Metabolome-Wide Association Study of Multiple Plasma Metals with Serum Metabolomic Profile among Middle-to-Older-Aged Chinese Adults

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ABSTRACT: Metal exposure has been associated with risk of various cardio-metabolic disorders, and investigation on the association between exposure to multiple metals and metabolic responses may reveal novel clues to the underlying mechanisms. Based on a metabolome-wide association study of 17 plasma metals with untargeted metabolomic profiling of 189 serum metabolites among 1992 participants within the Dongfeng−Tongji cohort, we replicated two metal-associated pathways, linoleic acid metabolism and aminoacyl-tRNA biosynthesis, with novel metal associations (false discovery rate, FDR < 0.05), and we also identified two novel pathways, including biosynthesis of unsaturated fatty acids and alpha-linolenic acid metabolism, as associated with metal exposure (FDR < 0.05). Moreover, two-way orthogonal partial least-squares analysis showed that five metabolites, including aspartylphenylalanine, free fatty acid 14:1, uridine, carnitine C14:2, and LPC 18:2, contributed most to the joint covariation between the two data matrices (12.3%, 8.3%, 8.0%, 7.4%, and 7.3%, respectively). Further BKMR analysis showed significant positive joint associations of plasma Al, As, Ba, and Zn with aspartylphenylalanine and of plasma Ba, Co, Mn, and Pb with carnitine C14:2, when all the metals were at the 55th percentiles or above, compared with the median. We also found significant interactions between As and Ba in the association with aspartylphenylalanine (*P* for interaction = 0.048) and between Ba and Pb in the association with carnitine C14:2 (*P* for interaction < 0.001). Together, these findings may provide new insights into the mechanisms underlying the adverse health effects induced by metal exposure.

The aminoacyl-tRNA biosynthesis pathway was not assigned under the Metabolism category and thus not able to be plotted on the KEGG global map.

KEYWORDS: *Multiple plasma metals, Untargeted metabolomics, Metal−metal interaction, Metabolome-wide association study, Environmental health*
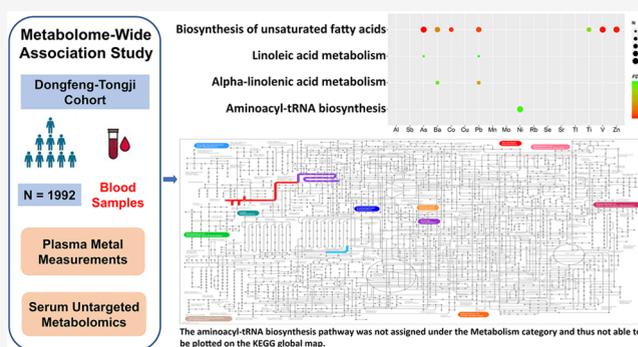
1. INTRODUCTION

Metals exist ubiquitously in the environment, and humans are exposed to multiple metals continually on a daily basis through ambient air and contaminated food or drinking water.[1] Some metals are essential to the human body, such as magnesium (Mg), iron (Fe), manganese (Mn), molybdenum (Mo), selenium (Se), copper (Cu), and zinc (Zn), playing important roles in maintaining certain biological functions at the homeostatic level,[2] while other metals, such as arsenic (As), cadmium (Cd), mercury (Hg), lead (Pb), and thallium (Tl), are nonessential. It has been shown by accumulating evidence that exposure to nonessential toxic metals, as well as an inadequate or excessive intake of essential metals, are both associated with the risk of various cardio-metabolic diseases, such as cardiovascular disease (CVD) and type 2 diabetes (T2D), and also cancers, apart from causing damages in their target organs or tissues.[2−5]

However, the mechanisms underlying these associations remain unclear.

Metabolomics refers to the systematic profiling of small-molecule metabolites in a biological sample, and metabolites might be able to serve as intermediate biomarkers connecting exposure and its adverse health effects, thus providing clues to the underlying mechanisms.[6] Such utility of the metabolomic technology has been exemplified in a recent study which revealed the mediating role of a lipid metabolite, sphingomyelin (40:3), in the association between Zn exposure and lung cancer

risk,[7] while a systematic metabolome-wide association study of mixed exposure to multiple metals could provide an even better understanding of metal toxicology. A number of studies have explored metabolic responses to exposure to one certain toxic metal,[8−15] such as As, Cd, or Pb, half of which were conducted among residents living in heavily polluted areas.[8,9,13,14] Several other studies also reported associations between exposure to multiple metals; with examination of mixed metal exposure which better characterized real-life exposure scenarios, these studies revealed various metal-associated metabolites which fell on metabolic pathways mainly involved in amino acid, lipid, and nucleotide metabolisms.[16−21] However, most of these studies were conducted among study populations with certain health conditions or concerns, including pregnant women,[16,17] mother−child pairs,[18] and residents living near industrial plants,[19,20] plus one study among Native Americans,[21] limiting generalizability of these findings to other populations, and were also limited in sample size (750 at most). Moreover, pathway enrichment of metal-associated metabolites was underexplored in previous studies.

In the present study, we conducted a metabolome-wide association study of 17 plasma metals with untargeted metabolomic profiling of 189 serum metabolites among 1992 participants within the Dongfeng−Tongji (DFTJ) cohort for exploration for metal toxicity in terms of circulatory metabolic responses, which could serve as a mechanistic link between metal exposure and the development of systematic diseases. Facilitated by novel statistical approaches such as two-way orthogonal partial least-squares (O2PLS)[22] and Bayesian kernel machine regression (BKMR),[23] we were able to evaluate the joint metabolomic association of mixed exposure to multiple metals and explore potential interactions between different metals.

2. METHODS

2.1. Study Population. The study population of this cross-sectional study was a subsample from the DFTJ cohort from two previously conducted nested case-control studies aiming to investigate risk factors for future risk of acute coronary syndrome (ACS) and T2D, respectively, both with sample sizes of 500 incident cases and 500 age- and sex-matched controls, among whom profiling of plasma metals and untargeted serum metabolomics were performed. In brief, the DFTJ cohort recruited 27,009 retirees from Dongfeng Motor Company (DMC) during September 2008 to June 2010. All participants took a physical examination, completed an interviewer-administered standardized questionnaire, and provided fasted blood samples at baseline.[24] A total of 2000 participants were included in the two nested case-control studies. The participants were all free of CVD, cancer, and severely abnormal electrocardiogram at baseline and also free of T2D for those within the nested case-control study for T2D risk. After excluding those with incomplete data of metals concentrations ($n = 8$), 1992 participants were finally included in this study. Informed consent was obtained from each participant, and the study was approved by the ethics committee of Tongji Medical College, Huazhong University of Science and Technology.

