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Human biomonitoring data analysis for metals in an Italian adolescents cohort: An exposome approach

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ABSTRACT

The first Italian human biomonitoring survey (PROBE – PROgramme for Biomonitoring general population Exposure) considered a reference population of adolescents, aged 13–15 years, living in urban and rural areas and investigated their exposure to metals. The study was expanded up to 453 adolescents living in the same areas of Latium Region (Italy) and blood samples were analyzed for 19 metals (As, Be, Cd, Co, Cr, Hg, Ir, Mn, Mo, Ni, Pb, Pd, Pt, Rh, Sb, Sn, Tl, V, and W) by sector field inductively coupled plasma mass spectrometry. The exposure assessment was contextualized following an exposome approach that considered several determinants related to the subjects, available environmental parameters and geo-coding of residence address.

To assess the influence of exposure determinants and modifiers on children biomarkers levels we used two independent methodologies. The first makes use of the so-called Environment-Wide Association Study (EWAS) methodology while the second was based on the application of a Generalized Liner Model (GLM) capturing co-exposures to pairs of key determinants. Based on our analysis, Hg and As were positively associated with dietary pathways (primarily linked to fish and to a lesser extent to milk consumption) while Cr showed a more complex interaction between co-exposure to different dietary pathways (milk and fish) coupled to proximity of residence to industrial activities. In addition to diet, socio-economic status of the mother revealed robust statistical associations with Cd, Ni and W biomonitoring levels in the respective children.

1. Introduction

For some years already, the scientific community agrees with the view that considering only exposure data coming from Human Biomonitoring (HBM) activities as parameters to describe the environmental burden of health risk is not enough. HBM studies often collect additional information besides sampling of blood, urine, or other biological matrices, mostly through administered questionnaires, and in some studies, also by matching analyses of, e.g., diet habits, drinking water, lifestyle factors (e.g. exposure to environmental tobacco smoke, alcohol consumption, diet, use of cosmetics, etc.), or occupational and environmental characteristics (e.g., living near specific emission sources, etc.). In the context of a holistic vision of the subject the exposome concept was developed as a tool for more complete exposure assessment in environmental health studies by integrating the internal environment of the human body, the specific external xenobiotics burden to which an individual is exposed and the social, cultural and ecological context, as well as the final potential health outcomes. The exposome is composed of every exposure to which an individual is subjected from conception to death, considering the nature of the exposures and their changes over the lifetime. Types of exposures that can be considered are internal, specific external and general external, and their measures in each of their domains may reflect to differing degrees on components of the exposome (Wild, 2012). With regard to heavy metals exposome, in the latest relevant review study, it was highlighted that exposure to heavy metals is a public health concern as even relatively low levels of metals, can disrupt normal development of the central nervous system, especially during fetal life and early childhood (Sarigiannis and Salifoglou, 2016). In addition, the risks of heavy metals have been explicitly addressed in many studies. The central nervous system is a common target for many environmental metals. These may interact to cause synergistic effects on central nervous system that are

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different from the main effects of exposure to each stressor individually. Children in Europe are often exposed to metals such as lead (Pb), arsenic (As), mercury (Hg), cadmium (Cd), and manganese (Mn) (Laborde et al., 2015). To better realize the societal cost of exposure to heavy metals, it has been estimated that the monetary cost in the EU associated to reduced IQ as a result of exposure only to Pb, amounts to almost 50 billion euro (Bierkens et al., 2012). Importantly, metal exposure rarely occurs in isolation and co-exposure to metals is likely to be the norm, thus, evaluating exposures on an individual chemical basis does not adequately account for the wide array of metal-related mixtures encountered in the environment (Sarigiannis and Hansen, 2012). Beyond co-exposure, there is toxicological evidence that metals interact in ways that imply greater than additivity effects, e.g. Mn interacts synergistically with Pb and Cd to inhibit Na^{+}/K^{+}-ATPase activity directly, leading to increase in Ca^{2+}, increase in ROS and oxidative stress with concomitant disruptions in neurotransmitter release and neuronal function (Henn et al., 2012). Beyond long term exposure effects, several studies have reported short-term health effects due to inadvertent exposure to lead in adults (Papanikolaou et al., 2005) and in children (Kokori et al., 1998, 1999). With regard to different sources of exposure to heavy metals, in a recent study in Romania, it was found that daily intake rates of metals through local vegetable consumption exceeded the limit values established by the European Food Safety Authority for Pb 1.2–2.4 times) and Cd 5.5–9.1 times) in industrially contaminated areas (Nedelescu et al., 2017). Besides diet, inhalation of traffic emitted particles represents an additional source of exposure, independently from the fuel type or the vehicle mileage (Golokhvast et al., 2015), while metallic impurities of multiwalled carbon nanotubes may be a potential source of exposure from both inhalation and skin routes (Vitkina et al., 2016).

A biomonitoring survey of the Italian population started in 2008 in the PROgramme for Biomonitoring of the Exposure (PROBE) for several metals in blood. In that context, a special survey was conducted on adolescents aged 13–15 years in cooperation with the National Association against Microcytemia (ANMI) during the annual screening for thalassemia among school children. Preliminary results of PROBE (Pino et al., 2012) provided for the first time reference data on the internal dose of metals for a cohort of Italian youngsters from an urban area. These results are now integrated with concentration on blood levels of 19 metals for a population of 453 adolescents living in the Latium region (Italy). Children and adolescents are identified as a particularly susceptible subgroup because their specific behavioral and dietary habits as well as their physical development makes them more vulnerable to adverse influences from the living environment.

In this study the exposome paradigm was used to analyze the HBM data of PROBE. The exposome methodology does not consider the existence of confounding factors, effectively taking into account all specific and non-specific exposure determinants and modifiers and all exposure biomarkers measured in the PROBE cohort adopting an unbiased (untargeted) approach towards the development of exposome-wide associations. That allowed the exploration of a larger parameter space when it came to exposure determinants. Linkage disequilibrium was tested in this large parameter space to identify putative causal relationships between exposure determinants/modifiers and measured exposure biomarker levels in human biosamples. Linkage disequilibrium, a concept borrowed from population genetics (Slatkin, 2008), is defined in this context as the non-random association of exposure determinants and modifiers for different exposure biomarkers. Exposure determinants/modifiers are considered as being in linkage disequilibrium when the frequency of association of different determinants differs from the expected value had these determinants been independent and associated randomly. The extent to which this happens is expressed quantitatively by the coefficient of linkage disequilibrium, D. Linkage disequilibrium can be affected by many factors such as location, behavioral traits, dietary patterns, seasonality, gender, etc. As a result, the pattern of linkage disequilibrium is a powerful signal of the processes underlying the structure of the exposome in a given population. It should be noted that the value of D is a property of the pair of exposure determinants/modifiers studied and not of the respective exposure biomarkers. Other pairs of determinants/modifiers of the same exposures may have different coefficients of linkage disequilibrium.

