Free Radical Biology and Medicine 194 (2023) 52–61

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Metabolomic patterns, redox-related genes and metals, and bone fragility endpoints in the Hortega Study

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A R T I C L E I N F O

Keywords:
Metabolomics
Bone mineral density
Osteoporosis-related bone fractures
Candidate genes
Metals
Redox

A B S T R A C T

Background: The potential joint influence of metabolites on bone fragility has been rarely evaluated. We assessed the association of plasma metabolic patterns with bone fragility endpoints (primarily, incident osteoporosis-related bone fractures, and, secondarily, bone mineral density BMD) in the Hortega Study participants. Redox balance plays a key role in bone metabolism. We also assessed differential associations in participant subgroups by redox-related metal exposure levels and candidate genetic variants.
Material and methods: In 467 participants older than 50 years from the Hortega Study, a representative sample from a region in Spain, we estimated metabolic principal components (mPC) for 54 plasma metabolites from NMR-spectrometry. Metals biomarkers were measured in plasma by AAS and in urine by HPLC-ICPMS. Redox-related SNPs (N = 341) were measured by oligo-ligation assay.
Results: The prospective association with incident bone fractures was inverse for mPC1 (non-essential and essential amino acids, including branched-chain, and bacterial co-metabolites, including isobutyrate, trimethylamines and phenylpropionate, versus fatty acids and VLDL) and mPC4 (HDL), but positive for mPC2 (essential amino acids, including aromatic, and bacterial co-metabolites, including isopropanol and methanol). Findings from BMD models were consistent. Participants with decreased selenium and increased antimony, arsenic and, suggestively, cadmium exposures showed higher mPC2-associated bone fractures risk. Genetic variants annotated to 19 genes, with the strongest evidence for *NCF4, NOX4* and *XDH,* showed differential metabolic-related bone fractures risk.
Conclusions: Metabolic patterns reflecting amino acids, microbiota co-metabolism and lipid metabolism were associated with bone fragility endpoints. Carriers of redox-related variants may benefit from metabolic interventions to prevent the consequences of bone fragility depending on their antimony, arsenic, selenium, and, possibly, cadmium, exposure levels.

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https://doi.org/10.1016/j.freeradbiomed.2022.11.007
Received 13 September 2022; Received in revised form 6 November 2022; Accepted 6 November 2022
Available online 9 November 2022
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Abbreviations

AAA	Aromatic amino acid
Asb	Arsenobetaine
BCAA	Branched-chain amino acid
BMD	Bone mineral density
BMI	Body mass index
eGFR	Estimated glomerular filtration rate
HDL	High-density lipoprotein
HWE	Hardy Weinberg equilibrium P value
LDL	Low-density lipoprotein
MAF	Minor allele frequency
mPC	Metabolic Principal Component
NMR	Nuclear Magnetic Resonance
PCA	Principal Component Analysis
P-int	P value of interaction
ROS	Reactive oxygen species
SNPs	Single nucleotide polymorphisms
VLDL	Very low-density lipoprotein

1. Introduction

Bone metabolism is a tightly regulated balance of bone resorption and formation [1]. The loss of bone homeostasis due to bone cells dysfunction and related regulatory factors alteration, leads to pathological bone remodeling, where bone resorption can take over bone formation, leading to osteoporosis and bone fragility [1]. Metabolism subproducts, such as amino acids, microbiota co-metabolites and lipoproteins, are emerging as potentially relevant bone health determinants [2–6]. For instance, mechanistic studies report that essential amino acids could stimulate osteoblast differentiation and promote bone synthesis [3]. Alternatively, lipoprotein profile has been linked to bone mass loss by promoting osteoclast formation and inhibiting osteoblast growth [2]. In addition, mounting evidence support that redox unbalance and inflammatory cytokines release can ultimately lead to bone deterioration [4], although the precises mechanisms are still not completely understood. Gut microbiota may determine osteoclast differentiation through redox mechanisms [6], and also, it can influence the digestive uptake of essential elements involved in bone remodeling [5]. However, previous studies evaluated the role of individual metabolites on bone homeostasis, but the joint influence of metabolites to form metabolic patterns on bone endpoints has not been reported. Importantly, previous studies have been mostly cross-sectional.

The aim of this study was to evaluate the prospective association of plasma metabolomic profiles summarized as metabolic principal components (mPCs) with newly diagnosed osteoporosis-related fractures (from now on called incident bone fractures), in the Hortega Study, a representative population from a region in Spain. Given the fact that bone loss is the main risk factor for osteoporosis-related bone fractures, in secondary analyses, we assessed the cross-sectional association between plasma metabolomic profiles and reduced bone mineral density (BMD). In addition, since antimony, arsenic, cadmium, cobalt, copper, selenium and zinc have been related to metabolic patterns and oxidative stress markers in our study population [7,8], and redox balance plays a key role in bone metabolism, we also assessed potential differential associations in population subgroups defined by metal exposure biomarkers levels, and by genotypes of redox-related genetic variants, including the *In silico* exploration of associated biological pathways. Given the high prevalence of osteoporosis and elevated burden of osteoporosis-related bone fractures, studies evaluating the influence of gene-environment interactions on bone fragility can help to identify new mechanisms and strategies relevant for bone disease prevention.

2. Materials and methods

2.1. Study population

The study population was made of adults older than 20 years, residents in the catchment area of Hospital Universitario Rio Hortega in Valladolid, in Spain, after a multi-stage complex sampling that it has been previously reported [9]. The Hortega Study examination visit and samples collection happened in 2001–2003. Participants were followed-up until November 30, 2015. As age is as a main determinant of bone status, especially for women, we included only participants over 50 years in the current analysis. Among the 775 participants older than 50 year, we excluded 28 missing BMD measurements, 163 missing metabolites levels, 64 missing dietary pattern intakes, 10 missing urinary cotinine levels, 1 missing alcohol intake and 2 missing smoking status. We further excluded 9 participants with a prevalent fracture at baseline and 31 participants considered lost during follow-up (6 participants did not use the healthcare system, 6 participants moved out of the catchment area, 6 participants died during follow-up and 13 participants could not be located) resulting in a total of 467 participants.

Participants signed an informed consent when joining to the study. Additionally, the research protocol was approved by the Ethics Committee of the Hospital Universitario Rio Hortega.

