



Urinary metals and metal mixtures and oxidative stress biomarkers in an adult population from Spain: The Hortega Study



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ARTICLE INFO

Handling Editor: Yong-Guan Zhu

Keywords:

Urine metals

Oxidative stress

Metal mixtures

Population-based

ABSTRACT

Introduction: Few studies have investigated the role of exposure to metals and metal mixtures on oxidative stress in the general population.

Objectives: We evaluated the cross-sectional association of urinary metal and metal mixtures with urinary oxidative stress biomarkers, including oxidized to reduced glutathione ratio (GSSG/GSH), malondialdehyde (MDA), and 8-oxo-7,8-dihydroguanine (8-oxo-dG), in a representative sample of a general population from Spain (Hortega Study).

Methods: Urine antimony (Sb), barium (Ba), cadmium (Cd), chromium (Cr), cobalt (Co), copper (Cu), molybdenum (Mo), vanadium (V) and zinc (Zn) were measured by ICPMS in 1440 Hortega Study participants.

Results: The geometric mean ratios (GMRs) of GSSG/GSH comparing the 80th to the 20th percentiles of metal distributions were 1.15 (95% confidence intervals [95% CI]: 1.03–1.27) for Mo, 1.17 (1.05–1.31) for Ba, 1.23 (1.04–1.46) for Cr and 1.18 (1.00–1.40) for V. For MDA, the corresponding GMRs (95% CI) were 1.13 (1.03–1.24) for Zn and 1.12 (1.02–1.23) for Cd. In 8-oxo-dG models, the corresponding GMR (95% CI) were 1.12 (1.01–1.23) for Zn and 1.09 (0.99–1.20) for Cd. Cr for GSSG/GSH and Zn for MDA and 8-oxo-dG drove most of the observed associations. Principal component (PC) 1 (largely reflecting non-essential metals) was positively associated with GSSG/GSH. The association of PC2 (largely reflecting essential metals) was positive for GSSG/GSH but inverse for MDA.

Conclusions: Urine Ba, Cd, Cr, Mo, V and Zn were positively associated with oxidative stress measures at metal exposure levels relevant for the general population. The potential health consequences of environmental, including nutritional, exposure to these metals warrants further investigation.

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1. Introduction

In occupational and general populations, exposure to non-essential metals, such as cadmium (Cd) and lead (Pb), through diet, ambient air and drinking water has been positively associated with mortality and health conditions such as cancer, cognitive outcomes and cardiovascular, bone and kidney disease (Nigra et al., 2016; Tellez-Plaza et al., 2018; ATSDR, 2012a; ATSDR, 2007; Chowdhury et al., 2018). For other metals, however, the body of evidence is not so extended. Occupational studies reported an association of barium (Ba) with blood pressure (Choudhury, 2001), and antimony (Sb) with respiratory illness (Potkonjak & Pavlovich, 1983), gastrointestinal effects (Taylor, 1966), dermatitis (White et al., 1993), altered electrocardiography readings and elevated blood pressure (Sundar & Chakravarty, 2010). Occupational inhalation of hard metal dust containing tungsten (W) and cobalt (Co) causes asthma and fibrosis (Lison, 1996; Moulin et al., 1998). Alternatively, essential metals deficiency has also been related to several diseases, such as renal and liver disease, diabetes or other chronic illnesses (Burk, 2002; Prasad, 2003). In addition, excessive exposure to essential metals, frequently present in vitamin and mineral supplements in addition to diet and polluted air, has also been related to adverse health effects (Sathawara et al., 2004).

Both essential and non-essential metals can participate in oxidative stress, an unbalanced state with increased reactive oxygen species (ROS) formation that can eventually lead to lipid peroxidation and cellular injury, including DNA damage. For instance, while copper (Cu) plays an important role in the catalytic activity of the anti-oxidant enzyme superoxide dismutase (SOD) (Fetherolf et al., 2017), unbound Cu directly enters Fenton reactions (Valko et al., 2005). Non-essential divalent metals such as Cd or Ba directly compete with divalent essential metals for protein binding sites, interfering in oxidative stress pathways (Imlay, 2014; Menon et al., 2016). The hexavalent form of chromium (Cr) [Cr (VI)] translocates through biological membranes and it is intracellularly reduced to pentavalent Cr (Cr [V]), tetravalent Cr (Cr [IV]) and trivalent Cr (Cr [III]) concomitantly with ROS generation (Rembacz et al., 2012). A few studies have linked high molybdenum (Mo) exposure with oxidative stress mechanisms, for instance by inducing changes in antioxidant enzymatic activities, alone or in co-exposure with Cd (Zhuang et al., 2016; Cao et al., 2016; Yang et al., 2016). In liver tissue from exposed rats, the antioxidant activity of GPx decreased with increasing Ba doses (Elwej et al., 2017). One possible mechanism for metal-related health effects is thus the generation of organ damage through the alteration of the redox balance. The association of metals with oxidative stress biomarkers, however, has seldom been investigated at exposure levels that are relevant for general populations. Moreover, there is a paucity of epidemiological studies evaluating the potential role of metal mixtures in oxidative stress.

A number of oxidative stress biomarkers have been used in epidemiological studies (Ilyasova et al., 2012). For instance, reduced glutathione (GSH) provides reducing equivalents for the enzymatic reaction mediated by glutathione peroxidase (GPx) resulting in oxidized glutathione (GSSG). Under oxidative stress conditions, GSSG will accumulate and the ratio GSSG/GSH will increase. Oxidized to reduced glutathione ratio (GSSG/GSH) thus reflects enzymatic activity of GPx and is considered a marker of oxidative stress at the cellular cytoplasm (Brigelius-Flohé, 1999). Malondialdehyde (MDA) and 8-oxo-7,8-dihydroguanine (8-oxo-dG) are, however, markers of early biological effects of ROS on lipids (mainly from cellular membranes) and DNA oxidation and repair, respectively (Halliwell & Gutteridge, 2015; Teichert et al., 2009). The purpose of the current analysis was to investigate the cross-sectional association of urinary metal concentrations (Sb, Ba, Cd, Cr, Co, Cu, Mo, vanadium [V] and zinc [Zn]) and metal mixtures with urinary GSSG/GSH, MDA, and 8-oxo-dG in the general population. To do so, we analyzed data from the Horteiga Study, a representative sample of adults from Valladolid (Spain).

