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ORIGINAL ARTICLE

Metabolome-wide association study of occupational exposure to benzene

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Abstract

Benzene is a recognized hematotoxin and leukemogen; however, its mechanism of action in humans remain unclear. To provide insight into the processes underlying benzene hematotoxicity, we performed high-resolution metabolomic profiling of plasma collected from a cross-sectional study of 33 healthy workers exposed to benzene (median 8-h time-weighted average exposure; 20 ppm_a), and 25 unexposed controls in Shanghai, China. Metabolic features associated with benzene were identified using a metabolome-wide association study (MWAS) that tested for the relationship between feature intensity and benzene exposure. MWAS identified 478 mass spectral features associated with benzene exposure at false discovery rate < 20%. Comparison to a list of 13 known benzene metabolites and metabolites predicted using a multi-component biotransformation algorithm showed five metabolites were detected, which included the known metabolites phenol and benzene diolepoxide. Metabolic pathway enrichment identified 41 pathways associated with benzene exposure, with altered pathways including carnitine shuttle, fatty acid metabolism, sulfur amino acid metabolism, glycolysis, gluconeogenesis and branched chain amino acid metabolism. These results suggest disruption to fatty acid uptake, energy metabolism and increased oxidative stress, and point towards pathways related to mitochondrial dysfunction, which has previously been linked to benzene exposure in animal models and human studies. Taken together, these results suggest benzene exposure is associated with disruption of mitochondrial pathways, and provide promising, systems biology biomarkers for risk assessment of benzene-induced hematotoxicity in humans.

Introduction

Benzene is a high production volume chemical with widespread environmental and occupational exposures. Epidemiologic research shows that benzene exposure increases risk of hematologic diseases, including aplastic anemia, leukemia and myelodysplastic syndrome, and limited evidence of chronic lymphocytic leukemia, non-Hodgkin lymphoma and multiple myeloma (1). Mechanistic studies show hematologic effects require oxidative metabolism to generate a range of reactive

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Abbreviations

BCAA	branched chain amino acid
BM	bone marrow
FDR	false discovery rate
HGB	hemoglobin
HILIC	hydrophilic interaction liquid chromatography
HRM	high-resolution metabolomics
HRMS	high-resolution mass spectrometry
HSC	hematopoietic stem cell
PC1	first principal component
PLT	platelets
RBC	red blood cell
ROS	reactive oxygen species
RPC	reverse phase chromatography
WBC	white blood cell.

products, including epoxides, phenol, hydroquinone, benzoquinone, muconaldehyde and related chemicals (2–4). The more reactive of these chemicals are proteotoxic (4) and genotoxic (5), especially causing mitochondrial dysfunction (6), but the precise disease mechanisms remain incompletely understood (7). Improved mechanistic understanding is essential to better understand health effects of benzene exposure in humans (3).

The metabolic effects of benzene exposure and the resulting disruption to underlying biological response pathways and homeostasis is unknown in humans. While the primary mechanism of benzene toxicity is thought to occur through multiple processes that include generation of reactive metabolites, increased oxidative stress and induction of stem cell dysregulation within the bone marrow (BM) niche, systems biology approaches that incorporate multiple levels of biological information to measure downstream biomarkers of these effects have been recognized as a promising tool in benzene risk assessment (8,9). High-resolution metabolomics (HRM) relies upon ultra-high-resolution mass spectrometry to improve sensitivity and coverage of endogenous metabolites as well as trace environmental chemicals in biologic samples (10,11). The methods are especially useful because they provide untargeted analysis of metabolites in 136 out of 154 human metabolic pathways (12), thereby enabling a relatively broad survey of potential biologic responses to exposure. Use of HRM with samples from carefully designed studies with documented exposures allows tests for associations of internal body burden with external exposures, metabolic response, risk biomarkers and health outcomes (10,13). Thus, application of HRM provides a key measure for toxicokinetics and toxicodynamics *in vivo*, and holds potential for insight into metabolic biomarkers that may connect benzene exposure to disease outcomes.

In the present study, we used HRM of plasma from a cross-sectional study of healthy workers exposed to benzene and unexposed controls to provide insight into the metabolic processes underlying benzene hematotoxicity. To evaluate early biological effects of exposure to benzene, we applied a metabolome-wide association study (MWAS) framework to test for mass spectral features associated with shift-averaged benzene exposure. Identified metabolic pathways were then tested for their relationship with benzene exposure biomarkers, as well as measures of effect related to hematopoetic pathways, including hematologic cell counts, and numerical chromosomal aberration rates for chromosomes 8 and 21. Results from this study support a link between benzene exposure and disruption in mitochondrial pathways and provide new insight into the application of systems biology approaches to study benzene toxicity in humans.

Materials and methods

Study population

Identification of factories and worker enrollment are described in detail elsewhere (14). Study subjects were enrolled in the Fall of 1992 in Shanghai, China and evaluated at the Shanghai Hygiene and Anti-Epidemic Institute. The study protocol was explained to all potential participants, and informed consent was obtained using Institutional Review Board-approved procedures. Data collected included age, gender, tobacco use, alcohol consumption, medical history and an occupational work history. Prior to the clinical visit, study subjects were asked to refrain from eating solid foods after dinner the night before and the morning of the clinical phase of the study. The following morning, a 27 ml sample of blood was obtained by venous phlebotomy. Plasma samples were then frozen at –80°C, shipped to the National Cancer Institute on dry ice, and stored continuously at –80°C until analysis. Studies of plasma collected >50 years prior to HRM analysis show storage at –80°C is sufficient for maintaining sample integrity and identifying biological effects of environmental exposures; therefore, sample storage length is not expected to impact the ability to identify benzene-associated biological response pathways (15,16).

Benzene exposure assessment

Details of personal benzene exposure assessment have been reported previously (14). In brief, individual exposure was monitored by organic vapor passive dosimetry badges (3M no. 3500. St. Paul. Minnesota), which were worn by each worker for a full shift on five separate days for a 1–2 week period prior to blood collection. Badges were analyzed by gas chromatography with flame ionization detection for benzene, toluene and xylene. Average exposure was calculated for each compound as the geometric mean of the five air measurements. Urinary benzene was determined by gas chromatography-mass spectrometry using head-space solid-phase microextraction (17); urinary phenol, hydroquinone, catechol, muconic acid and S-phenyl-mercapturic acid were measured as trimethylsilyl derivatives (18).