2. Methods

2.1. Sample treatment and analysis

The sample were stored at −20 °C and transported to laboratory in a deep-frozen (−86 °C) state. The entire experimental design was set to avoid or limit alterations in the analytical samples information. Briefly, a blood collection system specifically designed for trace metal analysis (7.5 mL SMonovette ® LH and Monovette®-needle, Sarstedt, Nümbrecht, Germany) was chosen, and an aliquot of 1 mL of blood sample was microwave (MW) digested with ultra pure nitric acid (Romil Ltd, Cambridge, UK) by means of a MW oven (Milestone ETHOS MEGA II, FKV, Bergamo, Italy). Subsequently samples were diluted with high purity deionised water (EASY Pure system, Barnstead, Dubuque, USA) and the analyses were carried out by a sector field inductively coupled plasma mass spectrometry (SF-ICP-MS, Element 2, Thermo Scientific, Bremen, Germany), in Low (m/Δm = 300), Medium (m/Δm = 4000) and High Resolution (m/Δm = 10,000) mode. Blanks and samples digestion was run together to account for possible contaminations during sample preparation. The validation protocol of the method included the limit of detection (LoD) and quantification (LoQ), linearity range and accuracy. Accuracy ranged between 91.2–109.4% for the CRM whole blood level 1 and 93.2–109.6% for the CRM whole blood level 2 (Seronorm, Billingstad, Norway). Finally, the expanded uncertainty budget, derived from reproducibility and accuracy/recovery with a contribution of 50% each – ranged from 10.9% (Hg, Pb) to 26.0% (Cd, Sb). The analytical method used is validated by ACCREDIA (the Italian National Accreditation Body) and the following validation performances were assessed: linearity, Limit of Detection (LoD) and Limit of Quantification (LoQ), specificity, accuracy (precision and trueness) and robustness (Pino et al., 2012).

2.2. Study population

A population of 453 adolescents aged 13–15 years (242 females and 211 males) living in Latium Region (Italy) were enrolled. The areas were Viterbo (VT), two areas in the Rome province (Fontenuova, FN and Monterotondo, MR), and the city of Rome (RM). The distribution of adolescents in the four sampled areas was as follows: 161 from VT, 131 from FN, 72 from MR and 89 from RM. Metals investigated were: As, Be, Cd, Co, Cr, Hg, Ir, Mn, Mo, Ni, Pb, Pd, Pt, Rh, Sb, Sn, Tl, V, and W. Non-fasting blood specimens were obtained for our purposes by ANMI during the annual screening for microcytemia in schools (2009); this was an excellent opportunity to ethically collect blood. The children parents gave their written consent and filled a questionnaire for each subject; the entire study design was approved by the Ethical Committee of the Italian National Institute for Health (ISS). The questionnaire key points included information that was used to stratify the population according to: sex, presence of dental fillings and/or braces, current use of piercings and tattoos, second hand smoke, frequency intake of fish, and milk, socio-economic status (SES) of the family that was derived merging the educational level and the occupational status of the parents. International Standard Classification of Occupations (ILO, 2012) was adopted to describe the parental occupations. From the questionnaires administered several types of information were obtained: 138 adolescents had dental braces and/or fillings while 49 got piercing and 93 adolescents had parents smoking at home. Relating to dietary habits, 265 adolescents consumed fish 1 time a week (1/w) and 81 twice a week or more (≥ 2/w) while 241 had milk every day (7/w), 63 from 4 to 6 times a week (4–6/w) and 99 from1 to 3 times a week (1–3/

Table 1
Characteristics of the population.

		Males	Females
Subjects	N	211	242
Brace N (%)		21 (10.0)	27 (11.2)
Fillings N (%)		38 (18.1)	52 (21.5)
Junk jewelry N (%)		24 (11.4)	149 (61.6)
Piercings N (%)		15 (7.1)	34 (14.0)
Second-hand smoke	Home N (%)	36 (17.1)	56 (23.1)
	Outdoor N(%)	31 (14.8)	58 (24.0)
	No N (%)	1 (0.5)	9 (3.7)
Fish	1/ week (%)	119(56.7)	146(60.3)
	> = 2/w (%)	40 (19.0)	41(16.9)
Milk	1–3 /w (%)	44(21.0)	34 (14.0)
	4–6 /w (%)	29 (13.8)	56 (23.1)
	7 / w (%)	116 (55.2)	149 (61.6)

w) (Table 1). Special attention has been paid to milk and fish consumption; heavy metals are lipophilic compounds with great mobility and persistence in the environment. As a result, they tend to accumulate in milk (Pilarczyk et al., 2013; Simsek et al., 2000) and fish (Jia and Wang, 2017; Vicente-Martorell et al., 2009) through the food web, due to both bioaccumulation and biomagnification. Milk and fish are very important types of food in terms of affecting human internal dose of metals such as mercury and/or arsenic (Freire et al., 2010). On top of that, milk, is an important dietary component for children and adolescents; moreover, in some of the study areas the consumed milk was produced from locally bred animals, living in volcanic sites and exposed to natural sources of As.

2.3. Environmental data

To have a more complete view of the metal exposome in the population of the study, metal concentrations were related to environmental data of air and water quality supplied by the Environmental Protection Agency of the Lazio region (ARPA, 2007). Kriging techniques were applied to derive spatially resolved concentration of chemicals in the outdoor air starting from data collected by air monitoring stations. Pollutants considered were NOx, NO, NO_2, CO, benzene, PM10. Arsenic levels in drinking water were derived from the database managed by ARPA Latium. Relating to water supply, a city map was prepared reporting water sources for the different boroughs, streets and squares.

Fig. 1. CORINE land cover classes used in this study (2006).

For each water source the As concentration was measured, and these values were attributed to subjects according to their home address. As concentrations in the water supplies of the subjects according to their home address is shown in Table S1 of the Suppl. Material.

