Supplementary Materials

Exposure to a mixture of four metals and associations with urinary oxidative stress biomarkers in Uruguayan adolescents

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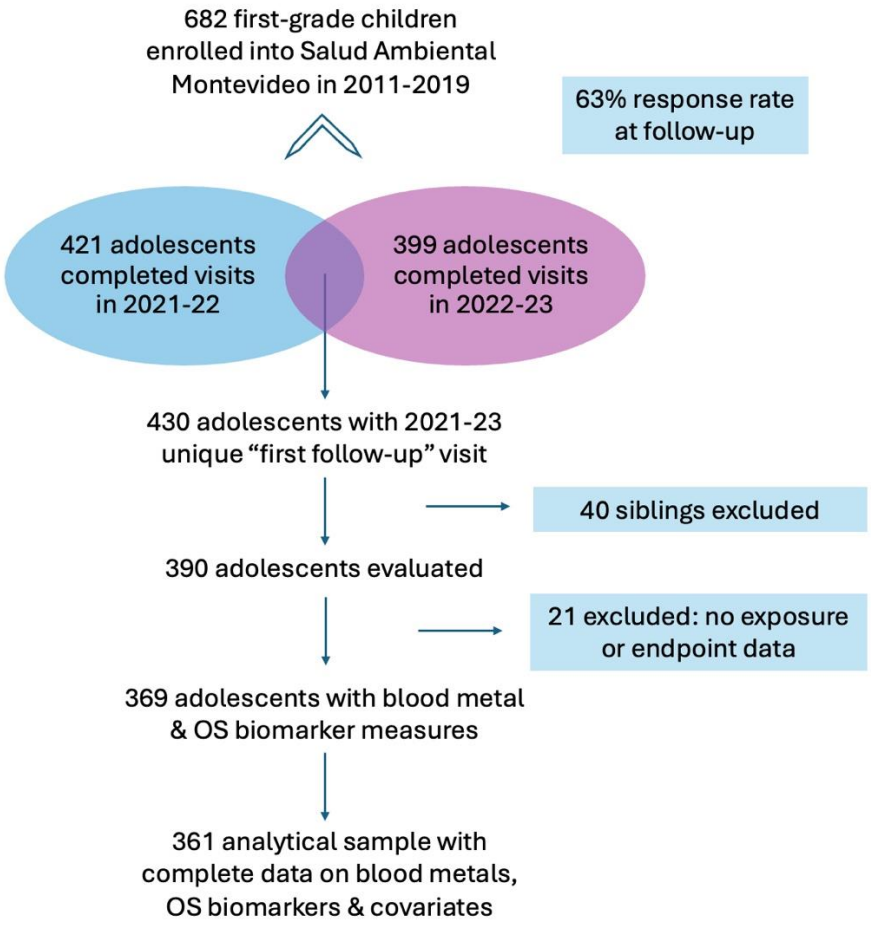


Supplementary Figure 1. Participant flow to establish study sample on metals and oxidative stress markers among Uruguayan adolescents.

Supplementary Table 1. Scoring criteria for Physical Activity Questionnaire for Older Children (PAQ-C)[1], for Uruguayan children

Question 1: Recent sports participation		
Sports/activities assessed	***Score assigned***	***Composite Question 1 score calculated by finding the mean of all activities.***
Jumping	1 = No participation	
Walking (for exercise)	2 = 1-2 times per week	
Biking	3 = 3-4 times per week	
Running	4 = 5-6 times per week	
Aerobics	5 = 7 times or more	
Swimming		
Dancing		
Soccer		
Volleyball		
Basketball		
Skating		
Horse Riding		
Other		
Question 2: Physical activity directly after school in last 7 days		
Score assigned	3 = 3-4 times per week	
1 = No participation	4 = 5-6 times per week	
2 = 1-2 times per week	5 = 7 times or more	
Question 3: Physical activity in the afternoon in the last 7 days		
Score assigned	3 = 3-4 times per week	
1 = No participation	4 = 5-6 times per week	
2 = 1-2 times per week	5 = 7 times or more	
Question 4: Physical activity during the previous weekend		
Score assigned	3 = 3-4 times per week	
1 = No participation	4 = 5-6 times per week	
2 = 1-2 times per week	5 = 7 times or more	
Question 5: Overall Physical Activity in during the last 7 days		
Score assigned	3 = 3-4 times per week	
1 = No participation	4 = 5-6 times per week	
2 = 1-2 times per week	5 = 7 times or more	
Calculation of final score.		
Final PA score calculated by finding the mean of all scores to provide overall physical activity, with higher scores indicating greater activity levels.		

[1]Adapted from Kent C. Kowalski, P. C., Rachel Donen. (2004). *The Physical Activity Questionnaire for Older Children (PAQ-C) and Adolescents (PAQ-A) Manual*

Blood collection and analysis
Fasting venous blood was collected into a lavender-top tube (~2 mL) containing an EDTA anticoagulant. The blood tubes were pre-screened and certified by the analyzing laboratory for trace element analysis. The tube was inverted 4-6 times to mix the blood with the anticoagulant, then stored in a refrigerator at 4°C. Samples were shipped monthly to the Trace Elements Section of the Laboratory of Inorganic and Nuclear Chemistry at the New York State Department of Health's Wadsworth Center. The Wadsworth Center is one of three Human Health Exposure Analysis Resource (HHEAR) laboratories designated by the NIH to support target analyses of biospecimens for environmental contaminants.

The samples were analyzed with a well-validated "direct blood dilution" method based on Inductively Coupled Plasma Tandem Mass Spectrometry (ICP-MS/MS) (Thermo Scientific™ iCAP™ TQ) and optimized for human biomonitoring studies [1] (Supplemental Table 1). The ICP-MS/MS instrument was calibrated with standards traceable to the National Institute of Standards and Technology (NIST). Three concentrations of internal blood-based quality control (QC) pools were included in each analytical run to ensure repeatability of conditions for each element measured. Method accuracy (% bias) and imprecision (±95% CI) for blood As, Cd, Hg and Pb were established by analyzing NIST Standard Reference Material (SRM) 955c Toxic Metals in Caprine Blood and 955d Metals and Metabolites in Frozen Human Blood [Supplementary Tables 2-5].