High-resolution metabolomics

Plasma samples were analyzed by liquid chromatography and Fourier transform high-resolution mass spectrometry (HRMS) (Dionex Ultimate 3000, Q-Exactive HF, Thermo Scientific) using a combination of hydrophilic interaction liquid chromatography (HILIC) with electrospray ionization (ESI) operated in positive mode and reverse phase chromatography (RPC) with electrospray ionization operated in negative mode (19). HRMS analysis and quality assurance and control procedures are described in detail in the supporting information. Uniquely detected ions consisted of *m/z*, retention time and ion abundance, referred to as *m/z* features. Prior to data analysis, *m/z* features were filtered to remove those with coefficient of variation ≥ 100% and more than 75% non-detected intensities. The remaining 11 293 and 8216 *m/z* features for HILIC and RPC, respectively were then $\log_2$ transformed and used for all statistical analyses.

Benzene MWAS

All statistical analyses were performed in R 3.4.0. MWAS was completed using a linear regression framework to identify *m/z* features exhibiting an exposure-response relationship with shift-averaged personal benzene levels. While cumulative benzene exposure is an important risk factor for cancer outcomes, the goal of our study was to evaluate early biological effects underlying benzene exposure. Therefore, exposure assignments for exposed workers were based upon shift-averaged benzene levels. Due to benzene levels exceeding the current Occupational Safety and Health Administration time-weighted exposure limit of 1 ppm_a for all exposed workers, the median exposure of 20 ppm was used to classify low- and high-exposed workers to identify exposure-associated metabolic changes. For each *m/z* feature, peak intensity was $\log_2$ transformed to test for association with benzene across the categories of exposure, which included controls, low exposed (<20 ppm_a) and high exposed (≥20 ppm_a), defined as a continuous variable (control = 0; < 20 ppm_a = 1; ≥20

ppm_a = 2). Exposure was defined as an independent variable with equal spacing between control, low and high exposure groups to enhance metabolite selection towards those showing dose-response while providing representative metabolic associations that differ between benzene exposed workers and unexposed controls (20).

The statistical model included adjustments for age (continuous), sex (factor), body mass index (continuous), smoking status (lifetime smoking of > 20 cigarettes, factor) and current alcohol use (≥1 average alcoholic drinks per week, factor). To account for multiple comparisons, we applied a Benjamini-Hochberg (21) false discovery rate (FDR) threshold of 20%, all results are presented using adjusted *p*-values. An FDR threshold of 20% was selected to balance the rate of type I and II errors, and when combined with a dose-response linear regression framework and permutation-based metabolic pathway enrichment has previously been shown to improve detection of biological activity and reduce false positives (20,22). Due to differences in metabolite annotation, we chose to apply a separate FDR for each HRM mode. Raw *P* values corresponding to the FDR threshold were: HILIC $P = 2.1 \times 10^{-3}$ and RPC $P = 9.2 \times 10^{-3}$.

Metabolite annotation

We first characterized the *m/z* features associated with benzene to identify exposure products by matching the mass for common positive (HILIC) and negative (RPC) electrospray ionization adducts of both known (7) and predicted metabolites at match accuracy of ± 5 ppm (± 10^6 × theoretical *m/z* mass) using xMSannotator (23). Potential benzene metabolites were predicted using BioTransformer, which predicts the site of metabolism, the reacting enzyme, and the resulting product structure(s) across a multicompartmental model that includes calculations with transformations from phase I, phase II, microbial and promiscuous enzymatic reactions (24).

The remaining *m/z* features not annotated as benzene metabolites were matched to a reference database of over 250 metabolites confirmed with MS^2 and co-elution studies. Additional features not matching these metabolites were annotated with the KEGG database (25), which provides information on 487 pathways containing 17 620 unique metabolites. Identities were assigned using evidence scoring provided in Mummichog (22) and ±5 ppm mass tolerance. Enriched metabolic pathways were selected using a Mummichog scoring threshold of 0.05, FDR < 20% and the presence of three or more metabolites associated with benzene exposure.

Metabolic correlation with exposure, hematopoetic cell counts and chromosomal changes

To characterize protentional biological response mechanisms underlying benzene toxicity in exposed participants, we used correlation-based network analysis to evaluate the relationship between the first principal component (PC1) of the pathways associated with benzene exposure, benzene exposure biomarkers and bioeffect measures previously characterized in this cohort. Exposure biomarkers included urinary benzene, phenol, catechol, hydroquinone and muconic acid, as well as HRM detected benzene-related metabolites (17,18). Bioeffect markers included hematologic cell counts [red blood cells (RBCs), hemoglobin (HGB), mean corpuscular volume, lymphocytes, platelets (PLTs) and white blood cells (WBC)], telomere length, and hyperploidy (HYPR-) and hypoploidy (HYPO-) rates for chromosomes 8 and 21, and total aneuploidy (SCA-) rates for chromosomes 8 and 21. For each Mummichog-identified pathway associated with benzene exposure, principal component analysis was completed using metabolite intensities from each pathway, and PC1 scores for each pathway were tested for correlation with exposure or bioeffect markers using a Pearson correlation coefficient. By using an integrated, single measure for each pathway represented by PC1 scores, this analysis enables characterization of the relationship between pathway impacts, benzene metabolites and pathological changes (i.e. immunological changes and genetic instability) potentially underlying benzene-related diseases. To visualize the relationship between metabolic pathways and the exposure and effect measures, correlation networks were generated based upon correlations with $|r| \geq 0.3$ and $P < 0.05$. Community detection was then performed using the multilevel community detection method (26) to identify clusters of pathways and biomarkers in each network, which were visualized using Cytoscape (27).

Results

Study population

Demographics, including age, sex, body mass index, current smoking and alcohol use status were comparable among the exposed and unexposed workers (Table 1). Measured benzene levels for exposed factory worker levels ranged from 1.6 ppm_a to 134 ppm_a.