While unrestricted data sharing is not allowed, the statistical code and data that support the findings of this study are available upon reasonable request by qualified researchers trained in human subject confidentiality protocols. The present study adhered to the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE).

2.2. Plasma metabolites levels

Non-fasting plasma metabolites levels were obtained by a Nuclear Magnetic Resonance (NMR) Spectroscopy. The samples were kept at 37 °C, acquiring a single-pulse pre-saturation experiment. For the process, 82 μl of D2O and 418 μl of blood plasma were mixed in a 5 mm NMR tube. 1H NMR spectra (using as reference the doublet of alanine at 1.478 ppm) were recorded using a Bruker Avance DRX 600 spectrometer (Bruker GmbH, Rheinstetten, Germany). After the assignment of resonances in the spectra, the metabolite peaks in the aliphatic region of the spectra were normalized to total aliphatic spectral area excluding residual water signal to calculate relative abundances, homogenize metabolites concentrations and avoid spectra variability.

Signals belonging to selected metabolites were quantified using semi-automated in-house MATLAB 6.5 (The MathWorks Inc., Natick, Massachusetts) integration and peak-fitting routines. Chenomx NMR Suite 4.5 software and two-dimensional NMR technology, including heteronuclear single quantum and homonuclear correlation spectroscopy, determine the quantification of the metabolomics.

In addition, 500 μl of blood plasma samples were shipped on dry ice to Biosfer Teslab (Reus, Spain) for an advanced lipoprotein profiling by using the LIPOSCALE® test, a commercially available methodology based on 2-D diffusion-ordered 1H NMR spectroscopy [70]. The lipoprotein profile characterization included the lipid content, size of the lipoprotein classes (very low-density lipoprotein [VLDL], low-density lipoprotein [LDL] and high-density lipoprotein [HDL]), and the particle concentration of its respective large, medium and small lipoprotein subclasses.

For statistical purpose, to consider the potential variability introduced by fasting time in metabolomic profile, we corrected plasma metabolites levels by fasting time (hours) by using linear regression recalibrated the resulting residuals to metabolites concentrations mean in those individuals that reported fasting conditions.

2.3. Bone fragility-related endpoints

Osteoporotic-related fractures. Fracture events occurring during the follow-up, were assigned using imaging tests, including X-ray, computerized radiography scan (CT) or magnetic resonance (MR), available from clinical records. A physician evaluated medical records of 14-year follow up [9] to exclude accidental fractures and determine if fractures were related to osteoporotic conditions, mostly detected in humerus, wrist, hip and spine location. In cases a bone fracture related to osteoporosis was detected, time to event was calculated from the date of the baseline exam until the date of diagnosis of a pathological fracture (years). For non-cases, time to event was computed from the date of the Hortega Study examination visit (2001–2003) until the administrative censoring day (November 30, 2015) or the date of the death if it happened during the follow-up.

Peripheral BMD. A peripheral densitometry (Peripheral Instantaneous X-ray Imaging System [PIXI]) was performed in the right calcaneus of the participants to calculate BMD. From continuous BMD in grams per square centimeters (g/cm^2) we obtained the T-score for each sex based on a reference Spanish population. Reduced BMD was defined as a T-Score below one standard deviation (-1 SD), which showed the highest sensitivity to detect osteopenia in our population comparing to central DEXA in hip [10]. The calibration and quality standards were ensured keeping in the limits set by the software periodically.

2.4. Metals biomarkers

Copper, selenium and zinc were measured in plasma by atomic absorption spectrometry (AAS) with graphite furnace at Cerba international Laboratories Ltd and antimony, arsenic, arsenobetaine, cadmium and cobalt were measured in urine using a 7500 cs model inductively coupled-plasma mass spectrometry (ICP-MS) (Agilent Technologies, Tokyo, Japan). The limit of detection (and corresponding coefficient variation [CV]) for plasma metals were 0.63 μg/dL (7.2%) for copper, 29.9 μg/L (5.6%) for selenium and 0.65 μg/dL (4.2%) for zinc, and for urine metals 0.003 μg/L (5.27%) for antimony, 0.024 μg/L (6.5%) for arsenic, 0.005 μg/L (5.2%) for cadmium and 0.001 μg/L (2.96%) for cobalt. The percentage of individuals below the limit of detection was 1.81% for antimony, 0.07% for cadmium and 0.14% for cobalt. The limit of detection for urine arsenobetaine (Asb) was 0.056 μg/L (9.7%) with 4.7% of participants with undetectable Asb values. No participants presented levels below the limit of detection for the rest of the metals.

Urine Asb concentrations were determined in a randomized subsample of 295 individuals. We conducted a Markov Chain Monte Carlo (MCMC) multiple imputation by Gibbs sampling using a linear model to determine the distribution of Asb in participants with missing completely at random data [12]. Arsenic levels were adjusted by Asb concentrations by regressing total urine arsenic on Asb using a residual-based method previously reported [11] to exclude organic arsenic mainly from seafood that is not toxic for human health. Urine Asb concentrations were determined in a randomized subsample of 295 individuals. The mean of total arsenic concentrations among participants with low Asb (defined as individuals with measured or imputed Asb levels below the second percentile of Asb distribution [4.72 μg/L]) was added to the residuals in order to obtain the levels of inorganic arsenic exposure. Urine metals biomarkers in μg/L were corrected by urine creatinine in g/L to consider urine dilution.

2.5. DNA isolation, SNP selection and genotyping

From the peripheral blood cells of participants recruited in the study, DNA was determined with the Chemagic System (Chemagen). The quality of DNA was corroborated using PicoGreen dsDNA Quantification Reagent (Invitrogen, Carlsbad, CA, USA). In an exhaustive search in SYNPS program, 341 single nucleotide polymorphisms (SNPs) from 79 related candidate genes involved in oxidative stress pathways were identified. We genotyped the SNPs through an oligo-ligation assay (SNPlex, Applied Biosystems, Foster City, CA) to genotype the SNPs. From the original 341 identified SNPs, we excluded 63 SNPs because did not have exactly 2 alleles and 18 SNPs with minor allele frequency (MAF) lower than 5% in the study population, resulting in 260 SNPs for the analyses. The mean coverage of the final 260 included SNPs was 96.9%. For relevant SNPs, we reported the specific genotyping coverage, MAF and Hardy Weinberg Equilibrium p-value (HWE).

