

Heavy metals interfere with plasma metabolites, including lipids and amino acids, in patients with breast cancer

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Abstract. The aim of the present study was to examine the association between plasma heavy metals and the metabolome in patients with breast cancer (BC), and the association with cancer development. Nuclear magnetic resonance was used to determine the metabolites involved and an inductively coupled plasma mass spectrometry system was used to quantify the heavy metals in the plasma samples. It was indicated that cadmium was significantly higher in the plasma of patients with BC compared with that in the control population (~15-fold increase). Chromium, arsenic and lead were also elevated in the plasma of patients with BC by ~3.24, 2.14 and 1.52 fold, respectively. A number of small molecules, including amino acids and salts, were altered in the plasma of patients with BC compared with the control population. Another notable finding in this investigation was that plasma lipid levels were elevated in patients with BC compared with those in the control population. The findings of the present study suggest that exposure to heavy metals, including cadmium, arsenic, chromium and lead, may influence blood lipid levels and other small molecule metabolites, which in turn may be involved in BC development. Further studies surrounding urinary heavy metals and the metabolome are required to further determine the impact of metals on metabolism and on BC development.

Introduction

Breast cancer (BC) is common in women and is one of the main causes of cancer-associated mortality in the female population worldwide, with an incidence rate of 1 in 8 women (13%) (1-3). Environmental contamination serves a vital role in cancer

development, as well as other diseases, including endometrial cancer and endometriosis (4,5). Reports show that >20% of global disease burden and >30% of disease burden in children is due to contaminated environments (2,3,5). Therefore, it has been speculated that a healthy environment would prevent or decrease the incidence of many diseases/disorders and ultimately reduce morbidity (4). A comprehensive understanding of the mechanisms involved in the etiology of BC and the identification of new biomarkers of its risk are key components for the improvement of BC prevention (6). Even though genetic modifications for BC have been widely investigated, further advancements are required to uncover the metabolic changes associated with this disease (7,8).

Metabolomics (one of the newest 'omics') is a rapidly developing branch of science and medicine aimed at identifying biomarkers for a number of human diseases or disorders. It has assisted in further understanding the underlying mechanisms of cancers and therefore treatment strategies (9). The pathophysiological status of biological systems can be reflected by changes in the metabolome, which may be owing to genetic alterations in metabolic pathways or changes in catabolism and enzymes activities (9,10). The metabolome is an amplified culmination of biological systems, as small alterations in enzyme activities may result in major changes in metabolite levels (10). Lipids are reported to be risk factors for BC reoccurrence and development (11,12). Amino acids have also been reported to be associated with BC development (13,14). Nuclear magnetic resonance (NMR) spectroscopy has been extensively used in metabolome studies, due to its ability to detect metabolites in intact tissues and even in *in vivo* (15). There are many advantages for NMR spectroscopy, such as the following: No need for sample purification; both hydrophilic and hydrophobic metabolites can be detected; quantitative analysis can be performed; a fast method (1-dimensional techniques, excluding solid-state NMR); small amounts of sample are required; a non-invasive and non-destructive method; and high reproducibility (9). Due to the abundance of hydrogen in nature (>99.98%), low relaxation time and an appreciable nuclear spin, proton nuclear (¹H) NMR is the most popular NMR technique applied in metabolome investigation (16); it can be used to detect metabolomic changes in cells, tissues

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or biofluids (17,18) and to provide novel insights into disease etiology or underlying mechanisms (7,19-21).

The metals chromium (Cr), cobalt (Co), copper (Cu) and nickel (Ni) are important trace elements for humans, since they are components of enzymes. However, at high concentrations the metals can cause serious issues, such as disease or toxicity, owing to their inhibition of enzyme activity (22-25). Some other non-essential heavy metals, such as cadmium (Cd), lead (Pb), mercury (Hg) and tin (Sn), are toxic at high levels, as they can block the functions of other essential metals (26). In addition, in natural conditions these elements cannot be decomposed, or may even be bioaccumulated and biomagnified in food chains (27,28). It has been reported that Cd, Cr, Ni, Cu, Pb and Hg are carcinogens (29,30). Furthermore, it has been recorded that Cd, Cr, Ni, Cu, Co, Pb and Hg can cause lung cancer; Cr can increase the probability of liver, larynx, esophageal, and gastrointestinal cancer; Cd and Ni can result in renal and prostate cancer; Cu can cause non-Hodgkin's lymphoma or skin cancer; Pb and Hg may increase the risk of glioma and stomach, prostate or bladder cancer; and Ni, Pb, Hg, and Cr (VI) may cause breast sarcoma and carcinoma (29,30). Environmental exposure to heavy metals may be mainly through the food chain, smoking and even drinking water (31,32). Heavy metal anthropogenic contamination comes mainly from power industries, waste deposits and even fertilizers (33).