Table 1. Demographic characteristics and benzene exposure level

	Exposed				
Subjects	Controls (*n* = 25)	Total (*n* = 33)	<20 ppm (*n* = 16)	>20 ppm (*n* = 17)	*P*[a]
Demographic characteristics					
Age, mean (SD)	34 (7)	34 (7)	34 (8)	34 (5)	0.82
BMI, mean (SD)	21 (2)	21 (3)	21 (2)	21 (4)	0.29
Smoking status, n (%)[b]	13 (52)	19 (58)	7 (44)	12 (71)	0.28
Current alcohol use, n (%)[c]	9 (36)	10 (30)	6 (38)	4 (24)	0.63
Sex, n (%)					
Female	11 (44)	17 (52)	5 (31)	12 (71)	0.22
Male	14 (56)	16 (48)	11 (69)	5 (29)	0.13
Benzene exposure					
Benzene air level, ppm	0.01 (0.01)	36.2 (34.7)	10.2 (5.9)	60.6 (32.6)	<0.0001
Minimum exposure, ppm	0	1.6	1.6	20.1	
Maximum exposure, ppm	0.05	134	19.6	134	
Urinary metabolites					
Benzene, µg/l (% detected)	0.08 (88)	28.8 (97)	9.7 (100)	47.9 (94)	0.0005
Phenol, µg/mg creatinine (% detected)	21.55 (44)	168.8 (97)	68.1 (100)	269.5 (94)	<0.0001
Hydroquinone, µg/mg creatinine (% detected)	2.91 (44)	34.1 (97)	19.3 (100)	48.9 (94)	<0.0001
Catechol, µg/mg creatinine (% detected)	3.8 (40)	29.2 (94)	10.3 (94)	46.9 (94)	<0.0001
Muconic Acid, µg/mg creatinine (% detected)	0.3 (44)	29.5 (97)	16.1 (94)	29.5 (97)	<0.0001

[a]P-values for comparison of controls to all exposed were determined by *t*-test.
[b]Smoking status defined as having smoked more than 20 cigarettes over the lifetime.
[c]Current alcohol use was defined as participants consuming an average of one or more alcoholic drinks per week.

MWAS of benzene

Following FDR correction, MWAS for HILIC identified 117 *m/z* features associated with benzene exposure (Figure 1A). Of these, 101 *m/z* features increased with benzene exposure, while 16 were decreased. RPC identified a greater number of *m/z* features associated with exposure, which included 361 *m/z* features. Of these, 258 showed a positive association with exposure, while 103 were negative (Figure 1B). Secondary MWAS analyses that considered differences between control and exposed workers showed minimal differences between the two approaches for exposure classification, with the monotonic trend test showing the greatest number of associated features. Additional sensitivity analysis using a single FDR threshold of 20% (raw $P = 0.0049$) for the combined dataset of HILIC and RPC showed almost identical pathway enrichment results, with only a small number of metabolites differing from the pathways. Thus, to simplify annotation, MWAS results from each analytical mode were characterized separately.

Identification of benzene metabolites

The *m/z* features associated with exposure were first characterized for the presence of known and predicted benzene metabolites by comparison to a list of 13 known benzene metabolites and 86 metabolites predicted using BioTransformer. Annotated benzene metabolites are shown in Figure 2. Two known benzene metabolites, including phenol ($q < 0.009$) and benzene diolepoxide ($q = 0.2$) showed a strong association with benzene exposure. Additional annotated metabolites showing a positive relationship with exposure and raw $p < 0.05$ but did not meet the FDR threshold included benzene ($q = 0.4$), EE-muconic acid ($q = 0.47$) and catechol ($q = 0.37$). In addition to phenol and benzene diolepoxide, which were accurately predicted by BioTransformer, additional metabolite matches included sulfation and hydrolysis products. Metabolites with $P < 0.05$ included two sulfation products ($C_6H_{10}O_{10}S$, $C_6H_8O_6S$) corresponding to four possible structures, and two hydrolysis products ($C_6H_{10}O_7$, $C_7H_8O_4$,). Predicted chemical structures are provided in Supplementary Figure 1, available at *Carcinogenesis* Online.

Biological response to benzene exposure

Following annotation of benzene-related metabolites, we used metabolic pathway enrichment to characterize endogenous changes associated with exposure (Table 2). The enrichment analysis identified 14 and 25 pathways for HILIC and RPC, respectively. Butanoate metabolism, fatty acid activation, glycosphingolipid metabolism and hexose phosphorylation were identified in both modes. Pathways were consistent with alterations in oxidative stress, mitochondrial pathways, signaling lipids and nucleoside metabolism.

To further characterize biological response to benzene exposure, we evaluated individual metabolites corresponding to oxidative stress and mitochondrial pathways. Metabolite changes associated with benzene were consistent with increased oxidation, including reduction in methionine ($\beta = -3.71$; $q = 0.12$), linoleic acid ($\beta = -2.35$; $q = 0.19$) and taurine ($\beta = -0.29$; $q = 0.075$), and increased levels of the oxidation products cystine ($\beta = 3.95$; $q = 0.08$), 12,13-epoxylinoleic acid radical ($\beta = 0.53$; $q = 0.075$) and 12,13-epoxy-9-alkoxylinolenic acid radical ($\beta = 4.08$; $q = 0.17$). Additional antioxidants were positively associated with exposure, including ascorbate ($\beta = 0.58$; $q = 0.04$) and α-ketoglutaric acid ($\beta = 0.19$; $q = 0.10$).

Metabolic associations with benzene exposure related to mitochondrial function included carnitine shuttle, fatty acid metabolism, glycolysis and glucogenesis. Metabolites from the carnitine and fatty acid pathways showed the greatest number of associations, and are listed in Table 3. Glucogenesis metabolites included matches to carbohydrates ($\beta = 0.58$; $q = 0.06$) that were not distinguishable with the chromatography method used and represent the combined signal of multiple compounds, including glucose, mannose, fructose, galactose and myo-inositol. Pyruvate ($\beta = 0.28$; $q = 0.04$) was also associated with benzene exposure.

Additional metabolites from branched chain amino acid (BCAA) metabolism, nucleoside metabolism and signaling lipids were annotated. BCAA related metabolites included the metabotoxin ketoleucine ($\beta = 0.18$; $P = 7.6 \times 10^{-5}$), hydroxyisobutyric acid ($\beta = 0.26$; $q = 0.07$), methylmalonate semialdehyde ($\beta = 0.19$; $q = 0.06$) and a match representing the combined intensity of leucine and isoleucine ($\beta = -0.14$; $q = 0.19$). Sphingosine-1-phosphate ($\beta = 0.39$, $q = 0.1$) and sphinganine 1-phosphate ($\beta = 0.26$; $q = 0.18$), which are signaling lipids and facilitate traffic and mobilization of hematopoietic stem cells (HSCs), were also associated with benzene exposure. Alterations in nucleoside metabolites from pyrimidine metabolism included decreased

