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Original article

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The association of urine metals and metal mixtures with cardiovascular incidence in an adult population from Spain: the Hortega Follow-Up Study

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Abstract

Background: The association of low-level exposure to metals and metal mixtures with cardiovascular incidence in the general population has rarely been studied. We flexibly evaluated the association of urinary metals and metal mixtures concentrations with cardiovascular diseases in a representative sample of a general population from Spain.

Methods: Urine antimony (Sb), barium (Ba), cadmium (Cd), chromium (Cr), cobalt (Co), copper (Cu), molybdenum (Mo), vanadium (V) and zinc (Zn) were measured in 1171 adults without clinical cardiovascular diseases, who participated in the Hortega Study. Cox proportional hazard models were used for evaluating the association between single

metals and cardiovascular incidence. We used a Probit extension of Bayesian Kernel Machine Regression (BKMR-P) to handle metal mixtures in a survival setting.

Results: In single-metal models, the hazard ratios [confidence intervals (CIs)] of cardiovascular incidence, comparing the 80th to the 20th percentiles of metal distributions, were 1.35 (1.06, 1.72) for Cu, 1.43 (1.07, 1.90) for Zn, 1.51 (1.13, 2.03) for Sb, 1.46 (1.13, 1.88) for Cd, 1.64 (1.05, 2.58) for Cr and 1.31 (1.01, 1.71) for V. BKMR-P analysis was confirmatory of these findings, supporting that Cu, Zn, Sb, Cd, Cr and V are related to cardiovascular incidence in the presence of the other metals. Cd and Sb showed the highest posterior inclusion probabilities.

Conclusions: Urine Cu, Zn, Sb, Cd, Cr and V were independently associated with increased cardiovascular risk at levels relevant for the general population of Spain. Urine metals in the mixture were also jointly associated with cardiovascular incidence, with Cd and Sb being the most important components of the mixture.

Key words: Urine metals, cardiovascular incidence, population-based, cohort study, BKMR

Key Messages

- Few epidemiological studies have evaluated the role of metal mixtures in cardiovascular disease (CVD).
- By using BKMR-P, a novel statistical tool that can accommodate correlated mixture components, we found that urine Cu, Zn, Sb, Cd, Cr and V, both individually and as a mixture, were associated with CVD.
- Public health interventions that prevent metal exposure may decrease the burden of metal-related CVD.

Introduction

Exposure to non-essential metals through diet, ambient air and drinking water has been positively associated with atherosclerotic endpoints in human studies.[1–3] Essential metals deficiency has traditionally been related to adverse changes in cardiometabolic factors,[4,5] and excessive exposure has also been related to adverse health effects, including cardiovascular disease (CVD).[6,7] The accumulated epidemiological evidence in support of a causal association of individual metals such as cadmium (Cd), copper (Cu) and lead (Pb) is strong.[3,8,9] The evidence evaluating the association of other non-essential and essential metals with cardiovascular incidence endpoints at exposure levels that are relevant for general populations is not sufficient.[1] Additional lines of research supporting the role of metals in cardiovascular risk include experimental studies,[1,2,10] which provide biological mechanisms that support the associations observed in epidemiological studies, and chelation trials showing a benefit of metal chelation in secondary CVD prevention.[11]

Some metals can vary jointly, possibly reflecting common exposure sources and metabolic pathways for those metals.[12,13] The individual components of metal mixtures may also interact differently with respect to different health endpoints.[12,14] Traditional approaches to handling metal mixtures in association studies, such as simultaneously adjusting models for several metals or introducing interaction terms, have methodological limitations.[14,15] Few epidemiological studies have so far evaluated the role of metal mixtures in health outcomes using methods that can more realistically capture the complexity of mixed exposures, especially when the health endpoint of interest is defined as time to event in survival settings.

Our objective was to evaluate the association between the exposure to individual metals available from an inductively coupled plasma mass spectrometry (ICP-MS) multielemental technique, including essential [cobalt (Co), Cu, molybdenum (Mo) and zinc (Zn)] and non-essential [antimony (Sb), barium (Ba), chromium (Cr), Cd and vanadium (V)] metals and metal mixtures, with CVD incidence in a representative sample of a general population from Spain. In this context, we implemented a novel Probit extension of Bayesian Kernel Machine Regression (BKMR-P) methods[16,17] to estimate non-linear and non-additive dose–response functions for a potentially high-dimensional set of correlated exposures accounting for uncertainty in the context of survival analysis.