Some heavy metals, such as Cd, have been found at high levels (20-30 $\mu\text{g/g}$ tissue) in breast tissue (34). Furthermore, heavy metals can accumulate in breast tissue, cause DNA damage and even increase tumor development (35). However, to the best of our knowledge, the association between plasma heavy metals and the metabolome in patients with BC is unknown, as is the association between plasma heavy metals and the metabolome in BC development. Therefore, the present investigation aimed to examine the metabolome and heavy metals present in the plasma of patients with BC at first diagnosis.

Materials and methods

Patients and plasma sample collection. Plasma samples from female patients with malignant BC ($n=105$; 50.22 ± 9.83 years) and age-matched healthy female controls ($n=35$; 49.76 ± 10.07 years) were collected from the Affiliated Tengzhou Central People's Hospital of Jining Medical University (Jining, China) between November 2017 and May 2018. Written informed consent was obtained from the patients and controls in the present study. The patients with BC and the control population were from the same local area of Tengzhou. Heavy metal contamination is relatively high in this area owing to the number of mining operations. The study was performed in accordance with the standards of the Institutional Ethical Committee and the Helsinki Declaration of 1975, as revised in 1983, and was approved by the Institutional Review Board of the Affiliated Tengzhou Central People's Hospital of Jining Medical University. The patients were chosen based on the following criteria: i) All patients were female; ii) all patients received positive pathology for BC; iii) all patients were in the early stages of BC (stages I-II), according to the clinical Tumor-Node-Metastasis staging method (6,7); iv) no patients

received pre-operative treatment, including adjuvant chemotherapy or radiotherapy; and v) patients did not have diabetes or any other diseases. The selected healthy controls included age- and sex-matched healthy subjects with no metabolic diseases and who were confirmed to have no breast lesions following a physical examination followed by mammography and breast ultrasonography. Prior to surgery in the patients and following overnight fasting for all subjects, 10 ml of venous blood was collected from each subject in a vessel tube, containing heparin as the anticoagulant, and was subsequently centrifuged ($1,500 \times g$ for 15 min at 4°C) to collect clear plasma. The plasma was then transferred into sterile vials and immediately stored at -80°C until further analysis.

NMR spectroscopy. Nuclear magnetic resonance (NMR) analyses were performed as described previously (36,37). Briefly, prior to the NMR spectroscopy, 200 μl of plasma sample was mixed with 80 μl D_2O solution containing sodium phosphate buffer (0.1 M, pH 7.4) and sodium 3-trimethylsilyl-2,2,3,3-d₄-propionate as an internal standard ($\delta=0$ ppm). The ^1H NMR spectra was acquired using a 600.13 MHz Bruker AV600 spectrometer (Bruker Corporation) with a 5-mm CryoProbe at 300 K. Nuclear Overhauser effect spectroscopy and a zg pulse sequence of ^1H NMR spectra and zgpg30 pulse sequence of J-resolved NMR spectra were used to acquire the NMR information (38). The low molecular weight metabolite (LMWM) model and the lipid molecules in lipoprotein particles (LIPO) model were used in this study, as previously described (37). The LIPO model provides information on lipoprotein lipids, and subclasses, which are acquired through the water-suppressed ^1H NMR spectrum. Alternately, the LMWM model suppresses most of the broad macromolecules and lipoprotein lipid signals, therefore improving the sensitivity of low molecular weight metabolites (37-41).

NMR spectral processing and analysis. The ^1H NMR spectra were processed by MestRe-C software (version 3.0; Mestrelab Research) as described previously (36,38). Briefly, the spectra were binned with a unit of 0.005 ppm between 0.2 and 10.0 ppm, and then integrated spectral intensity for each bin. The binned data were adjusted by generalized log transformation and mean-centered prior to multivariate analysis.

Multivariate analyses. The processed NMR datasets were examined by principal component analysis (PCA) and partial least squares discriminant analysis using the SIMCA-P10.0 10.0 software package (MKS Umetrics AB), as previously described (36,38).

Mineral element quantification. Determination of tissue mineral elements was performed as previously described (42,43). Briefly, 0.2 ml aliquots of plasma were transferred to 120-ml Teflon digestion vessels, followed by the addition of 5 ml of nitric acid. Analysis of metals in plasma was preceded by microwave digestion with concentrated nitric acid to destroy organic matter and mineralize the sample. The multi-element calibration standard was provided as 10 mg/l in 5% nitric acid and was not in the plasma matrix. An Agilent 7500 (Agilent Technologies, Inc.) inductively coupled plasma mass spectrometry system was used for simultaneous

determination of Cd, Cr, Arsenic (As), Pb and Hg. Positive ionisation mode was used (38). The voltage for the ion lens was set at 6 V; the argon gas flow rate in the spray chamber was 0.88 l/min; the power output for the RF generator was 1,100 W; the auxiliary gas flow rate was 1.2 l/min; and the nebulizer gas flow rate of the plasma was 16 l/min at room temperature. All the certified reference materials (in solution) were purchased from the National Institute of Metrology. Blank controls (n=3) underwent the same procedures (38).