2. Methods

2.1. Study population

The Horteiga Study is a population-based survey among adults residing in the catchment area of the Rio Horteiga University Hospital in Valladolid (Spain) in 1997–2003. In Spain, tertiary hospitals have assigned specific geographic areas for patient referral, and they integrate the network of primary care centers in each area. The study design and data collection methods have been previously described (Galan-Chilet et al., 2014). Briefly, the study recruited a total of 1502 participants. We excluded 27 participants with insufficient urine to measure metal or oxidative stress biomarkers, participants missing educational level information ($n = 8$), smoking status ($n = 15$) and urine cotinine ($n = 2$). We additionally excluded 10 participants with abnormal levels of urine metal concentrations (defined as 3 times the percentile 99 of each metal). A total of 1440 participants were finally included in this analysis. The research protocol was approved by Ethics Committee of the Rio Horteiga University Hospital and all participants provided written informed consent.

2.2. Metal biomarker levels

In 2013, urinary Sb, Ba, Cd, Cr, Co, Cu, Mo, V and Zn were measured in urine of the Horteiga Study participants using inductively coupled plasma mass spectrometry 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. 1 mL of urine was made up to 5 mL with 5% (v/v) of HNO₃ Ultra Trace Metals control and 100 ng/mL of Rh aqueous solution were added as internal standard for the samples and calibrants. Quality control of analysis was performed using the following reference materials: *Clincheck* Urine Control lyophilised (*RECIPE* Level I and II, and Standard Reference Material® 2670 for Urine Toxic metal from National Institute of Standards and Technology (NIST). 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. The specific percentages of participants with concentrations below the limit of detection (< 2% across all metals) and coefficients of variation (CV) (ranging from 1.57% for Zn to 7.26% for Cd) are shown in detail in Table S1. Undetected urine metal levels were replaced by the limit of detection divided by the square root of two (Hornung & Reed, 1990). We accounted for urine dilution by standardizing all metal concentrations by urine creatinine. Plasma Sb, Ba, Cd, Cr, Co, Cu, Mo, V, Cr and Zn were measured in 2010 by atomic absorption spectrometry with graphite furnace at Cerba International Laboratories Ltd. following a standardized protocol, including using commercially available reference materials (Scharlau standard solution for ICP), which are traceable to the corresponding NIST reference material. Additional internal controls (Seronom trace elements) were used for daily quality control. However, only plasma Cu and Zn are established biomarkers of exposure to these elements and had > 50% detected values (detection limits for plasma Cu and Zn were 0.63 and 0.65 µg/dL, respectively). The corresponding CV were 7.2 and 4.2% for Cu and Zn respectively. No individual had levels below the detection limit for plasma Cu and Zn.

2.3. Oxidative stress biomarkers

GSSG, GSH, MDA, and 8-oxo-dG were measured in urine. GSSG and GSH were analyzed by high-performance liquid chromatography (HPLC) (Brigelius et al., 1983; Navarro et al., 1997). MDA was analyzed by HPLC and spectrophotometric quantification of MDA thiobarbituric acid (TBA) at 532 nm (Moselhy et al., 2013). The amount of 8-oxo-dG in urine was measured by HPLC with electrochemical detection (HPLC-EC) (Espinosa et al., 2007; Li et al., 2013) with modifications (Borrego

et al., 2013). GSSG and GSH concentrations were measured in nmol and standardized by milligrams of protein content as determined by the Lowry method. The ratio GSSG/GSH was then reported as a percentage. MDA and 8-oxo-dG were corrected for urine dilution by using urine creatinine and were reported in nanomoles per millimoles of creatinine. Median (minimum, maximum) values of oxidative stress biomarkers in our study population were 0.66 (0.05, 8.53) nmol/mg protein for GSSG, 17.5 (0.12, 52.1) nmol/mg protein for GSH, 0.56 (0.07, 8.52) nmol/nmol creatinine for MDA, and 2.96 (0.10, 8.95) nmol/mmol creatinine for 8-oxo-dG. Urine creatinine was measured by the modified kinetic Jaffé method. The coefficients of variation for GSSG, GSH, MDA and 8-oxo-dG were, respectively, 11.4, 4.7, 5.5 and 11.9%.

2.4. Other variables

The interviews, physical examinations and collection of biospecimens were conducted by trained staff using standardized protocols. Socio-demographic (age, sex, race/ethnicity) and lifestyle (smoking status) information was collected using standardized questionnaires. Body mass index was calculated from measured weight in kilograms divided by the square of measured height in meters. Urine cotinine was measured by enzyme-linked immunosorbent assay (ELISA) (Kit “Análisis DRI® Cotinina”, Ref. 0395 Microgenics laboratories), with a limit of detection of 34 ng/mL (77% of participants below the limit of detection). Participants were considered to have diabetes mellitus if the level of fasting glucose was 126 mg/dL or higher, if hemoglobin A1c was 6.5% or higher, if they had been previously diagnosed of type 2 diabetes by a physician, or if they had a record of use of diabetes medications in the clinical history. Blood pressure was measured using a mercury sphygmomanometer. Systolic and diastolic blood pressure levels were the average of 3 readings measured at 5-min intervals. Serum creatinine was measured by the modified kinetic Jaffé method by isotope dilution mass spectrometry (IDMS) on a Hitachi 917 analyzer (Roche Diagnostics GmbH, Mannheim Germany). Estimated glomerular filtration rate (eGFR) was calculated from serum creatinine, age and sex using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formula (Levey et al., 2009).

2.5. Statistical methods

Metals, GSSG/GSH, MDA, and 8-oxo-dG concentrations were log-transformed for all analyses. Spearman's correlation coefficients were calculated to examine the bivariate correlations between pairs of metals after correction for urine dilution. Cut-offs for urine metals quantiles were estimated using sampling weights. We estimated the geometric mean ratio (GMR) and 95% confidence intervals (CI) for GSSG/GSH, MDA, and 8-oxo-dG by urine metal levels using linear regression in separate models. Urine metal concentrations were modeled as tertiles, comparing the highest two tertiles to the lowest one, and as log-transformed (continuous) variables, comparing the 80th versus the 20th percentiles. Statistical models were adjusted for sex, age, education (< high school, ≥ high school), smoking status (never, former and current smoker), cumulative smoking dose (pack-years), urine cotinine (< 34, 34–500, and ≥ 500 mg/dL), alcohol intake (mg/day), diabetes status (yes, no) and eGFR. In preliminary analyses, we conducted an additional adjustment model including body mass index, residence place (urban, rural), total cholesterol, HDL-cholesterol, lipid-lowering treatment (yes, no), systolic blood pressure, and antihypertensive treatment. We did not retain these variables in the final models because the point estimates did not substantially change (data not shown). P values for trend were obtained from Wald tests for log-transformed urine metal regression coefficients. To further explore the shape of the relationship between urine metal and oxidative stress biomarkers, we also modeled urine metal levels as restricted quadratic splines with knots at the 10th, 50th, and 90th percentiles. P values for non-linearity were obtained from Wald tests including the non-linear spline terms.