Methods

Study population

The Hortega Study is a population-based survey among adults aged 15–85 years residing in the catchment area of the Rio Hortega University Hospital in Valladolid (Spain). In Spain, tertiary hospitals have assigned specific geographical areas for patient referral, and they integrate the network of primary care centres in each area. The complex study design and data collection methods have been described elsewhere.[18] In summary, the Hortega Study was initiated in 1997 on a random sample from the administrative list of public health system beneficiaries assigned to the University Hospital Rio Hortega's catchment area in Valladolid (Spain), who received a mailed questionnaire (Phase I), followed by a pilot examination of randomly selected Phase I participants in 1999–2000 ($N = 495$, Hortega Phase II). In 2001–03, Phase II participants were re-examined, together with additional randomly selected Phase I participants who were examined *de novo*, making up the cross-sectional baseline examination (Hortega Phase III, $N = 1502$). Biological samples, including urine, plasma, serum and buffy coat for DNA extraction, were collected and stored at -80°C from all Hortega Phase III participants. Follow-up of Phase III participants (which established the Hortega Follow-up Study) was conducted through review of mortality and health registers in 2015.

We measured baseline urinary metal concentrations in 1502 Hortega Follow-up Study participants with stored urine samples. We excluded 149 participants with prevalent CVD, 15 with missing metals data, 73 with missing values in relevant covariates and 94 participants who were lost to follow-up, leaving a total of 1171 participants. The research protocol was approved by the Ethics Committee of the Rio Hortega University Hospital, and all participants provided written informed consent.

Urine metal levels

In 2013, levels of urinary arsenic (As), Sb, Ba, Cd, Cr, Co, Cu, Mo, Pb, selenium (Se), V and Zn were measured in the urine of the Hortega Study participants using ICP-MS with dynamic reaction cell on an Agilent 7500CEx ICP-OR-MS, following a standardized protocol at the Environmental Bioanalytical Chemistry Laboratory of the University of Huelva, Spain. We decided not to include urine Pb and Se in our analysis because they are not accepted biomarkers of exposure. In addition, total urine As in our population mainly reflects organic arsenic from seafood consumption,[19] which is not toxic. Information on inorganic arsenic from urine arsenic speciation data was only available in a small subset of our study population ($N = 295$). Thus, we did not analyse urine As either in this study. The limits of detection were: 0.003 μg/L for Sb, 0.015 μg/L for Ba, 0.0005 μg/L for Cd, 0.038 μg/L for Cr, 0.001 μg/L for Co, 0.043 μg/L for Cu, 0.01 μg/L for Mo, 0.008 μg/L for V and 1.31 μg/L for Zn. No participant showed undetectable metal concentrations in our study population. We accounted for urine dilution by standardizing all metal concentrations by urine creatinine.

Cardiovascular disease incidence

Health records were reviewed by a committee of two physician reviewers who adjudicated incident events. The primary outcome of this study was cardiovascular incidence, including both fatal and non-fatal events, which was defined as mortality or the first episode of hospitalization for any cardiovascular cause (International Classification of Diseases, 10th Revision (ICD-10) codes I00-I78). Secondarily, we also report findings for a combined endpoint for incident coronary heart disease (CHD) and stroke, and an additional endpoint for incident heart failure. The main reason for using a combined endpoint for CHD and stroke is that both reflect atherosclerotic disease and, also, that the specific number of events for each endpoint is relatively small ($N = 53$ for CHD and 56 for stroke). Detailed definitions of specific cardiovascular events follow the guidelines published by the 2014 American College of Cardiology and American Heart Association Task Force of Clinical Data Standards Committee.[20] Time to event was calculated as the difference between the date of the baseline examination and the date of the event, the date of death or 30 November 2015 (administrative censoring), whichever occurred first.

Other variables

The interviews, physical examinations and collection of biospecimens were conducted by trained staff using standardized protocols. Sociodemographic (age, sex, race/ethnicity, education, urbanization level), lifestyle (smoking status) and medication information was collected using questionnaires. We used a combination of laboratory, examination and interview data to define body mass index, urine cotinine, plasma total cholesterol, high-density lipoprotein (HDL) cholesterol, blood pressure (BP), systolic BP (SBP), serum creatinine, estimated glomerular filtration rate (eGFR) and diabetes mellitus of type 2 (see Supplementary Methods, available as Supplementary data at *IJE* online).

Statistical methods

We estimated hazard ratios (HR) and 95% confidence intervals (CI) of incident CVD, combined CHD and stroke, and heart failure by urine metal levels using weighted Cox

proportional hazards regression with a robust variance specification to obtain representative estimates from the underlying source population. Urine metal concentrations were introduced in the models as tertiles, to compare the third and second tertiles with the first tertile, or as log-transformed (continuous) variables, to compare cardiovascular incidence in the 80th versus 20th percentiles of the metal distribution. Statistical models were adjusted for sex, education (<high school, >=high school), smoking status (never, former, current smoker), cumulative smoking dose (0, 0–12, >12 pack-years), urine cotinine (<34, 34–500 and >=500 mg/dl), estimated glomerular filtration rate (<60, 60–90, >=90 ml/min/1.73 m^2), residence (urban or rural) and traditional cardiovascular risk factors [total cholesterol (mg/dl), HDL-cholesterol (mg/dl), lipid-lowering medication (yes/no), type 2 diabetes mellitus (yes/no) systolic blood pressure (mmHg) and blood pressure-lowering medication (yes/no)]. We also assessed non-linear relationships between the three endpoints and each metal using restricted quadratic splines with knots at the 10th, 50th and 90th percentiles. Further details of statistical methods for the estimation of summary trends and associations can be found in the Supplementary Methods.