Statistical analysis. Data were statistically analyzed with SPSS statistics software (version 22; IBM Corp.) and Student's t-test. Differences were compared for every parameter and data are presented as the mean \pm standard deviation. $P < 0.05$ was considered to indicate a statistically significant difference (38).

Results

Baseline characteristics of the study population. Table I shows the baseline characteristics of the patients with BC and the control population. There was no significant difference between the mean age of the patients with BC (50.22 \pm 9.83 years) and the control population (49.76 \pm 10.07 years). Similarly, there was no significant difference for average body mass index. Following diagnosis with BC at stages I or II, blood samples were drawn from the patients for analysis.

Plasma metabolome changes. The LMWM ^1H NMR spectra, the metabolic fingerprints of small molecules, from plasma metabolites of patients with BC and the control group are presented in Fig. 1A; while the LIPO ^1H NMR spectra, the metabolic fingerprints of large molecules, from plasma metabolites of patients with BC and the control group are presented in Fig. 2A. Chemical shift and peak multiplicity were used to assign the specific plasma metabolite (37-41).

The latent biochemical information from the ^1H NMR spectra were analyzed by partial least squares discriminant analysis. For the small molecules in the LMWM model, there was a clear separation between patients with BC and the control population based on score plots (Fig. 1B). For large molecules in the LIPO model, the score plots also indicated a distinct difference between patients with BC and the control population (Fig. 2B).

Numerous alterations in endogenous metabolites were discovered in the ^1H NMR spectra of plasma samples in both the LMWM and LIPO models. Fig. 3 shows the prominent small molecules, whose presence was greater in patients with BC in the LMWM model. Compared with the control population, six metabolites, namely leucine, propylene glycol, citrate, β -glucose, acetate and phenylalanine, were significantly elevated in patients with BC (Fig. 3). There were seven metabolites, namely arginine, glutamate, trimethylamine, lysine, α -glucose, tyrosine and histidine, with significantly decreased levels in patients with BC compared with that in the control group (Fig. 4).

It is noteworthy that the prominent large molecules (lipids or lipoproteins) were different in the LIPO model analysis between patients with BC and the control population. The levels of all prominent large molecules were increased (Fig. 5). These large molecules were high-density lipoprotein (HDL), HDL

Table I. Baseline characteristics of the study population.

Characteristics	Patients with BC (n=105)	Control group (n=35)
Mean age \pm SD, years	50.22 \pm 9.83	49.76 \pm 10.07
Mean BMI \pm SD, kg/m ²	24.32 \pm 3.39	24.11 \pm 3.89
Normal (18.5-24.9), n (%)	67 (63.81)	25 (71.43)
Preobese (25-29.9), n (%)	37 (35.24)	10 (28.57)
Obesity (\geq 30), n (%)	1 (0.95)	0 (0.00)
Stage, n (%)		
I	65 (61.90)	
II	40 (38.10)	

BMI, body mass index; BC, breast cancer.

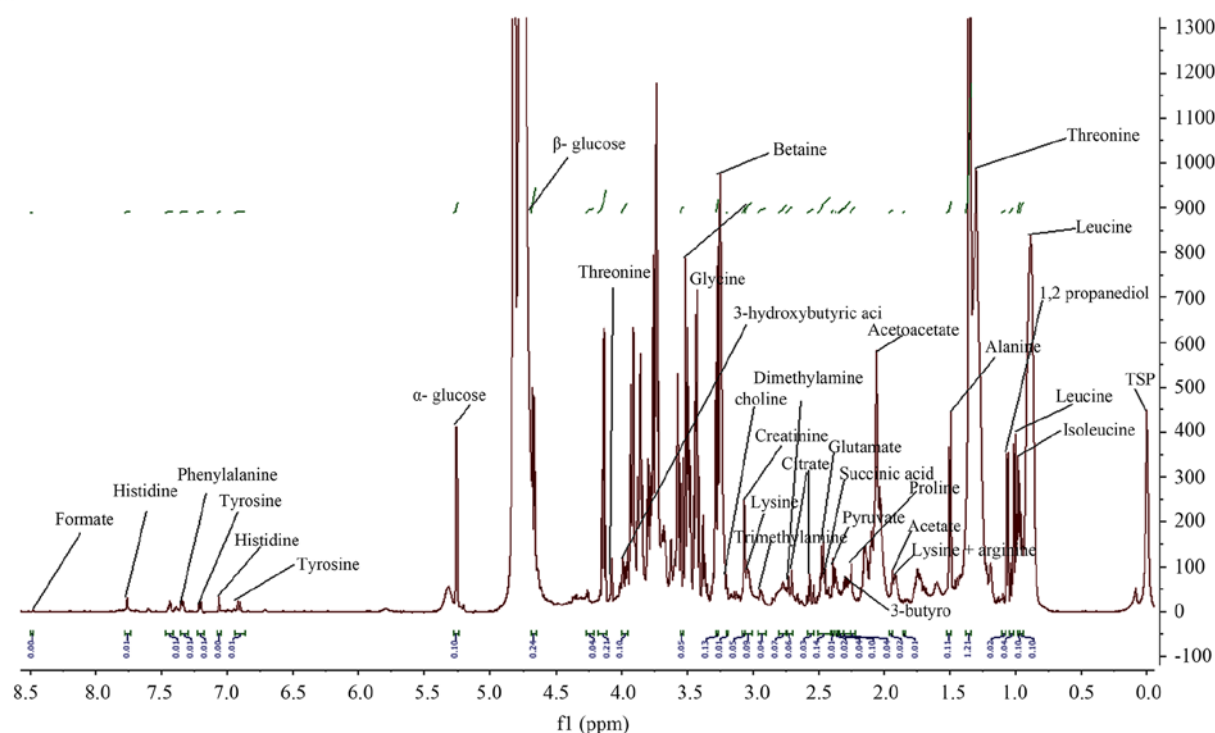
low-density lipoprotein (LDL) very low-density lipoprotein (VLDL)(-CH₃), lipid-CH₂-C=C-, lipid-C=C-CH₂-C=C- and lipid-CH=CH-. In addition, HDL was significantly elevated in patients with BC.