In addition, we conducted progressively adjusted multi-metal linear models. Our multi-metal models only considered as mixture components those metals that showed a statistically significant association with oxidative stress biomarkers in the single-metal models. However, with 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 linear regression settings (Bobb et al., 2015). In an attempt to address these challenges, when > 2 metals were statistically significant in single-metal models, we implemented a Bayesian Kernel Machine Regression (BKMR) method to confirm results from multi-metal models.

We also conducted principal components analysis (PCA) to evaluate relationships among the 9 metals irrespectively of the oxidative stress endpoints. PCA is widely used in environmental research to classify metals based on similarities of environmental sources (Loska & Wiechula, 2003; Pang et al., 2016). Principal components were estimated on the normally distributed z-score of the metal biomarker residuals after adjusting for urine creatinine, diabetes status and glomerular filtration rate. The normalized z-score was estimated by subtracting the residuals mean and dividing by the corresponding standard deviation as conducted by the “scale()” function in R. Varimax normalized rotation was applied to maximize the variances of the factor loadings across variables for each factor (Kaiser, 1958). We retained all principal components with eigenvalues ≥ 1.0. We subsequently fitted linear regression models with the PCA scores introduced as explanatory variables instead of the specific metals.

All statistical analyses were performed using R software (version 3.3.0). We used the “survey” package (Lumley, 2017) to account for the complex sampling design and survey weight. The R package BKMR conducts Bayesian inference for the model by using MCMC algorithm (Bobb et al., 2015).

3. Results

3.1. Descriptive statistics of urinary metals

Geometric mean urine levels were 5.2% for GSSG/GSH, and 0.49 and 2.69 nmol/mmol of creatinine for MDA and 8-oxo-dG, respectively (data not shown). In unadjusted analyses, geometric mean levels of urine essential metals were 0.24, 6.1, 24.5 and 171 µg/g for Co, Cu, Mo and Zn, respectively (Table S1). Geometric mean urine concentrations of non-essential metals were 0.07, 56.0, 0.36, 3.5 and 2.1 µg/g for Sb, Ba, Cd, Cr and V, respectively (Table S1). Older participants showed lower urinary concentrations of Co, Cr, and V, and higher concentrations of Cu, Ba, and Zn. Men had higher urinary concentrations of most evaluated metals compared to women. Among never smokers, Cd concentrations were higher in women (median 0.4 µg/g) than in men (median 0.3 µg/g) (data not shown). Compared with current smokers, never smokers had higher levels of Mo and Ba, and lower levels of Cd, Cr and V (Table 1). Cd increased with increasing pack-years smoked. Participants with reduced eGFR generally had lower urinary metal levels except for Cu, Zn and Ba (Table 1). After urine creatinine correction, we observed moderate to strong positive correlations between most metals (Fig. S1). The lowest correlation was observed between Sb and Ba (Spearman correlation = 0.33), while the highest was observed between Cr and V (Spearman correlation = 0.99).

3.2. Individual metals and oxidative stress

Increased urine levels of Mo, Ba, Cr and V were associated with increased GSSG/GSH in single-metal fully adjusted models (Table 2). In particular, the GMR (95% CI) of GSSG/GSH comparing the 80th to the 20th percentiles of metal distributions were 1.15 (1.03–1.27) for Mo, 1.17 (1.05–1.31) for Ba, 1.23 (1.04–1.46) for Cr and 1.18 (1.00–1.40) for V. Increased Zn and Cd were consistently associated with increased MDA and 8-oxo-dG (Table 2), although the association of Cd with 8-

Table 1
Median (IQR) of urine metal concentrations (µg/g) by participant's characteristics.