2.6. Other relevant variables

Participants reported sociodemographic data (sex, age and education) and lifestyle habits (diet, physical activity, alcohol, and tobacco intake) in an exhaustive questionnaire. Diet was assessed with semi-quantitative food frequency and two 24-h recall questionnaires. The type and frequency of walking and physically active hobbies, sports, or exercises were self-reported by the participants and measured in metabolic equivalents (METs) per minute/week based on standardized intensity scores. We categorized physical activity considering 3000 METs-minute/week, since it has been reported that lower risk for cardiovascular disease occurred specially above 3000 METs-minute/week. Smoking and drinking status were categorized in never, former and current consumer. Additionally, physical examination, and blood and urine samples collection were conducted by trained staff. We measured urine cotinine levels, the main metabolite of nicotine, used as a biomarker for tobacco smoke exposure, by enzyme-linked immunosorbent assay (ELISA) ("Análisis DRI® Cotinine" Kit, Ref. 0395 Microgenics laboratories to measured). A 77% of subjects had urine cotinine levels below the limit of detection (34 ng/ml). Serum creatinine was determined by the modified kinetic Jaffé method by isotope dilution mass spectrometry on a Hitachi 917 analyzer (Rocher, Boheringer, Germany). Kidney function was determined by estimated glomerular filtration rate (eGFR) based on serum creatinine using the abbreviated CKD-EPI equation. Body mass index (BMI) was calculated using measured weight (kilograms) divided by measured height squared (meters^2).

2.7. Statistical analysis

Descriptive analysis. We conducted a principal component (PC) analysis to group metabolites considering its contribution to clusters and similarities, and obtained four metabolic principal components (mPCs). After adjusting plasma metabolites concentrations by fasting time, we standardized a z-score on the fasting-adjusted residuals and calculated the varimax rotation to maximize the variances of the factor loading across variables. We selected rotated mPCs with eigen values > 2, which explained the ~80% of the variability. We described participants characteristics and mPCs scores by reporting mean and standard error of continuous variables, and relative and absolute frequency of categorical variables. Additionally, we described median and interquartile range of individual plasma metabolites by incident osteoporosis-related bone fracture categories.

Association between mPC and incident osteoporosis-related fractures. We used multi-adjusted Cox proportional hazards regression models for prospective analysis, including mPC levels, as independent variables, in separate models, and time to incident bone fractures, as dependent variable. Age was considered in the survival models as the time scale. Metabolic PC levels were introduced as categorical, comparing the two highest tertiles with the lowest, and as continuous, comparing the 80th to the 20th percentile. Models were progressively adjusted by potential confounders. In particular, model 1 was adjusted for sex, BMI (kg/m^2) high education (no, yes) and total triglycerides (mg/dL). Model 2 was Model 1 further adjusted for lifestyle habits and cardiovascular risk factors: cumulative-tobacco smoking (pack-years), smoking and alcohol consumption (never, former, current), urine cotinine levels (ng/mL), exercise (METs-min/week), eGFR (ml/min/1.73 m^2), lipid lowering medication (yes/no); and dietary fat, carbohydrate, and protein intakes

(gr/day). We conducted an additional analysis excluding participants with prevalent reduced BMD at baseline. Low vitamin D and calcium intake are well-known determinants of bone fragility [13]. In sensitivity analysis we conducted an additional model adjusting for calcium and vitamin D intake (mg/day). In secondary analysis we assessed the cross-sectional association between metabolic profile (for each mPC separately) with reduced BMD using logistic regression models. We also evaluated the main findings in subgroups defined by biologically relevant bone fragility determinants, such as sex, tobacco smoking, alcohol intake, eGFR and physical activity.

Interaction withredox-related genetic polymorphisms and metals. Since several metals have been related to plasma metabolomic profile and incidence of osteoporotic-related fractures in the Hortega Study [8,14], and these associations are potentially related to a redox activity of metals [7,15], we evaluated differential association in subgroups defined by median metal levels and genotypes of SNPs annotated to candidate genes involved in oxidative stress pathways. We used Cox regression models with interaction terms for each mPC by categorical metal exposure variables (below and above median metal concentrations) and individual SNPs assuming a continuous additive model (0, 1, or 2, minor allele dosage), respectively. In general, all the statistical interactions evaluated in this study are based on the pre-specified hypothesis of redox role of metals and candidate SNPs on bone metabolism. Nonetheless, given the relatively elevated number of evaluated SNPs (N = 260), for genetic interactions we determined a statistically significant Bonferroni corrected interaction P value of $<1.9 \times 10^{-4}$ [0.05/260 SNPs]. Interaction p values $\geq 1.9 \times 10^{-4}$ and $<1 \times 10^{-2}$ were considered suggestive.

Biological pathway analysis. We conducted enrichment and network analysis using pathways from Kyoto Encyclopedia of Genes and Genomes (KEGG) database with those genes annotated to SNPs with suggestive genetic interaction. The enrichment and network analysis provide a holistic view of genomic information integrated with biological pathways reported by KEGG, which provide significant links between accumulated biological and gene knowledge. The significance threshold for KEGG pathway enrichment was set to a P value ≤ 0.05 based on a two-sided hypergeometric exact test. We also described in an enriched network pathways within the KEGG database that contained at least one of the genes annotated to suggestive genetic interactions. We set a Kappa statistic threshold of 0.6 to determine KEGG terms interrelations (edges) and functional similarity groups based on shared genes between pathways.

Statistical analysis was conducted with "survey" package in R software (version 4.0.4) to account for the complex survey design. For pathway enrichment and network analysis of genes that showed gene-metal interactions we used Cytoscape (version 3.8.2) with the ClueGO (version 2.5.8) and CluePedia (version 1.5.8) plugins.

