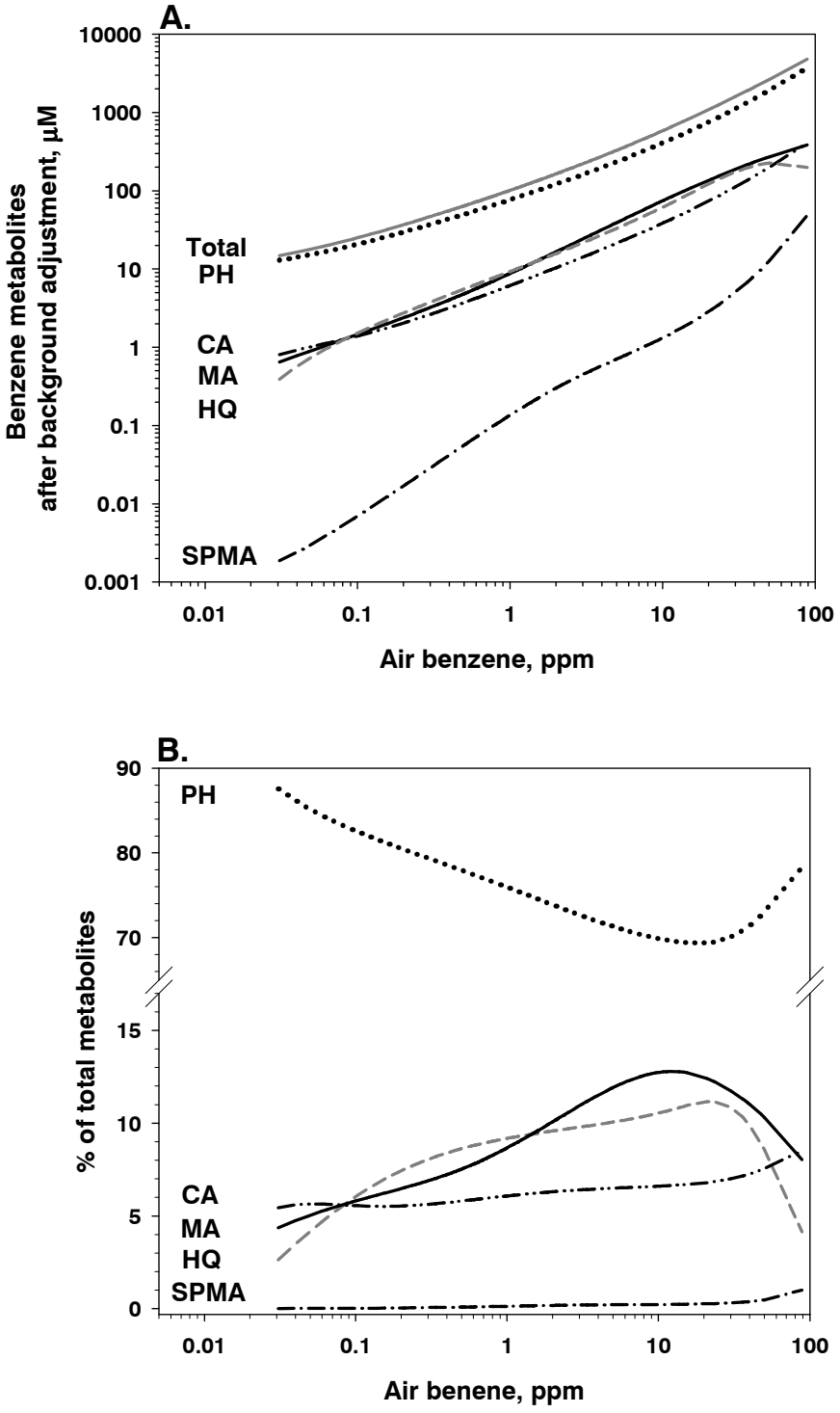

Figure 3.3. Dose-dependent production of benzene metabolites. Expected mean trends from natural spline models applied to subjects with benzene exposures between 0.03 and 88.9 ppm (n = 267). (A) Background-adjusted levels of benzene metabolites. (B) Molar fractions of benzene metabolites.

Molar fractions for MA and HQ increased 3-fold (4% to 12%) and 4-fold (3% to 11%), respectively, with benzene exposure from 0.03 to 20 ppm, and then decreased with benzene exposure above 20 ppm. The molar fraction of PH showed the opposite behavior, with reduction from about 88% to 68%, for benzene exposures between 0.03 and 20 ppm, followed by increases above 20 ppm. Molar fractions for CA and SPMA remained fairly constant, at about 6% and <1%, respectively, over the whole range of benzene levels, with some upwards curvature above 20 ppm.

As shown in Figure 3.4, dose-specific metabolite levels [values of $(\hat{Y}_{m,j|X_j} - Y_{m,b}) / X_j$], decreased with increasing benzene exposure for all but the minor product, SPMA. At air concentrations above 20 ppm, shifts in dose-specific metabolism are apparent, with decreasing values for MA and HQ and increasing values for PH, CA and SPMA. Uncertainties in model predictions of dose-specific metabolite levels are indicated in Figure 3.4 by iteration-trajectory plots, interquartile ranges, and 95% confidence intervals from the 500 realizations of bootstrap resampling. Although the 95% confidence intervals tended to be large for PH, HQ and CA at low benzene exposures (< 0.1 ppm), interquartile ranges were very modest for all metabolites over the entire range of predicted benzene exposures (0.03 – 88.9 ppm). Also, the median values from bootstrap analyses were very close to predictions from the NS models applied to the original 386 subjects in our study.

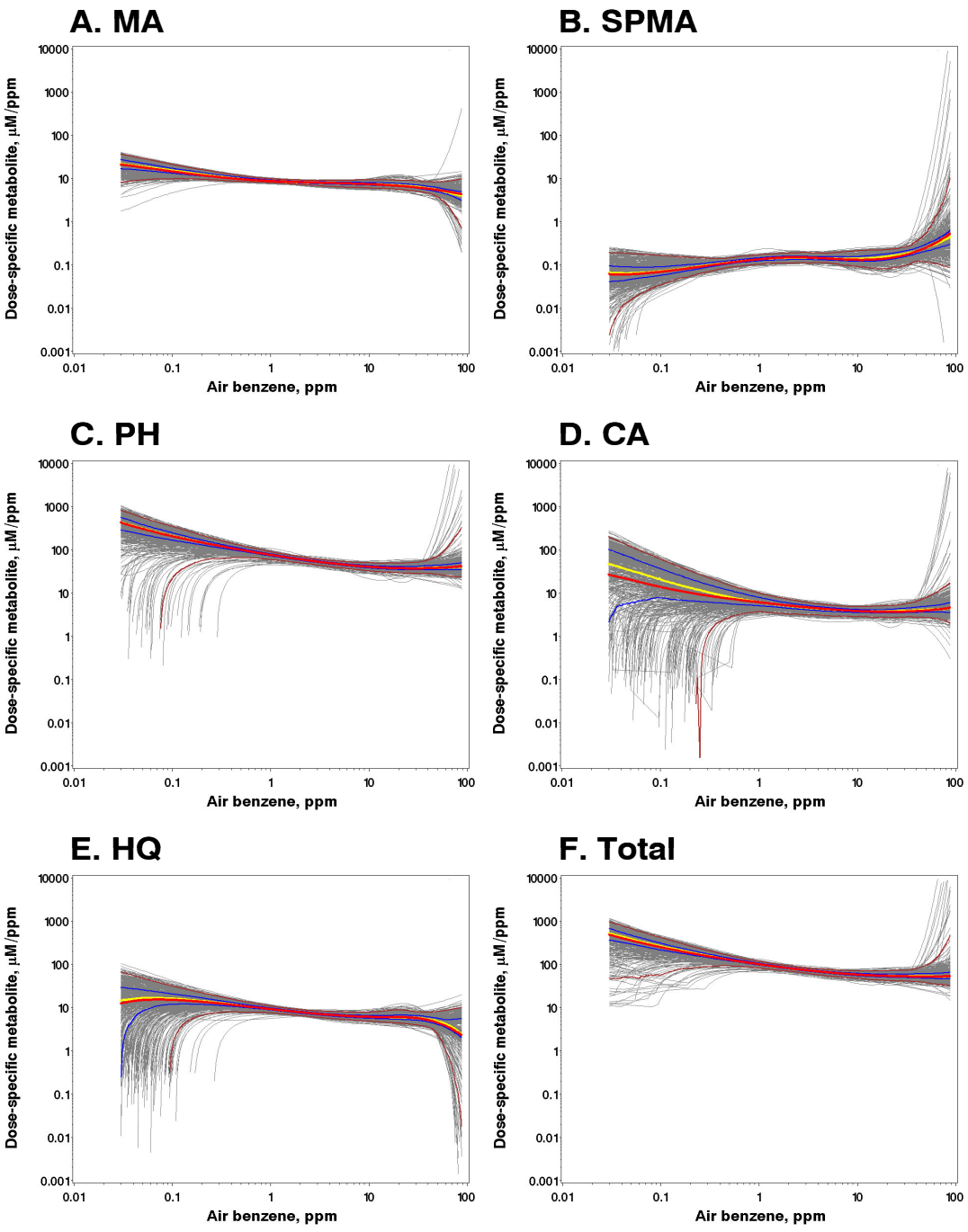

Figure 3.4. Dose-specific levels (µM/ppm) of benzene metabolites, predicted by natural spline models, for benzene exposures between 0.03 and 88.9 ppm. Expected mean trends are indicated by red curves. Yellow curves are the corresponding 50th percentiles of the sampling distributions of expected mean trends from 500 iterations of bootstrap resampling. The corresponding interquartile ranges (blue curves) and 95% confidence intervals (brown curves) of expected mean trends from bootstrap resampling are shown along with the individual iteration-trajectories (grey curves).

