Cross-Mediterranean Environment and Health Network

CROME-LIFE

ANNEX 11

Deliverable B.5.2

Technical report on quantitative health impact assessment (cancer and neurodevelopmental disorders)

LIFE ENVIRONMENT PROGRAMME

LIFE12 ENV/GR/001040

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Contact Person Editing Partner
Name: Prof. Dimosthenis Sarigiannis
Phone: +30 2310994562
Fax:
e-mail: sarigiannis@auth.gr

Authors Editing Partner
Name:
Phone:
Fax:
e-mail:

CROME-LIFE web site: http://www.crome-life.eu

Table of Content

Introduction

The overall objective of this task is the quantitative estimation of the effect on human health due to exposure to selected chemicals using the results derived from the application of PBTK model and /or biomonitoring data collected in Action B.2 or collected through targeted field campaigns executed in Action B.3. 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.

Methodology applied in CROME

Neurodevelopmental dissorders

Regression methods

A critical aspect in the study of neurobehavioural effects of environmental stressors is relative to the specific characteristic of the outcome. Usually a child's neuropsychological maturation is represented by continuous outcome variables, which give more information than dichotomous variables but are more easily affected by measurement errors. Developmental neurotoxicity is often evaluated by behavioral symptoms that, by their very nature, are endowed with a wide variability range and high sensitivity to both individual history (family, SES, nutritional factors) and characteristics of the neuropsychological test applied. As a matter of fact, most of prospective epidemiologic studies on neurotoxicity do not report clinically defined conditions (i.e. autism or learning disability) but rather atypical behavioral traits that range from increased/decreased anxiety and aggressiveness, to poorer motor or intellectual development in a significant proportion of exposed infants/children (Jurewicz et al., 2013).

While consideration of specific clinical diagnoses is indeed important, it is equally critical to include in the analysis also subclinical effects of environmental exposures on neurodevelopment or intermediate phenotypes like in the case of the autism spectrum. Even a small shift in the mean IQ score in a population will result in a substantial increase in the percentage of individuals with extremely low scores, with a significant impact on economic and health costs (Grosse et al., 2002).Subclinical effects can have profound population levels implications and can be assessed through global neurodevelopmental scales that evaluate multiple domains. The problem with large prospective cohort studies from general population is that the effects that are being sought are generally subtle effects of low-level exposure (White, 1993). Thus absence of detectable effects on most test batteries cannot be interpreted as an indication of no risk and the presence of detectable effects must meet certain criteria before a causal relationship can be established.

IQ tests have been used extensively in the study of certain types of toxicant exposures (especially lead and polychlorinated biphenyls). However, domain-specific neuropsychological tests have received more attention in recent years in behavioural toxicology because of their greater sensitivity to prenatal exposure to toxicants. The test batteries applied in the cohort included in CROME are widely validated . In addition, these tests provide more insight into the underlying central nervous system (CNS) damage that may be associated with exposures, since there is a

significant literature that links impaired performance within individual domains or patterns of impaired and intact performance across domains to specific types of brain damage (structural, neural system, neurotransmitter).

This knowledge is critical in that it may allow investigators to form hypotheses concerning the structural or functional elements of the CNS that may be affected by exposures. These hypotheses can serve as the basis for further investigations (e.g., omics analyses). They also may have value in examining the subtle effects of other types of exposure (e.g., stress, medications, drugs, dietary factors) as well as the interaction between several factors.

Regression methods are usually applied to analyse the influences of multiple environmental factors on neuropsychomotor development. Preliminary to regression models simple linear correlation is applied to identify the relation between exposure variables and between outcome variables. In a second step, multiple linear regression models are specifically adapt to identify what exposure variables are significantly associated with outcome variables and the extent of the effect (calculation of the Beta coefficient).

Environment-wide association studies (EWAS)

Environment-Wide Association Study (EWAS) is a population-based data analysis approach that correlates multiple environmental factors to disease. The EWAS approach has been introduced by Patel et al. (Patel et al., 2010) and it is based on Genome-Wide Association Study (GWAS). In GWAS multiple genetic factors are assayed along with phenotypic information on each individual. In particular, GWAS enhance the discovery of genetic variants that they can postulate about the function of the pathways discovered in diseased individuals. In other words, the genetic factors are the independent variables, and the phenotype is the dependent variable. The GWAS have been widely and effectively applied to emerge the missing heritability of complex diseases (Manolio et al., 2009). Especially, among a wide range of studies and researches, GWAS have used to provide new insights into type 2 diabetes aetiology (Frayling, 2007), to investigate genomic characteristics of trait/disease-associated SNPs (TASs) (Hindorff et al., 2009) , to map chromatin marks across cell types to systematically characterize regulatory elements, their cell-type specificities and their functional iterations (Ernst et al., 2011) and to examined the role of common genetic variation in schizophrenia (Ripke et al., 2011).

EWAS has been conducted to evaluated the hypotheses regarding the broad contribution of the environment to disease (Patel et al., 2010). In EWAS, the place of the genome domain is replaced by the envirome domain. The Envirome consists of the environmental factors which are the quantity of the individual exposures that has directly measured. Particularly, the measures can be the amount of a chemical substances in human tissues and organs, a self-report historical exposure and common well–being characteristics such as family and social economic status (SES). Hence, Patel et al. (2012 conducted EWAS to comprehensively and systematically explore and associate multiple environmental factors discovering and replicating robust correlations with serum lipid levels. Also, EWAS was used to evaluate possible additive role of both contaminants and lifestyle factors regarding Metabolic Syndrome revealing the existent of associations in the examined elderly population (Lind et al., 2013).

The EWAS framework can carried out a systematic sensitivity analyses, whereby validated factors are modeled under different assumptions or with additional covariates. Also, using a pair-wise validation method it is computed the correlation of dependence between the factors, revealing potential evidence for exposure or confounding route. The EWAS framework and analysis approach is based on the GWAS and the framework of EWAS were introduced by Patel et al. (2010. Firstly, in the proposed framework it is conducted an initial scan for environmental factors associated with the observed effects/variables through general linear modeling (e.g logistic regression). The model takes into consideration the factor which have been adjusted for known confounder considering the existent environmental association. Secondly, the false discovery rate is applied on multiple hypotheses. Finally, factors that it is deemed that significantly associated with the observed factors beyond the region of false discovery are "validated" in independent cohorts.

The first step of the analysis was to calculate the non-parametric correlation coefficient between socio-economic as well as environmental factors and neurological effect. The analyzed dataset consisted of continuous variables and categorical factors that were measured on a ratio scale. The standard score was applied to calculate the probability

of a score occurring within the existent normal distribution enabling the comparison of two scores that appeared different normal distributions. Additionally, the association between all possible factor pairs were checked for monotonic and non-monotonic relationship using scatter plots and Pearson`s correlation. At the same time, logistic regression (LR) has been used to associate each environmental and socio-economic factor with the neurological attributes adjusted for gender. Thus, every environmental and neurological factor (Xi) as well adjusted variables were computed using the following logistic function:

$$Y = a + b_i \cdot X_i + \varsigma \cdot Z$$

Where, "*a*" is constant and "b_i" is the effect size of that factor, adjusted by other variables and also it characterizes the effect of change in the observed variable. The association has been computed by the two-sided p-value for bi that tests the "null hypothesis". For the needs of the computation, part of the continuous factors were categorized based on histograms or were z-transformed in order to compare the effect sizes. The *p*–value was computed through chi2 test (χ^2 test) and Pearson correlation depending on the type of the factor.

Because of the non-monotonic relationships detected in the variable pairs, Hoeffding's D-statistics (Hoeffding, 1948) has been used to examine a wide variety of dependence structures beyond the association. 7,056 correlations between pairs of unique socio-economic and/or environmental factors as well as 3,948 correlations between pairs of unique neuropsychological and socio-economic/environmental factors were computed.

Next the false discovery rate (FDR) q-value versus the number of real discoveries for each correlation was estimated using the Benjamini-Hochberg step-down approach. The correlation was taken into consideration if the q-value was lower than 5% in at least 2 permuted subsets. It has to be highlighted that 10 permuted subsets were simulated from the original dataset. The original data was randomly permutated (without replacement) to 10 subsets of the 80% of the total data. Additionally, the two-sided p-value for an individual correlation from ρ was the fraction of correlations from the permutated dataset with the highest absolute value. Estimation of false discoveries was based on the "null distribution" of regression test statistics by shuffling the observer factor with permutation and refitting the regression models. The statistically significant parameters corresponding to the FDR level were validated using different subsets of the data. The significance level of the permutated data set was validated with restrictions at least equal to the FDR level of the initial dataset.

Finally, the correlation networks between independent factors were examined. The degree of dependency between validated factors was carried out by computing their raw correlation coefficient (Pearson`s ρ). These dependencies were illustrated with circular network plots which display the exposome networks. The permutated data that had produced the null correlation filtered the correlation to values within the 5th and 9th percentile of the distribution. Therefore, the networks shown in the respective Annexes report the most important correlations among the measured factors arranged in a circle and connected with arrows. The thickness of the lines represents the levels of correlation values and the color of the line represents the sign of the respective correlations (blue for negative and red for positive correlations).

All the calculations were executed in R Studio. In the Manhattan and Volcano plots the statistically significant values are reported with red points above the chosen level of significance ($p<0.05$).

Cancer

Lung cancer risk associated to PAHs

Exposure assessment

Personal exposure is equal to the average concentration of a pollutant that a person is exposed to over a given period of time, e.g. 1 day, 1 month or 1 year. If over the given period of time, T, the person passes through n locations, spending a fraction f_n of the period T in location n where the concentration of the pollutant under consideration is C_n, then the personal exposure for this period T, represented by the concentration ET, is given by Ott (1982:

$$E_T = \sum_n f_n \cdot C_n \qquad [1]$$

Microenvironments were distinguished in terms of time spent within them, and time-weighted factors were used, based on the time-activity data of the EXPOLIS study (Hänninen et al., 2004), which were further enhanced with data from the MTUS[1] database. The exposure factors used were cross-checked against the European Commission`s EXPOFACTS database[2]. Thus, in order to estimate exposure we used information on detailed time activity patterns, linking the several types and duration of activities to specific microenvironments.

By using detailed activity patterns and linking them to specific microenvironments an additional factor influencing the actual human intake of PMx, namely inhalation rate, was taken into account. Different types of activities demand different levels of effort that correspond to different inhalation rates. For the estimation of human exposure to PAHs, weighted average daily inhalation rate (IR_i, m^3/day) is calculated for each age group. Calculations refer to the time-activity pattern of individuals for a period of one week (weekdays and weekend), as given by Sarigiannis et al (2012). Eq. 2 is applied, where IR_j (m^3/hr) is the inhalation rate that corresponds to activity j and t_j is the duration of the activity.

$$IR_i = \frac{5 \sum_{j,weekday=1}^{n} (IR_{j,weekday} \times t_{j,weekday}) + 2 \sum_{j,weekend=1}^{n} (IR_{j,weekend} \times t_{j,weekend})}{7} \qquad [2]$$

HRT deposition

HRT particle deposition modeling is applied for the determination of PM deposition fraction (DF) to the three parts of the pulmonary system in order to estimate the internal dose of PAHs. Major mechanisms of PM deposition across HRT include diffusion, sedimentation and impaction. Secondary mechanisms involve interception and electrostatic deposition. Different HRT regions involve different deposition mechanisms, with regard to different PM size as follows:

- Naso-pharyngeal region (or upper respiratory tract – URT): impaction, sedimentation, electrostatic (particles > 1 μm)
- Tracheo-bronchial (TB) region: impaction, sedimentation, diffusion (particles < 1 μm)
- Pulmonary (P) region: sedimentation, diffusion (particles < 0.1 μm)

Several parameters affect HRT deposition, including PM properties (concentration and size distribution), air flow parameters (lung capacity and breathing frequency) and HRT physiology (structure and morphology). All of these parameters have been taken into account in the approach proposed herein.

HRT deposition was carried out using the Multiple Path Particle Deposition (MPPD) v. 2.1 model (de Winter-Sorkina and Cassee, 2002). Age-specific lung geometries representing 10 distinct ages from 3 months old to 21 years old are also provided. An idealized symmetric single-path model as well as a 5-lobe symmetric multiple-path model are available for use with each age setting (Mortensen, 1983a; Mortensen, 1988; Mortensen et al., 1983b). Software inputs include morphological parameters of pulmonary system – functional residual volume (FRC), tidal volume (TV), upper respiratory tract (URT) volume, as well as breathing frequency (BF) for each age group (Table 1). As MPPD results refer to a monodisperse distribution of particles, DF results are weighted by the use of volume distribution for average urban aerosols, given by Seinfeld and Pandis (2006.

Table 1. Age dependent HRT morphological parameters (de Winter-Sorkina and Cassee, 2002)

[1] MTUS: Multinational Time Use Study (http://www.timeuse.org/mtus/)

[2] EXPOFACTS: Exposure Factors database maintained by the European Commission`s Joint Research Centre (http://expofacts.jrc.ec.europa.eu/)

Age group	FRC (ml)	URT (ml)	BF (min^{-1})	TV (ml)
0-3 months	27.4	2.45	39	30.4
3-23 months	78.5	6.94	27	86.8
23 months-3 years	95.4	9.47	24	121
3-8 years	437	21.0	17	278
8-14 years	881	30.6	16	388
14-18 years	1935	37.4	15	447
18-21 years	1855	42.3	14	477

Cancer risk assessment

In its Provisional Guidance for Quantitative Risk Assessment of Polycyclic Aromatic Hydrocarbons (EPA/600/R-93/089, July 1993) and regional guidance, EPA recommends that a toxicity equivalency factor (TEF) be used to convert concentrations of carcinogenic polycyclic aromatic hydrocarbons (cPAHs) to an equivalent concentration of benzo(a)pyrene when assessing the risks posed by these substances. Calculation of the overall toxicity of the mixture of the 19 PAHs is done using Toxic Equivalent Factors (TEFs), based on the assumption that the *TEF* for B[a]P is equal to 1 (Nisbet and LaGoy, 1992).

TEQ values are calculated according to Eq. 3 using the median value of the measured concentrations, since the concentrations of individual compounds follow an asymmetric distribution:

$$TEQ = \sum_{i=1}^{19} (C_i \times TEF_i) \qquad [3]$$

Genotoxic effects of PAHs are estimated through inhalation cancer risk (*ICR*) assessment. *ICR* is expressed as a linear function of ambient *TEQ* concentration and $IUR_{B[a]P}$ (Eq. 4), as the exposure-cancer risk relationship is considered linear in the low dose region (EPA, 2005). California Environmental Protection Agency recommends an $IUR_{B[a]P}$ value of 1.1 10^{-3} $m^3/\mu g$ (CEPA, 2004).

$$ICR = TEQ \times IUR_{B[a]P} \qquad [4]$$

Equation 4 is adapted to include the exposure and dose parameters discussed earlier for each age group.

In order to calculate the risk of cancer that can be attributed to PAHs , we need to estimate the amount of *TEQ* deposited across the middle (tracheobronchial) and lower (alveoli) HRT regions (Bostrom et al., 2002). This is calculated as the sum of the products of the different size fractioned PM mass deposited across the different HRT regions, multiplied to the *TEQ* estimated for the specific size fraction.

$$TEQ_{uptake} = \sum_{1}^{n} PM_{Tr\_bronch-i} \cdot TEQ_{Tr\_bronch-i} + \sum_{1}^{n} PM_{Alveoli-i} \cdot TEQ_{Alveoli-i} \qquad [5]$$

The cancer risk function implemented is given in Equation 6, where BW_i is the average body weight of each age group and SF is the B[a]P slope factor, derived from the assumption that $IUR_{B[a]P}$ refers to a human of 70 kg inhaling 20 m^3 of ambient air per day. SF is equal to 3.85 10^{-6} (kg day)/ng B[a]P.

$$ICR = TEQ_{uptake} \cdot \frac{IR_i}{BW_i} \cdot SF \qquad [6]$$

Cancer risk assessment associated to exposure to dioxins and furans

Mixtures of PCDDs/PCDFs are complex environmental mixtures of 210 interrelated chemicals composed of different dioxins and furans. For mixtures PCDDs/PCDFs, the reference chemical is 2,3,7,8 – tetrachlorodibenzo-p-dioxin (2,3,7,8–TCDD) because it is the most toxic and best-studied of the 210 PCDDs/PCDFs. The toxicity equivalency factor (TEF) methodology was developed by the U.S. Environmental Protection Agency to evaluate the toxicity and assess the risks of a mixture of structurally related chemicals with a common mechanism of action. A TEF is an estimate of the relative toxicity of a chemical compared to a reference chemical. Toxic Equivalents, or TEQs, are used to report the toxicity-weighted masses of mixtures of PCDDs/PCDFs. The TEQ method of PCDDs/PCDFs reporting is more meaningful than simply reporting the total number of grams of a mixture of variously toxic compounds because the TEQ method offers toxicity information about the mixture. Within the TEQ method, each PCDDs/PCDFs compound is assigned a Toxic Equivalency Factor, or TEF. This factor denotes a given dioxin compound's toxicity relative to 2,3,7,8-TCDD, which is assigned the maximum toxicity designation of one. Other dioxin compounds are given equal or lower numbers, with each number roughly proportional to its toxicity relative to that of 2,3,7,8-TCDD. Developed by the World Health Organization, TEFs are used extensively by scientists and governments around the world (Van den Berg et al., 1998), finally expressing the so-called TEQ WHO (toxicity equivalent concentration in accordance with the methodology of the World Health Organization), that uses units of grams-TEQ. The EPA uses TEQ WHO to report emissions of PCDDs/PCDFs from known sources to the open environment in its Inventory of Sources of Dioxin in the United States and similar practices have been adopted worldwide, including all the data presented in this study. To obtain the number of grams-TEQ of a dioxin mixture, one simply multiplies the mass of each compound in the mixture by its TEF and then totals them.

EPA has classified 2,3,7,8-TCDD as a Group B2, meaning a probable human carcinogen (USEPA, 1985). With regard to 2,3,7,8-TCDD, EPA has calculated an inhalation cancer slope factor of $1.5 \cdot 10^5$ $(mg/kg/d)^{-1}$ and an inhalation unit risk estimate of 3.3 x 10^{-5} $(pg/m^3)^{-1}$ for 2,3,7,8-TCDD. A similar slope factor of $1.5 \cdot 10^5$ $(mg/kg/d)^{-1}$ has been proposed for orally administered 2,3,7,8-TCDD, which correspond to an oral unit risk factor of 4.5 $(\mu g/L)^{-1}$.

Cancer risk assessment associated to PCBs and organochlorine compounds

Polychlorinated Biphenyls (PCBs) are a family of man-made organic chemicals with a common structure (a pair of benzene rings) that vary primarily in their degree of chlorination. Each single form of PCB is called a congener, and is often identified by number (e.g., PCB 153). Depending on the number and position of chlorine atoms attached to the biphenyl ring structure, 209 different PCB congeners can be formed. PCB congeners can be divided into the coplanar, the mono-ortho-substituted PCBs, and other non-dioxin-like PCBs. The significance of this designation is that coplanar and some of the mono-ortho-substituted PCBs have dioxin-like toxicologic effects. The chlorination pattern of the PCBs determines the toxicity of the substance. A number of PCB congeners show dioxin-like toxicity. These PCBs have no more than one chlorine atom at the ortho-position (polychlorinated non-ortho and mono-ortho biphenyls). The phenyl rings of these molecules can rotate and adopt a coplanar structure, which leads to the same toxicity as the polychlorinated dibenzo-p-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs). A number of PCB congeners, however, have two or more of the ortho-positions in the biphenyl molecules occupied by chlorine molecules. For these, the two phenyl rings are not in the same plane, and these PCBs express non-dioxin-like toxicity.

Once released into the environment, PCBs adsorb strongly to soil and sediment. As a result, these compounds tend to persist in the environment, with half-lives for most congeners ranging from months to years. PCBs leach from soil slowly, particularly the more highly chlorinated congeners, and translocate to plants via soil insignificantly.

Cycling of PCBs through the environment involves volatilization from land and water surfaces into the atmosphere, with subsequent removal from the atmosphere by wet or dry deposition, then revolatilization. In the general population, inhalation of these airborne PCBs is one route of exposure, in addition to the food source of exposure to PCBs.

PCBs are metabolized by the microsomal monooxygenase system catalyzed by cytochrome P-450 to phenols (via arene oxide intermediates), which can be conjugated or further hydroxylated to form a catechol. Arene oxide intermediates are electrophilic in nature. They can covalently bind to nucleophilic cellular macromolecules (e.g., protein, DNA, RNA) and induce DNA strand breaks and DNA repair, which can contribute to the toxic response of PCBs. Additionally, arene oxide intermediates can be conjugated with glutathione and further metabolized to form methylsulfonyl metabolites, which have been identified in human serum and tissue samples and in laboratory animals. Binding of methylsulfonyl metabolites to some proteins may contribute to some of the toxic effects of PCBs. It has also been hypothesized that hydroxylated PCB metabolites could contribute to the toxicity of PCBs.

PCBs are classified by the U.S. EPA as B2, probable human carcinogens, based on liver tumors in adult rats. The World Health Organization International Agency for Research on Cancer (IARC) in 1998 classified PCBs as Group 2A, probably carcinogenic in humans.

Owing to the fact that dioxin-like compounds normally exist in environmental and biological samples as complex mixtures of congeners, the concept of toxic equivalents (TEQs) has been introduced for risk assessment and regulation. In applying this concept, relative toxicities of dioxin-like compounds in relation to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (i.e. toxic equivalency factors, TEFs) are determined from in vitro and in vivo studies.

The EPA has derived cancer potency estimates for oral exposure to PCBs. A range of upper-bound slope factors were calculated to represent the potency of representative classes of environmental PCB mixtures. A three-category approach is used that considers how environmental processes (partitioning, chemical transformation, and bioaccumulation) affect each exposure pathway or situation by altering the composition and cancer potential of the original PCB mixtures.

The highest slope factor (2.0 $(mg/kg/d)^{-1}$ is for the high risk and persistent category, which is used for pathways in which environmental processes are likely to increase risk, such as food chain exposure, sediment or soil ingestion, dust or aerosol inhalation, and exposure to dioxin-like, tumorpromoting, or persistent congeners. Due to the potential for higher sensitivity in early life, the highest slope factor is also used for all early-life exposures.

An intermediate slope factor (0.4 $(mg/kg/d)^{-1}$ is used for the low risk and persistence category, which is appropriate for exposure pathways in which environmental processes tend to decrease risk, such as drinking water ingestion of water soluble congeners, inhalation of evaporated congeners, and dermal exposure (because PCBs are incompletely absorbed through the skin).

The lowest slope factor (0.07 $(mg/kg/d)^{-1}$ applies to the lowest risk and persistence category, and is used when congener or homologue analyses of an environmental mixture verify that congeners with more than four chlorines comprise <0.5% of total PCBs, as well as the absence of dioxin-like, tumor-promoting, and persistent congeners. For the upper slope factor of 2 $(mg/kg/d)^{-1}$, doses corresponding to risk levels ranging from 10^{-4} to 10^{-7} are $5x10^{-5}$ to $5x10^{-8}$ mg/kg/day, respectively,

The Department of Human Health Services (DHHS) considers the evidence for the carcinogenicity of hexachlorobenzene in experimental animals sufficient, and this chemical is reasonably anticipated to be a carcinogen in humans. A cancer assessment for hexachlorobenzene is available on Integrated Risk Information System (IRIS, 2011) in which the chemical is assigned to U.S. EPA cancer weight-of-evidence Group B2, probable human carcinogen, on the basis that oral administration of hexachlorobenzene has been shown to induce tumors in the liver, thyroid, and kidney in three rodent species. IRIS (2011 presents an oral slope factor of 1.6 $(mg/kg/d)^{-1}$ and an inhalation unit risk $4.6x10^{-4}$ based on hepatocellular carcinoma in female Sprague-Dawley rats exposed orally.

Cancer risk assessment associated to arsenic (As) exposure

It is likely that metabolism of arsenic, like other toxic metals, is associated with the conversion of the most potentially toxic forms of this element to the less toxic form, followed by accumulation in or excretion from the cell. Two metabolic pathways for As(i) have been described, an enzymic arsenic reduction/methylation pathway (Buchet and Lauwerys, 1985) and an alternative pathway involving nonenzymatic formation of arsenic-glutathione complexes. Fig.2 next page depicts the Arsenic metabolic pathways used later in the PBPK model formulation.