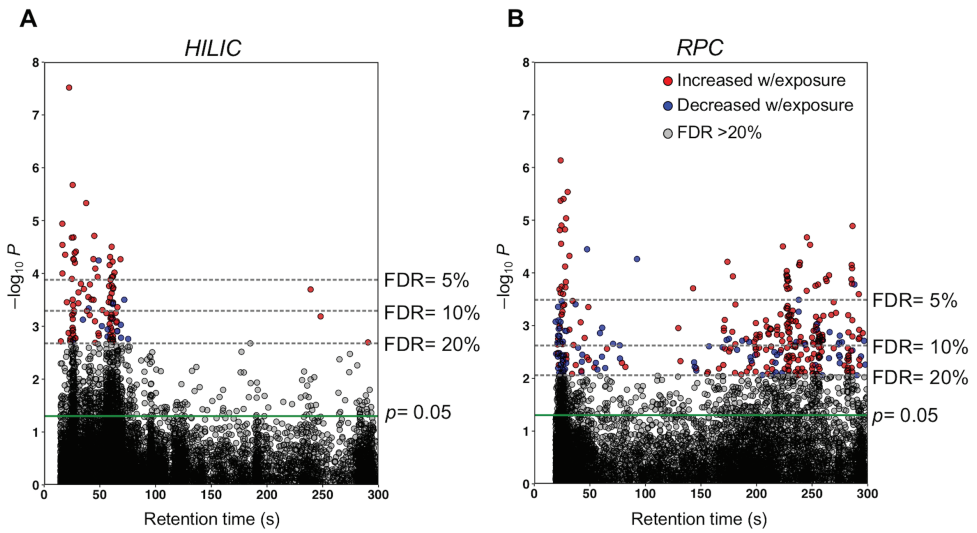

Figure 1. Results of benzene MWAS for (**A**) HILIC and (**B**) RPC.

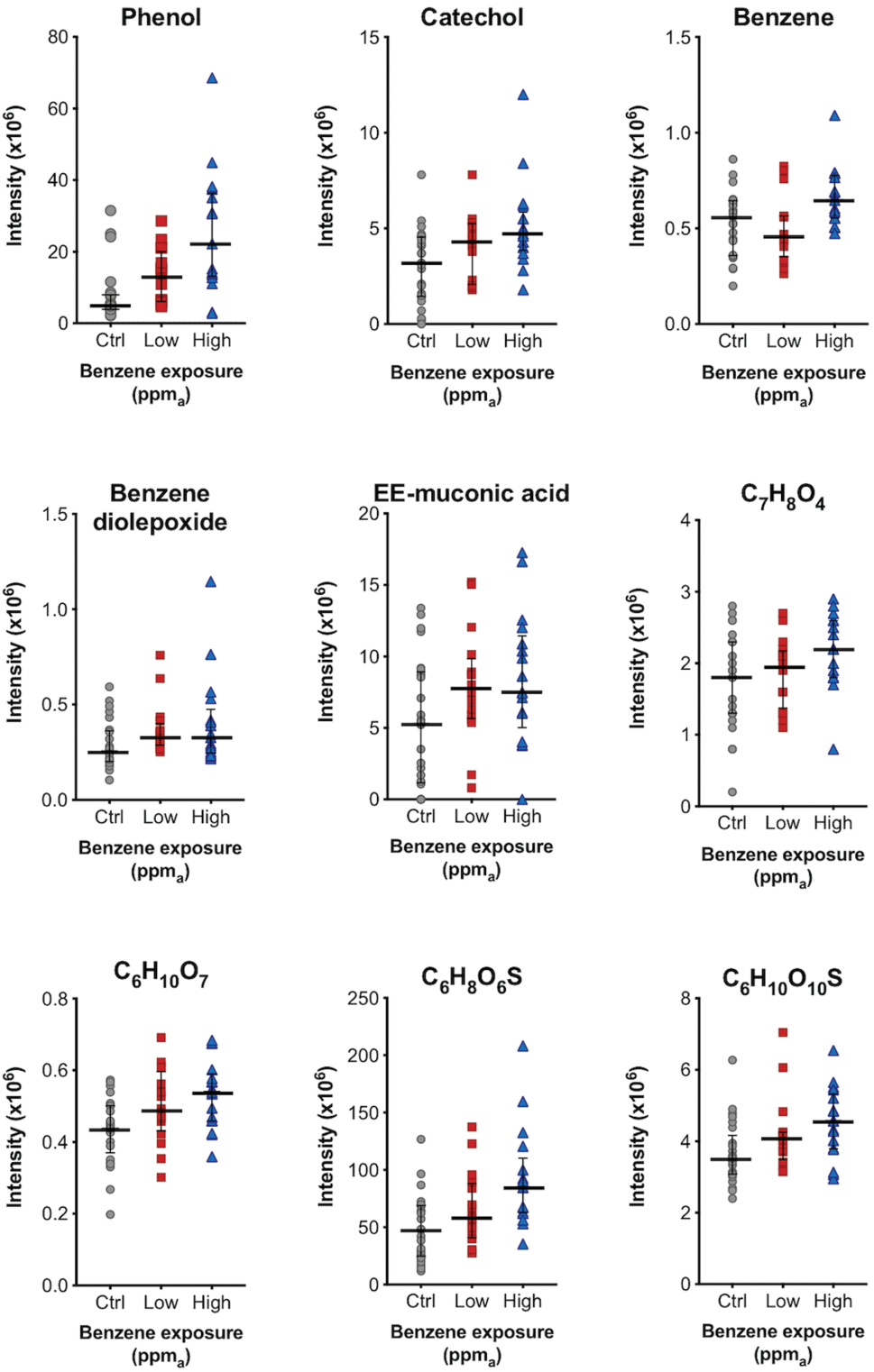

Figure 2. Known and predicted plasma benzene metabolites associated with benzene exposure measured by HRM. Metabolites labelled with a chemical formula were predicted using BioTransformer, and include two hydrolysis metabolites ($C_6H_{10}O_7$, $C_7H_8O_4$) and two sulfation products of benzene ($C_6H_{10}O_{10}S$, $C_6H_8O_6S$). Ctrl: Unexposed. Exposed individuals were classified as Low (<20 ppm$_a$) and High: (>20 ppm$_a$) based upon the median exposure level.

levels of the nucleic acids cytosine (β = –0.13; q = 0.19) and thymine (β = –0.11; q = 0.08), and elevated levels of deoxyribose (β = 0.21; q = 0.009), in addition to aspartate (β = –0.06; q = 0.18) and a match to the DNA synthesis molecules deoxyguanosine triphosphate or deoxyadenosine triphosphate (β = –4.2; q = 0.14).