2.2. Plasma Metals Measurements. Plasma concentrations of 23 metals, including aluminum (Al), antimony (Sb), arsenic (As), barium (Ba), cadmium (Cd), chromium (Cr), cobalt (Co), copper (Cu), iron (Fe), lead (Pb), manganese (Mn), molybdenum (Mo), nickel (Ni), rubidium (Rb),

selenium (Se), strontium (Sr), thallium (Tl), tin (Sn), titanium (Ti), tungsten (W), uranium (U), vanadium(V), and zinc (Zn), were measured using inductively coupled plasma-mass spectrometry (ICP-MS) following a previously published protocol.[25] We excluded three metals with detection rates less than 50% (tin [Sn], tungsten [W], and uranium [U]), and three other metals which were not well-represented by plasma level for exposure assessment (cadmium [Cd], chromium [Cr], and iron [Fe]).[25−27] Finally, 17 plasma metals were included in subsequent analysis. Table S1 shows the raw concentrations and LODs of 17 plasma metals, and values below the LOD were imputed with the LOD divided by 2.

2.3. Serum Untargeted Metabolomics. Serum metabolites were measured using ultrahigh performance liquid chromatography−mass spectroscopy (UPLC-MS) according to previously described methods.[28] Briefly, 50 μL serum samples were treated with 200 μL of methanol, spiked with internal standards, vortexed for 10 min, and centrifuged at 4 °C for 10 min at 500 g to remove the protein. Then, supernatant was filtered and stored at −80 °C until liquid chromatography−mass spectrometry (LC-MS) analysis. The sample was redissolved in 50 μL of methanol/water (1:4, v/v), vortexed for 10 min, centrifuged at 10 °C for 15 min at 800 g, and injected into an ACQUITY UPLC BEH C8 column (2.1 mm × 50 mm, 1.7 μm, Waters Corp.) or an ACQUITY UPLC BEH T3 column (2.1 mm × 50 mm, 1.8 μm, Waters Corp.) for positive and negative ion modes analyses, respectively. Meanwhile, a Waters ACQUITY UPLC system (Waters Corp.) coupled to a TripleTOF 5600 mass spectrometer (AB SCIEX) or a Q Exactive HF mass spectrometer (Thermo Fisher Scientific) were used at positive and negative ion modes, respectively. All samples were analyzed randomly and in batches of 10, and the quality control (QC) samples prepared by spiking the pooled serum samples with internal standards were analyzed at the beginning of each batch to check instrument performance. Detailed information on 11 internal standards is presented in Table S2. Metabolite identification was performed by matching mass-to-charge ratio (m/z), retention time (RT), and MS/MS information to an established in-house database.[29] After excluding metabolites with relative standard deviation (RSD) above 30% within QC samples[28] and those with detection rates below 50% within analytical samples, 189 annotated metabolites were retained for subsequent analysis, and missing values were imputed with the minimum normalized peak area value of the corresponding metabolite divided by 2.

2.4. Assessment of Covariates. Sociodemographic (age, sex, and education), lifestyle (smoking status, drinking status, and exercise status), and medical history (hypertension, hyperlipidemia, and diabetes status) information was obtained through interviewer-administered standardized questionnaires. Standing height, body weight, and blood pressure were measured during health examinations by qualified staff. The biochemical laboratory of Dongfeng General Hospital analyzed the fasting blood samples for fasting glucose and serum lipids following a standard procedure. Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared. Smoking status was categorized into current (smoking at least one cigarette per day for more than half a year), former, and never smoking groups. Drinking status was categorized into current (drinking at least once per week for more than half a year), former, and never drinking groups. Physical activity was defined as those who regularly exercised for at least 30 min no less than 5 days per week. Hypertension was defined if the

participant had blood pressure ≥ 140/90 mmHg, self-reported diagnosis of hypertension, or use of antihypertensive medication. Hyperlipidemia was defined if the participant had total cholesterol > 5.72 mmol/L or triglycerides > 1.70 mmol/L at the medical examination, self-reported diagnosis of hyperlipidemia, or use of lipid-lowering medication. Diabetes was defined if the participant had fasting glucose ≥ 7.0 mmol/L, self-reported diagnosis of diabetes, or use of antidiabetic medication.

2.5. Statistical Analysis. Baseline characteristics of the study participants were presented as mean ± standard deviation (SD) and frequency (percentages), and plasma concentrations of 17 metals were shown as median (interquartile range, IQR). To rectify the skewed distribution, both plasma metal concentrations and serum metabolite levels were natural log-transformed prior to further analyses. Spearman rank correlation analysis was performed to evaluate the correlations between metals. Multivariable linear regression models were used to investigate the associations of each individual metal−metabolite pair, with adjustments for age, gender, BMI, smoking status, drinking status, and physical activity. To account for multiple testing, a false discovery rate (FDR) correction was performed across 3213 association tests between 17 metals and 189 metabolites. Pathway analysis was conducted among significant metabolites associated with each metal (FDR < 0.05) using MetaboAnalyst 4.0.[30]

We applied O2PLS analysis[22] using the *OmicsPLS* R package to examine variation shared by all plasma metals and serum metabolites. In this analysis, variations within levels of the 17 plasma metals and within 189 serum metabolites were decomposed into three parts: joint part, orthogonal part, and noise part. The components of the joint part (i.e., joint components) represent the joint covariation between metal and metabolite data, while those of the orthogonal part indicate the unique systematic variations in each of the data matrices. The third noise part captures the unsystematic variation. We conducted 10-fold cross-validation to determine the number of components in the O2PLS model and selected the model with minimal prediction errors as the final model. By plotting the loadings of each metal/metabolite against the joint components, we aimed to identify the metabolites contributing most to the covariation between the two data matrices, indicated by the sum of squared loadings on the joint components (SS_{joint}), which might suggest the relative importance of the associations between each metabolite and integrated metal exposure.