In addition, we conducted progressively adjusted multiple-metal Cox models. In our multiple-metal models, we only show results for mixture components that showed statistically significant associations with cardiovascular incidence in the single-metal models. However, an elevated number of metal mixture components, strong correlations and non-linear and non-additive relationships between components can challenge the study of mixtures in traditional regression settings.[16] Thus, we applied BKMR-P in order to flexibly model the relationship between multiple metals and cardiovascular risk. BKMR-P is a kernel regression-based method which allowed us to examine: (i) the dose–response relationship between each metal and CVD, fixing other metals in the mixture to some given percentiles; (ii) the statistical interactions between metals within the mixture; (iii) the joint association between the whole mixture and CVDs; and (iv) for highly correlated metals, to apply a hierarchical variable selection procedure. The R package BKMR conducts Bayesian inference for the probit regression model (BKMR-P) by using a MCMC algorithm.[21] The posterior inclusion probabilities (PIP) obtained from the BKMR-P quantify the relative importance of each exposure in the model, as they are a ranking measure to see how much the data favour the inclusion of a variable in the model. Given the almost complete correlation between Cr and V in our data, we decided to use a hierarchical variable selection approach to introduce Cr and V in the model as a single group. This approach first estimates the probability of the group being included in the model (group PIP), and then the probability that each metal within the group is driving the effect of the whole group (conditional PIP). More details regarding the BKMR-P model specification can be found in the Supplementary Methods. In addition, more about theoretical and practical use of BKMR package can be found in Bobb *et al.* (2015)[16] and Bobb *et al.* (2018).[14] All statistical analyses and graphical displays were performed using R software (version 3.1.3).

Results

Descriptive analysis

Median urinary metal concentrations in our study population are shown in Table 1. In unadjusted analysis, participants with incident all-CVD showed a worse cardiovascular risk profile compared with participants without incident all-CVD, as they were older, with a lower level of education and with an increased likelihood of showing cardiovascular risk factors and treatments. Among ever-smokers, individuals with incident all-CVD showed a higher proportion of increased accumulated exposure to tobacco smoke. Participants who had a cardiovascular incidence event showed lower baseline levels of Co and Mo, and higher baseline levels of all the other metals (Table 1). Cu, Cr and V were highly correlated with all the other metals (Supplementary Figure S1, available as Supplementary data at *IJE* online). The Spearman correlation between Cr and V was especially high (r = 0.99). In our study population of individuals free of CVD at baseline, we observed 166, 90 and 46 incident cases of all-CVD, combined CHD and stroke, and heart failure, respectively (Table 2; Supplementary Tables S1 and S2, available as Supplementary data at *IJE* online).

Individual metals and cardiovascular disease

In single-metal models, the fully adjusted HRs for CVD comparing the 80th to the 20th percentiles of metal distributions (95% CI, *P*-value for trend) were 1.15 (0.91, 1.46), *P*-trend: 0.25 for Co, 1.35 (1.06, 1.72), *P*-trend: 0.02 for Cu, 1.18 (0.88, 1.58), *P*-trend 0.28 for Mo, 1.43 (1.07, 1.90), *P*-trend: 0.01 for Zn, 1.51 (1.13, 2.03), *P*-trend: 0.006 for Sb, 1.32 (0.96, 1.82), *P*-trend: 0.08 for Ba, 1.46 (1.13, 1.88), *P*-trend: 0.003 for Cd, 1.64 (1.05, 2.58), *P*-trend: 0.03 for Cr and 1.31 (1.01, 1.71), *P*-trend: 0.04 for V (Table 2). Cu, Zn, Sb, Cd, Cr and V were individually related to CVD. For combined CHD and stroke, the corresponding HRs (*P*-trend) were 0.92 (0.68, 1.25), *P*-trend: 0.60 for Co, 1.11 (0.80, 1.55), *P*-trend: 0.53 for Cu, 0.93 (0.63, 1.38), *P*-trend: 0.73 for Mo, 1.03 (0.72, 1.48), *P*-trend: 0.87 for Zn, 1.36 (0.90, 2.06), *P*-trend:

Table 1. Participants' characteristics by cardiovascular incidence status

	Total ($N = 1171$)	CVD ($N = 166$)	No CVD ($N = 1005$)
Age (years), mean (SD)	47.91 (17)	55.55 (15.86)	48.28 (17.02)
Sex, % male	51.7	46	52.8
Smoking status, %			
Never	46.3	53.6	45.1
Former	28.2	33.7	27.3
Current	25.5	12.7	27.7
Cigarettes pack-year, %			
0	47.1	54.8	45.9
0_12	27.2	12	29.7
≥12	25.7	33.1	24.5
eGFR, % <60 ml/min by 1.73 m^2	7.9	19.9	5.9
Residence, % urban	77.5	76.7	77.6
Education, % >high School	74.9	47.9	79.5
Cotinine (ng/ml), %			
<=34	74.7	85.5	72.9
34-500	5.7	4.2	5.9
>500	19.6	10.2	21.1
Total cholesterol (mg/dl), mean (SD)	204.01 (37.33)	210.02 (33.34)	201.74 (37.41)
HDL cholesterol (mg/dl), mean (SD)	52 (14.29)	49.54 (12.72)	52.47 (14.33)
Lipid-lowering medication, %	5.2	11.4	4.1
Systolic blood pressure (mmHg), mean (SD)	124.97 (18.21)	137.69 (17.99)	126.94 (19.33)
Blood pressure-lowering medication, %	14.7	36.2	11.2
Diabetes, %	6	19.3	4.6
Co (μg /g), median (IQR)	0.23 (0.13, 0.48)	0.20 (0.12, 0.26)	0.23 (0.13, 0.49)
Cu (μg/g), median (IQR)	6.06 (3.78, 9.74)	6.13 (4.58, 9.01)	6.02 (3.66, 9.58)
Mo (μg/g), median (IQR)	25.37 (13.41, 50.32)	19.96 (8.89, 45.61)	25.21 (13.17, 49.52)
Zn (μg/g), median (IQR)	183.16 (95.85, 341.73)	203 (110.13, 343.03)	176.67 (94.47, 326.38)
Sb (μg/g), median (IQR)	0.08 (0.03, 0.16)	0.10 (0.04, 0.19)	0.07 (0.03, 0.16)
Ba (μg/g), median (IQR)	58.44 (31.71, 103.61)	81.28 (41.14, 146.30)	57.64 (31.70, 102.21)
Cd (μg/g), median (IQR)	0.38 (0.23, 0.64)	0.41 (0.29, 0.69)	0.37 (0.22, 0.64)
Cr (μg/g), median (IQR)	3.58 (2.27, 5.88)	4.07 (2.96, 5.40)	3.46 (2.23, 5.87)
V (μg/g), median (IQR)	2.12 (1.32, 3.47)	2.41 (1.70, 3.28)	2.04 (1.29, 3.47)

For continuous variables, we show mean (SD) if normally distributed or median (IQR) otherwise. For categorial variables, percentages are shown.
SD, standard deviation; IQR, interquartile range; eGFR, estimated glomerular filtration rate; HDL, high-density lipoprotein.

0.14 for Sb, 1.30 (0.88, 1.93), *P*-trend: 0.19 for Ba, 1.19 (0.86, 1.65), *P*-trend: 0.29 for Cd, 1.48 (1.06, 2.08), *P*-trend: 0.02 for Cr and 1.43 (1.02, 2.01), *P*-trend: 0.04 for V (Supplementary Table S1, available as Supplementary data at *IJE* online). Cr and V were individually related to combined CHD and stroke. For heart failure, the corresponding HRs (*P*-trend) were 1.40 (0.94, 2.09), *P*-trend: 0.10 for Co, 2.17 (1.49, 3.16), *P*-trend: <0.001 for Cu, 2.35 (1.34, 4.11), *P*-trend: 0.003 for Mo, 1.88 (1.10, 3.20), *P*-trend: 0.02 for Zn, 1.88 (1.10, 3.24), *P*-trend: 0.02 for Sb, 1.27 (0.63, 2.55), *P*-trend: 0.50 for Ba, 3.95 (1.44, 10.88), *P*-trend: 0.008 for Cd, 1.43 (0.81, 2.53), *P*-trend: 0.21 for Cr and 1.39 (0.79, 2.44), *P*-trend: 0.25 for V (Supplementary Table S2, available as Supplementary data at *IJE* online). Cu, Mo, Zn, Sb and Cd were individually related to heart failure. The association for Cr in all-CVD models, and for Cd in heart failure models, was non-linear (Table 2; Supplementary Table S2, available as Supplementary data at *IJE* online).

In progressively adjusted multiple-metal Cox models, only Sb and Cd remained statistically significant after accounting for the other metals (Supplementary Table S3, available as Supplementary data at *IJE* online), whereas the associations of all CVD with Cu and Zn became null. The results for V and Cr in the multiple-metal models illustrate that including correlated variables in regression settings induces inflated associations and standard errors. In all-CVD models, the *P*-value for interaction by sex and smoking subgroups was non-statistically significant for all metals (data not shown).