In addition, method performance for blood As was monitored via successful participation in four External Quality Assessment Schemes (EQAS): Centre de Toxicologie du Québec's Interlaboratory Programs (QMEQAS); The UK TEQAS; The College of American Pathologists (CAP) Trace Metals, Whole Blood; and the New York State Department of Health's Biomonitoring Proficiency Testing Program for Trace Elements (NYSDOH PT). As a designated HHEAR lab hub, the Trace Elements Laboratory at Wadsworth follows all HHEAR (and CHEAR) QC protocols [2]. The Wadsworth HHEAR laboratory is also fully accredited under the Clinical Laboratory Improvement Amendments (CLIA). The method Limit of Detection (LOD) was established for each element by analyzing base (i.e., unspiked) blood pools at least 7 independent runs and multiplying the SD by k=3 following the IUPAC guidelines for a single laboratory method validation [3]. None of the participants had values below the LOD; 100% values were detectable. For the statistical analysis, the two measures of blood As were averaged for those participants with both measures collected.

Supplementary Table 2. ICP-MS/MS method validation data for blood arsenic using NIST Standard Reference Materials

Arsenic in Whole Blood SRM	Certified value ±U[a], µg/L As	NYS found value ±95% CI[b], µg/L As	% Bias
NIST 955c Toxic Metals in Caprine Blood			
NIST 955c Level 1	<5[c]	0.110 ±0.009	-
NIST 955c Level 2	21.66 ±0.73	20.22 ±0.12	-6.6
NIST 955c Level 3	52.7 ±1.1	51.6 ±0.3	-2.1
NIST 955c Level 4	78.8 ±4.9	77.8 ±0.7	-1.3
NIST 955d Metals and Metabolites in Frozen Human Blood			

NIST 955d Level 1	5.31 ±0.76	4.88 ±0.14	-8.1
NIST 955d Level 2	277.5 ±4.8	271 ±3.2	-2.3
NIST 955d Level 3	774 ±13	757 ±0.32	-2.2

[a]NIST uncertainty expressed as the Guide to Measurement Uncertainty (GUM) expanded uncertainty, U, the 95% confidence level, reflecting the combined effects of measurement uncertainty, blanks, and any systematic differences between techniques when more than one method was used to assign a value.

[b]Method imprecision estimated from found values and calculated as the 95% confidence interval from independent measurements (n=5) obtained during a June 2022 method validation study that was performed immediately before the analysis of project samples.

[c]NIST information value.

Supplementary Table 3. ICP-MS/MS method validation data for blood cadmium using NIST Standard Reference Materials

Cadmium in Whole Blood SRM	Certified value ±U[a], µg/L Cd	NYS found value ±95% CI[b], µg/L Cd	% Bias
NIST 955c Toxic Metals in Caprine Blood			
NIST 955c Level 1	0.0317 ±0.0062	<0.072	-
NIST 955c Level 2	2.16 ±0.22	2.14 ±0.03	-0.9
NIST 955c Level 3	5.201 ±0.038	5.17 ±0.05	-0.6
NIST 955c Level 4	10.26 ±0.11	10.15 ±0.12	-1.1
NIST 955d Metals and Metabolites in Frozen Human Blood			
NIST 955d Level 1	0.326 ±0.010	0.33 ±0.008	-0.3
NIST 955d Level 2	5.343 ±0.082	5.23 ±0.072	-2.1
NIST 955d Level 3	10.50 ±0.11	10.41 ±0.12	-0.9

[a]NIST uncertainty expressed as the Guide to Measurement Uncertainty (GUM) expanded uncertainty, U, the 95% confidence level, reflecting the combined effects of measurement uncertainty, blanks, and any systematic differences between techniques when more than one method was used to assign a value.

[b]Method imprecision estimated from found values and calculated as the 95% confidence interval from independent measurements (n=5) obtained during a June 2022 method validation study that was performed immediately before the analysis of project samples.

Supplementary Table 4. ICP-MS/MS method validation data for blood lead using NIST Standard Reference Materials

Lead in Whole Blood SRM	Certified value ±U[a], µg/dL Pb	NYS found value ±95% CI[b], µg/dL Pb	% Bias
NIST 955c Toxic Metals in Caprine Blood			
NIST 955c Level 1	0.424 ±0.011	0.416 ±0.010	-1.9
NIST 955c Level 2	13.950 ±0.080	13.71 ±0.11	-1.7
NIST 955c Level 3	27.76 ±0.16	27.33 ±0.18	-1.5
NIST 955c Level 4	45.53 ±0.27	44.92 ±0.49	-1.3
NIST 955d Metals and Metabolites in Frozen Human Blood			
NIST 955d Level 1	1.48 ±0.026	1.43 ±0.02	-3.4
NIST 955d Level 2	4.947 ±0.085	4.80 ±0.02	-2.9
NIST 955d Level 3	42.13 ±0.63	41.30 ±0.34	-2.0

[a]NIST uncertainty expressed as the Guide to Measurement Uncertainty (GUM) expanded uncertainty, U, the 95% confidence level, reflecting the combined effects of measurement uncertainty, blanks, and any systematic differences between techniques when more than one method was used to assign a value.

[b]Method imprecision estimated from found values and calculated as the 95% confidence interval from independent measurements (n=5) obtained during a June 2022 method validation study that was performed immediately before the analysis of project samples.

Supplementary Table 5. ICP-MS/MS method validation data for blood mercury using NIST Standard Reference Materials

Mercury in Whole Blood SRM	Certified value ±U[a], µg/L Hg	NYS found value ±95% CI[b], µg/L Hg	% Bias
NIST 955c Toxic Metals in Caprine Blood			
NIST 955c Level 1	0.017 ±0.011	<0.14	-
NIST 955c Level 2	5.42 ±0.66	5.22 ±0.34	-3.7
NIST 955c Level 3	17.8 ±1.6	18.2 ±0.21	-2.1
NIST 955c Level 4	35.4 ±0.20	33.6 ±0.59	-5.1
NIST 955d Metals and Metabolites in Frozen Human Blood			
NIST 955d Level 1	1.373 ±0.081	1.42 ±0.08	3.4
NIST 955d Level 2	6.83 ±0.33	6.59 ±0.31	-3.5
NIST 955d Level 3	55.3 ±1.6	53.4 ±2.0	-3.4

[a]NIST uncertainty expressed as the Guide to Measurement Uncertainty (GUM) expanded uncertainty, U, the 95% confidence level, reflecting the combined effects of measurement uncertainty, blanks, and any systematic differences between techniques when more than one method was used to assign a value.