	N	Essential metals				Non-essential metals				
		Co	Cu	Mo	Zn	Sb	Ba	Cd	Cr	V
Overall	1440	0.23 (0.13–0.48)	6.5 (4–10.4)	26.3 (13.9–51.7)	198 (102–367)	0.08 (0.03–0.16)	61.8 (35–111.5)	0.39 (0.23–0.65)	3.5 (2.2–5.8)	2.1 (1.3–3.4)
Age, years										
< 50	674	0.26 (0.14–0.63)	5.8 (3.7–9.6)	25.0 (13.2–49.4)	162 (87–316)	0.08 (0.04–0.17)	53.3 (28.8–92.4)	0.37 (0.22–0.64)	3.6 (2.3–6.2)	2.1 (1.4–3.6)
50–64	231	0.2 (0.13–0.34)	6.4 (3.8–9.2)	23.2 (12.7–46.4)	198 (117–323)	0.08 (0.03–0.18)	56.8 (34.4–103.8)	0.43 (0.26–0.67)	3.6 (2.2–5.8)	2.2 (1.3–3.3)
≥ 65	535	0.2 (0.12–0.39)	7.5 (4.6–12.2)	29.4 (15.4–54.6)	251 (130–468)	0.07 (0.03–0.14)	78.6 (46.8–138.9)	0.39 (0.23–0.66)	3.3 (2–5.4)	1.9 (1.2–3.1)
Sex										
Men	723	0.18 (0.12–0.33)	6.4 (4–10.4)	26.9 (14.8–49.6)	231 (134–398)	0.08 (0.04–0.16)	61.5 (34.1–109.7)	0.41 (0.25–0.67)	3.6 (2.3–5.9)	2.1 (1.4–3.5)
Women	717	0.29 (0.15–0.66)	6.5 (3.9–10.5)	24.8 (12.5–53.5)	157 (78–337)	0.07 (0.03–0.15)	62.1 (35.8–115.4)	0.37 (0.2–0.62)	3.4 (2.1–5.8)	2.0 (1.2–3.3)
Smoking status										
Never	671	0.23 (0.12–0.51)	6.6 (4.1–10.3)	27.5 (15–53.5)	195 (97–366)	0.07 (0.03–0.15)	65.7 (36–116)	0.34 (0.2–0.58)	3.3 (2.1–5.7)	1.9 (1.2–3.3)
Former	433	0.21 (0.13–0.46)	6.6 (3.9–10.8)	26.0 (13.9–49.8)	212 (114–395)	0.08 (0.04–0.17)	62.4 (34.9–116.2)	0.41 (0.26–0.7)	3.5 (2.2–5.9)	2.1 (1.3–3.4)
Current	336	0.24 (0.14–0.46)	6.2 (3.7–9.9)	24.4 (11.7–49.3)	185 (102–351)	0.08 (0.04–0.15)	53.8 (32.2–102.7)	0.46 (0.27–0.69)	3.8 (2.4–6)	2.3 (1.4–3.6)
Urine cotinine, ng/mL										
< 34	1106	0.22 (0.12–0.48)	6.5 (4–10.5)	26.4 (14.5–52.7)	201 (102–383)	0.07 (0.03–0.16)	64.1 (35.6–112.5)	0.37 (0.22–0.62)	3.4 (2.1–5.7)	2.0 (1.3–3.3)
≥ 34	334	0.24 (0.14–0.48)	6.2 (3.8–10)	25.8 (11.5–48.3)	193 (107–350)	0.08 (0.04–0.17)	56.1 (33.2–107.3)	0.44 (0.25–0.72)	3.7 (2.4–6.2)	2.2 (1.4–3.6)
Cumulative smoking, pack-year										
0	682	0.23 (0.12–0.51)	6.7 (4.1–10.3)	27.6 (15–53.9)	197 (97–369)	0.08 (0.03–0.16)	65.3 (36.4–116.1)	0.34 (0.2–0.59)	3.4 (2.1–5.7)	1.9 (1.2–3.3)
0–12	379	0.24 (0.14–0.53)	6.2 (3.7–10.2)	26.7 (13.5–53.1)	168 (97–356)	0.08 (0.04–0.17)	55.5 (33.3–109)	0.38 (0.23–0.64)	3.6 (2.4–6.7)	2.1 (1.3–3.8)
≥ 12	379	0.21 (0.13–0.38)	6.5 (4.1–10.7)	22.4 (12.3–45.3)	221 (121–388)	0.07 (0.03–0.14)	61.5 (34.5–107.6)	0.5 (0.3–0.78)	3.6 (2.2–5.5)	2.1 (1.3–3.2)
eGFR, mL/min per 1.73 m ²										
< 60	118	0.18 (0.12–0.38)	8.2 (4.4–16.3)	24.4 (12.3–44.5)	263 (137–525)	0.06 (0.02–0.14)	86.6 (52.4–149.9)	0.34 (0.22–0.62)	3.0 (1.7–4.7)	1.7 (1–2.7)
60–90	550	0.21 (0.13–0.38)	6.7 (4–10.3)	27.2 (14.5–51.9)	214 (117–397)	0.07 (0.03–0.14)	66.1 (39.4–119.8)	0.37 (0.22–0.62)	3.3 (2–5.4)	2.0 (1.2–3.2)
> 90	772	0.25 (0.14–0.55)	6.1 (3.9–9.7)	26.2 (13.7–52.7)	178 (92–336)	0.08 (0.04–0.17)	55.2 (30.6–97.3)	0.40 (0.23–0.67)	3.8 (2.4–6.3)	2.2 (1.4–3.7)
Diabetes status										
No	1321	0.23 (0.13–0.5)	6.4 (3.9–10.4)	26.6 (13.8–52)	190 (99–360)	0.08 (0.03–0.16)	60.8 (34.3–112.7)	0.40 (0.23–0.66)	3.6 (2.3–5.9)	2.1 (1.3–3.5)
Yes	119	0.17 (0.11–0.29)	6.9 (4.6–9.9)	24.4 (14.5–43.1)	294 (171–446)	0.07 (0.03–0.13)	69.7 (47.8–103.7)	0.35 (0.21–0.59)	2.8 (1.9–4.6)	1.7 (1.1–2.7)

Abbreviations: IQR, interquartile range; eGFR, estimated glomerular filtration rate.

oxo-dG was borderline significant. The GMR (95% CI) for MDA comparing the 80th to the 20th percentiles of Zn and Cd were, respectively, 1.13 (1.03–1.24) and 1.12 (1.02–1.23). The corresponding GMR (95% CI) for 8-oxo-dG were 1.12 (1.01–1.23) for Zn and 1.09 (0.99–1.20) for Cd. The dose responses were linear for most significant metals, except for Cr and V in GSSG/GSH models, and for Zn and Cd in MDA and 8-oxo-dG models. The flexible dose responses between each metal and the oxidative stress biomarkers are shown in Fig. S2.

3.3. Metal mixtures and oxidative stress

Given the almost complete correlation of Cr and V, we did not consider V for subsequent multi-metal analyses. In multi-metal models the association of Mo and Ba with GSSG/GSH disappeared after adjustment for Cr (Table S2). Conversely, the association of Cr and GSSG/GSH became stronger after adjustment for Mo and Ba. The results were confirmed in BKMR models, where after simultaneously entering Mo,

Ba and Cr, only Cr showed a clear dose-response (Fig. 1). The posterior inclusion probabilities (PIPs), which are a ranking measure to see how much the data favors the inclusion of a variable in the model (Tsangarides, 2004), were 0.05 for Mo, 0.24 for Ba and 1 for Cr (data not shown). There was no differential association of Cr with GSSG/GSH when other metals were fixed at the lower, medium and high range of concentrations (Fig. 1, Fig. S3). For MDA and 8-oxo-dG multi-metal regression models the association with Zn did not change after adjustment for Cd, whereas the association of Cd was attenuated and no longer statistically significant after adjustment for Zn (Table S3).

3.4. PC analysis and oxidative stress

The two first PCs (PC1 and PC2, respectively) showed an eigenvalue ≥ 1 (Fig. S4) and explained 41.2 and 15.2%, respectively, of the total variance in the joint distribution of metals. Based on the estimated loadings (Fig. S4), PC1 scores largely reflected non-essential metals

Table 2
GMR (95% CI) of urine GSSG/GSH, MDA and 8-oxo-dG associated with urine metals in adult participants from the Hortega Study (N = 1440).