3. Results

3.1. Descriptive analysis

In PCA, four mPCs explained 78.3% of the metabolites joint variability. We considered metabolites with loadings $>|0.5|$ as main contributors to each mPC concluding that increasing mPC1 reflected increasing non-essential and essential amino acids, including brained-chain (BCAA), and bacterial co-metabolites, including isobutyrate, trimethylamines and phenylpropionate, versus decreasing fatty acids and VLDL subclasses; increasing mPC2 reflected increasing essential amino acids, including aromatic (AAA), and bacterial co-metabolites, including isopropanol and methanol; increasing mPC3 and mPC4 largely reflected increasing LDL and HDL subclasses, respectively (Supplemental Table S1). In descriptive analysis, participants with incident bone fractures were mostly women, older, less obese, with lower eGFR and higher cholesterol levels. Additionally, participants with incident bone fractures also had lower levels of mPC1 and higher levels of mPC2, mPC3 and mPC4 at baseline (Table 1). Overall, participants with incident bone fractures had slightly higher levels of bacterial co-metabolites and lower levels of fatty acids, compared to participants with no incident bone fractures. Participants with incident bone fractures also had lower levels of lipoproteins, particularly LDL and VLDL subclasses (Supplemental Table S2).

3.2. Metabolic profiles and osteoporosis-related bone fractures

A total of 44 (9.4%) participants developed osteoporosis-related fractures over a total of 5112.4 person-years of cumulative follow-up. The mean follow-up time was 10.9 years. The adjusted hazard ratio (HR)(95%CI) of incident osteoporosis-related fractures comparing 80th to 20th percentile of mPC distribution was 0.32 (0.10, 0.97) for mPC1, 1.73 (1.23, 2.44) for mPC2, 0.87 (0.53, 1.45) for mPC3 and 0.44 (0.23, 0.83) for mPC4 (Table 2). Fig. 1 displays the flexible dose-response association for the evaluated metabolic patterns with the incident osteoporotic-related fractures, with mostly linear associations, except

Table 1
Participants characteristics and metabolomics patterns by fracture incidence status.

	Overall	Not incident bone fracture	Incident bone fracture
N	467	423	44
Female	49.0 (216)	49.3 (189)	69.4 (27)
Age, years	66.0 (0.3)	65.5 (0.4)	71.5 (1.7)
High education	53.8 (238)	53.8 (215)	53.5 (23)
Smoking status			
Never	54.1 (250)	53.8 (225)	56.9 (25)
Former	33.9 (171)	34.0 (155)	32.1 (16)
Current	12.0 (46)	12.1 (43)	10.9 (3)
Urine cotinine, mg/dL	183.7 (37.5)	187.0 (78.8)	147.6 (40.4)
Alcohol intake, gr/day	11.4 (1.14)	12.0 (1.24)	5.1 (1.78)
BMI≥30 kg/m^2	28.2 (134)	28.3 (123)	27.5 (11)
eGFR, mL/min/1.73m^2	11.5 (303)	10.5 (272)	22.5 (31)
Exercise <3000 METs-min/week	41.0 (260)	41.0 (239)	47.0 (21)
Lipid lowering medication	80.3 (0.78)	81.0 (0.81)	73.1 (3.05)
High cholesterol (mg/dL)	37.9 (176)	38.8 (163)	28.1 (13)
Metabolomic patterns			
mPC1	−0.23 (0.05)	−0.23 (0.05)	−0.23 (0.14)
mPC2	−0.10 (0.05)	−0.14 (0.05)	0.30 (0.13)
mPC3	0.18 (0.04)	0.18 (0.05)	0.19 (0.15)
mPC4	0.10 (0.04)	0.10 (0.05)	0.05 (0.12)

Abbreviations: BMI, body mass index; eGFR, estimated glomerular filtration rate; mPC, metabolomic principal component.
Data is expressed in mean (standard error [SE]) for continuous variables and, frequency (%[N]) for categorial variables.

Table 2
Hazard ratio (95%CI) of incident osteoporosis-related fractures by metabolic principal components (mPC1 to mPC4) (N = 467).

	Cases/No cases	Model 1	Model 2
mPC1			
Tertile 1	18/140	1.00 (Ref)	1.00 (Ref)
Tertile 2	14/145	0.50 (0.21, 1.19)	0.57 (0.24, 1.40)
Tertile 3	12/138	0.27 (0.10, 0.70)	0.35 (0.13, 0.89)
80th vs 20th[a]		0.27 (0.09, 0.84)	0.32 (0.10, 0.97)
p-value		0.02	0.04
mPC2			
Tertile 1	8/145	1.00 (Ref)	1.00 (Ref)
Tertile 2	14/136	1.58 (0.60, 4.17)	1.63 (0.61, 4.37)
Tertile 3	22/142	2.59 (1.04, 6.47)	2.94 (1.18, 7.33)
80th vs 20th		1.63 (1.15, 2.31)	1.73 (1.23, 2.44)
p-value		0.006	0.002
mPC3			
Tertile 1	20/147	1.00 (Ref)	1.00 (Ref)
Tertile 2	11/142	0.55 (0.23, 1.30)	0.62 (0.26, 1.51)
Tertile 3	13/134	0.71 (0.34, 1.47)	0.83 (0.36, 1.90)
80th vs 20th		0.84 (0.52, 1.36)	0.87 (0.53, 1.45)
p-value		0.48	0.59
mPC4			
Tertile 1	12/144	1.00 (Ref)	1.00 (Ref)
Tertile 2	18/137	1.12 (0.52, 2.43)	0.87 (0.35, 2.19)
Tertile 3	14/142	0.57 (0.24, 1.39)	0.51 (0.21, 1.27)
80th vs 20th		0.50 (0.28, 0.89)	0.44 (0.23, 0.83)
p-value		0.02	0.01

The 80th and 20th percentiles distributions were 0.65 and −0.97 for mPC1, 0.54 and −0.74 for mPC2, 0.94 and −0.57 for mPC3, and 0.86 and −0.68 for mPC4.
Model 1 adjusted for age (years), sex, BMI (kg/m^2), high education (no, yes) and total triglycerides (mg/dL).
Model 2 is model 1 further adjusted for cumulative-tobacco smoking (pack-years), smoking status (never, former, current), alcohol intake status (never, former, current), cotinine levels (ng/mL), exercise (METs-min/week), eGFR(ml/min/1.73 m^2), lipid lowering medication (yes/no), fat, carbohydrate, and protein intakes (gr/day).
Tertile cutoffs were −0.47 and 0.35 for mPC1; −0.39 and 0.27 for mPC2; −0.25 and 0.56 for mPC3 and -0.37 and 0.40 for mPC4.
mPCs scores were unitless.
[a] Association obtained from a regression models with mPC1 modelled as restricted quadratic splines with knots at the 10, 50 and 90 percentiles. The p-value of non-linearity was obtained from a wald-test of the spline terms. Other p-values in the table were obtained from a wald test of the regression coefficient for log-transformed urine arsenic and cadmium.