[b]Method imprecision estimated from found values and calculated as the 95% confidence interval from independent measurements (n=5) obtained during a June 2022 method validation study that was performed immediately before the analysis of project samples.

Measurement of oxidative stress biomarkers

F_2-isoprostanes

For purification via solid phase extraction (SPE), samples were thawed at room temperature for 20min, then 1mL was diluted with 2.5mL acetate buffer (1M, pH 4.0).

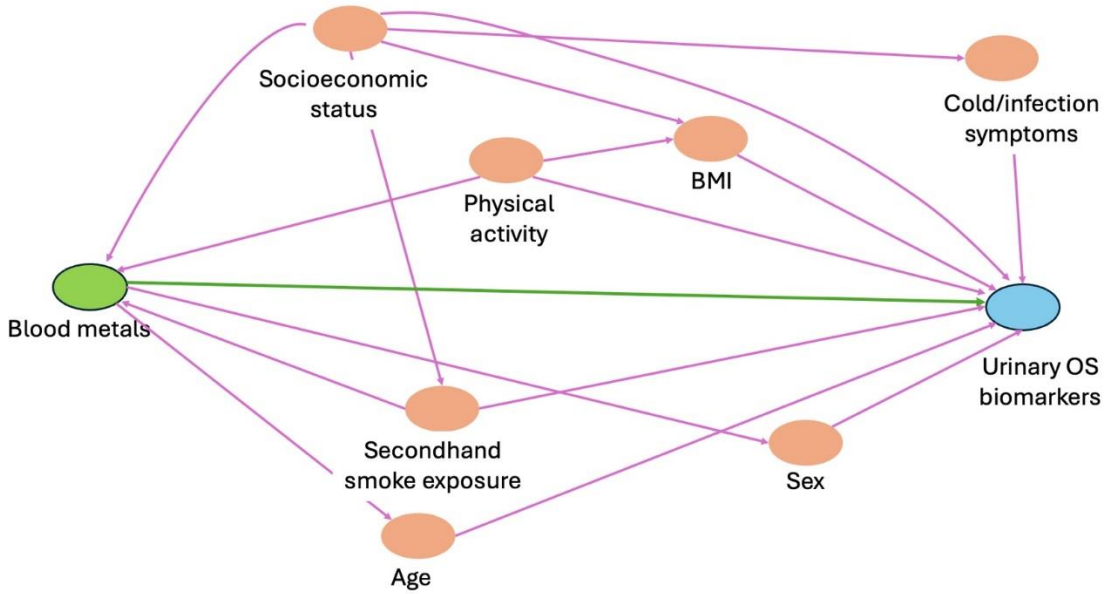
SPE C18 (500mg) affinity cartridges (Cayman Chemicals, MI, USA), attached to a vacuum manifold, were activated with 5mL methanol, and rinsed with 5mL ultrapure water. Diluted samples were run through the columns followed by 5mL of ultrapure water. Columns were dried and F_2-IsoP was eluted with 2mL methanol into glass vials. Vials were stored at -80ºC until ELISA analysis.

F_2-IsoP was quantified using a competitive ELISA assay (Cayman Chemicals, MI, USA). Methanolic SPE extracts were evaporated under a stream of nitrogen gas at 37ºC for ~40min and replaced with 1mL ELISA buffer. A 32-fold sample dilution in ELISA buffer was prepared in 1.7mL microfuge tubes. The F_2-IsoP standard was also prepared in the ELISA buffer (ranging 500 – 0.8 pg/mL). The wells of mouse anti-rabbit IgG coated plate were filled with standards, samples, 8-isoprostane-acetylcholine esterase (AChE) conjugate (8-isoprostane tracer), and antiserum. Control wells contained non-specific binding ELISA buffer and tracer onlyl;

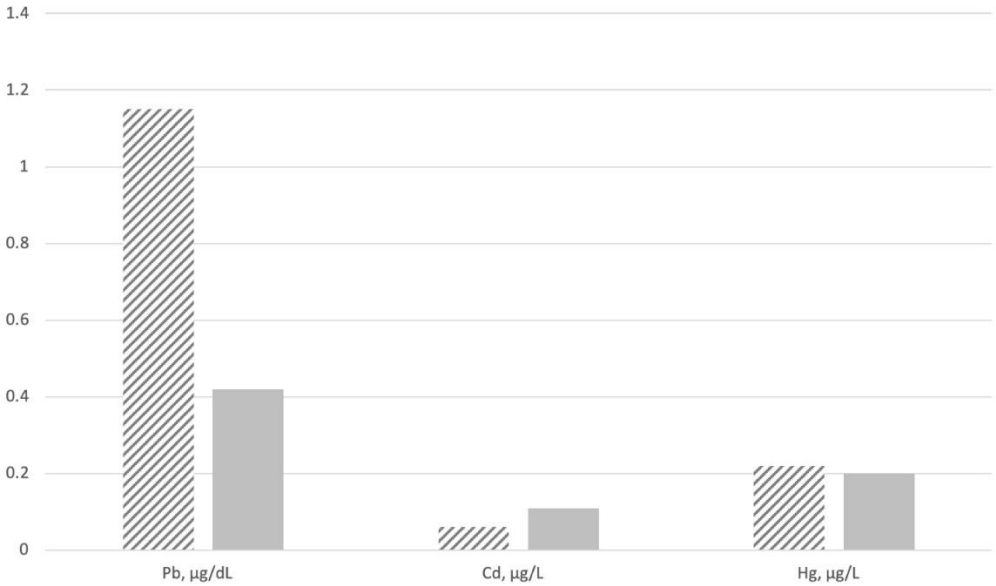
maximum binding wells contained ELISA buffer, tracer, and antiserum. Standard and samples were run in duplicate. The plate was sealed with plastic film and incubated at 4ºC for 18hrs. The next day, the plate was washed five times to remove unbound reagents. The plate was tapped onto a paper towel and 200µL Ellman's reagent was added. The plate was sealed with plastic film, covered with aluminum foil, and placed on an orbital shaker set to 300rpm for 90min at room temperature. A plate reader (BioTek Synergy HTX Multi-mode Plate Reader, Agilent Technology Inc., Winooski, Vermont) set to 412nm and 25ºC was used to read the absorbance. The data was plotted and reduced using Cayman's Competitive ELISA Double Calculations Excel workbook. The concentration of the tracer is held constant; the intensity of the color is proportional to the amount of tracer and inversely proportional to the amount of F_2-IsoP.