2.4. Data treatment and statistical analysis

The basic statistics of data relating to the study population include the 50th and 95th percentiles, geometric mean (GM) and the corresponding 95% confidence interval (95%CI). In the statistical evaluation values below the LoD were considered as LoD/2 and extreme values were excluded. This procedure was used to derive Reference Values (RVs) where the 95th percentile describes the upper value useful in health care and environmental policy. The adolescent cohort was also stratified by some characteristics including sex, residence area in turn associated to traffic intensity, presence of dental fillings and/or braces, piercings and tattoos, second hand smoke, fish and milk consumption, Socio Economic Status (SES); each variable was coded according to the corresponding levels applied in the questionnaires. For all comparisons and statistical analyses, the data base including the extreme values was considered. Differences for each metal concentration among subgroups based on the different variables were tested by the Mann-Whitney U or the Kruskall-Wallis test (depending on the number of levels for each grouping variable). Mann-Whitney *U* test with Bonferroni's correction was used for multiple comparisons, when appropriate. Significance level was set at $p < 0.05$. Statistical calculations were performed on STATA 8.1 (STATA Corporation, USA, TX). The geo-statistical analyses of the subjects were carried out based on their residence address in a Geographical Information System (GIS) and stored in a Geodatabase along with human biomonitoring data, diet habits, environmental and land cover data. For the latter, EEA (2006) land cover data (CLC200) with 100 by 100 m of spatial resolution has been used. It classifies the land cover according to the standard CLC nomenclature which includes 44 land cover classes, grouped in a three-level hierarchy. Five main categories are "artificial surfaces", "agricultural areas", "forest and semi-natural areas", "wetlands", "water bodies" (Fig. 1).

Land cover data downloaded from the EEA web site was imported into a GIS infrastructure and superimposed to the residence location of the anonymised study participants. On the basis of the residence location of the participants the corresponding land cover class was extracted to be used as a new variable which allowed us to gather information on the type of "environment" present where the participants spend most of their time given their age (i.e. at home) to verify whether associations between the characteristics of the type of environment where the subjects lived and internal concentration of metals exist.

A Generalized Linear Model (GLM) was used to investigate associations between human biomonitoring data and diet patterns (fish and milk) and land cover. The model took the following form:

$$HBMvalue = k + a_1C_f + a_2C_m + a_3LC + a_4C_f\cdot LC + a_5C_f\cdot C_m + a_6C_m \cdot LC + a_7C_f\cdot C_m\cdot LC$$

Where k is the intercept, C_f is fish consumption, C_m is milk consumption, LC is the land cover category and a_n are the regression coefficients.

The data were further analyzed following the Environment-Wide Association Study (EWAS) framework to discover potential associations among covariates and HMB data in an untargeted approach. A systematic sensitivity analyses was carried out, whereby validated factors were modeled under different assumptions or with additional covariates. Moreover, the correlation of dependence between the factors, revealing potential evidence for exposure or confounding route has been computed using pair-wise validation. The EWAS framework applied herein was introduced by Patel et al. (2010) in order to describe the correlation between multiple variables following the analytical concept of "unsupervised learning" (Coates et al., 2011). The EWAS framework follows an approach similar to the widely used Genome-Wide rijensAssociation Study (GWAS) (Butte and Kohane, 2000; Horvath, 2011). The EWAS approach provides the ability to seek emerging relationships between environmental factors and observed effects/variables. Thus the "relevance globe" of the study was created using the "relevance networks" methodology (Butte and Kohane, 2000) allowing a more interpretable view of the overall analysis results. In particular, an initial scan for environmental factors associated with the observed effects/variables was carried out through general linear modeling (e.g. logistic regression). The results of this initial scan are presented in Fig. S2 of the Suppl. Material. The non-parametric correlation coefficient for each pair of exposure factors used (e.g. biomonitoring data, participants' characteristics) was computed. Spearman correlation was applied to compute correlations between variables avoiding any *a priori* distributional assumptions for the variables. An estimation of the two-sided *p*-value for the correlations between each pair of variables was computed using a permutation-based approach. Permutation resampling validated the robustness of the analysis (Mielke Jr and Berry, 2007). Then, the false discovery rate was estimated for multiple hypotheses to identify factors that are significantly associated with the observed factors beyond the region of false discovery (Chirag Patel's Group, 2017).

The form of the logistic regression model was:

$$\mathrm{logit}(z) = c + a_{M\,ses}\cdot X_{M\,ses} + a_{F\,ses}\cdot X_{F\,ses} + a_f C_f + a_m C_m + a_{LC} LC + a_j X_j$$

where c is constant, C_f fish consumption, Cm is milk consumption, LC is the land cover category, X_{M_ses} is the mother SES, X_{F_SES} is the father SES, X_j is the use of jewelry, a_i are the regression coefficients, logit(z) is the inverse of the sigmoidal "logistic" function or logistic transform and z is the log-odds of the biomarker.

All calculations were performed in Ubuntu 16.04 using R Studio. More in detail, the package 'Hmisc' (Harrell Jr, 2008) was used, that provides important functionalities related to data analysis, variable clustering, utility operations, functions for computing sample size and power, importing and annotating datasets, imputing missing values and advanced table making. Moreover, the package 'permute' (Simpson et al., 2016) was used for permutation resampling and the package 'RCircos' (Zhang et al., 2013) for globe visualization. Last but not least, the EWAS analysis was enhanced by the 'X-Wide Association Analyses' package, for applying the Logistic Regression and the False Discovery Rate.

3. Results

3.1. Comparison between subgroups of adolescents

Reference values computed excluding extreme values (see Methods for details) in theverall group of adolescents, and in male and female subgroups, are presented in Table 2.

Observing the GM values, the two groups of adolescents were markedly different only for Pb, with males showing a concentration higher than females (10.7 vs 8.73 μg/L, respectively). Based on the Mann-Whitney or Kruskall-Wallis tests, the following statistically significant differences were pointed out: blood Hg, Ni and Pb were significantly different between genders, with males having higher levels of Ni ($p < 0.05$) and Pb ($p < 0.0001$), and lower levels of Hg ($p < 0.05$) than females. Relating to diet habits, As and Hg levels were significantly different in all groups based, as expected, on fish consumption ($p < 0.01$), while Co was associated with milk consumption ($p < 0.05$) (for all elements, the higher the consumption, the higher the metal concentration). Second-hand smoke was associated to higher levels of As ($p < 0.01$) and Pd ($p < 0.05$). A group of metals (As, Cd, Hg, Ir, Mn, Mo, Ni, Pb, Pt, Rh, Sb, Sn, U and V) showed significant differences based on residence areas: As, Hg, Mn, Mo, Pb, Sn and V were significantly higher ($p < 0.0001$) around Viterbo than in other areas, and Cd, Hg, Ir, Mn, Ni, Pb, Pt, Rh, Sb and U were significantly higher ($p < 0.01$) in Rome. Finally, socio-economic status of the family was

Table 2
RVs in blood for adolescents of Latium, Italy (μg/L).

