Cross-Mediterranean Environment and Health Network

CROME-LIFE

ANNEX 12

Deliverable B.6.1

Recommendations for policy makers

LIFE ENVIRONMENT PROGRAMME

LIFE12 ENV/GR/001040

http://www.crome-life.eu

Cross-Mediterranean Environment and Health Network

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Executive summary

This report aims at providing guidelines for the reproducibility and generalized application of the CROME-LIFE methodology proposed and applied in several case studies in the demonstration countries addressed in the project. It encompass recommendations for the environment and human biomonitoring sample collection, the processing of data, their statistical analysis as well as the reference document and the preliminary recommendations for policy answer at European National and Regional levels. The guidelines development will benefit of an already established close collaboration with the COPHES and DEMOCOPHES teams.

The report is structured into three main sections: the first one provides an overview of the protocols used along with the major difficulties encountered in the execution of the project case studies and the second one summarizes the data available as well as the major data gaps identified. Based on the these two sections, the third one summarizes the key findings and results derived from the application of the developed methodology and proposes recommendations for policy answers at European National and Regional levels. The recommendations proposed include suggestions for the optimal use the measurements available, for measurements to be made when data gaps are identified as well as for the statistical methods to be used for an optimal interpretation of the environmental and biomarker data.

Description of protocols followed and of the encountered difficulties in the implementation of the national studies

Greece

Oinofoita – Cr(VI)

50 individuals age stratified (4 to 65 years old) where recruited so as to evaluate current as well as past and cumulative exposures to Cr. For this purpose, both urine and hair samples were taken. Population covered the wider basin of Asopos area, reflecting the variability of exposures related to the different levels of Cr sampled in the environmental campaigns mentioned above. Hair samples were collected from the occipital region of the head. The samples of hair were obtained using stainless steel scissors from the nape of the neck. The hair samples were cut into approximately 0.5-cm pieces in length and mixed to make a representative hair sample.

The analytical method was based on the described by Afridi et al (2006) where biological samples were collected from a total of 56 long-term exposed steel production workers (PW), 35 quality control workers (QCW) and 75 unexposed normal controls (all male, age range 25 55 years). Hence, the working solution of Cr was prepared from certified standard solutions of all analytes under study in 2M nitric acid. All solutions were stored at 4ºC. In the case of each person, hair strands were washed with diethyl ether-acetone (3+1) mixture, non-ionic detergent solution (distilled water) and ultrapure water, respectively. After washing, the hair samples were dried at 80ºC for 6 h. Hair samples for each participant were placed in separate plastic envelopes, which indicated the identification (ID) number of the participant. Duplicate 0.5 mL of each certified urine samples, while 0.2 g of human hair samples BCR 397, were placed into 50-mL Pyrex flasks. A 5-mL volume of a freshly prepared mixture of concentrated HNO_3 -H_2O_2 (2:1, v/v) is added to each flask, and the solutions will be heated on an electric hot plate at 80ºC for 2-3 h, until the clear transparent digests is obtained. Final solutions was made up to 10 mL with 2M HNO_3. The final solutions were collected in polyethylene flask for determinations of Cr by ETAAS. Blank digestions were carried out. Duplicate samples of QCW, PW and normal controls were treated as described above. The calibration was periodically verified by analyzing the standard at the frequency of 10 readings. A microwave-assisted digestion procedure was carried out in order to achieve a shorter digestion time. For digestion of biological samples, duplicate samples of dried scalp hair (200 mg) three replicate samples of CRM 397, were weighed into Teflon PFA digestion vessels directly, to which 2 mL of HNO_3 and 1 mL of 30% H_2O_2 were added and left to stand for 10 min, then the vessels were sealed and placed in a PTFE reactor. This was then heated following a one-stage digestion programme (250 W, 15 min for hair samples). After cooling the digestion vessels in an ice bath for 20 min before opening, the resulting solution was evaporated almost to dryness to remove excess acid, and then diluted to 10.0 mL in volumetric flasks with 2M HNO_3. Blank extractions (without sample) were carried through the complete procedure of both methods. The concentrations were obtained directly from calibration graphs after correction of the absorbance for the signal from an appropriate reagent blank.

The methodology to identify Cr in urine samples was based on the one proposed by Scheepers et al. (2008). Spot urine samples were stored at 4 ^{0}C in the dark and transferred to a laboratory for further storage at −18 ^{0}C. The analysis of Cr is performed according to Lewalter, Korallus et al. (1985). Urine was diluted with a solution of magnesium nitrate with Triton-X and sulphuric acid (matrix modifier). Cr levels were determined at 357.9nm using electro thermal atomic absorption spectrometry, AAS (Solaar M, Thermo Analytical) with Zeeman background correction. The LOQ was 0.05 μg/L of urine and the coefficient of variation was 5.4% at 0.31μg/L.

Aspropyrogos – PCDDs/PCDFs

Blood samples were collected from 60 individuals aged 6 to 58 years old, living in the close proximity of the recycling plant. Approximately 50–80 ml of blood were collected from each individual. Blood samples were collected in polyethylene recipients. Immediately after sampling, blood samples were processed for serum separation, frozen right after separation and transported to the laboratory for measurement of PCDDs/PCDFs. The blood serum samples, remained frozen until they were analyzed at a temperature of -80 C.

All solvents used were residue analysis picograde and were purchased from Promochem (Germany). The isomers for the preparation of the 13C12 internal standard solutions were purchased from Wellington Laboratories (Canada). Carbosphere 80–100 mesh was purchased from Alltech (it was cleaned by elution with methanol and consequently with toluene for several weeks before usage). Alumina was Basic activity Super 1 for dioxin analysis, MP Biochemicals GmbH. The sulphuric acid impregnated silica gel was prepared as follows: Silica gel (100 g, 60–200 mesh, Merck) was activated in an oven at 200 C for at least 2 days and then mixed with concentrated sulphuric acid (44 ml).

Blood serum samples were subjected to a liquid–liquid extraction procedure consisting of mixing with sodium oxalate and methanol, followed by extraction steps with a combination of diethyl ether–petroleum ether. The organic layer of this extraction was evaporated and the lipid residue was dried and weighed in order to calculate the levels of lipids in serum and breast milk.

The labelled quantification standards were added to each sample before the extraction. They were toluene solutions containing a mixture of the $^{13}C_{12}$ isomers of all the 17 PCDDs/PCDFs congeners except OCDF.

The quantification of PCDDs/PCDFs, was performed by HRGC-HRMS (EI) in MID mode on a Trace GC gas chromatograph (ThermoFinnigan) coupled to a MAT-95 XP mass spectrometer (ThermoFinnigan) equipped with a CTC A 200S autosampler at 10 000 resolving power (10% valley definition). Instrumental conditions and purity control criteria were according to the EPA 1613B and EPA 1668A methods. The quantification of concentration and recovery was carried out by the isotopic dilution method. The limit of detection (LOD) for each congener was determined as the concentration in the extract which produced an instrumental response at two different ions to be monitored with a signal to noise ratio of 3:1 for the less sensitive signal. For TEQ calculations the WHO-98 toxicity equivalent factors (TEF) were used (Van den Berg et al., 1994).

Thessaloniki – PM-PAHs

Environmental data

Considering that there are a lot of data regarding the mean daily exposure to PM and PAHs in the area of Thessaloniki, it is of particular interest to identify the diournal variability of ambient air and indoor air to PM and to associate this to specific activities. This will allow us to further refine exposure estimates for the various groups and eventually the lung cancer risk estimates associated to PAHs exposure.

For this reason, time dynamic measurements of PM (in parallel to the existing equipment for gravimetric PM sampling and chemical analysis) was continuously monitored during the cold period of the years 2014-2015 and 2015-2016 using the Mini Laser Aerosol Spectrometer (Mini-LAS). With regard to the outdoor measurements, PM10 and PM2.5 samplers were installed at 3.5 m above ground, located at one traffic and one background site. The traffic site is surrounded by several streets with typical city heavy-traffic levels and the background site is located in an urban environment without direct traffic emissions. The measurements lasted for 2.5 months, covering the transition from the warm to the cold period (average daily temperatures 23 and 4 ^{0}C respectively).

Parallel measurements of indoor air quality (for PM10 and PM2.5) were carried out in 30 houses located close to the traffic and the urban background station respectively to validate the INTERA estimates. In order to identify the contribution of biomass burning in indoor air quality, targeted measurements for the effect of the open fireplaces operation on PM concentrations were carried out in a selected indoor location and under controlled conditions of air exchange rate (AER). More specific, within a third floor apartment of 140 m^2, extensive measurements of PM10 and PM2.5 were carried out, while a Particle Number Count sizer was used for measuring temporal variation of PM number and mass concentration.

Meteorological data needed for the study where provided by the local meteorological station from the traffic site.

PM2.5 and PM10 samples were collected using 6 low-flow air samplers (ENCO PM, TCR TECORA, Italy). The samplers used sampling heads meeting the EN 12341 (PM10) and EN 14907 (PM2.5), and operated at a flow-rate of 38 L/min, with a collection time of 24 h per sample. Samples were collected on PTFE membranes filters with PMP supporting ring (PALL Life Sciences, Ø 47mm, pore size 2 μm, USA). Teflon filters were analyzed gravimetrically for particle mass concentrations using an electronic microbalance with a sensitivity of ± 1 μg after 24-h equilibration at a temperature between 20 C and 23 C and a relative humidity (RH) between 30 and 40%. Each filter was weighed at least three times before and after sampling, and the net mass was obtained by subtracting the average of the pre-sampling weights from the average of the post-sampling weights. Differences among replicate weightings were <5 μg for the blanks and the samples. Prior to the start of the sampling campaign, the flow rate of the PM2.5 and PM10 samplers was calibrated. Field blank filters were also collected and used to correct for background concentrations or influences from handling and transport.

The same PM samplers 24 h sampling of PM10 and PM2.5 within 30 indoor location, with and without the operation of fireplace. Beyond these, particulate matter were also monitoring by handheld, portable Particulate Counter (Lasair II, Model. 525, Particle Measuring System). A built-in pump draws aerosol sample through the instrument with a flow rate of 0.01425 m^3/min. The resulting droplets are counted by a diode laser, 780nm. Lasair II enables particle number concentration measurements in the size classes 0.5-0.7μm, 0.7-1.0μm, 1.0-5.0μm, 5.0-10.0μm, 10-25μm and pm above 25.0 μm. Lasair II has been used during indoor measurements with and without the operation of fireplace. Particles number concentrations were transformed into mass concentration using the following formula:

$$C_{(\mu g/m^3)} = \frac{PNC \cdot d \cdot (D_{median})^3 \cdot \pi}{6}$$

Where ***PNC*** is the particle number concentration for the given range of particles diameter, ***ρ*** is the particles density, taken equal to 1 g/cm^3 and D_{median} is the median diameter for the selected class of particles.

Italy

The CROME Cross-Mediterranean study

Trieste - Metals and neuropsychological outcome in 7-year-old children

Within the CROME common study, the ISS team, in collaboration with the Burlo Garofolo Pediatric Hospital in Trieste, brought about a follow up study at the age of 7 years of 200 children born within the original NAC II PHIME cohort, established in the Friuli Venezia Giulia Region of Italy in 2007. Data concerning perinatal exposure to metals and metalloids, mother's lifestyle and health, socioeconomic factors and neuropsychological measures at 18 and 40 months are available for this cohort . At 7 years of age child`s hair, child`s urine and saliva sample of both mother and child (Oragene DNA self-collection kit) were collected at the time of appointment. Mothers completed a new questionnaire to update information on life-style factors. Children were subjected to neuropsychological testing by trained neuropsychologists. The neuropsychological tests administered included Wisc-IV (Wechsler Intelligence Scale for Children), Nepsy-II for assessment of sensorimotor, attention, learning capabilities, MT for assessment of reading skills, BVSCO2 for complete assessment of writing skills, CBCL - Child Behaviour Check List to identify behavioural and emotional problems in children.