	Essential metals					Non-essential metals				
	Co	Cu	Mo	Zn	Sb	Ba	Cd	Cr	V	
GSSG/GSH										
Tertile 1	1.00 (referent)	1.00 (referent)	1.00 (referent)	1.00 (referent)	1.00 (referent)	1.00 (referent)	1.00 (referent)	1.00 (referent)	1.00 (referent)	
Tertile 2	1.03 (0.88, 1.20)	0.98 (0.84, 1.15)	1.05 (0.90, 1.22)	0.83 (0.71, 0.96)	0.99 (0.85, 1.16)	1.02 (0.87, 1.19)	0.85 (0.74, 0.99)	1.03 (0.89, 1.18)	1.01 (0.87, 1.15)	
Tertile 3	1.01 (0.85, 1.19)	1.09 (0.93, 1.29)	1.15 (0.98, 1.35)	1.01 (0.86, 1.20)	1.00 (0.85, 1.17)	1.19 (1.01, 1.41)	1.06 (0.90, 1.26)	1.41 (1.20, 1.66)	1.37 (1.16, 1.61)	
p80 vs p20	0.98 (0.89, 1.08)	1.06 (0.95, 1.18)	1.15 (1.03, 1.27)	1.00 (0.88, 1.12)	0.99 (0.90, 1.10)	1.17 (1.05, 1.31)	1.08 (0.97, 1.19)	1.23 (1.04, 1.46) ^a	1.18 (1.00, 1.40) ^a	
p-Trend	0.72	0.29	0.01	0.86	0.81	0.005	0.19	0.02 ^a	0.05 ^a	
MDA										
Tertile 1	1.00 (referent)	1.00 (referent)	1.00 (referent)	1.00 (referent)	1.00 (referent)	1.00 (referent)	1.00 (referent)	1.00 (referent)	1.00 (referent)	
Tertile 2	0.97 (0.89, 1.06)	0.96 (0.88, 1.05)	0.98 (0.90, 1.07)	1.07 (0.98, 1.18)	0.92 (0.85, 1.01)	1.00 (0.91, 1.10)	0.97 (0.89, 1.07)	0.95 (0.87, 1.04)	1.01 (0.92, 1.10)	
Tertile 3	0.99 (0.90, 1.08)	1.06 (0.97, 1.16)	1.01 (0.92, 1.10)	1.11 (1.02, 1.21)	0.98 (0.90, 1.07)	0.99 (0.91, 1.08)	1.12 (1.03, 1.22)	0.94 (0.86, 1.02)	0.97 (0.89, 1.06)	
p80 vs p20	0.98 (0.94, 1.03)	1.04 (0.98, 1.09)	1.01 (0.96, 1.07)	1.13 (1.03, 1.24) ^a	1.00 (0.94, 1.05)	1.02 (0.96, 1.08)	1.12 (1.02, 1.23) ^a	0.98 (0.92, 1.03)	0.97 (0.92, 1.02)	
p-Trend	0.52	0.20	0.66	0.01 ^a	0.86	0.57	0.02 ^a	0.37	0.25	
8-Oxo-dG										
Tertile 1	1.00 (referent)	1.00 (referent)	1.00 (referent)	1.00 (referent)	1.00 (referent)	1.00 (referent)	1.00 (referent)	1.00 (referent)	1.00 (referent)	
Tertile 2	1.10 (1.01, 1.19)	1.07 (0.99, 1.17)	1.15 (1.06, 1.25)	1.08 (0.99, 1.18)	1.14 (1.05, 1.23)	0.98 (0.90, 1.06)	1.13 (1.04, 1.22)	1.06 (0.98, 1.15)	1.05 (0.97, 1.14)	
Tertile 3	1.04 (0.95, 1.13)	1.06 (0.97, 1.15)	1.02 (0.94, 1.11)	1.14 (1.04, 1.25)	1.03 (0.95, 1.12)	1.00 (0.91, 1.09)	1.07 (0.98, 1.17)	1.06 (0.97, 1.15)	1.02 (0.94, 1.11)	
p80 vs p20	1.03 (0.98, 1.09)	1.04 (0.99, 1.09)	1.02 (0.97, 1.07)	1.12 (1.01, 1.23) ^a	1.00 (0.94, 1.06)	1.00 (0.95, 1.05)	1.09 (0.99, 1.20) ^a	1.04 (0.99, 1.10)	1.03 (0.98, 1.09)	
p-Trend	0.22	0.15	0.51	0.03 ^a	0.98	0.94	0.08 ^a	0.13	0.26	

Models adjusted for age (years, splines), sex, education (< secondary education, ≥ secondary education), smoking status (never, former, current), urine cotinine (< 34, 34–500, ≥ 500 ng/mL), cumulative smoking (0, < 12, ≥ 12 packages year), alcohol consumption (g/day), diabetes status (no, yes) and estimated glomerular filtration rate (mL/min per 1.73 m²).

Tertiles cut-offs for essential and non-essential metals (µg/g): Co, 0.16 and 0.37; Cu, 4.55 and 8.45; Mo, 16.9 and 39.7; Zn, 124 and 276; Sb, 0.05 and 0.13; Ba, 40.4 and 82.9; Cd, 0.27 and 0.54; Cr, 2.61 and 5.01; V, 1.55 and 2.94.
p80 and p20 cut-offs for essential and non-essential metals (µg/g): Co, 0.61 and 0.12; Cu, 11.5 and 3.5; Mo, 58.8 and 11.4; Zn, 411 and 81; Sb, 0.19 and 0.03; Ba, 122.4 and 28.6; Cd, 0.75 and 0.20; Cr, 6.8 and 1.9; V, 4.0 and 1.2.

^a Non-linear associations with corresponding metals modeled at restricted quadratic splines.