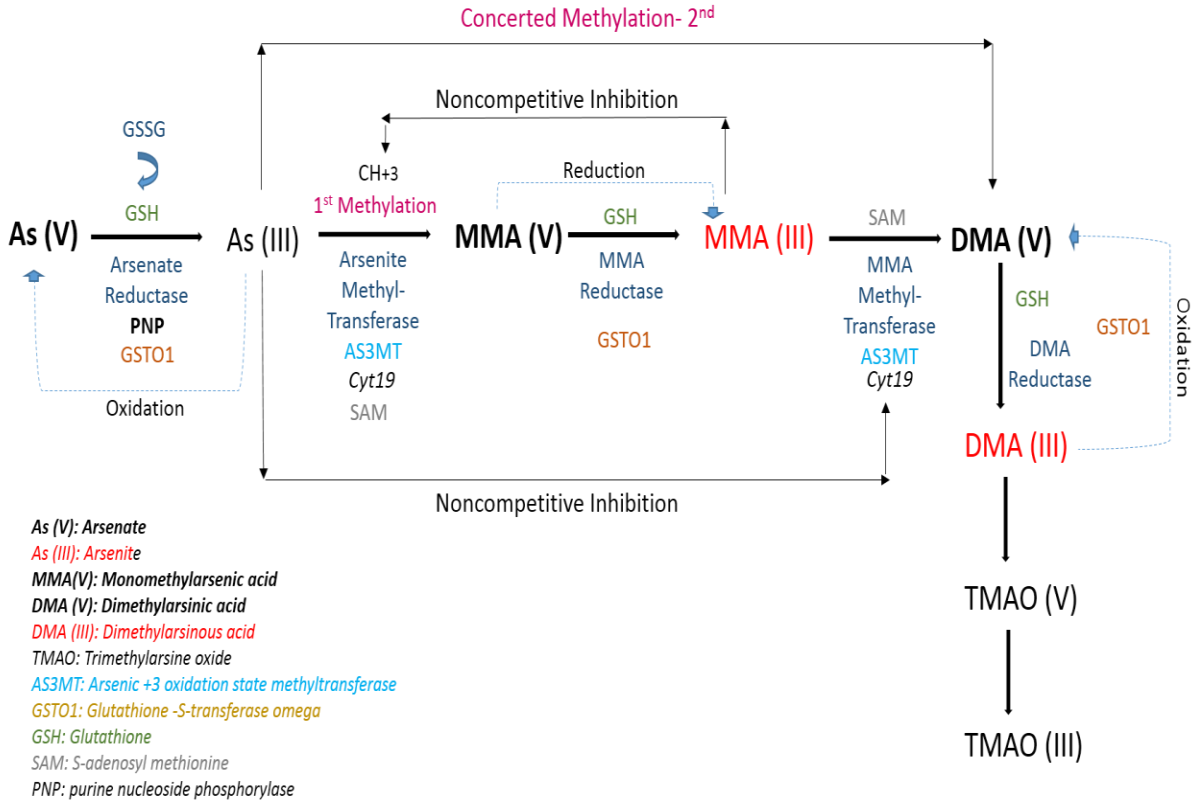


Figure 1. Arsenic metabolism pathways

As seen in Figure 1, Reduction of AsV produces AsIII which is a substrate for AS3MT to methylate, to form MMAV. MMAV is reduced to MMAIII which is methylated by AS3MT (or using SAM as a methyl donor) to form DMAV. DMAV is further reduced to form DMAIII. The modelled metabolic pathways included in addition rates of oxidation of trivalent arsenicals to their respective pentavalent forms. A third metabolic pathway has recently been described where involves initial binding of inorganic arsenic to sulfhydryl groups of cysteinyl moieties on proteins, followed by reductive methylation catalyzed by AsIII, AS3MT and using the methyl group donor SAM to form MMAV and DMAV (ATSDR, 2007) Quantitative description of arsenic metabolic pathways is further complicated by the inhibitory influence of metabolites on methylation (Easterling et al., 2002; Kenyon et al., 2001; Styblo et al., 1996).

Reduction of As^{V}, MMA, and DMA^{V} takes place very rapidly and can occur by either enzymatic or non-enzymatic mechanisms (El-Masri and Kenyon, 2008a; Zakharyan et al., 2005). Mitochondria can work as reactors, where they take up As^{V}, rapidly reduce it, and export the formed As^{III} (Németi and Gregus, 2002). Methylation of inorganic arsenic facilitates the excretion of inorganic arsenic from the body, as the end-products are readily excreted in urine, for this reason, the methylation of arsenic was viewed as a detoxification pathway (Buchet and Lauwerys, 1985). However, the methylation of inorganic arsenic may be a toxificaton-activation process, due to the great biological activity of trivalent methylated arsenic metabolites with proteins and even DNA (Kitchin, 2001).

A single enzyme has been identified, AS3MT, that catalyzes both the oxidative methylation of trivalent arsenicals and the reduction of pentavalent arsenicals (Waters et al., 2004a; Waters et al., 2004b) but also others enzymes support those processes, such as GST01 (Chowdhury et al., 2006; Zakharyan and Aposhian, 1999), which is widely distributed in human tissues. Recently a new enzyme AdoMet dependent methyltransferase (Thomas et al., 2004) has been reported.

Two genes are responsible for arsenic metabolism: human nucleoside phosphorylase (hNP) and human glutathione S-transferase omega 1-1 (hGST0 1-1), (Yu et al., 2003). Polymorphisms in those genes have been discovered. Studies in humans suggest the existence of a wide difference in the activity of methyl-transferases, and the existence of polymorphisms. Genetic polymorphism that have been examined include AS3MT, cystathione-β-synthase, Glutathione-S-transferase ┐1, ω1, methylenetetrahydrofolate reductase, and N-6 adenine- specific DNA methyltransferase 1 (ATSDR, 2007). Individuals with polymorphisms associated with a higher MMA: DMA ratio in urine may be more susceptible to arsenic-induced toxicity.

Children seem to have their own way dealing with arsenic. The first metabolic pathway is more active in adults than children, but the second methylation step is more active in children than adults (Chowdhury et al., 2003). Due to this reason, fetuses and babies may be protected by increased methylation of arsenic during pregnancy and breastfeeding (Gurbay et al., 2012). Fängström et al. (2008 found that arsenic in blood plasma does not pass easily through the mammary glands and arsenic in breast milk correlated negatively with DMA%. Thus, indicating that breast-feeding protects the infant from exposure to arsenic (Fängström et al., 2008). The same conclusion came also from Carignan et al. (Carignan et al., 2015).

Arsenic is excreted in the urine primarily through the kidneys. Humans excrete a cocktail of inorganic, monomethylated and dimethylated forms of arsenic. The pentavalent metabolites MMAV and DMAV are less toxic than arsenite or arsenate (ATSDR, 2011). Approximately 50% of excreted arsenic in human urine is dimethylated and 25% is monomethylated, with the remainder being inorganic. Other less important routes of elimination of inorganic arsenic include feces, incorporation into hair and nails, skin desquamation, and sweat. The whole-body biological half-life of ingested arsenic is about 10 hours, and 50-80% is excreted over 3 days (Casarett and Klaassen, 2008).

The toxicity of arsenic, including cancer, is most likely due to multiple mechanisms. The mechanisms responsible for the adverse effects associated with arsenic, probably occur through multiple independent and interdependent mechanisms (Duker et al., 2005; NRC, 2001). Two general types of mechanisms appear to be involved in arsenic-induced toxicity: (1) formation of reactive oxygen species (ROS). Arsenic can disrupt the oxidative phosphorylation, leading to free radical formation. Pentavalent arsenic may be transformed to a substitute for inorganic phosphate in glycolysis, leading to uncoupling of oxidative phosphorylation and loss of ATP formation (TOXNET, 2016). Arsenic-induced ROS generation has been associated with numerous effects on cellular targets (Hubaux et al., 2013), which can directly damage cellular components or lead to a cascade of effects in response to oxidative stress (alterations in intracellular oxidation/reduction reaction, decreased glutathione levels, lipid peroxidation, damage to proteins, disruption of mitochondrial membrane, genomic instability through damage to DNA). The current consensus in studies with cultured cells, experimental animals, and humans is the fact that arsenic causes oxidative stress through the generation of reactive oxygen species (Fujino et al., 2005; Kumagai and Sumi, 2007). (2) interaction of arsenic metabolites with cellular macromolecules. Arsenic can interfere with essential enzymatic functions and transcriptional events in the cells. Inorganic arsenic exerts epigenetic effects (Bodwell et al., 2006; Reichard et al., 2007). Trivalent species are more potent cytotoxicants, genotoxicants and inhibitors of enzymes compared to pentavalent arsenicals (El-Masri and Kenyon, 2008a). One of possible mechanisms for higher toxicity is the higher affinity for thiol compounds (Shiobara et al., 2001) and generation of reactive oxygen species (Nesnow et al., 2002). Exposure to inorganic arsenic has been shown to modify the expression of a variety of genes related to cell growth and defense, including the tumor suppressor gene p53, as well as to alter the binding of nuclear transcription factors (TOXNET, 2016).

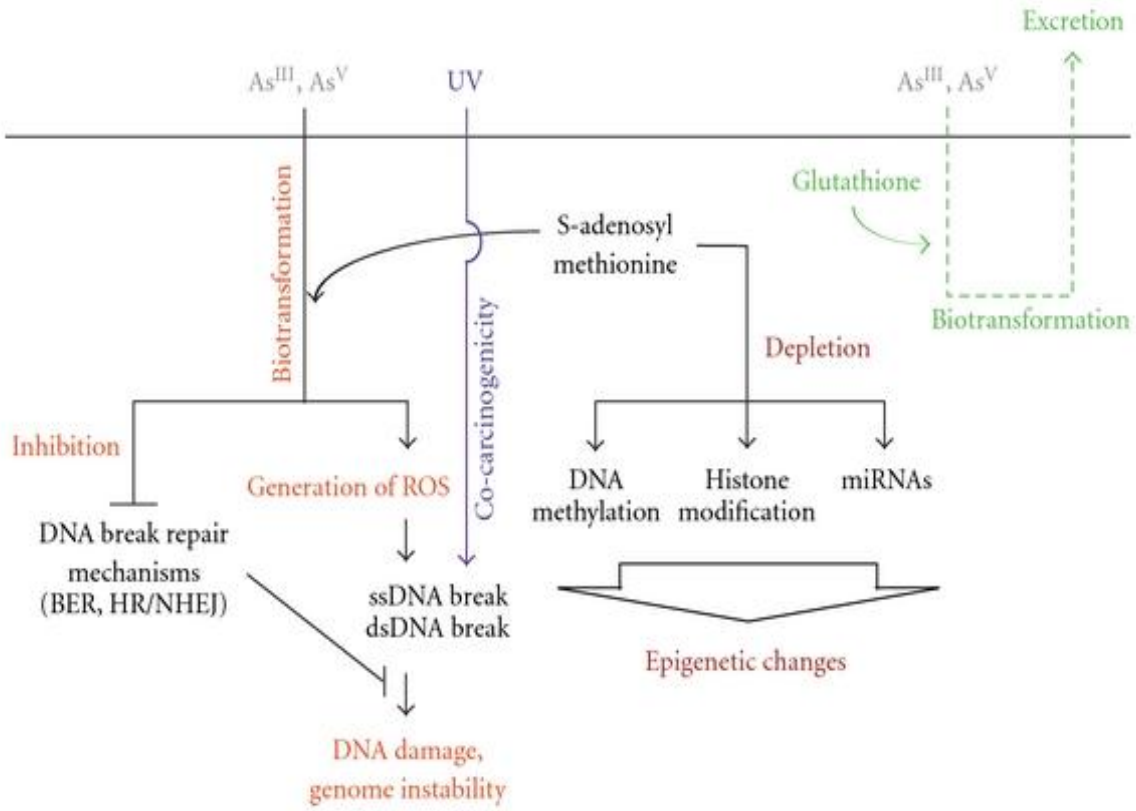


Figure 2. Carcinogenic mechanisms of arsenic transformation.

Figure 2 explains how ingested arsenic undergoes biotransformation process and how those can result to carcinogenic activity. (1) Biotransformation could lead to arsenic excretion, when conjugated with glutathione. (2) Biotransformation generates reactive oxygen species (ROS), that induce single-strand (ssDNA) and double-strand (dsDNA) breaks by inducing oxidative damage. The process can also inhibit DNA break repair mechanisms (Martinez et al., 2011). Additionally, ROS can act as co-carcinogens. Furthermore, the requirement of S-adenosyl methionine (SAM) for arsenic biotransformation can lead to depletion of SAM, which is the substrate for DNA methylation. Recently, a study showed that exposure to arsenic triggers a shift in microRNA expression and revealed an induction of cell cycle progression and failure of apoptosis supporting the idea of inorganic arsenic carcinogenic activity (Sturchio et al., 2014).

Unlike many carcinogens, arsenic is not a mutagen in bacteria and acts weakly in mammalian cells, but can induce chromosomal abnormalities, aneuploidy, and micronuclei formation. In vitro studies showed that As^{III} exposure to humans from drinking water can lead to the formation of micronuclei (Johnson, 2007). Arsenic can also act as a co-mutagen and/or co-carcinogen (Casarett and Klaassen, 2008). Although a large amount of research is available on arsenic`s mode of action, the exact nature of carcinogenic action is not yet clear (NRC, 2001). The proposed Mode of Action include alteration in DNA repair, change in DNA methylation, suppression of cell cycle check point protein (p53), altered expression of growth factor and oxidative stress.

Inorganic arsenic has been classified by the IARC (IARC, 1973) in Group 1 as carcinogenic to humans on the basis of increased incidence of cancers at several sites where people were exposed. IARC (2004) has classified arsenic as a known human carcinogen, associated with tumors of the skin, lung, and urinary bladder, and possibly kidney, liver, and prostate. A ranging risk of 10^{-4} to 10^{-7} was developed by EPA (ATSDR, 2007). An established association between human arsenic exposure and human cancer has been known for many years (Chen et al., 1992; Wu et al., 1989). A

clear dose-response relation between Arsenic and drinking water for cancer in kidney, lung and bladder has been reported in Argentina (Hopenhayn-Rich et al., 1998) and a high lung cancer mortality in Japan (Tsuda et al., 1995). Arsenic is contributing to cancer (Bernstam and Nriagu, 2000; Clewell et al., 1999) of the skin (Yu et al., 2000), lungs (Ferreccio et al., 2000; Lubin et al., 2000), kidney, liver and bladder (Bates et al., 1992; Chen and Wang, 1990; Smith et al., 1992). Trivalent methylated arsenicals are responsible for the toxicity and carcinogenicity of environmental arsenic (Hirano et al., 2004; Nesnow et al., 2002). MMA^{III} and DMA^{III} have been suggested as potential contributors to arsenic-induced carcinogenicity (Bernstam and Nriagu, 2000; Kitchin, 2001). DMA^{V} on the other hand, is a urinary bladder carcinogen and tumor promoter in rats (Cohen et al., 2006). The most common pathway of exposure to inorganic arsenic for the general population is via the drinking water. Early effects of exposure to arsenic in drinking water included pigmentation changes and hyperkeratosis (Alam et al., 2002; Mazumder et al., 1998; Smith et al., 2002). These skin lesions may develop into more serious and disabling forms, including cancer (Haque et al., 2003). In the table below, several endpoints concerning exposure to inorganic arsenic and cancer are summarized.

Table 2. Toxicological cancer endpoints for inorganic arsenic using evidence from human studies

Chronic exposure - Inhalation			
Exposure	LOAEL	Form	Ref.
1->30y	0.213M (serious) CEL: lung cancer	AsIII	(Enterline et al., 1987)
19.5y	0.064M (serious) CEL: lung cancer 0.064 mg/kg/day for liver and lung cancer corresponds to 4,48 mg/day for a weight of 70 kg	AsIII	(Enterline et al., 1987)
3m->30y	0.05M (serious) CEL: lung cancer (3.5mg/day)	AsIII	(Jarup et al., 1989)
1->30y	0.38M (serious) CEL: lung cancer	AsIII	(Lee-Feldstein, 1986)
>25y	0.29M (serious) CEL: lung cancer	AsIII	(Lubin et al., 2000)
14.8y	0.3M (serious) CEL: lung cancer	AsIII	(Welch et al., 1982)
Intermediate Oral Exposure			
0.5-14y	0.05 (serious) hyperpigmentation with keratosis, possibly pre-cancerous		(Huang et al., 1985)
4mo	0.06F (serious)persistent extensive hyperkeratosis of palms and soles		(Wagner et al., 1979)
Systemic Oral Exposure			
>8y	0.0012 (less serious) increased risk of premalignant skin lesions		(Ahsan et al., 2006)
4y	0.1 F (serious) de-pigmentation with hyperkeratosis, pre-cancerous	As(III)	(Bickley and Papa, 1989)

NS	0.009 (serious) hyperpigmentation with keratosis, pre-cancerous		(Guha Mazumder et al., 1988)
Cancer			
NS	0.0011 CEL: lung cancers (0.077 mg/day)		(Ferreccio et al., 2000)
NS	0.018 CEL: lung cancer mortality 1.26 mg/day		(Guo, 2004)
NS	0.018 CEL: bladder cancer		(Guo and Tseng, 2000)
>1y	0.0075 CEL: basal or squamous skin carcinoma (0.525 for 70kg)		(Haupert et al., 1996)
5y	0.033 CEL: lung, urinary tract cancer 2.31 mg/day for a weight of 70 kg	As(III)	(Tsuda et al., 1995)
22-34y	0.014 CEL: basal cell and squamous cell carcinomas of the skin, hemangio endothelioma of the liver 0.014 mg/kg/day for fatal liver tumor and 22 year of exposure, corresponds to 0.98 mg/day for a weight of 70 kg		(Zaldivar et al., 1981)

The diagram of the PBPK model developed for Arsenic is presented in Figure 3. The model estimates levels of arsenic and its metabolites in tissues and urine after oral and inhalation exposure to either As^{V} or As^{III}. There are two routes of exposure: oral and inhalation and several pathways, such as drinking water, cooking water, food consumption, smoking, breathing. The model, based on El-Masri and Kenyon`s model formulation, is composed of four individual PBPK models (see Fig. 4) for As^{V}, As^{III}, MMA and DMA linked together by the transformation of As^{III} to MMA^{V} and DMA^{V}, and the transformation of MMA^{III} to DMA^{V} (methylation). The inhibitory effects of As^{III} on the methylation of MMA^{III} to DMA^{V}, and MMA^{III} on the methylation of As^{III} to MMA^{V} were assumed to follow a non-competitive mechanism. This assumption can be based on studies from the literature (El-Masri and Kenyon, 2008b). Reduction of the pentavalent arsenicals is assumed to follow a first order reaction, because of the ubiquitous availability of thiols such as glutathione in most tissues (Hayakawa et al., 2005).

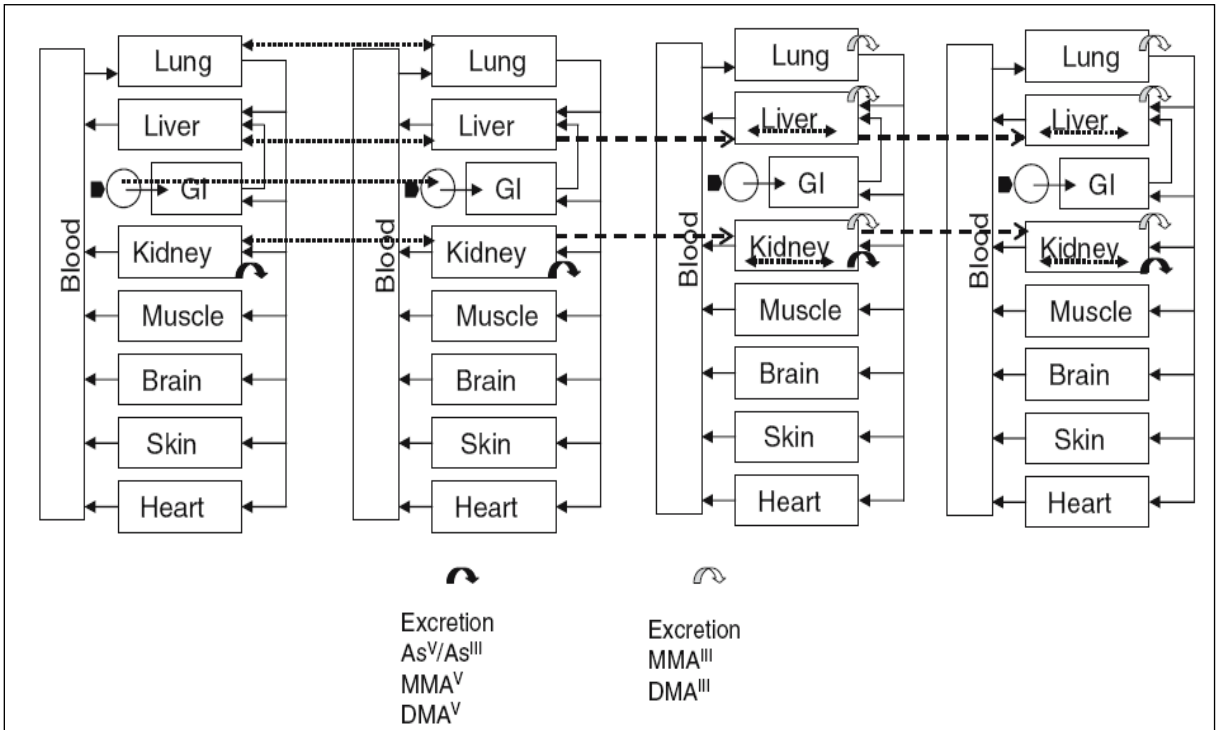


Figure 3. Schematic of the overall PBPK model for inorganic arsenic and methylated metabolites.

Despite all the information about carcinogenesis to human beings, development of a reliable animal model system for arsenic-induced carcinogenicity has been difficult (Ng et al., 1999), indicating marked variation in sensitivity towards arsenic toxicity between species (Vahter, 1999). The difficulties in this area could be due to species-specific differences in detoxification, metabolism, or uptake and accumulation in target tissues. There are major qualitative and quantitative interspecies differences, for example in methylation (Hsueh et al., 1998; Mann et al., 1996). Another example is the Trimethylarsine oxide (TMAO), the final metabolite of inorganic arsenic in some animal species, but has never been found in human urine (Yoshida et al., 1997). Some animal species even lack arsenic methylation capacity, perhaps as an adaptation mechanism (Casarett and Klaassen, 2008). Only in the last decade has the metal been demonstrated to cause cancer in animals under specific exposure scenarios.

Dose-response assessment, is the estimation of the relationship between dose or level of exposure to arsenic, and the incidence of an effect (Leeuwen, 2007). BBDR models provide the substrate for simulations that link mode of action research with predicted physiological consequences of exposures (Andersen et al., 2002). Once the internal doses are calculated via the PBPK model, the next step is to link the internal dose with the health point considered to assess the quantitative risk associated with the given exposure (Clewell et al., 2007; Conolly and Andersen, 1993). The result is a quantitative estimate of health risk relevant to specifics health end-points in the exposed population.

A risk assessment not taking into account the different species but considering only total arsenic, would lead to a considerable overestimation of the health risk related to arsenic exposure (Chain, 2009), therefore it is required to relate the toxicity of all the forms of arsenic found in the PBPK model, to the toxicity of the trivalent arsenic. An in vitro study with human epidermal keratinocytes showed the relative toxicities: $As^{III} > MMA^{III} > DMA^{III} > DMA^{V} > MMA^{V} > As^{V}$ (Vega et al., 2001). Among the different forms in which arsenic can be found, the most toxic is arsenite, followed by arsenate, then the two organic metabolites. However, more recent studies report that the trivalent form of MMA and DMA are likely to be as biologically active as arsenite. Thus, the toxicity order of Arsenic metabolites may be described as follows: DMA^{III}, $MMA^{III} > As^{III} > As^{V} > DMA^{V}$, $MMA^{V} > TMAO$. In general, the toxicity of pentavalent species is lower than that of trivalent by the order of 10^{-3} to 10^{-4} (Hirano et al., 2004; Vega et al., 2001). This may be explained by the faster uptake rate of As^{III} in endothelial cells (Hirano et al., 2003). .

Table 3. Relative toxicity of arsenic species to trivalent inorganic arsenic.