For the identified metabolites, we conducted two-stage BKMR analysis[31] using the *bkmr* R package to assess the joint associations of mixed metal exposure with these metabolites and also to search for the metals responsible for such joint associations. In the first stage, we included all 17 metals in the BKMR model and conducted a component-wise variable selection method with 25,000 iterations by a Markov chain Monte Carlo (MCMC) algorithm. Posterior inclusion probabilities (PIPs) were calculated for indication of the relative importance of each metal, and metals with PIPs > 0 were included in the second stage BKMR analysis, with the same settings of the Gaussian kernel function and tuning parameters as in the first stage, and the second stage BKMR model was considered as the final model. All BKMR models were adjusted for age, gender, BMI, smoking status, drinking status, and physical activity. Model convergence was achieved in all analyses, as indicated by trace plots with a good mixing of multiple MCMC chains and no discernible fluctuations in each single chain. We further tested possible interactions between

metals suggested by the BKMR analysis through multivariable linear regression models by incorporating multiplicative interaction terms.

To scrutinize the robustness of the findings, we conducted further sensitivity analyses of joint association between mixed metal exposure and metabolites in the BKMR analysis. First, we performed additional adjustment for potential confounding factors, including disease status of hypertension, hyperlipidemia, and diabetes at baseline, as well as future disease status which was categorized into three groups ("control", "ACS", and "T2D"). Second, considering that the study population was from two nested case-control studies, thus being a biased sample from the study cohort, we performed a sensitivity analysis within only the control participants ($N = 996$).

Statistical analyses were performed with R (version 4.1.2) or MetaboAnalyst 4.0 (https://www.metaboanalyst.ca/). The metabolic network of significantly metal-associated metabolic pathways shown in the graphical abstract was visualized using iPath 3.0 (https://pathways.embl.de/). A two-sided *P*-value < 0.05 was considered statistically significant.

3. RESULTS

3.1. Study Population Characteristics. Table 1 describes the baseline characteristics of the study participants. The study population had a mean age of 63.95 ± 7.25 years, 46.08% of them were male, and the average BMI was 24.67 ± 3.27 kg/m². Among the subjects, 420 (21.08%) were current smokers, 445 (22.34%) current drinkers, and 590 (29.62%) had received

Table 1. Baseline Characteristics of the Study Participants ($N = 1992$)

Characteristics[a]	Statistics
Age, years	63.95 ± 7.25
Male, *n* (%)	918 (46.08)
BMI, kg/m²	24.67 ± 3.27
High school or beyond, *n* (%)	590 (29.62)
Current smoker, *n* (%)	420 (21.08)
Current drinker, *n* (%)	445 (22.34)
Physical activity (yes), *n* (%)	1771 (88.91)
Metals[b] (μg/L)	
Al	48.24 (29.23, 96.01)
Sb	0.14 (0.09, 0.21)
As	2.03 (1.27, 3.74)
Ba	36.48 (23.24, 66.55)
Co	0.15 (0.12, 0.19)
Cu	959.34 (851.95, 1072.48)
Pb	13.10 (9.12, 21.19)
Mn	4.06 (2.97, 5.67)
Mo	1.34 (1.07, 1.73)
Ni	2.79 (2.03, 4.11)
Rb	351.14 (314.87, 391.80)
Se	64.22 (56.12, 74.75)
Sr	35.82 (30.33, 42.88)
Tl	0.14 (0.10, 0.18)
Ti	31.01 (25.01, 38.50)
V	0.69 (0.55, 0.99)
Zn	1191.05 (1000.32, 2903.34)