More in detail, 201 mothers completed questionnaires to obtain information on dietary habits of the child and family, socioeconomic variables, life style, occupational/residential exposure and fish consumption. Mothers were also subjected to the PSI (Parental Stress Index) test to evaluate the interaction of the psychological state of the mother with other environmental factors. PSI is specifically designed for the early identification of those features that may affect the normal development and functioning of the child, and it allows to identifying children with emotional and behavioral disorders and parents who are at risk of living in a dysfunctional their parental role (Guarino, Blasio et al. 2008).
Each child underwent neuropsychological testing by expert neuropsychologists at the premises of the Burlo Garofolo Pediatric Hospital in Trieste. Assessment took about four hours for each child and included the following tests:

A. Wisc-IV (Wechsler Intelligence Scale for Children) generates an IQ score which represents a child's general cognitive ability.
B. Nepsy-II is specific for assessment of sensorimotor, attention, learning capabilities
C. MT for assessment of reading skills
D. BVSCO2 for complete assessment of writing skills
E. CBCL - Child Behaviour Check List – identifies behavioural and emotional problems in children (attention deficit, hyperactive disorder, oppositional defiant disorder, conduct disorder, childhood depression, separation anxiety, childhood phobia, etc.).

An uncertainty of this study was the absence of a validated analytical method to be used to determine the single As species, other than total As. To fill this gap, we used a speciation analysis based on Ion Chromatography coupled to ICP-MS to determine and quantify six different As species in the 200 children: results showed that the high relative concentration of organic As (mainly as AsBet) reflected that the main As exposure route in children was seafood .Therefore, high concentrations of total As (which includes also organic As) do not necessarily indicate elevated exposure to iAs, which is the toxic form.
Additional source of uncertainty is questionnaire-based behavioural and dietary data. Uncertainties originating from external data exposure allow only approximate estimates of exposure patterns, but are an important indication for further investigations.

A limitation of the CROME common study, it has to be noted that the follow up has generated a selection bias, as it is well known that the likelihood of being lost to follow up is related to outcome status and exposure status. Families with a lower socioeconomic status are more likely to get out of a longitudinal study. Thus our sample is representative of more educated and healthier families, which possibly modifies the quality and quantity of environmental stressors that the child is exposed to.

Chemical analyses

Chemical analyses of biological samples collected following neuropsychological assessment consisted in measurements of the concentration of five metals reported as developmental neurotoxicants (mercury, lead, manganese, cadmium, arsenic) in either hair or urine.

Hair: Manganese (Mn) and Mercury (Hg) were analysed on 200 samples of human hair, collected in plastic bags and stored in a dryer until analysis. Before the analysis hair samples, put in glass bakers conveniently decontaminated in HNO3, were treated as follows: (i) three cycles of washes under continuous stirring for 30 min each in a mixture of 3:1 (v/v) ethyl ether-acetone (Sigma-Aldrich, St.Louis, MS, USA) to remove the sebaceous film from the hair, after each cycle hair samples were washed by high purity deionised water (EASY-pure UV, PBI, Milan, Italy) under stirring; (ii) a wash under stirring for 1 hr in 5% sodium ethylenediamine tetracetic acid (EDTA) (Sigma-Aldrich, USA) to bind the chemical elements present on hair surface and, (iii), repeated rinsing by high purity deionised water (EASY-pure UV, PBI, Milan, Italy). After drying in stove for 16h at 85 C, approximately 0.25 g of hair from each sample was then weighed. A mineralization cycle in ModBlock plate (ModBlock CPI International, Santa Rosa, CA, USA) with 4 ml of HNO3 at 60-70 C and 1 ml of H2O2 at about half an hour from the end was added. The Mn and Hg quantifications were performed using the inductively coupled plasma mass spectrometry (iCAP Qs ICP-MS, Thermo-Fisher, Bremen, Germany). The instrument configuration and operation parameters are shown in Table 1. The iCAP Qs ICP-MS used in this study was equipped with a PFA ST MicroFlow nebulizer (ESI, Omaha, NB, USA), a Peltier cooled quartz spray chamber (operating at 3 C), a 2.0 mm ID sapphire injector and a quartz torch with Ni sampler and skimmer cones. .

Table 1. Instrument configuration

RF power	1550 w
Argon gas flow (L/min)	Plasma 14, Auxiliary 0.8, Nebulizer 1.08
Collision cell gas	He at 4.2 mL/min
KED barrier	2 V
Analytical parameters	Dwell time 80 ms per peak, 50 sweeps, 3 replicates
Analytical masses	55Mn, 202Hg
Internal standard	115In

Urine: Urine samples (ca.100mL) were collected in polyethylene containers and stored at-20 C until analysis. The containers were previously decontaminated with diluted (10% v/v) ultrapure HNO3 (Normatom, Leuven, Belgium) and rinsed with high-purity deionized water (Barnstead EASY-Pure II, Dubuque, IA, USA). Urine were analyzed by a in-housevalidated method, which was previously accredited by the Italian accreditation body (ACCREDIA). Urine samples were diluted 1:5 (v/v) with high-purity deionized water before metal quantification. Metals were measured by sector field inductively coupled plasma mass spectrometry (SF-ICP-MS, Element 2, Thermo Fischer, Bremen, Germany) working at low resolution mode (LR, m/Δm = 300) for not interfered metals: 114Cd, 202Hg, 208Pb; at medium resolution mode (MR, m/Δm = 4000) for interfered metal: 55Mn; at high resolution mode (HR, m/Δm = 10,000) for heavily interfered metals: 75As and 82Se. The instrument was equipped with Ni sampler and skimmer cones, a Meinhard nebulizer, a water cooled spray chamber (Scott-type) and a guard electrode device. The matrix-matched calibration combined with the internal standardization by 115In and 69Ga (1 ng/mL in the analytical solutions) provides a satisfactory method for compensating for any residual matrix effects and to minimize instrumental drifts. To test the accuracy of the analytical procedure two certified reference materials of lyophilized human urine were used: Seronorm (Sero AS, Billingstadt, Norway) and ClinCheck (Recipe, Munich, Germany).

Statistics

The main exposures of interest were metals' levels in the blood (Hg, Mn, Pb, Cd, As) and hair (Hg, Mn) of 7-year old children . As in the original PHIME study fish intake during pregnancy by the mother was considered as the source of exposure to Hg, intake of different kind of fish by the child was estimated from the same detailed 138-item

FFQ completed by the mothers soon after delivery. The questionnaire included 7 quantitative questions on fish, which addressed the frequency of consumption of 150-gram servings of fish, crustaceans, and molluscs (cooked according to different recipes), and fish in oil. The frequency distribution of those variables was described by using absolute numbers and percentages of subjects in each category. For each fish item, conversion from categories of consumption into continuous intakes of fish servings was done by assigning to each category a consumption level equal to the median value for that category (eg, 2–4 times/week became 3 times/week). Overall fish intake was calculated by summing the estimated weekly intake of all fish types.

Distributions of fish intake and metals' concentration were represented by arithmetic means and SDs, quartiles, and minimum-maximum ranges. Geometric means for Hg concentration were also calculated. Normality of distribution was assessed by the Shapiro-Wilk test. MeHg concentration in biological samples, which was known in a subsample of the cohort, was compared with THg concentration among the corresponding subjects.

The main outcomes of interest were composite cognitive, social-emotional, and adaptive behavior scores. The distributions of neuropsychological scores were represented by arithmetic means and SDs, quartiles, and minimum maximum ranges. Differences between groups were assessed by the Wilcoxon rank sum test (for continuous variables)

and the $\chi 2$ test (for categorical variables). Crude associations between metals' concentrations and each neuropsychological score were analyzed using linear regression analyses.

Multivariate linear regression was used to assess the association between Hg and neurodevelopment, after adjustment for potential confounding variables. The following covariates are hypothesized to affect child neurodevelopment and were thus considered in the analyses: intake of fresh, frozen, and canned fish by the child and by the mother during pregnancy , the child`s sex, birth weight, gestational age, maternal intelligence quotient (IQ), maternal age at delivery, BMI before pregnancy, weight gain, marital status at delivery, socioeconomic (SES) index (adapted from Bennett et al28), size of the home, number of adults and children living in the home, cigarettes smoked and alcohol intake during pregnancy. Models analyzing cord blood also included concentrations of Cu, Zn, and Se.

Because the data for most covariates were derived from questionnaires, which can be incomplete, and to avoid loss of statistical power in multivariate analyses because of missing values, imputation was done for some variables, assuming that it was completely random. Among the covariates considered as potential confounders, only those associated with at least 1 neuropsychological outcome ($P < 0.10$) were included in the final models.

We used analyses stratified by the child`s sex to assess whether the effect of Hg on neurodevelopment differed in boys and girls.

Data were analysed by Pearson correlations between pairs of exposure and outcome variables and multiple regression analyses by using the SPSS software.

Latium Region - Levels of metals in adolescents

In the context of the PROBE (PROgramme for Biomonitoring of the Exposure), a biomonitoring campaign of the Italian population started in 2008 for the detection of several metals in blood, a special survey was addressed to the adolescents and conducted in cooperation with the National Association against Microcytemia (ANMI) during the annual screening for thalassemia among the school population. Within the national case study in Italy the protocol consisted in enlarging the first PROBE cohort to a total of 453 adolescents and in re-analyzing the whole biomonitoring data set , considering:

i) the evaluation of questionnaires to link metal levels in adolescents` blood and life style and demographic characteristics of each subject as: sex, residence area, traffic intensity, Socio Economic Status (SES), presence of dental fillings and braces, piercings and tattoos presence, second hand smoke, fish and milk consumption.

ii) the collection of air quality data (benzene, CO, NO_2, NO_X, O_3, PM_{10}, $PM_{2.5}$) from air quality monitoring stations nearest to the residence addresses of each subject, identified by GIS and supplied by Regional Environment Authority (ARPA, Latium Region) to link metal levels in adolescents` blood and levels of selected air quality markers.

iii) The collection of data on the As content in drinking water from the Viterbo area to link metal levels in adolescents` blood and exposure to As *via* water.

Population

An adolescent cohort of 453 subjects, aged 13-15 years, living in urban and rural areas of Latium region was enrolled for their exposure to metals. The adolescent distribution in the four sampled areas was: 160 from Viterbo (small town), 131 from Fontenuova and 72 from Monterotondo (rural semi-rural areas), and 89 from Rome (big town). Non-fasting blood specimens were obtained by ANMI during the annual screening for microcytemia in schools (2009). In blood sample 19 metals were investigated: As, Cd, Co, Cr, Hg, Ir, Mn, Mo, Ni, Pb, Pd, Pt, Rh, Sb, Sn, Tl, U, V, and W.

Analysis

Blood samples were stored at -20 C and in the laboratory 1 mL of blood sample out of ca. 1.5 mL collected was microwave digested (Milestone ETHOS MEGA II, FKV, Bergamo, Italy) with ultra-pure HNO_3 (Romil Ltd., Cambridge, UK) and subsequently diluted with high purity deionized water (EASY Pure system, Barnstead, Dubuque, USA). The sector field inductively coupled plasma mass spectrometry (SF-ICP-MS, Element 2, Thermo Scientific, Bremen, Germany) analyses were carried out to determine metals content.

The method used for blood analysis was in-house validated and accredited by ACCREDIA (the Italian Accreditation Body) and the following validation performances were assessed: linearity, Limit of Detection (LoD) and Limit of Quantification (LoQ), specificity, accuracy (precision and trueness), robustness and extended uncertainty (AOAC, 1998; Commission Decision 2002/657/EC; LGC, 2003; NATA, 2009; Thompson et al., 2002).

For each donor a questionnaire was also filled in order to collect information on:

- general data such as gender, piercings, junk jewelry, diet habits, drinking water, lifestyle factors (e.g. exposure to environmental tobacco smoke, alcohol consumption, use of cosmetics, etc.), dental fillings and braces, family occupational status. Socio economic status (SES) of the family was derived merging the educational level and the occupational status of the parents; the International Standard Classification of Occupations (International Standard Classification of Occupations, 2008) was adopted to describe the parental occupations. From the questionnaires administered several 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 the diet habits, 265 adolescents consumed fish 1 time a week (1/w) and 81 2 times 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/w).

The exposure assessment was completed by geo-referencing all subjects with their residence address location by GIS tools and considering also the lifestyle of the adolescents and their families.