Element	Subjects N	Percentiles		GM (95% CI)	HBM values		% over HBM I	% over HBM II
		50th	95th		HBM I	HBM II		
As	443	0.73	2.95	0.71 (0.66–0.78)				
Cd	431	0.3	0.6	0.29 (0.28–0.31)				
Co	445	0.09	0.28	0.09 (0.09–0.10)				
Cr	414	0.3	1.25	0.31 (0.29–0.34)				
Hg	436	0.83	2.05	0.78 (0.73–0.83)	5**	15**	0.9	0.4
Ir*	437	6.84	15	6.71 (6.39–7.05)				
Mn	449	7.46	16	7.22 (6.89–7.57)				
Mo	449	1.1	2.39	1.11 (1.06–1.16)				
Ni	411	1.02	2.6	0.94 (0.88–1.01)				
Pb	440	9.55	21.6	9.60 (9.16–10.06)	Suspended	Suspended		
Pd*	440	22.1	38.6	21.3 (20.4–22.2)				
Pt*	423	10.9	23.3	10.9 (10.4–11.4)				
Rh*	437	22.1	35.6	21.2 (20.5–22.0)				
Sb	425	0.39	0.78	0.37 (0.35–0.38)				
Sn	439	0.56	1.52	0.57 (0.53–0.60)				
Tl	442	0.04	0.09	0.04 (0.038–0.042)				
U*	445	4.85	14.3	5.09 (4.83–5.37)				
V	445	0.08	0.17	0.07 (0.066–0.073)				
W	444	0.03	0.08	0.03 (0.027–0.030)				

* ng/L.
** Children and adults.

positively associated with As concentration ($p < 0.01$).

Evaluation of blood metal levels in relation to land cover classification of living address of study participants shows higher values in urban areas for some metals such as Ir, Pt, Rh which are typically associated with road traffic (Table 3).

Indeed, increased use of PGEs (Platinum Group Elements) as components of autocatalytic converters of the motor vehicles resulted in higher levels of Pt, Pd, and Rh in urban areas all over the world (Bocca et al., 2003; Conti et al., 2008). Other metals showing higher concentration in urban areas were Sb, Sn and U. Metals like As, Cd, Co, Hg, Mn, Mo, Ni, Pd Tl, V and W were found at higher levels in small industrial areas especially located in the Northern area of Viterbo even though the relatively few occurrences (n = 28) does not allow us to draw robust conclusions.

Mn and U are metals associated with milk consumption. The primary source of Mn in the general human population is diet. Milk and milk products are the most diversified natural foodstuffs, containing more than 20 different minor and trace elements. Most of them such as copper, zinc, manganese and chromium, are also essential and very important for mammalian metabolism, growth and development. It is true that the amount of metals in pure milk and milk products is normally very small, but their contents may be significantly altered during manufacturing and packaging. The presence of a lot of trace metals in milk and milk products was investigated by Khan et al. (2014); they found that Mn concentration was higher than 100 ng/g (133.9 ± 0.47 in milk and 236.9 ± 0.39 in fruit mixed yogurt) while U was lower than 0.8 ng/g (0.76 ± 0.001 ng/g in milk) but higher in fruit mixed yogurt (1.47 ± 0.001 ng/g) (Khan et al., 2014).

Table 3
Internal doses (in μg/L) as a function of the land use.

	Urban	Industrial	Agricultural
As	1.15	1.49	1.13
Cd	0.36	0.38	0.23
Co	0.08	0.15	0.07
Cr	0.81	0.62	1.00
Hg	1.03	1.31	1.01
Ir	8.75	6.93	7.50
Mn	8.32	10.21	7.81
Mo	1.22	1.58	1.44
Ni	2.19	6.71	2.94
Pb	11.46	11.72	11.97
Pd	22.92	26.45	25.39
Pt	14.48	10.5	13.15
Rh	23.99	21.72	22.36
Sb	0.50	0.38	0.47
Sn	1.06	0.86	0.69
Tl	0.027	0.037	0.033
U	7.01	6.50	5.32
V	0.06	0.12	0.06
W	0.02	0.05	0.03
N of occurrence	303	28	111

Note: the highest (red), the lowest (green) internal dose values.

3.2. GLM analysis

The association of HBM values data with land cover, milk and fish consumption was investigated both considering them individually and in combination to explore potential synergistic effects. A GLM multivariate analysis has been applied to the dependent (HBM data) and categorical variables (fish and milk consumption and land cover). Results of the GLM analysis in terms of significance (*p value*) are reported in the Manhattan plot shown in Fig. 2. The figure shows the associations of the environmental/dietary factors that have been found to be linked to the internal dose of the metals examined based on all populations studied (i.e. $p < 0.05$). The red line represents a $p < 0.02$, denoting a more robust association. Results show robust statistical associations of Cr with the dietary pathways studies and land cover, showing that both out-of-region and local sources can be associated with the observed levels in the population. Other robust associations found were between W and co-exposure to milk and proximity to industrial activities and Hg with a more complex interaction between co-exposure to different dietary pathways (milk and fish) coupled with proximity to industrial activities. No pathway alone is dominant but the combined effect results in statistically significant associations with metal concentration levels in blood. The associations of Ni with dietary pathways (co-exposure to milk and fish) and Pt with fish and industrial activities and enhanced traffic even though statistically significant ($p < 0.05$), fail to meet the statistical robustness test (they are below the red line in Fig. 1). All other metals do not show statistically significant associations with dietary patterns and/or land cover information. Details on the associations among the single parameters (GLM without interaction terms among the independent variables) and the