Levels of five heavy metals in the blood. Five common heavy metals (those found at the highest levels in Tengzhou) were measured in the blood samples, including Cd, Cr, As, Hg, and Pb. The five heavy metals in order of relative concentrations ($\mu\text{g/l}$) from low to high were as follows: Cd, Hg, As, Pb and Cr. A total of 4 heavy metals, including As, Cd, Cr and Pb, were significantly increased in patients with BC compared with levels in the control population (Fig. 6). As showed the most significant ($P = 2.48 \times 10^{-9}$) increase in patients with BC. Cd showed the greatest increase (14.91 fold) of the metals in patients with BC following by Cr (3.24 fold), As (2.14 fold) and Pb (1.52 fold).

Discussion

BC is a common type of cancer affecting women worldwide. A number of factors are associated with BC development, including genetic background, diet, lifestyle, obesity, smoking, alcohol consumption and environmental contamination (44-47). Contamination of the local environment serves a vital role in BC development. Environmental metallic compounds have been identified as risk factors for development of BC (48) and a number of heavy metals have been reported to be risk factors for numerous types of cancer, including stomach and liver cancers (49-52). The World Health Organization has classified As, Cd and Ni as Group 1 human carcinogens (53). Pb, Hg and Cr have been established as human and animal carcinogens or cocarcinogens (29,30). In the present study, it was found that Cd levels were higher in patients with BC compared with those in the control population (15 fold); at the same time, Cr, As and Pb were also elevated in patients with BC by 3.24, 2.14 and 1.52 fold, respectively. This suggests that these four heavy metals may be involved in BC development. Patients with BC, in addition to the control population, were all situated in a local region area with many mines and where heavy metal environmental contamination has been reported to be high.