8-OHdG

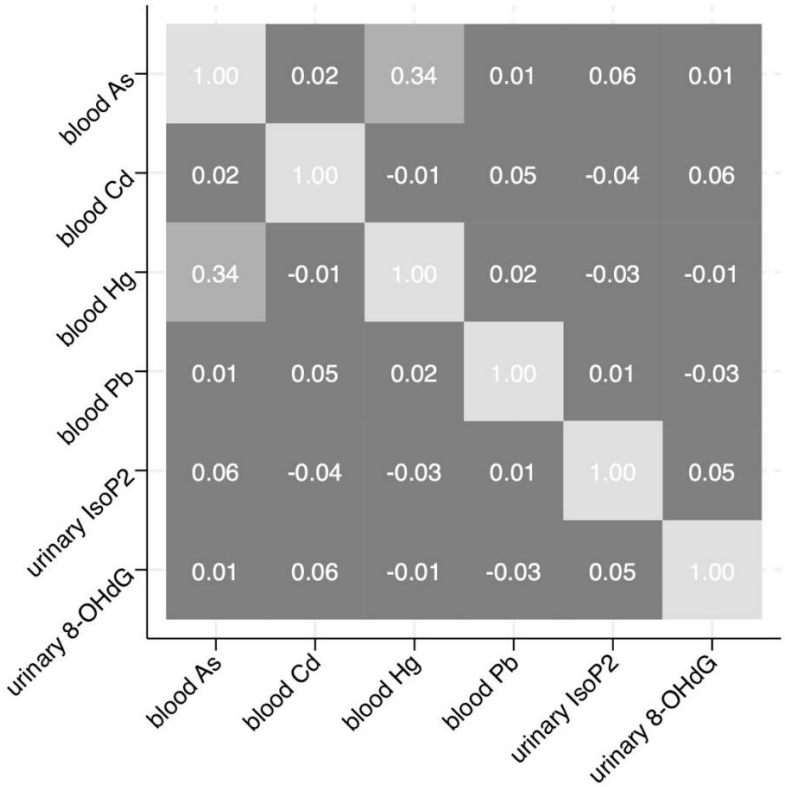
The samples were shipped on dry ice to Creative Proteomics (Shirley, NY, USA) and analyzed with an in-house ELISA kit for the detection of 8-OHdG, utilizing a standard range of 1-320 pg/mL and Thermo Scientific™ Multiskan™ FC Microplate Photometer. Samples were analyzed in triplicate. The standard (50µL each) was added to the designated well. Testing samples were diluted fivefold in the wells by adding 10µL of sample followed by 40µL sample dilution buffer. HRP-Conjugate reagent (100µL) was added to all, except the blank well. The plate was sealed with plastic film and incubated for 60min at 37°C. The wells were washed five times with the washing solution, dried by swing, and patted on paper towel. Color was developed in the dark for 15min at 37°C by adding chromogen solutions A and B to each well. The reaction was stopped with 50µL stop solution and absorbance was read at 450nm within 15min. The blank was subtracted from the results. Each 8-OHdG oncentration was estimated from a XY-scatter plot and regression relating absorbance the the standard concentration.



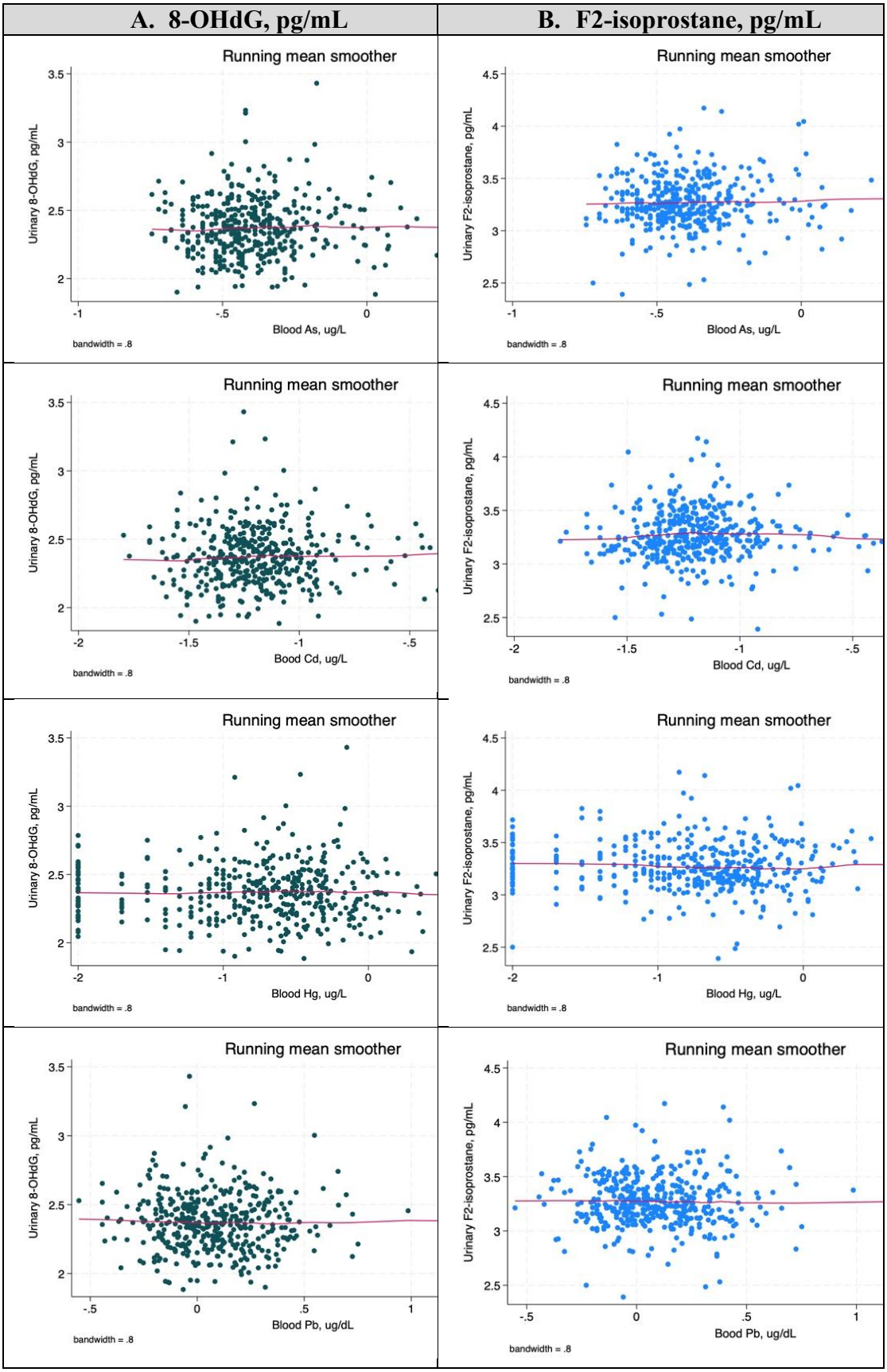
Supplementary Figure 2. Directed Acyclic Graph to select covariates for the study on metals and oxidative stress in Uruguayan adolescents.