The main limitation of the PROBE adolescent study was the lack of information on the health status of the subjects monitored and the the impossibility to contact again the same subjects. Without this information, we were unable to linking metals content in adolescents` blood and potential effects on adolescents` health.

Environmental data

To have a view of the exposure in the exposome sense, metal concentrations were related to environmental data of air and water quality supplied by Regional Environment Authority (Latium Region). Kriging techniques were applied to derive spatially resolved concentration of chemicals in the outdoor air starting from data collected by air monitoring stations. Chemicals considered were NOx, PM10, PM 2,5, benzene, CO, NO_2, O_3.

Statistical analysis

The basic statistics of data relating to 453 adolescents included percentiles, geometric mean (GM) and the 95% confidence interval for the geometric mean (CI, GM). Geometric mean represents better the central tendency of data if the distribution is asymmetric instead of the median index. In the statistical evaluation values below the LoD were taken into account as LoD/2 and extreme values were excluded. This procedure was used to derive Reference Values (RV) where the 95th percentile describes the upper value useful in health care field 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, SES. Each variable was coded according to the levels applied in the questionnaires: number of dental fillings and/or braces, piercings and tattoos (0: no; 1: yes), second hand smoke (0: no; 1: at home; 2: outdoor), frequency intake of fish (0: never; 1: 1 time/week; 2: >=2 times/week) and milk consumption (0: never; 1: 1-3 times/week; 2: >3 times/week; 3: 7 times/week), SES of the family (0: low; 1: medium; 2: high). For all the comparison and other statistical analysis the data base, included the extreme values, was considered. Differences for each metal concentration among subgroups based on the different variables were tested by Mann-Whitney U test, or Kruskall-Wallis or Wilcoxon 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 a $p<0.05$. Statistical calculations were performed by STATA statistical software Release 8.1 (STATA Corporation, TX). To perform geo-statistical analysis all the 453 subjects were geo-referenced on the basis of their residence address in a GIS system and stored in a Geodatabase along with human biomonitoring data, diet habits, environmental data and land cover. Generalized Linear Model (GLM) and the environment-wide association study (EWAS) were used to investigate the associations between human biomonitoring data, life-style, diet patters (fish and milk) and land cover. EEA 2006 (http://www.eea.europa.eu/data-and-maps/data/corine-land-cover-2006-raster-3) land cover at high spatial resolution (100 m) was used to analyze possible spatial relationships between the type of land use where adolescents were living and the metals concentration in blood.

Slovenia

National HBM data and identifying sources of exposure

National HBM programme was first conducted in Slovenia in the period between 2007 and 2015 and its execution was based on the legislation for the implementation of the HBM programme in Slovenia (defined in the Article 49 Act of Chemicals of the Chapter IX). The programme included collection of biological samples to monitor internal exposure of inhabitants of different areas of the country, but it didn`t include monitoring of external exposure. Within the CROME, several environmental campaigns along with the collection of ancillary data were designed and executed to fill data gaps on external exposure. Having additional data collected and linked to the existing HBM data, CROME contributed significantly to the long-term objectives and policy requirements of the HBM programme in Slovenia.

The targeted environmental campaigns included collection of tap water samples from 108 location across Slovenia and sampling of moss from 102 locations in Slovenia as a bio-indicator of air quality and deposition. Air quality (AQ) data was complemented with data from AQ monitoring network (O3, PM2.5 and PM10) on a European scale and data on emissions and releases from point and diffuse sources (NOx, SOx, CO, ammonia NH3, carbon dioxide CO2 and PM10). Chemical composition of soil was obtained from interpolated geochemical map of Slovenia. Additionally, we collected information on build environment from Cadastre of public infrastructure. As a proxy for fertilizer use, modelled nitrogen and phosphorous surpluses were obtained from the Geological Survey of Slovenia.

Specific uncertainties related to individual proxies are the following. In the case of soil geochemical composition, layers used are a result of spatial interpolation based on point data. Nitrogen and phosphorous surpluses are estimates only based on prevailing land use in the area. In case of European scale AQ data these have a very coarse resolution and as such do not provide an insight detailed enough into exposure of an individual and differences between different population groups, respectively. Similar, in the absence of actual data on traffic density, type of road infrastructure in the vicinity of individuals leaving environment was used.

The collected environmental data along with the questionnaire-based surveys of behavioural and dietary habits of the HBM study population supported the derivation of overall exposure patterns of the population across the country. The main difficulties encountered were differences in spatial resolution between different environmental databases and certain parameters used as proxies (e.g. nitrogen surplus as a proxy for artificial fertilizer use) which increases uncertainty of environmental data in compare to precise data on internal exposure. Specific uncertainties related to individual proxies are the following. In the case of soil geochemical composition, layers used are a result of spatial interpolation based on point data. Nitrogen and phosphorous surpluses are estimates only based on prevailing land use in the area. In case of European scale AQ data these have a very coarse resolution and as such do not provide an insight detailed enough into exposure of an individual and differences between different population groups, respectively. Similar, in the absence of actual data on traffic density, type of road infrastructure in the vicinity of individuals leaving environment was used.

Additional source of uncertainty is questionnaire-based behavioural and dietary data. Uncertainties originating from external data exposure allow only approximate estimates of exposure patterns, but are an important indication for further investigations.

Cross-Mediterranean study

As part of the Cross-Mediterranean study, Slovenian population of children recruited at birth within the PHIME project has been followed-up. Aiming to assess the association between prenatal exposure to mercury (Hg) and neurodevelopment/neurobehaviour, the children were re-assessed neuropsychologically using Weschler Intelligence Scale for Children (WISC) at 7-8 years of age. As it is well known, that several factors can modulate the neuropsychological outcome, among them co-exposure to other metals/elements and genetic factors, the protocol included besides WISC testing also determination of Hg and other relevant elements (Pb, Mn, Cd, As, Se, Zn, Su) in biological matrices and analysis of gene polymorphisms, based on which certain individuals may have a predisposition to more adverse health effects than those reported in the general population.

Polymorphisms that were selected for the CROME Cross-Mediterranean protocol, are on genes that code for enzymes involved in absorption, distribution, metabolism or elimination of metals and some also directly in brain development processes (e.g. synaptogenesis). To this date, genotyping analysis along with the internal exposure and neuropsychological assessment were completed on a limited sample size, as is available for Slovenian study population (n=179). Such small sample number doesn`t allow for stratification of the collected data by genotype. Association between prenatal/childhood exposure and intelligence (WISC) score will be further assessed as database is completed with data from other study populations (Croatia, Italy).

BPA levels in children and their mothers and fathers

Bishenol A (BPA) levels in urine of 6-11 years old children, their mothers and fathers that were obtained for Slovenian population in the framework of a demonstration project DEMOCOPHES, were evaluated for the first time within the CROME, with regard to risk assessment. Based on the urinary data, daily intake for the individuals was back- calculated using a physiology based biokinetic (PBBK) model properly parameterized for BPA, coupled with an exposure reconstruction algorithm. Re-running the PBBK model in forward mode allowed the estimation of biologically effective dose (free plasma BPA) and the respective daily area under the curve (AUC). Finally, risk characterization ratio was derived using both external and internal dose metrics.

Spain

Cross-Mediterranean study - Sabadell - Metal exposure in pregnant women

As part of the INMA research network (Childhood and Environment) (Guxens, Ballester et al. 2012) 657 pregnant women were recruited in their 12th week medical visit in the Sant Fèlix Primary Care Center II (Sabadell), between 2004 and 2006. Recruitment involved only those women that lived in Sabadell, were older than 16 years, had a singleton pregnancy, volunteered for the program and wanted to give birth at the Hospitals of Sabadell or Terrassa (a nearby city). Women suffering from chronic diseases, having impaired communication or that become pregnant by assisted reproduction were excluded. After obtaining the consent from the admitted women, questionnaires were administered by trained interviewers in the 12th and 32th weeks of pregnancy. This population provides a representative example of the urban populations in the Spanish Mediterranean areas.

Urine samples were collected in 100 mL polypropylene containers in the first and third trimester of pregnancy from 489 pregnant women of this cohort. The samples were stored in polyethylene tubes at -20ºC until further processing. This research project was approved by the Research Ethics Committee of the CREAL. To maintain confidentiality, participant information was encoded.

Analysis of urine samples

Prior to Q-ICP-MS analysis, the samples were digested and diluted to oxidize and remove organic matter and to minimize the concentrations of inorganic solids (Krachler, Radner et al. 1996, Castillo, Martinez et al. 2008). Three mL of each urine sample, 3 mL of Instra-Analysed 65% HN03 (J.T. Baker, Germany) and 1.5 mL of Instra-Analysed 30% H2O2 (Baker) were introduced in Teflon vessels. The mixtures were left in an oven at 90ºC overnight. After cooling, the vessels were opened and then placed on a heating plate at 250 C to evaporate off the nitric acid. Once dried, the resulting solid samples were dissolved in 3 mL of 4% HN03, placed in 7 mL glass bottles and subsequently stored in a refrigerator until instrumental analysis. Before analysis, an internal standard of 10 ppb of In was introduced and depending on sample density they were diluted to 30 mL or 60 mL with MilliQ water to avoid non-spectral interference.

Q-ICP-MS analysis was performed by an X-SERIES II (Thermo Fisher Scientific) instrument. Specific isotope ions for Co and Ni were selected in order to avoid potential calcium interferences from the sample matrix. Cl atoms may also potentially interfere in the determination of As and Se. In these cases the collision/reaction cell technique should be added to the instrumental methods but no interferences were observed in the present samples and these cells were not used. Instrumental limit of detection (LOD) for all metals was 0.2 ng/mL attending to the most reliable lowest calibration point. The two samples corresponding to the first and third trimesters of each subject were digested and analyzed at the same time. One MilliQ water blank was processed in each batch of samples to control for possible contamination. If there was any contamination, thorough cleaning of all material was performed and digestion was repeated. Field samples were also obtained by analysis of Milli Q water which was previously stored in the containers used for maternal urine bottles and transported together with the samples.

A Bio-Rad Level 1 (Lyphochek Urine Metals Control 1-69131; Marnes-la-Coquette, France) urine reference was extensively used to evaluate the developed methodology, as it contains metal concentrations close to those in the urine samples from the study cohort. This reference material provided certified values for As, Cd, Co, Cr, Cu, Mn, Ni, Pb, Sb, Tl, Zn and Se. Prior to digestion, the lyophilized reference urine samples were reconstituted with 25 mL of MilliQ water as recommended by the manufacturer.

All glassware and polypropylene material was thoroughly cleaned by soaking in 10% nitric acid for 24 h, followed by rinsing three times with MilliQ water. The Teflon vessels were cleaned after each use by rinsing with 10% nitric acid three times, and, following the last rinse, leaving them in the oven at 90ºC overnight. Finally, the vessels were rinsed with a large volume of MilliQ water.

Creatinine was determined at the Echevarne laboratory of Barcelona by the Jaffé method (kinetic with target measurement, compensated method) with Beckman Coulter© reactive in AU5400 (IZASA®).

Statistical analyses

Descriptive statistical parameters were initially computed. Values for mean, standard deviation (SD), median and P91 were calculated for the metal concentrations. Normality was checked by the Kolmogorov-Smirnov test. The metal concentrations between the first and the third trimesters were compared using Spearman correlations and paired Mann-Whitney hypothesis tests. The individual ratios between the metal concentrations during the third and first trimesters were also calculated. Mean, standard deviation and median values for these ratios were computed. All statistical analyses were performed using Stata 12.0 software (Stata Corporation, College Station, Texas).

Asturias - Exposure to organohalogen compounds

The cohort of study was established in Asturias by the Preventive Department of the University of Oviedo, as part of the INMA –Infancia y Medio Ambiente (Environment and Childhood) Project (Guxens, Ballester et al. 2012). 494 pregnant women were recruited between May 2004 and June 2007. Deliveries took place between October 2004 and February 2008 at the reference hospital San Agustin, in Avilés (Asturias, Spain). 326 cord blood samples were successfully collected from assistance to 485 childbirths within the cohort population. 308 mother-umbilical cord blood paired samples were finally available as consequence of this project. Placental tissues were collected in a subset of 50 women. We present data of POP concentration for the 308 paired samples available and 50 placenta samples. The characteristics of the mothers from this group of 308 paired samples did not show significant differences from the whole recruited group. The study protocol was approved by the Ethics Committee of the reference hospital, and informed consent was obtained for every participant.