maybe for mPC1. In secondary analysis, the corresponding odds ratio (OR)(95%CI) of reduced BMD were 0.38 (0.18, 0.81) for mPC1; 1.30 (1.00, 1.70) for mPC2; 1.12 (0.80, 1.56) for mPC3; and 1.14 (0.80, 1.63) for mPC4 (Supplemental Table S3). In flexible dose-response mPC3 was positively associated with reduced BMD at extreme levels (Supplemental Fig. S1). Also, we conducted sensitivity analysis by excluding participants with prevalent reduced BMD at baseline resulting in 14 cases with fractures and 258 non-cases with similar trend in the flexible dose-response (Supplemental Fig. S2). In additional sensitivity analysis, fully adjusted models further adjusted for calcium and vitamin D intake (mg/day) showed essentially identical results (data not shown). In subgroups analysis by well-established determinants of bone fragility, the p-values for interaction were not statistically significant (Supplemental Table S4).

3.3. Interaction analysis with redox-related metals and genetic variants

The association between mPC2 and incident osteoporosis-related fractures showed statistically significant interactions by urine antimony and arsenic and plasma selenium biomarkers. Specifically, the association with mPC2 was stronger in participants with increased levels of urine antimony (HR[95%CI]: 2.72 [1.62, 4.58] and 1.06 [0.54, 2.07] for participants above and below 0.07 μg/g, respectively) and arsenobetaine-adjusted arsenic (corresponding HR[95%CI]: 3.24 [1.78, 5.89] and 0.96 [0.49, 1.87] for participants above and below 6.81 μg/g, respectively). At a suggestive statistical significance level (p-interaction 0.07), increasing urine cadmium levels were associated with stronger association of mPC2 with bone fractures risk (HR[95%CI] was 2.15 [0.83, 5.58] and 1.09 [0.51, 2.32] for participants above and below 0.41 μg/g). The corresponding association was, however, stronger in participants with lower plasma selenium levels (HR[95%CI]: 1.17 [0.59, 2.30] and 2.66 [1.44, 4.92] for participants above and below 85.3 μg/L, respectively) (Supplemental Table S5).

In gene-metabolism interaction analyses, 3 SNPs displayed statistically significant interaction at the Bonferroni threshold ($<1.9 \times 10^{-4}$) including, for mPC1, rs6752058, annotated to the closest gene *XDH* (HR of bone fracture [95%]: 0.09 [0.03, 0.31], 0.65 [0.17, 2.49] and 4.46 [0.63, 31.78] for 0, 1 and 2 minor alleles, respectively); and for mPC2, rs2072712, annotated to the closest gene *NCF4* (corresponding HR was 2.30 [1.56, 3.40], 0.63 [0.35, 1.11] and 0.17 [0.05, 0.56)]); and rs3017887, annotated to the closest gene *NOX4* (corresponding HR was 2.16 [1.57, 2.96], 0.51 [0.27, 0.96] and 0.12 [0.03, 0.47]). We also report suggestive interactions (P value $< 10^{-3}$) between mPCs and incident fractures by 33 SNPs annotated to 19 candidate genes (Supplemental Table S5).

3.4. Biological pathway analysis of genes related to interacting SNPs

In overall KEGG enrichment analysis (out of the union set of genes from redox-related SNPs that showed suggestive interactions with mPCs), "Diabetic cardiomyopathy" and "Renin-angiotensin system" were the most enriched biological pathways (Supplemental Fig. S3). *AGTR1*, followed by *AGT*, interacting with mPC4 were the genes more frequently associated with several biological pathways, mainly involved in fluid balance, kidney function and blood pressure regulation. Other genes, such as, *COX6B1* and *COX8C* (interacting with mPC4), *COX7A2* (interacting with mPC3); and *NDUFS1*, and *NDFUS2* (interacting with mPC4 and mPC2), contributed to enriched pathways related to neurological diseases, such as Huntington, Parkinson or Alzheimer disease, in addition to pathways involved in cardiac conditions.

