Fluoride exposure disrupts creatinine homeostasis and undermines the reliability of urinary metabolomics normalization

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ABSTRACT

Urinary creatinine is widely used to adjust for dilution in studies of urinary biomarkers and metabolomics, yet its stability under environmental exposures is insufficiently evaluated. We analyzed data from U.S. adolescents (NHANES, n = 2378) and a cross-sectional sample of older Chinese adults (n = 475) to assess whether fluoride exposure disrupts urinary creatinine homeostasis and compromises its effectiveness as a normalization factor. In NHANES, participants with missing urinary fluoride measurements, abnormal urine albumin-to-creatinine ratios, or extreme fluoride values were excluded. In both populations, urinary fluoride demonstrated a nonlinear inverted L-shaped association with creatinine, characterized by a steep increase at lower exposure levels followed by a plateau. To quantify the downstream implications for metabolomic analyses, we compared results from unadjusted data with three dilution-control strategies: creatinine adjustment, specific gravity adjustment, and osmolality adjustment. Creatinine normalization markedly altered metabolite identification and pathway enrichment outcomes: 47.34 % of differential metabolites reversed their expression direction, and 24.72 % changed statistical significance. Enriched pathways shifted as well—from amino acid metabolism and circadian rhythm in the unadjusted analysis to lysine degradation and pyrimidine metabolism after creatinine adjustment. In contrast, specific gravity– and osmolality-based normalization produced results highly concordant with the unadjusted data in both metabolite directionality and pathway structure. Together, these findings indicate that fluoride exposure perturbs creatinine homeostasis and, consequently, the use of creatinine as a normalization factor can introduce substantial bias in urinary metabolomics. Caution is therefore warranted when applying creatinine adjustment in environmental epidemiology, particularly in populations with fluoride exposure.

1. Introduction

Urine, as a non-invasive and easily obtainable biological specimen, is widely used in metabolomics, environmental epidemiology, and toxicology research (Khamis et al., 2017; Li et al., 2025). Due to individual variability in hydration and excretion rates, urine composition is subject to considerable dilution effects, necessitating normalization strategies to enhance data comparability and interpretability.

Creatinine (CR, referring to urinary creatinine in this study), an endogenous metabolite derived from muscle metabolism, has long been employed as the primary internal reference for urine normalization based on the assumption of its stable production and excretion (O'Brien et al., 2017). In metabolomics, CR concentration is used to adjust metabolite signal intensities to account for differences in injection volume and urinary dilution (Li et al., 2022a). Similarly, CR normalization is widely adopted in quantifying urinary biomarkers such as KIM-1 and NGAL (Helmersson-Karlqvist et al., 2013, 2016).

In environmental exposure assessment, urinary CR is commonly used to normalize concentrations of metals and other analytes (Wang et al., 2020a; Nisse et al., 2017). However, recent studies have shown that CR excretion is not constant and can be influenced by various factors including age, sex, nutritional status, muscle mass, renal function, and environmental exposures (Kalantari and Bolton, 2013; Barr et al., 2005). This variability is particularly pronounced in sensitive populations such

as pregnant women, children, and the elderly, introducing potential bias when using CR as a universal normalization factor in environmental health research (Moriguchi et al., 2005; Andersen et al., 2010; Lee et al., 2021). These concerns raise the possibility that CR may be susceptible to perturbation by environmental toxicants, thereby undermining its validity as a dilution-adjustment marker.

Fluoride, a prevalent environmental contaminant, is commonly found in drinking water, toothpaste, and industrial emissions (Srivastava and Flora, 2020). Substantial evidence from animal and epidemiological studies suggests that fluoride can impair renal function by inducing tubular epithelial injury and disrupting CR filtration and excretion processes (Wang et al., 2020b; Santoyo-Sanchez et al., 2013; Tian et al., 2019; Song et al., 2014). Analyses of National Health and Nutrition Examination Survey (NHANES) data further indicate associations between fluoride exposure and multiple renal function indicators, including eGFR, BUN, and urinary albumin, implying that fluoride may compromise the validity of CR as a proxy in exposure assessments (Malin et al., 2019). Importantly, populations vulnerable to fluoride exposure—such as pregnant women, children, and the elderly—also exhibit greater variability in CR excretion, potentially amplifying standardization errors and undermining the interpretability of metabolomics and urinary biomarker findings (Helte et al., 2021; Ibarluzea et al., 2023; Barberio et al., 2017).

Given these considerations, understanding whether fluoride exposure alters CR homeostasis is essential for evaluating the reliability of CR-based normalization in metabolomics and biomarker studies. To rigorously assess this relationship, we analyzed two physiologically distinct human populations—adolescents from NHANES and older adults from endemic fluorosis areas in China. Adolescents are in a growth phase, with creatinine production likely not yet at adult peak levels because their muscle mass is still developing; compared with adults, their musculature has not fully matured (Trowbridge et al., 1982). In contrast, older adults commonly exhibit reduced muscle mass (sarcopenia), declining renal function, and diminished metabolic capacity, all of which can influence CR generation and excretion (Sabatino et al., 2021). Moreover, these two groups differ substantially in fluoride exposure: NHANES adolescents are typically exposed to low environmental levels, whereas older Chinese adults have experienced long-term moderate-to-high exposure. Leveraging these contrasts allows us to evaluate the robustness and generalizability of fluoride–CR associations across diverse physiological stages and exposure scenarios.

In this context, we integrated NHANES adolescent data and elderly Chinese individuals to systematically evaluate the impact of fluoride exposure on CR homeostasis and its implications for normalization in metabolomics. We further explored potential biological mechanisms via pathway enrichment analysis. To our knowledge, this is the first study to propose, from both mechanistic and methodological perspectives, that fluoride exposure may compromise urine normalization by destabilizing CR, highlighting the need for caution when using CR as a universal normalization factor in environmental exposomics.