3.5. Discussion

This study of 386 workers in Tianjin, China represents the most extensive set of measurements reported to date for paired air and urine samples from benzene-exposed workers and matched controls. We previously published the empiric relationships between urinary metabolite levels and benzene exposure, based upon groups of these same subjects who had been aggregated by their exposure levels (Kim *et al.*, 2006b). Those preliminary analyses defined crude shapes of the exposure-biomarker relationships, which showed evidence of background levels of phenolic metabolites and MA (from dietary sources and smoking) and also of partial saturation of metabolic enzymes. However, the preliminary analyses did not permit expected metabolite levels to be predicted at given air concentrations of benzene, nor did they allow effects of age, gender and other covariates to be estimated, after adjusting for benzene exposure. In the current study, we found that GLM+NS models were ideal for characterizing the continuous relationships between metabolite levels and benzene exposures (Figure 3.2) and for testing effects of demographic factors (Table 3.2). Moreover, using GLM+NS models, we can avoid problems that have plagued generalized additive models that apply backfitting algorithms (Dominici *et al.*, 2002; Ramsay *et al.*, 2003; He *et al.*, 2006).

Although various spline regression models have been applied to investigate covariates in time-to-health-effects or survival analyses (Durrleman and Simon, 1989; Hess, 1994; Herndon and Harrell, 1995; Heinzl and Kaider, 1997; Heuer, 1997; Little *et al.*, 2000; Royston, 2000; Zhang *et al.*, 2000; Dominici *et al.*, 2002; Jemal *et al.*, 2002; Binquet *et al.*, 2003; European Collaborative Study, 2003; HEI, 2003; Samoli *et al.*, 2003; Vogt *et al.*, 2003; Mar *et al.*, 2005; Samoli *et al.*, 2005), we are unaware of any such applications involving

human metabolism or exposure-biomarker relationships. Since GLM+NS models are flexible and robust, they offer great promise as tools for investigating human metabolites and other biomarkers of environmental toxicants.

We used GLM+NS models to test for effects upon metabolite levels of several demographic factors (Table 3.2). Of the covariates considered, only age and/or gender consistently showed significant effects among the 5 metabolites, after adjustment for benzene exposure. Male subjects had exposure-adjusted metabolite levels that were about 20% lower than those of females. (For example, males had $100\times\left(1-e^{-0.209}\right)=18.9\%$ less HQ than females at a given exposure level). Likewise, levels of 4 metabolites (MA, SPMA, PH, and HQ) diminished with age, at rates between 1.1 and 1.9 percent per year of life (Table 3.2). This range is the same as that reported for albumin adducts of benzene oxide and 1,4-benzoquinone in a different sample of Chinese workers (Rappaport *et al.*, 2002). Although we expected that co-exposure to toluene (a competitive inhibitor of benzene for CYP metabolism) would affect metabolite levels, this was not the case, possibly due to the relatively low toluene concentrations in our study (median, 3.29 ppm ; 10^{th}–90^{th} percentile range, 0.012– 21.9 ppm n =326).

After adjusting for benzene exposure, smoking subjects had about 40% higher levels of HQ and CA than nonsmokers (Table 3.2). Since significant smoking effects were not observed for MA, SPMA and PH, we attribute this result to the uptake of HQ and CA *per se* from cigarette smoke (Hecht *et al.*, 1981; IARC., 1986; McDonald *et al.*, 2001; McCue *et al.*, 2003). To quantify the contributions of HQ and CA derived per cigarette, we regressed the logged levels of HQ and CA on self-reported smoking frequencies in 131 control subjects, who provided this information. This resulted in the following relationships:

ln(HQ, μM) = 1.79 + 0.021(cig./d) ($p < 0.01$) and ln(CA, μM) = 2.41 + 0.011(cig./d) ($p = 0.19$). Based upon these models, smoking 20 cigarettes would result in 52% more HQ [i.e., $100\times(1 - e^{(0.021\times20)})\%$] and 20% more CA than observed in nonsmoking control subjects.

Although background-adjusted levels of all metabolites increased monotonically with benzene exposures up to about 30 ppm (Figure 3.3A), the molar fractions for PH, HQ and MA changed continuously with increasing air concentrations (Figure 3.3B) while those for CA and SPMA remained relatively constant. This indicates that the competing CYP-mediated pathways (Figure 3.1) were sensitive to the air levels of benzene inhaled by these subjects. Below 20 ppm, molar fractions of HQ and MA increased with exposure, while those of PH decreased with exposure; above 20 ppm the opposite behavior was observed. Since production of HQ and MA was preferred to that of PH (below 20 ppm), we infer that PH and oxepin were either higher-affinity substrates than benzene for the particular CYP enzymes or were more accessible to these enzymes. This conjecture is supported by studies showing that K_M values for CYP-mediated metabolism of PH and oxepin were smaller than those of benzene in tissues from humans and/or animals (Medinsky *et al.*, 1989; Schlosser *et al.*, 1993; Snyder *et al.*, 1993; Seaton *et al.*, 1994). Above 20 ppm, the second CYP oxidation steps, leading to HQ and MA, appear to have become increasingly saturated; this led to the buildup of PH and, to lesser extents, of CA and SPMA (other products of a single CYP-oxidation step) (Henderson *et al.*, 2005a).

Using mean trends from the NS models (Figure 3.4) we investigated dose-specific levels (μM/ppm) of the 5 benzene metabolites and their sum (total metabolites). The spaghetti plots from the 500 bootstrap realizations were dense along the observed mean trends, and interquartile ranges were relatively small. Wide 95% confidence bands were observed below

about 0.1 ppm, due to the large relative errors from background subtraction in this region, particularly for PH, HQ and CA, which have important dietary and endogenous sources (McDonald *et al.*, 2001; National Library of Medicine, 2003; EPA, 2006). However, given the narrow interquartile ranges, our conclusions regarding the mean trends should be reasonable. Overall, expected median values of dose-specific levels of total metabolites decreased about 9-fold between 0.03 and 88.9 ppm of benzene. For benzene exposures below 20 ppm, the decreasing trends were more pronounced for PH and CA (7 – 11-fold) than for HQ and MA (2 – 3-fold), reflecting the apparent preference for metabolism of the latter metabolites at low exposures (described above). At benzene exposures above 20 ppm, the trends accelerate downwards for MA and HQ and turn upwards for PH and CA, consistent with results from previous investigations of workers heavily exposed to benzene (Rothman *et al.*, 1998; Waidyanatha *et al.*, 2001; Waidyanatha *et al.*, 2004). The mean trend for the minor product, SPMA, increased over the observed range of exposures and, therefore, displayed completely different behavior than those of the major metabolites.

Interestingly, about 90% of the reductions in dose-specific metabolite levels occurred below about 3 ppm for each major product. This behavior was unexpected, given current toxicokinetic models which indicate that saturable benzene metabolism should not be observed below about 3 – 10 ppm in humans (Travis *et al.*, 1990; Rappaport *et al.*, 2005). Thus, our results suggest that current toxicokinetic models for benzene are not accurate for air concentrations below 3 ppm.

It is also important to point out that human health risks associated with benzene exposure are based upon linear extrapolation from epidemiology studies involving workers exposed, on average, to air concentrations of tens to hundreds of ppm (Hayes *et al.*, 1997;

Savitz and Andrews, 1997). Our results indicate that persons exposed to air concentrations less than 0.1 ppm metabolize benzene about 9-times more efficiently that such heavily exposed workers (see Figure 3.4F). Since the toxic effects of benzene are thought to result from metabolism, this suggests that the health risks associated with low and very low benzene exposures can be considerably greater than those currently predicted from occupational studies.

CHAPTER 4.