Arsenic form	AsIII equivalent mole (for 1 mole of compound)	Toxicity
Arsenite – As(III)	1	1
Arsenate – As(V)	1	1/35
Trivalent Monomethylarsonate MMA(III)	0.605	1
Pentavalent Monomethylarsonate MMA(V)	0.536	1/85
Trivalent Dimethylarsinate DMA(III)	0.620	1
Pentavalent Dimethylarsinate DMA(V)	0.543	1/85

Steps for using PBPK model estimated Internal Dose in Dose-Response Model for arsenic was (Andersen et al., 2005):

1) Identify toxic effects in people, and determine health endpoints from experimental data associated with arsenic exposure
2) Use an appropriate PBPK model to estimate the internal tissue dose metric for various routes of administration, at various doses, for specific exposure scenarios
3) Development of a dose-response model based on the relationship between internal dose and health points.
4) Estimate the probability of the health risks in humans based on the internal tissue dose calculated during human exposures

Step 3 allows us to develop and parameterize a three-stage model (administered dose-internal dose-cancer probability) for cancer growth that links internal doses to health risk probability. In developing BBDR models it is necessary to evaluate the effect of dose on biological parameters of the model. The effects can be described empirically, as has usually been done, or mechanistically. For the cancer models the stochastic aspect involves some probability of division, death, or mutation that occurs randomly (Andersen et al., 2002). Trying to quantify the relation between dose and response probability, it is useful to decompose the relation between exposure and health risk probability. In this case one relation links the administered dose to the internal dose of arsenic and its metabolites, the other links internal dose to cancer probability. In probabilistic terms it can be explained as follows (Armitage and Doll, 1954).

The dose-response relation between exposure and risk can be denoted by $(p||x)$ that indicates that the probability of cancer at time t is determined by the history of arsenic exposure x up to time t. The risk depends on exposure, or else, the history $\{y\}$ of inorganic arsenic in different organs. This situation can be diagrammed as $\{x\}^{\rightarrow}\ \{y\}^{\rightarrow}\{p\}$. This means $\{x\}$ determines$\{y\}$and $\{y\}$ determines $\{p\}$. Thus, the dose-time-response relation $(p||x)$ may be written as by $(p||x) = (p||y) * (y||x)$. The $(y||x)$ component corresponds to the relation of a PBPK model (mapping the exposure dose history $\{x\}$ into time courses $\{y\}$ of inorganic arsenic in different organs) and$(p||y)$ represents an internal dose-response function. The general curve which better describes such relationship is in the form of Hill equation (Cox and Ricci, 1992):

$$P(y) = 1 - e^{(-by+cy^2+dy^3)} \qquad (3)$$

where: *P(y)* = lifetime probability of the health effect, *y* = biologically effective dose of the toxicant at the target organ (internal dose), *b, c, d* = parameters calculated fitting a multistage model to the experimental dataset.

The most common way for calculating mortality (or any other toxic effect) through a dose-effect relationship, is to relate mortality to the pollutant concentration. The pathology model for arsenic uses two different equations for deriving the prevalence of fatal cancer within a given population. These include the Hill equation and an exponential equation (Ling and Liao, 2007) alternatively to the Hill equation:

$$P = 1 - \exp\left[-\left(a + b \cdot C_{H,i}^{C}\right)\right] \quad (4) \quad \text{and,}$$

$$P = \frac{P_{MAX} \times C_{H,i}^{n}}{EC_{50,i}^{n} + C_{H,i}^{n}} \quad (5)$$

Where: P = prevalence of the health effect, P_{MAX} = human maximum prevalence of those exposed to the contaminant, $C_{H,i}^{n}$= internal arsenic concentration in human target organ i (μg/g), $EC_{50,i}^{n}$ = 50% effect concentration (μg/g) of P_{MAX} for target organ, a, b, c = parameters calculated fitting a multistage model to the experimental dataset. , n = Hill coefficient which is a measure of cooperativity, an n>1 represent a sublinear (sigmoidal) response indicating positive cooperatively, and n<1 represent a subpralinear response.

Cancer risk assessment associated to hexavalent chrome (Cr(VI)) exposure

A review was performed of the carcinogenic dose responses of 14 hexavalent chromium (Cr(VI)) compounds. The molecular entity that drives the carcinogenicity of these compounds is the Cr(VI) ion, which is released when the substances solubilise and dissociate. Chromium(VI) causes lung tumours in humans and animals by the inhalation route and tumours of the gastrointestinal tract in animals by the oral route. These are both local, site-of-contact tumours – there is no evidence that Cr(VI) causes tumours elsewhere in the body. A clear mode of action (MoA) for these tumours has not been established. The overall body of evidence indicates that Cr(VI) is genotoxic in vivo, resulting in the formation of DNA adducts and oxidative DNA damage. However, clear evidence of mutagenicity in vivo in the target tissues (lung and intestine) by relevant routes of exposure is lacking. This supports the contention that Cr(VI) is only weakly mutagenic in vivo and that its mutagenicity is most likely to be only one contributory factor in the carcinogenic process, together with tissue injury/irritation/inflammation and cell proliferation. However, there is insufficient evidence to exclude a genotoxic mode of action and therefore a threshold cannot be assumed.

Dose-response relationships were derived by linear extrapolation. Extrapolating outside the range of observation inevitably introduces uncertainties. As the mechanistic evidence is suggestive of non-linearity, it is acknowledged that the excess risks in the low exposure range might be an overestimate.

Information from epidemiological and mechanistic studies indicated that the carcinogenic potency of Cr(VI) compounds to the lung is greater for substances of high and moderate solubility in comparison to the insoluble chromates. However, quantifying any differences in lung carcinogenic potency for Cr(VI) compounds of different solubility is not possible with the currently available data. Therefore, the proposed lung cancer risk estimates should be applied to inhalation exposures to aerosols of highly soluble, slightly soluble and insoluble Cr(VI) compounds, accepting that they will perhaps overestimate risks in the case of exposure to insoluble chromates. Inhalation exposures to Cr(VI) compounds are to a range of particle sizes. Air sampling from the plants of the cohort studies (Baltimore and Painesville) that have demonstrated a clear association between exposure to Cr(VI) and lung cancer show that workers in these cohorts were exposed to respirable-sized particles. It is therefore possible to differentiate between the risk assessments for respirable Cr(VI) particles and the non-respirable fraction of the inhalable dose, given that sufficiently detailed data on exposures is available. The "respirable fraction" (E(R)) is defined as the portion of inhalable particles (E(I)) that enter the deepest part of the lung, the non-ciliated alveoli. For this fraction, the particle diameter corresponding to 50% sampling efficiency (D50) is given as 4 μm.

Larger inhaled particles that are deposited in the upper respiratory tract are cleared by the mucociliary escalator and swallowed. It therefore seems reasonable to associate the "inhalable, non-respirable fraction" of Cr(VI) inhalation exposure with the potential for an increased risk of cancer of the small intestine.

For exposure by the oral route, tumours of the small intestine were observed in animals dosed with soluble Cr(VI) compounds. There is no information on the oral carcinogenic potential of chromates of lower solubility, but it is expected that these will be less bioavailable. As noted above, and in the absence of further information, the proposed small intestine cancer risk estimates should be applied to exposures to highly soluble, slightly soluble and insoluble Cr(VI) compounds, accepting that they will perhaps overestimate risks in the case of exposure to slightly soluble and insoluble chromates.

The risk estimates for oral exposure are based on the analyses by USEPA (2012). The USEPA selected the NTP bioassay in rats and mice (NTP, 2008) for dose-response assessment because it was a well-conducted lifetime animal study of Cr(VI) carcinogenicity via ingestion, and no other adequate studies of Cr(VI) carcinogenicity by the oral route were available. In the mouse study, exposure to sodium dichromate dihydrate in drinking water for 2 years resulted in significant increases in the incidences of neoplasms of the small intestine in males and females at doses ≥ 2.4 and ≥ 3.1 mg Cr(VI)/kg bw/day, respectively. The mouse was determined to be the most sensitive because tumor incidences were statistically significantly elevated at lower doses and a greater response was exhibited by the mice at the two highest doses. In order to derive an oral cancer slope factor (CSF), BMD (benchmark dose) modelling was carried out using USEPA`s BMDS (USEPA, 2000). The multistage model was fitted to the data and the BMDL10 (lower 95% confidence bound of the dose corresponding to a BMR of 10% extra risk) was estimated. The CSF was then calculated by dividing the BMR10 (0.1) by the BMDL10 and then converting this slope value to human equivalents. A BMDL10 of 0.9 mg/kg bw/day was identified in males, leading to a CSF of 0.09 $(mg/kg\ bw/day)^{-1}$ in males. The animal CSF values were then converted in human CSF values by multiplying them for the mouse allometric scaling factor (~ 6), resulting in a human oral CSF values for tumours of the small intestine of 0.5. These CSFs were further adjusted by applying age-derived assessment factors (assuming higher sensitivity during childhood) resulting in an average lifetime CSF of 0.8 per mg/kg bw/day for the general population.

Based on an exposure for 70 years (24h/day, every day) and an 89-year life expectancy and against a human background cumulative lifetime intestinal cancer risk of 9 –16 per 1000 for the German population, an excess lifetime intestinal cancer risk = 8×10^{-4} per µg Cr(VI)/kg bw/day was finally estimated.

Application in the population under study

Neurodevelopmental effects

Italy

*The I NAC II cohort*Introduction and study design

The Northern Adriatic Cohort II (NAC II) is a prospective mother-child cohort established in 2007 in a coastal area of Friuli Venezia Giulia Region, to investigate the association between prenatal mercury (Hg) exposure from maternal fish consumption and child neurodevelopment. The NACII cohort represents an extraordinary source of information to address in a developmental perspective the potential interactions of chemical and non-chemical stressors with genetic and epigenetic factors. At present, a biological bank consisting of biological matrices collected at birth from mother-child pairs, a data bank of neuropsychological outcomes measured at different developmental stages (18 months, 40 months, and 6-7 years), as well as a wide range of potential explanatory variables (including housing conditions and socioeconomic indicators, diet habits, child postnatal exposures, maternal lifestyle during pregnancy and breastfeeding period) are available. In total, 900 pregnant women were originally enrolled in the cohort; 767 (85%) of these women remained in the study at delivery, 632 children underwent neuropsychological assessment testing at age 18 months (82%), 470 (70%) at age 40 months.

Several data have been already collected within the NAC II cohort: mother`s hair, mother`s blood (Italy and Croatia), cord blood, cord tissue and meconium have been sampled at birth, breast milk and mother`s hair 1 month after

birth. Hair samples have been analysed for mercury (total Hg and MeHg for the samples having more than 1000 ng/g total Hg in hair), blood and breast milk for mercury (total Hg and MeHg for the samples having more than 1000 ng/g total Hg in hair), cadmium, lead and arsenic, as well as for essential elements (selenium, zinc, copper). Children were then tested for neurodevelopment (Bayley III test) at the age of 18 and 40 months. Detailed questionnaires about health and socioeconomic data of child and her/his family are also available.

CROME has supported a new research protocol focused on the follow up of 200 children born within the NAC II PHIME cohort, at the age of 7 years. The new biomonitoring campaign started in August 2014. Child`s hair, child`s urine and saliva sample of both mother and child (Oragene DNA self-collection kit) have been collected from each child-mother pair at the time of appointment. Mothers have completed a new questionnaire to update information on life-style factors, and children (now at the age of 7) were subjected to neuropsychological testing by trained neuropsychologists. Mothers were also subjected to PSI (Parenting stress index) interview. Chemical analyses of biological samples collected following neuropsychological assessment consisted in measurements of the concentration of five neurotoxic metals (mercury, lead, manganese, cadmium, arsenic) in either hair or urine. 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 assessment of writing skills, CBCL - Child Behaviour Check List to identify behavioural and emotional problems in children.

Results of the follow-up:

Metals concentration in urine and hair; correlation with fish intake

Data concerning concentration of the metals in study in both urine and hair of the children are reported in *Table 4*. As for Hg and Pb, values are within the reference values established by the PROBE survey for adolescents aged 13-15 in blood (see below).

More in detail, when considering the chemical exposure measured at the different ages, we found a significant correlation between Hg in cord blood and Hg in mother`s hair during pregnancy; both are correlated to Hg in hair at 7 years that in turn is correlated with Hg in urine at the same age (Table 5). On the whole, these results suggest that the maternal life style is among the factors influencing Hg levels in 7 year-old children. This is also supported by the significant correlation found between fish consumption of the mother during pregnancy and breastfeeding and total Hg levels in urine and hair at 7 years ($r = 0.26$; $p < 0.001$) (Table 6 and Table 7).

There are signficant correlations between metals internal concentrations (Table X), which could be possibly attributable to coexistence of different metals in fish. The link between consumption of all kinds of fish by the child and levels of metals in urine/hair is supported by the finding that Hg levels (both urine and hair) are related to consumption of canned fish (mainly tuna fish). Mn levels in urine was significantly related to fresh fish and fried crustacean consumption ($r = 0.23$, $p = 0.002$; $r = 0.33$, $p = 0.001$); finally Pb levels are related to consumption of fried fish, crustacean or molluscan (data not shown)

Table 4. NAC-II cohort follow-up at 7 years (n = 200): mean concentration of the five neurotoxic metals in urine (A) and hair (B).

(A)	As	Cd	Hg	Mn	Pb	Se
mean	35,752	0,47	0,48	0,20	0,717	38,94
SD	97,787	0,38	0,29	0,30	0,448	20,94
rSD%	273,518	79,6	60,4	152,4	62,557	53,79

min	1,200	0,07	0,23	0,06	0,145	6,53
max	920,165	3,10	1,89	3,45	3,230	129,30
(B)			Hg	Mn		
mean			0,76	0,21		
SD			0,66	0,17		
rSD%			86,90	80,36		
min			0,06	0,04		
max			4,09	1,09		

Table 5. Correlation among all metals in urine at 7 years

		As7	Cd7	Hg7	Mn7	Pb7	Se7
As7	Correl. Pearson	1	.111	.061	-.042	.018	.188**
	P		.117	.393	.553	.801	.008
	N		200	200	200	200	200
Cd7	Correl. Pearson		1	.134	.062	.196**	.378**
	P			.058	.380	.005	.000
	N			200	200	200	200
Hg7	Correl. Pearson			1	-.077	.081	.360**
	P				.278	.253	.000
	N				200	200	200
Mn7	Correl. Pearson				1	.020	.008
	P					.779	.913
	N					200	200
Pb7	Correl. Pearson					1	.167*
	P						.018

	N						200
Se7							1

*Table 6. Correlation between Mn and Hg values in child's hair and those found in different matrices in the perinatal stages (Cord blood, Maternal venous blood in pregnancy, Maternal hair and Breast milk 1st month). *p<0.05 **p<0.01*

Perinatal Hg exposure	THg child hair
	Pearson correlation
Cord_blood_THg	0,277**
Cord_blood_MeHg	0,190
M_vblood_THg	0,310**
M_vblood_MeHg	0,200
M_hair_THg	0,461**
M_hair_MeHg	0,178
M_milk_MeHg	0,238
M_milk_THg	0,218**

Table 7. Correlation between perinatal exposure to Mn in different matrices (maternal venous blood, cord blood and breast milk at 1 month postpartum) and Mn at 7 years

Perinatal exposure to Mn	Mn child hair 7 years
	Pearson correlation
Cord_blood_Mn	0,143
M_vblood_Mn	-0,067
M_milk_Mn	0,215*

Perinatal exposure to Mn	Mn child urine 7 years
	Pearson correlation
Cord_blood_Mn	0,262**
M_vblood_Mn	0,239**
M_milk_Mn	-0,022

Table 8. Reference values established on the basis of data collected in different biomonitoring studies in children and adolescents (blood)

Blood metals (μg/L), GM							
Metal	GerES IV [a]	Health Canada [b]		NHANES [c]	FLESH II [d]	PROBE [e]	
		M	F			M	F
As		0.58	0.61			0.71	0.72
Cd	0.14	0.16	0.18	<0.14	0.21	0.29	0.29
Hg	0.26	0.29	0.33	0.49		0.77	0.78
Mn		9.44	10.6		9.66	7.24	7.22
Mo		0.70	0.65			1.10	1.11
Ni		0.62	0.63		1.25	0.94	0.94
Pb	14.5	8.8	7.1	<0.42	14.8	9.60	8.75

a - German Federal Environment Agency, 2008; adolescents aged 11-14
b - Health Canada, 2010; children aged 12-19
c - Fourth National Report on Human Exposure to Environmental Chemicals, CDC, 2009; children aged 12-19
d – FLESH II, adolescents aged 14-15
e - PROBE survey, adolescents aged 13-15

Table 9. Multiple linear regression with Hg levels in hair at 7 years as dependent variable

Coefficient[a]								
Model		Coefficients not standardized		Coefficient standardized	t	Sign.	Collinearity	
		T	Standard Error	Beta			Tolerance	VIF
1	(Costant)	-.520	.654		-.795	.428		
	Ed_titles_mom	.213	.079	.229	2.690	.008	.840	1.191
	Child_sex	-.229	.123	-.151	-1.860	.066	.921	1.085
	Raven_QI_Mother	-.001	.005	-.011	-.124	.902	.808	1.237
	Canned fish	.162	.045	.300	3.635	.000	.889	1.125
	WCANNED	-.055	.066	-.068	-.832	.407	.906	1.104
	Home_size	.098	.066	.119	1.489	.140	.951	1.052
	M_hair_THg	.158	.051	.361	3.351	.001	.524	1.908

	Cord_blood_THg	.005	.017	.029	.268	.789	.526	1.903
a.: Hg_child's hair_								

The levels of Hg in child's hair at 7 years is influenced by the educational level of the mother, consumption of canned fish by the child (mainly tuna fish, rich in Hg) and levels of Hg in the mother's hair at birth. Nor scores of the mother in the Raven test(IQ score) or consumption of canned fish during pregnancy by the mother and socioeconomic index such as Home Size are related to the dependent variable.

Association between metals' concentration and neuropsychological scores at 7 years

When considering the correlation between levels of metals in urine and hair at 7 years and the different neuropsychological scores obtained in the selected test batteries, no major effects of total Hg (or other metals) levels in urine on the different behavioral functions scored (scale MT, CBCL, NEPSY-II, WISC) was found. At variance, Hg in hair is positively related with cognitive scores in Wisc-III (the higher the Hg levels the better the performance) but also with CBCL scores, with children with higher Hg levels showing the worse behaviour (more anxiety and retreat).

Mn as measured in child's hair produced adverse effects ($r=0.16$, $p<0.04$) on the five Wisc-III score, with statistically significant decrement of the general IQ and of verbal comprehension.

As expected we confirmed a positive association between maternal IQ and IQ of the child assessed in the WISC-III test, as well as a positive correlation between IQ previously assessed at 18 and 40 months and that assessed at 7 years, and a positive effect of higher socioeconomic status on the child's IQ.

These analyses confirm the results of other large epidemiological studies on the neurobehavioral toxicity of prenatal/neonatal mercury exposure at low doses. In absence of significant impairments of behavioral functions at different ages, the results highlight the complex interaction between multiple environmental exposures, dietary and life style factors, and support the usefulness of the integrated CROME approach.

Table 10. Significant ($p<0.05$) association between metal biomarkers and neuropsychological scores at 7 years

Neuropsychological assessment *	Nepsy II	MT	WISC III	CBCL
Educational title of the mother			**Positive effect**	
Urine	**Cadmium + Arsenic + Selenium +**	----------	**Arsenic +**	----------
Hair	----------	**Manganese -**	**Manganese –**	**Mercury -**

* Nepsy II: sensorimotor, attention, working memory; MT: reading and writing skills; WISC III: general cognitive scores and intelligence (IQ); CBCL: behavioural and emotional problems reported by teachers and parents.

+ child's performance improved; - child's performance decreased

Evaluating developmental trajectories

Following evaluation of the association between metals/metalloids levels and neuropsychological performances at 7 years, we decided to consider the role of previous exposure to the same chemical compounds. Data concerning metals concentration in venous blood of the mothers in pregnancy (20th week), in cord blood at birth and in brest milk analysed at the end of the first month of life were made available by the database of the PHIME study. In Table 11 the mean concentrations of the metals in study are reported.

Table 11. Mean concentration of metals and metalloids in pregnancy (20-30 wks),at birth and on first month of postnatal life

	As	Cd	Hg	Mn	Pb	Se	Cu	Zn
Mother blood in pregnancy (ng/ml)								
Mean	2.20	0.36	3.32	10.12	11.49	109.21	1581.70	5009.02
SD	2.66	0.23	2.97	3.47	5.48	17.99	281.46	795.69
Cord blood (ng/g)								
Mean	2.39	0.15	5.73	40.79	11.70	116.44	712.40	2445.17
SD	3.13	0.13	4.57	14.89	5.96	26.63	141.5	597.57
Breast milk first month (ng/ml)								
Mean	0.70	0.13	0.30	2.61	1.28	18.34	521.60	2739.19
SD	1.31	0.34	0.40	1.34	2.14	7.20	228.20	1388.29

With the aim to describe the developmental trajectories of the 200 children enrolled in the follow up, we reanalysed the effects of the previous exposure on the performance of these same children in the Bayleys scales carried out at 18 and 40 months. Main results are shown in Table 12

Table 12. Neuropsychological scores through Bayleys scales at 18 and 40 months in relation to perinatal exposure, with correction for life style factors

Behavioural domains		
Age at testing	18 months	40 months

	Cognition	*Language*	*Motor*	*Cognition*	*Language*	*Motor*
Period of exposure/matrix						
Pregnancy/mother blood	Copper -	Copper -	Copper –	——	Zinc -	——
Birth/cord blood	Zinc +	————	Copper +	Zinc – Copper +	——	Cadmium-
First month of life/breast milk	——	————	————	Zinc –	———	Zinc – Cadmium –

* fish consumption during pregnancy (20th week and follow up at 1st month postpartum), maternal smoking, mother educational level; + positive effects on the outcome, - negative effects on the outcome.

In agreement with previous published studies in the PHIME cohort , Hg did not influence psychomotor scores at 18 or 40 months, while micronutrients exerted significant modulation on these same scores.Interestingly, in general both Zinc and Copper delayed psychomotor development, depending also on the time window of exposure.

The study of the association between perinatal exposure and neuropsychological functions at 7 years revealed a complex picture, where different metals modulate negatively or positively the scores of the children in the different domains considered (see Table 13).

Table 13. Significant (p<0.05) association between metal biomarkers in the perinatal stage and neuropsychological scores at 7 years

Neuropsychological assessment *	Nepsy II	MT	WISC III	CBCL
Period of exposure/matrix				
Pregnancy/ mother blood	----------	**Selenium +** **Zinc -**	**Selenium + (IQ)** **Zinc -**	**Selenium -**
Birth/ cord blood	**Mercury –** **Cadmium -** **Arsenic +**	**Selenium +** **Cadmium -**	----------	**Arsenic -**
First month of life/breast milk	**Manganese -** **Lead -** **Arsenic +** **Selenium +**	**Manganese -** **Lead -**	**Manganese -** **Cadmium -**	----------

* Nepsy II: sensorimotor, attention, working memory; MT: reading and writing skills; WISC III: general cognitive scores and intelligence (IQ); CBCL: behavioural and emotional problems reported by teachers and parents.

Summary of major results and conclusions

- 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 neurpsychological outcome to all the NAC II cohort.

Spain

The INMA study

Evaluation of the potential neurotoxic effect of lactation exposure to organochlorine pollutants

The first part of this contribution was aimed to evaluate whether lactational exposure to PCB-153, the PCB congener with the highest levels and detection rate, DDE and HCB during specific postnatal periods, as estimated with a PBPK model, is associated with decrements in mental and psychomotor functions.

Study population

This study was based on the cohorts from Gipuzkoa – Basque Country, Sabadell – Catalonia, and Valencia – Valencian Country belonging to the INMA-INfancia y Medio Ambiente (Environment and Childhood) – Project (Guxens et al., 2012b). All regions followed the same protocol and started recruiting pregnant women between 2003 and 2008 (Sabadell N=657, Valencia N=855, Gipuzkoa N=638). Pregnant women coming for their first trimester routine antenatal care visit in the main public hospital or health centre of reference and who fulfilled the inclusion criteria (age above 16 years, to have a single pregnancy, intention to deliver in the reference hospital, and no problems of communication) were invited to participate. Protocol details are described elsewhere (Guxens et al., 2012b). Out of the initial 2150 mother-children pairs enrolled, 339 had no information on maternal POP levels, 247 were not assessed for mental and psychomotor development and 295 had no information on variables needed in the PBPK model (sex, birth weight, type and duration of breastfeeding, maternal age at delivery, maternal age at the time of blood draw, concentration of lipids in maternal serum, age of the child at the time of neuropsychological development assessment, and weight of the child at each month of postnatal life during the 1st year of life). Out of the 1269 remaining children, 10 were diagnosed with pathologies and were excluded, as well as those flagged by psychologists because of difficulties and suboptimal cooperation at the time of the evaluation (N=75). Finally, 9 children had missing information on co-variables used in the adjusted models, so these were excluded as well, resulting in a total of 1175

participants in the present study. This study was conducted with the approval of the hospital ethics committees in the participating regions and written informed consent was obtained from the parents of all children.