2. Methods

2.1. Study population

2.1.1. NHANES population study

Data were obtained from the 2015–2016 cycle of NHANES, which was approved by the Research Ethics Review Board of the National Center for Health Statistics (NCHS), U.S. Centers for Disease Control and Prevention. All participants provided written informed consent. A total of 9971 individuals participated in NHANES during 2015–2016. We excluded 7653 participants without urine fluoride (UF) measurements, as UF concentration was the primary exposure variable, and missing UF data could lead to exposure misclassification. According to NHANES documentation, UF testing was conducted in a subsample of participants, and missingness occurred at random with respect to age, sex, and

major health characteristics; therefore, this exclusion was unlikely to introduce systematic bias. We further excluded 29 participants with abnormal urinary albumin-to-creatinine ratios and one participant with an extreme UF value well beyond the population distribution. The final analytic sample comprised 2408 participants with valid, non-zero sample weights. Demographic information—including age, sex, race/ethnicity, and the income-to-poverty ratio—was collected through household interviews. Height and weight were measured during the mobile examination center visit. Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared. Missing BMI values (n = 30) were imputed using sex- and age-specific median values. For missing income-to-poverty ratio data, values were imputed based on household income questionnaire responses. For individuals with missing responses (n = 149), the overall median value was used; for the remaining cases (n = 77), the median of the corresponding income group was applied. A flowchart depicting the participant selection process is provided in Fig. 1.

2.1.2. Chinese older adult population

In 2024, a cross-sectional survey was conducted among 475 elderly residents in Zhaodong City, Heilongjiang Province, China, to assess health status and lifestyle factors. The study protocol was approved by the Ethics Committee of the Endemic Disease Control Center at Harbin Medical University, and all participants provided written informed consent. Information was collected through standardized questionnaires, covering demographic characteristics (age, sex, ethnicity, education level, household income), lifestyle factors (smoking, alcohol consumption, dietary habits), and detailed data on drinking water exposure, including water source, duration of use, and daily intake. Medical history and current disease conditions were also recorded. To assess skeletal fluorosis, anteroposterior X-ray images of the elbow and knee joints were obtained using a portable digital medical diagnostic X-ray system (Beijing Lang'an Imaging Technology Co., Ltd.). Diagnosis was made according to the Diagnostic Criteria for Endemic Skeletal Fluorosis (WS/T 192–2008), based on a combination of radiographic findings, fluoride exposure history, and clinical symptoms.

2.2. Measurement of UF, CR, urine specific gravity, and urine osmolality

In the NHANES population, UF was measured using an ion-selective electrode (ISE) method, conducted by the Inorganic and Radiation Analytical Toxicology Branch (IRATB) of the Division of Laboratory
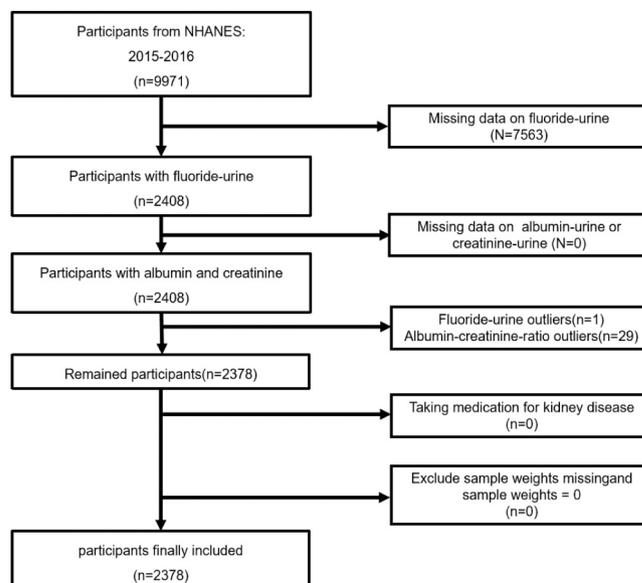

Fig. 1. Participant selection flow chart.

Sciences, National Center for Environmental Health. The limit of detection (LOD) for UF was 0.144 mg/L; values below the LOD were replaced with LOD/$\sqrt{2}$. Approximately 94 % of participants had UF levels above the LOD. Creatinine was measured using an enzymatic endpoint method performed by the University of Minnesota, with a reportable limit of 5 mg/dL. In the elderly Chinese population, UF was measured according to the national standard of China, "Determination of Fluoride in Urine by Ion-Selective Electrode Method" (WS/T 89–2015), at Harbin Medical University. The linear detection range for UF was 0.1–10 mg/L. Creatinine was measured using a creatininase oxidase method, also conducted by Harbin Medical University, with a detection limit of 30 μmol/L. Urine specific gravity was measured using reflectance photometry and completed by China Servicebio Company. Urine osmolality was measured using the freezing-point depression method and completed by China Guangce Research Institute.

2.3. Urine sample preparation and metabolomics profiling in the Chinese older adult population

In this study, a 1:1 case–control design was applied based on age and sex to compare participants with and without skeletal fluorosis. A total of 25 cases and 25 matched controls were selected for metabolomic analysis. Untargeted metabolomic analysis was conducted by Calibra Lab at DIAN Diagnostics (Hangzhou, Zhejiang, China) on their CalOmics metabolomics platform.

All urine samples were immediately aliquoted after collection and stored at −80 °C until analysis to prevent metabolite degradation. For sample preparation, each urine sample was extracted with methanol at a ratio of 1:4 (urine: methanol), vortexed for 3 min, and centrifuged at $4000 \times g$ for 10 min at 20 °C. The supernatant was collected, divided into four aliquots of 100 μL each, dried under a stream of nitrogen, and reconstituted in an appropriate solvent prior to instrumental analysis.