GENETIC POLYMORPHISMS AND BENZENE METABOLISM IN HUMANS EXPOSED TO A WIDE RANGE OF AIR CONCENTRATIONS

[Kim S, Lan Q, Waidyanatha S, Johnson AB, Vermeulen R, Smith MT, et al. Pharmacogenet Genomics 2006 (submitted)]

4.1. Abstract

Using generalized linear models with natural-spline smoothing functions, we detected effects of particular metabolizing genes and gene-environment interactions on levels of benzene metabolites in 250 benzene-exposed and 136 control workers in Tianjin, China (for all subjects the median exposure was 0.694 ppm and the 10^{th} and 90^{th} percentiles were 0.006 and 7.79 ppm, respectively). We investigated 5 urinary metabolites [*E, E*-muconic acid (MA), *S*-phenylmercapturic acid (SPMA), phenol (PH), catechol (CA) and hydroquinone (HQ)] and 9 SNPs of metabolizing genes [cytochrome P450 2E1 (CYP2E1), NAD(P)H: quinone oxidoreductase (NQO1), microsomal epoxide hydrolase (EPHX1), glutathione-*S*-transferases (GSTT1, GSTM1 and GSTP1) and myeloperoxidase (MPO)]. After adjusting for covariates, including gender, age and smoking status, *NQO1*2* affected all five metabolites, *CYP2E1* (-1054C>T) affected all metabolites except CA, *EPHX1* (Ex3-28T>C

or Ex4+52A>G) affected CA and SPMA, and GSTT1 and GSTM1 affected SPMA. Significant interactions were also detected between benzene exposure and all four genes and between smoking status and NQO1 and EPHX1. 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 SPMA, and provide additional evidence that SNPs of NQO1, CYP2E1, and EPHX1 are functional in humans exposed to benzene.

4.2. Introduction

Benzene is an important industrial chemical that is ubiquitous in the environment due to vaporization from petroleum products and combustion of hydrocarbons (IARC, 1982; Wallace, 1996; ATSDR, 1997). Occupational exposures to benzene can cause blood disorders, including aplastic anemia, myelodysplastic syndrome, and acute myelogenous leukemia (Aksoy, 1985; Yin *et al.*, 1989; Rothman *et al.*, 1996a; Savitz and Andrews, 1997). Significant decreases in the numbers of white blood cells and platelets have been reported in workers exposed to less than one ppm of benzene (Lan *et al.*, 2004). 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 (Ross, 2000) (Snyder, 2000a; Snyder, 2002).

Since the pioneering work of Parke and Williams' (Parke and Williams, 1953b; Parke and Williams, 1953a), the metabolism of benzene has been extensively investigated (reviewed in (Ross, 2000) (Snyder, 2002)). The major metabolic pathways, shown in Figure 4.1, 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), as well as 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 oxepin followed by ring-opening), and 1,2- and 1,4-benzoquinone (BQ) (from spontaneous or peroxidase-mediated oxidation of CA and HQ, respectively).

As shown in Figure 4.1, 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) (Gilmour *et al.*, 1986; Snyder and Hedli, 1996; Witz *et al.*, 1996), microsomal epoxide hydrolase (EPHX) catalyzes the hydrolysis of BO to initiate the CA pathway (Penning and Sharp, 1990; Snyder and Hedli, 1996), various glutathione-S-transferases (GSTs) catalyze production of SPMA (Verdina *et al.*, 2001), and NAD(P)H: quinone oxidoreductase (NQO1) and peroxidases (notably myeloperoxidase (MPO)) are thought catalyze transformations between CA and HQ and the corresponding quinones (1,2-BQ and 1,4-BQ, respectively) (Snyder and Hedli, 1996; Smith, 1999; Snyder, 2002; Ross, 2005).

It has been speculated that polymorphic genes of the above enzymes predispose some individuals to benzene toxicity through metabolism (Seaton *et al.*, 1994; Rothman *et al.*, 1997; Nedelcheva *et al.*, 1999). 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 (Lan *et al.*, 2004). Yet, although GSTT1

polymorphisms have been shown to affect production of the minor metabolite SPMA (Sorensen *et al.*, 2003; Sorensen *et al.*, 2004; Qu *et al.*, 2005), 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.

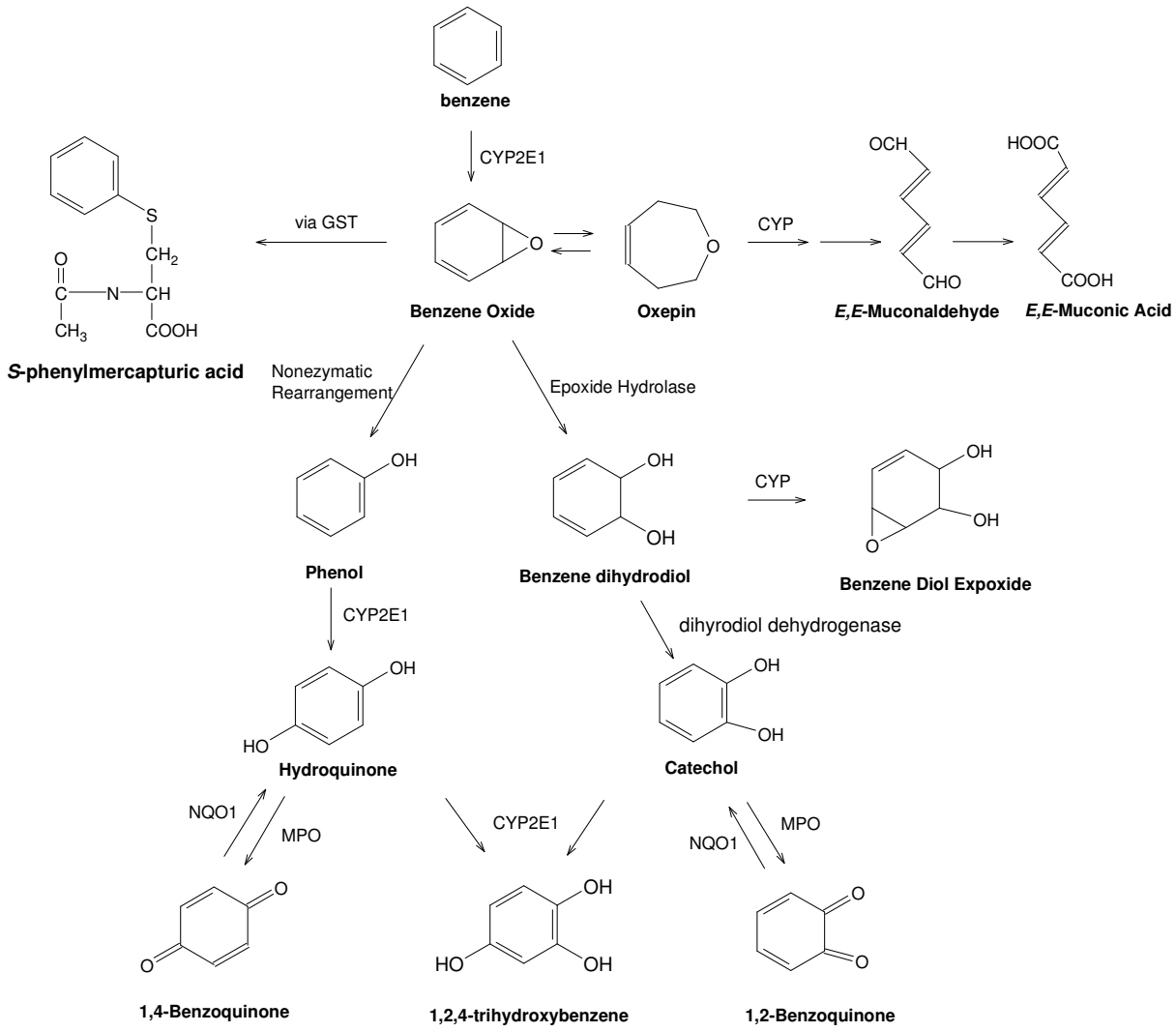

Figure 4.1. Simplified metabolic scheme for benzene showing major pathways and metabolizing genes.

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, as well as of 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 gender, smoking status, and body mass index (BMI) (Kim *et al.*, 2006b; Kim *et al.*, 2006a). Using generalized linear models (GLM) with natural spline (NS) smoothing functions, we were able to elucidate the effects of gender, BMI, and smoking status, after adjustment for benzene exposure (between 0.01 and 88.9 ppm) in a sample of 386 benzene-exposed and control workers in Tianjin, China (Kim *et al.*, 2006a). 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 GSTs on urinary levels of PH, MA, HQ, CA and SPMA in these workers.

4.3. Materials and Methods

4.3.1. Study Population, and Air and Biological Sample Collection

Exposed and control subjects were recruited with informed consent from two shoe-making factories and three clothes-manufacturing factories, respectively, in Tianjin, China as described in Lan *et al.* (Lan *et al.*, 2004), and benzene exposure and workplace characteristics have been previously described in detail (Lan *et al.*, 2004; Vermeulen *et al.*, 2004; Kim *et al.*, 2006b). After excluding 4 control subjects, who were missing values of metabolite and/or exposure measurements, the samples included 250 exposed subjects and 136 control subjects. Exposed and control subjects were frequency matched by gender. Table 4.1 shows summary statistics for the gender, age and smoking status of participants.