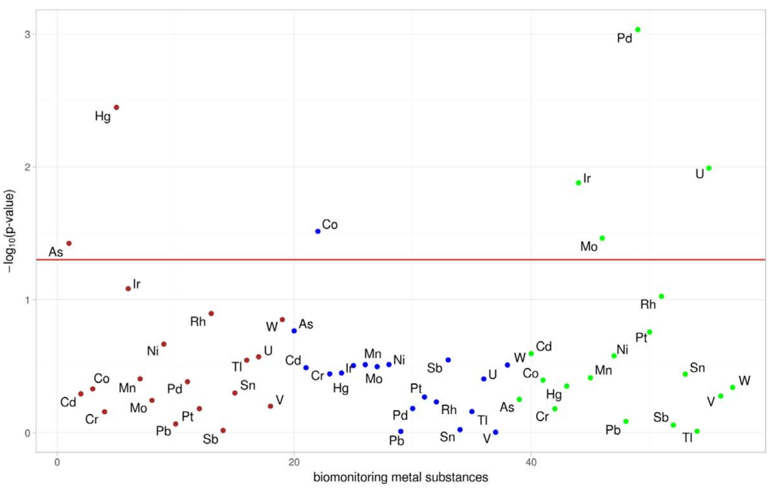

Fig. 2. GLM results – Manhattan plot of between-subjects effects.

biomonitored levels are given in Fig. S1 of the Suppl. Material.

3.3. EWAS analysis

EWAS analysis confirmed the associations found with GLM providing further insight on the role of other exposure determinants and modifiers on the HBM values. Results reported in the heat-map and in correlation network plots (Fig. 3) showed statistically significant positive associations ($p < 0.05$) between fish consumption and As and Hg levels and between milk consumption, passive smoking and Co levels. Biomonitoring levels of metals in children are inversely associated with their socio-economic status (SES). More specifically the socio-economic status of the mother revealed a statistically robust negative association ($p < 0.05$ and in some cases $p < 0.01$) with Cd, Ni and W biomonitoring levels of the children. The use of costume jewelry showed a statistically significant positive association ($p < 0.05$) with Cd and Pt levels.

4. Discussion

4.1. General considerations

This study showed that the metal concentrations in blood in a cohort of adolescents living in two areas of the Latium region are associated with different types of exposure sources. All metals analyzed in this study are environmental pollutants of high concern for human health. Their distribution in the environment is a result of natural processes (volcanoes, erosion, spring water, bacterial activity) and/or anthropogenic activities (fossil fuel combustion, industrial and agricultural processes) (Florea, 2005; Florea et al., 2004). However, in

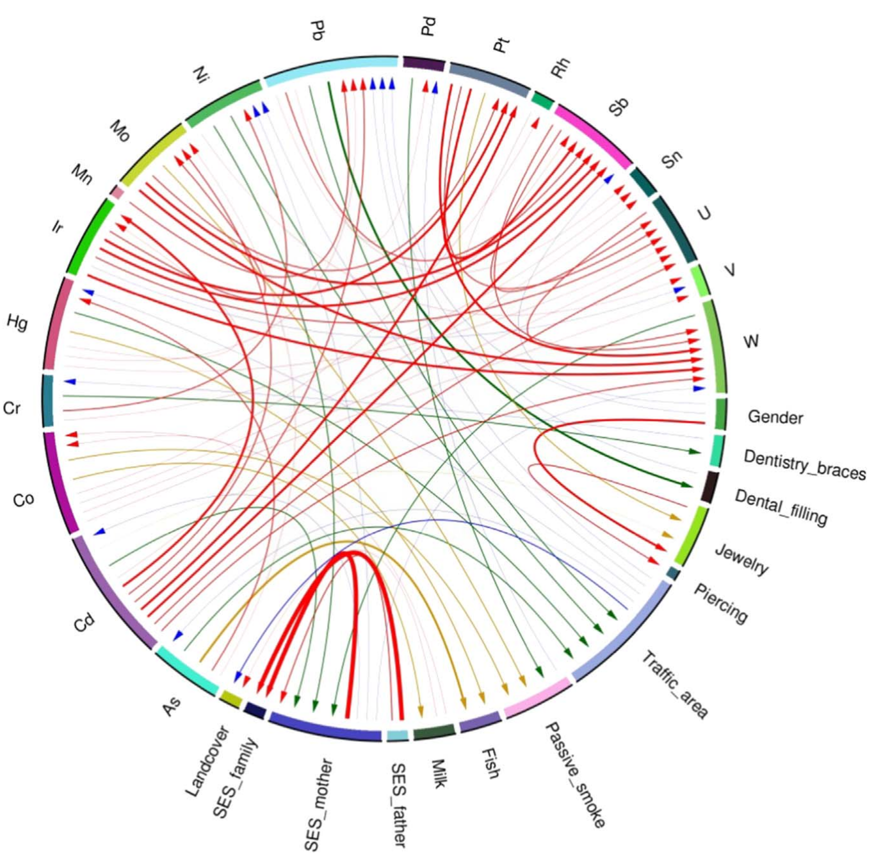

Fig. 3. EWAS correlation globe plot. Blue and red lines show the correlations (negative and positive respectively) between covariates. The green and the dark yellow lines are subset of the blue and red lines respectively showing only the significant associations between HBM data and exposure determinants/modifiers. These subsets have been calculated taking into account the q value of the EWAS analysis, applying as validation method the false discovery rate (Benjamini and Hochberg, 1995) on 4 random subsets of the examined dataset. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

addition to environmental sources, also other factors such as individual lifestyle, diet, socio-economic factors contribute to the levels of metals in the human body. Such information coming from the questionnaire administered to all the adolescents enrolled was used to compare HBM data with individual exposure determinants variables. Some metals (As, Cd, Hg, Pb) are in the top 10 of the Priority List of Hazardous Substances of Agency for Toxic Substances and Disease Registry (ATSDR), and are considered to pose the most severe potential threats to human health due to the combination of their frequency, toxicity, and potential for human exposure. The list also includes Co, Cr and Ni (ATSDR, 2016). As, Cd and Pb have also been suggested as components of a mixture for use in interaction profile studies (ATSDR, 2004). Concurrent exposure to Pb, Cd, or As may produce additive or synergistic interactions or even new effects that are not seen in single component exposures (Wang and Fowler, 2008). Trace metals like As, Cd, Cr (VI) and Ni are carcinogens and listed in Group 1 (Carcinogenic to humans) of the International Agency for Research on Cancer (IARC) list, others, such as Pb are considered as probable carcinogens to humans (Group 2A) (Jarosinska and Gee, 2007). As, Hg, Ni, Mn and Pb can have neurotoxic effects, in particular on the development of children also at low concentrations (Davidson et al., 2004). In this study data showed significantly higher concentrations of Pb in the male group. Several authors have suggested that Pb-related neurotoxic effects seem to be more pronounced in boys than in girls (Burns et al., 1999; Jedrychowski et al., 2009; Vermeir et al., 2005). A study conducted recently on young children (aged three to six years) reported that boys with elevated blood Pb levels may present deleterious cognitive effects more than girls related to a more pronounced negative impact on executive function than on reading readiness. These findings support recent research on adults indicating that exposure to Pb is related to atrophy within the prefrontal cortex and that estrogen and estradiol may act as neuroprotectants against the negative impact of neurotoxins (Khanna, 2015).