A



B

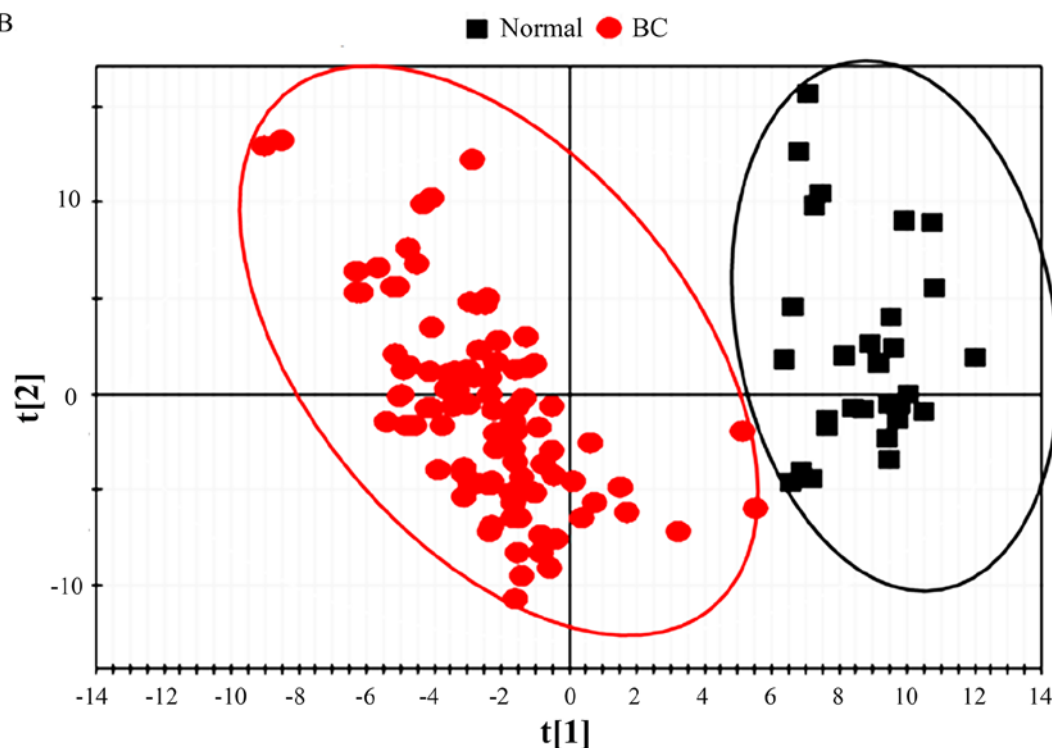


Figure 1. ^1H NMR analysis of small molecule metabolites. (A) NMR spectral characteristics and the metabolic contents of the LMWM model analysis. (B) Score plot of the principal component analysis model from the analysis of ^1H NMR spectra of plasma for LMWM from patients with BC and the control population. NMR, nuclear magnetic resonance; LMWM, low molecular weight metabolite; BC, breast cancer; TSP, trimethylsilyl propionate.

It has been reported over the last 10 years that heavy metals cause a number of issues, such as immunodeficiency, osteoporosis, neurodegeneration, organ failure and cancer (22). Previous studies have also reported potential associations between heavy metals and estrogen-dependent disorders,

including pre-term deliveries, spontaneous abortions, endometrial cancer and BC (22-24). The general population is mainly exposed to heavy metals through environmental contamination. Sources of environmental Cd exposure to the general population include cigarette smoking, dietary sources and