Methods of sampling air and urine were also previously described (Lan *et al.*, 2004; Vermeulen *et al.*, 2004; Kim *et al.*, 2006b). Briefly, personal full-shift air measurements were matched with post-shift urine samples from exposed and control workers. Of the 386 subjects in this analysis, 139 had repeated measurements of air and urine, making a total of 617 matched air/urine samples. Among subjects with repeated measurements, the median number of paired air and urine samples was three (range: 2 – 4). Information about height, weight, smoking status and other relevant factors were obtained by questionnaire (Lan *et al.*, 2004).

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

Table 4.1. Demographics, benzene exposure and other characteristics of study population (*n* = 386).

	Female		Male		All	
Occupational exposure*						
Controls	84	(61.8%)	52	(38.2%)	136	(100.0%)
Exposed	164	(65.6%)	86	(34.4%)	250	(100.0%)
Benzene exposure†						
Air benzene (ppm)	0.770	(0.006–7.74)	0.616	(0.006–9.21)	0.694	(0.006–7.79)
Age (y)†	31	(21–44)	24	(20–39)	28	(21–43)
BMI (kg/m^2)†	21.8	(18.7–27.0)	22.0	(18.4–26.5)	21.9	(18.7–26.9)
Current smoking status*						
Non-smoker	240	(81.4%)	55	(18.6%)	295	(100.0%)
Smoker	8	(8.8%)	83	(91.2%)	91	(100.0%)
Toluene exposure*						
Low (≤1.79 ppm)	130	(67.4%)	63	(32.6%)	193	(100.0%)
High (>1.79 ppm)	118	(61.1%)	75	(38.9%)	193	(100.0%)

* Number (percent). † Median (10^{th}–^{90}th percentiles)

4.3.2. Measurements of Air and Urinary Analytes

Measurements of analytes in air and urine were described previously (Vermeulen *et al.*, 2004; Kim *et al.*, 2006b; Kim *et al.*, 2006a). Briefly, benzene and toluene were measured in air using passive personal monitors (Organic Vapor Monitors, 3M, St. Paul, MN) followed by solvent desorption and gas chromatography (Vermeulen *et al.*, 2004). Air measurements of benzene and toluene were below limits of detection (LOD, nominally 0.2 ppm for benzene and 0.3 ppm for toluene) and were missing for all control subjects and for some exposed subjects (missing values, $n = 23$; measurements below the LOD for exposed subjects: $n = 70$ for benzene, $n = 67$ 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 (Kim *et al.*, 2006a). As summarized in Table 4.1, the median air level of benzene was 0.694 ppm, the 10^{th} percentile level was 0.006 ppm and the 90^{th} percentile was 7.79 ppm. The median air level of toluene was 1.79 ppm.

Urinary benzene was determined by gas chromatography-mass spectrometry (GC-MS) using head-space solid-phase microextraction according to Waidyanatha *et al.* (Waidyanatha *et al.*, 2001). 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.* (Waidyanatha *et al.*, 2004). 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 LOD/2 = 0.591 nM was imputed to these samples (Hornung and Reed, 1990).

4.3.3. Genotyping

We selected 9 single nucleotide polymorphisms (SNPs) in 7 genes coding for enzymes that presumably play a role in benzene metabolism, based on the evidence of functionality in experimental or human studies (described in the discussion section).

Table 4.2. Distributions of SNPs among subjects in the study.

Gene name SNP region SNP ID (Notes)	Genotype	No. subjects	(%)	Presumed phenotype	Reference
CYP2E1	C/C	239	(62.1)	Active	(Marchand *et al.*, 1999)
-1054C>T	C/T	127	(33)	Less active	
Rs2031920	T/T	19	(4.9)	Least active	
(ascribed to *RsaI*)					
NQO1	C/C	105	(27.3)	Active	(Ross *et al.*, 2000)
Ex6+40C>T	C/T	173	(44.9)	Less active	
Rs1800566	T/T	107	(27.8)	Inactive	
*(NQO1*2)*					
NQO1	C/C	359	(93.7)	Active	(Pan *et al.*, 1995)
Ex4-3C>T	C/T	24	(6.3)	Less active	(Eguchi-Ishimae *et al.*, 2005)
Rs4986998	T/T	0	(0.0)	Least active	
*(NQO1*3)*					
MPO	G/G	297	(77.3)	Active	(Williams, 2001)
-642G>A	G/A	76	(19.8)	Less active	
Rs2333227	A/A	11	(2.9)	Least active	
GSTM1	+/+	28	(7.4)	Conjugator	(Hsieh *et al.*, 1999)
del{GSTM1}	+/-	141	(37.2)		
n/a	-/-	210	(55.4)	Null	
GSTT1	+/+	33	(8.6)	Conjugator	(Hsieh *et al.*, 1999)
del{GSTT1}	+/-	187	(48.8)		
n/a	-/-	163	(42.6)	Null	
GSTP1	A/A	224	(58.6)	Conjugator	(Morgan and Smith, 2002)
Ex5-24A>G	A/G	142	(37.2)		
Rs947894	G/G	16	(4.2)	Less active	
EPHX1	T/T	143	(38)	Normal	(Jourenkova-Mironova *et al.*, 2000)
Ex3-28T>C	T/C	176	(46.8)		(Lodovici *et al.*, 2004)
Rs1051740	C/C	57	(15.2)	Slow metabolizer	
(Y113H)					
EPHX1	A/A	302	(79.9)	Normal	(Jourenkova-Mironova *et al.*, 2000)
Ex4+52A>G	A/G	71	(18.8)		(Lodovici *et al.*, 2004)
Rs2234922	G/G	5	(1.3)	Rapid metabolizer	
(H139R)					

As summarized in Table 4.2, the following SNPs were chosen: *CYP2E1* (-1054C>T, contributes to *CYP2E1*5B*, aka -1053), two alleles of *NQO1* [*NQO1*2* (Ex6+640C>T) bearing P187S and *NQO1*3* (Ex4-3C>T) bearing R139W], *MPO* (-642G>A, aka -463 promoter variant), *GSTM1*, *GSTT1*, and *GSTP1* (Ex5-24A>G) bearing I105V, and two alleles of *EPHX1* (Ex3-28T>C and Ex4+52A>G) bearing Y113H and H139R, respectively. Genotyping was performed with an ABI 7900HT detection system using TaqMan end point reads as described on the website, http://snp500cancer.nci.nih.gov (Packer *et al.*, 2004). The numbers of subjects with each SNP of the various metabolizing genes are summarized in Table 4.2. Quality control procedures have been described previously (Lan *et al.*, 2005). In brief, blind replicate samples were randomly interspersed throughout the study sample plates and showed intrasubject agreement >99% for all genotype assays.

4.3.4. Statistical Analyses

Relationships between levels of the urinary metabolites and the corresponding air concentrations of benzene were examined using GLM+NS models, as previously described (Kim *et al.*, 2006a). 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) (Burnham *et al.*, 2002). (The candidate knots in the 5-knot model were 0.001, 0.009, 0.512, 1.54 and 11.3 ppm of benzene in air, corresponding to the 5^{th}, 27.5^{th}, 50^{th}, 72.5^{th} and 95^{th} percentiles (Harrell, 2001), respectively). To avoid overparameterization, non-significant knots for each exposure-metabolite relationship were removed by stepwise elimination using a value of $p < 0.10$ for retention (PROC REG of SAS) (Kim *et al.*, 2006a). For subjects 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 SNPs and their interactions with benzene exposure and smoking status, after adjusting for the following covariates: gender (0, female; 1, male), age (centered around the estimated mean value of 29.8 y, n = 386), smoking status (0, nonsmoker; 1, smoker), BMI (centered around the estimated mean value of 22.5 kg/m^2, n = 384). We also investigated effects of toluene exposure (0, low exposure; 1, high exposure; median as a cut-off point, 1.79 ppm) on levels of each metabolite.

Potential effects of SNPs 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 biologically plausible of explanatory variables (Burnham *et al.*, 2002). Modeling was performed using PROC GLMSELECT of SAS/STAT with the selection/stop option of AICc (Burnham *et al.*, 2002; SAS Institute., 2005).

In coding each SNP, the homozygous wild-type was defined as the reference group. Data for *EPHX1* (Ex4+52A>G), variant homozygotes and heterozygotes were combined in the analysis because the former contained only five subjects (1.3%) (Lan *et al.*, 2005). Tests for Hardy-Weinberg equilibrium (HWE) among subjects were conducted based on 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* (-642G>A) (p = 0.04). The quality control data were rechecked and the precision of genotyping for this SNP 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 SNPs in the final models.