Multiple deuterated compounds were used as internal standards to correct for signal drift and ensure quantitative accuracy. The internal standard mixture—composed primarily of deuterated amino acids, fatty acids and sugars (e.g., d_3-leucine, d_{35}-octadecanoic acid, d_7-glucose)—was added in equal amounts to every sample and to the pooled quality control (PQC) samples. The retention time and signal intensity of each internal standard were continuously monitored throughout the analytical run to ensure instrument stability; when abnormal drift was detected, corrective quality-control actions (e.g., re-sampling and re-analysis) were implemented.

Rigorous quality-control procedures were applied: a pooled QC sample, prepared by proportionally mixing aliquots from all study samples, was injected once every ten study samples to monitor system stability; process blanks were included to detect potential contamination. Non-targeted detection was performed on a UPLC–MS/MS platform (Waters ACQUITY 2D UPLC coupled with Thermo Q Exactive) using four chromatographic–mass spectrometric methods (POSa, POSb, NEGa, NEGb) with C18 or HILIC columns under different ionization modes.

2.4. Statistical analysis

2.4.1. Statistical analysis in the study populations

All statistical analyses were performed using SPSS (version 25.0) and R (version 4.2.3), with two-sided $P < 0.05$ considered statistically significant. For the NHANES population, MEC weights were applied to account for the complex multistage sampling design. Urinary fluoride concentrations were divided into quartiles based on the 25th, 50th, and 75th percentiles: Q1 (0.102–0.306 mg/L), Q2 (0.306–0.517 mg/L), Q3 (0.517–0.807 mg/L), and Q4 (0.807–4.484 mg/L). The distribution of CR and covariates across quartiles was assessed for normality. Creatinine exhibited a skewed distribution, and log-transformation did not normalize the histogram; thus, CR levels were summarized using the median and interquartile range (IQR). Group differences were evaluated

using non-parametric tests (Wilcoxon rank-sum and Kruskal–Wallis tests). For both the NHANES and Chinese older adult populations, the dose–response relationship between UF and CR was examined using restricted cubic spline (RCS) models. When evidence of nonlinearity was observed, piecewise weighted linear regression models were fitted, with turning points determined from the spline curve. Covariates included age, sex, race/ethnicity, and BMI in both the NHANES and Chinese older adult cohorts.

2.4.2. Metabolomics data analysis in the Chinese older adult population

All statistical analyses were performed with R language (R version 3.4.1; Rstudio version 1.4.1717; mixOmics version 6.10.9). After pre-processing of raw data and data quality control inspection, ion peaks were extracted using proprietary in-house IT hardware and software. Metabolites were identified by searching an in-house library generated from running reference standards commercially purchased or obtained from other sources. Identification of metabolites in samples requires strict matching of three criteria between experimental data and library entry: narrow window retention index (RI), accurate mass with variation less than 10 ppm and MS/MS spectra with high forward and reverse searching scores. Peak area for each metabolite was calculated using area-under-the-curve. Before statistical analysis, raw peak areas were normalized to adjust for system fluctuation among different run days. The normalized peak areas were then log-transformed (log2) to reduce data distribution skewness and be in approximate normal distribution (Gaussian distribution). Missing values in peak area matrix were imputed by using the minimal detection value of a metabolite among all samples. Significantly changed metabolites between case and control groups were found by parametric (student's t-Test, ANOVA) or non-parametric (Wilcox's rank test, Kruskal-Wallis, etc.) statistical methods. Multivariate analysis approaches including principal component analysis (PCA). The pathway enrichment analysis was conducted using MetPA based on KEGG database and Pathview. Only significantly different metabolites with associated KEGG ID were included in this analysis. Significance analysis of pathway enrichment was completed by hypergeometric test.

The confidence levels of metabolite identification were classified according to the Metabolomics Standards Initiative (MSI): Level 1 indicates metabolites confirmed with authentic reference standards, providing the highest confidence; Level 2 (*) indicates metabolites not confirmed with reference standards but supported by literature and database evidence, representing high-confidence identification; Level 3 indicates metabolites identified based on literature and database evidence without reference standards, considered relatively reliable. The confidence level of each metabolite is provided in Supplementary Material 2.

For univariate analysis, the Benjamini–Hochberg (BH) method was applied to correct for multiple testing, controlling the false discovery rate (FDR) at 5 %. If any metabolites remained statistically significant after BH correction, the adjusted p-values were used for subsequent analyses. In cases where no metabolites passed the FDR threshold, the original unadjusted p-values were used for downstream analyses.

3. Results

3.1. Nonlinear association between fluoride exposure and CR levels in the NHANES population

Table 1 presents the weighted demographic characteristics. Participants were categorized into quartiles based on UF concentrations. As shown in Table 2, there were significant differences in CR levels across UF quartiles, with a clear increasing trend corresponding to higher UF concentrations. We have provided the unweighted baseline feature tables S1 and S2 in the supplementary file.

We applied RCS modeling to explore and visualize the dose–response relationship between UF and CR (Fig. 2A). The results indicated a

Table 1
Weighted baseline characteristics of ungrouped study subjects for UF.