Metal mixtures and cardiovascular disease

The BKMR-P analysis, which allowed the evaluation of individual metals while appropriately accounting for

Table 2. HR (95% CI) for CVD incidence associated with urine metals in adult participants of the Hortega Study ($N = 1171$)

	Tertile 1, Non-cases/cases	Tertile 2, Non-cases/cases	Tertile 3, Non-cases/cases	p80 vs p20	*P*-trend
Essential metals					
Co	338/60	328/61	339/45		
	1.00 (Referent)	1.22 (0.82, 1.81)	1.29 (0.83, 1.99)	1.15 (0.91, 1.46)	0.25
Cu	351/30	322/69	332/67		
	1.00 (Referent)	2.40 (1.48, 3.88)	1.87 (1.17, 3.00)	1.35 (1.06, 1.72)	0.02
Mo	335/52	338/54	332/60		
	1.00 (Referent)	0.98 (0.65, 1.47)	1.12 (0.73, 1.71)	1.18 (0.88, 1.58)	0.28
Zn	352/30	331/56	322/80		
	1.00 (Referent)	1.47 (0.91, 2.40)	1.93 (1.21, 3.08)	1.43 (1.07, 1.90)	0.01
Non-essential metals					
Sb	350/51	331/55	324/60		
	1.00 (Referent)	1.41 (0.93, 2.13)	1.57 (1.04, 2.36)	1.51 (1.13, 2.03)	0.006
Ba	341/35	334/54	330/77		
	1.00 (Referent)	1.17 (0.74, 1.85)	1.25 (0.80, 1.94)	1.32 (0.96, 1.82)	0.08
Cd	356/36	327/63	322/67		
	1.00 (Referent)	2.27 (1.44, 3.57)	2.31 (1.47, 3.65)	1.46 (1.13, 1.88)	0.003
Cr	345/51	330/62	330/53		
	1.00 (Referent)	1.37 (0.90, 2.09)	1.63 (1.08, 2.46)	1.64 (1.05, 2.58)*	0.03*
V	346/53	327/64	332/49		
	1.00 (Referent)	1.45 (0.97, 2.18)	1.51 (1.00, 2.29)	1.31 (1.01, 1.71)	0.04

Models adjusted for sex, education (<high school, >=high school), smoking status (never, former and current smoker), cumulative smoking dose (0, 0–12, >12 pack-years), urine cotinine (<34, 34–500 and >=500 ng/ml), estimated glomerular filtration rate (ml/min per 1.73m^2), residence (urban or rural), HDL cholesterol level (mg/dl), total cholesterol level (mg/dl), dyslipidaemia treatment (yes/no), hypertension treatment (yes/no), diabetes mellitus of type 2 (yes/no) and systolic pressure (mmHg). Tertiles cut-offs for essential and non-essential metals (μg/g): Co, 0.15 and 0.36; Cu, 4.52 and 8.35; Mo, 16.88 and 40.21; Zn, 123.01 and 269.38; Sb, 0.05 and 0.13; Ba, 39.65 and 83.11; Cd, 0.27 and 0.53; Cr, 2.63 and 4.97; V, 1.56 and 2.94. p80 and p20 cut-offs for essential and non-essential metals (μg/g): Co, 0.57 and 0.11; Cu, 11.37 and 3.44; Mo, 58.91 and 11.34; Zn, 395.03 and 79.22; Sb, 0.19 and 0.03; Ba, 123.02 and 28.34; Cd, 0.75 and 0.19; Cr, 6.67 and 1.97; V, 3.98 and 1.17.

*Statistically significant non-linear associations were estimated modelling the corresponding metals in the regression models as restricted quadratic splines.

correlated exposures, estimated that Sb and Cd individually showed the higher PIPs, consistent with the single-metal models, followed by Cu and Zn (Table 3). Cr and V were related to CVD as a group (group PIP of 0.74), but there was no evidence to support that one of the two drives the effect of the whole group, since the conditional PIPs show a value of 0.5 for both (Table 3) (i.e. the MCMC algorithm selects Cr as the most important component of the group in half of the iterations and V in the other half). Supplementary Figure S2, available as Supplementary data at *IJE* online, shows that the shape of the metal-specific dose–responses are approximately linear, except possibly for Cd.

Figure 1 shows the estimated difference in the probit of incident CVD hazard when all the predictors are fixed to different percentiles, as compared with when they are all fixed to the 50th percentile, supporting a strong and linear positive association of the whole mixture with CVD incidence. Supplementary Figures S3 and S4, available as Supplementary data at *IJE* online, aim to evaluate potential interactions between the metals within the mixture by evaluating differential associations for the specific metals when fixing all the other metals (Supplementary Figure S3) or a second given metal (Supplementary Figure S4) to the 25th, 50th and 75th percentiles. We observed no evidence for interactions between metals given the similar point estimates (Supplementary Figure S3) and the parallel dose–

Table 3. PIPs of individual metals in the BKMR-P model

Metal	Group PIP	**Conditional PIP**[a]
Co	0.56	NA
Cu	0.68	NA
Zn	0.63	NA
Ba	0.56	NA
Mo	0.57	NA
Cd	0.70	NA
Sb	0.81	NA
Cr	0.74	0.53
V	0.74	0.47

Models adjusted for sex, education (<high school, >=high school), smoking status (never, former and current smoker), cumulative smoking dose (0, 0–12, >12 pack-years), urine cotinine (<34, 34–500 and >=500 ng/ml), age, estimated glomerular filtration rate (ml/min per 1.73m^2), residence (urban or rural), HDL cholesterol level (mg/dl), total cholesterol level (mg/dl), dyslipidaemia treatment (yes/no), hypertension treatment (yes/no), diabetes mellitus of type 2 (yes/no) and systolic pressure (mmHg). NA, Not available.