[a]Continuous variables are presented as mean ± SD, and categorical variables are shown as frequency (percentages). [b]Plasma concentrations of 17 metals are presented as median (IQR).
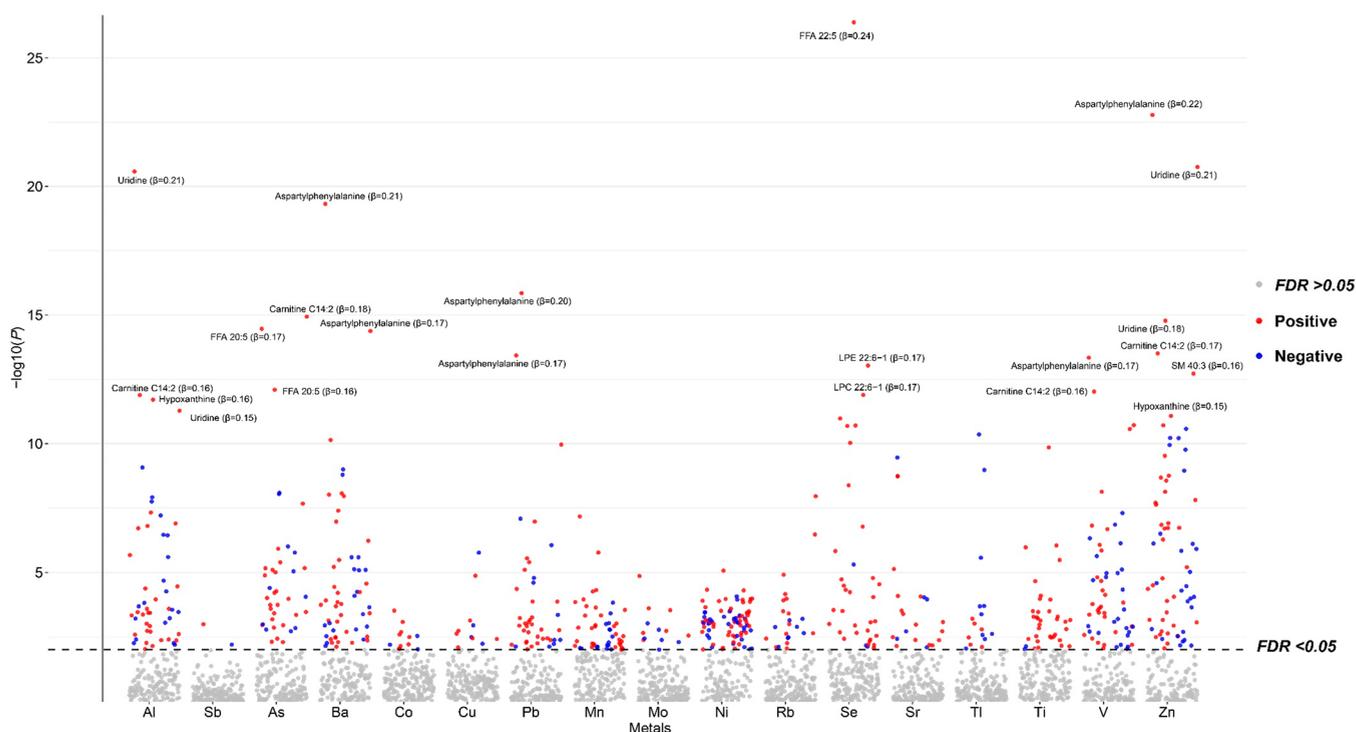
Figure 1. Scatter plot demonstrating the associations between 17 metals and 189 metabolites. *P* values were derived from single-metal multivariable linear regression models which adjusted for age, gender, BMI, smoking status, drinking status, and physical activity. False discovery rate (FDR) correction was performed across 3213 association tests between 17 metals and 189 metabolites.
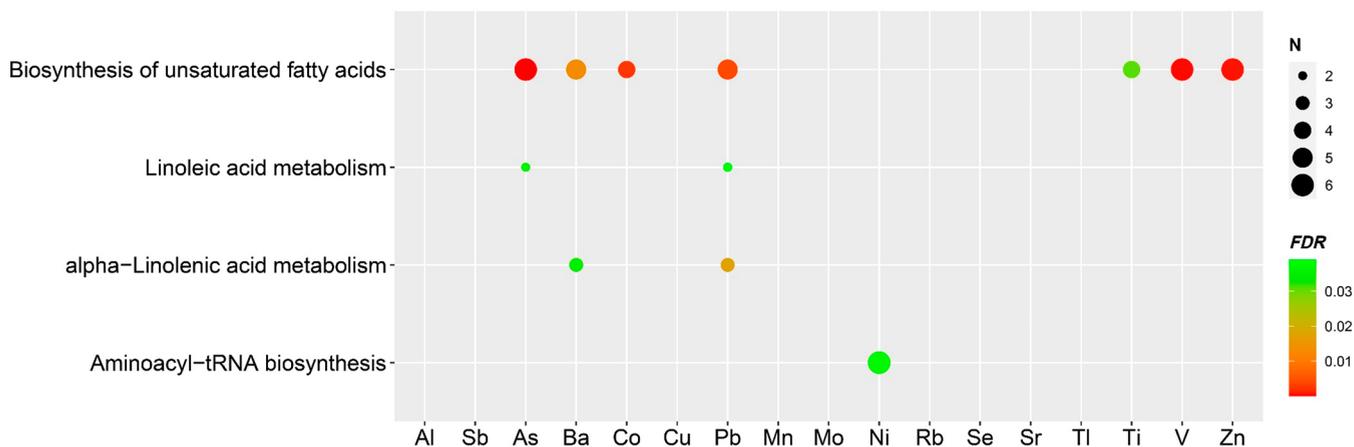

Figure 2. Metabolic pathways associated with plasma metals. Sizes of circles were mapped to the number of significant metabolites in the pathways, and color was mapped to the FDR values (FDR < 0.05).

education in high school or beyond, while 1771 (88.91%) took regular exercise. The median (IQR) of plasma metal concentrations were 48.24 (29.23, 96.01) μg/L for Al, 0.14 (0.09, 0.21) μg/L for Sb, 2.03 (1.27, 3.74) μg/L for As, 36.48 (23.24, 66.55) μg/L for Ba, 48.24 0.15 (0.12, 0.19) μg/L for Co, 959.34 (851.95, 1072.48) μg/L for Cu, 13.10 (9.12, 21.19) μg/L for Pb, 4.06 (2.97, 5.67) μg/L for Mn, 1.34 (1.07, 1.73) μg/L for Mo, 2.79 (2.03, 4.11) μg/L for Ni, 351.14 (314.87, 391.80) μg/L for Rb, 64.22 (56.12, 74.75) μg/L for Se, 35.82 (30.33, 42.88) μg/L for Sr, 0.14 (0.10, 0.18) μg/L for Tl, 31.01 (25.01, 38.50) μg/L for Ti, 0.69 (0.55, 0.99) μg/L for V, and 1191.05 (1000.32, 2903.34) μg/L for Zn. The pairwise correlation coefficients within all 17 metals ranged from −0.06 to 0.74, and 10 pairs of metals had correlation coefficients over 0.6, which were Al−Ba,

Al−Pb, Al−Mn, Al−V, Al−Zn, As−Ba, Ba−Pb, Ba−Zn, Pb−Zn, and V−Zn (all *P* values < 0.01, Figure S1).