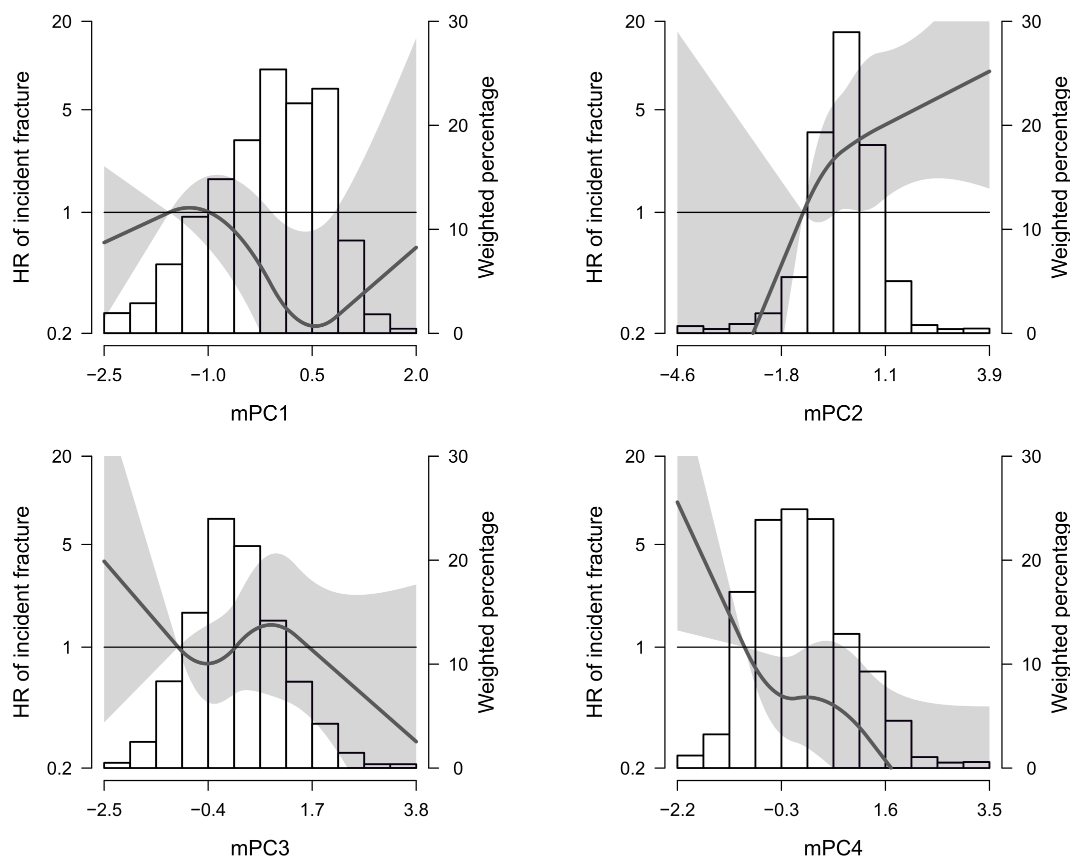

Fig. 1. Hazard ratio (95% CI) of incident osteoporosis-related fractures by metabolic principal components (mPC1 to mPC4) (N = 467). The solid curve and shades lines represent the hazard ratio (95% confidence interval) of incident osteoporosis-related fractures based on restricted quadratics splines with knots at the 10th, 50th and 90th percentiles of mPC1 (−1.57, −0.08 and 0.85), mPC2 (−1.28, −0.1 and 0.97), mPC3 (−1.02, 0.13 and 1.37) and mPC4 (−1.01, 0.05 and 1.42)(unitless mPC scores), respectively. The reference value was set at the 10th percentile of mPC. Models were adjusted for sex, BMI (kg/m^2), high education (no, yes), cumulative-tobacco smoking (pack-years), smoking status (never, former, current), alcohol intake status (never, former, current), cotinine levels (ng/mL), exercise (METs-min/week), eGFR(ml/min/1.73 m^2), lipid lowering medication (yes/no), total triglycerides (mg/dL), and fat, carbohydrate, and protein intake (gr/day). The histogram represents the weighted frequency distribution of mPC1, mPC2, mPC3 and mPC4 in the study sample.

4. Discussion

In our population-based study, mPC1, reflecting non-essential and essential amino acids -including BCAA (isoleucine, leucine and valine) -, and bacterial co-metabolites (isobutyrate, trimethylamines and phenylpropionate), versus fatty acids and VLDL; and mPC4, reflecting HDL subclasses, were inversely associated with incident osteoporosis-related bone fractures. In addition, mPC2, reflecting other essential amino acids -including AAA (tyrosine and tryptophan) -, and bacterial co-metabolites (isopropanol and methanol), was positively associated to incident osteoporosis-related bone fractures. Individuals with increased antimony, inorganic arsenic and, suggestively, cadmium, and decreased selenium biomarkers levels showed a stronger association between mPC2 and incident bone fractures. Genetic variants annotated to genes with a putative role in endocrine, neurological and cardiovascular pathways, including *NCF4, NOX4* and *XDH,* showed differential metabolic-related bone fractures risk.

Identifying new strategies for bone fragility prevention is needed, given the elevated burden of bone disease in the elderly. While some studies have revealed a role of individual metabolites in bone physiology, heterogenous findings makes hard to clarify the relationship between classes of metabolites, including amino acids, lipids and microbiota co-metabolism, with BMD fragility. In the following sections, we assess the consistency of our findings with current evidence according to individual groups of metabolites. Nonetheless, previous findings in the literature must be taken with caution, given the fact that in other studies the evaluated metabolites were assessed individually, and may have served as a proxy for other correlated metabolites.

4.1. Amino acids

An evolving body of epidemiological studies supports that amino acids could influence bone turnover regulation [16–22]. While essential amino acids, including BCAA, could play a beneficial role stimulating osteoblast growth, AAA could promote osteoclast activity and bone resorption [20,21], which is overall consistent with our findings. Evidence evaluating the association between amino acids and reduced BMD is still scarce, given the lack of studies. In this regard, only two prospective studies from China (N = 1070 and 2997) [21,23] have studied BCAA exposure in incident bone fracture, with no statistically significant results. Regarding AAA exposure, both Chinese studies and another study from the US (N = 5187) observed inverse associations between serum [21,23] and dietary [18] tryptophan with incident bone fractures, contrary to our results.

For reduced BMD, two studies from China [21] and United Kingdom [17] reported positive association between serum and dietary BCAA with central BMD, consistently with our findings. In cross-sectional studies from Australia [16] and China [18], a null association between serum and dietary AAA with central BMD was reported. Inconsistently with our findings, two studies -from Mexico (N = 602) and China (N = 2997)- reported a positive association of leucine levels and reduced BMD [19] and an inverse association between tryptophan and reduced BMD [21].

4.2. Microbiota co-metabolism

Gut microbiota balance contributes to maintain the intestinal mucosal barrier, which has a key role in the absorption of essential elements needed for bone metabolism [5]. In addition, intestinal microbiota dysfunction promotes inflammation, and releases cytokines which ultimately stimulate osteoclast differentiation and increase bone resorption biomarkers [6]. Many studies have identified taxonomic changes in microbiota composition in individuals with BMD loss [24–29]. However, evidence from studies that evaluate the role of microbiota in incident fracture, in addition to BMD, is limited. Only a prospective study from Japan (N = 38) reported an association between gut microbiota diversity and incident osteoporosis-related fractures in postmenopausal women [30]. Probiotics have been proposed as therapeutic targets in osteoporosis with mixed results in clinical trials [31, 32].

4.3. Lipoproteins

While a substantial number of epidemiological studies report associations between HDL and LDL, and bone health endpoints with mixed results [33–38], mechanistic studies suggest that cholesterol promotes the bone turnover. Specifically, oxidized LDL cholesterol and HDL deficiency could have a detrimental effect in osteoblastic activity [1,2].

Our findings are supportive of an inverse association between HDL subclasses with incident osteoporosis-related fractures. However, a meta-analysis of three prospective studies showed that HDL below 40 mg/dL (categorical variable) was associated with lower risk of bone fractures [34]. Our results are also not consistent with a cross-sectional study from China (mean serum HDL ∼58 mg/dL), which reported that HDL was positively associated with bone fractures self-reported by the participants [39], and a mendelian randomization study which reported a positive association with prevalent fractures in an European cohort [40]. Alternatively a substantial number of studies have evaluated BMD loss as an endpoint, supporting a detrimental effect of extreme HDL levels in bone health [37,38,41–47], except for a number of studies that reported a beneficial [44,48–51] or null [33,35,36,52–54] role of serum HDL in central BMD.