Variables	Level	Overall
n		54442457
Age (years) (mean (SD))		12.99 (3.86)
Household income to poverty ratio (mean (SD))		2.43 (1.56)
UF (mg/L) (median [IQR])		0.52 [0.31, 0.81]
CR (mg/dL) (median [IQR])		110.00 [62.00, 163.00]
Gender (n (%))	Female	26267309 (48.2)
	Male	28175148 (51.8)
Race (n (%))	Mexican American	8349881 (15.3)
	Non-Hispanic Asian	2577885 (4.7)
	Non-Hispanic Black	7367154 (13.5)
	Non-Hispanic White	28292573 (52.0)
	Other Hispanic	4943616 (9.1)
	Other Race - Including Multi-Racial	2911349 (5.3)
Bmi_category (n (%))	Normal weight	32381804 (59.5)
	Obese	10724431 (19.7)
	Overweight	9672757 (17.8)
	Underweight	1663465.0 (3.1)

significant inverted L-shaped nonlinear association (P for nonlinearity < 0.001). Specifically, CR levels rose rapidly with increasing UF concentrations below 0.51 mg/L, while the trend plateaued at concentrations above this threshold. Based on this inflection point, we conducted piecewise weighted linear regression. After adjusting for age, sex, BMI, race/ethnicity, and income-to-poverty ratio, each 1 mg/L increase in UF was associated with a 224.33 mg/dL increase in CR (95 % CI: 175.10, 273.57; P < 0.001) in the lower exposure range (UF ≤ 0.51 mg/L). In contrast, for UF > 0.51 mg/L, the regression coefficient was 41.41 mg/dL (95 % CI: 11.12, 71.70; P = 0.02).

3.2. Validation of the UF–CR association in the Chinese older adult population

In the Chinese older adult population, the median age was 63 years

(IQR: 55.8–69.0), with 35.5 % male and 64.5 % female participants. The median UF concentration was 1.25 mg/L (IQR: 0.88–1.81), and the median CR level was 21.41 mg/dL (IQR: 12.51–28.91).

After adjusting for age, sex, and BMI, RCS modeling revealed a significant nonlinear association between UF and CR (P for nonlinearity < 0.001). As UF increased, CR followed an inverted L-shaped trajectory, with an inflection point observed near UF = 1.02 mg/L, suggesting a potential threshold or physiological adaptation in CR excretion under increasing fluoride exposure (Fig. 2B). Among covariates, both age (P = 0.001) and sex (P < 0.001) were significantly associated with CR, while BMI was not significant (P = 0.53). Subsequently, piecewise linear regression analysis was conducted based on the identified inflection point to evaluate UF's effect on CR across exposure strata. After adjustment for age, sex, and BMI, UF levels below 1.02 mg/L were associated with a significant increase in CR: for each 1 mg/L increase in UF, CR increased by 20.83 mg/dL (95 % CI: 13.93–27.73; P < 0.001). At UF levels ≥ 1.02 mg/L, the increasing trend persisted but with a reduced effect size (β = 3.27 mg/dL; 95 % CI: 1.79–4.75; P < 0.001). In addition, age was negatively associated with CR (β = –0.21 mg/dL; P < 0.001), and male participants had significantly higher CR levels compared with females (β = 6.38 mg/dL; P < 0.001).

3.3. Impact of fluoride exposure on the effectiveness of CR normalization in metabolomic analyses

A total of 995 urinary metabolites were detected, encompassing a wide range of metabolic pathways. According to Super Pathway classification, amino acids accounted for the largest proportion (28.74 %), followed by xenobiotics (24.82 %) and lipids (19.10 %). Other categories included incompletely annotated compounds (6.13 %), nucleotides (5.43 %), cofactors and vitamins (4.72 %), carbohydrates (4.22 %), energy metabolites (4.22 %), peptides (1.31 %), and a small proportion of hormones and secondary metabolites. This distribution suggests that the metabolomic platform used in this study offered broad coverage of amino acid, lipid, and xenobiotic pathways, supporting its capacity to capture fluoride-associated metabolic alterations.

To evaluate the influence of CR normalization on metabolite detection, we compared the data before and after CR adjustment. PCA revealed that PC1 explained 26.73 % of the variance before normalization, with a discernible group separation between cases and controls. After normalization, the explanatory power of PC1 declined to 19.42 %, and group separation was reduced accordingly (Figure S1). Heatmap visualization showed that, prior to adjustment, a broad range of metabolites—particularly amino acids, lipids, and xenobiotics—were upregulated in the case group, indicating pathway activation
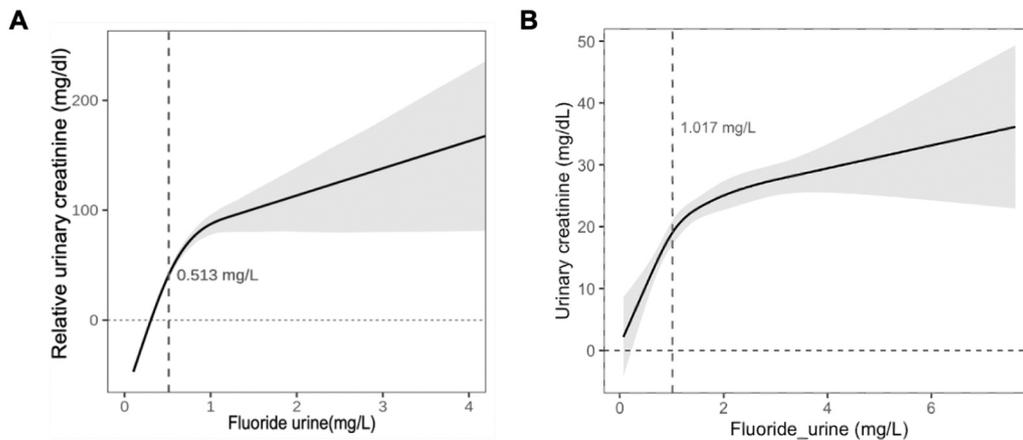
Table 2
Weighted baseline characteristics of the UF subgroup study population.