3.2. Associations of Each Individual Metal with Metabolites. The 189 annotated metabolites were across 12 classes, including 22 amino acids and derivatives, 22 carnitine and acyl carnitines, 37 fatty acids and derivatives, 4 carbohydrates and conjugates, 4 purines and derivatives, 8 bile acids, 8 other organic acids, 4 benzene and derivatives, 38 lysophosphatidylcholines (LPC) and phosphatidylcholines (PC), 19 lysophosphatidylethanolamines (LPE) and phosphatidylethanolamines (PE), 8 phosphosphingolipids, and 15 other metabolites. Regression coefficients from multivariable linear regression analyses to evaluate the associations between each individual metal−metabolite pairs are provided in Table S3, and FDR < 0.05 was considered statistically significant. The *P* values
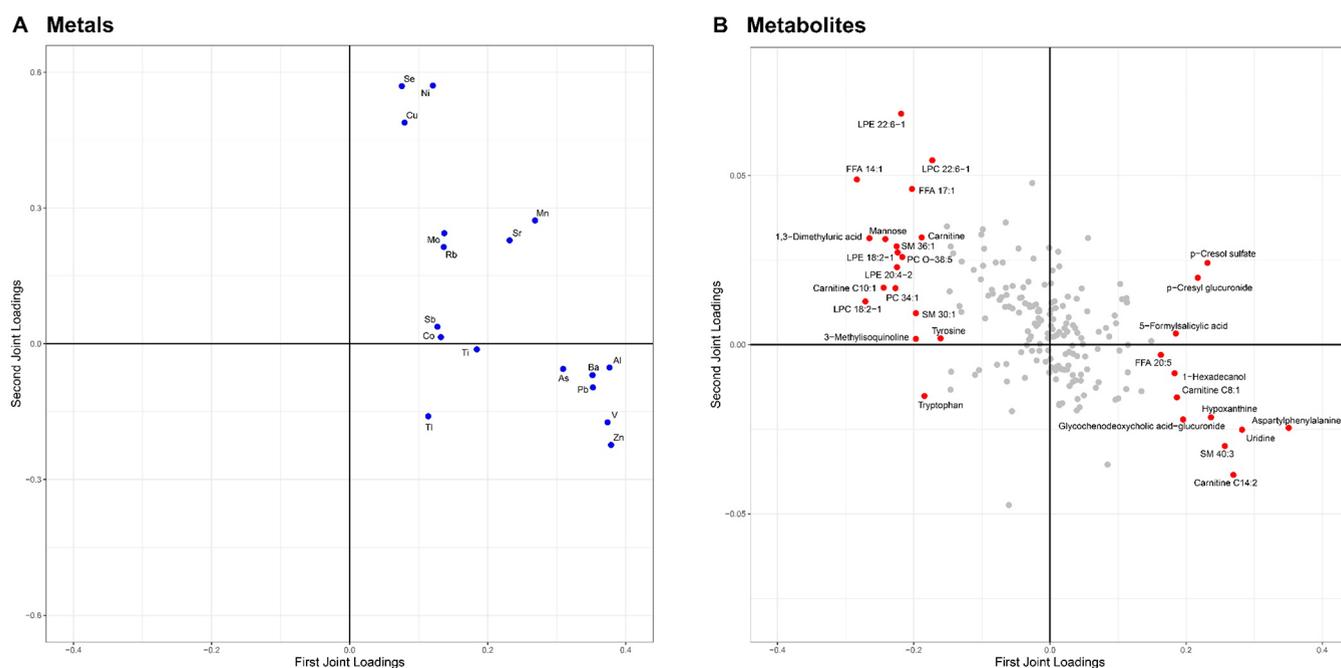
Figure 3. O2PLS modeling of metal−metabolite associations. The number of components included in the O2PLS model were selected by 10-fold cross-validation, and the model contains 2 joint, 10 X-orthogonal, and 10 Y-orthogonal components. (A) Loading plot for plasma metals. (B) Loading plot for serum metabolites. In this display, the relative position of each point in the two panels indicates whether regression coefficients for a given pair of metals and metabolites positively or negatively correlates to each other, while the sum of the squared loadings on the two joint components (SS_{joint}) indicates the magnitude of the association of the metal (metabolite) with metabolome (metallome). All 17 metals and the top 30 metabolites are labeled. O2PLS: two-way orthogonal partial least-squares.
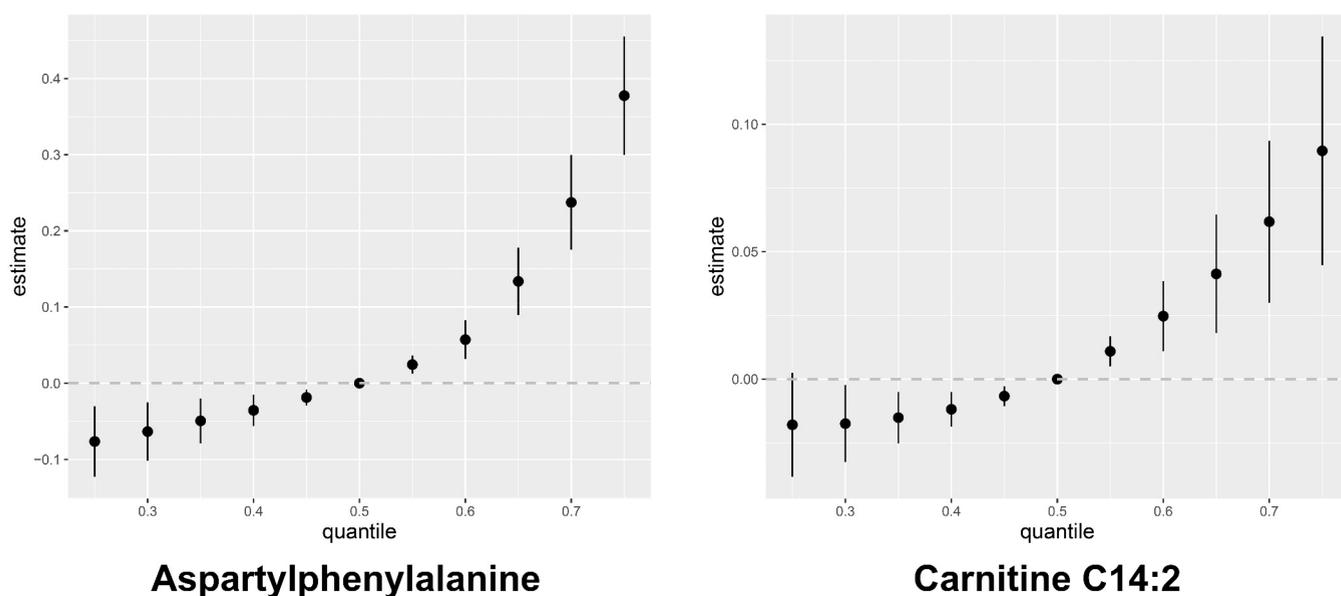
Figure 4. Joint associations of metal mixtures with metabolites by two-stage Bayesian kernel machine regression (BKMR) analyses. The figure plots the estimated change in metabolite concentrations when all the metals at particular percentiles (*x*-axis) were compared to all the metals at their 50th percentile. The BKMR model adjusted for age, gender, BMI, smoking status, drinking status, and physical activity. For aspartylphenylalanine, the BKMR model included a mixture of Al (PIP = 0.09), As (PIP = 0.16), Ba (PIP = 0.37), and Zn (PIP = 0.99). For carnitine C14:2, the BKMR model included a mixture of Ba (PIP = 0.65), Co (PIP = 0.18), Mn (PIP = 0.05), and Pb (PIP = 0.88).