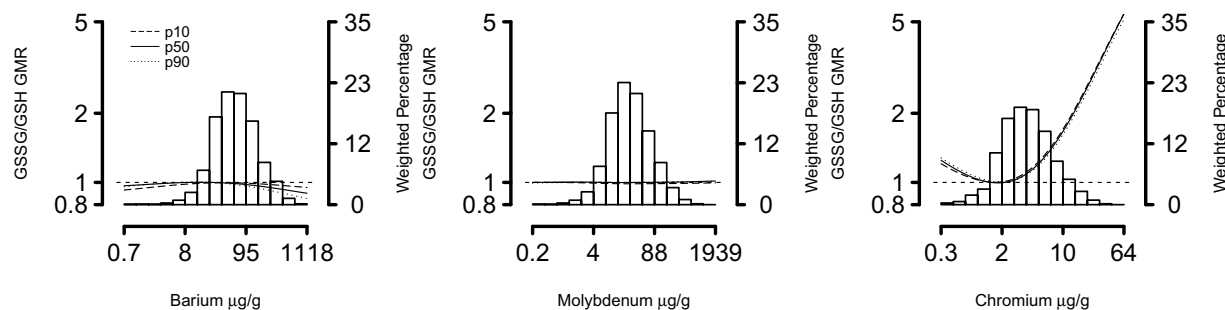


Fig. 1. GMR of GSSG/GSH by Ba, Mo and Cr concentrations when the other two metals are fixed at their 10th, 50th and 90th percentiles.

Flexible dose responses were estimated from a BKMR model adjusted for age (years, splines), education (< secondary education, ≥ secondary education), sex, smoking status (never, former, current), urine cotinine (< 34, 34–500, ≥ 500 ng/mL), cigarette packages per year (0, 0–12, ≥ 12), alcohol consumption (g/day), type 2 diabetes status and estimated glomerular filtration rate (mL/min per 1.73 m²). The reference value (GMR = 1) was set at the 10th percentile of the urine metal distributions (21.2, 7.5 and 1.5 µg/g for Ba, Mo and Cr, respectively). The 10, 50 and 90th percentiles of urine metal distributions were, respectively, 21.8, 58.7 and 200.9 µg/g for Ba, 8.0, 25.2 and 105.4 µg/g for Mo and 1.5, 3.5 and 9.9 µg/g for Cr. The histogram represents the frequency distribution of urine metals in the study sample. For a given percentile of urine metal distribution, the corresponding GMR is interpreted as the expected percentage change in the geometric mean of GSSG/GSH levels associated with changing metal exposure levels from that percentile to the reference (10th percentile). For example, model estimates suggest that the geometric mean of GSSG/GSH levels of participants in the 90th percentile of urine Cr distribution (10 µg/g) is ~50% higher (i.e. GMR ~1.50) compared to participants in the 10th percentile (1.5 µg/g), after accounting for adjustment factors.

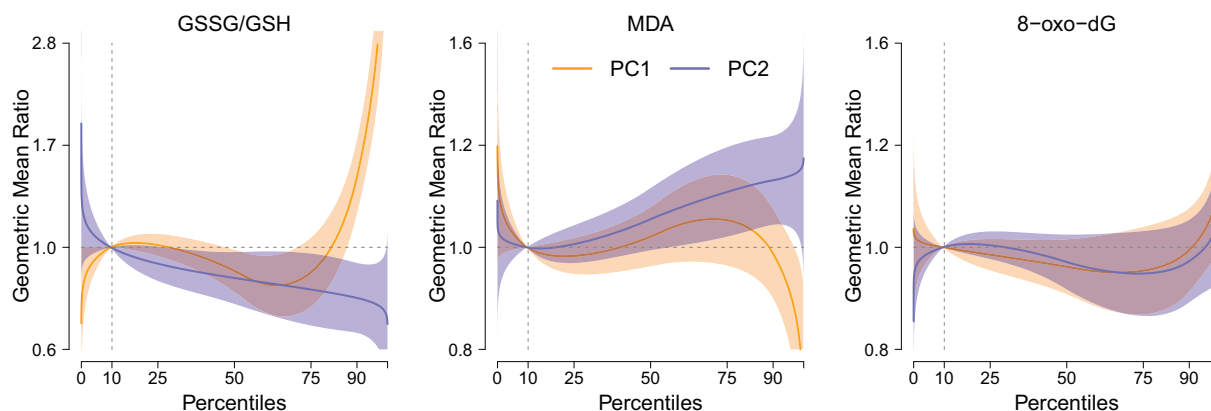


Fig. 2. Dose-response relationship of oxidative stress biomarkers by urine metals principal components.

Abbreviations: PC1, first principal component, largely reflecting non-essential metals; PC2, second principal component, largely reflecting essential metals. Lines (shaded areas) represent adjusted geometric mean ratios (95% confidence intervals) for each oxidative stress biomarker estimated from linear regression models where the first two metal principal components were considered explanatory variables and introduced as restricted quadratic splines (knots at 10th, 50th and 90th percentiles) of the individual scores. The reference value was set at 10th percentile of each principal component (dashed line). Statistical models were adjusted for age (years, splines), sex, education (< secondary education, ≥ secondary education), smoking status (never, former, current), urine cotinine (< 34, 34–500, ≥ 500 ng/mL), cumulative smoking (0, < 12, ≥ 12 packages year), alcohol consumption (g/day), diabetes status (no, yes) and estimated glomerular filtration rate (mL/min per 1.73 m²).

including Sb-Cd-Cr-V. Similarly, PC2 scores largely reflected essential metals including Co-Cu-Mo-Zn (Fig. S4). In linear regression models including the individual PC scores as independent variables, the GMR (95% CI) of GSSG/GSH comparing the 80th vs 20th percentiles of PC1 distribution was 1.35 (1.20–1.51) (Table S4). The corresponding GMR (95% CI) of GSSG/GSH and MDA comparing the 80th vs 20th percentiles of PC2 distribution were 0.84 (0.75–0.94) and 1.08 (1.02–1.15), respectively (Table S4). Fig. 2 shows the corresponding flexible non-linear dose responses and associated confidence intervals. We found no significant associations between metal PC and 8-oxo-dG levels.