Integration with exposure, hematopoetic cell counts and chromosomal changes

Network analysis showed pathways identified with Mummichog were correlated with at least one benzene metabolite detected in either blood or urine, as well as hematological and chromosomal

Table 2. Enriched metabolic pathways associated with benzene exposure

Metabolic pathway	No. significant metabolites	Total number of metabolites detected in pathway	P[a]	Q[b]
		HILIC-positive		
Carnitine shuttle	10	31	0.00050	0.021
N-Glycan degradation	4	7	0.00051	0.021
Keratan sulfate degradation	3	7	0.00070	0.021
Hexose phosphorylation	4	19	0.00087	0.021
Glycosphingolipid biosynthesis—ganglioseries	3	12	0.0012	0.021
Butanoate metabolism	4	24	0.0013	0.021
Fructose and mannose metabolism	4	24	0.0013	0.021
Starch and sucrose metabolism	3	15	0.0018	0.026
Glycolysis and gluconeogenesis	4	37	0.0046	0.044
Sialic acid metabolism	3	27	0.0091	0.070
Fatty acid activation	3	27	0.0091	0.070
Glycosphingolipid metabolism	3	28	0.010	0.070
Aminosugars metabolism	3	30	0.013	0.080
Galactose metabolism	3	33	0.019	0.10
		RPC-negative		
Fatty acid activation	10	16	0.00039	0.012
De novo fatty acid biosynthesis	9	18	0.00040	0.012
Glycerophospholipid metabolism	12	37	0.00047	0.012
Phytanic acid peroxisomal oxidation	4	6	0.00055	0.012
Valine, leucine and isoleucine degradation	8	23	0.00059	0.012
Fatty acid metabolism	5	12	0.00078	0.014
Methionine and cysteine metabolism	10	37	0.00090	0.014
Heparan sulfate degradation	3	6	0.0017	0.017
Chondroitin sulfate degradation	3	6	0.0017	0.017
Omega-3 fatty acid metabolism	3	6	0.0017	0.017
Beta-alanine metabolism	4	11	0.0018	0.017
Histidine metabolism	5	17	0.0022	0.019
Glycosphingolipid metabolism	7	28	0.0024	0.019
Alanine and aspartate Metabolism	5	20	0.0046	0.030
Butanoate metabolism	5	20	0.0046	0.030
Linoleate metabolism	5	21	0.0059	0.036
Caffeine metabolism	3	10	0.0075	0.044
Glycine, serine, alanine and threonine metabolism	7	36	0.011	0.059
Urea cycle/amino group metabolism	7	38	0.015	0.077
Glutamate metabolism	3	13	0.019	0.087
Hexose phosphorylation	3	14	0.025	0.11
Phosphatidylinositol phosphate metabolism	4	21	0.025	0.11
Propanoate metabolism	3	15	0.032	0.13
Aspartate and asparagine metabolism	8	50	0.036	0.14
Pyrimidine metabolism	7	45	0.045	0.16

[a]Mummichog enrichment score, which is based upon comparison of pathway enrichment for randomly sampled *m/z* features to those identified in the MWAS analysis.

[b]Adjusted *P*-values were calculated to estimate the false discovery rate. Only pathways with ≥3 metabolites, Mummichog $P < 0.05$ and FDR < 20% were considered.

bioeffect markers (Figure 3). Hydroquinone and two predicted benzene metabolites were not correlated with PC1 for any of the pathways. Hematological biomarkers not correlated with pathways or benzene metabolites included lymphocytes and mean corpuscular volume, while HYPO8 was the only chromosomal measure not present. Community detection analysis identified four clusters, which were largely based upon benzene metabolite compartment, and measurements of hematologic or genomic instability measures. The first cluster, which included WBC and reactive benzene metabolites detected using HRM, clustered with fatty acid pathways, and glycerophospholipids. Cluster 2 consisted of chromosomal damage measures HYPR8, HYPR21, SCA821 and SCA21, and urinary benzene metabolites. Pathways associated with this cluster included amino acid metabolism pathways related to oxidative stress, nucleotide metabolism, and microbiome pathways. Cluster 3 was the smallest in the network, and included a catabolic amino acid pathway correlated with both known and predicted metabolites of benzene measured using HRM, as well as two bioeffect measures of genomic instability. The fourth cluster, which included urinary levels of benzene and a predicted hydrolysis metabolite measured in blood, included the largest number of pathways. Hematological measures included HGB and RBC, which are highly correlated, and PLT. The pathways present within this cluster are from a variety of biological processes, including BCAA metabolism, mitochondrial energy production and carnitine metabolism, and carbohydrate metabolism. Of all clusters, HGB, RBC, PLT, urinary benzene and the predicted hydrolysis metabolite showed the

Table 3. Fatty acid oxidation metabolites associated with benzene exposure

Metabolite annotation	Adjusted r^2	β (95% confidence interval)	*q*
	Carnitine shuttle		
Tetradecanoyl carnitine	0.28	0.34 (0.19 to 0.48)	0.033
Palmitoylcarnitine	0.24	0.29 (0.16 to 0.42)	0.035
Vaccenyl carnitine	0.26	0.31 (0.17 to 0.45)	0.035
Hexadecenoyl carnitine	0.23	0.31 (0.17 to 0.45)	0.035
Carnitine	0.30	0.4 (0.2 to 0.6)	0.057
Clupanodonyl carnitine	0.29	0.2 (0.1 to 0.31)	0.078
Heptadecanoyl carnitine	0.24	0.46 (0.19 to 0.73)	0.16
Stearoylcarnitine	0.21	4.35 (1.76 to 6.93)	0.18
	Fatty acid metabolism		
Dihomo-gamma-linolenic acid	0.24	0.49 (0.28 to 0.7)	0.017
Octanoic acid	0.28	0.16 (0.09 to 0.24)	0.035
Docosahexaenoic acid	0.22	0.43 (0.22 to 0.64)	0.033
Oleic acid	0.15	0.16 (0.07 to 0.24)	0.078
Butyric acid	0.20	0.26 (0.12 to 0.41)	0.068
Stearidonic acid	0.16	0.53 (0.23 to 0.83)	0.075
Arachidonic acid	0.14	0.31 (0.13 to 0.49)	0.076
Eicosapentaenoic acid	0.18	0.4 (0.17 to 0.63)	0.084
9(S) or 13(S)-HPOT	0.07	4.08 (1.2 to 6.97)	0.17
Arachidic acid	0.21	3.62 (1.06 to 6.18)	0.17
Stearic acid	0.11	0.24 (0.07 to 0.41)	0.18
Linoleic acid	0.17	−2.36 (−4.06 to −0.65)	0.19
Palmitic acid	0.06	0.33 (0.09 to 0.57)	0.19

largest number of correlated pathways, which include 26, 26, 19, 18 and 24, respectively. Interestingly, the predicted metabolite in cluster 4 exhibit the highest degree of pathways correlations of all other benzene metabolites. While separate clusters were identified using the community detection algorithms, it is important to recognize the high-degree of connection within this network, most likely due to the moderate to high correlation between the different exposure and bioeffect measures.