[a]Conditional PIPs are only estimated for multi-exposure groups.

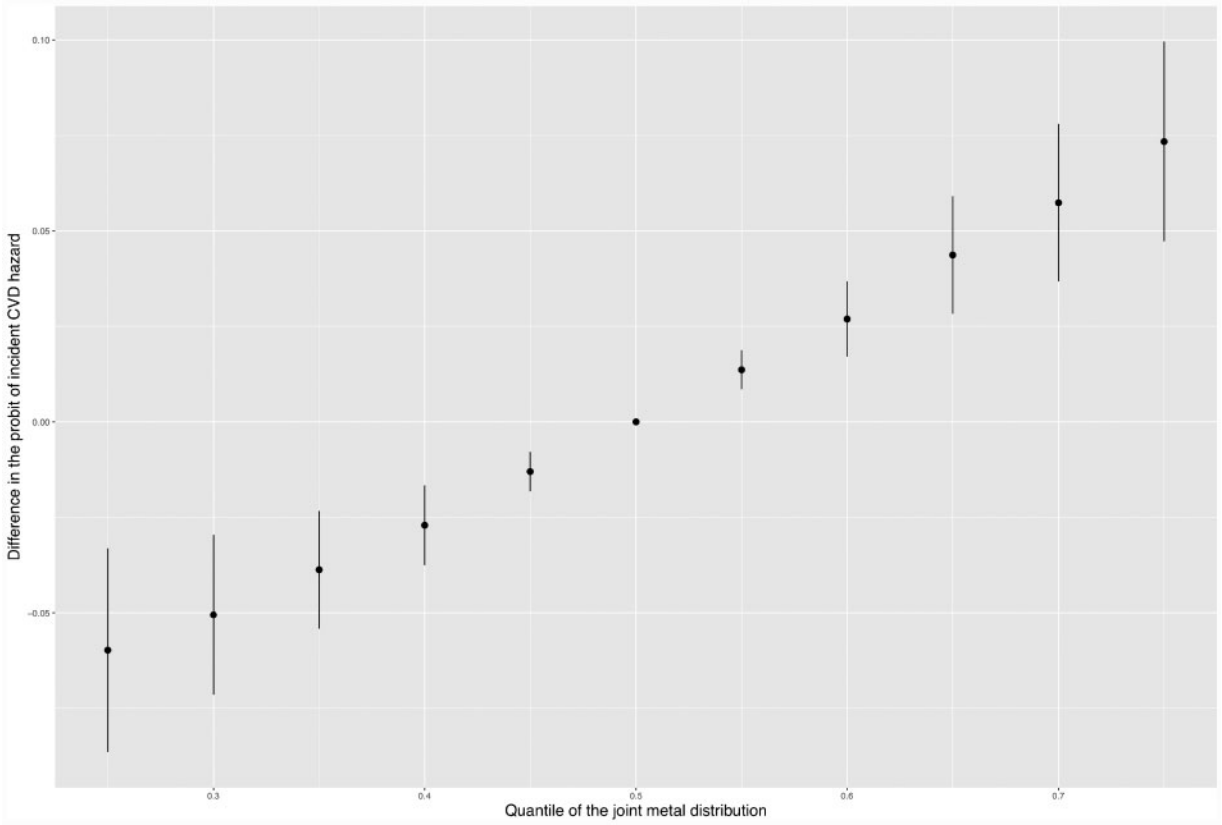

Figure 1. Overall association of the metal mixture with CVD incidence (difference in the probit of incident CVD hazard and 95% credibility intervals) when all predictors are at a particular percentile compared with the value when all of them are at their 50th percentile. BKMR-P models adjusted for sex, education (<high school, >=high school), smoking status (never, former and current smoker), cumulative smoking dose (0, 0-12, >12 pack-years), urine cotinine (<34, 34–500 and >=500 ng/mL), age, estimated glomerular filtration rate (mL/min per 1.73m^2), residence (urban or rural), HDL cholesterol level (mg/dl), total cholesterol level (mg/dl), dyslipidaemia treatment (yes/no), hypertension treatment (yes/no), diabetes mellitus of type 2 (yes/no) and systolic pressure (mmHg).

responses (Supplementary Figure S4). For descriptive purposes only, we show dose–responses of Cu, Zn, Cd and Sb with CVD incidence (relative risk or ratio of marginal probabilities predicted from the BKMR-P model) when all the other metals are fixed to their 10th, 50th and 90th percentiles (Supplementary Figure S5, available as Supplementary data at *IJE* online). The figure suggests that the dose–response with CVD incidence for each metal remains the same when all the other metals are fixed to their 10th or 50th percentiles but is attenuated when they are fixed to higher concentrations (90th percentile).