With regard to other environmental parameters, after analyzing the environmental data available for this study, we decided not to include them in the statistical analysis so as not to lose statistical power of the results. Indeed, air quality data were available for a limited number of monitoring stations (i.e. 6 in Rome and 2 in Viterbo) located close to each other; as such they did not allow the derivation of a concentration field large enough to correspond to most of the HBM data available. Interpolation of the AQ data and extraction of their values to the HBM data locations (i.e. residential address of the study participants) resulted in a number of valid data equal to 87 out of 453 HBM existing data (19.2%). In addition, most of these 87 data are located in a very small portion of the interpolated AQ concentration field, thus characterized by similar levels of AQ concentration levels. Moreover, data measured in the different monitoring stations showed a limited range of variability, that resulted in limited spatial variability of the air quality levels. Similar considerations were made for As in drinking water. In this case measured data were available only for the Viterbo Province, which includes 156 out of 453 HBM available data (ca. 34%). On top of that, the respective measured As data showed a limited variability ranging from 10 to 24 μg/L, with 117 out of 135 (86.7%) arsenic concentration samples lying in the range between 10 and 12 μg/L. Thus, in order not to limit the statistical significance of the investigated associations and to avoid misleading conclusions about exposure determinants, we decided to investigate the parameters and their interaction terms of the most complete datasets.

Among these, a main source of exposure is diet, in particular for children (Kroes et al., 2002). Dietary patterns of children may contribute to a higher exposure to contaminants present in food. Apart from having higher exposure, children also have different physiology than adults. There are specific periods in their development when exposure to a chemical, physical, or biological agent may result in adverse health outcomes (Jarosinska and Gee, 2007).

In this adolescent cohort, As (p: < 0.001) and Hg (p: < 0.005) were found to be associated with fish consumption. Human exposure to As comes from contaminated water and soil as well as from food rich in As species; however blood As levels are not as well correlated with drinking water concentrations as urine levels (Valentine et al., 1979). Arsenic and other metals could originate mainly from natural sources related to volcanic activity. Some studies reported enhanced biological availability of trace metals in the marine community (Ag, Se, Al, Fe, Mn, Sr, Ti and Zn) and penguin feathers (Pb) collected in Deception Island where concentration values of dissolved and particulate As in fresh water were higher in samples collected in or near local spring waters (de Ferro et al., 2013). A study based on the content of metals in hair of people from two areas in Azores, one with volcanic activity and the other without volcanic activity since a million years, reported that humans chronically exposed to volcanic emissions show high concentrations of essential and non-essential trace metals in scalp hair. This study concluded that this type of exposure may be as harmful as living close to industrial facilities. Children (0–14 years) from Furnas (volcanic area) presented higher (Mann–Whitney U, $p < 0.05$) concentrations of Cd and Pb, than those from Santa Maria (no volcanic area) (Amaral et al., 2008). Considering drinking water, the guidelines of the WHO, the US Environmental Protection Agency as well as the European Commission recommend a maximum As concentration level of 10 μg/L (Fawell and Mascarenhas, 2011). Citing the precautionary principle, some authors recently have called for a further lowering of the current standard (Sauvé, 2014). Several studies have reported associations between low-level As exposure (< 100 μg/L in drinking water) in the population (including pregnant women and young children) and cognitive, behavioral, or motor/sensory function in children (Tsuji et al., 2015; Wasserman et al., 2011). A study conducted on 201 children in Bangladesh suggested that As in water was associated with reduced intellectual function, following a dose-response relationship. Children exposed to water with As levels > 50 μg/L achieved significantly lower Performance and Full-Scale scores than children with water As levels < 5.5 μg/L (Wasserman et al., 2004). Arsenic contamination of drinking water is a public health problem in several areas in Italy due to the volcanic origin of the territory. Arsenic values in drinking water were chronically between 20 and 50 μg/L, in large areas of Italy (e.g. Toscana, Lombardia, Lazio, Campania), and since 2003 the Italian Government requested several derogations from the EU in order to perform structural interventions on the water supply system, declaring in October 2010 an official "state of emergency" for water supply in 128 Italian municipalities, 60 of which are located in the Viterbo province, the northern part of the Lazio region. Due to the long derogation period, the implementation of mitigation measures was delayed for several years and the population did not modify their food or drinking water habits. The particular hydrogeological characteristics of the Viterbo area (Angelone et al., 2009; Dall'Aglio et al., 1994) contributed to an exposure of the local population to As at low-medium levels for a long time. The area of Viterbo province is characterized by the presence of a volcanic system where a continuous basal aquifer flows within Plio-cene-Pleistocene sedimentary rocks with very high concentrations (up to 130–370 μg/L) of As (Baiocchi et al., 2011; Baldi et al., 1973; Preziosi et al., 2010). This volcanic aquifer covers an area of 5500 km^2 and supplies water for human consumption (about 150,000 inhabitants) and local agricultural activities. In particular, our results show that children living in Viterbo had higher levels of As ($p < 0.01$), Hg ($p < 0.05$), and V ($p < 0.001$), even though they frequently belong to middle and high SES families, than the children residing in the other 3 areas. Thus, in the evaluation of the exposure to As, location of residence, if associated with SES, could be a confounding factor ($p < 0.01$): indeed, when accounting for residence location alone such association appears to be no longer significant.

For the general population, the main source of Hg exposure is fish consumption followed by fruit and vegetables in the diet. Daily intake of total Hg for the adult population is estimated to be below 0.015 mg/day. It has been reported that fish consumption is positively related to

Table 4
Comparison of blood metal concentration (μg/L) of several biomonitoring campaigns.