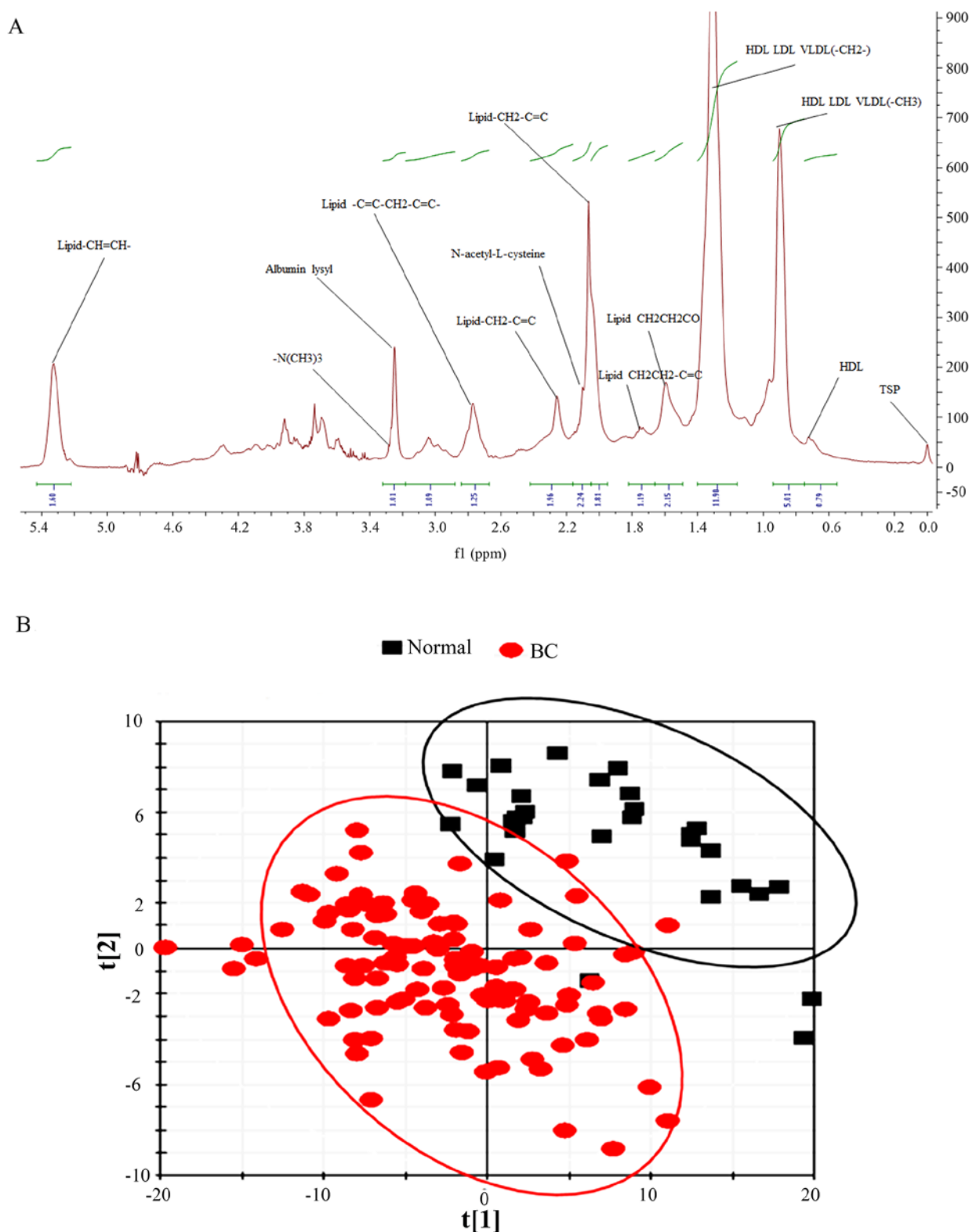


Figure 2. ¹H NMR analysis of large molecule metabolites. (A) NMR spectral characteristics and the metabolic contents of the lipoprotein lipid and albumin model analysis. (B) Score plot of the principal component analysis model from the analysis of ¹H NMR spectra of plasma for large molecules from patients with BC and the control population. NMR, nuclear magnetic resonance; BC, breast cancer; HDL, high-density lipoprotein; LIPO, lipid molecules in lipoprotein particles; LDL, low-density lipoprotein; VLDL, very low-density lipoprotein; TSP, trimethylsilyl propionate.

drinking water (31,32). Cd has been found in surface water and even ground water (54). Meanwhile, epidemiological studies have found potential associations linking Cd exposure and BC development (22-24). It is reported that Cr exposure is a

risk factor for BC development (55). In addition, Cr has been identified as a potential risk factor for lung cancer, and cancer of the buccal cavity, pharynx, esophagus and NHL, exclusively in women who smoke tobacco, drink Cr-laden water and eat

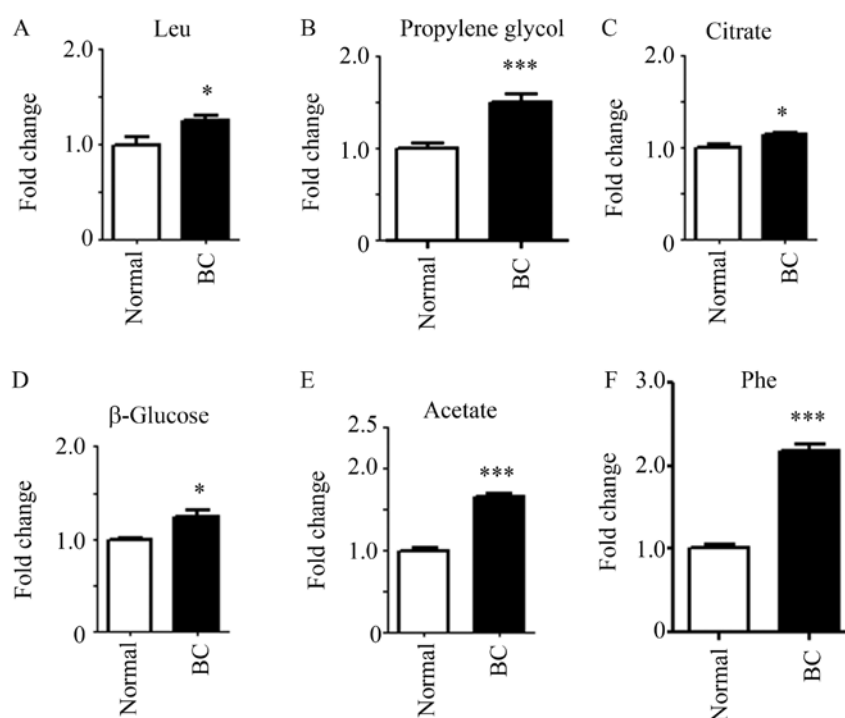


Figure 3. Small molecule metabolites elevated in the plasma of patients with BC. (A) leucine, (B) propylene glycol, (C) citrate, (D) β-glucose, (E) acetate and (F) phenylalanine in the plasma of patients with BC and the control population. n=35 for control population; n=105 for patients with BC. *P<0.05 and ***P<0.001 vs. normal. BC, breast cancer; Leu, leucine; Phe, phenylalanine.