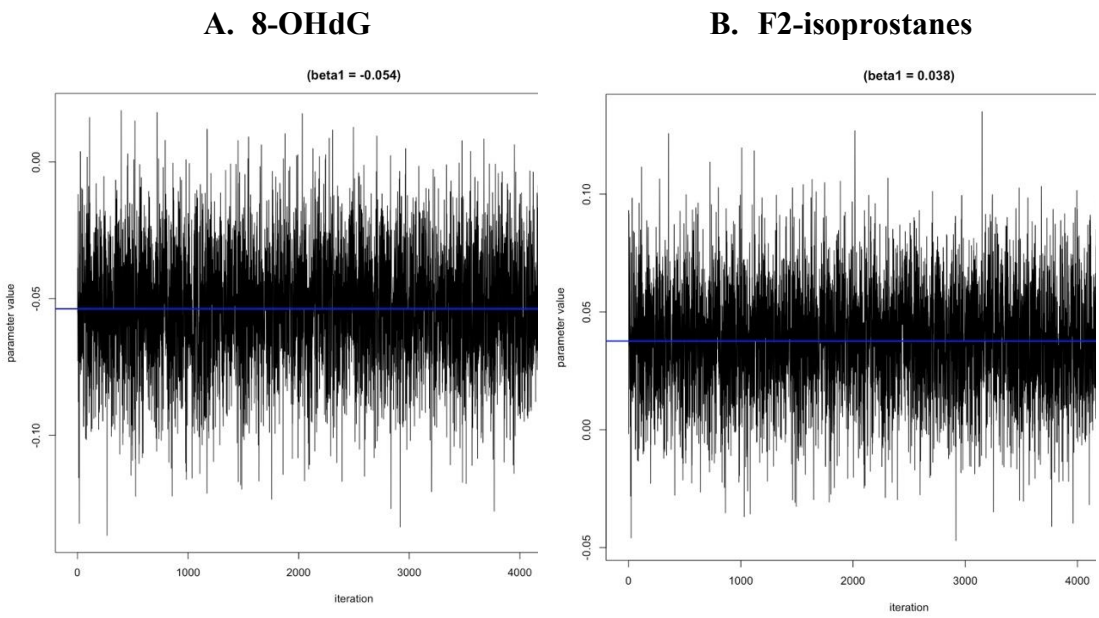
Supplementary Figure 3. Median blood metal levels among 361 MOX study participants with a mean age of 12 years (gray, cross-hatched bars), and children aged 10-14 years participating in the 2017 to March 2020 National Health and Nutrition Examination Survey (light gray bars).



Supplementary Figure 4. Correlation matrix for blood metal concentrations and urinary oxidative stress biomarkers (adjusted for specific gravity) among 361 adolescents from Montevideo, Uruguay.