Discussion

Benzene is a recognized hematotoxin and has been associated with increased risk of leukemia and other blood disorders; however, the metabolic changes linking exposure to disease in humans is uncertain (7). In this study, we applied untargeted HRM to identify metabolic effects of benzene exposure workers with relatively high exposures, well above the current standards in both China and the USA. Metabolic alterations associated with benzene were consistent with fatty acid oxidation, energy metabolism and increased oxidative stress, suggesting exposure can influence changes in metabolic pathways that have been linked to HSC control and mitochondrial dysfunction. Integration of these pathways with measures of benzene exposure, hematologic measures and chromosomal aberrations provide additional evidence of the role of these pathways in benzene toxicity, and provide new insight into potential metabolic processes underlying benzene exposure and disease risk. While exposure levels experienced by all workers are higher exposures than those experienced by the general public, the use of this population can provide key insight into systems biology-based biomarkers of benzene exposure, disease risk factors and early biological effects of exposure.

Through MWAS, we identified metabolic features that showed an exposure-response relation with occupational benzene exposures. Results of the untargeted analysis provided accurate mass matches to unmetabolized benzene and several benzene metabolites, including phenol, benzene diol epoxide, EE-muconic acid, catechol, sulfation products and four hydrolysis metabolites. To our knowledge, this is the first report that benzene diolepoxide has been detected in human serum/plasma and reinforces the finding of a benzene diolepoxide adduct of Cys34 in serum albumin from benzene-exposed workers (4). Unmetabolized benzene was higher in the control group compared to low exposed workers, which was unexpected due to the high levels of exposure. This result could be due to the increased variability present when using HRMS to detect low level, environmental compounds, and could be further affected by poor suitability of HRM methods for detection of volatile compounds. Previously, biomonitoring studies using benzene metabolites have measured these compounds in urine (2,18,28), which may provide a more accurate representation of occupational exposure burden than measuring metabolites in blood.

Model systems studies showed benzene toxicity to mitochondria (6,29), and human studies have demonstrated that occupational exposure is associated with increased mitochondrial DNA copy number (30). Formation of reactive quinones and increased reactive oxygen species (ROS) is one mechanism suspected to underlie benzene toxicity, with mitochondria a major intracellular source and primary target of ROS effects. Results from Cys34 adductomics in benzene exposed and control workers showed increased levels of Cys34 oxidation product, derived from ROS, and adducts of reactive carbonyl species derived from peroxidation of microsomal lipids (4). In the present study, pathway enrichment analysis provided considerable evidence for benzene-associated effects on mitochondrial function and oxidative stress. Combined results for both columns showed that 11 fatty acid pathways were altered by benzene exposure. Among these, the carnitine shuttle is essential for mitochondrial fatty acid transport. Because fatty acids are needed for HSCs and control of stem cell self-renewal (31), the results imply that benzene exposure could cause hematotoxicity through impairment of mitochondrial fatty acid metabolism and related bioenergetics. This interpretation is supported by widespread effects on glycolysis and hexose phosphates, and mitochondrial