Child neuropsychological assessment

Children`s mental and psychomotor development was assessed at around 14 months of age (range 11-21 months) using the BSID-I (Bayley, 1977). The mental scale consisted of 163 items that assessed age-appropriate cognitive development in areas such as performance ability, memory, and first verbal learning. The psychomotor scale consisted of 81 items assessing fine and gross motor development. All testing was done in the health care centre in the presence of the mother by eight specially trained psychologists who were blind to exposure levels or any other information. To limit inter-observer variability, we applied a strict protocol, including training sessions where inter-observer differences were quantified and three sets of quality controls (inter-observer-reliability-tests) undertaken during the fieldwork. A high inter-rater reliability, estimated in 12 children through intra-class correlation analyses, was observed with coefficients of 0.90 for mental test scores, and 0.91 for psychomotor test scores. Raw scores were standardized for child`s age in days at test administration using a parametric method for the estimation of age-specific reference intervals. The parameters of the distribution were modeled as a fractional polynomial function of age and estimated by maximum likelihood. Residuals were then standardized to a mean of 100 points with a standard deviation of 15 points to homogenize the scales (Guxens et al., 2012a).

Prenatal exposure assessment

Concentrations of PCB-153, DDE and HCB were measured in maternal serum samples taken between the 7th and the 26th week of pregnancy (median=12.9 weeks) from peripheral veins. Serum samples were stored in crystal tubes at -20ºC (Sabadell and Gipuzkoa) or at -80ºC (Valencia) and analyzed by gas chromatography using methods described elsewhere (Goni et al., 2007). The limits of detection (LOD) were 0.071 ng/ml in Sabadell and Gipuzkoa and between 0.01 and 0.071 ng/ml in Valencia. International intercalibration exercises showed that differences of levels between regions were not due to lab differences. For comparison purposes, values in Valencia below 0.071 ng/ml were set as non-detected. Samples with non-detectable levels were then set at a value below the LOD using univariate simple imputation sampling. Exposures were expressed on a lipid basis in ng/g lipid using the method described in Phillips et al. (1989.

Postnatal exposure estimation - PBPK modelling

The PBPK model used to simulate postnatal exposure to POPs in this study is detailed in a previous paper (Verner et al., 2009). In a nutshell, this PBPK model is a mathematical representation of the absorption, distribution, metabolism and excretion of POPs in both the mother and child. For any exposure scenario and individual physiology, the model can generate blood and tissue level vs. time profiles. Tissue volumes and lipid composition, blood flows and breast milk composition and daily intake were scaled according each mother and children weight, height, age and gender. Breast milk POPs levels are estimated based on the maternal blood POP levels measured during the prenatal period. Maternal daily oral dose throughout her life is estimated through an iterative optimization process so that simulated blood levels match those measured in the study. This oral daily dose continues during the postnatal period. Both the duration of exclusive (period during which the child is only fed with breastmilk) and partial breastfeeding (period during which the child is fed both breast milk and other types of food) were abstracted from questionnaires administered at the time of neuropsychological testing at age of 14 months of the child. In order to characterize breast milk intake during partial breastfeeding in this population, we used data from 382 mother-child pairs enrolled in the region of Sabadell for which we collected information on the percentage of food intake attributable to breastfeeding during the period when children were fed both breast milk and other solids/liquids. In this subset of the population, breast milk represented 71% of total food intake during the first month of the partial breastfeeding period and 55 % during the last month. These percentages were independent of the duration of partial breastfeeding. Therefore, we defined breast milk intake during this period as a constant decrease from 71% to 55% of the daily intake in exclusively breastfed children over the period of partial breastfeeding.

We traced body weight profiles for the first year of life based on month-by-month weight data. Participants were weighed on average 6 times (ranging from 2 to 12) between birth and 12 months of age during their visits to the pediatrician. Missing birth weight values were estimated by linear regression based on weight at 1 month (n=3). The Jenss-Bayley (van Dommelen et al., 2005) growth model was used to estimate missing monthly weight data based on available measurements. Height profiles were based on standard growth curves and children`s height at 13.8 months of age (range = 11.4-19.5). For each child, we simulated blood POP level profiles for the first year of life. We extracted the blood concentration at each month for a total of 12 exposure estimates. The PBPK model was coded in ACSLX software (Aegis Technologies Group, Inc., Huntsville, AL, USA). A script was developed to extract data from databases and compile results in Microsoft Excel 2010 spreadsheets (Microsoft, Redmond, WA, USA).

Other variables

Information on co-variables was extracted from the questionnaires answered by the mothers during the 1st and the 3rd trimester of pregnancy and at age 14 months of the child: maternal age, education and region of birth, maternal smoking during pregnancy, parity (first child or not), day care attendance, maternal consumption of total fish during pregnancy and maternal and paternal social class (defined using the UK Registrar General`s 1990 classification according to occupation by ISCO88 code; non-manual – professionals, managers and technicians, other non-manual – skilled manual and non-manual jobs, manual – semiskilled and unskilled jobs). Maternal pre-pregnancy body mass index (BMI), gestational age and weight at birth were collected from clinical records or reported by mothers.

Statistical methods

We log-transformed POP concentrations for statistical analyses. Coefficients for the association between the different POPs concentrations and the BSID`s mental and psychomotor scores were estimated using linear regression models, where each exposure was introduced individually. Given the high correlation of simulated children`s blood levels between contiguous months of postnatal life, four periods of three months were created, where monthly levels were averaged to better untangle the importance of each period of exposure and to be able to adjust one exposure for the other (multi-exposure model) whilst avoiding problems of collinearity. However, we also assessed the role of postnatal exposure at each month during the first 12 months. Linearity of the association between POPs and BSID`s mental and psychomotor scores was assessed by using Generalized Additive Models (GAM); associations between DDE and mental and psychomotor test scores were not linear (p-gain for non-linearity <0.05 and 0.13, respectively). For this reason, DDE levels were included in the models as a binary variable, where the median was used as a cut-off. Potential confounders /Covariates were considered using a backward selection procedure; those showing associations with p-value<0.05 with mental or psychomotor test scores or those that resulted in a change in estimate of ≥10% in the linear regression models, were retained in the models. All the analyses were done with STATA 10 (Stata Corporation, College Station, Texas, USA).

POP concentrations

There were no differences in POPs levels between participants and non-participants. None of the participants had a gestational age below 37 weeks and the percentage of children with low birth-weight (≤2500 g) was much lower. Participants were breastfed for a longer period and had better mental and psychomotor BSID scores.

Table 14. Geometric mean (GM) and the 25th and the 75th concentrations (ng/g lipid) of the exposure to PCB153, DDE and HCB during prenatal (measured in maternal blood) and postnatal life (estimated through PBPK modelling[a]).

	PCB153			DDE			HCB		
	GM	25th	75th	GM	25th	75th	GM	25th	75th
Prenatal exposure	39.81	28.19	60.65	132.47	76.61	204.57	42.74	24.60	77.58
Postnatal exposure estimations									
1st three-month period	60.32	34.69	115.48	199.17	107.16	356.48	62.29	34.64	121.72
2nd three -month period	60.85	26.86	148.14	199.11	94.39	447.22	60.49	26.51	150.14
3rd three -month period	54.87	20.97	152.51	177.86	72.86	463.17	52.41	20.53	143.03
4th three -month period	49.63	18.32	144.91	159.28	61.27	417.79	45.45	17.00	129.15

[a]Postnatal exposure was estimated month by month from the 1st until the 12th month of postnatal life, however, in the present table estimations have been grouped into four-month periods (N=1175), which are the periods used in the main analysis.

PCB-153, DDE and HCB were detected in a high proportion of maternal serum samples (95.1%, 99.5% and 91.2% of the samples, respectively). DDE was the compound present in the highest concentrations in maternal serum, approximately three times higher than PCB-153 or HCB (Table 1). Regarding estimations obtained with the PBPK model, the highest levels of POPs, on average, were reached around the 3rd month of life for DDE (GM=206.6 ng/g lipid) and HCB (GM=63.9 ng/g lipid) and within the 4th month of life for PCB153 (GM=62.9 ng/g lipid) (full data not shown). The correlation between prenatal and estimated postnatal concentrations of the different POPs decreased with increasing child age. Correlations between different compounds showed that prenatal DDE concentrations were poorly correlated to PCB-153 or HCB concentrations (r=0.11 and 0.22, respectively), whereas the correlation between PCB-153 and HCB was 0.45. However, the correlation between compounds increased across subsequent months of exposure; at 12 months of age there was a correlation of 0.74 and 0.72, respectively, between DDE and PCB-153 between DDE and HCB, whereas the correlation between PCB-153 and HCB was 0.82.

The associations between postnatal estimates of exposure and BSID`s mental and psychomotor test scores were not statistically significant for any of the three-months periods assessed (Table 15), whereas increasing prenatal PCB-153 concentrations were significantly associated to a lower psychomotor score (adjusted βcoefficient [95%CI]= -1.36 [-2.61, -0.11]). Associations observed between PCB-153 and psychomotor test scores during prenatal life weakened gradually across periods of postnatal life (1st period: -0.41 [-1.37, 0.54]; 2nd period: -0.07 [-0.79, 0.65]; 3rd period: 0.02 [-0.63, 0.68]; 4th period: 0.05 [-0.58, 0.68]). This same pattern was observed with the mental test scores, although no significant association with pre- or postnatal exposure was found (Table 2). Similar results were obtained when analyses were performed using monthly blood levels. No association was found between postnatal exposure to DDE or HCB and any of the two scores assessed (Table 15), neither with prenatal exposure.

Table 15. Associations between prenatal and postnatal exposure[a] (divided into three-month periods) and mental and psychomotor scales (N=1175).

	Mental scale[b]			Psychomotor scale[c]		
	Crude β coefficient (95%CI)	**Adjusted β coefficient (95%CI)**	**p-value**	**Crude β coefficient (95%CI)**	**Adjusted β coefficient (95%CI)**	**p-value**
PCB153						
Prenatal exposure	0.20 (-1.08, 1.48)	-0.39 (-1.83, 1.06)	0.60	-1.16 (-2.41, 0.09)	-1.36 (-2.61, -0.11)	0.03
Postnatal exposure estimations						
1st three -month period	0.21 (-0.75, 1.18)	-0.23 (-1.25, 0.78)	0.65	-0.20 (-1.14, 0.74)	-0.41 (-1.37, 0.54)	0.39
2nd three -month period	0.27 (-0.45, 1.00)	-0.03 (-0.77, 0.72)	0.94	0.09 (-0.61, 0.80)	-0.07 (-0.79, 0.65)	0.85
3rd three -month period	0.31 (-0.34, 0.97)	0.06 (-0.62, 0.73)	0.86	0.17 (-0.48, 0.81)	0.02 (-0.63, 0.68)	0.94
4th three -month period	0.34 (-0.30, 0.97)	0.10 (-0.56, 0.75)	0.77	0.18 (-0.45, 0.80)	0.05 (-0.58, 0.68)	0.89
DDE						
Prenatal exposure (<123.22 vs ≥123.22)	0.60 (-1.17, 2.37)	0.86 (-0.92, 2.63)	0.34	0.46 (-1.27, 2.19)	0.50 (-1.23, 2.22)	0.57
Postnatal exposure estimations						

1st three -month period (<199.79 vs ≥199.79)	0.14 (-1.61, 1.87)	0.27 (-1.49, 2.03)	0.76	0.52 (-1.18, 2.22)	0.46 (-1.24, 2.16)	0.59
2nd three -month period (<230.94 vs ≥230.94)	-0.44 (-2.16, 1.28)	-0.50 (-2.24, 1.24)	0.57	-0.38 (-2.07, 1.30)	-0.51 (-2.20, 1.18)	0.55
3rd three -month period (<213.51 vs ≥213.51)	-0.32 (-2.04, 1.40)	-0.43 (-2.17, 1.31)	0.63	-0.02 (-1.70, 1.66)	-0.13 (-1.82, 1.55)	0.88
4th three -month period (<192.61 vs ≥192.61)	0.06 (-1.67, 1.78)	-0.03 (-1.77, 1.71)	0.97	0.18 (-1.51, 1.86)	0.07 (-1.62, 1.75)	0.94
HCB						
Prenatal exposure	0.79 (-0.22, 1.81)	0.66 (-0.44, 1.76)	0.24	-0.68 (-1.67, 0.31)	-0.70 (-1.69, 0.29)	0.17
Postnatal exposure estimations						
1st three -month period	0.68 (-0.20, 1.56)	0.43 (-0.49, 1.34)	0.36	-0.13 (-0.98, 0.73)	-0.23 (-1.09, 0.64)	0.61
2nd three -month period	0.57 (-0.12, 1.27)	0.35 (-0.36, 1.07)	0.33	0.12 (-0.56, 0.80)	0.01 (-0.67, 0.70)	0.97
3rd three -month period	0.57 (-0.07, 1.20)	0.37 (-0.28, 1.02)	0.26	0.19 (-0.44, 0.81)	0.09 (-0.53, 0.72)	0.77
4th three -month period	0.57 (-0.04, 1.19)	0.39 (-0.24, 1.01)	0.23	0.20 (-0.40, 0.80)	0.11 (-0.49, 0.71)	0.72

[a]PCB153 and HCB were introduced in the model as continuous variables and DDE was introduced as a categorical variable (median as a cut-off). bMental scale model; crude models already adjusted for sex and region. Adjusted model for: region of study, sex, gestational age, day-care attendance, birth weight, maternal social class and maternal region of birth. For prenatal exposure the model was also adjusted for predominant breastfeeding. cPsychomotor scale model; crude models already adjusted for region of study. Adjusted model for: region of study, gestational age, paternal social class.

Models including prenatal and postnatal PCB-153 exposures jointly showed similar results to those obtained when only one of these exposures was included in the model. For instance, when the 1st period of postnatal exposure was included in the model together with prenatal exposure the association between prenatal exposure and psychomotor score was β(95%CI)=-2.24 (-4.18, -0.31) compared to β(95%CI)=-1.36 (-2.61, -0.11) when postnatal exposure was left out of the model, whereas no association with postnatal exposure was found. The same occurred when including prenatal and the 2nd, the 3rd or the 4th period of postnatal exposure in the model.

Postnatal exposure estimations to PCB153, DDE and HCB

On average, children`s estimated blood POP levels were higher during the first months of postnatal life than those measured during their prenatal life; due to breastfeeding, levels increased during the first 3 to 4 months of life, and started to decrease from the 4th-5th month. In Catalonia, it is around this time when most of the mothers return to work and thus stop breastfeeding or start to combine breastfeeding with other types of foods. Results in the present cohort also show that the correlation between prenatal and subsequent estimated postnatal concentrations of the different POPs decreased with increasing months of life.

The low correlation between prenatal DDE and PCB-153 or HCB in the present population can be explained by the different sources of exposure to these compounds. In Spain, DDE was extensively used in agriculture and for pest control, whereas PCB153 was used in industrial processes. HCB was used as a fungicide in agriculture but has also been released in the environment as an unintended product of industrial combustion processes. Diet is another differential exposure factor; for instance, fish is a major source of PCB-153, but not of DDE, for which meat, fruit and cereals seem to be more important sources of exposure in the present population (Llop et al., 2010). However, as the main source of exposure to all these compounds during postnatal life is breastfeeding, the correlation among them increases.

Neurotoxic effects of exposure to postnatal PCB153, DDE and HCB

With the exception of the study published by Verner et al. (2010), previous studies evaluating neurotoxic effects of postnatal exposure to POPs at around one year of age assessed postnatal exposure by measuring POPs levels in breast milk or, in order to improve exposure estimations, by multiplying the levels measured in breast milk by the number of weeks of breastfeeding (Gladen et al., 1988; Huisman et al., 1995; Koopman-Esseboom et al., 1996; Pan et al., 2009; Wilhelm et al., 2008a; Wilhelm et al., 2008b). None of these studies observed an association between postnatal exposure to PCBs (Gladen et al., 1988; Huisman et al., 1995; Koopman-Esseboom et al., 1996; Pan et al., 2009; Walkowiak et al., 2001; Wilhelm et al., 2008a; Wilhelm et al., 2008b) or DDE (Gladen et al., 1988; Pan et al., 2009) and different domains of neuropsychological development at ages between 6 and 24 months of age. Postnatal effects of HCB have never been assessed in previous studies. In the present study, where postnatal exposure assessment has been improved by applying PBPK models, no associations were found with any of the three-month or month-by-month periods of postnatal exposure. In fact, the associations observed during prenatal life weakened gradually across periods of postnatal life. No association with mental score was found. These results contrast with those obtained elsewhere (Verner et al., 2010), the only other study applying PBPK models, where an association between blood PCB-153 levels around the 4th month of life and infants` ability to control their activity (Behaviour Rating Scale of the BSID test) at the age of 11 months was detected. However, there are some methodological differences that could explain this discrepancy; for instance, the domains assessed in this study were different from those evaluated in the present study. Additionally, prenatal and postnatal levels of exposure were higher in the Inuit study population (on average, mothers breastfed their children for 156 days in the Inuit cohort vs. 106 days in the present cohort). On the other hand, we should note that, although BSID is one of the most widely used tools available in Spanish to assess neuropsychological development at such young ages, it has sometimes shown a low predictive value for later performance on general cognition and intelligence tests (Bayley, 1977). For instance, a study assessing effects of postnatal PCB exposure (calculated by multiplying milk PCB levels by the weeks of breastfeeding) did not observe any associations with BSID measures at 7, 18 and 30 months of life. However, significant negative effects of postnatal exposure were detected when children were assessed with the Kaufman Assessment Battery for Children at age 42 months (Walkowiak et al., 2001). Thus, evaluation of both pre- and postnatal exposure to these compounds will be interesting at older ages in the present study population, when brain is more developed, phenotypes better expressed and children have acquired more neuropsychological abilities that can be tested with more precise and reliable tests.

The role of breastfeeding

Postnatal exposure to POPs during the first year of life is primarily due to lactational exposure (Jorissen, 2007). Through breast milk, children are also exposed to beneficial compounds such as long-chain polyunsaturated fatty acid (LC-PUFA), as shown by a previous study including 504 children of the INMA birth cohort study in the region of Sabadell, which revealed mental score to be better among children with higher LC-PUFA levels and longer breastfeeding period. However, psychomotor development was not improved (Guxens et al., 2011). In the present study it is still possible that we see no effect of postnatal exposure to PCB-153 (or other POPs) because of the counterbalancing beneficial effects of breastfeeding, or maybe because the critical window of exposure for these outcomes is, in fact, prenatal life. To test for that, in the present study we adjusted the model for psychomotor scores by including prenatal and postnatal exposures together and results were similar to those obtained with models including one exposure estimate at the time. This seems to indicate that prenatal exposure probably is the most critical window of exposure or, at least, more critical than any other period of postnatal life for these outcomes.

Strengths and limitations

The present study has some limitations, but also strengths compared to previous papers. No preterm children or with low birth weight were represented. However, we do not think this is affecting or biasing the association between the exposure and neuropsychological development of the children. Indeed, it might be helping to better detect the effects of such exposure, because we are avoiding important factors related to worse neuropsychological development such as being a preterm child, with low birth weight, and coming from lower social and educational classes.

We have not measured postnatal exposure at age 14 months to validate the PBPK model simulations in our study population. However, Verner et al. (2009 validated the model and showed good correlations between measured and estimated values. The PBPK approach provides estimates of POP levels at any time point during the first year of life, a serious advantage over other metrics such as the multiplication of breast milk levels by the weeks of breastfeeding that only give an overall estimate of postnatal exposure. It is thus a potential tool for future epidemiological birth cohort studies evaluating the health effects of POP exposure during the first years of life. However, this model needs to be validated for other POPs such as PBDEs for which substantial postnatal exposure also occurs and may exhibit different chemical properties such as transfer rates from maternal blood to other biological matrices (Costa and Giordano, 2011; Frederiksen et al., 2010).

When models including prenatal PCB-153 exposure were adjusted for any of the postnatal three-month periods of exposure, associations between psychomotor score and prenatal PCB-153 remained, whereas no association with postnatal exposure was observed. Although the study population differed a little (N=1391 vs 1175), we obtained consistent results to those found by Forns et al. (2012a between prenatal PCBs and psychomotor score.

Our study population is the largest to date (N=1775). Previous studies on the effects of postnatal exposure to POPs ranged from 168 (Verner et al., 2010) to 858 participants (Gladen et al., 1988). Additionally, we were able to perform PBPK models because we had complete information on the type and duration of breastfeeding during the first year of life, as well as complete information on other important variables needed to perform such models and to control for potential confounding.

Conclusions

Despite the fact that breastfeeding increases children`s blood POP levels during postnatal life, results from this study suggest that deleterious effects of PCB-153 on early brain development, particularly on psychomotor development, could mainly be attributable to prenatal exposure to low levels of POPs.

The glutamatergic system and neurodevelopment

The second part of this contribution was devoted to assess how the glutamatergic system is targeted by pollutants and what is the influence of the process in neurodevelopment in the early life period. Glutamate is a non-essential amino acid that is important for the normal growth and development of the fetus during pregnancy. Glutamate is produced by the fetal liver using maternal glutamine, and the excess of glutamate from the fetal circuit is removed by glutamate transporters (namely excitatory amino acid transporters, EAAT) in placenta (Battaglia, 2000; Noorlander et al., 2004). Glutamate is also the major excitatory neurotransmitter in the central nervous system of mammals and it is present in human fetal brains at concentrations that did not vary during the third trimester of pregnancy (Girard et al., 2006). It mediates key aspects of normal brain function such as sensory information, motor coordination, emotions and cognition. However, it has neurotoxic effects when it is present in excess (Danbolt, 2001).On the other hand, the amino acid aspartate shares the same uptake transport than glutamate. Although its role as a neurotransmitter has not yet been established, recent studies suggest that it may play neuro modulatory functions at developmental stages by acting on glutamate receptors (Errico et al., 2012; Ota et al., 2012).

Mercury has been reported to inhibit glutamate transport in neural cells and to increase glutamate release into the extracellular space of neural cells and platelets (Allen et al., 2001; Borges et al., 2007; Fonfria et al., 2005). Specific attention should be paid to methylmercury because: i) it has recognized properties as a human neurodevelopmental toxicant (Grandjean and Landrigan, 2006); ii) poisoning incidents with methylmercury have demonstrated the potential of this pollutant as neurotoxicant and its serious health consequences (Castoldi et al., 2008; Ekino et al., 2007; Grandjean et al., 2010); iii) millions of people are nowadays chronically exposed to this contaminant and there is epidemiological (Marsh et al., 1987; Pinheiro et al., 2007; Yokoo et al., 2003) and experimental (Castoldi et al., 2008; Farina et al., 2009; Vendrell et al., 2010; Vendrell et al., 2007) evidence of the toxic effects of chronic

exposure to this metal especially for the nervous system; and iv) previous results showed elevated levels of this pollutant in umbilical cord blood, where 70% of newborns had mercury levels above US EPA recommended level, i.e. 5.8 µg/L of MeHg (Ramon et al., 2008).

Moreover, organochlorine compounds (OCs) such as PCBs and dieldrin have also been described to alter glutamate neurotransmission (Briz et al., 2010; Llansola et al., 2010; Mariussen and Fonnum, 2001; Piedrafita et al., 2008; Stavenes Andersen et al., 2009), which may lead to neurotoxicity. Prenatal exposure to some of them are of high concern since levels above the limit of quantification have been found in a significant percentage of samples of several mother-child cohorts including the INMA cohort of Valencia (Vizcaino et al., 2011a; Vizcaino et al., 2010). Furthermore, neuropsychological studies on these children reveal an association between PCB prenatal exposure and impaired neuropsychological and psychomotor development (Cheslack-Postava et al., 2013; Fernandez et al., 2012; Forns et al., 2012a; Forns et al., 2012b; Gascon et al., 2013; Sagiv et al., 2012). The aim of the present study is to address whether mercury and some OCs alter glutamate uptake in human placental membranes and, thus, influence the levels of excitatory amino acids in cord blood. For this purpose, 40cord blood samples with known concentrations of mercury and seven organochlorines, including PCBs, DDTs, hexachlorobenzene (HCB), and beta-hexachlorocyclohexane (βHCH) have been analyzed for their content in two excitatory amino acids, glutamate and aspartate. The associations between these amino acids and the OCs and mercury have been analyzed. Binding assays with human placental homogenates exposed to methylmercury and OCs have also been performed.