UF (mg/L)	Level	Quartile1 (0.102–0.306)	Quartile2 (0.306–0.517)	Quartile3 (0.517–0.807)	Quartile4 (0.807–4.484)	p
n		13615404	13697116	13563064	13566874	
Age(years) (mean (SD))		13.37 (3.82)	13.11 (3.73)	12.99 (3.85)	12.49 (3.98)	0.08
Household income to poverty ratio (mean (SD))		2.44 (1.54)	2.38 (1.54)	2.50 (1.57)	2.41 (1.58)	0.63
CR (mg/dL) (median [IQR])		49.00 [32.00, 78.12]	104.00 [68.00, 137.00]	130.00 [90.00, 184.00]	164.03 [117.00, 215.17]	< 0.001
Gender (n (%))	Female	8011644 (58.8)	6809024 (49.7)	5867734 (43.3)	5578907 (41.1)	0.001
	Male	5603760 (41.2)	6888092 (50.3)	7695329 (56.7)	7987967 (58.9)	
Race (n (%))	Mexican American	2129052 (15.6)	2272393 (16.6)	1881121(13.9)	2067315 (15.2)	0.01
	Non-Hispanic Asian	1030427 (7.6)	698216 (5.1)	423756 (3.1)	425487 (3.1)	
	Non-Hispanic Black	1112849 (8.2)	1843828 (13.5)	1698055 (12.5)	2712422 (20.0)	
	Non-Hispanic White	7246294 (53.2)	6979977 (51.0)	7632482 (56.3)	6433820 (47.4)	
	Other Hispanic	1395293 (10.2)	1340269 (9.8)	1061315 (7.8)	1146739 (8.5)	
	Other Race	701490 (5.2)	562434 (4.1)	866335 (6.4)	781090 (5.8)	
Bmi category (n (%))	Normal weight	8459228 (62.1)	7867773 (57.4)	7876513 (58.1)	8178290 (60.3)	0.60
	Obese	2377324 (17.5)	2719987 (19.9)	3084077 (22.7)	2543044 (18.7)	
	Overweight	2382864 (17.5)	2581973 (18.9)	2181640 (16.1)	2526280 (18.6)	
	Underweight	395987 (2.9)	527383 (3.9)	420834 (3.1)	319260 (2.4)	

Fig. 2. Associations between UF and CR (A) The RCS results of the NHANES database (B) The RCS results of the Chinese Elderly Database.

(Figure S2). After CR normalization, these differences were attenuated, and some metabolites even reversed in direction (Figure S3), suggesting that normalization may introduce systematic bias under certain exposure scenarios, thereby masking true biological signals.

Differential metabolite analysis corroborated these findings: 132 differential metabolites (124 upregulated, 8 downregulated) were identified before adjustment, compared with 148 (1 upregulated, 147 downregulated) after adjustment. Among them, 47.34 % showed a complete reversal in expression direction, and 24.72 % changed in

statistical significance. Only 17 metabolites remained significantly different across both conditions, of which 9 exhibited opposite expression trends (Fig. 3). Notably, CR levels themselves showed an inverse trend between groups before and after normalization (Figure S4), further suggesting that conventional CR-based adjustment may obscure or distort true metabolic differences and introduce misinterpretation of exposure effects.

Pathway enrichment analysis highlighted the impact of normalization strategies. Before adjustment, enriched pathways were primarily
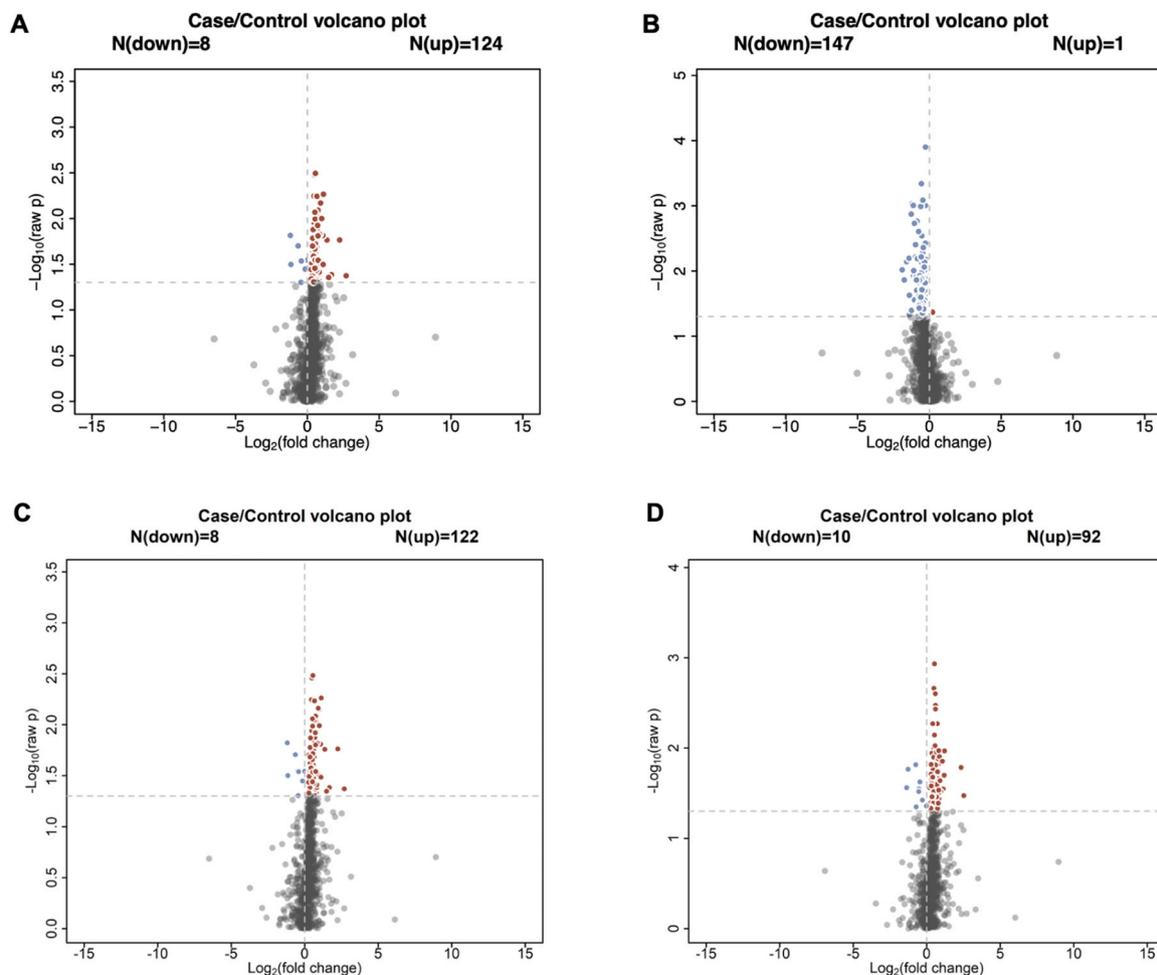