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

4.4. Results

4.4.1. GLM+NS Models and Covariate Effects

The following NS smoothing functions were used for the 5 benzene metabolites:

MA: $E[\ln(Y_{MA,j}) \mid \ln(X_j)] = 0.754 + 0.127[\ln(X_j)] + 0.005[\ln(X_j)\text{-}\xi_1]_+^3 - 0.019[\ln(X_j)\text{-}\xi_3]_+^3$,

SPMA: $E[\ln(Y_{SPMA,j}) \mid \ln(X_j)] = \text{-}6.65 - 0.119[\ln(X_j)] + 0.030[\ln(X_j)\text{-}\xi_1]_+^3 - 0.040[\ln(X_j)\text{-}\xi_2]_+^3$,

PH: $E[\ln(Y_{PH,j}) \mid \ln(X_j)] = 4.38 + 0.053[\ln(X_j)] + 0.005[\ln(X_j)\text{-}\xi_2]_+^3$,

CA: $E[\ln(Y_{CA,j}) \mid \ln(X_j)] = 3.02 + 0.098[\ln(X_j)] - 0.005[\ln(X_j)\text{-}\xi_1]_+^3 + 0.011[\ln(X_j)\text{-}\xi_2]_+^3$, and

HQ: $E[\ln(Y_{HQ,j}) \mid \ln(X_j)] = 1.72 - 0.016[\ln(X_j)] + 0.004[\ln(X_j)\text{-}\xi_1]_+^3 - 0.123[\ln(X_j)\text{-}\xi_5]_+^3$.

where $E[\ln(Y_{m,j})|\ln(X_j)]$ is the conditional mean value of $\ln(Y_{m,j})$ representing the natural-log transform of the level of the m^{th} metabolite level in the j^{th} subject at $\ln(X_j)$, the corresponding (logged) air concentration of benzene (ppm), and ξ_i is the location of the i^{th} knot (in log-scale of benzene exposure): ξ_1 = ln(0.001 ppm), ξ_2 = ln(0.009 ppm), ξ_3 =

ln(0.512 ppm), ξ_4 = ln(1.54 ppm), ξ_5 = ln(11.3 ppm). The 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 4.3 – 4.7 for the 5 benzene metabolites. Referring to the non-genetic effects, results are similar to those reported previously without adjustment for SNPs (Kim *et al.*, 2006a). Female subjects had higher levels of MA, PH, CA and HQ than males ($p < 0.05$) and younger subjects (below 30 yr) had higher levels of MA, PH and HQ than older subjects. 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 co-exposure to toluene.

Table 4.3. Parameter estimates for the final model of *E,E*-muconic acid (MA). [The dependent variable was the natural logarithm of the MA concentration, (μM); n = 382, R^2=85.0%]

Independent variable	Description	Parm. est.	Std. err.	*p*-value	Cumulative ΔR^2 (%)
Intercept		**0.937**	0.197	<.0001	
Age	Centered at mean (29.8 yr)	**-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)×*NQO1*2*	ln(X_j) × **1/*2,* less active	**-0.076**	0.027	0.005	0.37
	ln(X_j) × **2/*2*, least active	**-0.070**	0.029	0.016	
NQO1*2	**1/*2*, less active	**-0.174**	0.099	0.081	0.38
	**2/*2*, least active	**-0.184**	0.110	0.094	
ln(X_j) × *CYP2E1*	ln(X_j) × C/T, less active	0.001	0.024	0.955	1.00
	ln(X_j) × T/T, least active	**-0.150**	0.042	<0.001	
CYP2E1 (-1054C>T)	C/T, less active	0.037	0.087	0.667	1.01
	T/T, least active	**-0.406**	0.191	0.034	

Legend: ln(X_j) represents the natural logarithm of the air benzene concentration (ppm) in the j^{th} subject.

Table 4.4. Parameter estimates for the final model of *S*-phenyl mercapturic acid (SPMA). [The dependent variable was the natural logarithm of the SPMA concentration, (μM); n = 365, R^2=82.0%]

Independent variable	Description	Parm. est.	Std. err.	*p*-value	Cumulative ΔR^2 (%)
Intercept		**-4.811**	0.685	<.0001	
Age	Centered at mean (29.8 yr)	-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	0.31
	-/-, null	**-0.590**	0.248	0.018	
ln(X_j) × *EPHX1*	ln(X_j) × (A/G or G/G, faster)	**0.101**	0.051	0.049	0.55
EPHX1 (Ex4+52A>G)	A/G or G/G, faster	0.234	0.187	0.213	0.56
ln(X_j)×*NQO1*2*	ln(X_j) × **1/*2*, less active	**-0.149**	0.049	0.003	0.98
	ln(X_j) × **2/*2*, least active	-0.069	0.053	0.199	
ln(X_j) × *CYP2E1*	ln(X_j) × C/T, less active	**0.114**	0.045	0.011	1.44
	ln(X_j) × T/T, least active	-0.038	0.076	0.617	
CYP2E1 (-1054C>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	1.94
	Smoker × **2/*2*, least active	**1.041**	0.413	0.012	
NQO1*2	**1/*2*, less active	**-0.654**	0.203	0.001	2.01
	**2/*2*, least active	**-0.646**	0.227	0.005	
ln(X_j) × *GSTT1*	ln(X_j) × +/-, less active	-0.035	0.069	0.614	2.63
	ln(X_j) × -/-, null	**-0.143**	0.070	0.041	
GSTT1	+/-, less active	**-0.592**	0.251	0.019	4.86
	-/-, null	**-1.444**	0.258	<.0001	

Legend: ln(X_j) represents the natural logarithm of the air benzene concentration (ppm) in the j^{th} subject.

Table 4.5. Parameter estimates for the final model of phenol (PH). [The dependent variable was the natural logarithm of the PH concentration, (μM); n = 382, R^2=64.8%]

Independent variable	Description	Parm. est.	Std. err.	*p*-value	Cumulative ΔR^2 (%)
Intercept		**4.645**	0.100	<.0001	
Age	Centered at mean (29.8 yr)	**-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	**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) × *CYP2E1*	ln(X_j) × C/T, less active	0.026	0.022	0.230	2.75
	ln(X_j) × T/T, least active	**-0.141**	0.038	<0.001	
CYP2E1 (-1054C>T)	C/T, less active	0.037	0.079	0.640	3.08
	T/T, least active	**-0.613**	0.174	0.001	

Legend: ln(X_j) represents the natural logarithm of the air benzene concentration (ppm) in the j^{th} subject.

Table 4.6. Parameter estimates for the final model of catechol (CA). [The dependent variable was the natural logarithm of the CA concentration, (μM); n = 370, R^2=57.2%]

Independent variable	Description	Parm. est.	Std. err.	*p*-value	Cumulative ΔR^2 (%)
Intercept		**3.072**	0.285	<.0001	
Age	Centered at mean (29.8 yr)	<0.001	0.004	0.997	
Sex	Male	**-0.272**	0.087	0.002	
Smoking	Smoker	**0.567**	0.127	<.0001	
BMI	Centered at mean (22.5 kg/m^2)	-0.007	0.009	0.448	
ln(X_j)×*NQO1*2*	ln(X_j) × **1/*2*, less active	-0.037	0.023	0.113	0.67
	ln(X_j) × **2/*2*, least active	**-0.058**	0.025	0.021	
NQO1*2	**1/*2*, less active	**-0.186**	0.089	0.036	0.96
	**2/*2*, least active	**-0.237**	0.097	0.015	
EPHX1 (Ex4+52A>G)	A/G or G/G, faster	**0.143**	0.076	0.061	1.43
ln(X_j) ×*EPHX1*	ln(X_j) × (T/C, slow)	**-0.039**	0.021	0.063	2.22
(Ex3-28T>C)	ln(X_j) × (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 (Ex3-28T>C)	T/C, slow	-0.019	0.086	0.827	3.36
	C/C, slower	0.096	0.118	0.420	

Legend: ln(X_j) represents the natural logarithm of the air benzene concentration (ppm) in the j^{th} subject.

Table 4.7. Parameter estimates for the final model of hydroquinone (HQ). [The dependent variable was the natural logarithm of the HQ concentration, (μM); n = 382, R^2=72.6%]

Independent variable	Description	Parm. est.	Std. err.	*p*-value	Cumulative ΔR^2 (%)
Intercept		**1.758**	0.118	<.0001	
Age	Centered at mean (29.8 yr)	**-0.011**	0.004	0.006	
Sex	Male	**-0.302**	0.082	<0.001	
Smoking	Smoker	**0.473**	0.089	<.0001	
BMI	Centered at mean (22.5 kg/m^2)	-0.010	0.009	0.248	
NQO1*2	**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) × *CYP2E1*	ln(X_j) × C/T, less active	-0.008	0.020	0.676	1.25
	ln(X_j) × T/T, least active	**-0.125**	0.035	<0.001	
CYP2E1	C>T, C/T, less active	-0.036	0.072	0.617	1.77
	C>T, T/T, least active	**-0.657**	0.160	<.0001	

Legend: ln(X_j) represents the natural logarithm of the air benzene concentration (ppm) in the j^{th} subject.