and directions of associations between 17 metals and 189 metabolites are depicted in Figure 1. The numbers of metabolites significantly associated with individual metal were 51 for Al, 2 for Sb, 39 for As, 54 for Ba, 12 for Co, 11 for Cu, 42 for Pb, 49 for Mn, 13 for Mo, 85 for Ni, 23 for Rb, 36 for Se, 21 for Sr, 16 for Tl, 37 for Ti, 55 for V, and 66 for Zn. Among these

associations, 34 of them achieved *P* values < 1.00×10^{-10}, and the strongest association was that between Se and FFA 22:5 with a *P* value of 9.96×10^{-25}. We conducted pathway analyses among metabolites significantly associated with each metal (FDR < 0.05), and results are shown in Figure 2 and Table S4. Metal-associated metabolites were primarily enriched within
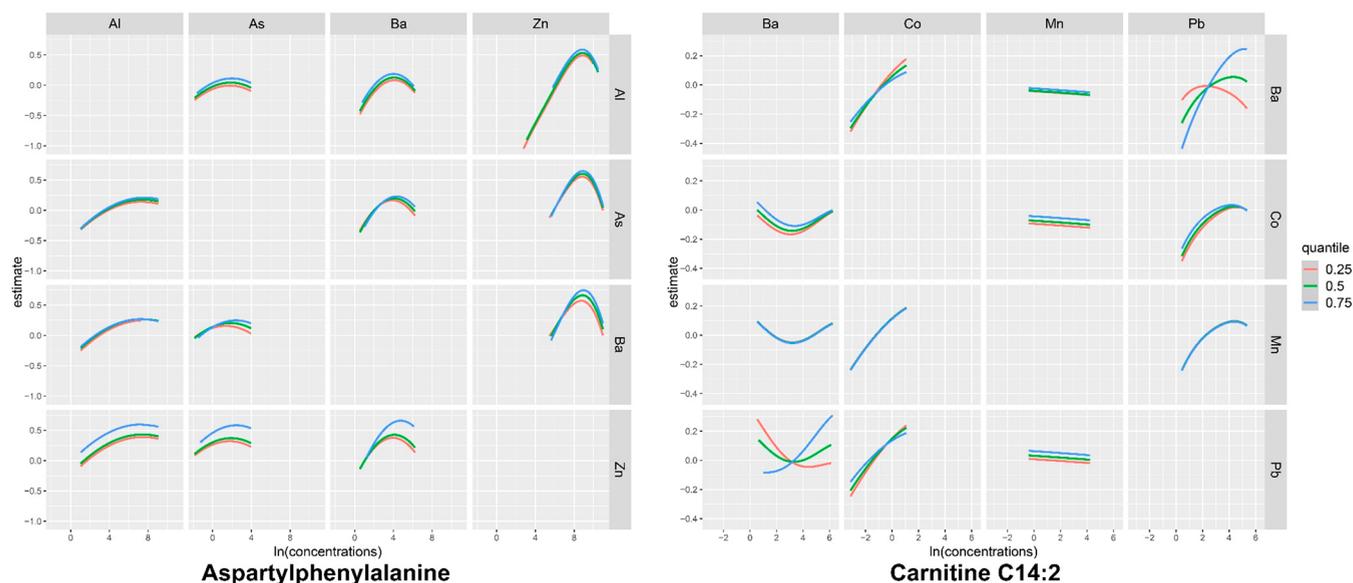
Figure 5. Bivariate exposure−response functions of each metal (shown by column) and metabolite concentration by two-stage Bayesian kernel machine regression (BKMR) analyses, holding concentrations of one metal (shown by row) at different quantiles (25th, 50th, and 75th) and other metals at medians. The BKMR model adjusted for age, gender, BMI, smoking status, drinking status, and physical activity. For aspartylphenylalanine, the BKMR model included a mixture of Al, As, Ba, and Zn. For carnitine C14:2, the BKMR model included a mixture of Ba, Co, Mn, and Pb. The multivariable linear regression models validated the interactions between As and Ba (P for interaction = 0.048) and Ba and Zn (P for interaction = 0.15) for aspartylphenylalanine and the interactions between Ba and Pb (P for interaction < 0.001), Ba and Co (P for interaction = 0.39), and Co and Pb (P for interaction = 0.30) for carnitine C14:2.

pathways related to lipid metabolism and amino acid metabolism with enrichment ratios from 12.00 to 27.93 (FDR < 0.05). In detail, seven metals were associated with the biosynthesis of unsaturated fatty acids pathway (As, Ba, Co, Pb, Ti, V, and Zn), two metals with the linoleic acid metabolism pathway (As and Pb), two metals with the alpha-linolenic acid metabolism pathway (Ba and Pb), and one metal with the aminoacyl-tRNA biosynthesis pathway (Ni).

3.3. Associations of Mixed Metal Exposure with Metabolites. We constructed the O2PLS model for examination of variation shared by all plasma metals and serum metabolites, and the model structure is depicted in Figure S2, which shows that the joint part explained 27.2% of the metal variation and 35.2% of the metabolite variation. The permutation test (n = 10,000) shows that the model was fitted with high reliability (P < 0.05, Figure S3). We then plotted the loadings of each metal/metabolite against the two joint components (Figure 3), from which the top five metabolites contributing most to the joint covariation between the two data matrices and in associations with the integrated metal exposure were identified, including aspartylphenylalanine, tetradecenoic acid (free fatty acid, FFA 14:1), uridine, carnitine C14:2, and LPC 18:2 (SS_{joint} = 12.3%, 8.3%, 8.0%, 7.4%, and 7.3%, respectively).