	Country	Year	Age	Total			Males			Females		
				N	GM	P95	N	GM	P95	N	GM	P95
As	Canada[a]	2007–2009	12–19	945	0.59	1.83	489	0.58	1.66	456	0.61	1.96
	This study	*2009*	*13–15*	*453*	*0.71*	*2.95*	205	0.71	2.82	238	0.72	3.08
Cd	Belgium[b]	2007–2011	14–15	200	0.21	0.41*						
	Germany[c]	2003–2006	12–14	460	0.14	1.25						
	USA[d]	2011–2012	12–19	1129	–**	0.90	3968	0.25	1.48	3952	0.304	1.53
	Canada	2007–2009	12–19	945	0.17	1.45	489	0.16	1.54	456	0.18	1.32
	This study	*2009*	*13–15*	*431*	*0.29*	*0.60*	200	0.30	0.63	231	0.29	0.57
Cr	Belgium	2007–2011	14–15	200	0.26	0.50						
	This study	*2009*	*13–15*	*414*	*0.31*	*1.25*						
Hg	Germany	2003–2006	12–14	456	0.26	1.0						
	USA	2011–2012	12–19	1129	0.41	2.25	3968	0.48	1.90	3952	0.69	4.03
	Canada	2007–2009	12–19	945	0.31	2.25	489	0.29	2.29	456	0.33	2.23
	This study	*2009*	*13–15*	*436*	*0.78*	*2.05*	*207*	*0.76*	*1.96*	*229*	*0.80*	*2.05*
Mn	Belgium	2007–2011	14–15	200	9.66	13.8*						
	Canada	2007–2009	12–19	945	9.97	16.3	489	9.44	14.7	453	10.6	17.28
	USA	2011–2012	12–19	1129	10.1	16.6	3968	8.74	14.9	3952	9.96	17.8
	This study	*2009*	*13–15*	*449*	*7.22*	*16.0*	*210*	*7.17*	*15.3*	*239*	*7.26*	*16.3*
Mo	Canada	2007–2009	12–19	945	0.68	1.31	489	0.70	1.35	456	0.65	1.26
	This study	*2009*	*13–15*	*449*	*1.11*	*2.38*						
Ni	Belgium	2007–2011	14–15	200	1.25	1.66*						
	Canada	2007–2009	12–19	945	0.63	1.78	489	0.62	1.77	456	0.63	1.80
	This study	*2009*	*13–15*	*411*	*0.94*	*2.60*	*188*	*1.00*	*2.63*	*223*	*0.90*	*2.50*
Pb	Belgium	2007–2011	14–15	200	14.8	25.1*						
	Germany	2003–2006	12–14	460	14.5	30.5						
	USA	2011–2012	12–19	1129	5.54	13.1	3968	11.3	36.8	3952	8.42	25.9
	Canada	2007–2009	12–19	945	8.00	16.4	489	8.8	17.9	456	7.1	14.6
	This study	*2009*	*13–15*	*440*	*9.60*	*21.6*	*205*	*10.7*	*22.1*	*235*	*8.73*	*20.6*
Tl	Belgium	2007–2011	14–15	200	0.027	0.034*						
	This study	*2009*	*13–15*	*442*	*0.04*	*0.090*						
U ng/L	Canada	2007–2009	12–19	941	< 5	10.0	489	–	< 5	452	–	10.0
	This study	*2009*	*13–15*	*445*	*5.09*	*14.3*	*208*	*5.29*	*15.5*	*237*	*4.91*	*13.7*

* 90th Percentile.
** Not calculated: proportion of results below limit of detection too high.
[a] Health Canada (2010). Report on human biomonitoring of environmental chemicals in Canada. In: Results of the Canadian Health Measures Survey Cycle 1(2007–2009). Health Canada, Ottawa, Ontario.
[b] Vrijens et al. (2014). Trace metal concentrations measured in blood and urine of adolescents in Flanders, Belgium: reference population and case studies Genk-Zuid and Menen. Int J Hyg Environ Health. 217(4–5):515-27.
[c] German Federal Environment Agency (2008). German Environmental Survey on Children 2003/06 (GerES IV). Federal Environment Agency (Umweltbundesamt)/Dessau-Roßlau Robert Koch-Institut (RKI), Berlin.
[d] Centers for Disease Control and Prevention (2015). Fourth Report on Human Exposure to Environmental Chemicals, Updated Tables, (February 2015). Atlanta, GA: U.S. Department of Health and Human Services, Centers for Disease Control and Prevention.

the blood levels of mercury (BHg) (Love et al., 2014); however, the toxic properties and target organs of Hg depend upon its chemical species. Exposure to methyl Hg, particularly in the prenatal period, has neurotoxic effects on the development of a child's central nervous system; yet gender difference was observed in the neurotoxicity of this metal. The Hg concentration of BHg in this survey (GM: 0.78 μg/L) is slightly higher than the results of a cross-sectional nationally representative survey (2009–2012) of the US population (NHANES), where the BHg geometric mean concentration was 0.50 ± 0.02 μg/L among seafood consumers and 0.27 ± 0.01 μg/L among those who did not consume seafood (Nielsen et al., 2015). Probably this result is due to a greater consumption of fish in people from the Mediterranean area and to the existence of sub-marine volcanos.

Children may be particularly susceptible to the combined effect of neurotoxic substances as suggested by findings from studies on the effects of As and Mn exposure on children's intellectual function. Beside the fact that As per se is a well-known neurotoxicant affecting the central nervous system, some studies have reported the combined effect of As and Pb on the neuropsychological development of children (Calderon et al., 2001). Higher blood Pb levels were also found in children from Rome (urban area; Pb mean: 82.36 μg/l) than children from industrial and rural areas (48.23 and 35.99 μg/l, respectively) in a study carried out on school children (113 girls, 96 boys), aged 6–12 years, from urban, industrial and rural areas in Fez city. Blood Pb levels were associated with infancy in the urban area (Laamech et al., 2014).