4.4.2. Effects of Genetic Polymorphisms

After adjusting for exposure and covariates, the following SNPs 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* for all metabolites, *CYP2E1* for all metabolites except CA, *GSTT1* and *GSTM1* for SPMA, *EPHX1* (Ex4+52A>G) for SPMA and CA, and *EPHX1* (Ex3-28T>C) for CA. The interaction between *CYP2E1* and benzene exposure accentuated the effects of polymorphic forms of this gene on levels of MA, SPMA, PH and HQ among subjects exposed to higher benzene concentrations (Tables 4.3, 4.4, 5 and 4.7). For each of these metabolites, subjects having 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. 4.2A, indicate that homozygous variants produced appreciably less metabolite than heterozygotes or homozygous wild types at air concentrations greater than 0.1 ppm (Tukey's test, $p < 0.05$). Similar behaviors were observed for PH, where significant departure was observed above 0.2 ppm, and for MA above 2 ppm. For SPMA, subjects with both variant alleles of *CYP2E1* had the lowest metabolite levels at benzene concentrations between 0.02 and 88.9 ppm; however, the difference was not statistically significant ($p > 0.05$).

Subjects with at least one variant allele of *NQO1*2* had lower levels of all metabolites than homozygous wild types or heterozygotes (Tables 4.3 – 4.7). This gene was also found to interact strongly with either benzene exposure (for MA, SPMA, and CA) and/or smoking status (for PH and SPMA). These interactions with benzene exposure resulted in subjects with at least one variant allele of *NQO1*2* having lower levels of CA above 0.8 ppm ($p < 0.05$, Fig. 4.2B), lower levels of MA above 6 ppm ($p < 0.05$), and lower levels of HQ over

the entire range of exposure (significance, $p < 0.1$). Among nonsmokers, subjects with *NQO1*1/*1* produced more SPMA above 0.5 ppm (Fig. 4.2C, $p < 0.05$) and more PH over the entire range of exposure (Fig. 4.2D, $p < 0.01$) than subjects with *NQO1*2/*2*. However, among smokers, these effects were diminished (Fig. 4.2D and 2E). No effects were observed for *NQO1*3* 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 subjects (Table 4.4). A significant interaction was also observed between benzene exposure and *GSTT1* null subjects, such that these workers produced increasingly less SPMA at higher air concentrations (see Fig. 4.1E). No effect of polymorphic forms of *GSTP1* was observed on SPMA levels.

Subjects with variant alleles of *EPHX1* (Ex3-28T>C) had lower levels of CA, particularly among smokers, than homozygous wild types (see Table 4.6). Due to the interaction of *EPHX1* (Ex3-28T>C) with benzene exposure, this effect was accentuated among smokers at air concentrations above 0.8 ppm ($p < 0.05$) (Fig.4.1.F). Subjects with at least one variant allele of *EPHX1* (Ex4+52A>G) had higher CA levels than homozygous wild types ($p = 0.06$). Interestingly, subjects with variant *EPHX1* (Ex4+52A>G) also had higher levels of SPMA at benzene concentrations above 6 ppm ($p < 0.1$).

No effects were observed for polymorphic forms of *MPO*.

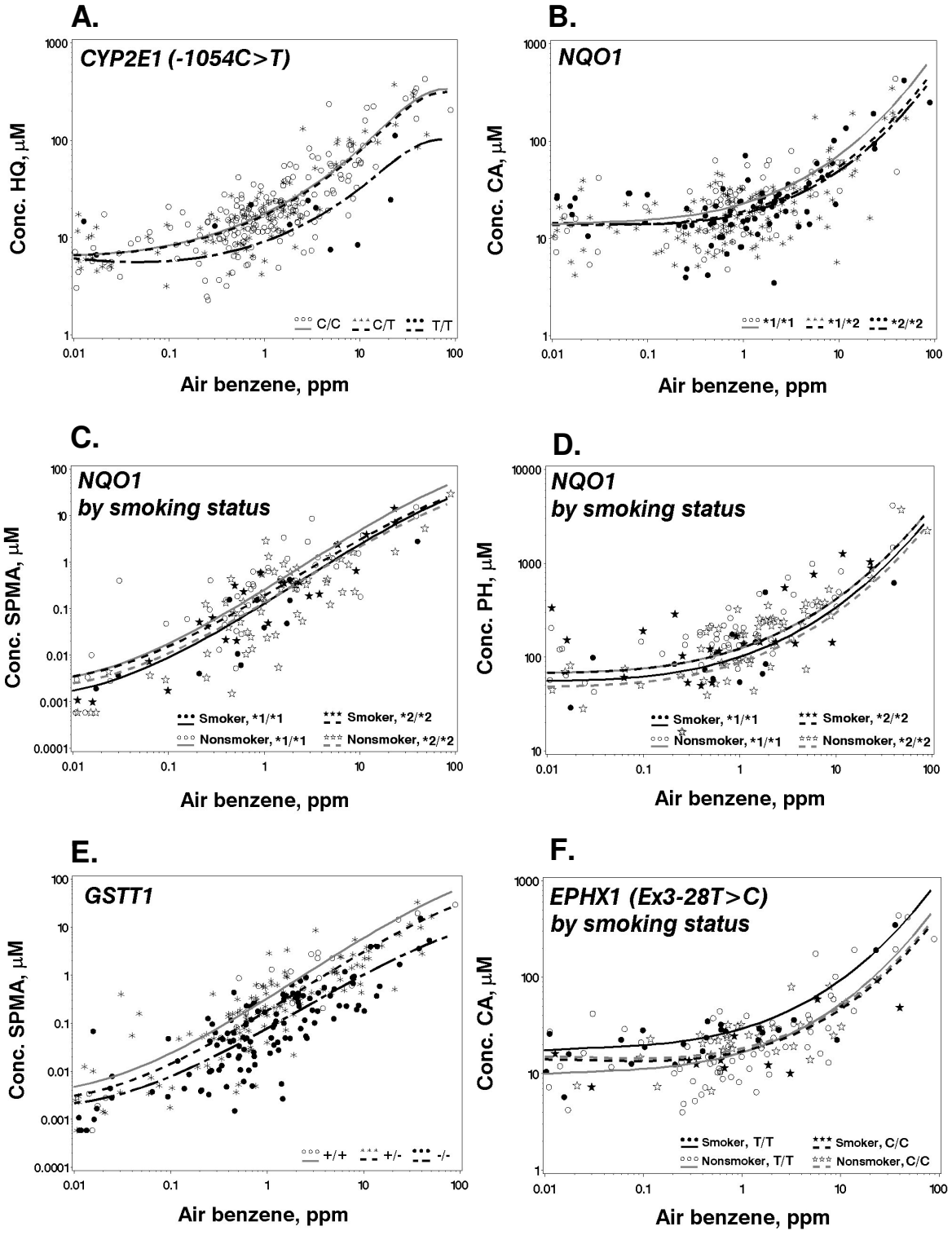

Figure 4.2. Effects of genetic polymorphisms on urinary metabolites of benzene in humans (representative plots). Each panel depicts effects of a particular SNP on levels of a given metabolite at versus the air concentration of benzene.

4.5. Discussion

Although the hematotoxicity of benzene was reported more than a century ago, the mechanism is not yet fully understood (Ross, 2000). It has been speculated that genetic and life-style factors can influence the toxic effects of benzene, but current evidence is far from conclusive (Ross, 2000; Snyder, 2000a; Snyder, 2002). Rothman *et al.* reported that heavily benzene-exposed workers who were rapid chlorzoxazone metabolizers (a measure of CYP2E1 phenotype) and also possessed variant *NQO1*2*, were at elevated risk of benzene-poisoning (Rothman *et al.*, 1997). More recently, Wan *et al.* (Wan *et al.*, 2002) reported increased benzene poisoning in workers with variant *NQO1*2* as well as in those with null-type *GSTT1* or *CYP2E1 DraI*, and Lan *et al.* (Lan *et al.*, 2004) found lower white blood cell counts in workers with variants of *NQO1*3* and *MPO* (-642G>A). Other studies of polymorphisms among benzene-exposed workers reported that subjects with variant *NQO1*2* had increased DNA single-strand breaks (Garte *et al.*, 2005) but decreased aneuploidy (Kim *et al.*, 2004) 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 (*DraI* or *RsaI*) (Kim *et al.*, 2004).

In the present study, we focused upon the effects of metabolizing genes on production of 5 prominent metabolites (MA, SPMA, PH, CA and HQ). These metabolites are not 'biological effect markers' (Wogan, 1992) *per se*, but rather reflect primary metabolism in the liver, which appears to be a necessary prelude to benzene-induced toxicity in target organs (Ross, 2000; Snyder, 2002). 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 ppm) (Rossi *et al.*, 1999;

Verdina *et al.*, 2001; Sorensen *et al.*, 2003; Sorensen *et al.*, 2004; Fustinoni *et al.*, 2005b), no measurements of air exposure (Hsieh *et al.*, 1999; Wan *et al.*, 2002; Avogbe *et al.*, 2005; Testa *et al.*, 2005), small numbers of subjects (Rossi *et al.*, 1999; Sorensen *et al.*, 2003; Kim *et al.*, 2004; Sorensen *et al.*, 2004), and difficulties in adjusting for covariates and nonlinear effects of exposure (Garte *et al.*, 2005; Qu *et al.*, 2005). Since benzene exposures were typically very low in prior studies, only SPMA and MA (metabolites with high specificity for benzene) tended to be measured, and the only consistent effect of any SNP was that of lower SPMA levels in subjects with *GSTT1 (null deletion)* (Sorensen *et al.*, 2003; Sorensen *et al.*, 2004; Qu *et al.*, 2005).