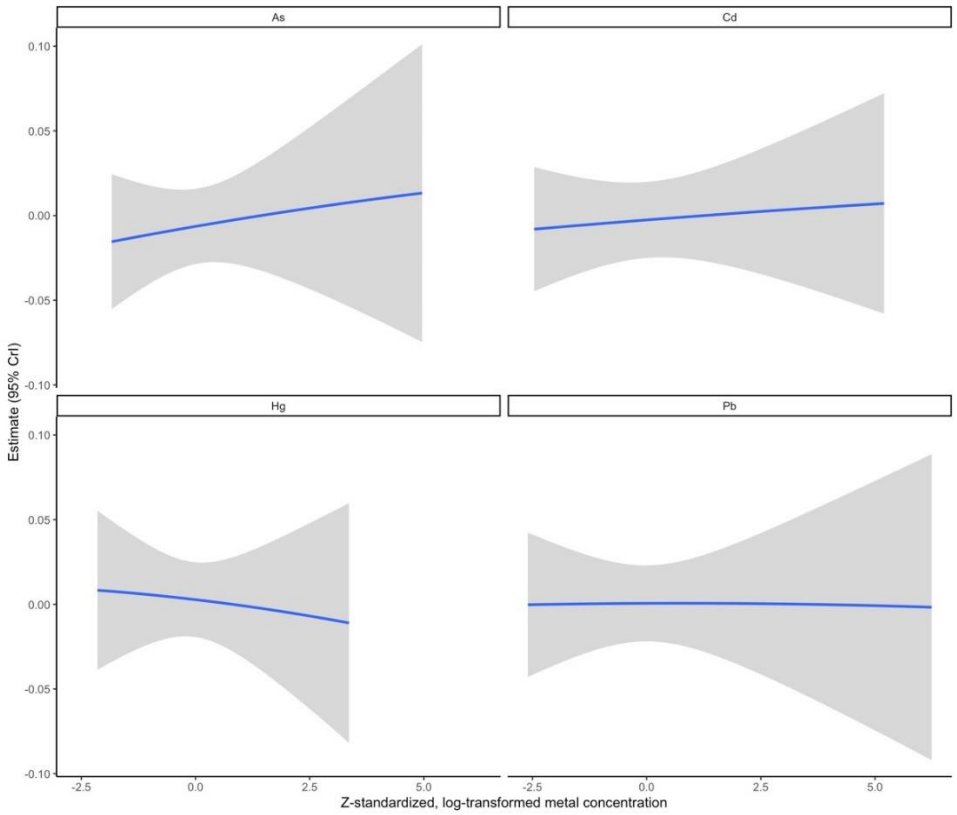


Supplementary Figure 5. Lowess curves to illustrate the relationship between blood metal concentrations (log10-transformed) and urinary OS biomarker levels (adjusted for specific gravity of urine, then log10-transformed).

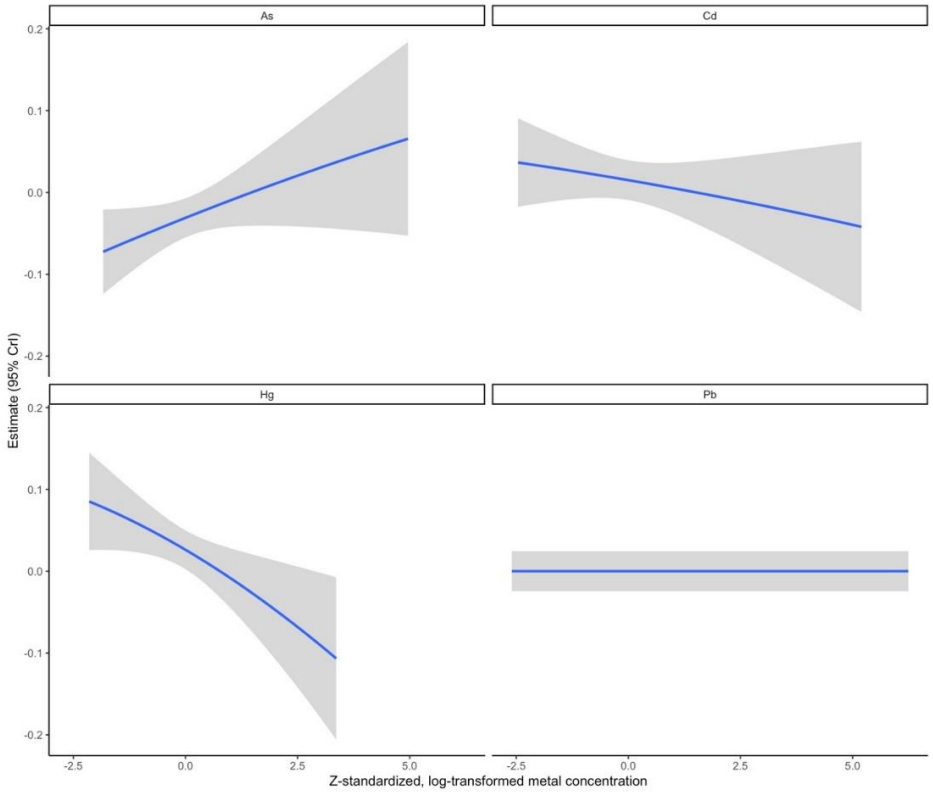


Supplementary Figure 6. Trace plots for BKMR models relating a metal mixture (blood arsenic, cadmium, mercury and lead) and urinary biomarkers of oxidative stress in Uruguayan adolescents. Model was run with 10,000 iterations.