Fig. 3. Volcanic map results of metabolomics differences test in Chinese elderly people (A) Raw data (B) Creatinine-adjusted (C) Specific gravity-adjusted (D) Osmolality-adjusted. N (down) and N (up) indicate the number of down-regulated and up-regulated metabolites, respectively.

related to cysteine and methionine metabolism, circadian rhythm, and longevity regulation—mechanisms linked to sulfur cycling and endocrine function (Fig. 4A). After adjustment, enrichment shifted toward dopaminergic synapse, lysine degradation, protein digestion and absorption, and pyrimidine metabolism, indicating a transition toward neurotransmitter and nucleotide metabolism pathways (Fig. 4B). These shifts underscore the potential of CR normalization to reduce sensitivity in detecting specific exposure-related biological responses. Collectively, our findings suggest that CR-based normalization may introduce systematic bias under certain exposure conditions, affecting the stability of differential features and the interpretation of metabolic pathways.

In contrast, the results obtained after CR normalization showed markedly lower concordance with both the raw and SG/OSM-normalized profiles. Specifically, CR adjustment produced the largest number of metabolite direction reversals, the greatest shifts in statistical significance, and the lowest overlap in enriched pathways with the unadjusted data. Whereas SG and OSM normalization largely maintained the core metabolic signatures related to sulfur metabolism, endocrine regulation, and cardiometabolic signaling, CR normalization generated a pathway structure that deviated substantially from this pattern. This divergence reflected reduced preservation of exposure-related metabolic signals when creatinine was used as the adjustment factor under fluoride exposure conditions. The SG-normalized results are shown in Fig. 3C and Fig. 4C, and the OSM-normalized results are shown in Fig. 3D and Fig. 4D.

We also considered the presence of "exposed but unaffected" individuals in the analysis. Some control participants had urinary fluoride levels > 1.6 mg/L, while some cases had UF < 1.6 mg/L, reflecting the cumulative nature of skeletal fluorosis and short-term fluctuations in urinary fluoride. Their inclusion may attenuate metabolic differences between groups. To address this, we performed a sensitivity analysis excluding control samples with UF > 1.6 mg/L and case samples with UF < 1.6 mg/L, followed by BH correction. The results showed directionality consistent with the original analysis, and enrichment of fluoride-related pathways, supporting the biological plausibility of our findings. The urinary fluoride distribution between cases and controls and the sensitivity analysis results are provided in Figure S5.

Collectively, these results demonstrate that CR normalization exerts the greatest impact on metabolite directionality, statistical significance, and pathway interpretation, whereas SG and OSM normalization maintain higher fidelity to the underlying metabolic structure of the raw data under fluoride exposure.