We detected several effects of SNPs on benzene metabolite patterns and interactions with benzene exposure that have not been reported previously (Tables 4.3 – 4.7). Our study had substantially more subjects (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.

Subjects with *NQO1*2* had lower levels of all 5 metabolites in our study. Since NQO1 catalyzes two- or four-election reductions of quinones (Nebert *et al.*, 2002) (Traver *et al.*, 1997; Siegel *et al.*, 1999; Ross *et al.*, 2000), it is reasonable that levels of CA and HQ would be lower in subjects with *NQO1*2* (less active), as we observed (Tables 4.6 and 4.7). When combined with evidence that less active forms of NQO1 are associated with benzene

poisoning and DNA damage (Rothman *et al.*, 1997; Wan *et al.*, 2002; Lan *et al.*, 2004; Garte *et al.*, 2005), 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 (Subrahmanyam *et al.*, 1990; Subrahmanyam *et al.*, 1991; McDonald *et al.*, 1994; Snyder and Hedli, 1996; Snyder, 2002). The fact that levels of the other three metabolites (MA, SPMA, and PH) were also lower among subjects with *NQO1*2*, suggests a more general role for NQO1. Furthermore, the interaction effects between *NQO1*2* and both benzene exposure and smoking status point to induction of NQO1 by reactive benzene metabolites or other reactive species (De Long *et al.*, 1987; Moran *et al.*, 1999; Ross, 2005). Such induction could come about via either the antioxidant or xenobiotic response element in the NQO1 promoter region (Ross, 1997; Smith, 1999; Jaiswal, 2000). We found that that *NQO1*2* but not *NQO1*3* was associated with lower levels of benzene metabolites and that SNPs of MPO did not affect metabolism. In contrast, we previously reported in the same study the presence of greater hematotoxicity in workers having the combination of *NQO1*3* (*C/T*, less active) and MPO (*A/A*, more active) (Lan *et al.*, 2004). These contrasting results 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).

We observed significant effects of *CYP2E1* variants on levels of MA, SPMA, PH, and HQ (Tables 4.3, 4.4, 5 and 4.7). Controversy has surrounded the relationship between the genotype and phenotype of CYP2E1, an important gene that metabolizes small molecules of toxicological interest, including benzene (Hayashi *et al.*, 1991; Lucas *et al.*, 1995; Carriere *et al.*, 1996; Kim *et al.*, 1996; Nedelcheva *et al.*, 1999; Wang *et al.*, 1999; Wormhoudt *et al.*,

1999; Carere *et al.*, 2002; Le Marchand *et al.*, 2002) (Ingelman-Sundberg *et al.*, 2006) (Seaton *et al.*, 1994; Seaton *et al.*, 1995; Bolt *et al.*, 2003). There is some evidence that a variant type *CYP2E1* (-1054C>T, also referred to as *Rsa*I-), is associated with decreased CYP2E1 activity *in vivo* (Carriere *et al.*, 1996; Powell *et al.*, 1998; Marchand *et al.*, 1999; Wang *et al.*, 1999; Choi *et al.*, 2003; Fustinoni *et al.*, 2005b). In the present study, we found that subjects with variant *CYP2E1* (-1054C>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 ppm for HQ, PH and MA (Tables 4.3, 4.5 and 4.7 and Fig. 4.2A). This interaction effect could point to induction of *CYP2E1* (-1054C>T) by benzene exposure, or to more rapid saturation of metabolism among homozygous variant subjects (Nedelcheva *et al.*, 1999). Our results substantially support evidence from prior studies that *CYP2E1* (-1054C>T) mutations functionally reduce the metabolism of CYP2E1 substrates.

Subjects with variant alleles of *EPHX1* (Ex3-28T>C) produced lower levels of CA in our study (Table 4.6 and Fig. 4.2F). EPHX1 enzymes hydrolyze epoxides through the formation of hydroxyl alkyl-enzyme intermediates (Morisseau and Hammock, 2005). 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 subjects (Lovern *et al.*, 1999; Nebert *et al.*, 2002). Two polymorphisms have been identified; one, in exon 3, decreased enzymatic activity 50% *in vitro* while the other, in exon 4, increased activity 25% (Hassett *et al.*, 1994). Our findings that workers with variant allele(s) of *EPHX1* (Ex3-28T>C) had lower levels of CA than homozygous wild-types while

those with at least one variant of *EPHX1* (Ex4+52A>G) had marginally higher CA levels, support the *in vitro* results. We also detected significant interactions between *EPHX1* (Ex3-28T>C) and both benzene exposure and smoking status. The gene-smoking interaction tended to accentuate differences in CA levels between smoking subjects who had different alleles of *EPHX1* (Ex3-28T>C) and to obscure effects among nonsmokers (Fig 4.2F). The gene-exposure interaction produced differences in CA levels that could be distinguished between smokers having homozygous wild- and variant-types of *EPHX1* (Ex3-28T>C) at benzene concentrations above one ppm. Our results are intriguing in light of recent epidemiologic studies indicating that, among smokers, fast EPHX1 metabolizers had greater risks of colorectal adenomas (Cortessis *et al.*, 2001; Huang *et al.*, 2005) than slow metabolizers. Interestingly, fast metabolizers of *EPHX1* (Ex4+52A>G) also had marginally higher levels of SPMA at benzene concentrations above 6 ppm ($p < 0.10$). Although the mechanism for production of SPMA is not yet established (Snyder *et al.*, 1993; Henderson *et al.*, 2005a), the apparent effect of EPHX1 on SPMA levels may offer clues regarding formation of this minor benzene metabolite (Morisseau and Hammock, 2005).

Among the GST isozymes, both GSTM1 and GSTT1 affected the production of SPMA (Table 4.4, Fig. 4.2B), with subjects having variant forms of these enzymes producing lower levels. Of the two isozymes, GSTT1 produced more substantial effects, based upon evidence ratios (Burnham *et al.*, 2002) (data not shown), and produced different profiles for each combination of alleles (Fig. 4.2B). This finding of is consistent with previous studies (Sorensen *et al.*, 2003; Sorensen *et al.*, 2004; Qu *et al.*, 2005). No effect of GSTP1 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 4.3 – 4.7 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 non-genetic covariates explained between 53 and 84% of the variability in metabolite levels. The corresponding percentages of variability explained 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.

Table 4.8. SNP effects on benzene metabolites at various levels of benzene exposure. [Least-squares-mean ratios of *variant/variant* to *wild/wild*].

Metabolite	SNP	Air concentration (ppm)			
		0.1	**1**	**10**	**100**
E,E-Muconic acid (MA)	*CYP2E1* (-1054C>T)	0.941	0.666	0.472	0.333
	*NQO1*2*	0.976	0.832	0.708	0.603
S-Phenylmercapturic acid (SPMA)	*CYP2E1* (-1054C>T)	0.675	0.619	0.567	0.519
	EPHX1 (Ex4+52A>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* (Nonsmokers)	0.614	0.524	0.448	0.382
	*NQO1*2* (Smokers)	1.74	1.48	1.27	1.08
Phenol (PH)	*CYP2E1*	0.75	0.542	0.392	0.283
	*NQO1*2* (Nonsmokers)	0.714	0.714	0.714	0.714
	*NQO1*2* (Smokers)	1.22	1.22	1.22	1.22
Catechol (CA)	*EPHX1* (Ex4+52A>G)	1.15	1.15	1.15	1.15
	*NQO1*2*	0.902	0.789	0.69	0.603
	EPHX1 (Ex3-28T>C, nonsmokers)	1.29	1.10	0.938	0.8
	EPHX1 (Ex3-28T>C, smokers)	0.685	0.584	0.498	0.425
Hydroquinone (HQ)	*CYP2E1* (-1054C>T)	0.692	0.518	0.389	0.291
	*NQO1*2*	0.849	0.849	0.849	0.849

Regarding interindividual differences in metabolism that would be expected for a given SNP, Table 4.8 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 4.3 – 4.7. These ratios represent the mean fold-ranges for variant/referent that would be expected for each SNP after adjusting for benzene exposure as well as covariates and other genetic effects. Since several gene-environment interactions were identified, the tables cover benzene concentrations between 0.1 and 100 ppm and differentiate between smokers and nonsmokers when appropriate. The values shown in Table 4.8 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 2- to 3.5-fold would only be anticipated for most metabolite-gene combinations when persons were exposed to very high benzene concentrations (100 ppm). The exception to this rule is the large effect of GSTT1 on SPMA production, where homozygous referents would typically have SPMA levels 3- to 8-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 (Sorensen *et al.*, 2003; Sorensen *et al.*, 2004; Qu *et al.*, 2005).