Population

Informed consent was obtained from all participants and the study was approved by the Hospital La Fe (Valencia) Ethics Committee. The study population included 40 mother-child pairs among the 787 deliveries of the INMA-Valencia cohort (Guxens et al., 2012a). Selection criteria of the samples were based on availability of cord blood and placenta samples for the determinations as well as on total mercury (T-Hg) concentrations. Thus, the consecutive 20 children with the highest levels of exposure to T-Hg and the sucessive 20 with the lowest levels were selected. All children in the highest group had levels of mercury >20 µg/L and, in the case of the group of lower levels, all children had <6.6 µg/L of mercury. The selected samples also had cord blood analyses ofsome OCs, including HCB, βHCH, 4,4´-DDT, 4,4´-DDE, and PCBs 138, 153 and 180. Total cholesterol and triglycerides were determined by means of enzymatic techniques, calculating total serum lipid concentrations (Phillips et al., 1989). The pollutant levels of the INMA-Valencia cohort,the laboratory analytical methods and qualitycontrol procedures are described elsewhere (Lopez-Espinosa et al., 2010; Ramon et al., 2011; Ramon et al., 2008; Vizcaino et al., 2011b; Vizcaino et al., 2010). Levels of these contaminants (n=40) have been described in Table 16.

Umbilical cord and placenta samples

Umbilical vein blood samples were collected using venipuncture of cord vessels before the placenta was delivered. Serum and plasma samples were obtained after whole blood centrifugation at 2500 rpm and separated into aliquots of 1 mL.Placentas collected at delivery were examined, and pieces of maternal and fetal sides were immediately dissected. Both cord and placentasamples were coded, frozen and stored confidentially and anonymously at -80 ºC until processed.

Table 16. statistics of the pollutants and amino acids analyzed in cord blood. INMA-Valencia cohort, 2004–2006

Pollutants (ng/mL)	LOD	Whole sample (n=40)						Spanish (n=34)			Latin American (n=6)		
		≥LOD (%)	GM	(95% CI)	P 25	P 50	P 75	P 25	P 50	P 75	P 25	P 50	P 75
T-Hg	2.0	65.0	9.2	(5.5; 15.4)	1.4	13.8	42.8	1.4	32.5	43.3	1.4	1.4	5.7*
4,4′-DDT	0.009	47.5	–	–	<LOD	<LOD	0.07	<LOD	<LOD	0.06	<LOD	0.03	0.08
4,4′-DDE	0.016	100.0	0.51	(0.41; 0.64)	0.30	0.54	0.80	0.28	0.48	0.76	0.55	1.13	1.46*
HCB	0.035	92.5	0.21	(0.14; 0.30)	0.12	0.29	0.49	0.14	0.34	0.58	0.03	0.11	0.24*
β-HCH	0.008	82.5	0.07	(0.04; 0.11)	0.05	0.13	0.16	0.06	0.14	0.17	0.03	0.07	0.12
PCB 138	0.031	90.0	0.08	(0.06; 0.10)	0.04	0.10	0.13	0.06	0.10	0.13	0.02	0.02	0.16*
PCB 153	0.020	95.0	0.10	(0.08; 0.13)	0.06	0.12	0.19	0.09	0.16	0.20	0.01	0.04	0.07*
PCB 180	0.018	95.0	0.07	(0.05; 0.09)	0.04	0.09	0.12	0.06	0.10	0.13	0.01	0.02	0.04*
Σ_3PCBs	–	–	0.27	(0.21; 0.34)	0.20	0.36	0.46	0.23	0.39	0.46	0.05	0.10	0.29*
Σ_7OCs	–	–	1.30	(1.08; 1.55)	1.00	1.47	1.82	0.98	1.54	1.85	1.12	1.40	1.64
Amino acids (μmol/L)													
Glutamate	1	100	38.0	(32.0; 45.0)	26.8	35.3	55.6	26.6	33.9	54.9	26.7	56.2	74.0
Aspartate	1	100	6.8	(5.3; 8.6)	3.9	5.3	11.2	3.7	4.9	9.0	5.3	15.1	31.9

CI: confidence interval; GM: geometric mean; HCB: hexachlorobenzene; HCH: hexachlorocyclohexane; LOD: limit of detection; PCBs: polychlorobiphenyls; P: percentile; $\sum_3$PCBs: sum of three PCBs (PCB 138, 153, and 180); S7OCs: sum of the seven organochlorine compounds; T-Hg: total mercury.
* $p < 0.05$, p-value from Mann–Whitney test comparing pollutants and amino acids concentrations according to origin.

Chemicals

Sacarose, Tris-HEPES, glacial acetic acid and protease inhibitor cocktail (P8340) were purchased from Sigma (St. Louis, USA).Chemical standards, methylmercuric chloride, PCB 138 and βHCH were from ICN (Cleveland, OH, USA), Dr. Ehrenstoner GmbH (Augsburg, Germany), and National Physical Laboratories UK (Teddington, United Kingdom), respectively. [3H]-D-aspartate (27 Ci/mmol) was from Amersham Life Sciences (Buckinghamshire, UK).Optiphase 'Hisafe` 2 liquid scintillation cocktail wasfrom Wallace Oy (Turku, Finland).The chemicals were dissolved and diluted in Tris-acetate buffered solution or in dimethyl sulfoxide solution (DMSO). When dissolved in DMSO, a 200 concentration was prepared, thus the concentration of DMSO in the testing solution was 0.5%. Controls contained the same amount of DMSO, when so required.

L-glutamic acid, L-aspartic and o-phthalaldehyde/-mercaptoethanol (50:50) reagent solutions were from Sigma (St. Louis, USA). Ortho-phosphoric acid was from Merck. All other chemicals were from Sigma (St. Louis) and Merck (Darmstadt, Germany). Primary antibodies goat polyclonal for the rat excitatory amino acid transporter 2 (EAAT2) (1:1000) and mouse monoclonal anti-Na+/K+-ATPase 1 (1:200) were from BD Biosciences and Santa Cruz Biotechnology, Inc, respectively. Secondary anti-goat horseradish peroxidase–conjugated (HRP) and HRP linked anti-mouse (1:1500) were from Molecular Probes.

Amino acid determination

Glutamate and aspartate cord plasma concentrations were determined by high performance liquid chromatography (HPLC)-fluorimetric analysis (Waters, Milford, MA; and Applied Biosystems, Foster City, CA) (Lopez-Gil et al., 2007; Reynolds et al., 2002). In brief, plasma samples were thawed and an aliquot of 25 μL was mixed with 475 μL of 0.14 M perchloric acid. After 10 minutes in an ice bath, the sample was centrifuged during 2 minutes at 14000 x g and 4ºC (x2). The supernatant was filtered through millex®-HV filters (0.45 μm) and divided in aliquots that were frozen at -80ºC until the analysis.Amino acids were derivatized with o-phthalaldehyde/-mercaptoethanol reagent solution (50:50) for 5 minutes and injected into a reverse-phase C18 column (Tracer Nucleosil C18 5-μm particle size, 10 x 0.4 cm; Teknokroma). The separation of the amino acids was performed using a mobile phase gradient consisting of 100 mM sodium phosphate dissolved in mili-Q water adjusted to pH 6.4 with ortho-phosphoric acid (Merck) and 28% methanol, followed by a lineal gradient up to 80 % methanol in water for 4 minutes. The system was kept on 80% methanol in water for 2 minutes and went back to the initial conditions in 5 minutes using a lineal gradient. Mobile phase flow was 0.8 ml/min. Both mobile phases were continuously degassed with helium. Samples were injected every 20 minutes. The amount of glutamate and aspartate was quantified by fluorescence detection (Excitation at 360 nm; Emission at 450 nm) with an external standard method using L-glutamic acid and L-aspartic acid dissolved in 0.14 M perchloric acid. A 9 point standard curve was run with each series of HPLC analysis and linearity was assured (r2>0.99). The plasma samples have been analyzed in triplicate-quintuplicate, in different

assays during a two month period. A control solution of 1μM glutamate was analyzed every each 10 samples to confirm that assay parameters remained constant. The mean of the intra-assay coefficient of variation (CV) was 5.5% for 8 experiments, and the inter-assay CV was 9.1%.

Table 17. Association between glutamate and pollutant levels in cord blood[a,b].

Pollutants (ng/mL)	n	Unadjusted models			Adjusted for lipids[c]		
		Change (%)[d]	(95% CI)	p	Change (%)[d]	(95% CI)	p
$\log_{10}$ T-Hg	34	8	(−18; 42)	0.56	4	(−20; 35)	0.77
T-Hg < 6.6	14	Ref				Ref	
T-Hg > 20	20	13	(−23; 66)	0.53	6	(−26; 53)	0.74
4,4'-DDT < LOD	18	Ref				Ref	
4,4'-DDT > LOD	16	−26	(−48; 7)	0.11	−16	(−42; 23)	0.37
$\log_{10}$ 4,4'-DDE	34	−36	(−66; 23)	0.17	−41	(−68; 7)	0.08
$\log_{10}$ HCB	34	−28	(−51; 6)	0.09	−29	(−50; 2)	0.06
$\log_{10}$ β-HCH	34	−18	(−37; 8)	0.16	−24	(−41; −2)	0.04
$\log_{10}$ PCB138	34	−39	(−69; 19)	0.14	−51	(−73; −10)	0.02
$\log_{10}$ PCB153	34	−27	(−58; 28)	0.26	−33	(−60; 13)	0.13
$\log_{10}$ PCB180	34	−41	(−72; 22)	0.15	−48	(−73; 2)	0.06
$\log_{10}$ (Σ_3PCBs)	34	−50	(−76; 5)	0.06	−56	(−77; −13)	0.02
$\log_{10}$ (Σ_7OCs)	34	−48	(−74; 3)	0.06	−54	(−76; −14)	0.02

CI: confidence interval; HCB: hexachlorobenzene; HCH: hexachlorocyclohexane; LOD: limit of detection; PCBs: polychlorobiphenyls; P: percentile; Ref: reference category; $\sum_3$PCBs: sum of three PCBs (PCB 138, 153, and 180); $\sum_7$OCs: sum of the seven organochlorine compounds; T-Hg: total mercury.

a Latin American women were excluded from analysis (n = 6), because of remarkable differences in mercury and OC concentrations from the Spanish ones.

b Linear regression models between glutamate and pollutants, where all variables were log10-transformed.

c Lipids were included as a separate term in the models.

d Coefficients were exponentiated ($(10^{coef} - 1) \times 100$) and interpreted as the percentage change in glutamate by every 10-fold increase in the pollutant level.

Table 18. Association between aspartate and pollutant levels in cord blooda,b.

Pollutants (ng/mL)	n	Unadjusted models			Adjusted for lipids[c]		
		Change (%)[d]	(95% CI)	p	Change (%)[d]	(95% CI)	p
$\log_{10}$ T-Hg	34	13	(−20; 59)	0.47	10	(−22; 55)	0.57
T-Hg < 6.6	14	Ref			Ref		
T-Hg > 20	20	31	(−18; 110)	0.25	26	(−22; 103)	0.33
4,4'-DDT < LOD	18	Ref			Ref		
4,4'-DDT > LOD	16	−35	(−59; 2)	0.06	−31	(−58; 12)	0.12
$\log_{10}$ 4,4'-DDE	34	−28	(−68; 64)	0.43	−32	(−70; 53)	0.34
$\log_{10}$ HCB	34	−22	(−53; 28)	0.32	−23	(−53; 27)	0.30
$\log_{10}$ β-HCH	34	−19	(−42; 14)	0.22	−23	(−46; 8)	0.13
$\log_{10}$ PCB138	34	−30	(−70; 63)	0.40	−40	(−75; 41)	0.23
$\log_{10}$ PCB153	34	−14	(−57; 75)	0.67	−19	(−60; 64)	0.55
$\log_{10}$ PCB180	34	−45	(−78; 37)	0.19	−50	(−80; 24)	0.13
$\log_{10}$ (Σ_3PCBs)	34	−39	(−77; 59)	0.30	−44	(−78; 45)	0.22
$\log_{10}$ (Σ_7OCs)	34	−47	(−78; 30)	0.16	−51	(−80; 18)	0.11

CI: confidence interval; HCB: hexachlorobenzene; HCH: hexachlorocyclohexane; LOD: limit of detection; PCBs: polychlorobiphenyls; P: percentile; Ref: reference category; ∑3PCBs: sum of three PCBs (PCB 138, 153, and 180); ∑7OCs: sum of the seven organochlorine compounds; T-Hg: total mercury.

a Latin American women were excluded from analysis (n = 6), because of remarkable differences in mercury and OC concentrations from the Spanish ones.

b Linear regression models between aspartate and pollutants, where all variables were log10-transformed.

c Lipids were included as a separate term in the models.

d Coefficients were exponentiated ((10coef - 1) x 100) and interpreted as the percentage change in aspartate by every 10-fold increase in the pollutant concentration.

Placental plasma membrane homogenates preparation

The placental plasma membrane homogenates were prepared following a described protocol (Jimenez et al., 2004) with some modifications. Firstly, fetal placental pieces of the fetal face were thawed, and the amount needed was taken (~10 g was used for each assay). Placental tissue was chopped into small pieces, washed with 0.9 % NaCl

solutionto remove blood and filtered through gauze.Then, the tissue was homogenized eight times (30 sec each time) at low speed with a B.Braun Potter Shomogenizer in three volumes of ice-cold buffer A (250 mM sacarose, 10 mM Tris-HEPES adjusted at pH 7.4 with glacial acetic acid) and a protease inhibitor cocktail (1:200) was added after homogenization. The homogenate was centrifuged at 1000 x g for 10 min and the supernatant was removed and retained. The pellet was resuspended in buffer A and homogenized for 30 sec five times. This second homogenate was centrifuged again at 1000 x g for 10 min and the supernatants from both centrifugations were combined. The supernatant was then centrifuged at 10000 x g for 15 min. The pellet corresponding to the mitochondria was discarded and the supernatant was centrifuged at 100000 x g for 1h 20 min with an L-90K ultracentrifuge (Beckman Coulter Optime™); the pellet was resuspended in 2 mL of buffer A. After that, protein content was determined.

Table 19. Association between glutamate and OCs at high (>20 mg/L) or low (<6.6 mg/L) levels of T-Hg[a,b].

Pollutants (ng/mL)	Unadjusted models				Adjusted for lipids[c]			
	Change (%)[d]	(95% CI)	*p*	*p* (inter)	Change (%)[d]	(95% CI)	*p*	*p* (inter)
4,4′-DDT > LOD				0.16				0.16
Model 1 (T-Hg < 6.6)	0	(−52; 109)	1.00		29	(−56; 276)	0.61	
Model 2 (T-Hg > 20)	−40	(−60; −11)	0.02		−33	(−55; 0)	0.05	
$\log_{10}$ 4,4′-DDE				0.12				0.05
Model 1 (T-Hg < 6.6)	−65	(−87; −8)	0.04		−73	(−90; −29)	0.01	
Model 2 (T-Hg > 20)	−2	(−64; 169)	0.97		0	(−59; 145)	1.00	
$\log_{10}$ HCB				1.00				0.92
Model 1 (T-Hg < 6.6)	−44	(−69; 1)	0.05		−43	(−69; 6)	0.07	
Model 2 (T-Hg > 20)	−44	(−77; 37)	0.19		−39	(−72; 34)	0.20	
$\log_{10}$ β-HCH				0.75				0.44
Model 1 (T-Hg < 6.6)	−30	(−54; 6)	0.09		−34	(−57; 2)	0.06	
Model 2 (T-Hg > 20)	−39	(−71; 30)	0.19		−49	(−73; −5)	0.03	
$\log_{10}$ PCB138				0.06				0.07
Model 1 (T-Hg < 6.6)	−70	(−88; −25)	0.01		−75	(−90; −40)	0.01	
Model 2 (T-Hg > 20)	14	(−63; 249)	0.81		−12	(−68; 143)	0.80	
$\log_{10}$ PCB153				0.91				0.74
Model 1 (T-Hg < 6.6)	−51	(−80; 20)	0.11		−56	(−82; 12)	0.08	
Model 2 (T-Hg > 20)	−45	(−88; 147)	0.41		−40	(−84; 127)	0.43	
$\log_{10}$ PCB180				0.21				0.54
Model 1 (T-Hg < 6.6)	−60	(−85; 6)	0.06		−60	(−86; 17)	0.09	
Model 2 (T-Hg > 20)	10	(−71; 325)	0.88		−33	(−81; 132)	0.50	
$\log_{10}$ (Σ_3PCBs)				0.52				0.65
Model 1 (T-Hg < 6.6)	−71	(−89; −23)	0.02		−71	(−89; −21)	0.02	
Model 2 (T-Hg > 20)	−42	(−92; 325)	0.57		−55	(−92; 156)	0.35	
$\log_{10}$ (Σ_7OCs)				0.26				0.23
Model 1 (T-Hg < 6.6)	−76	(−91; −35)	0.01		−77	(−91; −39)	0.01	
Model 2 (T-Hg > 20)	−39	(−85; 145)	0.46		−44	(−84; 88)	0.33	

CI: confidence interval; LOD: limit of detection; HCB: hexachlorobenzene; HCH: hexachlorocyclohexane; PCBs: polychlorobiphenyls; p(inter): P-value for the interaction between T-Hg and the OCs; $\sum_3$PCBs: sum of three PCBs (PCB 138, 153, and 180); $\sum_7$OCs: sum of the seven organochlorine compounds; T-Hg: total mercury.

a Latin American women were excluded from analysis (n = 6), because of remarkable differences in mercury and OC concentrations from the Spanish ones.

b Linear regression models between glutamate and OCs, where all variables were log10-transformed.

c Lipids were included as a separate term in the models.

d Coefficients were exponentiated ($(10^{coef} - 1) \times 100$) and interpreted as the percentage change in glutamate by every 10-fold increase in the pollutant level.

Glutamate transport

Methylmercury, PCB138 and βHCH were tested against glutamate transport in placenta based in the following facts: i) methylmercuryis the most neurotoxicant form of mercury and accounts for ≥90% of total mercury in cord blood in populations with high levels of exposure (Ramon et al., 2011) and ii) PCB138 and βHCH levels were associated with glutamate levels in cord blood.

Glutamate transporter EAAT2 expression was determined by Western Blot following current protocols as the one described elsewhere (Briz et al., 2010; Fonfría et al., 2005). 20 µl of placental homogenates containing 5.4 µg of protein were used. They were boiled during 5 minutes and then were subjected to SDS-polyacrylamide gel

electrophoresis using 12% polyacrylamide resolving gel at 100 mA for 1.5h-2h. Proteins were transferred into a nitrocellulose membrane and incubated with 5% non-fat dry milk in Tris-Buffered Saline Tween-20 (TBS-T) (20 mM Tris-HCl [pH 7.6], 140 mM NaCl, and 0.1% Tween-20). Membranes were incubated overnight at 4ºC with a goat polyclonal anti-rat EAAT2 (1:1000; BD Biosciences) in TBS-T solution containing 5% bovine serum albumin (BSA). After membrane washing, they were incubated with anti-goat horseradish peroxidase–conjugated (HRP) secondary antibodies (1:1500; Molecular Probes). A monoclonal anti-Na+/K+-ATPase 1 (1:200; Santa Cruz Biotechnology, Inc.) and a secondary HRP linked anti-mouse (1:1500) antibodies were used to control for the amount of protein loaded. The membranes were washed and incubated for 4 min in a chemiluminescent solution (Immun-Star HRP kit; Bio-Rad, Hercules, CA).

Glutamate transport was determined as previously described (Fonfria et al., 2005; Galofre et al., 2010) as [3H]-D-aspartate uptake, since D-aspartate is a non-metabolizable glutamate analogue. The binding of [3H]-D-aspartate to EAAT transporters in human placenta has been performed following the method described for human brain frozen tissue and platelets (Borges et al., 2007; Cross et al., 1987) with some modifications. In brief, placental tissue homogenate containing 150 µg protein was incubated in 1mL reaction mixture containing 50 mM Tris-acetate buffer (pH=7.4), 200 mM NaCl, and 20 nM [3H]-D-aspartate (1 TBq/mmol) in presence or absence of methylmercury, PCB138, βHCH and glutamate, at different concentrations. Incubation was carried out at 25ºC for 1 hour. Reaction was stopped by rapid vacuum filtration onto Whatman GF/C fibers filters using Brandell Cell Harvester equipped with 24-well probe. Filter sheets were soaked in ice-cold incubation buffer prior to use. Each individual filter was placed in 3ml vial scintillation vials with 2 mL of scintillation cocktail; the activity in each vial was determined by liquid scintillation spectrometry.

Statistical analysis

Descriptive analysis included geometric means, 95% confidence intervals (CIs), and percentiles, for contaminants and outcomes. Response and exposure variables were right-skewed, thus they were log10-transformed to approach normality. The association of glutamate and aspartate with contaminants was analyzed using linear regression analyses adjusting by serum lipids. Organochlorine compounds with detection frequency < 50% were analyzed as a dichotomous variable (below or above thelimit of detection), while mercury was analyzed both as a continuous and a dichotomous variable (low versus high mercury concentrations). Due to high differences in contaminant concentration between Spanish and Latin American (n=6) women (Table 16), main analyses were performed excluding women from Latin America. Interaction between OCsand T-Hg on glutamate was also assessed by including the product term between T-Hg (in two categories: low versus high level of exposure) and OCs (in logarithmic scale). All analyses were performed with SPSS statistical software package version 17 for Windows (SPSS Inc., Chicago, IL). Statistical significance was set at $p < 0.05$.

Results of [3H]-D-aspartate binding assays are expressed as mean ± SD. Concentration-response curves from two to three independent experiments, each one performed using triplicates for each concentration point, were fitted to sigmoid curves using the GraphPad Prism (GraphPad Software Inc, San Diego, CA, USA). At least, 6 concentrations of each pollutant were used to define the curve.

Concentrations of contaminants and amino acids in cord samples

Table 16 shows the concentrations of T-Hg and OCs detected in the cord samples expressed in ng/mL. Briefly, median concentrations of T-Hg, HCB, βHCH, 4,4´-DDE, and ∑PCBs were 13.8, 0.29, 0.13, 0.54, 0.36 ng/mL, respectively. Latin American women had much lower levels of mercury, HCB and PCBs, and higher levels of DDE. Due to these remarkable differences, we decided to exclude these women from the main analyses (Table *23* - Table 25). Table 14 also shows the concentrations of glutamate and aspartate levels (medians: 35 and 5.3 µmol/L) in cord plasma. They were positively correlated (Pearson r = 0.75, p<0.001).

Association between amino acid levels in human umbilical plasma and toxic compounds.

The relationships between glutamate and aspartate and pollutant levels in cord blood when excluding Latin-American women (n=34) are shown in Table 25 and Table 26. We found a significant negative relationship between glutamate and the sum of the three PCBs, and the seven OCs in cord blood after adjusting for lipids (p = 0.02 in both cases) (Table *23*). More specifically, βHCH and PCB138 showed a significant negative relationship with glutamate, with a decrease of 24 and 51% (p-values of 0.04 and 0.02, respectively) in glutamate levels for each 10-fold increase in the contaminant concentration. HCB, 4,4`-DDE and PCB180 showed a negative trend, although they did not reach statistical significance. On the other hand, no significant relationship was found between T-Hg concentration and the levels of glutamate in the blood samples. Finally, we did not find any significant relationship for aspartate and the pollutants analyzed in our samples, however the positive (for T-Hg) and negative (for OCs) trend found for glutamate was kept in the case of aspartate Table 26 and Table 27). Patterns were similar in the analyses including the whole sample (n=40), but, only the sum of the 7 OCs remained statistically significant.

We analyzed if the association between OCs and glutamate levels varied according to T-Hg exposure. Samples were stratified in two models consisting of low (< 6.6 μg/L) and high (> 20 μg/L) T-Hg exposure. Table 19 shows the association between OCs and glutamate levels stratified by T-Hg exposure. There was an interaction pattern (p<0.10) in the case of 4,4`-DDE or PCB138, with a significant inverse association between glutamate and 4,4`-DDE or PCB138 (coefficients: -73 and -75 μmol/L, respectively; p = 0.01) with low T-Hg concentrations and a lack of relation at high T-Hg concentrations.