4.2. Health risks associated with the current biomonitoring levels

To view the HBM data in a health risk assessment context the concentration measured can be also compared with available health-related biological exposure values, as the Human-BioMonitoring values (HBM: HBM I and HBM II) established by the German Human Biomonitoring Commission (Schulz et al., 2011). HBM values are derived based on toxicological, epidemiological studies or toxicokinetic extrapolation that provides a concentration of a substance or its metabolites corresponding to tolerable intake doses. Among the elements determined in blood in this study, only Hg and Pb had health reference limits. The HBM I limits (alert value) and HBM II (action value) for blood Hg are respectively 5 μg/L and 15 μg/L in children, while the HBM values for Pb in children have been suspended by the European Commission as they were considered arbitrary and not justified to establish an effect threshold for Pb blood levels. In the present study, the geometric mean values of Hg were 0.76 and 0.80 μg/l for males and females, respectively, i.e. much lower than the HBM I and HBM II and only in 4 (0.9%) adolescents Hg concentrations exceeded the HBM I limit of 5 μg/l and in 2 (0.4%) adolescents the limit of 15 μg/l out of 453. These findings suggested that the risk for health impairment in the studied adolescents is not to be expected for these metals.

A further approach for interpreting HBM results for specific chemicals in a health risk context can be the translation of guidelines exposure into Biomonitoring Equivalents (BEs). The latter are defined as the concentration of a chemical or metabolite in a biological medium (blood, urine, human milk, etc.) consistent with defined exposure guidance values or toxicity criteria that could be reference concentrations (RfD and RfCs), minimal risk levels (MRLs) and tolerable daily intakes (TDIs). Thus, the definition of BE is functionally similar to the HBM-I value definition from the German HBM Commission. A second BE level, $BE_{POD,}$ has also been defined as an estimate of the concentration of a chemical or its metabolites in a biological medium which is consistent with the human-equivalent Point of Departure (POD) for risk assessment. The BE_{POD} value corresponds to an intermediate value in risk assessment (Hays et al., 2008). Among the metals found in blood in this study only for Cd BEs and BEs_{POD} have been established, namely: (i) 1.7 and 5.3 μg/L respectively based on the US EPA reference dose (RfD); and, (ii) 1.4 and 4.4 μg/L respectively based on the ATDSR minimal risk level (MRL) (Hays, S.M. 2008). The blood Cd concentration obtained in this study had a GM of 0.26 μg/L, very far below the established BEs and the BEs_{POD}. Thus a follow-up risk assessment in this adolescent cohort is of low priority.

4.3. Comparison with other HBM campaigns

For some metals it is possible to make a comparison of results obtained in this study with values observed in blood of adolescents sampled in other biomonitoring campaigns carried out, e.g., in Belgium (FLESH II) (Vrijens et al., 2014), Germany (43-GerES IV, 2008), USA (CDC, 2015) and Canada (Health Canada, 2010) (see Table 4). Blood Hg was slightly higher both as GM (0.78 μg/L) and 95th percentile with respect to data from all three countries (0.26, 0.41, 0.31 μg/L in Germany, USA and Canada, respectively) while Mo and Ni levels (1.11 and 0.94 μg/L) were slowly higher than the concentrations found in adolescents in Canada (Mo: 0.68 μg/L and Ni: 0.63 μg/L) but lower than in Belgium for Ni (1.25 μg/L) in terms of GM. Mn levels were similar in all surveys, while overlapping results were observed for Tl and U. Unfortunately, it is not possible to compare the blood levels of Be, Co, Cr, Ir, Pd, Pt, Rh, Sb, Sn, Tl, V and W in youngsters as they were not quantified by the abovementioned international surveys. Comparable results were observed between the As concentration in Italian adolescents and Canadian adolescents aged 12–19 years; a similar trend was found for Cd and slightly lower Pb blood (9.60 μg/L) levels in the Italian adolescents with respect to the results reported in Belgium (14–15 years: 14.8 μg/L), Germany (12–14 years: 14.5 μg/L), but higher than the concentrations found in the USA (12–19 years: 5.54 μg/L) and Canada (8.00 μg/L). Comparable concentrations were found also for Hg in all cohorts examined.

5. Conclusions

This HBM study provided information regarding the levels of 19 metals in blood of adolescents (13–15 years) living in urban and rural areas in the Latium region (Italy). The results showed that the adolescents exposure levels are rather consistent with the concentrations found in other countries. Higher concentrations were observed for As, Cr, Mo, Ni confirming the environmental effect, as these adolescents are living in volcanic areas.

Two different advanced statistical approaches have been employed aiming at the determination of associations between children biomonitoring data and exposure determinants/modifiers. From both types of statistical analysis, it was confirmed that Hg and As are positively associated with dietary pathways (primarily to fish consumption and to a lesser extent to milk consumption) while Cr shows a more complex interaction between co-exposure to different dietary pathways (milk and fish) coupled to proximity of residence to industrial activities. Socio-economic status of the mother revealed robust statistical associations with Cd, Ni and W biomonitoring levels in the respective children. Other robust associations were found between W in biospecimens and co-exposure to milk and residence proximity to industrial areas and between Cd and Pt and the use of costume jewelry. Other metals such as Ir, Pt, Rh, Sb and Sn found in biosamples were strictly related to living in urban areas (mostly linked to traffic-related exposures). Concerning health risk, the levels of metals found in human biological samples in this study did not indicate a health risk when considering the health reference limits, as provided by HBM, HBM II and BE.

A key limitation of our study was our inability to consider exposure to metals through inhalation of airborne particulate matter. Even though the respective data were incorporated in the study database the available data covered only a limited part of the study territory, limiting thus the statistical power of the results. The HBM values of the metals studied in PROBE were low, revealing no significant health risk. Thus, the above limitation in data usage does not hamper the health risk assessment related to exposure of the children and adolescents in the PROBE cohort to metals. Nonetheless, an important lesson drawn from our study is the need for speciation analysis of the metals adsorbed in atmospheric particulate matter and for a well pondered spatial reconfiguration of the regulatory networks for air pollution monitoring in line with population-based HBM surveys. Coupling well-designed longitudinal HBM surveys with adequately spatialized environmental monitoring networks would result in a monitoring instrument that protects adequately public health.

The application of the exposome paradigm, i.e. the search for environment-wide associations without introducing any kind of bias in the analysis and without pre-considering certain parameters as confounders revealed linkages between exposure variables (metal levels in human biospecimens), specific lifestyle choices and the respective environmental contamination. This approach allowed identifying correlations between exposure determinants and human biomonitoring levels of the metals investigated in the study, while unraveling the co-correlations in the exposure determinant space. Thus, on the one hand the identification of sources of exposure and apportionment of the relative contribution to specific exposures become possible. On the other, this comprehensive data analytics framework supports efficiently the identification of causal relationships between exposure sources, determinants and biomonitoring levels.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.envres.2017.08.012.

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