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 9 SNPs investigated, *NQO1*2* affected all five metabolites, *CYP2E1* (-1054C>T) affected all metabolites but CA, *EPHX1* (Ex3-28T>C or Ex4+52A>G) affected CA and SPMA, and GSTT1 and GSTM1 affected SPMA. Significant interactions were detected between benzene exposure and all four genes and between smoking status and

NQO1 and EPHX1. Results are generally consistent with prior 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.

CHAPTER 5.

DISCUSSION AND CONCLUSIONS

5.1. Summary and Conclusions

Although the toxicity of benzene has been linked to its metabolites, the dose-related production of metabolites is not well understood in humans, particularly at low exposure levels. The present study was intended to elucidate the profiles of the main benzene metabolites in humans exposed to a wide range of benzene levels, and the effects of genetic and other factors upon metabolite levels. In order to do so, we analyzed a dataset containing air and metabolite levels along with personal information (including SNPs of key metabolic enzymes) from 389 subjects exposed to benzene in either workplace or general environment.

5.1.1. Dose-related Patterns of Human Benzene Metabolism

As exposure increased, the levels of benzene metabolites also increased. We used step-functions to depict nominal exposures which were associated with a statistically significant increase in metabolite levels for subjects grouped by their exposure levels (n=30 per group). While there were marginal increases in MA, PH, CA and HQ at air benzene levels as low as 0.02 ppm, levels of benzene metabolites were demonstrably different from background levels at about 0.2 ppm for MA and SPMA, 0.5 ppm for PH and HQ, and 2 ppm for CA (Fig. 2.2).

After adjustment for the background levels of benzene, MA, PH, CA and HQ showed significant downward trends of dose-related production [*i.e.*, metabolite levels per ppm of benzene], whereas SPMA had an upwards trend between 0.027 and 15.4 ppm. The transitions were particularly accentuated at lower exposure (0.027 – 0.274 ppm), where a 16-fold reduction for CA, 4.4-fold for PH, 1.8-fold reduction for MA and 1.6-fold reduction for HQ. Thus, it appears that metabolism shifted away from CA and PH at low doses in favor of MA and HQ, which are the only major benzene metabolites requiring two CYP oxidations (Figure 2.1). When exposures exceeded about one ppm, the dose-related profiles of PH, MA, HQ, and CA became quasi-parallel, suggesting that metabolism of benzene to BO had become rate-limiting.

5.1.2. Modeling Human Metabolism of Benzene

In order to verify the dose-related production of benzene metabolites, we needed a statistical model to address covariate effects on metabolites after adjustment for the nonlinear relationship between urinary metabolites and air benzene. From this perspective, a GLM+NS model was constructed. Based on analysis of the molar fractions of 5 major benzene metabolites (Fig.3.3B), CYP-mediated metabolic pathways favored MA and HQ below 20 ppm and favored PH above 20 ppm. Mean trends of dose-specific levels (μM/ppm benzene) of MA, PH, HQ, and CA decreased with increasing benzene exposure. Noticeably, about 90% of the reductions in dose-specific levels (from 0.03 to 88.9 ppm) occurred below about 3 ppm for each major metabolite. GLM+NS model also allowed us to detect significant effects of gender, age and smoking status on benzene metabolites. The level of each metabolite was about 20% or higher in females. Likewise, the levels of 4 metabolites (MA, SPMA, PH, and HQ) diminished with age, at rates between 1.1 and 1.9 percent per year of

life. The levels of HQ and CA were greater in smoking subjects (Table 3.2). Overall, these results indicate that benzene metabolism is highly nonlinear with increasing benzene exposure above 0.03 ppm (even after adjustment of background metabolites of benzene). Therefore, current human toxicokinetic models may not accurately predict benzene metabolism below 3 ppm.

5.1.3. Genetic Polymorphisms and Benzene Metabolism in Humans

We extended the GLM+NS model to investigate the effects of genetic polymorphisms of key enzymes for benzene metabolism including CYP2E1, NQO1, MPO, GSTs and EPHX1, after adjustment for age, gender, BMI and smoking status. The following SNPs showed significant effects on various metabolites, either as main effects or as interactions with benzene exposure and/or smoking: *NQO1*2* for all metabolites, *CYP2E1* for all metabolites except CA, *GSTT1* and *GSTM1* for SPMA, *EPHX1* (Ex4+52A>G) for SPMA and CA, and *EPHX1* (Ex3-28T>C) for CA. Interestingly, variant or deficient alleles of all genes [except *EPHX1* (Ex4+52A>G)] appeared to be associated with lower levels of benzene metabolites relative to homozygous wild alleles. While it was expected that the levels of CA and HQ would be lower in subjects with *NQO1*2* (less active), lower levels of MA, SPMA, and PH among subjects with NQO1*2 seemed to be associated with a more general role for NQO1. We also found that *NQO1*3* and *MPO* did not affect metabolism. As for *CYP2E1* (-1054C>T), significant differences in metabolite levels between homozygous wild-types and homozygous variants weres detected at benzene exposures in the range of 0.1 – 2 ppm for HQ, PH and MA (Tables 3, 5 and 7 and Fig. 2A). This interaction effect may reflect induction of *CYP2E1* (-1054C>T) by benzene exposure, or more rapid saturation of metabolism among homozygous variant subjects. Our results substantially support evidence

from previous studies that *CYP2E1* (-1054C>T) mutations functionally reduced the metabolism of CYP2E1 substrates. *GSTT1* affected the production of SPMA with a clear gene-dose relationship. While *GSTM1* showed similar effects on SPMA, there was no effect of *GSTP1* on SPMA. Subjects with variant allele(s) of *EPHX1* (Ex3-28T>C) had lower levels of CA than homozygous wild-types while those with at least one variant of *EPHX1* (Ex4+52A>G) had marginally higher CA levels, supporting previous *in vitro* results. Also, differences in CA levels seemed distinguishable between homozygous wild- and variant-types of *EPHX1* (Ex3-28T>C) among smokers exposed to benzene at one ppm or more. It is of interest that fast EPHX1 metabolism is a risk factor among smokers. Although we detected many influential SNP effects on benzene metabolites, the portion of SNP effects as explanatory variables appeared limited, that is, less than 5% of the total variation of the level of any benzene metabolite could be explained by SNPs.

5.2. Implications and Suggestions for Future Research

In the present study, we observed highly nonlinear relationships with regards to dose-specific production of benzene metabolites. Considering that MUC and HQ are thought to be the most hematotoxic metabolites of benzene (Snyder, 2004), and that metabolism of benzene appears to favor these species at lower exposure (Fig. 3.3B, Fig. 3.4) the general population might be at higher risk of benzene-induced health effects than generally thought from linear extrapolation of risks from studies involving highly exposed subjects. Also, the overall rate of metabolism, expressed as excretion of total benzene metabolites per ppm of benzene exposure points to more efficient metabolism at lower exposure (Fig. 3.4F). Based upon the results from the NS basis functions defined in Chapter 2 for total metabolites we

estimate that the percentage of the absorbed benzene dose that was excreted as urinary metabolites diminished from about 90% at 0.7 ppm, to 56% at 1 ppm, to 46% at 3 ppm. These calculations assume a ventilation minute volume of 7.5 l/min (Krishnan and Andersen, 2001), that 50% of the inhaled benzene is retained (Nomiyama and Nomiyama, 1974), an 8-h working day, and a 1 ml/min urine generation rate (Ritschel *et al.*, 1999). These apparent dose-related shifts in benzene metabolism need to be reproduced in other populations.

Age, gender, and smoking were important explanatory variables for our models of urinary benzene metabolites after adjusting for benzene exposure (Chapter 2). Considering that AML tends to be a disease of children and young adults (EPA, 2002), it is possible that enhanced benzene metabolism in younger persons might be a risk factor for this disease. Also, the fact that females appear to metabolize more benzene than males should also be considered as a potential risk factor for leukemia, although rates of AML in the U.S. tend to be greater for males than females of all races (Xie *et al.*, 2003). Finally, since cigarette smoke appears to contain significant quantities of benzene, HQ and CA (see Chapter 2), links between smoking and leukemia (McDonald *et al.*, 2001) could well reflect exposures to benzene and its metabolites.

We identified relatively small effects of SNPs of metabolizing genes on levels of urinary metabolites, which primarily reflect metabolism in liver (Chapter 3). Since liver is not a target organ, the roles of SNPs operating in the target blood-forming tissues might be different (Lan *et al.*, 2004). Therefore, our results should be considered as reflecting primary rates of benzene metabolism and not necessarily as reflecting particular activation and deactivation pathways in target cells. It is important that future studies consider the possible

interactions between primary (liver) metabolism and subsequent transformations in target tissues to more fully elucidate the mechanism(s) of benzene induced hematotoxic effects.

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