Data and sample collection

Maternal blood samples were collected during the first trimester of gestation (median = 12 weeks; range = 10-13 wks).Whole cord blood samples were collected using venipuncture of cord vessels before the placenta was delivered. Maternal and cord serum were collected after centrifugation for 10 minutes, separated into aliquotes of 1 ml and stored at −80 C until analyses. The whole placenta was collected immediately after delivery. Half of the placenta, including maternal and fetal sides and central and peripheral parts, was placed in a glass container of a mixer (Büchi Mixer B-400 Büchi Laboratories AG, Flawil, Switzerland) for its homogenization. Once homogenized, aliquots of 25 g were stored and frozen at −80ºC. Pregnant women completed two detailed in-person questionnaires (weeks 10–13 and 28–32) on anthropometric and sociodemographic characteristics and lifestyle variables.

Laboratory analyses

The laboratory analytical methods and quality control procedures for the analysis of POPs have been described elsewhere (Vizcaino, Arellano et al. 2009, Grimalt, Howsam et al. 2010). Concentrations of 7 PCB congeners (PCB28, PCB52, PCB101, PCB118, PCB153, PCB138 and PCB180), α-HCH, β-HCH, γ-HCH, δ-HCH, HCB, PeCB, 2,4`-DDT, 4,4`-DDT, 2,4`-DDE, 4,4`-DDE, 2,4`-DDD, 4,4`-DDD and 14 PBDE congeners (BDE17, BDE28, BDE47, BDE66, BDE71, BDE85, BDE99, BDE 100, BDE153, BDE154 BDE138 BDE 183 and BDE 190 and BDE 209) were analyzed in placental, maternal and cord serum samples.

Briefly, 1 mL of serum or 1 gr of placental tissue were spiked with the surrogate standards tetrabromobenzene (TBB) and decachlorobiphenyl (CB 209) and vortex stirred for 30 s at 2,000 rpm. n-Hexane (3 mL) was added, followed by concentrated sulfuric acid (2 mL). After reaction, the mixture was stirred for 30 s and the supernatant n-hexane phase was separated by centrifugation. The remaining sulfuric acid solution was re-extracted twice with 2 mL of n-hexane (each by 30 s stirring and centrifugation). The combined n-hexane extracts (7 mL) were additionally cleaned with sulfuric acid (2 mL, stirring 30 s). Then, the n-hexane phase was separated by centrifugation and reduced to a small volume under a gentle nitrogen stream. The extract was transferred to gas chromatography (GC) vials using four 25 μl rinses of isooctane. CB 142, BDE 118 (20 μl) and [13C]-BDE 209 (10 μl) were added as internal standards before injection. Organochlorine compounds (OCs) were determined by GC with electron capture detection (GC-ECD). BDE congeners were analyzed by GC coupled to mass spectrometry in chemical ionization mode and negative ion recording.

Total cholesterol and triglycerides were determined in maternal and cord serum samples using colorimetric enzymatic methods in the General Biochemistry Laboratory of Hospital San Agustin. The samples were processed using a Roche Diagnostics COBAS C711. Total serum lipids concentrations were calculated as described by Phillips et al (1989) using the following formula:

$$TL = (2.27 * TC) + TG + 62.3 \text{ mg} \cdot \text{dL-1}$$

Placental lipids were determined gravimetrically. 1 gr of placental tissue was homogenized in 5 ml of chloroform:methanol:hydrochloric acid (20:10:0.1) (v/v/v). After repeating the process, 10 ml of 0.1 N HCl were added and centrifuged at 3000 rpm for10 min. The organic phase was then collected; the non-organic phase was re-extracted and added to the first extraction product. Total lipid content was determined after drying the organic extracts under a nitrogen stream to constant weight and total lipid were expressed in grams of lipid per gram of placenta (Lopez-Espinosa, Lopez-Navarrete et al. 2008).

Validation of analytical results (including POPs and total lipid concentrations) was made by analysis of reference material obtained from the Artic Monitoring and Assessment Program (AMAP). We participate regularly in the AMAP Ring Test Proficiency Program for POPs in human serum (Centre de Toxicologie Institut National de Santé Publique du Québec, Québec, Canada) and the laboratory results usually are within 20% of the consensus values, including lipid concentrations.

Data analysis

POP levels were expressed in ng/mL. They were also adjusted to total serum lipid concentrations (ng/g lipid). Values of half detection limit were assigned when measurable analyte concentrations were not found. Spearman correlation and scatter plots were used to examine associations between POP levels in placenta, maternal and cord serum. Placental transfer was evaluated by calculation of the concentrations ratios between paired samples for each compound on ng/mL and ng/g lipid:

$$Rcm= Cuc/Cm; \quad Rpm= Cp/Cm$$

where Cuc is the umbilical cord concentration, Cm is the maternal concentration and Cp is the placental concentration. Correlations and concentration ratios were calculated excluding non detected values. Values exceeding three times the standard deviation of the mean were considered outliers and consequently excluded from the ratio calculations. The ratios of each compound were only calculated if there were at least 10 paired samples above the detection limit (Needham, Grandjean et al. 2011). Regression analyses with forcing regression to 0 were also calculated. No major differences were found between these options (data non shown). Therefore, only median concentration ratios are reported.

Gestational weight gain

GWG was defined as the difference between the last recorded weight during pregnancy and the mother`s self-reported pre-pregnancy weight. To avoid possible heteroscedasticity and non-linearity effects, GWG was calculated from weekly GWG rates (wGWG) during pregnancy, which were calculated for each week based on differences between weight measurements from prenatal visit records and the self-reported pre-pregnancy weight, divided by the gestational age at each measurement. The average (± SD) number of weight measurements per mother was 6.5 ± 2.7. Self-reported pre-pregnancy body weight was replaced with the first clinical record of body weight if it was measured before 8 weeks of gestation, and the second measure was not recorded before 15 weeks (n=1) (Nielsen et al., 2006). Missing or implausible self-reported pre-pregnancy weight (when wGWG was >1.5 kg/week or <-1 kg/week) was imputed by linear extrapolation when two GWG were recorded before 15 gestational weeks (n=4).

Total rate of weekly GWG (twGWG) was calculated as the difference between the last weight at the end of pregnancy and the self-reported pre-pregnancy weight divided by gestational age. For women whose last weight measurement was recorded during 37.5-42 weeks of gestation, we used the measurement taken closest to 39 weeks (wGWG39). This measurement was not necessarily the last, since GWG stalls at the end of pregnancy.

For 194 women who did not have a body weight measurement recorded during 37.5-42 weeks of gestation, we calculated twGWG39 from their last measured weight. Specifically, for woman i and week k:

$$twGWGi39 = mean(wGWG39) + \{[twGWGik - mean(wGWGk)]\ [sd(wGWG39)/sd(wGWGk)]\} \quad [1]$$

The mean(wGWG39), mean(wGWGk), sd(wGWG39), and sd(wGWGk) values were obtained from the wGWG distribution of a pooled INMA cohort encompassing pregnant women from Asturias, Gipuzkoa, Sabadell, and Valencia (n=2,413) The wGWG distributions in the Asturias and the reference INMA cohorts were similar involving higher wGWG and lower heteroscedasticity (SD) at higher gestational age which is in agreement with previous studies (Dietz, Callaghan et al. 2006, Kleinman, Oken et al. 2007, Nohr, Timpson et al. 2009).

Finally, total GWG was calculated as the product of gestational age in weeks and twGWG39. This variable was modeled as a continuous variable (total kg) and as a categorical variable (recommended, inadequate, or excessive) as defined by the Institute of Medicine (Rasmussen, Stene et al. 2009) according to pre-pregnancy BMI. Specifically, recommended GWG is 11.25-15.75 kg for women classified as having normal pre-pregnancy BMI (as defined by the World Health Organization), and as 12.60-18.00 kg, 6.75-11.25 kg, and 4.95-9.00 kg for women classified as underweight, overweight, and obese, respectively.

Other variables

Gestational age was calculated from the date of the last menstrual period (LMP) reported at recruitment and was confirmed using ultrasound examination in week 12 of gestation. If reported gestational age and ultrasound determination differed by more than 6 days (12.9% of participants), it was recalculated from the crown-rump length using an early ultrasound measurement (Westerway, Davison et al. 2000).

Age, pre-pregnancy BMI, parity, education level, social class, fish intake and previous breastfeeding history were considered potential confounding factors because of their possible associations with GWG and POP serum concentrations (Sarcinelli, Pereira et al. 2003, Vizcaino, Grimalt et al. 2011, Glynn, Miller et al. 2012). Pregnant women completed two detailed in-person questionnaires (weeks 10–13 and 28–32) and provided information on parity, age, social class (defined from a widely used Spanish adaptation of the international ISCO88 coding system (Vrijheid, Casas et al. 2012), education level, pre-pregnancy BMI (based on measured height at recruitment and self-reported pre-pregnancy weight) and previous breastfeeding history. Dietary information was collected from a validated semiquantitive food frequency questionnaire of 101 food items (Vioque, Navarrete-Munoz et al. 2013) and was focused on dairy products, meat, vegetables, fruits and fish. Total fish consumption included lean fish, oily fish, canned tuna fish, seafood as well as processed fish, mixed fried fish, and dried or smoked fish.

Existing data and data gaps encountered

Greece

Oinofoita – Cr(VI)

To the best of our knowledge, there are no systematic measurements of Cr(VI) before 2007. Since 2007, three independent sets of hexavalent chromium measurements are available for the Oinofyta area. These include:

- a study of the Institute of Geology and Mineral Exploration (IGME 2008) during the period November 2007 to February 2008, which detected 35 samples (out of 87) taken from different wells in the same area, where levels above 10 μg/l with a maximum value 156 μg/l were detected;
- a study conducted by the faculty of the Geology and Geo-environment department of the University of Athens (Ch. Vasilatos, I. Megremi et al. 2008) during the period September 2008 to December 2008, in which Cr(VI) levels ranged from 41 up to 53 μg/l in three samples taken from the public drinking water supply of Oinofyta; and

- repeated measurements by the Oinofyta municipality in the public drinking water supply during the period July 2007 to July 2010, in which there are 13 measurements with levels above 10 μg/l and with a maximum value of 51 μg/l

According to official Oinofyta municipality authorities, in early 2009 the main drinking water supply of Oinofyta was diverted to receive water from Mornos lake (reservoir) which is part of the drinking water supply network of the city of Athens. Therefore, more recent measurements made by the Oinofyta municipality (June 2009- July 2010) record relatively lower levels of Cr(VI) (<0.01-1.53 μ g/l).

Furthermore, in the study carried out by Economou-Eliopoulos et al. (2011), groundwater samples from the Asopos aquifer showed a wide spatial variability, ranging from <2 to 180 ppb Cr total content [almost same to the Cr(VI)-values] despite their spatial association. The presence of Cr(VI)-contaminated ground water at depths >200m is attributed to a direct injection of Cr(VI)-rich industrial wastes at depth rather than that Cr(VI) is derived from the Asopos river or by the interaction between water and Cr-bearing rocks.

Human biomonitoring data are the critical data missing for the application of the CROME methodology in Asopos basin. Given that environmental concentrations and actual exposure have been decreased after 2007, we need to identify recent, as well as past exposure. For this purpose, a combination of biomonitoring data will be applied, including urine samples (for assessing current exposure levels), as well as hair samples for assessing exposure burden from the past. For the purposes of the analysis, a sample of 20 residents will be collected.

Aspropyrogos – PCDDs/PCDFs

In order to estimate the risk related to the PCDDs/PCDFs emitted during the fire, it was critical to estimate the long term internal burden of exposure associated to this event. The need for addressing long term exposure is associated to the fact that PCDDs/PCDFs are bioaccumulative and persistent with a half-life time of almost 7.5 years in humans. Hence, it is critical to translate the actual uptake during the accidental event (that lasted for a few days) into a long term (lasting for many years) internal exposure burden. The only scientifically sound way to translate these external doses into internal exposure to the target tissues was carried out with physiology based biokinetic (PBBK) models. To be able to perform this type of calculation, it was critical to be able to identify (a) the background exposure levels to PCDDs/PCDFs and (b) the additional burden of exposure due to this accidental event.