4. Discussion

4.1. Fluoride exposure substantially disrupts the stability of CR excretion

In both the NHANES adolescent and Chinese older adult populations, we consistently observed a significant inverted L-shaped nonlinear dose–response relationship between UF and CR levels, with CR rising rapidly at low UF concentrations and reaching a plateau at higher concentrations. In NHANES adolescents, the inflection point was approximately 0.51 mg/L UF, whereas in the Chinese older adults it was around 1.02 mg/L UF. This nonlinear pattern, replicated across two independent populations, provides robust evidence that fluoride exposure can directly disrupt CR excretion. The difference in inflection points
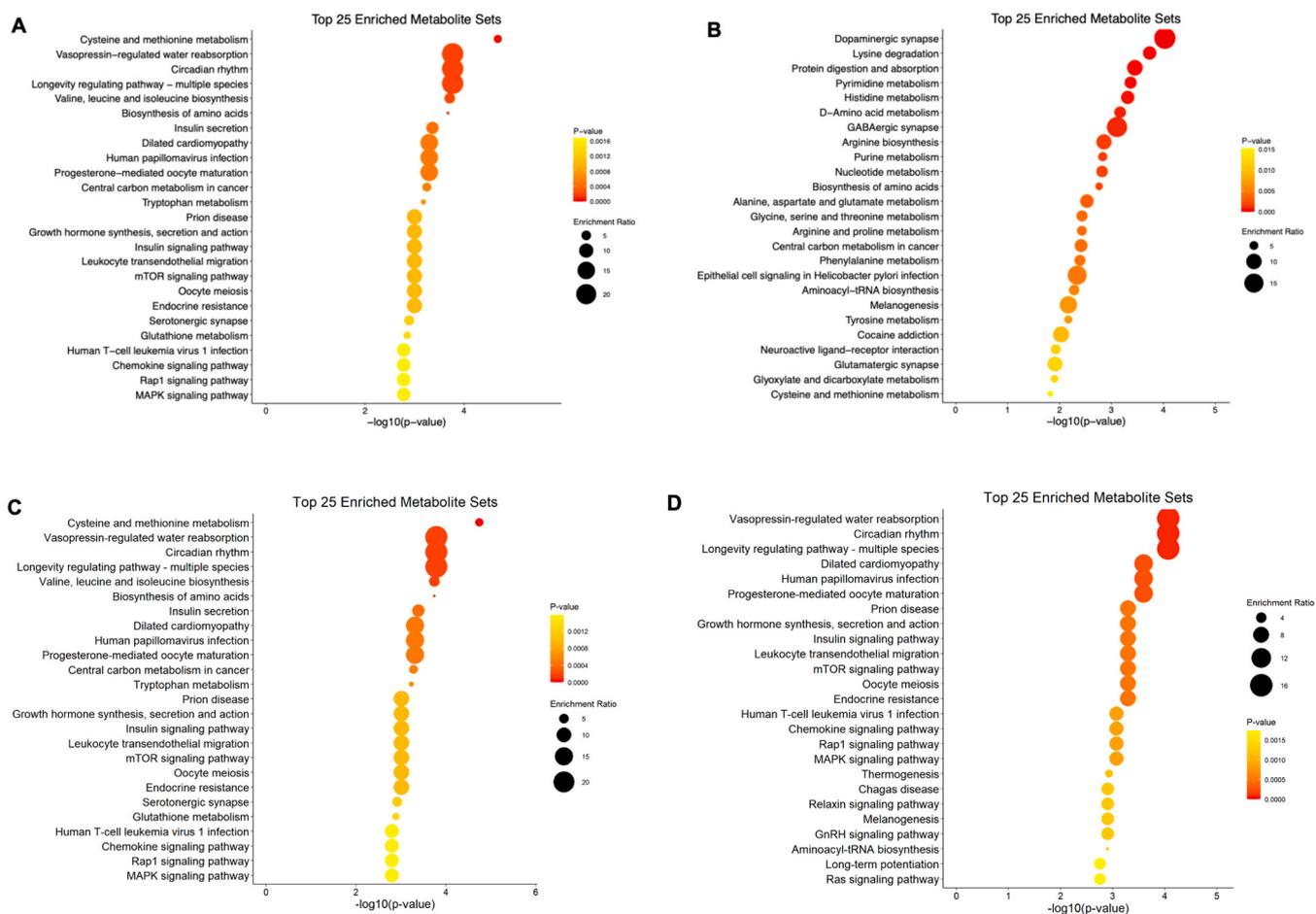

Fig. 4. Bubble plot of top 25 enriched metabolic pathways in metabolomics (A) Raw data (B) Creatinine-adjusted (C) Specific gravity-adjusted (D) Osmolality-adjusted.

between the populations may reflect variations in age structure, muscle mass, renal function, skeletal fluoride accumulation, and physiological adaptation. These observations suggest that the stability of CR as a urinary dilution correction factor may be compromised under conditions of fluoride exposure.

Traditionally, CR has been widely used in metabolomics, environmental epidemiology, and toxicology to adjust for urine dilution, based on the assumption that CR production is stable and not significantly influenced by exogenous exposures (Khamis et al., 2017; Li et al., 2025). However, both epidemiological and animal studies indicate that fluoride can impair proximal tubular function, disrupt ion channel activity, and alter bone and muscle metabolism, potentially leading to dynamic changes in CR excretion (Santoyo-Sanchez et al., 2013; Çetin and Yur, 2016; Luo et al., 2017). These mechanisms may lead to dynamic changes in CR excretion, undermining the assumption of its stable elimination. Previous studies have indicated that CR excretion is susceptible to various endogenous and exogenous factors, such as urine flow rate, hydration status, age, sex, body size, and diet. This variability is particularly pronounced in individuals with unstable renal function or under environmental exposure conditions (Goldstein, 2010; Gaines et al., 2010; O'Brien et al., 2016). Therefore, using CR for normalization under fluoride exposure may not only fail to correct for dilution bias but also introduce new sources of systematic error.

4.2. Risks of signal attenuation and underestimation of causal effects induced by CR normalization

Our results further demonstrate that CR normalization substantially interferes with the extraction of metabolomic signals. In the unnormalized data, skeletal fluorosis cases and controls were clearly separated in the PCA model, with differential metabolites predominantly enriched in amino acid, lipid, and xenobiotic pathways. After CR normalization, PC1 variance decreased from 26.73 % to 19.42 %, group separation was markedly reduced, and some differential metabolites reversed direction. In differential metabolite analysis, 47.34 % of metabolites showed complete direction reversal, 24.72 % changed in statistical significance, and only 17 metabolites remained significant under both conditions, among which 9 exhibited opposite trends. These results indicate that CR normalization can obscure true biological signals and potentially mislead exposure-phenotype interpretations. These observations are consistent with previous studies showing that when a normalization factor covaries with the exposure, CR adjustment can attenuate or distort exposure–phenotype associations (Chen et al., 2013; Bulka et al., 2017). Our study provides empirical evidence of this phenomenon using concrete metabolomic data, highlighting the need for caution when using CR as the sole normalization factor under fluoride exposure conditions.