A. 8-OHdG

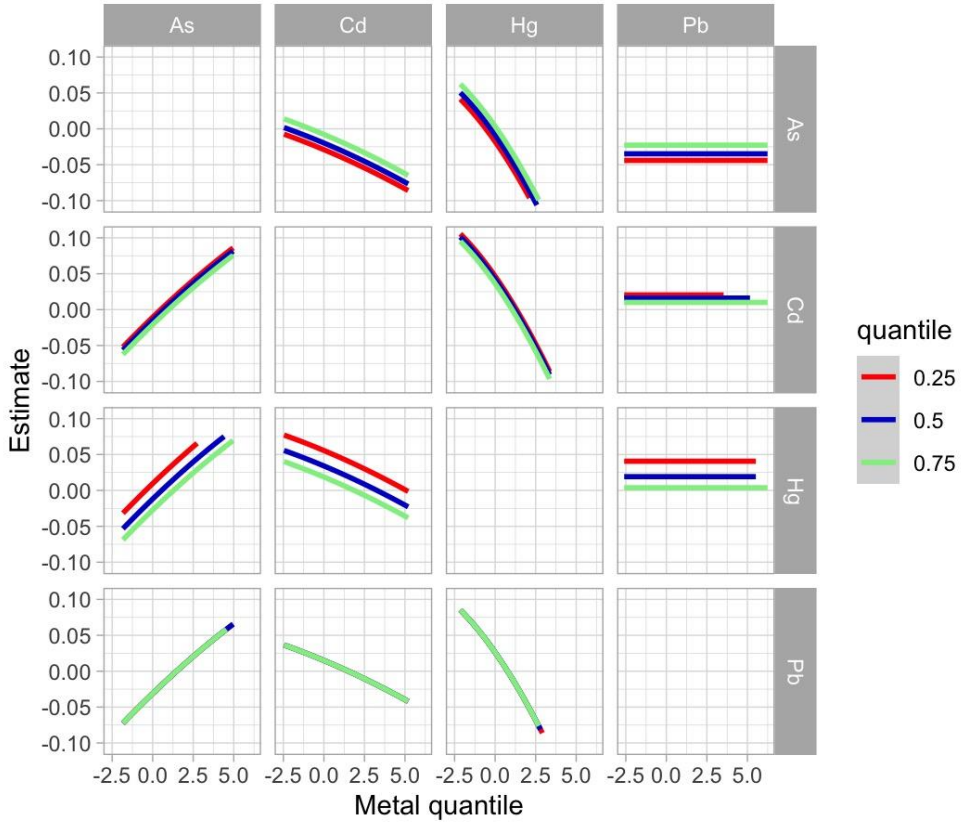


B. F2-isoprostanes

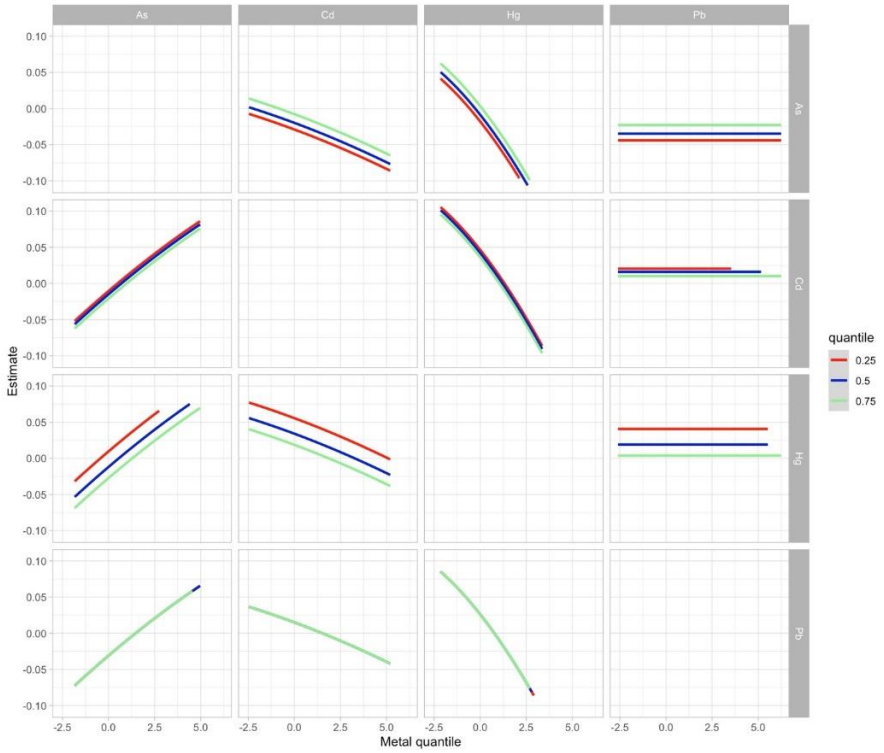


Supplementary Figure 7. Exposure-response plots for the association between each metal and OS biomarkers, when other trace metals are held at their median values and covariates are held constant. The models were adjusted for age, sex, BMI, SES, illness symptoms, physical activity score and secondhand smoke exposure.