3.5. Sensitivity analysis

We conducted a number of sensitivity analyses. Selenium (Se) is a metalloid with a key role as cofactor of GPx, the enzyme that oxidizes reduced glutathione (GSH) into oxidized glutathione (GSSG). Since plasma Se has been previously associated with oxidative stress biomarkers in our study population (Galan-Chilet et al., 2014), we additionally adjusted the single-metal models for plasma Se with essentially identical results (data not shown). Other studies reported that the

extent of proteinuria might alter metal ion content in urine (Khan et al., 2015). We thus further adjusted the single-metal models for urine albumin to creatinine ratio with unchanged results (data not shown). Plasma Cu and Zn are better-established biomarkers of exposure compared to urine (Nordberg et al., 2007), as Cu excretion is very low and Zn is mainly excreted through feces, not urine. Consequently we conducted additional analyses using plasma Cu and Zn instead of urine biomarkers both in the single-metal and multi-metal models (see Tables S2 and S3). The GMR (95%CI) comparing the 80th to the 20th percentiles of plasma Cu distribution were 1.20 (1.09, 1.33) for GSSG/GSH and 0.76 (0.69, 0.85) for MDA. The corresponding GMR (95%CI) in plasma zinc models were 1.22 (1.02, 1.46) for GSSG/GSH, and 1.42 (1.30, 1.54) for 8-oxo-dG (see Tables S2 and S3). Because increased urinary Zn and decreased serum Zn levels have been observed in individuals with diabetes (Hagglof et al., 1983), we evaluated the dose-response of plasma and urine Zn with oxidative stress biomarkers in the subset of participants without diabetes with consistent results compared to the overall (see Fig. S5). Since smoking is a source of metals and it contains multiple chemicals which could be potentially associated with oxidative stress (Centers for Disease Control and

Prevention, 2010), potential residual confounding by smoking is a concern. In addition to the extensive adjustment for tobacco exposure-related variables, we also conducted restricted analysis among never smokers. The point estimates for the associations of Cr and V with GSSG/GSH and Zn with MDA and 8-oxo-dG were similar in never smokers although the confidence intervals became wider, as expected given the smaller sample size (N = 671) (Table S5). Finally, urban or rural residency might be an important source of variation for metal exposures as natural and anthropogenic sources could be different (Davis et al., 2009). Accordingly, we repeated PCA by subgroups residing in rural and urban areas in separate analyses, obtaining similar PCs (data not shown).

4. Discussion

Increased urine Ba, Cr, Cd, Mo, V and Zn were associated with increased oxidative stress biomarkers at the levels seen in a general population from Spain. The associations, however, seemed to be driven by non-linear relations of Cr with GSSG/GSH, and Zn with MDA and 8-oxo-dG models. In PCA, scores reflecting essential metal concentrations (i.e. PC2) were inversely associated with GSSG/GSH but positively associated with MDA, whereas scores mostly reflecting non-essential metal concentrations (i.e. PC1) were positively associated with GSSG/GSH.

Biomarkers measured in urine are commonly used to assess metal exposure and internal dose as they integrate multiple exposure sources, including air, water and food (Aitio et al., 2007). The metals considered in the present study have relatively short half-lives in urine, and are thus, mostly considered reliable biomarkers of recent exposure (Nigra et al., 2016). Urine Cd, however, is generally accepted as a biomarker of long-term exposure, with half-life component of 10–30 years (Nordberg et al., 2007). Nevertheless, under chronic conditions of exposure, even urine metal biomarkers with relatively short half-lives can also serve as biomarkers of long-term exposure (Navas-Acien et al., 2009).

Urine concentrations of different metals might be related with each other due to common environmental sources and similarities in metabolism. While in descriptive analysis all pair-wise correlations between metals were positive, in unsupervised principal component analysis non-essential and essential metals showed a trend towards clustering separately in PC1 and PC2, respectively, after accounting for urine creatinine and renal function. PC1 (non-essential metals) could be reflecting exposure from tobacco smoke, which is known to contain several chemicals including Sb, Ba, Cd, Cr or V, and others (Bernhard et al., 2005; Richter et al., 2009). In post-hoc analysis, however, the pair-wise correlation between metals among never smokers with undetectable cotinine levels was similar to the overall (data not shown). Thus PC1, may also reflect common exposure sources other than tobacco smoke, including dietary sources. Alternatively, PCs could also be pointing to biological interactions and common metabolic pathways for non-essential and essential metals, including competitive interactions during absorption and transport (Flanagan et al., 1984). In our data, younger participants, men and participants exposed to tobacco smoke had higher levels of PC1, while older participants showed higher PC2 levels (Table S6). Occupational sources of non-essential metals cannot, thus, be discarded.

We found several associations between urine metal levels with markers of oxidative stress that are commonly used in population-based studies (Ilyasova et al., 2012). Available epidemiological studies evaluating potential redox effects of metals have been generally small (Ellis et al., 2012; Engström et al., 2010; Franken et al., 2017; Wong et al., 2005; Pesch et al., 2015) or limited by the lack of biomarkers of metal exposure (Garcon et al., 2004; Aranda et al., 2017; Gromadzińska et al., 1996; Ambreen et al., 2014; Zhang et al., 2016). Briefly, higher concentrations of oxidative stress markers have been related to occupational metal exposure (Garcon et al., 2004; Ambreen et al., 2014), increased metals intake from seafood (Aranda et al., 2017), and metal present in air pollution particles (Zhang et al., 2016). In small samples

of healthy volunteers (N = 178) (Ellis et al., 2012) and pregnant women (N = 212) (Engström et al., 2010) urine Cd was associated with urine 8-oxo-dG. In 600 teenagers from Belgium (Franken et al., 2017), 142 school children from Taiwan (Wong et al., 2005) and 238 occupationally exposed welders (Pesch et al., 2015), increased urinary Cr was associated with increased 8-oxo-dG biomarkers. Large population-based studies using both metals and oxidative stress biomarkers are needed to confirm our main findings in other general populations.

A number of mechanistic studies support the role of non-essential metals on oxidative stress. In particular, Cd can interfere with the activity of cysteine-rich antioxidant proteins such as GSH (Ballatori, 1994), metallothioneins (Jin et al., 1998) and Cu/Zn SOD (Ghosh et al., 2013). Cd has also been shown to increase MDA and lipid peroxidation and decrease GSH in some mice tissues (Müller, 1986; Djukić-Cosić et al., 2008) and to increase MDA and GSH-Px activity *In vitro* (Yang et al., 2003). However, in our data, the associations of cadmium with MDA and 8-oxo-dG, were substantially attenuated after adjustment for Zn. Interestingly, in experimental settings, Cd exposure changed the expression of Zn transporters (Nemmiche & Guiraud, 2016). A protective role of Zn on Cd-related toxicity has been previously reported (Jacquillet et al., 2006; Lin et al., 2014). These evidences support a biological connection in Cd and Zn metabolism and transport.