4.3. Impact of CR normalization on mechanistic interpretation through pathway identification

Beyond statistical features, we further observed substantial shifts in the direction of enriched metabolic pathways before and after CR normalization. In the unnormalized data, enriched pathways were primarily associated with energy and endocrine-related mechanisms, such as cysteine and methionine metabolism, the sulfur cycle, circadian rhythm, and longevity regulation—pathways potentially influenced by fluoride exposure. After CR correction, however, the significantly enriched pathways shifted toward dopaminergic synapse activity, protein digestion, and pyrimidine metabolism, indicating a "drift" in mechanistic interpretation. This finding suggests that CR normalization not only affects the identification of differential metabolites but may also distort the interpretation of exposure-related mechanisms at the pathway level. This issue of miscalibrated mechanistic inference has also been highlighted in a systematic NMR-based metabolomics study by Li et al., who recommended against using CR as the sole normalization

factor when it covaries with the exposure variable (Li et al., 2022b).

4.4. Caution in the use of CR normalization in epidemiological studies

Our findings indicate that CR normalization should be applied with caution in environmental exposure studies, particularly when the exposure factor may influence renal function or CR metabolism. Under fluoride exposure, CR excretion exhibits nonlinear behavior with population-specific inflection points (approximately 0.51 mg/L UF in NHANES adolescents and 1.02 mg/L UF in Chinese older adults), suggesting that CR may no longer satisfy the assumption of stable, exposure-independent production.

Metabolomic analyses show that CR normalization substantially alters the identification of differential metabolites, reverses their expression trends, and shifts pathway enrichment, potentially leading to underestimation or misdirection of exposure–phenotype relationships. Similar issues have been documented in previous studies. For example, a 2022 systematic comparison using Finnish cohort data evaluated seven urine metabolomics normalization strategies and found that, while CR normalization could partially adjust for urine dilution, it significantly altered the estimated associations between clinical phenotypes (e.g., BMI, mean arterial pressure) and metabolites in regression models, suggesting potential underestimation or directional shifts in exposure–phenotype relationships (Li et al., 2022b).

Therefore, unless the independence and stability of CR can be clearly validated in a given research context—particularly under conditions of exogenous exposure or fluctuating renal function—CR should not be used as the sole normalization method. Researchers should carefully assess the mechanistic validity of CR normalization in the context of exposure pathways, individual physiological status, and potential covariation with the exposure variable, to avoid systematic bias and misinterpretation of exposure effects in both statistical and mechanistic analyses.

4.5. Comparison of normalization strategies and recommendations

Given the limitations of CR normalization, we evaluated the performance of multiple alternative normalization strategies. Our results indicate that, compared with CR adjustment, specific gravity (SG) and osmolality (OSM) normalization better preserved the directionality of metabolite changes, the significance of differential metabolites, and core metabolic pathways, including sulfur metabolism, endocrine regulation, and cardiometabolic signaling. The metabolite reversals, shifts in statistical significance, and pathway alterations observed with CR normalization were not seen with SG or OSM, suggesting that these methods are more robust when the normalization factor covaries with the exposure variable.

Furthermore, combining CR correction with MS total useful signal (MSTUS) normalization has been shown to improve the stability and consistency of metabolomic data (Chen et al., 2013). Other potential strategies include total ion current (TIC) normalization and reference metabolite-based approaches (e.g., IS-PSEURID), which have demonstrated higher robustness in populations with environmental exposures or fluctuating renal function (Chen et al., 2013; Li et al., 2022b).

Overall, environmental epidemiology studies should tailor normalization strategies to the specific exposure context, individual physiological status, and characteristics of the normalization factor. By accounting for dilution effects and exposure-related variability, integrating multiple normalization methods can enhance both the reliability and biological interpretability of metabolomic findings. Our results suggest that, under fluoride exposure, relying solely on CR normalization may introduce systematic bias, whereas SG, OSM, or combined normalization strategies provide a more robust approach for metabolomic data correction.

4.6. Strengths and limitations

This study has several limitations. First, both population datasets were cross-sectional, restricting causal inference. Nevertheless, the reproducibility of the inverted L-shaped UF–CR association across two independent populations reinforces the robustness of the observed nonlinear pattern. Second, the metabolomics analysis was conducted in a relatively small sample (25 cases and 25 controls). However, rigorous quality control procedures, convergent results across sensitivity analyses, and coherent pathway-level signatures reduce concerns about statistical instability. Finally, although multiple normalization strategies were compared, non-targeted metabolomics inherently carries analytical variability; future studies incorporating targeted platforms and longitudinal exposure assessments will be essential to refine mechanistic interpretations.

5. Conclusions

In conclusion, our findings demonstrate that fluoride exposure markedly disrupts the stability of urinary creatinine excretion, challenging a fundamental assumption underlying creatinine-based dilution adjustment in biomonitoring and metabolomics. When creatinine covaries with the exposure of interest, its use as a normalization factor can attenuate, obscure, or even invert exposure-related metabolic signatures and pathway interpretations. By contrast, specific gravity and osmolality normalization retained signal directionality, statistical robustness, and pathway coherence under fluoride-induced renal and metabolic perturbation. These results highlight the need to select dilution-adjustment strategies based on exposure biology and mechanistic validity rather than convention, providing methodological and biological insights relevant to environmental fluoride research.

CRediT authorship contribution statement

Shirui Yan: Investigation. **Rui Zhang:** Investigation. **Zhe Mo:** Funding acquisition. **Gazala Zafar:** Investigation. **Nian Gao:** Investigation. **Lei Wu:** Visualization, Investigation. **Junrui Pei:** Funding acquisition, Data curation, Conceptualization. **Ailin Li:** Writing – original draft, Visualization, Investigation. **Xinxiao Li:** Investigation. **Di Wu:** Investigation. **Minghan Luo:** Investigation. **Xiaowei Wang:** Investigation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2025.119555.

Data availability

Data and analytical code can be made available to others on request after publication. All requests should be made via email to the corresponding author.

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