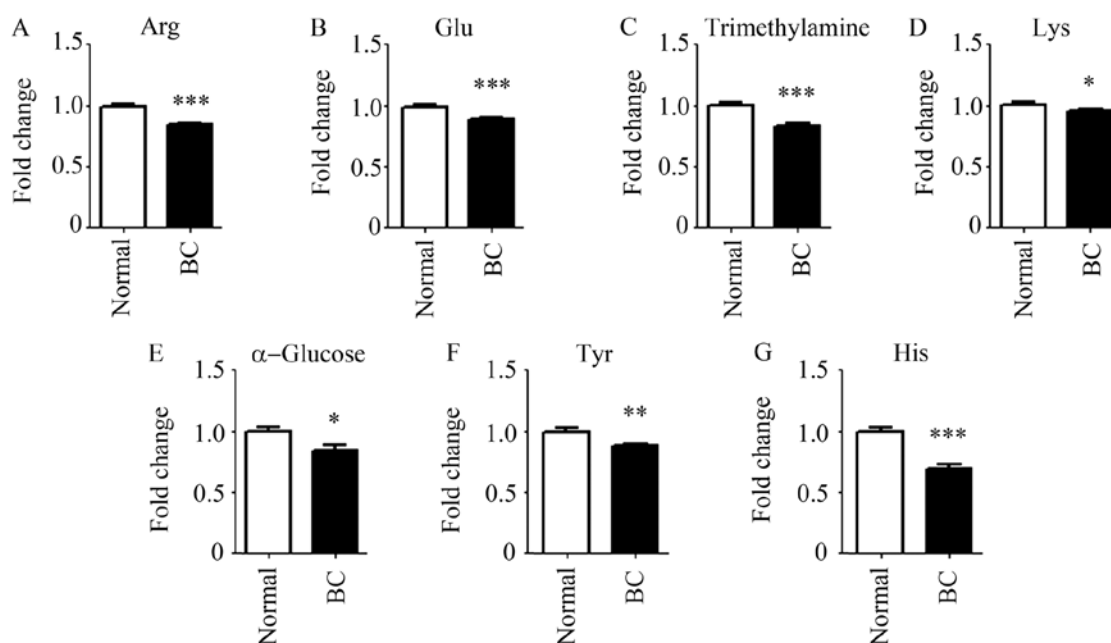


Figure 4. Small molecule metabolites decreased in the plasma of patients with BC. (A) arginine, (B) glutamate, (C) trimethylamine, (D) lysine, (E) α-glucose, (F) tyrosine and (G) histidine in the plasma of patients with BC and the control population. n=35 for control population; n=105 for patients with BC. *P<0.05, **P<0.01 and ***P<0.001 vs. normal. BC, breast cancer; Arg, arginine; Glu, glutamate; Lys, lysine; Tyr, tyrosine; His, histidine.

Cr-laden vegetables (56,57). Exposure to As is mainly through food, water and inhalation of sawdust or smoke from burning As-treated materials (58). Exposure of the general population to As is associated with the development of breast, skin, lung, bladder, liver and kidney cancer (54). It has been found that As is a potential risk factor for the development of BC in patients with the *BRCA1* gene (33).

Another notable finding in the present study was the elevation of plasma lipids in patients with BC compared with that in the control population. Usually, lipids are responsible for cardiovascular disease (11). However, more recently it has been discovered that circulating lipids are potential risk factors for BC development (11,12). The cofactors of hyperlipidemia for BC include a short breastfeeding period and

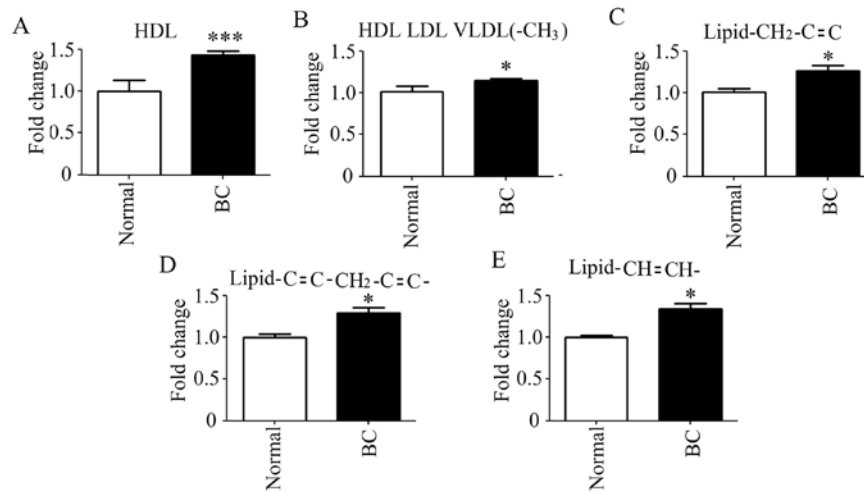


Figure 5. Large molecule metabolites elevated in the plasma of patients with BC. (A) HDL, (B) HDL LDL VLDL(-CH₃), (C) Lipid-CH₂-C=C, (D) Lipid-C=C-CH₂-C=C- and (E) Lipid-CH=CH- in the plasma of patients with BC and the control population. n=35 for the control population; n=105 for patients with BC. *P<0.05 and ***P<0.001 vs. normal. BC, breast cancer; HDL, high-density lipoprotein; LDL, low-density lipoprotein; VLDL, very low-density lipoprotein.