Decrease of glutamate transport in human placenta by environmental contaminants

We analyzed placental plasma membrane homogenates for the expression of EAAT2 in order to assure the presence of glutamate transporter in our placental samples. [3H]-D-aspartate bindingin placental homogenates was inhibited by glutamate with a 50% inhibitory concentration (IC50) of 6.3 ± 1.7 μM. We tested the effects of PCB138 and βHCH, since they were associated with levels of glutamate, as well as methylmercury, although the association between T-Hg and any of the amino acids did not reach statistical significance.PCB 138 and βHCH partially inhibited [3H]-D-aspartate binding with IC50 values of 14.2 ± 1.2 nM and 6.9 ± 2.9 nM, respectively. Despite the high affinity of PCB138 and βHCH for the glutamate transport they did not completely inhibit it. The maximum inhibitory effectsobserved were 46 ± 14 % and 55 ± 1 % for PCB138 and βHCH, respectively, and they were attained at concentrations ≥ 1 μM. Methylmercury completely inhibited [3H]-D-aspartate binding in placental homogenates with an IC50 value of 4.9 ± 0.8 μM.

The roles of glutamate and aspartate.

The values of glutamate and aspartate in human cord blood determined here were in agreement with other results obtained in similar studies (Cetin et al., 2005; Jauniaux et al., 2001). We found a significant negative correlation between the glutamate concentration in cord blood and the levels of PCB138 and βHCH, once adjusting for lipid content and excluding Latin American women from the study. Also, PCB180, HCB, and 4,4`-DDE seem to have a tendency to diminish the glutamate levels in cord blood. Finally, the sum of the three PCBs and all OCs also decreased significantly the levels of glutamate. With regards to mercury we did not find an association with glutamate levels.

We also showed that human placenta expressed glutamate transporters by determining both the EAAT2 protein expression and the inhibition of [3H]-D-aspartate binding by the amino acid glutamate. We have determined the protein expression of EAAT2 in our placental samples because it manages 90% of the glutamate uptake and it is found both in the syncytiotrophoblast layer of the placenta and in endothelial cells of fetal blood vessels (Danbolt, 2001; Noorlander et al., 2004). To the best of our knowledge, we demonstrated for the first time that methylmercury inhibits glutamate transport in human placental tissue, like it does in platelets and neural cells (Aschner et al., 2000; Borges et al., 2007; Fonfria et al., 2005). It should be noted that inorganic and organic mercury species inhibit glutamate transport with different potencies (Fonfria et al., 2005). We have determined the inhibition of glutamate transport by methylmercury becauseit is the most abundant mercury specie in cord blood accounting for ≥90% of total mercury in the analyzed samples and it represents the most neurotoxic form of

mercury. We also found that PCB 138 and βHCH inhibited glutamate transport in human placentas with high affinity (~ 10 nM) but low efficacy (~ 50 % of maximum inhibition). The findings with PCB 138 are in agreement with those reporting that other higher chlorinated ortho-PCBs partially inhibit the uptake of glutamate in rat brain synaptosomes (Mariussen and Fonnum, 2001; Stavenes Andersen et al., 2009). Also, PCB 138 impairs glutamate-related pathways in cultured cerebellar neurons with a high potency (Llansola et al., 2010). With respect to βHCH, as far as we know, there are no reports on its effect on glutamate transport.

Due to the inhibition by methylmercury and OCs of EAATs in the placenta, we expected to find an increase of the glutamate concentration in umbilical cord blood in relation to these pollutants. The lack of a significant association for Hg and glutamate levels could be due to the fact that the highest levels of T-Hgfound in the samples (66 μg/L 0.33 μM) are about ten times lower than the concentrations of methylmercury that significantly inhibit glutamate uptake (IC50 value: 4.9 μM). With regards to PCB138 and βHCH, their maximum concentrations in cord blood (1.1 and 4.2 nM, respectively) were similar to their IC50 values against glutamate uptake in human placenta. However, since these two compounds did not produce a complete inhibition of the glutamate transport, there would be still enough glutamate transport activity to avoid an increase of extracellular glutamate. Many factors might contribute to the observed decrease in glutamate levels in the cord blood, like altered synthesis/degradation of glutamate in the fetal liver or transamination of branched chain amino acids to provide glutamate in the placenta (Neu, 2001). In this way, a proteomic study revealed a reduction in the glutamate synthesizing enzyme glutaminase after amphipod chronic exposure to PCB 77 and 169 (Leroy et al., 2010). However, to the best of our knowledge, there are no reports on whether PCB138 or βHCH interacts with glutamate metabolic pathways.

The group of Latin American women was excluded from the study since the mother's country of origin appears to be an important determinant for patterns of mercury and OC concentrations in this cohort (Llop et al., 2010; Ramon et al., 2011) which is also relevant for the concentrations of these pollutants in-utero (Vizcaino et al., 2010). If we take into account the possible interaction between the contaminants, the presence of two different groups in the studied population may distort the output result.

Population is exposed to several pollutants at the same time, which may have compensatory, additive or synergic effects. Therefore, we wonder whether the interaction between pollutants could affect the output of the present results. Our results showed that PCB138, and 4,4`-DDE were inversely associated with glutamate for the samples with low T-Hg concentrations (6.6 μg/L) in cord blood, but not for the samples with high T-Hg concentrations, suggesting compensatory effects between OCs and mercury. We hypothesize that PCB138 (and probably other OCs) might firstly decrease glutamate in cord blood by disrupting glutamate synthesis/degradation pathways at any step, and this effect may be prevalent in the samples with low T-Hg levels. However, at high T-Hg levels glutamate may accumulate due to the inhibition of glutamate transport, which eventually compensate for the effects of OCs. In this way, there are experimental evidences that methylmercury counteract with PCBs when supplied in complex mixtures. Thus, it has been reported that methylmercury attenuated hearing and learning deficits induced by exposure to PCBs in developing rats (Piedrafita et al., 2008; Powers et al., 2009).

The developing fetal compared to adult brain is more vulnerable to the effects of toxic agents because of differential exposure, physiologic immaturity, and a longer lifetime over which disease initiated in early life can develop (Castoldi et al., 2008; Grandjean and Landrigan, 2006; Perera et al., 1999). The possible subtle changes in glutamate concentration related to environmental toxicant exposure could be associated with neurological deficits observed in children exposed to persistent environmental pollutants since glutamate is a key amino acid for the development of neural functions (Grandjean and Landrigan, 2006). In fact, recent studies performed on children up to 2 years of age found an inverse association between PCB 138 concentrations and impairment of psychomotor development (Forns et al., 2012b). It should be stressed that glutamate exerts a crucial role on motor coordination through the activation of metabotropic glutamate receptors (Nakao et al., 2007). In addition, Gascon et al. (2013 highlighted the negative role of prenatal exposure to PCB in comparison to postnatal exposure including breast-feeding. However, no association was found between mercury concentrations and mental or psychomotor development in two-years old

children (Llop et al., 2012). Other studies reported adverse effects of mercury on visual responses and linguistic pathways, however levels of exposure were higher than the ones reported in the present work (Ramirez et al., 2003; Yorifuji et al., 2013).

Conclusions

The levels of glutamate in umbilical cord blood were inversely associated with PCB138 and β-HCH concentrations, and to the sum of three PCBs and all OCs. A compensatory effect between PCB138 and 4,4`-DDE with mercury was found in accordance with previous animal experiments. We also found that methylmercury, PCB138 and β-HCH inhibit glutamate transport in human placentas. The affinity of these compounds against [3H]-D-aspartate binding was β-HCH ≥ PCB 138 > methylmercury, however complete inhibition was only attained by the latest.

Assessment of the neurotoxic effects of metal exposure in newbonrs

This third part of this contribution is aimed to investigate whether prenatal exposure to several metals with a potential neurotoxic effect was associated with neuropsychological development of children at the age of 4 in an Inma birth cohort. Specifically, we measured concentrations of 7 metals presents in human tissues (Co, Cu, As, Cd, Sb, Tl and Pb) in maternal urine samples collected at 12th and 32th weeks of pregnancy. Since the vulnerability of the brain could vary widely over the course of pregnancy (Mendola et al., 2002), this design could allow us to disentangle if the metal exposure may be particularly important during the first or third trimester of pregnancy.

Study design

This analysis used data from the population based cohort established in the city of Sabadell as part of the INMA (Environment and Childhood) Project (Guxens et al., 2011). A total of 657 eligible women (≥16 years, intention to deliver at the reference hospital, ability to communicate in Spanish or regional languages, singleton pregnancy, unassisted conception) were recruited during the first prenatal visit (1st trimester of pregnancy). A total of 619 (94.2%) children were enrolled at birth, and 553 (84.2%) were followed-up until their fourth year of life and performed the neuropsychological test. The study was approved by the Clinical Research Ethical Committee of the Municipal Institute of Healthcare (CEIC-IMAS) and all the participating mothers gave informed consent.

Neuropsychological testing

Neuropsychological assessment was conducted in 553 children at the age of 4 (range 4.1- 5.6). A standardized version of the McCarthy Scales of Children's Abilities (MSCA) adapted to the Spanish population was used to assess the cognitive development. The general cognitive scale (which includes verbal, perceptual-performance and quantitative scales) was examined. In addition, we included a new MSCA score of executive function by the reorganization of the MSCA subtests (Julvez et al., 2011). All testing was done in the health care center by 1 specially trained psychologist. The psychologist was not aware of any exposure information. The psychologist also flagged children difficult to evaluate because of less than optimal cooperation who were classified as having neuropsychological tests of uncertain quality and excluded from data analyses (n = 12). Some other child conditions, such as feeling seek during examination (n = 29), not sleeping well the night before examination (n = 39), mood changes during the last days (n = 17), visiting regularly a psychologist (n = 48) or being diagnosed as having a neuropsychological disorder (n = 27) were reported by the mothers and taken in consideration for statistical analyses as covariates. MSCA raw scores were standardized to a mean of 100 and a standard deviation (SD) of 15 to homogenize all the scales.

Children`s teachers filled-in two different questionnaires to assess social competence development and attention deficit and hyperactivity disorder (ADHD) symptomatology. Social competence was evaluated using the California Preschool Social Competence Scale (CPSCS) (Julvez et al., 2008), a test particularly designed to evaluate of social competence in children aged from 2.5 to 5.5 years. The CPSCS items cover a wide range of behaviors such as response to routine, response to the unfamiliar, following instructions, making explanations, sharing, helping others, initiating activities, giving direction to activities, reaction to frustration, and accepting limits. Raw scores were

standardized to a mean of 100 and SD of 15. We assessed the ADHD symptomatology, using the ADHD Criteria of Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (ADHD-DSM-IV) form (American Psychiatric Association 2002). ADHD-DSM-IV consists of a list of 18 symptoms categorized under two separate symptom groups: inattention (nine symptoms) and hyperactivity/impulsivity (nine symptoms). Each ADHD-DSM-IV item is rated on a 4-point scale (0 = never or rarely, 1 = sometimes, 2 = often, or 3 = very often). We analyzed the inattention scale (IA) and the hyperactivity/impulsivity scale (HI) as continuous variables. Higher scores indicate higher symptomatology (worse score).

Urinary metals determination

Urine samples (80 mL) were collected in the first and third trimester of pregnancy from 485 women. The samples were stored in polyethylene tubes at -20ºC until further processing. They were analyzed by inductively coupled plasma quadruple mass spectrometry (Q-ICP-MS). Prior to instrumental analysis they were digested and diluted as follows: 3 mL of urine were introduced in Teflon vessels together with 3 mL of Instra-Analysed 65% HNO3 (J.T. Baker, Germany) and 1.5 mL of Instra-Analysed 30% H2O2 (Baker) and they were left in an oven at 90ºC overnight. Once evaporated, the resulting solid samples were dissolved with 3 mL of 4% HNO3 dilution, placed in 7 mL glass bottles and subsequently stored in a refrigerator until instrumental analysis. An internal standard of Indium (10 ppb) was introduced and depending on sample density samples were diluted with MilliQ water to 30 mL or 60 mL to avoid spectral interferences. Q-ICP-MS analysis was performed by a X-SERIES II device from Thermo Fisher Scientific. One MilliQ water blank was processed in each batch of samples to control for possible contamination. Instrumental limit of detection (LOD) for all metals was 0.2 ng/mL. Metal urine concentrations were standardized to creatinine content determined at the Echevarne laboratory of Barcelona (kinetic with target measurement, compensated method) with Beckman 157 Coulter© reactive in AU5400 (IZASA®).

Other variables

Information on parental education, social class, country of birth and maternal smoking during pregnancy was obtained through questionnaires administered during the 1st and 3rd trimesters of pregnancy. Parental educational level was defined using three categories: primary or less, secondary school, and university. Parental social class based on occupation was derived from the longest-held job reported during pregnancy or, for those mothers not working during their pregnancy, the job most recently held. When social class could not be derived, the last job of the father was used. Nine social class categories were created according to the set of National Occupational Codes-94 and regrouped into three categories: I+II for managers, technicians, and associate professionals (non-manual), III for other non-manual workers, and IV+V for skilled, semi-skilled and unskilled manual workers (Domingo-Salvany et al., 2000). Information related to the child's gestational age, sex and birth weight was obtained from clinical records. In subsequent interviews at 6 and 14 months, data on breastfeeding practices were collected. All questionnaires were administered face-to face by trained interviewers. Information on maternal diet was obtained in the first trimester of pregnancy using a 101-item semiquantitative validated food frequency questionnaire (Vioque et al., 2007). The exposure to NO2 and benzene during pregnancy was also measured (Aguilera et al., 2010). Finally, at the child`s age of 4 years, we assessed parental intelligence and mental health using Similarities subtest of the Weschler Adult Intelligence-Third Edition (WAIS-III) (Weschler, 2001) and the Revised Symptom Checklist (SCL-90-R) (Martínez-Azumendi et al., 2001), respectively.

Statistical analysis

Metal urinary concentrations below the LOD were assigned a value of of LOD. Metals concentrations were examined in tertiles, comparing the 3rd vs the 1st tertile. This categorical approach allowed us to explore the effects of metals on neuropsychological development between extreme groups. We used linear regression models to analyze the MSCA and CPSCS scores. Negative binomial regression models were used for IA and HA scales of ADHD-DSM-IV, to account for over-dispersion. We fitted multiple linear regression models for each pair outcome-metal adjusting for all the covariates that fulfilled the confounding criteria. Covariates retained in the model were those showing associations with neuropsychological tests with p-values of <0.05 or those whose inclusion resulted in a change in the regression coefficient of the metals ≥ 10%.

In a next step, we performed sensitivity analyses to assess the robustness of our results. A series of models were run to assess the effect of additionally adjusting one by one each of the variables related in the literature to metals distribution such as fish intake, smoking and traffic air pollution (NO2 and benzene) during pregnancy to minimize the likelihood of residual confounding (Garcia-Esquinas et al., 2011; Jain, 2013; Marti-Cid et al., 2008; Steiner et al., 2007).

Results

Our analysis was based on 377 children with complete information on neuropsychological development assessment and metals exposure. A description of the characteristics of the study population is shown in Table 20. The mean age of assessment was 4.4 years. Forty-nine percent of children were females. Forty-three percent of mothers had secondary educational level while 33% had university degree. More than 50% of fathers belonged to a manual social-class. We also studied the differences between participants (n=377) (those with completed data on neuropsychological development and metals exposure) and non-participants (n=192) (those without complete data on neuropsychological development and metals exposure). Non-participants in this study only differed from participants in maternal education. Non-participant mothers had lower educational level than non-participants. No differences were found in variables such as child`s sex, maternal social class or country of origin.

Table 20. Neuropsychological scores and sociodemographic characteristics of participants (n=377)

	P50	(p25-p75)
Neuropsychological tests		
Age at examination (yrs)	4.43	(4.33 - 4.53)
General cognitive scale (MSCA)	100.90	(91.51 - 110.29)
Executive function score (MSCA)	100.21	(91.19 - 110.52)
Social competence (CPSCS)	102.37	(91.05 - 110.85)
Inattention scale (DSM-IV-ADHD)	2	(0 - 5)
Hyperactivity/Impulsivity scale (DSM-IV-ADHD)	2	(0 - 5)
Sociodemographic characteristics		
Sex, female (%)	49.09	
Birthweight (gr)	3290	(2980 - 3520)
Gestational age (weeks)	39.86	(38.86 - 40.71)
Maternal social class (%)		
CSI+II	22.6	
CSIII	32.5	
CSIV+V	44.9	
Maternal education (%)		
Primary	24.3	
Secondary	42.8	
University	32.9	
Paternal social class (%)		
CSI+II	24.4	
CSIII	18.4	
CSIV+V	57.2	
Paternal education (%)		
Primary	33.8	
Secondary	42.9	
University	23.3	
Maternal country of origin (%)		
Spain	91.6	
Others	8.4	
Paternal country of origin (%)		
Spain	89.8	
Others	10.2	
Duration of any breastfeeding (months)	25.86	(13.00 - 43.43)
Maternal smoking during pregnancy, yes (%)	27.4	

MSCA: McCarthy Scales of Children Abilities; CPSCS: California Preschool Social Competence Scale; DSM-IV-ADHD: ADHD Criteria of Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition.

In Table 21 concentrations of metals in urine samples during first and third trimester of pregnancy are shown. All metals excepting Tl were detected in more than 60% of urine samples during both trimesters. All metals exhibited

statistically significant differences between both periods (p<0.001) except Tl, Pb and As. Nevertheless, all of them showed statistically significant correlations between measurements in both stages (p<0.001 for the rest). I would add that correlations go from 0.2 to 0.6. Metals with the lowest median concentrations during both periods were Tl (0.14 ug/g creatinine 1st trimester; 0.13 ug/g creatinine 3rd trimester) and Sb (0.36 ug/g creatinine 1st trimester; 0.28 ug/g creatinine 3rd trimester). The metal with the highest median concentration was As (32 ug/g creatinine 1st trimester; 36 ug/g creatinine 3rd trimester).

We found a negative effect for the highest levels of cobalt at the 1st trimester on the executive function score (Coefficient (Coef) = -4.92; 95% Confidence Interval (CI) = -8.36 to -1.49) but not for the levels of cobalt at the 3rd trimester (Coef = -3.08; 95%CI = -6.69 to 0.54). There is a negative trend for the effects of cobalt on global cognitive score, but these effects were not statistically significant. It has also been observed a negative association between the highest levels of lead on general cognitive scale at the 3rd trimester (Coef = -3.87; 95%CI = -7.64 to -0.10). This negative association was not found for the levels of lead at the 1st trimester. We found positive coefficients for Sb on general cognitive scale and executive function score during the two periods, although these coefficients were not statistically significant. We observe positive coefficients for the highest levels of As on both general cognitive and executive function scores, but not statistically significant.

The results for the CPSCS were inconclusive at all. None of the metals were associated with this scale. We only found a greater risk to increase the HI scale in those children exposed to the highest levels of As, although the association was marginally significant (Incidence Rate Ratio = 1.45; 95%CI = 1.00 to 2.11). No more associations were found for the rest of metals and ADHD-DSM-IV scales. The inclusion of some variables in the final multivariable models such as fish intake during pregnancy, smoking during pregnancy and traffic air pollution (NO2 and benzene) did not materially change the results.

Impact of metal exposure on neurodevelpment

To our knowledge, this is the first study to estimate the effects of a complete set of metals measured in two different time-periods of pregnancy on child neuropsychological development during preschool period. The results of the present study suggest that prenatal exposure to high levels of lead and cobalt may negatively affect the cognitive development of the child during preschool period. The negative effects of cobalt seem to be more important during the 1st trimester of pregnancy, whereas the negative effects of lead were greater in the 3rd trimester of pregnancy. No more associations were found between the rest of metals analyzed (copper, arsenic, cadmium, antimony and thallium) and the child neuropsychological development at the age of 4 years.

In this longitudinal birth cohort study, we measured concentrations of a large set of heavy metals on maternal urine samples collected at 12th and 32th weeks of pregnancy: cobalt, copper, arsenic, cadmium, antimony, thallium and lead. Concentrations of most of metals were statistically different between both stages. Nevertheless, correlations of all metals between first and third trimester are significant, likely reflecting the absence of major changes in metal exposure along pregnancy. Most of the metals measured showed concentrations which are similar to those reported in previous and current studies worldwide, especially from non-contaminated sites (Benes et al., 2002; Komaromy-Hiller et al., 2000). However, it is important to remark that the effect of lead withdrawal from gasoline was clearly observed when comparing our concentrations with those reported for Italian population during the last eighties (Minoia et al., 1990).

Table 21. Concentrations of metals in urine samples (µg/g creatinine) during first and third trimester of pregnancy

	1st trimester			3rd trimester				
	% >LOD	Median	(p25-p-75)	% >LOD	Median	(p25-p-75)	difference p†	Spearman rho
Co	73.6	0.43	(0.19-0.86)	84.3	1.28	(0.69-1.94)	<0.001	0.38***
Cu	100	11.59	(8.14-16.79)	100.0	14.38	(9.53-20.27)	<0.001	0.21***
As	99.8	32.98	(16.86-71.62)	99.8	36.84	(19.47-76.68)	0.731	0.24***
Cd	90.1	0.58	(0.41-0.89)	87.5	0.55	(0.36-0.83)	<0.001	0.56***
Sb	73.7	0.34	(0.19-0.59)	64.8	0.28	(0.16-0.53)	<0.001	0.40***
Tl	19.7	0.14	(0.09-0.20)	17.2	0.13	(0.09-0.19)	<0.1	0.21***

Pb	98.9	3.83	(2.56-5-56)	100.0	3.81	(2.67-6.24)	0.255	0.46***

LOD: limit of detection; †Difference p represents the p-value difference between the concentrations of 1st trimester vs 3rd trimester

The neurotoxic effects of high levels of lead exposure on the developing brain have been extensively reported in the past decades (Grandjean and Landrigan, 2006). Since lead is a ubiquitous pollutant in the ecosystem, efforts to reduce exposure have been applied. Despite of this reduction in the levels, lead is still considered a primary environmental hazard on child health (Costa et al., 2004). Some authors have stated that "no level of lead exposure appears to be 'safe' and even the current 'low' levels of exposure in children are associated with neurodevelopmental deficits" (Bellinger, 2008). There are recent evidences about the negative effect of prenatal exposure to very low-levels of lead on cognitive development during childhood (Jedrychowski et al., 2009; Schnaas et al., 2006). In the present study, we observed a negative effect on general cognitive scale for those children prenatally exposed to high levels of lead. These deleterious effects seem to be particularly important during the third trimester of pregnancy exposure. These results are not in accordance with a previous study in that the authors reported a greater negative effect for the levels of lead in the first trimester than second or third trimester levels. They hypothesized that lead could affect the neural differentiation process which is primarily a first-trimester event. However, our findings seem to be pointed out that lead exposure could specially affects other critical processes of brain development which are markedly important during the second and third trimesters such as myelination and synaptogenesis (Johnston and Goldstein, 1998; Mendola et al., 2002). Our results support the results of a cohort study in Mexico in which the authors found that exposure to lead during the third trimester of pregnancy may produce "lasting and possibly permanent effects" because this period may constitute a critical period for subsequent intellectual child development (Schnaas et al., 2006).

Our results also provided evidence of an adverse effect of prenatal exposure to cobalt during the first trimester on neuropsychological development of preschoolers, particularly on executive function. Cobalt is a relatively rare magnetic element which is an essential oligoelement which enters in the composition of vitamin B12 (Barceloux, 1999; Lauwerys and Lison, 1994). From general population, diet is the main source of exposure to cobalt. Although cobalt is an essential element, at high concentrations it is toxic (Kubrak et al., 2011). Increases in cobalt ions can directly induce DNA damage, interfere with DNA repair, and lead to DNA–protein cross linking and sister chromatid exchange (Calderon-Garciduenas et al., 2013; Hengstler et al., 2003; Leonard et al., 1998). In animal studies, it has been found that excessive exposure to cobalt during embryonic period causes oxidative stress in brain and other tissues (Kubrak et al., 2011). The detrimental effects of cobalt are higher during the 1st trimester of pregnancy. This might indicate that exposure to cobalt could affect critical processes of brain development that occur during the 1st trimester such as neural migration and differentiation (Mendola et al., 2002). To date, the possible developmental neurotoxic effect of cobalt in humans has not observed. Further research in this birth cohort study is needed to disentangle if this finding is maintained in the future.