Similarly, for LDL the epidemiologic evidence offers mixed results. Our results are consistent with a meta-analysis of three prospective and two cross-sectional studies [34] and two other small cross-sectional studies in women from China (mean LDL ∼108 mg/dL) [39] and Turkey (mean LDL 138 mg/dL) [55], but not with a Japanese study in women [56]. Nonetheless, we observed a flexible dose-response pointing to a positive association between extreme LDL levels and reduced BMD, which is in accordance with studies supporting increased LDL cholesterol as a factor risk for BMD loss [37,38,48,49,57]. Conversely, other studies did not found a positive association between serum LDL cholesterol and central BMD loss [33,35,36,42,43,51–53,58–61].

Interestingly, in our study population, mPC1, reflecting decreasing VLDL, was inversely associated with incident bone fractures. This finding is novel as only one cross-sectional study has evaluated the association between VLDL with prevalent bone fractures with null results [55]. Epidemiological studies assessing the association of VLDL and bone loss are mostly limited to female populations and small sample size [55,62–64]. Similar to our results, two studies found negative correlation between VLDL and central BMD in women [62,63]. However, contrary to our results, a study did not find statistically significant results, and another small cross-sectional study reported a protective role of plasma VLDL in BMD loss [55,64]. Nevertheless, in these studies models did not consider dietary patterns and lipid-lowering medication, which are known metabolomic profile determinants.

Table 3
Hazard ratio (95%CI) of incident osteoporosis-related fractures comparing the 80th to the 20th percentile of metabolic principal component 2 (mPC2) distribution by combined categories of relevant metals and genotypes (3- way interaction) (N = 467).

Metals	Minor allele dosage	rs2072712 (*NCF4*)			rs3017887 (*NOX4*)		
		Cases/Non-cases	HR (95%CI)[a]	P-int	Cases/Non-cases	HR (95%CI)[a]	P-int
Plasma selenium, μg/L							
≤85.29	0 allele	186/281	3.81 (3.50, 4.14)	<0.001	195/272	3.16 (2.89, 3.46)	<0.001
	1 allele	37/430	1.04 (0.81, 1.34)		28/439	0.67 (0.57, 0.79)	
	2 alleles	3/464	0.29 (0.02, 3.73)		1/466	0.14 (0.01, 3.17)	
>85.29	0 allele	195/272	1.59 (0.84, 3.02)		196/271	1.69 (1.04, 2.75)	
	1 allele	38/429	0.14 (0.04, 0.50)		39/428	0.25 (0.03, 1.96)	
	2 alleles	2/465	0.01 (0.00, 0.07)		0/467	0.04 (0.00, 0.65)	
Urine antimony, μg/g							
≤0.07	0 allele	185/282	1.55 (1.37, 1.76)	<0.001	194/273	1.31 (1.20, 1.43)	<0.001
	1 allele	39/428	0.48 (0.28, 0.85)		33/434	0.71 (0.48, 1.06)	
	2 alleles	4/463	0.15 (0.01, 1.85)		1/466	0.38 (0.01, 10.20)	
>0.07	0 allele	200/267	3.52 (2.03, 6.11)		201/266	3.77 (2.40, 5.92)	
	1 allele	36/431	1.03 (0.44, 2.39)		34/433	0.28 (0.04, 1.94)	
	2 alleles	1/466	0.30 (0.10, 0.86)		0/467	0.02 (0.00, 0.31)	
Urine arsenic adjusted by Asb, μg/g							
≤6.81	0 allele	186/281	1.35 (1.14, 1.60)	<0.001	191/276	1.36 (1.24, 1.48)	<0.001
	1 allele	34/433	0.23 (0.11, 0.47)		31/436	0.02 (0.02, 0.03)	
	2 alleles	3/464	0.04 (0.00, 0.61)		1/466	0.00 (0.00, 0.01)	
>6.81	0 allele	194/273	4.36 (2.41, 7.88)		199/268	3.98 (2.36, 6.69)	
	1 allele	40/427	1.39 (0.62, 3.09)		36/431	1.50 (0.62, 3.62)	
	2 alleles	2/465	0.44 (0.17, 1.16)		0/467	0.56 (0.18, 1.75)	
Urine cadmium, μg/g							
≤0.41	0 allele	194/273	1.43 (1.23, 1.65)	<0.001	198/269	1.52 (1.34, 1.73)	<0.001
	1 allele	35/432	0.71 (0.41, 1.24)		31/436	0.19 (0.11, 0.35)	
	2 alleles	3/464	0.36 (0.03, 4.40)		1/466	0.02 (0.00, 0.30)	
>0.41	0 allele	191/276	3.54 (2.08, 6.02)		197/270	2.69 (1.79, 4.05)	
	1 allele	40/427	0.45 (0.14, 1.39)		36/431	0.72 (0.38, 1.35)	
	2 alleles	2/465	0.06 (0.01, 0.26)		0/467	0.19 (0.09, 0.43)	

Abbreviations: Asb, arsenobetaine; P-int, P value of interaction.
Model was adjusted for sex, BMI (kg/m2), high education (no, yes), cumulative-tobacco smoking (pack-years), alcohol intake (g/day), exercise (METs-min/week), eGFR(ml/min/1.73 m^2), total triglycerides (mg/dL), fat, carbohydrate, and protein intakes (gr/day). mPCs scores were unitless.
[a] For a 80th to the 20th percentile of mPC2 distribution (0.54 and 0.74, respectively) comparison.

4.4. Interactions with redox related-genetic variants and metals

Studies evaluating the association between joint metabolites with BMD endpoints and related fractures are scarce. While a prospective study involving Framingham Offspring Study (N = 1552) and Hong Kong Osteoporosis Study participants (N = 634) identified 27 plasma metabolites with LASSO methods -mostly amino acids and lipids- [65], metal biomarker and genetic variants were not included in the analysis. Our findings suggest, however, that the interaction of metabolic patterns and redox-related metal exposure biomarkers and genes may be a relevant bone fragility determinant.