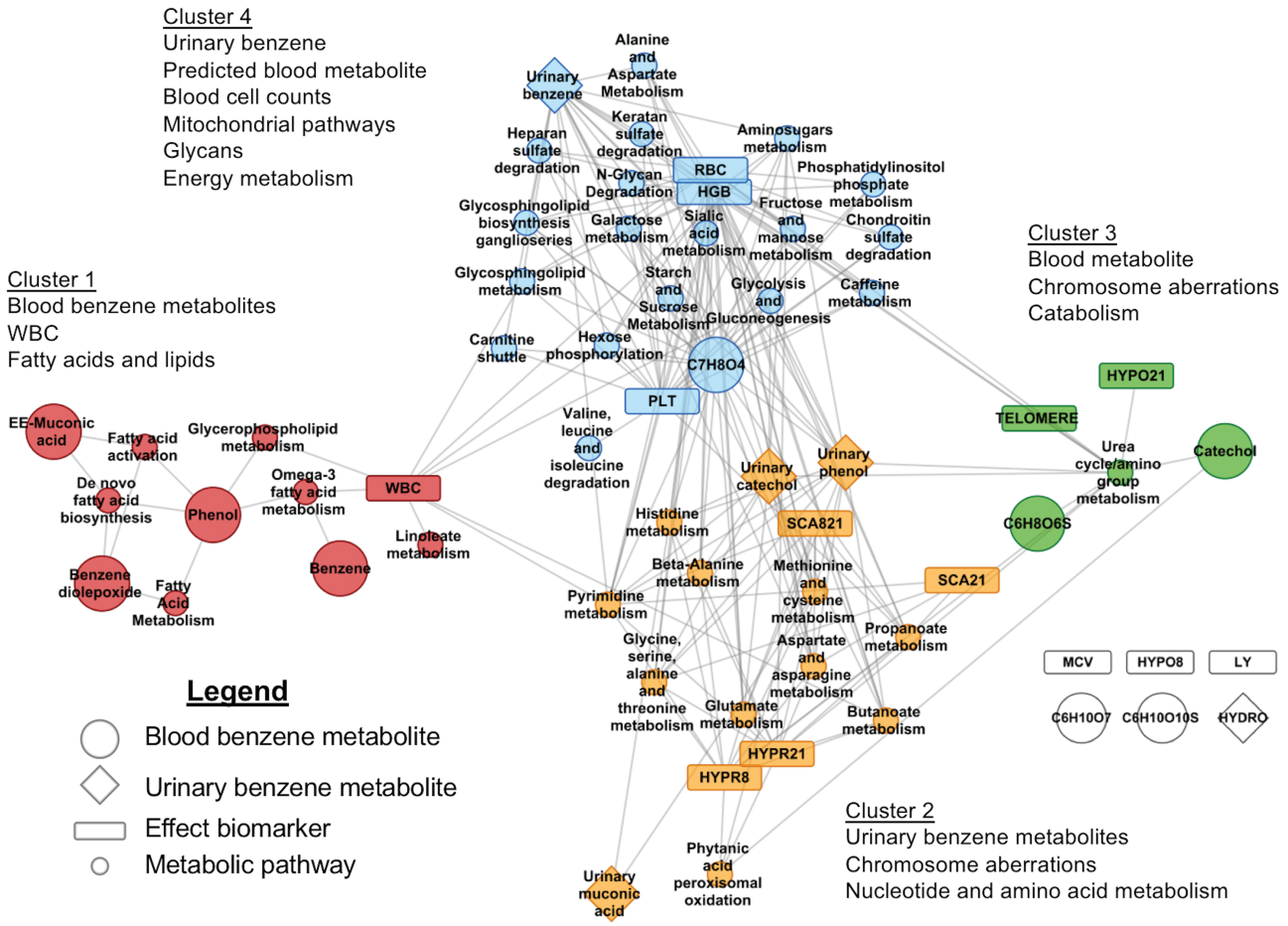

Figure 3. Correlation network analysis of benzene metabolites detected in blood and urine, hematologic cell counts and chromosomal measures, and PC1 for metabolic pathways that were associated with benzene exposure from the MWAS analysis. Community-based cluster detection was used to identify closely related nodes and identify potential relationships between benzene metabolites, metabolic pathways, and biological response measures previously characterized in this cohort, and is indicated by node color. Unfilled nodes were not correlated with any of the pathways at $|r| \geq 0.3$ and $P < 0.05$. Circle, HRM detected benzene metabolite in blood; diamond, urinary benzene metabolite, rectangle, metabolic pathways associated with benzene exposure; HGB, hemoglobin; RBC, red blood cell count; LY, lymphocyte count; MCV, mean corpuscular volume; WBC, white blood cell count; PLT, platelet count; HYPO8, hypoploidy rate for chromosome 8; HYPO21, hypoploidy rate for chromosome 21; HYPR8, hyperploidy rate for chromosome 8; HYPR21, hyperploidy rate for chromosome 21; SCA8,21, total structural aberrations chromosomes 8;21; SCA21, total structural aberrations chromosome 21.

pathways of BCAAs and butanoate metabolism. Peripheral blood gene expression of benzene exposed workers exposed to lower levels than the present study has also identified association with gene ontology categories related to apoptosis, adenosine triphosphate synthesis coupled proton transport and cholesterol transport, while gene expression pathway enrichment identified changes in oxidative phosphorylation, apoptosis, fatty acid metabolism and nucleotide excision repair (32). Together, the results provide evidence that mitochondrial dysfunction is a central response to benzene exposure.

Decreases in the antioxidants taurine and methionine, in addition to elevated cystine (CySS) further support increased oxidative stress. Taurine is an oxidized form of hypotaurine, which functions as a protective antioxidant through oxidation of the sulfinic group (33). Methionine is active in immune regulation, polyamine metabolism, DNA methylation (34) and glutathione formation, which is one route for detoxification of reactive benzene quinones. (3) In humans, the major extracellular thiol/disulfide redox couple is cysteine and its disulfide form, CySS (35). Increased oxidation of this couple has been shown to disrupt HSC quiescence, which has the potential to exhaust the HSC pool and causes hematologic failure (36). Decreased repopulating capacity of human HSCs has been observed to be associated with increased ROS and DNA damage; the oxidative DNA damage increases expression of cell-cycle inhibitors, causing HSCs to undergo premature senescence and consequently leading to the functional impairment of HSCs (37). Observed decreases in linoleic acid are also consistent with oxidative stress-related changes. Polyunsaturated fatty acids are susceptible to lipid peroxidation, which occurs through oxidant attack at carbon-carbon bonds (38). Elevated levels of hydroxylinoleic acid metabolites support increased formation of ROS associated with benzene, where lipid peroxidation products are easily reduced to corresponding hydroxy acids. While these results suggest oxidative stress is a key biological response pathway to benzene exposure, changes in oxidative stress profiles have been linked to a wide range of environmental exposures not associated with increased risk of hematologic diseases. Therefore, the combination of additional toxic effects, such as reactive benzene metabolite inhibition of TOPO-1 mediated DNA repair, may contribute to the hematotoxic properties of benzene.

Eight metabolites from carnitine shuttle and 14 fatty acid metabolites were associated with benzene exposure. Animal models investigating the effects of benzene on changes in

expression of key enzymes in the fatty acid oxidation pathway showed benzene alters expression of carnitine fatty acid transport and β-oxidation enzymes, resulting in mitochondrial dysfunction, and oxidative stress, in addition to decreased levels of BM carnitine (39). As discussed above, fatty acids are needed for HSCs and control of stem cell self-renewal, with the promyelocytic leukemia-peroxisome proliferator-activated receptor δ (PPAR-δ)-fatty acid oxidation pathway controlling HSC cell fate through the regulation of HSC asymmetric division (31). Inhibition of this pathway or mitochondrial fatty acid oxidation induces loss of HSC maintenance and has been shown to significantly decrease the number of quiescent leukemia progenitor cells in primary human acute myeloid leukemia samples (40). In a metabolomic study of BM cells and plasma collected from mice exposed to benzene, acetyl-carnitine was negatively associated with exposure in BM cells, in addition to plasma acetyl-carnitine and palmitoyl-carnitine (41). In a follow-up study, the authors found that acetyl-carnitine intervention can partially reduce benzene damage to BM and decrease DNA damage by reducing benzene-induced H_2O_2 and ROS (42). Although not meeting the FDR threshold of 20% in the present study, acetyl-carnitine did show a positive relationship with exposure at $P = 0.003$. Alterations in carnitine and related fatty acid oxidation pathways were also identified in metabolite profiles of benzene-exposed workers with low WBC counts (43). In this study, the authors used untargeted HRMS to characterize metabolomic differences in exposed workers and unexposed controls. Carnitine, which showed a positive significant association with exposure in the present study, was identified as one of the significant metabolites downregulated in benzene exposed workers with low WBC counts. Additional evidence of changes in fatty acid oxidation were also present, including alterations in gene expression related to mitochondrial fatty acid transport and fatty acid β-oxidation. Taken together the results suggest benzene exposure results in altered fatty acid transport and oxidation. In combination with recent studies identifying the carnitine system as a possible source of metabolic flexibility for cancer cells, this could represent a mechanism underlying the relationship between benzene exposure and hematopoietic cancers.