It is worth noting that in a large population-based sample of children, we collected biological samples at two different points in time during pregnancy (at 12th and 32th week of pregnancy) and analyzed a large number of metals. We also have standardized neuropsychological measurements of cognitive development at age 4, and collected data on a variety of potential socio-demographical factors including parental education, social class, intelligence quotient or mental health. For the MSCA assessment, several quality controls were introduced and the psychologist received extensive training to this end. This study, however, was limited by a number of factors. Probably the main limitation is related to the type of sample analyzed, particularly for lead. We analyzed the concentrations of metals in urine samples. This could hinder the comparability of our results to the previous studies since most of the articles analyzing the effects of lead and other metals on neuropsychological development are based on blood samples. Urine is the preferred non-invasive matrix in heavy metals biomonitoring (Esteban and Castano, 2009). The variability of urine volume and chemical concentration are the main drawbacks of urine measurements that can be corrected by using creatinine concentration (Barr et al., 2005), as performed in the present study. In addition, we only could analyze 377 subjects with information on metals and neuropsychological development. There were no differences between participants and non-participants in terms of socio-demographical variables, which suggest that selection bias was minimal.

Slovenia and Croatia

Prenatal mercury exposure, neurodevelopment and apolipoprotein E genetic polymorphism. Longitudinal cohort study in the Mediterranean region

The aim of the study was the evaluation of the association between prenatal exposure to mercury (Hg) and neurodevelopment of the child, considering genetic polymorphism of apolipoprotein E (Apoe) and other relevant confounders that could modulate the association between Hg and neurodevelopment (Snoj Tratnik et al., 2017a; Snoj Tratnik et al., 2017b).

Study design, study population and sample collection

This prospective cohort study was set within a 5-year integrated project PHIME. Mother-child pairs have been recruited during pregnancy or at birth, in 4 Mediterranean countries: Italy, Greece, Croatia and Slovenia. In total, 1700 pairs was aimed. Within the CROME study, assessment of potential genetic modulation of neurodevelopmental effects was performed on population groups from Croatia (the coastal city of Rijeka and its county) and Slovenia (the city of Ljubljana and its surroundings) were included. Women eligible for recruitment (permanent residents of the study areas for at least 2 years, were at least 18 years of age, and had no absence from the study area for more than 6 weeks during pregnancy, no history of drug abuse, no serious health problems or complications of pregnancy, and no twin gestation) were approached for consent during their hospital stay for delivery (Slovenia) or during routine visits between 34 and 38 gestational weeks (Croatia). Recruitment took place at the Maternity Hospital of the University Medical Centre of Ljubljana (Slovenia) and at the University Hospital of Rijeka (Croatia). In Slovenia, maternal hair and cord blood were collected at or immediately after birth, in Croatia maternal hair, maternal blood and maternal urine were collected between 34th week of pregnancy and birth, and cord blood at birth. In both study populations, breast milk was sampled one month after delivery and at the same time mothers were asked to complete a questionnaire. At 18 months of age, children were tested for cognitive, language and motor performance using Bayley-III assessment. The study design, including the setting, recruitment, criteria for exclusion, questionnaires, biological sampling and outcome assessment is described in detail by Valent et al. (2013. In this first stage, 601 pregnant women were recruited from the central Slovenia region and 243 from Rijeka, Croatia. During pregnancy no adverse health effects were observed in either group of pregnant women.

In the further follow-up, 179 children and their mothers from Slovenia were re-sampled at children`s age of 7-8 years to obtaine blood, urine and hair samples for elemental analysis; and saliva (Oragene DNA self-collection kit) for genotyping analysis. The children were re-assessed for neuropsychological performance using Wechsler Intelligence Scale for Children (WISC-IV). Mothers completed a questionnaire to update information on their and child`s life-style, diet, living environment and potential exposure.

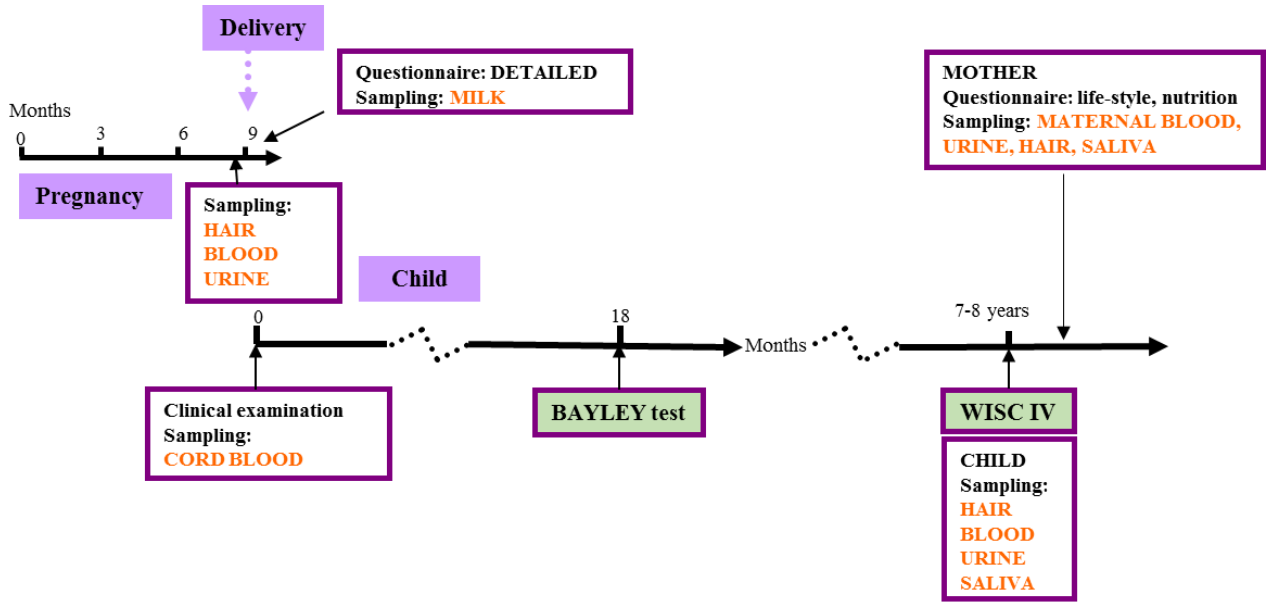


Figure 4. Study Design: recruitment, sample collection and psychological assessment at 18 month and 7-8 years of age

Figure 5. Study areas

Multielemental analysis of biological samples by ICP-MS: follow-up at 7-8 years of age

An aliquot of 0.2 mL of blood or urine sample was diluted with 2 mL of an alkaline solution containing Triton X-100, ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA), 1-Butanol and Internal standard Mix (Agilent). Measurements of prepared solutions were made by Inductively Coupled Plasma Mass Spectrometer (ICP-MS 7700ce, Agilent). Reference material Seronorm trace elements whole blood L-1 and L-2 (Sero), Seronom Urin L-1 and L-2 (Sero) were used to check the accuracy of the results. The limit of detection for Hg, Cd, Pb, As, Se, Cu, Zn and Mn were 0.035, 0.09, 0.76, 0.12, 0.96, 6.12, 48.3 and 0.54 µg/L sample, respectively.

DNA and mRNA extraction - genotyping and gene expression analysis

In the first stage, the DNA was extracted from the existing samples of children and their mothers from the Slovenian and Croatian population. Children`s DNA was isolated from cord tissue using a QIAamp DNA Mini Kit, QIAGEN, ZDA, following the instructions for DNA purification from tissues provided in the QIAamp DNA Mini and Blood Mini Handbook 04/2010. Maternal DNA was isolated from leukocytes in peripheral blood using the High Pure PCR

Template Preparation Kit (Roche), following the manufacturer's instructions. Samples were diluted (1:20) with ultrapure water (UltraPure DNase/RNase-Free Distilled Water, Sigma-Aldrich). The isolated DNA was then genotyped for apolipoprotein E (Apoe), genotyping was performed using TaqMan® pre-designed SNP genotyping assay small scale with C_3084793_20 for rs429358 and C_904973_10 for rs7412 (Applied biosystems, Foster City, Ca, ZDA). Genotyping was carried out using the Roche LightCycler® 480 II (Trdin, 2015). In addition, mRNA extracts from the blood samples of these children were tested for differentiated gene expression levels of key neuroinflammatory markers using the Microarrays SurePrint G3 Human Geb Exp v3 Array Kit 8x60K in a SureScan Microarray scanner in the AUTH premises.

In the second stage, following the re-sampling of children at 7-8 years of age, DNA was extracted from saliva samples of children and their mothers using ethanol precipitation protocol and prepIT®•L2P reagent for the purification of genomic DNA from the Oragene® collection kit. Maternal DNA was additionaly isolated from leukocytes in peripheral blood using the High Pure PCR Template Preparation Kit (Roche). Isolated DNA was used to genotype the polymorphisms:

1) *directly involved in brain development*: brain derived neurotrophic factor (*BDNF:* rs6265), paraoxonase (*PON1*: rs662), and catechol O-methyltransferase (*COMT*: rs4680), coproporphyrinogen oxidase (*CPOX:* rs1131857).

2) *important for metabolism of metals*: metallothionenins (*MT-1a*: rs11076161, *MT-1B*: rs2070839, *MT-1B*: rs8052334, *MT-1E*: rs2070836, *MT-1F*: rs2291956, *MT-1G*: rs12315, *MT-2a*: rs10636, *MT-2a*: rs1610216, *MT-1x*: rs2301234, *MT-3*: rs11644094, *MT-3*: rs28754593, *MT4*: rs396230, *MTF1*: rs3748682, *MTF1*: rs4653329, *DMT1*: rs224589), arseno-methyl-transferases (*AS3MT*: rs3740393, *AS3MT*: rs1046778, *AS3MT*: rs7085104, *AS3MT*: rs7897654, *AS3MT*: rs3740400, *AS3MT*: rs11191439), delta-aminolevulinic acid dehydratase (*ALAD*: rs1805313 and rs818708), vitamin D receptor (*VDR*: rs2228570 and rs739837), selenoprotein P (*SEPP1-2*: rs3877899 and rs7579) and thioredoxin reductase (*TXNRD1*: rs11111979 and rs1128446).

Neuropsychological assessment

Bayley Scales of Infant and Toddler Development, Third Edition (Bayley-III)

Child neurodevelopment was assessed at 18 (range, 16–20) months of age using a standardised individually administered developmental assessment instrument the Bayley Scales of Infant and Toddler Development, Third Edition (Bayley-III) (Bayley, 2006). Cognitive, Language (Receptive and Expressive), Motor (Fine and Gross) Scale were administered according to the standardised procedure. Both the scaled and composite scores were calculated. As suggested by Aylward (Aylward et al., 2013), composite scores <85 or scaled sores <7 should be considered indicative of disabilities when categorizing the results at Bayley III. The tests were conducted by trained psychologists (Croatia) and by trained psychology students (Slovenia); all of them were certified after attended a two-day workshop on the administration and scoring of Bayley III. In Slovenian group the interrater concordance was calculated for 39 children. Two raters rated each child during their assessment session. The Krippendorf's Alpha coefficients were calculated for each scale. They were 0.984 for Cognitive Scale, 0.988 and 0.961 for Receptive Language and Expressive Language Scale respectively and 0.943 and 0.954 for Fine Motor and Gross Motor Scale respectively.

The Wechsler Intelligence Scale for Children (WISC IV)

The Wechsler Intelligence Scale for Children - Fourth Edition (Wechsler, 2003) is one of the most widely used measures for the assessment of intellectual abilities in school-age children and adolescents. It has 10 core and five supplemental subtests. The subtests (represented with scaled scores: mean = 10; standard deviation = 3) can be clustered into composite quotients for four indices (represented as standard scores: mean = 100; standard deviation = 15): the Verbal Comprehension Index (VCI), the Perceptual Reasoning Index (PRI), the Working Memory Index (WMI) and the Processing Speed Index (PSI). These ten subtests also result in the calculation of a global composite measure, the Full Scale Intelligence Quotient (FSIQ).

Questionnaires

One month after delivery, mothers were given a questionnaire to obtain information about their socio-demographic and health status, nutritional habits (detailed food frequency questionnaire, particularly for fish consumption), life-style (smoking), potential exposure and residential and occupational history. A supplementary questionnaire was administered approximately 18 months after delivery. It assessed changes in residence, maternal marital and occupational status, anthropometric measures and developmental milestones of the child, breastfeeding history, child intake of fish, diseases, and day care attendance. Data obtained from the questionnaires was used as covariates assessing the association between Hg exposure and neuropsychological outcome.

Questionnaire filled out by mothers at 7-8 years of child`s age included information on child`s life-style, physical activity, diet, living environment, potential exposure and socio-economic status.

Results: internal exposure and main sources of exposure

In the overall PHIME birth cohort, internal exposure was the lowest in Slovenian mother-child pairs (Med 297 ng/g hair and 1.5 ng/g cord blood) and the highest in Greek (Med 1120 ng/g hair and 5.8 ng/g cord blood), and the overall Hg levels were associated significantly with the frequency of fish consumption, while with amalgam fillings only in the Slovenian population. The results have been published by Miklavcic et al. (2013. The low level in the Slovenian population comply well with the levels observed in fish from the Slovenian market reported by Miklav i et al. (2011.

Among Slovenian and Croatian populations, higher prenatal Hg exposure was observed in Croatian population (Table 6), which was due to higher fish intake in Croatian versus Slovenian population. Significant and positive correlation was observed between cord blood Hg levels and fish consumption frequency (r_S=0.45, p<0.001); in contrary, correlation between cord blood Hg and number of amalgam fillings was insignificant (r_S=-0.08, p=0.224).

Table 22. Prenatal exposure in Slovenian and Croatian population.

Biomarker	SLOVENIA GM (95% CI) min-max, n	CROATIA GM (95% CI) min-max, n	All GM (95% CI) min-max, n	p-value
Cord blood Hg (ng/g)	1.58 (1.42-1.74), 0.16-10.0, n=237	3.41 (2.96-3.94), 0.79-32.3, n=123	2.05 (1.87-2.25) 0.16-32.3, n=360	<0.001
Maternal hair Hg (ng/g)	273 (244-306), 24-2057, n=228	598 (505-708), 19-8710, n=129	361 (326-401) 19-8710, n=347	<0.001

The results of internal exposure re-assessed at child`s age of 7-8 years is presented in the Table 23

Table 23. Internal exposure at 7-8 years of child`s age. Element concentration (µg/L) in blood of mother-child pairs from Slovenia. n – sample number, GM-geometric mean, 95% CI-95% confidential interval, min-minimum value, max-maximum value. Different letters indicate significante difference between children and mothers (p<0.05).

Element concentration (µg/L)	Children (7-8 years) GM (95% CI) min-max, n	Mothers GM (95% CI) min-max, n
	BLOOD	BLOOD
Hg	0.47 (0.38-0.59)[a] <LOD-4.17, n=139	1.12 (1.00-1.27)[b] 0.11-10.2, n=169
As	0.38 (0.32-0.45)[a] 0.03-7.02, n=139	0.66 (0.57-0.76)[b] 0.11-19.0, n=170
Pb	9.72 (9.16-10.3)[a] 3.42-28.8, n=139	10.8 (10.1-11.6)[b] 3.76-69.0, n=170
Mn	8.42 (7.95-8.92)[a]	8.25 (7.83-8.70)[a]

Element concentration (μg/L)	Children (7-8 years) GM (95% CI) min-max, n	Mothers GM (95% CI) min-max, n
	BLOOD	BLOOD
Cd	1.97-21.4, n=139 0.12 (0.11-0.14)[a] <LOD-1.33, n=138	2.39-21.6, n=170 0.41 (0.37-0.46)[b] <LOD-5.24, n=170
Se	82.3 (79.9-84.7)[a] 42.2-133, n=139	105 (103-107)[b] 68.7-142, n=170
Zn	3840 (3713-3970)[a] 1925-6915, n=139	5316 (5186-5448)[b] 3561-8582, n=170
Cu	674 (656-692)[a] 326-1023, n=139	625 (608-642)[b] 395-1569, n=170

Table 24. Element concentration (μg/L or μg/g crea) in urine of mother-child pairs from Slovenia. n – sample number, GM-geometric mean, 95% CI-95% confidential interval, min-minimum value, max-maximum value. Different letters indicate significante difference between children and mothers (p<0.05).

Element	Children (7-8 years) GM (95% CI) min-max, n		Mothers GM (95% CI) min-max, n	
	Urine (μg/L)	Urine (μg/g crea.)	Urine (μg/L)	Urine (μg/g crea.)
Hg	0.07 (0.05-0.09)[a] <LOD-3.26, n=176	0.13 (0.10-0.16)[a] <LOD-10.7, n=170	0.12 (0.10-0.15)[b] <LOD-3.12, n=177	0.25 (0.21-0.30)[b] <LOD-10.8, n=176
As	3.58 (3.04-4.23)[a] 0.26-255, n=176	6.80 (5.85-7.92)[a] 0.98-189, n=170	3.37 (2.78-4.09)[a] 0.02-121, n=177	7.11 (6.00-8.42)[a] 0.08-238, n=176
Mn	0.21 (0.19-0.25)[a] <LOD-7.72, n=176	0.42 (0.33-0.54)[a] <LOD-75.9, n=170	0.15 (0.13-0.17)[b] <LOD-1.93, n=177	0.32 (0.26-0.40)[a] <LOD-5.17, n=176
Cd	0.05 (0.04-0.06)[a] <LOD-0.78, n=176	0.10 (0.08-0.11)[a] <LOD-2.77, n=170	0.09 (0.08-0.11)[b] <LOD-1.64, n=177	0.20 (0.17-0.24)[b] <LOD-5.58, n=176
Se	11.5 (10.2-13.1)[a] 1.34-58.9, n=176	21.9 (20.4-23.5)[a] 6.06-125 (n=170)	7.47 (6.43-8.69)[b] 0.03-95.3, n=176	15.9 (14.5-17.4)[b] 0.11-165 (n=176)

Two mothers exceeded HBMI value for Hg in blood, whereas none exceeded HBMII value. In urine, values higher than HBMI were observed in one child and in two mothers (values expressed per creatinine), while none exceeded HBMII values. As for the other elements, most of the values were below the established population-based or health-based reference values. In case of Cd in urine, one child and 3 mothers exceeded HBMI value and none HBMII value. In mothers, Cd blood levels were significantly associated with smoking, smoking mothers having higher levels (GM=1.08 μg/L, n=27) than non-smoking (GM=0.34, n=143) (p<0.001). In non-smoking mothers, passive smoking was associated marginally significantly with blood Cd levels only in mothers exposed to tobacco smoke in a car (p=0.090), while urinary Cd was associated with exposure to tobacco smoke while visiting their friends. In children, exposure to tobacco smoke in a car was associated with urinary Cd (p=0.085), but not with blood Cd.

As expected, Hg in children`s and maternal blood was associated strongly with frequency of fish or seafood consumption: seafood-total (r_s=0.36 and r_s=0.31, p<0.001), fresh fish (r_s=0.43 and r_s=0.34, p<0.001), canned fish

(r_s=0.24, p=0.005 and r_s=0.19, p=0.016) and shellfish and molluscs (r_s=0.38 and r_s=0.27, p<0.001); but not with frozen fish consumption (r_s=0.02, p=0.771 and r_s=0.06, p=0.407) or fresh water fish (r_s=0.08, p=0.349 and r_s=0.01, p=0.930).

Also Hg in urine was associated significantly with consumption of different types of fish and other seafood (0.20<r_s<0.28), but certain differences were observed depending on the gender. Association between urinary Hg and fresh fish consumption was significant only in girls (r_s=0.37, p<0.001), while shellfish and molluscs consumption only in boys (r_s=0.25, p=0.024). Canned fish consumption correlated significantly with urinary Hg in both, girls and boys (r_s=0.26, p=0.016 and r_s=0.22, p=0.043). In mothers, no significant association was observed between urinary Hg and sefood consumption.

Presence of amalgam fillings in children was not associated with Hg blood levels significantly, but it was with urinary Hg levels (p<0.001), and the same was observed in mothers (p<0.001). Among children, the association between urinary Hg and amalgam fillings was significant only in girls (r_s=0.41, p=0<0.001), while insignificant in boys (r_s=0.18, p=0.102).

Broken Hg-containing thermometer or energy-saving bulb in participant`s home was not associated with increased Hg concentration in blood or urine neither in children nor mothers.

Among the investigated elements, those that correlated significantly (p<0.05) between cord blood and blood at 7-8 years of age were: total Hg (r=0.41) and Mn (r=0.46). Those that correlated significantly between child`s and maternal blood were: total Hg (r=0.36), As (r=0.36) and Mn (r=0.43). In urine, elements that correlated significantly between maternal and child samples were: Hg (r=0.33) and As (r=0.39).

Within the same matrices, significant correlations were observed between Hg, Se and As in bith, maternal and child`s blood; and between essential elements (Mn, Se, Zn and Cu) in child`s blood, but not in maternal.

As expected, mothers had significantly higher Hg levels in both, blood and urine (both p<0.001). Within the mother-child pair, there was a good correlation observed for blood Hg (r=0.35) and also for urine Hg levels expressed per creatinine (r=0.32).

Results: genotyping

Apolipoprotein E (*Apoe*) genotyping was performed on Slovenian and Croatian children (n=360). Most children had the 3/ 3 genotypes, considered as a wild (reference) genotype (72 %); the frequency of genotypes 2/ 2 and 2/ 3 was 9 %, and the frequency of susceptible genotypes 3/ 4 and 4/ 4 was 18 % (Trdin, 2015).

BDNF, PON1, CPOX and *COMT* genotyping was so far performed on 179 children from Slovenia. The frequencies are given in the *Table 25*.

Table 25. Frequencies of genetic variants of BDNF, PON1, CPOX and COMT polymorphic genes in 7-8 years old children.

	BDNF rs6265	PON1 rs662	CPOX rs1131857	COMT rs4680
Wild type	105 (66%)	97 (56%)	123 (72%)	52 (30%)
Heterozygote	51 (32%)	64 (37%)	45 (26%)	84 (48%)
Variant	3 (2%)	11 (6%)	4 (2%)	39 (22%)

Genotyping of polymorphisms involved in ADME processes of metal(loid)s was so far performed only on the population of Croatian mothers (DNA isolated from samples obtained within PHIME study), polymorphisms analysed were the following: *MT-1a, MT-1B, MT-1B, MT-1E, MT-1F, MT-1G, MT-2a, MT-2a, MT-1x, MT-3, MT-3, MT4, MTF1, MTF1, DMT1, AS3MT, AS3MT, AS3MT, AS3MT, AS3MT, AS3MT, ALAD, ALAD, VDR, VDR*. Analysis of genotypes with respect to metal(loid)s concentrations in different matrices will be performed when the laboratory analysis are completed.

Results: neuropsychological outcome (Bayley-III and WISC IV)

Among the Bayley-III scores (Table 26), cognitive scores differed between the Slovenian and Croatian population significantly (p<0.001), Slovenian children having an average score of 114.3 (±13.0) and Croatian 106.7 (±13.2). Language and motor scores did not differ significantly between the studied populations. Cognitive and motor composite scores were marginally significant (p=0.096 and 0.098, respectively) while the fine motor scaled score was significantly lower in apolipoprotein E 4 carriers than in 2 and 3 carriers (p=0.044). The language composite score did not differ significantly between the genotypes (p=0.885). The majority of the children had assessment scores within the normal limits for cognitive, language and motor scale. Six, 20 and 5 children had delayed performance in cognitive, language and motor composite scales, respectively.

Table 26. Bayley Scores of the Slovenian and Croatian children, tested at age 180 months (n=360).

	Cognitive	Language	Motor	Fine-motor	Gross-motor
Bayley-III Score (mean ± SD)	111.7 ± 13.6 60-145	106.0 ± 14.4 50-144	107.4 ± 10.3 58-142	12.2 ± 2.2 4-19	10.3 ± 1.99 2-19

So far, 179 children from the existing PHIME cohort have been re-assessed for neuropsychological performance using WISC-IV test (Table 27). Among them, there was no significant difference observed in neither of the WISC scores between apolipoproteine E 4 carriers and 2/ 3 carriers; marginally significant difference was observed in FSIQ score between *PON1* wild type and carriers of variant allel (carriers of variant allel having higher slightly FSIQ score; p=0.089), whereas for other three SNPs (*BDNF, CPOX, COMT*) no significant difference was observed between the genotypes.

One of the children tested at 7-8 years of age had very low FSIQ score (<69), also PRI and WMI, while two children had very low VCI. There were 10 children with low FSIQ score (70-89), 15 had low VCI, 7 low PRI, 22 low WMI and 12 low PSI. In the average score range (90-109), there were 77 (FSIQ), 96 (VCI), 69 (PRI), 95 (WMI) and 92 children (PSI). The scores above average (110-129) were observed in 81 (FSIQ), 55 (VCI), 92 (PRI), 61 (WMI) and 63 (PSI) children. High scores (>130) were observed in 10 (FSIQ), 11 (VCI), 10 (PRI) and 12 (PSI) children.