To be able to estimate the additional risk posed by exposure to the accidental event utilizing the INTEGRA platform the following data were needed:

- Data on ambient air levels of PCDDs/PCDFs during the accidental event were obtained by various measurements of PM and analysis of PCDDs/PCDFs in the particle and gaseous phase.
- HBM data for estimating the background exposure to the exposed population. In practice, the HBM data of the local population collected from a previous study (before the accident) where used to estimate the equivalent background exposure that results in the corresponding HBM data; the additional exposure of the measured PCDDs/PCDFs was added to these background levels for a duration of 6 days.

Thessaloniki PM-PAHs

There are plenty of detailed environmental data related to PM concentrations and chemical speciation. This data incorporated to advanced modelling framework described above will allow the accurate assessment of external and internal exposure to PM. Exposure to PAHs and consequently to PMs, will be verified by PAHs metabolites measurements in urine. Health data for the relevant health endpoints mentioned in will be retrieved by the Hellenic Statistical Authority and the hospitals of the wider Metropolitan area of Thessaloniki.

In order to fill the biomonitoring gaps, a biomonitoring sampling campaign took place in winter of 2014-2015 in Thessaloniki, aiming to capture the effect of biomass burning in PM and PAHs exposure. Overall 50 of non-smoker individuals aged 8 to 64 will be sampled, covering a wide spatial distribution of the city. This will be necessary, since differences in exposure are expected to be found based on the levels of the performed activities, the ventilation conditions, as well as the respective distribution of PM and PAHs levels in the city.

Italy

Trieste - Cross Mediterranean Study

Existing data derived from the follow-up of the Italian birth cohort while indicating that internal concentration of metals and metalloids is associated with neuropsychological maturation of 7-year old children, highlight the complex interaction between multiple environmental exposures, dietary and life style factors. One major question which arises is relative to the exposure history of the children enrolled in the follow-up: what is the role of earlier exposure to the same metals/metalloids on neuropsychological performances at 7 years? The design of the original PHIME study consisted in collection of biosamples from mothers and their newborns during pregnancy (venous blood and hair), at birth (cord blood) and at 1 month post-partum (breast milk). In general the existing data base contained several information in order to apply a multivariate model taking into account the different factors that might have influenced the neuropsychological functioning at school age. On these bases, for each of the 200 children we were able to use also available data relative to perinatal levels of metals and essential elements in all these matrices. Altogether, we eventually obtained a picture where both levels of metals such as Mercury and Lead and essential elements such as Zinc, Selenium and Arsenic in the fetal and neonatal stage influence neuropsychological performances as measured at 7 years. The extent as well as the direction of the effects depend on the kind of matrix analysed and on time of measurement (see Del 5.2 for details).

A first important gap in knowledge concerned the role of dietary habits other than fish consumption: what other food items may concur to metal exposure in children? No updated indications concerning the levels of metals in food items in the areas where the population under study lives are available. A report on the national levels of some metals in fish and seafood product in Italy has been published in 2012 (Pastorelli, Baldini et al. 2012). Some information useful for evaluation of this specific issue may be made available by Slovenian and Croatian partners/subcontractor. A 3-day-diary assessing the quality and quantity of food items consumed by the children in the 3 days preceding the neuropsychological testing has been carried out at the follow-up but data have not been made available within the end of the CROME project. We will possibly have these data analysed within June 2017.This aspect is particularly important as some children presented very high urinary concentration of arsenic (As). Concentration of As may be interpreted in a risk-assessment context differently if As arises from exposure to iAs species (As(III) and As(V)), methylated As (MMA and DMA) or organic As (AsBet and AsChol). Thus the measurement of total As concentration (which contains inorganic and organic As) cannot be used solely to explain the biological and toxic effects of As.

However, the concentrations of the sum of As species [iAs+DMA+MMA] exceeded the levels that are consistent with non-cancer risk levels in some children (21%); this part of population will require principal attention. Moreover, information on other food sources, mainly rice and water consumption is still needed to better understand if other sources might contribute to the concentration of inorganic As in children urine.

A second gap concerns the lack of information on the environmental sources of metals/metalloids exposure other than diet. The Regional Agency for Environmental Protection (ARPA) of Friuli Venezia Giuli Region assures continuous monitoring of air and water. Data concerning chemical and microbiological characteristics of drinking water as well as air pollution, in particular with respect to VOCs, PM10, NO2 and benzene concentration in different areas of the Trieste municipality are available but so far they have not been included in data analysis. As geolocalization of children enrolled in the study is available we have reconstructed exposure through reverse dosimetry so as to estimate the possible contribution of environmental sources to metals/metalloids levels measured in children during the period of the follow-up. The scarcity of air pollution monitoring in the Trieste municipality did not allow us to differentiate among children exposure levels to air pollutants. In addition, we do not have any information on indoor pollution.

A third gap in knowledge regards the issue of specific gene vulnerability: it has been suggested that relatively common gene variations may confer susceptibility to even low dose of Hg. Might this be a factor that increase the risk for a subset of children is an issue worth of further investigation. As saliva has been collected from each child at the time of assessment, we plan to perform the genome-wide analysis of this sample provided we will receive funding from grant applications.

Finally, since children have been assessed at different life stages, there is the need to identify new methodological tools for tracing developmental trajectories to unravel the complex interplay among different metals and micronutrients and their effects on neuropsychological development as detected through different test batteries.

Italy

Latium Region - Level of metals' in adolescents

A first gap in knowledge refers to the fact that in the PROBE adolescent study only data on the ematological characteristics of each individual together with information derived from questionnaire (see below) were available For the specific aim of the campaign originally promoted by AMNI, no information on the health status of the adolescents understudy was collected.

Another gap of knowledge was the potential association between the information obtained by the questionnaire regarding life style and/or the demographic characteristics of each adolescent and metals content in adolescents` blood. To fill this gap, we applied statistical calculations to higlights differences in metal levels subdividing the PROBE adolescent cohort according to variables as sex, residence area, traffic intensity, Socio Economic Status (SES), presence of dental fillings and braces, piercings and tattoos presence, second hand smoke, fish and milk consumption.

Another lack of information was the potential association between air quality data in the Latium region understudy and metals content in adolescents` blood. To fill this gap, we asked to the Regional Environment Authority (ARPA) of the Latium Region the level of some air quality markers, as benzene, CO, NO_2, NO_X, O_3, PM_{10}, $PM_{2.5}$, collected in the same period of biological specimen sampling from stations near, as much as possible, to the residence addresses of each adolescent .

Also the association between the content of metals in the water and metals content in adolescents` blood was missing. To fill this gap, we asked to the Regional Environment Authority (ARPA) of the Latium Region the level of As concentration in the drinking water of the region where adolescents were living.

Once these data were collected, we applied models that included the levels of metals in adolescents` blood, adolescents` life-style, environmental data (water, traffic, land cover, air) and GIS data.

Slovenia

National HBM data

Existing data of the national HBM programme includes measurements of selected elements and organic pollutants in biological samples:

- Blood (women): Pb, Cd, Hg, As, Cu, Zn, Se (additional data: haemogram, creatinine, TSH)
- Blood (men): Pb, Cd, Hg, As, Cu, Zn, Se, organochlorinated pesticides, marker PCBs (28, 52, 101, 138, 153, 180) (additional data: haemogram, triglycerides, cholesterol, creatinine, TSH)
- Breast milk: Pb, Cd, Hg, As, Cu, Zn, Se, organochlorinated pesticides, marker PCBs (28, 52, 101, 138, 153, 180), (additional data: triglycerides, cholesterol)
- Urine: Pb, Cd, Hg, As, Cu, Zn, Se (additional data: markers of kidney function, creatinine)
- Hair: total Hg

Questionnaire data obtained along with the internal exposure:

- general part: age, body weight and height, education, occupation;
- basic characteristic of home: type of building, years the house was built, type of type of heating, type of water supply, traffic density;

- health conditions: disease if any, medications, number of amalgam fillings;
- life-style and nutritional habits: smoking, passive smoking, use of computer, hobbies, consumption of vegetables, fruit, nuts, milk, cheese, eggs, poultry, game, other meat, fish and seafood, tea, coffee, alcohol, type of oil used, daily water consumption, consumption of supplements;
- questions regarding pregnancy and lactation period: any problems during the pregnancy, smoking during pregnancy, alcohol intake, breastfeeding, baby`s gender, birthweight, current weight.

Environmental and ancillary data obtained within CROME:

Three major groups of ancillary data were identified and collected: 1) information describing environmental status, 2) information on releases of contaminants from various sources, and 3) data supporting characterization of the built environment in living surrounding of HBM participants.

- Drinking water quality in 10 locations across Slovenia (Sb, As, Cu, Ba, Cd, Cr, Ni, V, Pb, Co, Al, Mn, Mo, Fe, Zn, Sn, Sr, Hg and Rb)
- Air quality: bioindicators (As, Cd, Cu, Hg, Pb, Zn, Mn and Se in moss *Hypnum cupressiforme* in 102 locations across Slovenia), AQ monitoring network data (O3, PM2.5 and PM10)
- Geochemical composition of soil (Al, Ca, Fe, K, Mg, Na, P, Ti, Ba, Cd, Cr, Cu, La, Mn, Mo, Nb, Sc, Sr, Th, V, Y, Zn, Zr, and Hg)
- Nitrogen and phosphorous releases
- Build environment: distances to three types of roads according to classification in Slovenia (Highway/Motorway, Main Roads and Regional roads), and length of all roads (in meters) within three buffer zones (50, 100 and 500 m); area (in square meters) of three land use feature in 500 m surroundings of individual subjects address: CLC_3xx (Corine Land Cover classes 3.1+3.2+3.3), CLC_Urban_green (Corine Land Cover classes 2.1+2.2) and Fields_gardens

Internal exposure to selected elements identified in the Slovenian population as identified by the reported data analysis was a result of life-style determinants, particularly, for Cd (smoking and diet) and Hg (fish consumption and amalgam fillings), and of environmental exposure, which is of most concern in the area of Mežica Valley (Pb) and the former mercury mining town of Idrija (Hg). Linking HBM and environmental databases confirmed geo-dependant environmental nature of Pb and Hg exposure in the two cases, while the rural area related Cd was not associated with activities related to farming, namely artificial fertilizing. Apart of the Pb-smelter related exposure, public water supplies seem to be an important source of Pb (and also Mn). As a possible source of Pb exposure in the urban environment, emission of particulate matter from residential sources and roads was revealed.

Cross Mediterranean study

Internal exposure of birth cohort established in the Mediterranean area included levels of Hg, Cd, Pb, As, Se, Cu, Zn and Mn and showed low to moderate internal exposure in children and generally sufficient status of essential elements. Main source of exposure to Hg overall was fish consumption, however in Slovenian population amalgam fillings were an important source as well. The Bayley-III assessment at 18 months of age did not reveal any adverse effects on cognitive, language and motor development, however some decrease in the fine motor scores has been observed with increasing Hg exposure during pregnancy.

Following the CROME methodology, genotyping of some relevant gene polymorphisms was done and among the polymorphisms analysed, apolipoprotein E showed some differences in examined association between internal exposure and neurodevelopmental outcome: we found negative association between cognitive scores and Hg exposure in the carriers of apolipoproteine ε4 allele, but not in others (data on Slovenian and Croatian cohort).

Re-assessing the children at 7-8 years of age, we demonstrated positive association between Hg in hair or blood and cognitive scores as assessed by WISC assessment (data from Slovenian and Italian cohort), but worse behaviour (more anxiety and retreat) with higher Hg levels (data from Italian cohort). Associations will be further re-assessed once the follow up is completed also in the Croatian cohort. Having sufficient samples size, this will allow to stratify regression modelling by different genotypes and also by gender. Besides apolipoproteine, association between Hg and WISC scores will be stratified by different variants in the following genes that have been so far analysed for Slovenian study population: metallothionenins, arseno-methyl-transferases, delta-aminolevulinic acid dehydratase, vitamin D receptor, selenoprotein P and thioredoxin reductase.