Sensitivity analyses

We conducted a number of sensitivity analyses. Abnormally increased excretion of Zn in urine has been observed in diabetes.[22,23] Thus, we evaluated the association of urinary zinc and CVD after excluding patients with prevalent diabetes, with consistent findings (Supplementary Figure S6, available as Supplementary data at *IJE* online). Also, we conducted a sensitivity analysis by considering non-creatinine-standardized metals (μg/L) as independent variables in the regression models with separate adjustment for urine creatinine (g/L) in the CVD incidence models. The direction of the associations remained unchanged, but the point estimates were somewhat attenuated. In addition, the associations for Cu, Cr and V in single-metal models became non-statistically significant (data not shown).

Discussion

We found that urine Cu, Zn, Sb, Cd, Cr and V were positively related to cardiovascular incidence, individually and as a mixture. The major contributors to the cardiovascular associations of the mixture, however, were Sb and Cd. We did not observe any evidence of interactions within the mixture components. In BKMR-P, the dose–response for both the whole metal mixture and specific metals appeared to be fairly linear, except maybe for Cd.

Metal biomarker interpretation

Urine concentrations of metals are typically used to assess metal exposure, as they integrate multiple exposure

sources such as food, drinking water and air.[24,25] The metals considered in the present study have relatively short half-lives in urine, so they are mostly considered reliable biomarkers of recent exposure. The exceptions are urine Cd, which is an accepted biomarker of long-term exposure as it is believed to also reflect accumulation in the kidneys although it also has a short-term component,[26] and urine Zn, which is known to increase in urine in the presence of diabetes.[27] In sensitivity analysis however, the observed positive association of urine Zn with all-CVD incidence essentially remained unchanged in participants without diabetes at baseline. Under chronic conditions of exposure, even urine metal biomarkers with relatively short half-lives can serve as a proxy of long-term exposure.[28] Some variability exists when comparing urinary metal concentrations in this study with those in other studies around the world. For example, the Aragon Workers Health Study from Spain presents lower Cd concentrations but higher Sb concentrations.[29] The National Health and Nutrition Examination Survey (NHANES) from the USA presents higher concentrations of urinary Sb, Co and Mo and lower concentrations of Cd and Ba.[30] Lower Cd concentrations in the USA are likely related to a lower prevalence of smoking in the USA compared with Spain. In Asia, a case–control study from Wuhan (China) showed lower levels of V, Cr, Co, Cu, Zn and Ba and higher levels of Mo, Cd and Sb.[31] Cd-contaminated soils and rice are common in Asia[32] and could be a reason for the higher Cd levels in that study. However, caution is needed when comparing metal concentrations across geographical locations, as cross-laboratory variability, which is independent of exposure, cannot be discarded.

Essential metals

Although Cu deficiency has traditionally been related to increased cardiovascular incidence,[33,34] a recently published meta-analysis [8] concluded that Cu was associated with an increased risk of CVD and CHD. Moreover Wilson disease, a rare disorder of Cu metabolism resulting in an abnormal accumulation of Cu in several body tissues,[35] has been linked to cardiac complications.[36,37] At the exposure levels seen in our study population, urine Cu was also consistently related with increased cardiovascular incidence.

Zn deficiency has typically been related to increased cardiovascular risk in some observational studies[1,38] and clinical trials.[39] Zn supplementation was suggested to be an anti-inflammatory and antioxidant agent in randomized clinical trials conducted on individuals with low Zn profiles.[40,41] In those trials, Zn supplementation downregulated the production of atherosclerosis-related cytokines and molecules in elderly participants,[41] and decreased the levels in plasma of thiobarbituric reactive substances (TBARS) in individuals with diabetes.[40] Long-term micronutrient supplementation (including both Zn and Cu supplementation) reduced left ventricular volume and increased left ventricular ejection fraction in elderly patients with chronic heart failure due to left ventricular systolic dysfunction.[42] However, the results from these trials must be interpreted cautiously given the small sample sizes and the focus on specific subgroups, such as elderly patients, that are more likely to have nutritional deficiencies.[43] Overall, our positive associations for Zn and Cu in single-metal models do not support supplementation with these metals for CVD prevention and control.

Non-essential metals

The positive association of Cd and Sb with incident CVD has been consistently observed in other study populations.[1,8] In a meta-analysis of 14 studies,[8] the pooled relative risk (95% CI) of CVD comparing the highest with the lowest tertile of urine Cd levels was 1.33 (1.09, 1.64). Elevated urine Sb levels have also been associated with increased risk of heart disease mortality,[44,45] although the number of studies is small. The essentiality of Cr, especially Cr(III), and V, has been debated given controversial results for Cr(III) and V supplements for glycaemic control in diabetic patients.[46–50] We considered them non-essential metals, as the evidence supporting Cr and V essentiality is insufficient. For Cr(VI), the evidence is clear that it is a non-essential toxic metal related to adverse health effects, including cancer.[51] Our study did not conduct speciation. Although our data suggest that Cr could be a cardiovascular risk factor, few studies have evaluated the association of these metals with hard cardiovascular outcomes. More prospective studies are needed to confirm our findings.