In BKMR analysis for joint associations of plasma metals with each of the top five metabolites, which were aspartylphenylalanine, FFA 14:1, uridine, carnitine C14:2, and LPC 18:2, we included metals which obtained overzero PIPs in the first-stage analysis for construction of the second-stage BKMR model (Table S5). The number of metals included for the joint association analysis of the five top metabolites were four, four, one, four, and two, respectively. In the second-stage BKMR analysis, we observed significant positive joint association of coexposure to Al, As, Ba, and Zn with aspartylphenylalanine (Figure 4) and significant positive joint association of

coexposure to Ba, Co, Mn, and Pb with carnitine C14:2 (Figure 4), when all the metals were at the 55th percentiles or above, compared with the median, while no significant joint association was observed for FFA 14:1, uridine, and LPC 18:2 (results not shown). Specifically, for the positive joint association with aspartylphenylalanine, Zn (PIP = 0.99) was the greatest metal contributor, and bivariate exposure−response functions suggested synergistic interactions between As and Ba (P for interaction = 0.048; Figure 5). For the positive joint associations with carnitine C14:2, Ba (PIP = 0.65) and Pb (PIP = 0.88) were the vital contributors, and bivariate exposure−response functions suggested synergistic interactions between Ba and Pb (P for interaction < 0.001; Figure 5).

In the sensitivity analysis with further adjustment for hypertension, hyperlipidemia, and diabetes at baseline, as well as future disease status, all results remained robust (Figures S4 and S5). When the BKMR analysis was restricted to only control participants (N = 996), results were also similar except that the interaction between As and Ba in the association with aspartylphenylalanine became insignificant (P for interaction = 0.11), possibly owing to the reduced sample size (Figures S6 and S7).

4. DISCUSSION

In this metabolome-wide association study of 17 plasma metals, we characterized metabolic profiles associated with each of the metals, from which we uncovered two novel pathways associated with metal exposure, which were biosynthesis of unsaturated fatty acids and alpha-linolenic acid metabolism. In terms of individual metals, we found Pb was significantly associated with not only the two novel pathways but also the linoleic acid metabolism pathway, which had been reported associated with other metals.[21] These novel associations between Pb and metabolic pathways had not been reported in previous studies, except that there were also observations of associations of Pb

exposure with other pathways within lipid metabolism.[15,21] With respect to As, we observed As was significantly associated with one of the two novel pathways, biosynthesis of unsaturated fatty acids, and we also found a novel association between As and the linoleic acid metabolism pathway. These associations between As and metabolic pathways were reported for the first time. However, associations between As exposure and other pathways within lipid metabolism had been reported,[21] and metabolites within As-associated pathways identified in our study such as FFA 20:1, LPC 16:0, and LPC 14:0 were found to be associated with As exposure among residents living in a high-arsenic area[9] and among pregnant women.[10] Of note, plasma Ba was also significantly associated with both of the two novel pathways within lipid metabolism, even though research on association between Ba exposure and metabolomes among people is scarce, with only negative results from a study of 232 pregnant women.[17] At a nominal significance level, we also found associations of plasma Pb and As with the glycerophospholipid metabolism pathway, and similar associations had also been reported in previous studies of white veterans[15] and Native American,[21] although blood Pb and urinary As were measured, respectively, in the two studies. Consistent with our findings of notable disturbed lipid metabolisms in association with plasma Pb, As, and Ba, there was also evidence from animal studies that Pb exposure disrupted lipid metabolism through increasing transcription of genes involved in FFA and triglyceride synthesis,[32] and As exposure induced lipid metabolism disorder through regulating the ERK/PPAR signaling pathway,[33] while Ba exposure also caused lipid peroxidation via disruption of antioxidant defense systems.[34] Previous epidemiological studies also reported associations between Pb and As exposure and risk of CVD,[35,36] in which dyslipidemia played a cardinal role. As for Ba, epidemiological research on the associated health effects is scarce, and only a cross-sectional study showed that lipid peroxidation may mediate the association between Ba exposure and heart rate variability alteration, a cardiovascular risk factor.[37] Taken together, our study provided an important line of evidence from a population-based cohort that disruptions in lipid metabolism, particularly the metabolism of unsaturated fatty acids, might underlie the mechanism of cardio-metabolic risk induced by exposure to various nonessential toxic metals, including Pb, As, and Ba.

Apart from Pb, As, and Ba, we also found that metabolites associated with plasma Co, Ti, V, and Zn were all enriched in the pathway of unsaturated fatty acids biosynthesis, further highlighting the involvement of the metabolism of unsaturated fatty acids in metal exposure-related health effects. The top metabolite hits within this pathway for each metal were FFA 18:2, FFA 18:0, FFA 20:5, and FFA 20:5, respectively. Although no research has reported the biosynthesis of unsaturated fatty acid pathways associated with any of the four metals, three metabolites involved in the pathway, including FFA 18:2, FFA 20:4, and FFA 22:6, were identified in association with Zn exposure in a previous nested case-control study, also within the DFTJ cohort,[7] and we also found suggestively significant association between plasma Zn and the metabolism pathway of another unsaturated fatty acid, linoleic acid, consistent with another previous report.[21] Epidemiological studies have shown associations of exposure to Co, Ti, V, and Zn with cardiometabolic diseases such as dyslipidemia, T2D, and metabolic syndrome,[38–40] and there was also evidence from experimental studies of disruptive effects of Co, Ti, V, and Zn on lipid metabolism.[41–44] Our findings provided novel evidence

suggesting that Co, Ti, V, and Zn exposure might exert adverse health effects by interfering with the biosynthesis of unsaturated fatty acids.

Furthermore, we found plasma Ni was significantly associated with the aminoacyl-tRNA biosynthesis pathway, which had been reported with other metals,[45] with arginine being the top metabolite hit on this pathway in our study. Notably, among the 17 plasma metals examined in this study, Ni was associated with the largest number of metabolites, totaling 85 metabolites across 11 classes, and six of the Ni-associated metabolites fell within the aminoacyl-tRNA biosynthesis pathway. Although there was no previous report of such association, two amino acids involved in the aminoacyl-tRNA biosynthesis pathway, glutamine and alanine, were found to be associated with Ni exposure in a previous metabolomic study among pregnant women,[16] and an experiment study also found that exposure to Ni could affect amino acid metabolism in HepaRG cells.[41] From epidemiological studies, ample evidence showed that Ni exposure was associated with the risk of lung cancer, and there was also a report of association with CVD risk.[46] Our study further identified that it might be aminoacyl-tRNA biosynthesis related to amino acid metabolism that Ni exposure was associated with, which could be a mechanism underlying the adverse health outcomes related with Ni exposure.