Due to the good agreement between Hg internal levels and frequency of fish consumption, it is presumed that the source of Hg exposure in the study population is mainly fish, but it is not entirely clear what is the contribution of inorganic Hg exposure to the overall negative effect that Hg has on neurodevelopment/neurobehaviour. Speciation analysis in biological samples that are on-going will help reveal this.

Spain

Sabadell study - Metal exposure

The median age of the mothers at the time of their last menstrual period was 31 years, ranging between 20 and 40 years. Their mean body mass index before pregnancy was 23.77 kg/m2 (standard deviation 4.53 kg/m2, median 22.44 kg/m2, range 16.69-40.77 kg/m2). Overweight and obese women encompassed 18.1% and 8.4%, respectively. The proportion of primiparous mothers was 49%, 41% had another infant and 10.2% had more than two infants (Fort, Cosin-Tomas et al. 2014).

Ni, Cu, Zn, As, Se, Mo, Cd, Cs and Pb were detected in more than 90% of samples, whereas Co and Sb were detected in more than 65% in the first and the third trimester (Fort, Cosin-Tomas et al. 2014). Tl was the only element detected in less than 20% of the samples. The differences in metal concentrations between the urine samples collected in both periods were statistically significant for all metals except Ni, As, Tl and Pb (median values: 32 (1st)/35 (3rd) µg/g creatinine for As, 3.9/3.9 for Ni, 0.14/0.13 for Tl, 3.8/3.9 for Pb). The concentrations of Co, Cu and Zn were higher during the third trimester (median values: 0.45 (1st)/1.3 (3rd) µg/g creatinine for Co, 12/15 for Cu, 256/290 for Zn). The opposite was found for the concentrations of Mo, Se, Cd, Sb and Cs (median values: 55 (1st)/44 (3rd) µg/g creatinine for Mo, 10/8.7 for Se, 0.61/0.54 for Cd, 0.36/0.28 for Sb and 8.0/6.8 for Cs).

Consistently with these differences, comparison of the individual concentrations revealed that more mothers had higher concentrations of Se, Mo, Cd, Sb, Cs and Tl in the first than in the third trimester (53-64%) (Fort, Cosin-Tomas et al. 2014). On the other hand, more mothers exhibited higher concentrations of Co, Ni, Cu and Zn in the third than in the first trimester (55-82%). The concentration ratios between the third and first trimesters were consistent with these observed differences. Co, Cu and Zn showed the higher third-first trimester median concentration ratios and Mo, Se, Cd and Sb had the lower.

The first and third trimester concentrations of all metals were significantly correlated. The Spearman coefficients ranged between 0.16 (Mo) and 0.60 (Zn). The degree of significance of these correlations was $p < 0.001$ in most cases (Co, Ni, Zn, Se, As, Cd, Sb, Cs and Pb) and $p < 0.01$ in others (Cu, Mo) (Fort, Cosin-Tomas et al. 2014).

Exposure to organohalogen compounds

The concentrations of organochlorine pollutants newborn cord serum samples were at least one order of magnitude higher than those of PBDEs (Vizcaino, Grimalt et al. 2014). 4,4`-DDE was the pesticide found at highest concentration (median = 180 ng/g lipid) and was observed in 99.7% of the samples, followed by HCB (median 50 ng/g lipid) in 97.6 % of the samples. ß-HCH was the dominant HCH isomer (median = 17 ng/g lipid; 90.5 % of the samples). PCB153 was the most abundant PCB congener (median = 47 ng/g lipid) followed by PCB138 (median = 31 ng/g lipid) and PCB180 (median = 27 ng/g lipid). Total BDEs in cord serum ranged from <LOD to 816 ng/g lipid with a median of 3.9 ng/g lipid. BDE153 was the most frequent congener (43%) followed by BDE47 (36.5 %). BDE209 was only detected in 14.9% of the samples but it was the BDE congener found at highest concentration when detected (mean= 4.1 ng/g lipid).

Mean maternal age was 31.4 ± 4.2 years. 40.2% of the mothers had a university degree which was about the same proportion (43.3%) of those only having completed secondary school. 55% of the mothers belonged to the lowest social groups. Primiparous women constituted the largest group (63.1%) and the mean of gestational age was 39.6 ± 1.4 weeks. Standardized BMI categories showed that 21.9% of the mothers were overweight and 6.5% were obese before pregnancy. On average, gestational weight was 14.1 ± 5.2 kg (range: -2.9 kg – 34 kg). There was an inverse association between GWG and pre-pregnancy BMI (Spearman $r = -0.16$ $p < 0.01$). Most mothers did not conform to the IOM guidelines. 55% of overweight and obese women exceeded recommended GWG compared to 37% of normoweight and underweight women. The proportions of women gaining less GWG than recommended ranges were 9%, 30%, 14% and 20% for those underweight, normoweight, overweight and obese.

Bivariate analyses

Significant associations between maternal determinants and some but not all measured POPs were observed (Vizcaino, Grimalt et al. 2014). Negative correlations between all POPs analyzed and GWG were found. When using the IOM weight categories, mothers with inadequate GWG had children with higher POP cord serum concentrations than mothers with recommended or excessive GWG. A positive association between pre-pregnancy BMI and HCB and β-HCH concentrations was observed. All OC concentrations in cord serum but not PBDEs were statistically significantly associated with increasing maternal age. Fish consumption was the only maternal dietary item associated to POPs, involving higher total PCBs, total BDEs HCB and ß-HCH concentrations in newborns with maternal fish consumption above the median. Total PCB and HCB levels were higher among neonates from women with high education level (university grade) than from mothers with secondary or primary degrees, while 4,4`-DDE levels were higher in neonates of women with primary education. Concerning social class, higher levels of PCBs and HCB were found in children of mothers from the highest level (groups whereas no regular trend was observed for the other contaminants analyzed. Significant associations with parity or previous maternal feeding history were only observed for total PCBs.

Multivariable analyses

The associations between GWG and POP concentrations in cord serum were also inverse in the multivariate models (Vizcaino, Grimalt et al. 2014). Specifically, GWG was inversely associated with total PCBs, 4,4`-DDE and ß-HCH, and, at the edge of significance, for HCB, but GWG was not associated with total BDEs or 4,4`-DDT. Concerning pre-pregnancy BMI, only cord serum HCB concentrations in underweight mothers showed a significant negative association, although all other OCs were lower in underweight women. Other statistically significant predictors of POP concentrations in cord serum were age (ΣPCBs, 4,4`-DDT, HCB and β-HCH), education level (4,4`-DDE) and fish consumption (ΣPCBs, ΣBDEs, HCB and β-HCH). Social class, parity, and breastfeeding were not significant predictors in the multivariable models.

When models were stratified by maternal pre-prenancy BMI categories, associations between GWG and POPs appeared to differ across BMI groups. Newborns of underweight women tended to have higher OC levels with higher GWG. This was opposite to newborns from normal weight, overweight, or obese women, who tended to have lower OC levels with higher GWG. In contrast, newborns of underweight women tended to have lower PBDE levels with higher GWG. Grouping by the IOM categorical variable showed that the adjusted geometric mean cord blood POP concentrations from children of mothers with inadequate GWG were higher than those in children whose mothers had the recommended GWG. No differences were found between mothers with recommended or excessive GWG. These findings suggest that negative associations between POPs and GWG modelled as a continuous variable may have been driven by the positive association between POPs and inadequate weight gain during pregnancy.

Sensitivity analyses

The calculation method for twGWG39 showed a good agreement between predicted and observed twGWG39 values when there was a measure available in the third trimester (Vizcaino et al., 2014). Estimated errors in the twGWG39 calculations decreased as the gestational week of the last weight measurement increased, with median relative percentage differences in estimated values relative to observed values of 6% for twGWG36 (n = 123), 7.9% for twGWG33 (n = 52), and 9.5% for twGWG30 (n = 12), and 12.3% for twGWG27 (n = 7) .No significant changes were found in the results when preterm births (n=9) were excluded (data not shown), or when data from mothers who did not have weight measurements after 28.5 weeks of gestation (n = 7) were excluded as well. Associations between GWG and POPs were comparable when we excluded maternal pre-pregnancy BMI or excluded maternal POPs concentration as a model covariate, or modeled the relative difference in newborn concentrations compared with maternal concentrations. Overall, associations of GWG with individual PCB and PBDE congeners were consistent with associations of GWG with total PCBs and total BDEs.

Analysis of the results and recommendations for policy answers

Country specific recommendations

Greece

Oinofoita – Cr(VI)

Urinary levels range between 0.1 and 1.7 (mean value of 0.7) μg/L, while hair levels vary between 0.1 and 4.2 (mean value of 1.3) μg/g. Slightly higher values were observed in adults, potentially related to higher amounts of tap water consumption. Cr levels in hair showed higher variability that the respective urinary levels, however, this is somehow expected. The lack of highly sensitive methods and the limited weight of the material used (50-200 mg, instead of 2-5 ml for biological fluids such as urine and blood, or even 10 ml, which is the case for breast milk) act as limiting factors for the widespread use of this matrix. In addition, parameters such as external deposition of chemicals, or hair pigmentation have been amended as potential confounders.

The correlation between urinary and hair Cr levels is quite good indicating that both matrices seem able to capture the long term exposure related to exposure to multiple sources of Cr.

Overall, exposure to Cr(VI) in the Asopos Basin seems to be lower than in the previous years, as a result of the counter measures actions that relate to tap water. This was also verified by the low levels of Cr identified in the various biological matrices sampled of the relevant population.

Main recommendations:

- Strict regulation about effluents disposal
- Changing the local water supply
- Periodic control of drinking water quality
- Prohibiting irrigation and drinking water consumption from underground wells
- Use of urinary and hair as biological matrices to asses Cr long-term exposure
- Implementation of a national HBM program to assess internal exposure for key pollutants geographically covering major part of Greece

Aspropyrogos – PCDDs/PCDFs

Upper-bound total TEQ values were calculated for each category of congeners assuming that non-detected individual congener concentrations are equal to their corresponding limit of detection. The results of the analysis of the 60 samples, indicated that exposure to PCDDs/PCDFs fumes resulted in exposure levels that are higher than the ones identified in previous studies, hence indicating that the intake of the population was significant. These results could be the basis for further analysis of the toxicokinetic behavior, as well as for cancer risk assessment.

The analysis of the distribution of the PCDDs/PCDFs blood levels between children and adults, as well as between exposed and non-exposed population indicated that the accidental event resulted in significant exposure to the population, raising the blood concentrations of adults to almost twice levels. Although the respective change could not be identified in children due to lack of previous measurements, it is expected that the accidental event would have a higher contribution to children, since newer generations always have lower internal background levels of PCDDs/PCDFs. However, this hypothesis should be further explored through toxicokinetic analysis of this data and development of an internal dosimetry associated cancer risk slope factor.

Main recommendations:

- Allocation of recycling facilities away from populated areas
- Limit the amount of material to be recycled according to the legislation
- Implementation of a national HBM program to assess internal exposure for key pollutants geographically covering major part of Greece

Thessaloniki PM-PAHs

From the results of the analysis of the urinary samples, it was identified that urinary 1-OH pyrene levels ranged from 0.05 to 0.80. It has to be noted that higher 1-OH pyrene levels were identified in children. This is explained by the fact that the actual uptake (bodyweight normalised) of PAHs is higher in children. The differences of the distributions of both age groups are explained by the higher inhalation rate (bodyeight normalized) of children, as a result of the higher metabolism, as well as of the more intensive activities performed daily by the children. In addition, children commute more often than the adults, thus they are spending more time outdoors, where PM levels are usually higher.

It has also to be highlighted, that higher 1-OH pyrene levels were identified in the west area of the city compared to the east-center area. These results also corroborate with the findings of previous analysis of the ambient air PAHs analysis in the metropolitan area of Thessaloniki, where exposure to PAHs TEQ was higher in the western side. It has also to be noted, that higher difference (of almost 3 times) of the actual TEQ had been identified within the two locations, while the difference in 1-OH pyrene levels is lower (almost twice). This is attributed by the fact that TEQ is calculated taking into account the toxicity of the individual PAHs identified in the ambient air particles, hence, each compound contributes to the overall TEQ through a fraction, defined by its TEF. On the contrary, 1-OH pyrene is a metabolite indicating the overall burden of exposure to PAHs, irrespective of their toxicity.