A. 8-OHdG

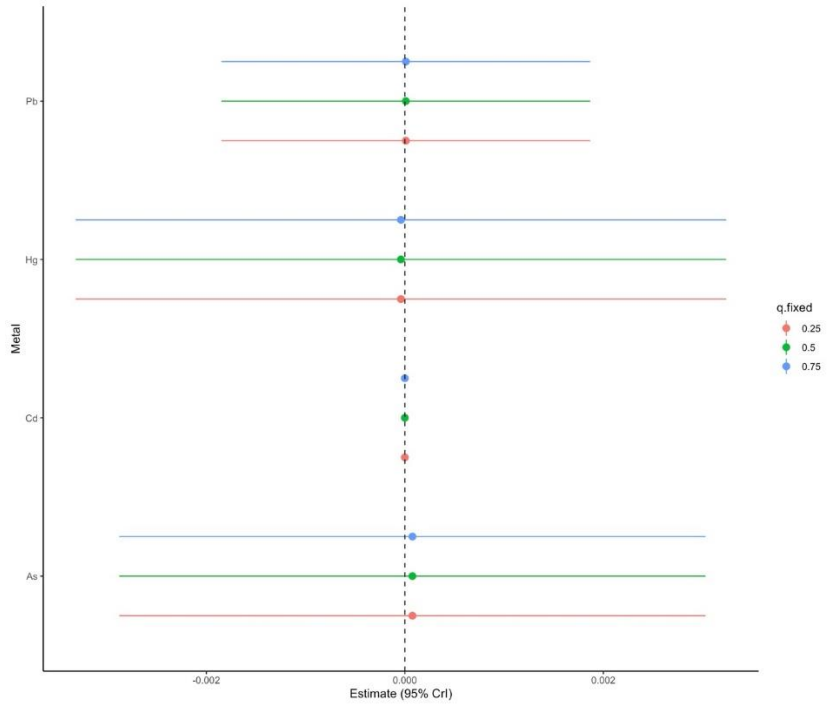


B. F2-isoprostanes

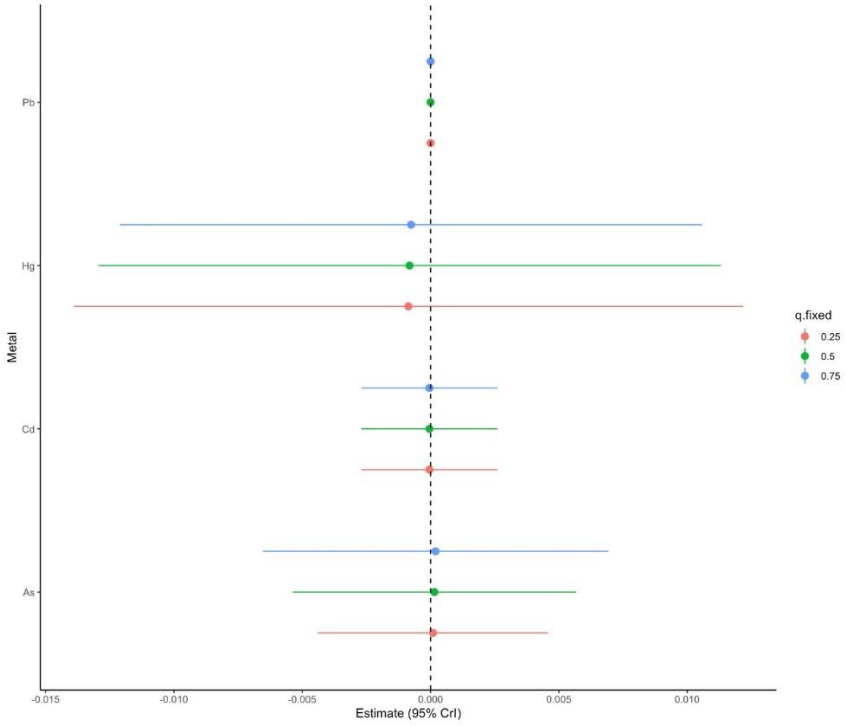


Supplementary Figure 8. Bivariate exposure-response plots for metal pairs, when the second metal is fixed at the 25th, 50th and 75th percentile of its distribution and when covariates are held constant. The models were adjusted for age, sex, BMI, SES, illness symptoms, physical activity score and secondhand smoke exposure.

A. 8-OHdG



B. F2-isoprostanes



Supplementary Figure 9. Exposure-response plots for each metal when the distribution of the other metals together were held at the 25th, 50th, and 75th percentiles and when the covariates were held constant. The models were adjusted for age, sex, BMI, SES, illness symptoms, physical activity score and secondhand smoke exposure.

Supplementary Table 6. Comparison of urinary F2-isoprostanes and 8-OHdG determination methods and distributions in urine obtained from studies on metal mixtures and oxidative stress

Author (year)	Setting	Population sample	OS biomarkers in urine	Temp. of storage	Solid phase extraction	Method	Urine dilution adjustment	Statistic	Statistic, explanation	Units
This study	Uruguay	Children 12.1 ± 2.2 years	F2-isoprostanes	-80	Yes	EIA Kit	Specific gravity	1.8 (1.4, 2.5)	Median (IQR)	ng/mL
			8-OHdG	-80	No	LC-MS	Specific gravity	232.5 (170, 310)	Median (IQR)	pg/mL
Kordas et al, 2018[4]	Uruguay	Children 6-8 years	F2-isoprostanes	-20	Yes	EIA Kit	Specific gravity	1.1 (0.3, 3.8)	Median (5%, 95%)	ng/mL
			8-OHdG	-20	Yes	EIA Kit	Specific gravity	39.6 (11.8, 115.2)	Median (5%, 95%)	pg/mL
Killian et al, 2020[5]	Taiwan	Children 4-8 years	F2-isoprostanes	-70	Yes	LC-MS	Creatinine	7.90 ± 4.10	Mean ± SD	µg/g creatinine
			8-OHdG	-70	Yes	LC-MS	Creatinine	11.14 ± 9.84	Mean ± SD	µg/g creatinine
Liu et al, 2024[6]	China	Children 1-6 years	8-OHdG	-80	No	UHPLC-MS/MS	—	Boys: 2.20 (1.43, 3.40)	Median (IQR)	µg/L*
							—	Girls: 2.61 (1.43, 3.87)	Median (IQR)	µg/L*
Pizzino et al, 2014[7]	Italy	Children 12-14 years	8-OHdG	-20	No	EIA Kit	—	Exposed: 71.49	Geometric mean	ng/mL
							—	Unexposed: 67.87	Geometric mean	ng/mL
Zhang et al, 2022[8]	China	Pregnant women	F2-isoprostanes	—	No	HPLC-MS	—	8.98 (5.53, 17.1)	Median (IQR)	µg/L*
			8-OHdG	—	No	HPLC-MS	—	8.54 (5.84, 12.6)	Median (IQR)	µg/L*
Ashrap et al, 2021[9]	Puerto Rico	Pregnant women	F2-isoprostanes	-80	No	GC/NICI-MS	Specific gravity	2.0 (1.3, 2.9)	Median (IQR)	ng/mL
Kim et al, 2019[10]	USA	Pregnant women	F2-isoprostanes	-80	Yes	EIA Kit	Specific gravity	182.5 (85.3, 341.7)	Geometric mean (IQR)	pg/mL
			8-OHdG	-80	Yes	EIA Kit	Specific gravity	122.6 (62.8, 216.2)	Geometric mean (IQR)	pg/mL

*µg/L=ng/mL

Supplementary Table 7. Posterior inclusion probabilities (PIP) from a BKMR model testing the relationship between heavy metals in blood and urinary OS biomarkers among Uruguayan adolescents (n=361). The models were adjusted for age, sex, BMI, SES, illness symptoms, physical activity score and secondhand smoke exposure

Metal in blood	Modeling F2-isoprostanes	Modeling 8-OHdG
Arsenic	0.012	0.006
Cadmium	0.007	0.001
Mercury	0.051	0.007
Lead	0.000	0.003

Supplementary Table 8. Estimated overall association between arsenic, cadmium, mercury and lead mixture and urinary OS biomarkers among Uruguayan adolescents (n=361). The models were adjusted for age, sex, BMI, SES, illness symptoms, physical activity score and secondhand smoke exposure

Quantile	8-OHdG Estimate ± SD	F2-isoprostanes Estimate ± SD
0.25	-0.001 ± 0.008	0.016 ± 0.009
0.30	-0.001 ± 0.006	0.012 ± 0.007
0.35	-0.001 ± 0.005	0.007 ± 0.005
0.40	-0.0005 ± 0.003	0.006 ± 0.003
0.45	-0.0004 ± 0.002	0.002 ± 0.001
0.50	0	0
0.55	0.0003 ± 0.001	-0.002 ± 0.002
0.60	0.0005 ± 0.003	-0.005 ± 0.003
0.65	0.001 ± 0.004	-0.006 ± 0.004
0.70	0.001 ± 0.006	-0.008 ± 0.006
0.75	0.002 ± 0.008	-0.013 ± 0.008

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