Results from our multi-metal analyses supported that elevated Cr exposure might drive the observed positive associations for GSSG/GSH, overall and also in stratified analysis among never smokers. However, in our data we could not separate Cr from V, another metal with a reported ability to generate ROS (Shi et al., 1996), given their almost complete correlation. V and Cr are metals that naturally occur in minerals and in fossil fuel deposits (ATSDR, 2012b; ATSDR, 2012c). Co-exposure to both metals in the population may be possible following exposure from fuel combustion, contaminated sources, such as in mining, welding and steel alloy industry-related settings, and hip joint implant material releases (ATSDR, 2012b; ATSDR, 2012c; Dunstan et al., 2005; Reis et al., 2014). The sources of exposure in our study population, however, are unclear, and we do not have a good explanation for the elevated correlation of Cr and V in our data. *In vitro* and *In vivo* studies have demonstrated that Cr (VI), induces oxidative stress by enhancing the production of ROS (Valko et al., 2005; Travacio et al., 2000; Bagchi et al., 2001). In other *in vitro* studies, glutathione (GSH) reduced Cr (VI) to Cr (III) leading to a GSH depletion (Wiegand et al., 1984). Alternatively, the essentiality of Cr (III) and V and potential use as supplements, however, have been discussed given some experimental studies and small clinical trials suggesting that Cr and V supplementation may decrease oxidative stress (Preet et al., 2005; Xie et al., 2014; Jamilian et al., 2016; Anderson et al., 2001). The role of dietary Cr and V, including supplements, in preventing chronic diseases such as diabetes and cardiovascular disease is a source of ongoing debate (Nigra et al., 2016).

With respect to essential metals, the role of Zn and Cu in the antioxidant defense system has been generally accepted (Prasad et al., 2004). However, both Zn and Cu deficiency and excess can cause oxidative damage (Valko et al., 2005; Nordberg et al., 2007). In particular, *In vivo* and *In vitro* studies found that high Zn intake is related with pro-inflammatory processes by reducing Cu absorption (Marreiro et al., 2017). Other studies support that changes in the ratio Cu/Zn can participate in the oxidative stress regulation, although the results are mixed (Mezzetti et al., 1998; Duan et al., 2010). Interestingly, in our sensitivity analysis, plasma Cu levels (median 95.3 µg/dL) were inversely associated with 8-oxo-dG after adjusting for plasma Zn (median 79.6 µg/dL). Alternatively, plasma Cu was positively associated with GSSG/GSH, independently of Cr. Indeed, it is known that redox active essential metals like Fe, Cu, or Co undergo redox cycling reactions and can produce reactive radicals (Valko et al., 2005). Different biomarkers of oxidative stress may be related in different ways with different essential metals.

In addition, although our results were compatible with a positive

association of urine Zn above ~100 µg/g (25th percentile) with MDA and 8-oxo-dG, a number of clinical trials evaluating the efficacy of essential metal supplementation in oxidative stress support an inverse association with oxidative stress. However, most available trials have focused on populations with likely Zn deficiency or elevated Zn demand (Monget et al., 1996; Faure et al., 1995; Barbosa et al., 2009; de Ribeiro et al., 2016), or Zn supplementation was combined with other vitamins and minerals (Monget et al., 1996; Barbosa et al., 2009; Galan et al., 1997). Our findings of Zn being related to increased MDA and 8-oxo-dG levels in a general population from Spain were confirmed in sensitivity analysis among participants without diabetes, and do not support the beneficial effects of Zn supplementation, especially in zinc-repleted populations. Overall, our PCA results also support the notion that excessive exposure to essential metals levels may increase oxidative stress in some biological processes.

Our study has several limitations in addition to the cross-sectional design, which does not allow us to establish the temporality of the observed associations. First, urinary levels of GSSG/GSH and 8-oxo-dG may be confounded by inter-individual variability in antioxidant and DNA repair capacity, which may not be related with enhanced oxidative stress and damage (Ilyasova et al., 2012). For instance, Cd inhibited, and down-regulated the expression of the human 8-oxoguanine-DNA glycosylase-1 (OGG1), which removes 8-oxoG adducts from DNA (Youn et al., 2005). Second, variations in renal function might influence both metal and oxidative stress concentrations in urine. However, we found statistically significant associations of individual metals and metal mixtures with oxidative stress biomarkers in models adjusted for eGFR and diabetes. Finally, total urine Cr levels do not provide information of individual Cr compounds (as Cr [III] or Cr [VI]). Detailed analyses with different Cr species levels are needed to better understand the association of Cr with the different oxidative stress biomarkers. Strengths of this study include the rigorous laboratory methods with extensive quality control, the reliability of urine as biomarker of exposure for several of the metals studied and the availability of plasma Cu and Zn levels, the novel application of methods to evaluate the joint role of metals in oxidative stress, and the representative sampling that allows the generalization of our findings to a general population from Spain.

5. Conclusions

In conclusion, increased urine metals levels, specifically Ba, Cd, Cr, Mo, V and Zn, were associated with increased oxidative stress measures in a representative sample of a general population from Spain. Our findings, thus, support the hypothesis that oxidative damage may partly explain the associations of elevated metals with adverse health outcomes. Further research is needed to confirm potential different roles of essential and non-essential metals in oxidative stress in different cellular compartments at different exposure levels.

Declarations of interest

None.

Acknowledgements

This work was supported by the Strategic Action for Research in Health Sciences [CP12/03080, PI10/0082, PI13/01848, PI07/0497 and PI11/00726]; GRUPOS 03/101, PROMETEO/2009/029 and 2005/027, AMP07/075 and ACOMP/2013/039 from the Valencia Government; GRS/279/A/08 from Castilla-Leon Government; European Network of Excellence Ingenious Hypercare (EPSS-037093) from the European Commission; Retics (PREDIMED RD06/0045/0006); CIBER Fisiopatología Obesidad y Nutrición (CIBERobn) [CIBER-02-08-2009, CB06/03 and CB12/03/30016] and CIBER de Diabetes y Enfermedades Metabólicas Relacionadas (CIBERDEM CB07/0/018). The Strategic

Action for Research in Health sciences, Retics, CIBEROBN and CIBERDEM are initiatives from Carlos III Health Institute Madrid and the Spanish Ministry of Economy and Competitiveness and co-funded with European Funds for Regional Development (FEDER). The authors declare they have no actual or potential competing financial interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envint.2018.11.055>.

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