The additional environmental and biomonitoring data obtained in the frame of CROME-LIFE, provided additional information towards the association between exposure and effects, for a population heavily exposed to biomass burning for space heating particulates and to the main carcinogenic compounds adsorbed, namely PAHs.

The additional measurements of ambient air PM diurnal variability, provided us the ground for improving exposure estimates, by deriving data related to the peaks of exposure and how these peaks correspond to the various activities and inhalation rates at a given time. In this way, exposure estimates are pretty refined, since it has been found that ambient air peaks do not contribute as expected to the overall daily exposure, since people tend to relax or to sleep at that hour, hence the actual uptake rate is low. In addition, an instrument that provides time-dynamic data, allows the identification of the contribution of specific exposure sources, such as the operation of an open fireplace.

The biomonitoring data on the other hand, provided valuable information on the validation of the PAHs internal dose estimates, calculated starting from ambient air exposure data and accounting for PM size distribution and PAHs TEQ actual uptake. The biomonitoring results confirmed that both (a) children are exposed to higher levels of PAHs compared to adults and (b) people leaving in the west side of Thessaloniki are exposed to higher amount of PAHs, highlighting the socioeconomic component of exposure to biomass burning for space heating emitted toxic components.

Main recommendations:

- Lowering the taxation of oil for space heating
- Introduction of newer pellet boilers
- Incentives for improving thermal behavior of households
- Strengthen monitoring system of air pollution

- Implementation of a national HBM program to assess internal exposure for key pollutants geographically covering major part of Greece

Italy

Cross-Mediterranean Study

At 7 years if age , some children presented very high levels of As. Levels of Hg, Cd and Pb were also related to consumption of some type of fish, or to dietary factors (consumption of canned fish, fried fish). We found mildly positive effect of Hg concentration in child`s hair on some neuropsychological test at 7 years Hg levels in the hair were positively related to higher scores in CBCL, indicating that both externalizing (anxiety) and internalizing (depression) behaviours were increased in children. Manganese levels (related to fish consumption as well) in the hair of the children were significantly associated to lower levels of the general IQ and verbal comprehension with the higher levels of Hg. Life style factors exerted a significant effect on neuropsychological scores, which are positively influenced by 1) fish consumption, 2) maternal IQ and educational level of the parents.

A significant role in neuropsychological functioning is also attributable to perinatal exposure. The reanalysis of the data on metals' concentrations in the different matrices highlights the importance of all the exposures occurred during postconceptional development, and suggest that together with neurotoxic metals, essential elements such as Zn, Cu and Se concur to modulate neurobehavioural development.

These results point to the need of extending the biomonitoring and evaluation of the neuropsychological outcome to all the NAC II cohort. The area of Trieste is included by law since 1998 among the National Contaminated Sites. This implies that pollution in these areas should be monitored and that interventions should be put in place to eliminate sources of pollution or at least reduce the concentrations of major pollutants in air, soil, water within acceptable levels. Main sources of pollution in Trieste are represented by the emissions from the iron plant, traffic and harbour activities. Release of heavy metals, particulate and benzopyrene has been recorded over years, and the study SENTIERI (whose responsible is the ISS) pointed to a higher occurrence of some kinds of cancer in the population living nearby the industrial plant. However, to the best of our knowledge, no biomonitoring program for exposed population has been put in place so far; the data collected since 2007 by the PHIME study in the Italian Northern Adriatic birth cohort and then by the CROME common study represent a valuable set of information as for levels of exposure to children of the Trieste municipality to metals and metalloids in the past ten years. The findings described in D5.2 evidence that exposure to neurotoxic metals is a major concern for children`s health. On the basis of the results of the CROME common study, the following specific recommendations could be proposed at a local level:

1. setting in place a programme of biomonitoring of the population living nearby the industrial plants/activities, with specific attention to vulnerable groups (children, adolescents, pregnant women). Biomonitoring should be paralleled by evaluation of health outcomes related to growth, obesity, metabolic disorders, asthma/allergies and neurodevelopmental/neuropsychiatric disorders;
2. empowering the monitoring system of air pollution;
3. monitoring indoor pollution in the school of the Trieste municipality;
4. control of the drinking water quality; control of soil and dust concentration of heavy metals, with specific attention to gardens and playgrounds
5. assessment of metals` concentration in samples of the local fish and in samples of vegetables collected in the local markets and grown in the Trieste area.

Italian case study

The 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, for their exposure to metals. This study investigated 453 adolescents living in the 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 in an exposome approach considering several determinants related to the subjects, available environmental parameters and geo-coding of residence address location. 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 Linear Model (GLM) capturing co-exposures to pairs of key determinants.

The results obtained in the 'original` PROBE adolescents study represented the reference values (RVs) for 19 metals in a particular susceptible population, the adolescents. The RVs produced are very usefull as a comparison term for other biomonitoring data in adolescents produced worldwide, and for tracking potential higher exposures or contaminations.

Moreover, results in the PROBE adolescent cohort showed that As and Hg were found associated with fish consumption.

Regarding As, the As contamination of drinking water is a public health problem in several Italian areas due to the volcanic origins of the territory. In particular, the area of Viterbo province is characterized by the presence of a volcanic system where a continuous basal aquifer flows within Pliocene-Pleistocene sedimentary rocks with very high concentrations (up to 130–370 μg/L) of As. The results in the PROBE adolescents` survey, show that children living in Viterbo area had higher levels of As (and also of Hg and V).

Regarding Hg, the concentration in blood of PROBE adolescents were slightly higher than in those reported in the cross-sectional nationally representative survey (NHANES) for the US population; probably this result is due to a greater consumption of fish in Italian adolescents from the Mediterranean area and to the existence of sub-marine volcanos.

As far as concern sex differences, we found blood Pb significantly higher in males than in females; this result is of relevance considering that elevated blood Pb levels may present deleterious cognitive neurotoxic effects that can be more pronounced in men than in women.

Another notable result was the higher values for some metals such as Ir, Pt, Rh in adolescents` blood living in urban areas respect to those living in rural ones; these metals are typically associated with road traffic, so PROBE adolescents living in urban areas are more prone to a higher exposure to traffic-derived metals.

Also by means of the GLM and EWAS approaches, we 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 (SES) of the mother revealed robust statistical associations with Cd and Ni blood levels in the respective children. Other associations were found between Cd and Pt and the use of costume jewelry.

To view the human biomonitoring data produced within the PROBE study in a health risk assessment context, the concentration measured were compared with available health-related biological exposure values, as the HumanBioMonitoring values (HBM: HBM I and HBM II) established by the German Human

Biomonitoring Commission, and the Biomonitoring Equivalents (BEs). Related to health risk, the levels of metals found in adolescents` blood did not indicate a health risk when considering the health reference limits. Thus a follow-up risk assessment in this adolescent cohort is of low priority.

On the basis of the PROBE adolescent study data, policies at the local level should pose special attention to the people living in volcanic areas (Viterbo) with:

- a periodic control of drinking water quality and the connected water bearings,
- control on foods and plantations in the same areas, which may reflect watering with high-level As water

For people living in urban areas (Rome):

- monitoring of outdoor air pollution with a more detailed scheme of air quality monitoring stations monitoring of air in the traffic zones in each municipality;

For all, special attention to vulnerable groups (children, adolescents, pregnant women) has to payed.

Promoted by the local authorities, biomonitoring programme should be implemented by national health and environment authorities in areas with high As levels in the drinking water, in parallel with assessment of health outcomes as suggested for the Trieste study.

Slovenja

On the basis of the CROME-LIFE results, the following specific recommendations could be proposed at a local/national level

- Implementation of the national HBM framework the data collected and assessment made provided an exposure profile for the Slovenian population residing in 12 regions across the country. Future surveys will be based on the outcomes of the current one, taking into account also problems encountered during the recruitment itself and missing/insufficient data.
- Having the data on internal exposure geographically covering major part of Slovenia, we can plan future survey much better. Using existing data and statistical power calculation, the sample size that allows us to detect environmentally relevant differences in exposure and time-trends can be efficiently determined, which is an important advantage in risk assessment.
- Data analysis based on the HBM results shows that collecting only invasive matrices is not an option in order to realistically assess internal exposure. We showed that the levels for certain environmental pollutants determined only in urine do not reflect the real exposure. This is the case particularly for Cd and POPs.
- For the long-term implementation of HBM, strengthening of national infrastructure is of crucial importance. This includes establishment of a national biobank for long-term biological samples storage, data management and storage, establishment of a database platform, which would include health- and environmental-related data and its management.
- Conducting HBM programmes, response rate is a key component in the success of a programme and the obtained data. For this, communication strategy should be well defined and continuously implemented in the levels of general public, policy makers, regulatory agencies and also on a clinical level. In the long- term, interdisciplinary involvement is very important, bringing together ministries for environment, health, agriculture, economy, and science.

Spain

Metal exposure

Exposure to metals in pregnant women has been assessed from their urine composition collected in the first and third trimesters of pregnancy using a newly developed digestion protocol for Q-ICP-MS analysis (Fort et al., 2014a). All metals except Ni, As, Th and Pb showed statistically significant concentration differences between these two periods. The concentrations of all metals in the first and third trimesters were significantly correlated which reflect the absence of major changes of metal inputs in the studied women during pregnancy. The significant concentration differences between these two sampling periods may respond to metabolic pregnancy changes. Accordingly, the measurements of the studied trace metals in urine provide representative estimates of exposure during the whole pregnancy period.

Mediterranean populations, such as the one represented by the Sabadell cohort, have higher total urinary arsenic concentrations than other European or North American populations (Fort, Grimalt et al. 2014). Consistently, the pregnant women from the Mediterranean region considered in this study have high arsenic levels. The high seafood consumption typical of these populations is the main cause for these high arsenic concentrations. A strong association between total urinary levels of arsenic and total seafood consumption is observed in the two different stages of pregnancy examined (first and third trimesters). Specifically, lean fish consumption is the main contributor to the intake of this metalloid. No significant differences in arsenic excretion are observed between these two periods and the observed small differences are mainly related to changes in lean fish consumption between the first and third trimesters. Seafood arsenic has been demonstrated to be mainly composed of organic species that are inert, not toxic and rapidly excreted. Therefore, these high levels of urinary arsenic found in Mediterranean populations are not cause of health concern. Assessment of the origin of arsenic in general population is important for evaluation of possible health effects in the exposed population. The approach described in the present study provides the necessary information for estimation of the possible toxic effects associated to arsenic accumulation.

Cobalt levels in maternal urine have been observed to rise significantly from the first to the third trimesters, probably due to the iron decrease along pregnancy (Fort, Grimalt et al. 2016). A significant negative correlation has been found between hemoglobin and urine cobalt concentrations in the third trimester, as well as between the differences between hemoglobin levels and urine cobalt concentrations between these two trimesters. This association has been previously reported in adolescents, women and hemochromatosis patients, but the present study is the first in which this trend is observed during pregnancy. Cobalt enhances transcription of erythropoietin, leading to higher red cell production. Higher absorption of this metal may tend to counterbalance iron depletion during last stages of pregnancy, when basal metabolic rate is high and 90% of fetal growth occurs and iron requirements are increased. This mechanism may be useful to contribute to fulfill the oxygen demand of these processes that are crucial for proper fetus development. However, the present results recommend the implementation of monitoring programs of cobalt concentrations in pregnant women from populations occupationally or environmentally exposed to this metal. This strategy could allow to anticipating possible deleterious effects for the mother or fetus as consequence of enhanced cobalt accumulation.

Atmospheric inputs are possibly responsible for the observed differences in urine Sb concentrations from pregnant women living in urban areas (Fort, Grimalt et al. 2016). The occurrence of this metal in the atmosphere has been attributed to traffic activity as consequence of its use in brake linings. The associations of Sb content in urine of pregnant women with seasonality, physical activity and traffic intensity near their homes is consistent with some dependence of the intake of this metal from atmospheric sources. These associations suggest that despite the estimated dietary inputs of this metal are somewhat higher than the estimated inhalation intake, the atmospheric inputs of Sb may be significant for the overall incorporation of this metal in populations of modern urban areas, e.g. in pregnant women.