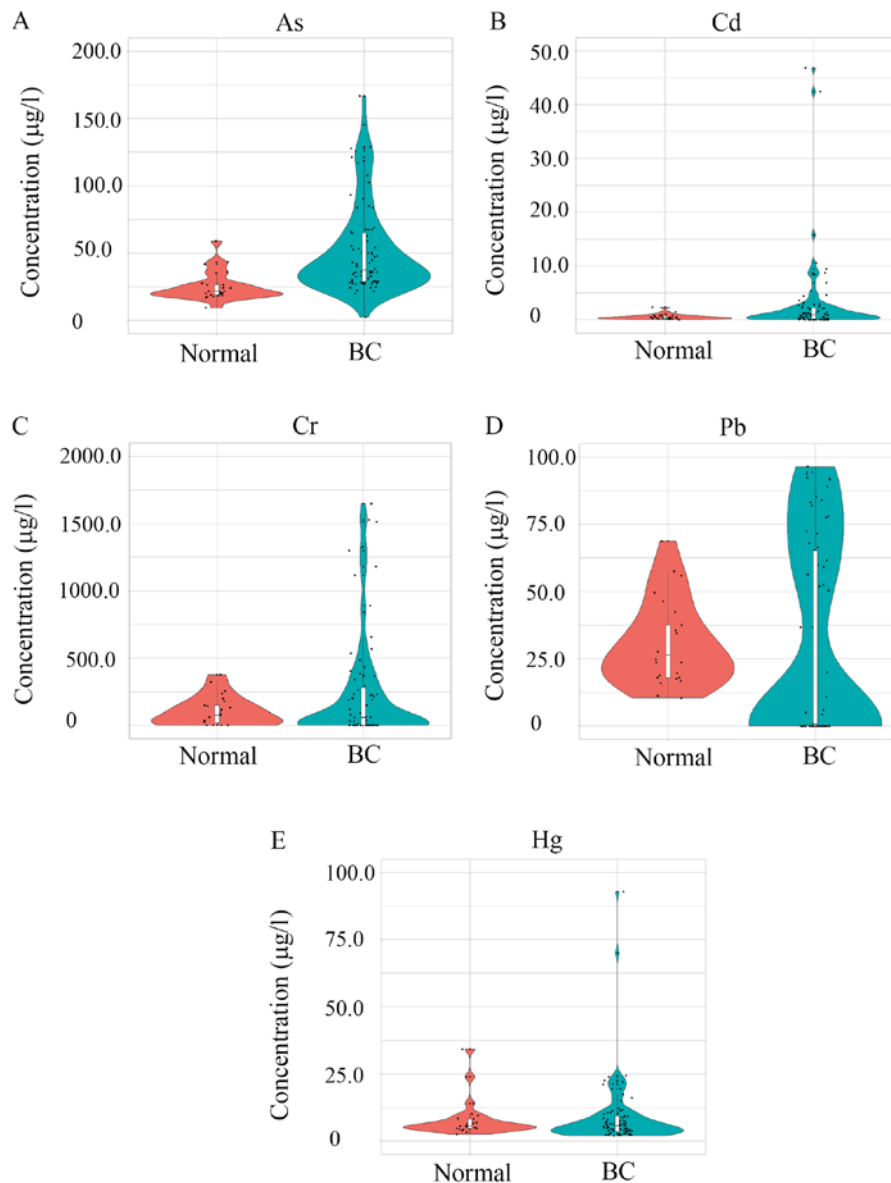


Figure 6. Content of five heavy metals in the plasma of patients with BC and the control population. (A) As, (B) Cd, (C) Cr, (D) Pb and (E) Hg in the plasma of patients with BC and the control population. BC, breast cancer; Cd, cadmium; Pb, lead; Hg, mercury; As, Arsenic; Cr, chromium.

mutations in the *BRCA1* and *BRCA2* genes (59). Furthermore, it has been reported that lipids are the risk factors for BC recurrence. Overall, hyperlipidemia, high serum cholesterol, LDL-cholesterol and triglyceride levels were found to be increased in patients with BC compared with the levels in the control population (60).

Environmental heavy metal exposure serves a vital role in the disturbance of lipid metabolism in humans (61). Elevated blood Cd concentration is reported to be a potential risk factor for dyslipidemia (62). The association of blood Cd and dyslipidemia is not dependent on lifestyle and BMI (62). Furthermore, blood Cd was found to be a more valid biomarker for dyslipidemia compared with Cd in urine (61). Blood Cd level is not only associated with the increased prevalence of dyslipidemia, but also with the elevated prevalence of high total cholesterol, high triglyceride, high LDL-cholesterol and low HDL-C (62).

Epidemiological studies show that As exposure is associated with cardiovascular diseases, including coronary heart disease and peripheral arterial heart disease (62,63). As can influence the blood concentration of apolipoproteins, which indicates that As may be a potential risk factor for dyslipidemia-associated diseases (63). Another study suggested that As can mediate dyslipidemia and electrolyte retention in rats (64).

The findings of the present study suggest that environmental exposure to heavy metals, such as Cd, As, Cr and Pb, may influence blood lipid levels and other small molecule metabolites, which in turn may be involved in BC development. However, a limitation of this study was the small population size for both the patients with BC and the control patients. Further studies to examine urinary heavy metals are required to understand the impact of heavy metals on metabolism and finally on BC development.

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Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

Authors' contributions

LL and MZ provided key intellectual input into the conception and design of the study, and assisted in the writing of the original manuscript. YM and WW performed the experiments. WZ and YM analyzed the data and contributed to the writing of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was performed in accordance to the Institutional Ethical Committee and the Helsinki Declaration of 1975, as revised in 1983, and was approved by the Institutional Review Board of the Affiliated Tengzhou Central People's Hospital of Jining Medical University. Written informed consent was obtained from the patients and controls in the present study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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