Metal mixtures

In our data, there was a strong and significant association of the mixture as a whole with all-CVD incidence. Few epidemiological studies have accounted for the potential correlation between metals in the analysis of metals-related health effects.[16,52–56] A systematic review[1] identified three studies evaluating the association of metals and CVD when other metals were adjusted in the regression models.[53–55] Urine Cd and Pb were positively associated with cardiovascular conditions in models adjusted for Ba, Co, Mo, tungsten and other heavy metals.[53] Toenail Cr was associated with decreased CVD prevalence in men after adjustment for Se and mercury.[54] Urine tungsten was positively associated with stroke in models that included Mo and Co as covariates.[55] In a more recent study from China,[56] plasma

titanium, As and Se were associated with CHD in models adjusted for aluminum and Ba. Overall, these studies used traditional multiple-metal regression models to deal with metal mixtures, which cannot accommodate high-order interactions and elevated correlations between metals or identify deviations from linearity. None of these studies formally evaluated the joint association of metal mixtures and cardiovascular incidence, including the identification of relevant components of the mixture.

In addition to Cd and Sb being the most relevant components for cardiovascular risk in our data, our results suggest that Cu and Zn are also related to CVD, given their relatively high PIPs and the positive dose–response association in BKMR-P models (Table 3; Supplementary Figure S2, available as Supplementary data at *IJE* online). These contributions, however, could not be detected by the Cox multiple-metal models, since standard Cox models lead to unstable results when introducing highly correlated variables. Overall, our findings suggest that public health interventions should be put in place to control for metal exposure in the general population, especially for non-essential metals. Regarding the almost complete correlation between Cr and V, although it was possible to fit Cox regression selecting one of the two metals arbitrarily to represent the contribution of the group, we attempted to use BKMR-P to simultaneously introduce both exposures and let the model disentangle whether one of them is driving the effect of the group on the outcome, with inconclusive results.

Strengths and limitations

Our study has several limitations. For instance, metal concentrations in urine might be influenced by variations in renal function and/or urine dilution. We adjusted our models for eGFR and diabetes to account for differences in renal function, and also divided urine metal concentrations by urine creatinine to correct for urine dilution. Creatinine, however, is associated with age, gender, race/ethnicity and muscle mass.[57] Another limitation is the moderate sample size, which allowed us to detect associations for the combined all-CVD endpoint, but was limited for analysis based on the combined CHD and stroke endpoint. We may also lack power to detect statistical interactions. In BKMR-P analysis, however, we observed parallel dose–responses of the evaluated metals under different exposure levels of the other metals within the mixture, especially for Cd and Sb (Supplementary Figure S4, available as Supplementary data at *IJE* online), suggesting confounding rather that interaction. Additional epidemiological studies evaluating CVD, including fatal and non-fatal endpoints in larger studies, are needed to confirm our findings, especially among never-smokers. Finally, BKMR-P is subject to potential unmeasured confounding, just as in any other regression approach. This is a typical limitation of all observational studies.

A strength of our study is that we employed BKMR-P, a novel statistical tool to evaluate potential cardiovascular effects of mixtures in a survival setting, using a sample of a general population from Spain. This statistical approach allowed us to overcome important limitations of statistical tools traditionally used in epidemiological studies, such as non-linearity, model mis-specification and high-order interactions. We were able to flexibly quantify and visualize the individual and joint effects of a mixture of metal compounds, as well as the contribution of individual metals to the mixture, without coarsening the continuous exposures and avoiding multicollinearity of highly correlated variables.

Our work contributes to the scientific literature in several ways. First, it adds to the body of evidence that supports that some metals are relevant for CVDs,[1,8] the world's leading cause of death.[58] Second, this is to our knowledge the first time that a BKMR approach has been applied to probit regression and time-to-event real data in the scientific literature. Other strengths of this study include the rigorous laboratory methods with extensive quality control, the availability of biomarkers of exposure which integrate exposure from all sources and the representative sampling design.

In conclusion, exposure to the mixture of metals considered in this study was associated with an increased risk of CVD. Our results, however, need to be reproduced in larger cohorts that estimate the effects of metal mixtures in CVD at exposure levels that are relevant for general populations, as human exposure to metals can be reduced through regulation and public health interventions. Evaluating food policies, promoting public and private non-smoking environments and limiting the pollutant emissions of contaminating industries are some actions that could decrease the burden of CVD associated with metals.

Supplementary Data

Supplementary data are available at *IJE* online.

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