When considering the integrated exposure of all 17 metals, we found through O2PLS analysis that the metabolites most strongly associated with integrated metal exposure were aspartylphenylalanine, FFA 14:1, uridine, carnitine C14:2, and LPC 18:2, which might serve as nonspecific metabolic biomarkers of metal exposure. Consistent with such observation, these five metabolites were each significantly associated with 10, 6, 13, 11, and 11 metals, respectively, in the analysis of individual metals. Of the five metabolites, aspartylphenylalanine is a dipeptide product of an angiotensin-converting enzyme (ACE) and a marker for ACE activity.[47] FFA 14:1, also called myristoleic acid, is a monounsaturated fatty acid (MUFA) and has been found associated with increased risk of T2D in a cohort s study.[48] Uridine is a pyrimidine nucleoside and plays a pivotal role in regulating energy homeostasis, disruption of which may contribute to metabolic disease.[49] Carnitine C14:2 is a long-chain acylcarnitine, involved in transporting cytosolic fatty acids across the inner mitochondrial membrane for β-oxidation and energy metabolism and has been found associated with both T2D and prediabetic states in a cross-sectional study.[50] LPC 18:2 belongs to lysophosphatidylcholine, a major class of glycerophospholipids in human plasma and has been identified as an independent predictor of incident coronary heart disease and T2D.[51,52] Three of the five metabolites were involved within pathways of lipid metabolism, the most notable pathways revealed in this study as associated with metal exposure, while the remaining aspartylphenylalanine and uridine were involved in metabolism of amino acids and nucleotides, respectively. Apart from these two metabolites, we also identified a number of other metal-associated metabolites involved in amino acid metabolism such as arginine, iso-leucine, tyrosine, phenyl-alanine, and tryptophan, as well as those involved in nucleotide metabolism such as hypoxanthine, uric acid, and 1,3-dimethyluric acid, and these two pathways had been previously reported to be associated with exposure to Pb among residents living in heavily polluted areas,[14] to Co among pregnant women,[45] and to As, Sb, Mo, and Se among Native Americans.[21] Even though we did not observe an enrichment of metal-associated metabolites within these two pathways in the present

study, possibly due to the limited coverage of related metabolites by our metabolomic platform, our results still provided support of the associations of amino acid metabolism and nucleotide metabolism pathways with metal exposure.

Through two-stage BKMR analysis of the five top metabolic markers of metal exposure identified in our study, which further accounted for intercollinearity within different metals, we found significant positive joint association of plasma Al, As, Ba, and Zn with aspartylphenylalanine and also positive joint association of plasma Ba, Co, Mn, and Pb with carnitine C14:2, with Zn and Ba plus Pb being the major contributors in the joint associations. We also found significant synergistic interactions between As and Ba in the association with aspartylphenylalanine and between Ba and Pb in the association with carnitine C14:2. There has been experimental evidence from Wistar rats that exposure to Ba and Pb both could induce lipid oxidation,[34,53] which might be one of the mechanisms underlying the synergistic Ba−Pb interaction found in our study, while mechanistic clues for the synergistic As−Ba interaction are still lacking. The novel metal−metal interactions in associations with metabolites found in our study suggested that coexposure to Ba and Pb and to As and Ba may lead to greater adverse health effects and need extra attention in real-life exposure scenarios, and further investigations are warranted to examine these interactions in other study populations.

Our study has several strengths. First, this study is the largest study to date investigating the association between exposure to multiple metals and metabolic responses, with a sample size close to that in total of previous studies, and examination of 17 plasma metals and 189 metabolites annotated by matching to authentic chemical standards, which enabled the uncovering of a number of novel pathways associated with metal exposure. Second, we applied new statistical methods such as BKMR analysis which evaluated associations between mixed metal exposure and metabolites and also revealed novel evidence of interactions between As and Ba and between Ba and Pb, respectively, in their associations with aspartylphenylalanine and carnitine C14:2. Last but not the least, we collected detailed data on demographic characteristics, lifestyle factors, and medical information, making possible the comprehensive adjustment for potential confounders in our statistical analysis.

There were also several limitations in our study. First, we only conducted a single measurement of plasma metals, which may not represent the long-term exposure levels. However, our previous study has evaluated the reproducibility and intra-individual variability of metal concentrations, and moderate to high reproducibility was observed for most metals.[25] Second, our study population was based on two nested case-control studies, thus selection bias might exist. Nevertheless, results from sensitivity analysis with additional adjustment of prevalent and future disease status and that within only control participants remained basically unchanged. Third, for the 17 metals examined in this study, we took a total measure in plasma as exposure assessment, while As has different forms with different toxicities, which may also lead to different metabolic responses that could be masked by a total measurement, and future studies with measurement of different As forms are needed to address this limitation. Fourth, participants in this study were middle-aged and elderly Chinese, which may limit generalizability to other populations, and causality cannot be established owing to the cross-sectional study design.

In this study, we replicated two metal-associated pathways, linoleic acid metabolism and aminoacyl-tRNA biosynthesis,

with novel metal associations, and uncovered two novel pathways associated with metal exposure, which were biosynthesis of unsaturated fatty acids and alpha-linolenic acid metabolism. Furthermore, we identified five metabolites as nonspecific metabolic markers of metal exposure and found evidence of interactions between As and Ba in association with aspartylphenylalanine and between Ba and Pb in association with carnitine C14:2. Our findings may provide new insights into the mechanisms underlying the adverse health effects induced by metal exposure.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.2c05547.

> Distribution and Spearman rank correlations of plasma metal concentrations (Table S1, Figure S1), detailed information on internal standards in untargeted metabolomics (Table S2), metabolites associated with individual metals (Table S3), results of pathway analysis (Table S4), overview of O2PLS model structures (Figure S2), permutation tests for O2PLS analysis (Figure S3), PIPs from the first stage of BKMR analysis (Table S5), sensitivity analyses of BKMR analysis (Figures S4−S7), and comparisons of metal concentrations and findings between our study and previous studies (Tables S6 and S7) (PDF)

> Metabolite, class, β (95% CI), P, FDR, and HMDB ID (XLSX)

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