To visualize the relationship between exposure, bioeffect markers and metabolic alterations associated with benzene, we applied correlation network analysis for samples from exposed workers only. Integrating exposure measures and bioeffect markers linked to disease outcomes with MWAS has the potential to enhance understanding of how functional molecular mechanisms interact due to environmental exposures The results from this network analysis further support benzene-induced alterations in oxidative stress metabolism and HSC proliferation. Methionine and cysteine metabolism was correlated with benzene metabolites suggesting changes in antioxidant defense pathways. Pyrimidine metabolism, which is a key pathway for DNA replication and RNA synthesis, was correlated with benzene and phase I metabolites. Uncontrolled growth of tumors has been linked to increased nucleotide metabolism, while pyrimidine catabolism induces terminal differentiation toward monocytic lineage for controlling aberrant cell proliferation in leukemic cells (44). Benzene metabolites were further correlated with glycolysis and bioenergetic pathways, signaling lipids and amino acid metabolism, providing additional evidence that benzene exposure results in dose-associated changes to a wide range of metabolic processes. Clustering analysis suggests individual benzene metabolites may result in pathway-specific metabolic changes, with benzene metabolites measured in blood present in one cluster, while urinary benzene and urinary benzene metabolites were present in two separate clusters. Differences in benzene metabolite clustering could be due metabolic effects at the site of benzene metabolism, or from differences in exposure time scale measure in blood or urine. Future studies with longitudinal sampling would be needed to further investigate how benzene metabolism alters the metabolome.

Network correlations for bioeffect markers and metabolic pathways were consistent with suspected mechanisms for benzene toxicity. Hyperploidy rates for both chromosomes and structural aberrations were clustered with pathways related to nucleotide metabolism, oxidative stress and amino acids. Hyperploidy has previously been linked to benzene exposure and is associated with defects in mitotic spindle assembly and dynamics, centrosome amplification, cell-cycle regulation, chromatid cohesion and telomere metabolism (45). These defects may be caused by mutation, epigenetic dysregulation, or altered expression of the genes involved in these processes. Aneuploidy has also been observed in rapid proliferation of HSC following depletion of BM cells (46) and could be a marker of HSC-related cell proliferation after benzene exposure, which is consistent with decreased blood cell counts observed in exposed workers. As discussed above, nucleotide metabolism contributes to control of cell proliferation, which is consistent with the presence of pyrimidine in this cluster. Increased oxidative stress has also been observed to override spindle checkpoint-dependent arrest (47). Additional fatty acid pathways were clustered with WBCs. These results support a potential role in benzene related alterations to these pathways, and loss of HSC maintenance. The two remaining clusters included blood cell counts and telomere length; as expected, the highly correlated measure of HGB, PLTs and RBCs cells clustered together and showed additional enrichment in glycolysis, additional glycation pathways carnitine metabolism and BCAAs, suggesting benzene exposure results in alterations to bioenergetic pathways for these cell types.

We acknowledge some limitations in this work. First, this is a limited sample population of 58 individuals. However, this population was exposed to relatively high level of benzene that was characterized in detail by personal air monitoring and has been linked to substantial alterations in several known or established biomarkers related to benzene exposure (14). Second, this study was focused on known benzene and endogenous metabolites because the limited sample availability precluded in-depth structural characterization of non-identified metabolites. Third, benzene toxicity is well-recognized to occur within the BM niche. By measuring metabolites in blood, we cannot separate contributions from other organ systems and effects of metabolic adaption in other tissues. Future studies that apply these methods to benzene exposure within the BM niche will be needed to determine if the metabolic effects identified in this study are likely to directly contribute to relevant benzene-induced biological changes. Fourth, samples were collected and stored at −80°C for 30 years prior to analyses. While systematic evaluation of metabolite stability over this timeframe is not possible, studies of blood samples collected >50 years prior to HRM analysis show consistent storage at −80°C is sufficient for maintaining sample integrity. Blood samples were collected from exposed and unexposed workers within the same geographical region and timeframe using identical clinical procedures and stored continuously at −80°C. Thus, we do not expect differences in sample integrity among exposed or unexposed workers, nor would those differences be expected among exposed workers experiencing low and high exposures.

The results of this observational study are correlative in nature, and cannot be assumed to represent direct causal associations. While it is not possible to account for all unknown confounders, such as differences due to diet, exposures outside the workplace, diurnal variation and unmeasured co-exposures, the participant enrollment was designed to reduce the influence of confounding factors. Participants were enrolled from factories within the same geographical regions and comprehensive exposure monitoring was employed to identify locations with minimal exposure to other VOC's, controls were frequency-matched on age (<5 year interval) and gender, and fasted blood samples were collected during the morning of the clinical visit. By identifying metabolites showing significant associations with benzene exposure levels, we further reduce the possibility that effects were due to unknown confounders, although this does not rule out false-positive findings that can occur by chance. As such, it is important to replicate these findings in populations with comparable benzene exposure to this study population as well as among populations with exposure levels < 1 ppm to benzene to determine if the observed effects are present, or other alternations are identifiable. Despite these limitations, the results from this study provide important and novel insights into metabolic effects that may contribute to toxicological mechanisms underlying benzene exposure and identify new pathways for testing in model systems.

Conclusion

Untargeted HRM profiling identified metabolic changes suggesting benzene exposure induces oxidative stress and alters pathways related to mitochondrial dysfunction and HSCs. In addition, molecular markers previously shown to be associated with benzene exposure were correlated with endogenous metabolic processes related to hematopoietic diseases and cancer. The results, therefore, show that HRM provides a useful approach to link occupational exposures to metabolic perturbations and obtain insight into mechanisms underlying environmental contributions to disease.

Supplementary material

Supplementary data are available at *Carcinogenesis* Online.

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Conflicts of interest

M.T.S. has received consulting and expert testimony as a consultant and expert witness in legal cases involving exposure to benzene and its relationship to the development of hematopoietic malignancies on behalf of the plaintiffs. The other authors declare they have no competing financial interests.

Data availability statement

Participant covariates and untargeted metabolomics data underlying this article are available on the Metabolomics Workbench (Study ID: ST001918; DOI: http://dx.doi.org/10.21228/M8F70B).

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