Cu is also used in brake linings but the high predominance of inputs of this essential metal from dietary components make unlikely the significance of the atmospheric urban inputs in the overall human intake (Fort, Grimalt et al. 2016). This is consistent with the lack of statistical significance of the observed differences in Cu urine concentrations when grouped according to atmospheric pollution indicators.

Exposure to organohalogen compounds

The distributions of most OCs between maternal serum and cord serum and maternal serum and placenta are significantly correlated (Vizcaino and Crawford 2015). In general, the highest relative concentrations are found in maternal serum and the lowest in cord serum. These distributions are consistent with a predominant maternal source that transfers the pollutants to the placenta and the fetus. However, these distributions do not correspond to pollutant passive diffusion among the three types of tissues according to their lipid content. Conversely, they require an active transplacental transfer of the compounds possibly in association to the transport of enzymes through the membranes.

The compounds that can be metabolically transformed, namely 4,4`-DDT and several PBDEs, have been observed to accumulate selectively in cord blood (Vizcaino and Crawford 2015). Once these are able to reach the fetus they are better preserved than in the maternal tissues. This difference evidences a low capacity of fetal metabolism for the degradation of organic pollutants which may lead to the accumulation of pollutants that usually are found in minor concentrations in adults or in mothers. POP exposure assessment studies of newborns may overlook the effects of some of these pollutants if they only consider maternal determinations.

GWG influences the accumulation of POPs in newborns (Vizcaino, Grimalt et al. 2014). Neonatal concentrations of all POPs were lower in association with increasing GWG after adjustment for potential confounders. Other predictors such as maternal age, fish consumption during pregnancy, and educational level might be also relevant for the accumulation of POPs in utero. On average, mothers whose gestational weight gain was below IOM recommendations gave birth to newborns with higher POP concentrations than mothers who met or exceeded the weight gain recommendations (between 9%-

30% higher than the recommended gain). Accordingly, the IOM recommendations for GWG during pregnancy may be beneficial for reducing POP exposures in newborns. These findings and previous results on the potential association between GWG and birth outcomes (Nohr, Rasmussen et al. 2009) support the incorporation of GWG as covariate in epidemiological studies of effects of POPs on children`s health. IOM recommendations for GWG have also clinical value for primary care as women can reduce POP concentrations in their newborns.

General methodological recommendations

Recommendations on how to optimally use the measurements available

The biomonitoring data collected in the Mediterranean countries participating in CROME represent a valuable piece on information to fill the knowledge gap relative to the internal exposure of general population and vulnerable population groups (pregnant women, children and adolescents) to different classes of environmental contaminants (heavy metals, metalloids, organohalogenates, PCDDs/PCDFs, PM- PAHs). Whereas the mean values are in general within the reference values established by previous US and EU biomonitoring studies in general population, these same data indicate that exposure to toxic metals, POPs and VOCs is ubiquitory in vulnerable population subgroups such as children in the Mediterranean area. Given the uncertainty to establish a "safe" level for most of these chemicals with respect to their implication in developmentally-based diseases such as asthma, allergies, obesity and neurodevelopmental disorders, it is mandatory that the measurements obtained are used for studying the environment/health association in an integrated perspective. Biomonitoring data should thus be integrated with more complete information of the population in study, including exposures from diet, lifestyles and behaviors. The allostatic role of socio economic status in modulating the effects of chemical exposure should be also carefully considered. The potential enhancement/modification of chemicals` adverse effects on human health by climate change is another issue to be included in risk assessment. The exposomic approach will help to improve strategies aimed at preventing and treating diseases.

Recommendations for measurements to be made when data gaps are identified

Based on the results o far obtained in the four countries of the CROME consortium, main data gaps refer to i) measuring more precisely sources of pollutants in study to prevent undesirable exposure in populations; ii) estimating or quantifying the risk for health outcomes (cancer and neurodevelopmental disorders) .

These two gaps can be addressed in different ways: for assessment of external exposure, local and national environment and health authorities should foresee at least for areas/sites at major risk for environmental pollution:

1. A periodical monitoring of selected contaminants (depending on the environmental criticalities of the area in study) in samples of soil, dust, seawater, water used for watering in gardens and ;
2. Integration of the data base of biomonitoring studies with data derived from regional data base on air and drinkable water quality;
3. Periodical analysis of selected contaminants in food items (including not only vegetables but also meat, fish, dairy products and drinking water). Analysis should include speciation analytical approach to differentiate the organic forms of some metals (i.e., the non-toxic As form) from the inorganic ones (i.e., the toxic As form).
4. Use of personal sensors for more accurate exposure assessment associated to a lifestyle pattern.

For assessment of internal exposure and associated health outcomes, collection of biomonitoring data including vulnerable population subgroups should be implemented. Wherever national or local registries for neurodevelopmental disorders or other chronic conditions of the general population are available, the link with morbidity and /or mortality should be assessed, to calculate a potential increase of the disease or mortality risk. Financial support from the health or environmental authorities at national or local level for the detection of health outcomes in infants and children. It is desirable that prospective studies are initiated in the different countries as they give information on the low-dose and delayed effects of most of the pollutants under scrutiny in the CROME network.

Finally, complete information on supplementation recommended for pregnant women is needed, as the Trieste study showed that some essential elements (Zn, Cu or Se) might lead to the development of adverse effects on children neurodevelopment.

Identification of the statistical methods recommended for an optimal interpretation of the environmental and biomarker data

Application of multivariate analyses are of course recommended for interpretation of the link between environmental and biomarker data,. In particular the approach indicated by the EWAS may be specifically suited to evaluate the hypotheses regarding the broad contribution of the environment to disease (Patel, Bhattacharya et al. 2010). Internal doses are coupled to health impacts on the local population through advanced statistical methods to derive the dose – response functions which account for differences in exposure patterns, susceptibility differences and inter-individual variation (due to lifestyle, age, sex or physiological status) in health response. The approach uses as a starting point the biomarker values measured in different biological matrices (urine and/or blood) to estimate through the application of the lifetime generic PBTK model the biological effective dose in the target tissue which is consistent with the biomarker level measured. To estimate the health impact we will use a statistical approach based on survey-weighted logistic multivariate regression adjusted for different covariates (age, sex, socio-economic status (SES) etc.) linking internal doses with health effect considering the interdependence of the covariates (using as metric an analogy of the "linkage disequilibrium" metric used in genome-wide association studies). Although the exposure-response formula will be derived from the existing environmental/biomonitoring and health data, these will be used to estimate the expected health impacts for further population groups starting from the biomonitoring data itself.

Recommendations on the operational HBM framework including definition of strategy for recruitment, definition of national methods and related QC aspects for sampling specimen including choice of collectors, conditions of handling, preservation, sending, storing and national communication plan for all stages of the project (from recruitment to dissemination of results at individual and collective level).

There are many methodological recommendations that can be advanced in order to implement efficient and useful HBM campaigns. One critical aspects concerns the matrices to be collected to assure reliable exposure assessment:

Invasive matrices
Blood is the most frequently used invasive matrix to determine biomarkers as blood is a universal link between all tissues of the organism (Paustenbach and Galbraith 2006). The invasive character of sampling however often negatively affects participation rates, and there are ethical issues involved in using blood. Often special consent needs to be obtained from both participants and local and national ethical oversight committees to use blood as a biomonitoring matrix. Additionally, the blood volume that can be collected normally is limited. This makes the use of blood for biomonitoring in children suboptimal. Blood analysis is often carried out for substances that are slowly excreted from the organism (Polkowska, Koz owska et al. 2004).

An important advantage of using blood as a matrix is that concomitant with exposure markers, also many relevant biomarkers of effect can be determined in blood. Combining both exposure and effect markers in a biomarker battery, makes relating exposure, dose, effect and health impact much more relevant.

Non-invasive matrices
Urine probably is the most used matrix in which biomarkers are measured. The collection and analysis of urine carries no associated risks, sample volumes can be large and samples are obtained for different age classes, including little children with minimal impact (Kozlowska, Polkowska et al. 2003, Polkowska, Koz owska et al. 2004, Bradman and Whyatt 2005). Unfortunately, for many biomarkers, urine is not the most reliable indicator of exposure because it often contains excreted metabolites instead of parent compounds (Paustenbach and Galbraith 2006). Because chemicals are often slowly excreted over the

course of hours or days after exposure, also toxicokinetic factors may hamper the usability of urine as a matrix. Although this can be reduced by collecting 24-hr samples rather than single spot samples, the timing of sample collection remains an essential aspect of biomonitoring using urine as a matrix (Barr, Wilder et al. 2005, Kissel, Curl et al. 2005). Urinary creatinine concentrations, specific gravity and osmolarity are common methods for adjusting dilution of urine samples. The most commonly used method is creatinine adjustment that involves dividing the analyte concentration by the creatinine concentration. Guidelines for creatinine adjustment and proper data interpretation are available in literature (WHO 1996, Barr, Wilder et al. 2005).

Cord blood, amniotic fluid and breast milk provide an overview of the pollutant load of mothers, and at the same time provide relevant information on the *in utero* or early life exposure of babies (Shen, Main et al. 2007).With the current interest in *in utero* and childhood exposure reflecting windows of extreme vulnerability, these matrices deserve extensive attention when preparing HBM programs. Potentially problematic issues using cord blood or amniotic fluid may arise from the fact that sampling is not always straightforward as obviously collection of HBM samples is not the first priority at time of delivery. Breast milk is a reliable matrix to monitor the presence of fat-soluble contaminants such as polychlorinated biphenyls (PCB), brominated flame retardants (BFR) or dioxins ((Uehara, Peng et al. 2006, Shen, Main et al. 2007, Yu, Palkovicova et al. 2007)) and may be the most important route of exposure to contaminants.

However, it needs to be stressed that HBM in itself cannot replace environmental monitoring and modeling data. Most often, environmental monitoring data for different environmental compartments (air, water, food, soil) provide better insight into potential sources, hence allowing the development of more informed and appropriate risk reduction strategies. At the same time, mathematical approaches to describe the pharmacokinetic and toxicokinetic behavior of environmental agents (generally referred to as PBPK models) offer a more mechanistic insight into the behavior and fate of environmental agents following aggregate exposure (Indirect EDR-relationship in). As biomarker data also reflect individual accumulation, distribution, metabolism and excretion (ADME) characteristics of chemicals, HBM data offer an excellent opportunity for the validation of these PBTK models. Ultimately, combining both lines of evidence to assess exposure prove to be optimal for relating complex exposure to environmental agents to potential adverse health effects assessment.

Conclusions

Overall summary of the country specific and the methodological recommendation presented above

A list of high priority key elements of a primary preventive approach should foresee strategic interventions at national and local level to:

- *Reduce exposure to environmental hazards* including air pollutants, and environmental and industrial pollutants, especially heavy metals such as mercury and lead.
- *Improve building materials with lower emissions* in houses, schools and workplaces.
- *Reduce exposure to parental and other sources of environmental tobacco smoke* in utero, infancy and childhood.
- *Ensure adequate dietary intake of key nutrients, including folic acid and other vitamins and minerals*, among women of childbearing age, to protect against neural tube conditions and other consequences of vitamin deficiencies. As there is an association between neonatal exposure to POPs and inadequate GWG during pregnancy. Encouraging pregnant women to meet the recommended IOM guidelines for GWG may reduce the accumulation of POPs in newborns.

At a more specific level, we recommend to:

- *Implement of national and EU HBM framework*, the data collected and assessment made, provided an exposure profile for general and vulnerable population subgroups. Using existing data and statistical power calculation, the sample size that allows us to detect environmentally relevant differences in exposure and time-trends can be efficiently determined, which is an important advantage in risk assessment.
- *Associate Biomonitoring with evaluation of health* outcomes related to growth, obesity, metabolic disorders, asthma/allergies and neurodevelopmental/neuropsychiatric disorders;
- *Establishment of national biobanks for long-term biological samples storage*, data management and storage, establishment of a database platform, which would include health- and environmental-related data and its management.
- *Planning communication strategies* well defined and continuously implemented at the levels of general public, policy makers, regulatory agencies and also at clinical level. In the long- term, interdisciplinary involvement is very important, bringing together ministries of environment, health, agriculture, economy, and scientific research.

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