In particular, we identified that individuals at increased arsenic, antimony, and cadmium, and decreased selenium levels, were at increased metabolic-related bone fractures risk, which is compatible with the hypothesis that the redox unbalance exerted by these elements can promote the bone loss [1], and also in line with previous studies conducted in our study population where most of these metals were related to metabolomic compounds [8] and decreased selenium was associated with higher fracture incidence following a U-shape dose-response [7].

Similarly, most interacting SNPs annotated to genes such as *NCF4* and *NOX4,* which encode a protein related to NADPH oxidase activity that increases reactive oxygen species (ROS) production, and *XDH,* which encodes the xanthine dehydrogenase involved in the oxidative metabolism of purines, are consistent with previous studies showing that NADPH oxidase 4 expression was upregulated in participants with reduced BMD [66]. In post-hoc analysis, we conducted 3-way interaction analysis between mPC2 and incident osteoporosis-related fractures by combinations of most interacting redox-related metals (antimony, arsenic, cadmium and selenium) and SNPs [rs2072712 (*NCF4*) and rs3017887 (*NOX4*)] showing that these variants may reduce bone loss, being this protective role attenuated if high exposure to toxic metals or deficient selenium status (Table 3). Functional studies are needed to confirm the protective role of these variants on the bone tissue.

Interestingly, our bioinformatic biological pathway enrichment analysis suggests a connection of bone metabolism with other chronic diseases. Among the contributing genes, *AGTR1* and *AGT* are involved in renin-angiotensin-aldosterone system, which regulates blood pressure and fluid balance maintenance; and *NDUFS1*, *NDUFS2, COX6B1, COX7A2* and *COX8A,* have a role in the mitochondrial respiratory chain. Most of them are overexpressed in kidney tissue [67], which is essential for Vitamin D activation and consequently in calcium and phosphate metabolism. Diabetic cardiomyopathy was the most enriched biological pathway, which has a microvascular component that could be related to the well-known microvascular renal complications of long-term diabetes. Indeed, in a study with male participants with diabetes, bone mass was associated with ventricular diastolic function [68]. Moreover, bone metabolism has been related with several neurological diseases and arterial and valve calcification [2,69].

4.5. Limitations and strengths

Our study is not exempt of limitations. First, menopause status was not available in our study population. Nevertheless, the criteria to include participants over 50 years in our analysis makes unlikely that menopause status influences our results. Another limitation is the sample size, which allowed us to detect associations of metabolic patterns with incident fractures and reduced BMD, but it was underpowered for genetic interaction analysis. While we could detect some differential associations by genotypes in candidate genes, even after applying a conservative Bonferroni correction to the statistical significance threshold, larger studies are needed for replication of findings. The panel of available candidate genes in our study population provided a focused view of potential gene-metal interactions based on genes encoding proteins with a well-known role in redox pathways. However, extended genotyping efforts are needed to entertain a full exploration of the genome to identify additional gene-metal interactions which can be relevant for bone health, including, for instance, interactions with vitamin D receptor polymorphisms. Strengths of this study include the survey design, which makes our sample representative of a general population from a region in Spain, and the availability of a unique set of determinations including an extended panel of metabolites, metals and SNPs. Moreover, we interrogated the interacting role of redox-related metals and SNPs with bone fragility endpoints, which yielded a novel sight of potential effects of metabolism on bone health.

5. Conclusions

In conclusion, in our study population, specific metabolic patterns were prospectively associated with bone fragility endpoints, supporting the hypothesis that bone remodeling is influenced by amino acids, lipids and microbiota co-metabolism. Our data support that carriers of specific redox-related genotypes may have differential metabolic-related bone fragility risk, and may benefit from intensified preventive interventions depending on arsenic, antimony and selenium status. While our gene-environment interaction results need to be reproduced in other prospective studies given the limited sample size, they point to interventions that could contribute to mitigate the burden of bone disease and other chronic diseases in ageing populations.

Acknowledgments

Authors' contribution

M.G-F, Z.R–H, M.G-P, M.T-P, D.M, J.R and JC.M-E contributed to the preparation of the research data, statistically analysis, and writing of the manuscript. J.R and JC.M-E gave access to the Hortega Study data used in this analysis and contributed to the study design. AB.G-G, FJ.C contributed to the genotyping. D.M and N.A contributed to metabolomic profiling. T.G-B and JL.G-A contributed to measure metal levels. JL.P–C, LS.B·F and JC.M-E reviewed clinical records and assigned osteoporotic related endpoints.

All authors have contributed to the interpretation of the findings and to the contents of the manuscript.

Disclaimer

The opinions and views expressed in this article are those of the authors and do not necessarily represent the official position of the Instituto de Salud Carlos III (Spain).

Funding statement

The work was funded by theó State Research Agency at the Ministerio de Ciencia Innovación y Universidades of Spain (PID2019-108973RB-C21, PID2019-108973RB-C22 and PCIN-2017-117); the Generalitat Valenciana of Spain (IDIFEDER/2021/072, GRUPOS 03/101, PROMETEO/2009/029, ACOMP/2013/039, IDIFEDER/2021/072 and GRISOLIAP/2021/119); the EU Joint Programming Initiative Healthy Diet Healthy Life (HDHL) GUTMOM (INTIMIC-085); the Strategic Action for Research in Health sciences [CP12/03080, PI15/00071, PI10/0082, PI13/01848, PI14/00874, PI16/01402, PI17/00544 and PI11/00726]; CIBER Fisiopatología Obesidad y Nutrición (CIBEROBN) (CIBER-02-08-2009, CB06/03 and CB12/03/30016); the Castilla-Leon Government (GRS/279/A/08) and European Network of Excellence Ingenious Hypercare (EPSS- 037093) from the European Commission. The Strategic Action for Research in Health sciences and CIBEROBN are initiatives from Carlos III Health Institute Madrid and co-funded with European Funds for Regional Development (FEDER). M.G-P received the support of a fellowship from "la Caixa" Foundation (ID 100010434, fellowship code LCF/BQ/IN18/11660001). The funding bodies had no role in the design of the study and collection, analysis, and

interpretation of data and in writing the manuscript.

Declaration of competing interest

N.A is stock owner of Biosfer Teslab, which commercializes the lipoprotein profiling described in the present manuscript and has a patent for lipoprotein profiling. All other authors declare no competing interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.freeradbiomed.2022.11.007.

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