Table 27. WISC IV Scores of the Slovenian children, tested at age 7-8 years (n=179).

	VCI	PRI	WMI	PSI	FSIQ
WISC IV Score (mean ± SD)	106.0 ± 14.0 55-142	111.2 ± 12.7 65-147	102.5 ± 11.7 65-129	107.6 ± 13.8 73-141	109.6 ± 12.6 59-138

FSIQ correlated significantly with the cognitive composite score of the Bayley test (r=0.45, p<0.001). One of the 3 Slovenian children, that had delayed cognitive performance as assessed by Bayley, had very low FSIQ score, the other two were normal according to FSIQ score.

Results: association between internal exposure and neurodevelopment

Based on the multiple regression modelling, adjusting for all potential confounders and related covariates, Hg in mother´s hair and in cord blood was observed not to predict Bayley scores but a moderate beneficial effect of fish consumption in pregnancy was observed. Other chemical elements were not found to be associated with the outcomes (PHIME, 2006).

Re-assessment of association between prenatal Hg exposure and neuropsychological outcome was done using intelligence score data (FSIQ assessed by WISC IV) at 7-8 years of age. Results are presented in the Table 28 and Table 29. FSIQ scores vs. cord blood Hg levels (Table 28) and FSIQ scores vs. blood Hg levels at age of the child 7-8 years (Table 29). In accordance with the finding in the overall PHIME cohort, we observed positive association between cord blood Hg levels and FSIQ. This positive effect might be due to fish consumption, however no association between frequency of fish consumption in pregnancy and FSIQ was observed. Food frequency consumption data in general has much higher uncertainty in compare to data of laboratory analysis (e.g. Hg concentration), which might

explain the lack of association. Besides Hg, the model revealed marginally significant positive association for breastfeeding and negative association for smoking during pregnancy. Boys were observed to have marginally significantly higher scores than girls, and music extra-culicular activity was found to be positively associated with the scores.

Table 28. WISC-IV total score (FSIQ) association with total Hg in cord blood (ln ng/g) using multiple linear regression. Coefficient – estimate of change in intelligence score (FSIQ). CI 95% - 95% confidence interval.

FSIQ score *(Model R^2 adj.=0.09, p=0.015)*	**Coefficient**	**Std. coefficient (β)**	**CI 95%**	**p-value**
Cord blood total Hg (ln)	**2.98**	**1.32**	**0.36; 5.60**	**0.026**
Education (prim./second. vs. tertiary)	1.44	2.18	-2.88; 5.76	0.511
Music activities (yes vs. no)	**5.48**	**2.22**	**1.08; 9.87**	**0.015**
Language activities (yes vs. no)	4.34	2.72	-1.05; 9.72	0.113
Breastfeeding (≤10 months vs. >10 months)	4.17	2.21	-0.21; 8.54	0.062
Smoking during pregnancy (yes vs. no)	-6.99	3.70	-14.3; 0.33	0.061
Child's gender (boys vs. girls)	-3.87	2.20	-8.21; 0.48	0.080
Amalgam feelings during pregnancy (yes vs. no)	-5.91	4.16	-14.1; 2.32	0.158
Cord blood Pb (ln)	-1.45	2.47	-6.35; 3.44	0.558
Cord blood Mn (ln)	-2.11	3.10	-8.24; 4.02	0.497

Fitting child`s blood Hg concentration at 7-8 years of age into the regression model, no association with FSIQ was revealed. Similarly, association was not observed neither for urinary Hg concentration, nor for other metal(loid)s. Among the co-variates and confounders used in the model, significant positive association between FSIQ and breasfeeding was confirmed and negative association between FSIQ and smoking during pregnancy (Table 29).

Table 29. WISC-IV total score (FSIQ) association with total Hg in child` blood (ln ng/g) using multiple linear regression. Coefficient – estimate of change in intelligence score (FSIQ). CI 95% - 95% confidence interval.

FSIQ score *(Model R^2 adj.=0.11, p=0.011)*	**Coefficient**	**Std. coefficient (β)**	**CI 95%**	**p-value**
Child's blood total Hg (ln)	0.04	0.93	-1.80; 1.87	0.967
Education (prim./second. vs. tertiary)	4.11	2.25	-0.34; 8.57	0.070
Music activities (yes vs. no)	4.10	2.30	-0.45; 8.65	0.077
Language activities (yes vs. no)	5.23	2.27	-0.52; 10.6	0.075
Breastfeeding (≤10 months vs. >10 months)	**5.01**	**2.25**	**0.74; 9.73**	**0.028**
Smoking during pregnancy (yes vs. no)	**-10.8**	**4.07**	**-18.9; -2.75**	**0.009**
Child's gender (boys vs. girls)	-3.65	2.37	-8.34; 1.04	0.126
Amalgam feelings during pregnancy (yes vs. no)	-7.78	4.31	-16.3; 0.76	0.074
Child's blood Pb (ln)	-0.31	3.19	-6.62; 6.00	0.922
Child's blood Mn (ln)	-0.27	3.53	-7.27; 6.73	0.939
Child's blood Cd (ln)	1.78	1.58	-1.34; 4.90	0.261
Child's blood Se (ln)	7.29	7.30	-7.17; 21.7	0.320

Results: association between internal exposure and neurodevelopment by genotype

In the first phase of assessment of potential genetic modulation, 361 mother-child pairs were included from Slovenian (n=237) and Croatian (n=124) population, having the complete datasets for internal exposure, neurodevelopment assessment at 18 months of age (Bayley-III), APOE genotyping analysis and questionnaire variables.

Table 30 shows associations between exposure to Hg during pregnancy (Hg level in cord blood) and neurodevelopment of children for three main scales – cognitive, language and motor, and for fine and gross motor subtests. The models were constructed for the total population adjusted for Apoe genotype and separately for Apoe 4 allele carriers and those without the 4 allele (2, 3 carriers).

Hg in cord blood showed a negative correlation with cognitive composite score in 4 carriers, which was significant in the fully adjusted model (Table 30) and marginally significant in the model with no adjustment for Se and Pb cord blood levels. Furthermore, a marginally significant interaction between the genotype and Hg levels was observed for this domain (Table 30). In the motor domain, increasing cord blood levels were significantly associated with a decrease in the fine motor scaled score, estimates of change were similar among the genotypes. An association between cord blood Hg levels and language domain showed no significant effect, but with βestimates being more negative in 4 carriers than in non-carriers (Table 30).

Furthermore, adjusting for Se and Pb levels revealed a marginally significant positive association between serum Se levels and language composite score in all subjects (coeff=7.6, p=0.094), while the association was insignificant in 4 carriers (coeff=15.0, p=0.316). A negative association between cord blood Pb levels and outcome was observed for the motor domain in 2 and 3 carriers (coeff=-3.2, p=0.035), but not in 4 carriers (β=4.4, p=0.222). The association was insignificant in all subjects (coeff=-2.17, p=0.111). Also fine motor scale was negatively associated with cord blood Pb levels in all subjects (coeff =-0.64, p=0.020), in 2 and 3 carriers (coeff=-0.82, p=0.008), but not in 4 carriers (coeff=0.73, p=0.294).

Table 30. Bayley-III score associations with total Hg in cord blood (ln ng/g) using multiple linear regression. β – estimate of change in neurodevelopment score. CI 95% - 95% confidence interval.

		Coeff. (CI 95%), p-value, Bayley vs. total Hg in cord blood (ln)				
Subjects	**n**	**Cognitive**	**Language**	**Motor**	**Fine motor**	**Gross motor**
All	283	-1.41 (-3.47, 0.66), 0.181	-1.03 (-3.46, 1.40), 0.406	-1.16 (-2.76, 0.44), 0.153	**-0.33 (-0.66, -0.01), 0.043**	-0.10 (-0.41, 0.22), 0.553
ε2, ε3 carriers	232	-0.49 (-2.76, 1.78), 0.949	-0.59 (-3.42, 2.45), 0.682	-0.84 (-2.68, 1.00), 0.369	-0.29 (-0.66, 0.08), 0.122	-0.06 (-0.41, 0.29), 0.742
ε4 carriers	51	**-5.44 (-10.7, 0.19), 0.043**	-2.61 (-7.41, 2.20), 0.276	-2.54 (-5.94, 0.86), 0.139	-0.51 (-1.16, 0.13), 0.117	-0.26 (-1.09, 0.57), 0.528
*Interaction p**	*283*	*0.052*	*0.181*	*0.275*	*0.353*	*0.418*

Remark: Model adjusted for country and particular socio-demographic characteristics (mother`s age at delivery, child`s gender, birth weight, educational level of the mother, smoking during pregnancy) and serum Se and cord blood Pb. *p-value for interaction term (*Apoe**Hg) in the models with all subjects.

These results indicated that even low-to-median Hg exposure in children with generally normal neurodevelopmental outcome can result in lower cognitive and fine motor scores at 18 months of age as assessed by Bayley-III. The Hg-associated decrease in cognitive sores was observed in children carrying at least one *Apoe* 4 allele, while the decrease in fine motor scores was independent of the *Apoe* genotype. The results are published by Snoj Tratnik et al. (2017b.

Apoe polymorphism has been studied extensively in relation to neurodegenerative diseases and *Apoe* 4 allele has been recognized as one of the genetic risk factors associated with Alzheimer`s disease (Buttini et al., 1999), but the role of *Apoe* polymorphism has been increasingly recognized also in neurodevelopment. APOE is believed to promote myelination, synaptogenesis or other fatty acid/cholesterol mediated processes associated with neurodevelopment (Mauch et al., 2001; Wright et al., 2003). There are only few studies addressing Hg exposure, *Apoe* and neurodevelopment, and in general, decreased performance is observed in relation to 4 allele (Ng et al., 2013; Ng et al., 2015; Woods et al., 2014). However, the study of Woods et al. (2014 revealed impaired performance in learning and memory in boys that were carriers of 4 allele, while in girls this allel was associated with increased performance in attention and motor domains.

The mechanism for the observed association is yet to be identified, however, the difference in Hg-associated decrease between the genotypes may be explained by the *Apoe* role in Hg metabolism. The APOE 4 isoform is without cysteins, whereas the 3 isoform contains one cysteine and one arginine, and the 2 isoform two cysteines. Cysteine contains one thiol (–SH) group which can conjugate with metals and help remove them from essential cellular binding sites. Our data showed that mean cord blood Hg levels were indeed marginally significantly higher in 4 carriers than in those without 4 allele. However, this difference should be taken with care as it might be masked by the levels of other elements (e.g. Se) and as cord blood levels cannot be directly used to quantify levels in the brain. Also, the difference between boys and girls seems to play an important role in metabolism of metals and also in relation to neurodevelopmental outcome. Based on the marked gender differences in urinary Hg as a function of seafood consumption observed in the present follow-up study, Hg demethylation/elimination processes seem to differentiate significantly between boys and girls.

In addition to the observed negative Hg-related associations, adjusting for selenium (Se) and lead (Pb) levels in regression modelling revealed a positive influence of Se on the language domain and a negative influence of Pb on motor domain, but not in the subgroup of children carrying the 4 allele. These observations are in accordance with the literature (Ng et al., 2013; Skroder et al., 2015; Wright et al., 2003) and demonstrate importance of monitoring co-exposure to other potentially neurotoxic elements.

Other associations observed:

- negative association: **CB-Pb** and **motor score** in ε4 non-carriers: -3.2 (CI 95%: -6.1, -0.23), p=0.035
- positive association: **CB-Se** (serum) and **language** in All: 7.6 (CI 95%: -1.3, 16.6), p=0.094
- Higher language scores in girls, All: 7.6 (CI 95%: 3.45, 10.6), p<0.001
- Hg-related decrease in **cognitive performance**, but only among ***Apoe* 4 carriers**
- gene-metal interaction indicated for cognitive score
- Hg-related decrease in **fine motor scores** was independent of the genotype

The mechanistic explanation of the effect of Apoe 4 in lower neurodevelopmental scores was provided by the gene expression analysis. A key finding of this study was that Apoe polymorphisms resulted in increased neuroinflammation; these results may reflect an inappropriate immune response to environmental factors, like the one induced by Hg exposure. Considering the key role of microglia as sensors of pre- and post-natal environmental stimuli and its involvement in the regulation of synaptic connectivity, maturation of brain circuitry and neurogenesis under increased inflammatory status could eventually result in late life neurodevelopmental problems. However, not all the all markers within proinflammatory, anti-inflammatory, repair, and immunoregulatory classifications were equally modulated by Apoe genotype; among the neuroinflammatory markers, the ones that were mostly modulated, were the ones considered as pro-inflammatory such as TNFa and IL-1b, as illustrated in Figure 6. Overall, these results, provide a mechanistic explanation on how different genetic variants are more susceptible to environmental insults. A possible explanation is that the induction of oxidative stress induced by Hg exposure, acts synergistically with the pre-inflammatory status predisposed in Apoe 4 genotypes.

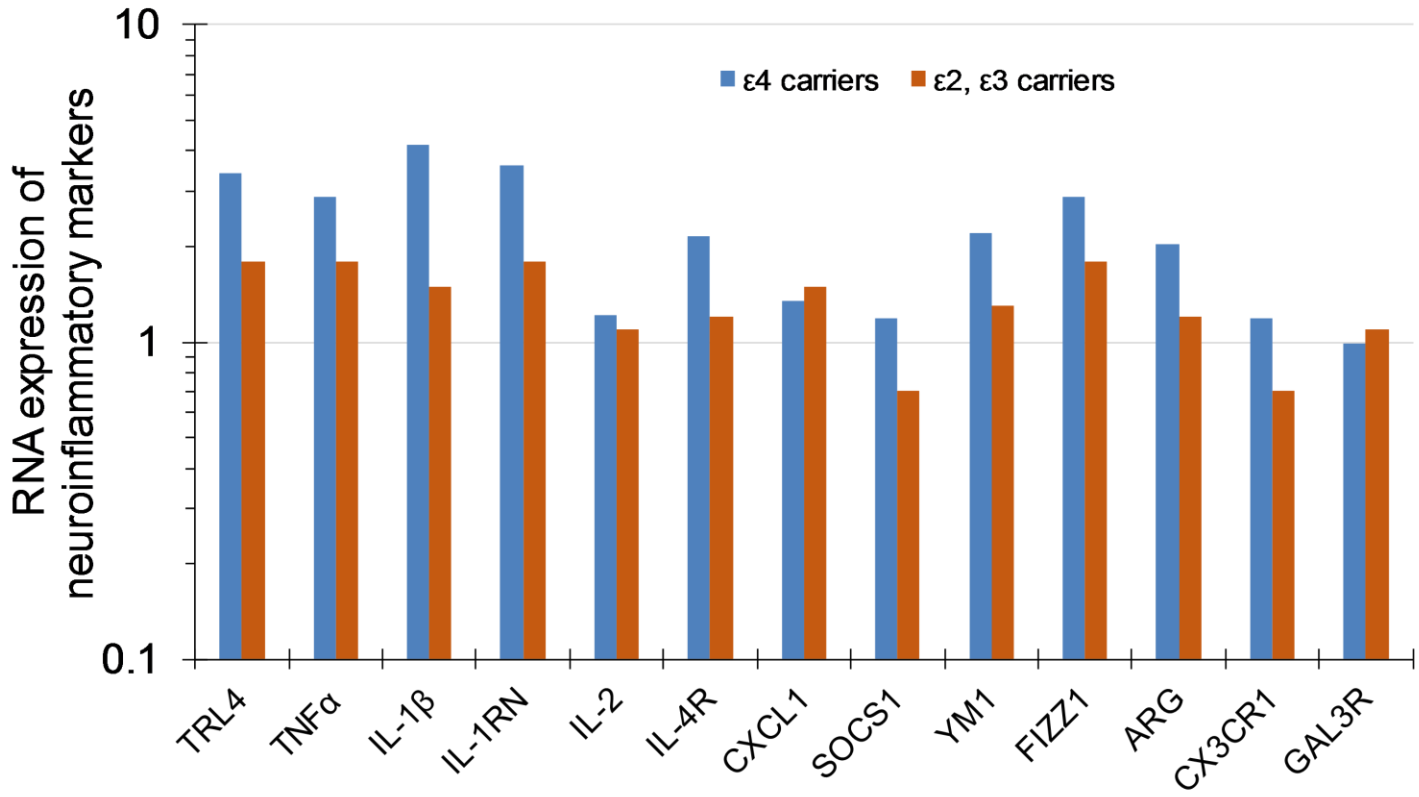


Figure 6. Differences in the mRNA expression of neuroinflammatory markers among the various Apoe carriers

Conclusions

Levels of Hg and other metals in the birth cohort from Mediterranean area showed low to moderate internal exposure in children and their mothers and generally sufficient status of essential elements. 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 increased Hg exposure during pregnancy. Based on the genotyping that was done so far, we found negative association between cognitive scores and Hg exposure in the carriers of apolipoproteine 4 allele, but not in others (data on Slovenian and Croatian cohort).

The follow up performed so far 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.

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.

The effect of waste management in child neurodevelopment

Introduction

Assessment of the health impacts related to hazardous waste is a major scientific challenge with multiple societal implications. Health impacts related to the operation of various waste management options have been investigated up to now only using associations of exposure proxies with specific health endpoints. Characteristic examples are the associations observed between cancer prevalence and the presence of incinerators (Forastiere et al., 2011) or the link between residence proximity to landfills and adverse birth outcomes (Elliott et al., 2001). In addition, several review studies have been made available in the last ten years (Giusti, 2009; Hossain et al., 2011; Porta et al., 2009). The conclusion of these reviews is not definitive, with some difficulties in interpreting data from primary studies due to non-homogeneous design, and lack of accurate exposure information and control of potential confounders.

Although these studies provide some rough evidence of the association between adverse health outcomes and proximity of the population to hazardous waste, they do not provide established mechanistic links between environmental exposure and disease burden. Causality is very difficult to establish in these associations, thus prohibiting decision-makers from exploring cost-effective waste management options with the certainty that their choices will bear the expected improvement in terms of protecting the health of man and the environment. The same occurs for other types of hazardous waste, like the ones disposed of in various environmental media (e.g. air, soil, surface waters) from industrial activities resulting in contamination of such media with hexavalent chromium, mercury or PCBs. It is critical to evaluate and, if possible, quantify what is the actual burden on overall morbidity and mortality in the relevant population from exposure to hazardous waste. This would support targeted policy interventions for protecting public health and at the same time help develop the optimal technological options that minimize the cost:benefit ratio.

Given that waste management options are reflected in the type of compounds emitted and the respective health effects, it is important to keep in mind the share of the various waste management options employed across Europe. In the 28 Member States, 28% of municipal waste was recycled, 15% was composted (through aerobic and anaerobic processes), while 26% and 31% were, respectively, incinerated and disposed of in landfill. However, there is a large variability in approaching waste management across the EU; in Greece, landfilling accounts for 80.7%, composting only for 3.7% and recycling for 15.5%, while countries which recently joined the EU are characterized by similar figures. In addition, informal activities around waste collection, sorting, treatment and disposal, and illegal flows and trafficking of hazardous waste represent a serious challenge. While the extent of the problem is largely unknown, some data and anecdotal evidence suggest that such activities are not uncommon. Informal waste management activities can provide income and support the livelihoods of families and local communities. Yet, the price in terms of direct health impact for those involved is likely to be very high. Severe questions of health inequality and environmental justice arise, as the people engaged in informal waste management are socially disadvantaged in other respects. A characteristic example in Europe is the Roma population, who have been repeatedly reported to be involved in informal waste handling in order to recover high-added value metals such as copper (WHO, 2016).

Overall, the importance of health effects of waste management and disposal activities has also been extensively recognized by the World Health Organisation (WHO), where the need for multisite cohort studies and refined current risk estimates has been highlighted in the last Workshop on Waste and health, organized by WHO on October 5-6 of 2017 in Bonn, Germany. (WHO, 2016). In this workshop, Prof. Sarigiannis, the coordinator of CROME was invited as an expert on the issue of waste management and human health. Prof. D. Sarigiannis, based on the outcomes of CROME-LIFE, made clear proposals on waste management policy actions in Europe to ensure health and sustainability in his capacity as WHO advisor on integrated health impact assessment. This will be used as input to the forthcoming Ministerial Conference of the WHO (13-15 June 2017 in Ostrava, Czech Republic). A major problem that was highlighted in the WHO workshop, is the impact of landfilling in human health.

Based on the above and the extensive experience in CROME related to heavy metals and children neurodevelopment, it was of particular interest to estimate the impact of landfilling in child neurodevelopment across EU. Overall the study aimed at the collation of data, related to the identification of children population living close to landfilling,

existing biomonitoring data related to heavy metals exposure, as well as the influence of other factors such as diet and sociodemografic components.

Data used in the analysis

Exposure factors

For the association, several exposure factors have been investigated, including:

- Exposure to heavy metals, including:
  - Cd, Hg and As in urine
  - Pb in blood
  - Mn and Hg in hair
- Additional proxies of exposure, such as
  - Distance from the contaminated sites. For this purpose, data obtained from D-WASTE HELLAS LTD regarding the spatial distribution of landfills across EU were used. The precise allocation of landfills, in collaboration with the CROME geodatabase, allowed the identification of the distance of the exposed individuals from the respective nearest landfill.
  - Concentration of heavy metals in the soil of the child address

Exposure and effect modifiers

Additional factors considered as exposure and effects modifiers were included as well. These included:

- Sociodemographic parameters such as
  - Socioeconomic status
  - Mother education
  - Father education
  - Stress events
- Child anthropometric parameters and post-delivery factors
  - Child body mass index
  - Child gender
  - Breastfeeding
- Presence of micronutrients, minerals and vitamins
  - Se in the mother plasma during pregnancy, delivery and in cord blood
- Detailed dietary habits
  - Consumption of meat products (pork meat, beef, lamb, sausages)
  - Consumption of fish
  - Consumption of sea food
  - Consumption of poultry (eggs, chicken)
  - Consumption of dairy products (milk, yogurt)
  - Consumption of nuts

- Consumption of fruits
- Consumption of vegetables
- Consumption of snacks (biscuits, chocolates)

Health outcomes investigated

The health outcomes considered in this study are relevant to the neurodevelopmental disorders in children estimated following the administration to the children or their parents and teachers the following four test batteries.

The **Child Behavior Checklist** (Achenbach and Rescorla, 2001), also called the Achenbach System of Empirically Based Assessment, is a report form to screen for emotional, behavioral, and social problems. The CBCL's questions are associated with problems on a syndrome scale in eight different categories: anxious/depressed, withdrawn/depressed, somatic complaints, social problems, thought problems, attention problems, rule-breaking behavior, and aggressive behavior. The CBCL also has a scale set to show scores associated with disorders from the Diagnostic and Statistical Manual of Mental Disorders (American Psychiatric Association., 2000): anxiety, oppositional defiant disorder, conduct problems, somatic problems, affective problems, and attention deficit disorder. Many studies have demonstrated a high rate of reliability between the scales of the CBCL and actual psychological diagnosis (Warnick et al., 2008).

The **Cambridge Neuropsychological Test Automated Battery** (CANTAB); it has been used to assess neurocognitive performance in modeling studies of Chronic Fatigue Syndrome (CFS) (Capuron et al., 2001; Robbins and Sahakian, 2002). CANTAB has modules for several neurocognitive functions and processes including psychomotor and motor speed, reasoning and planning abilities, memory and attention, and frontal, temporal and hippocampal dysfunctions. Thus, it allows assessment of neuro-cognitive dysfunctions associated with neurologic disorders, phannacologic manipulations, and neuro-cognitive syndromes.

The **Social Responsiveness Scale** (SRS); it is often used to measure Autism Spectrum Disorders (ASD) severity. The Social Responsiveness Scale (SRS) is a parent and teacher-completed screening questionnaire measures social ability of children from 4 years to 18 years old. It is used primarily to measure Autism Spectrum Disorders (ASD) severity. Although SRS is frequently referred to as a measure of "social impairment," many SRS items describe other core features of ASD, including communication deficits and repetitive behaviors (Constantino et al., 2000), as well as symptoms not exclusively related to ASD diagnostic criteria (Grzadzinski et al., 2011).

The **Wechsler Intelligence Scale for children – Fourth Edition** (Wechsler, 2003); it is an individually administered measure of intelligence intended for children aged six years to 16 years and 11 months. WISC-IV yields measures of general intelligence as reflected in both verbal and nonverbal (performance) abilities and specific indices including verbal comprehension, perceptual reasoning, working memory and processing speed.

Results

EWAS analysis

Data clustering

For clustering the various exposure related data, the two different clustering techniques were used. The results are graphically illustrated in Figure 7 and Figure 8 respectively.

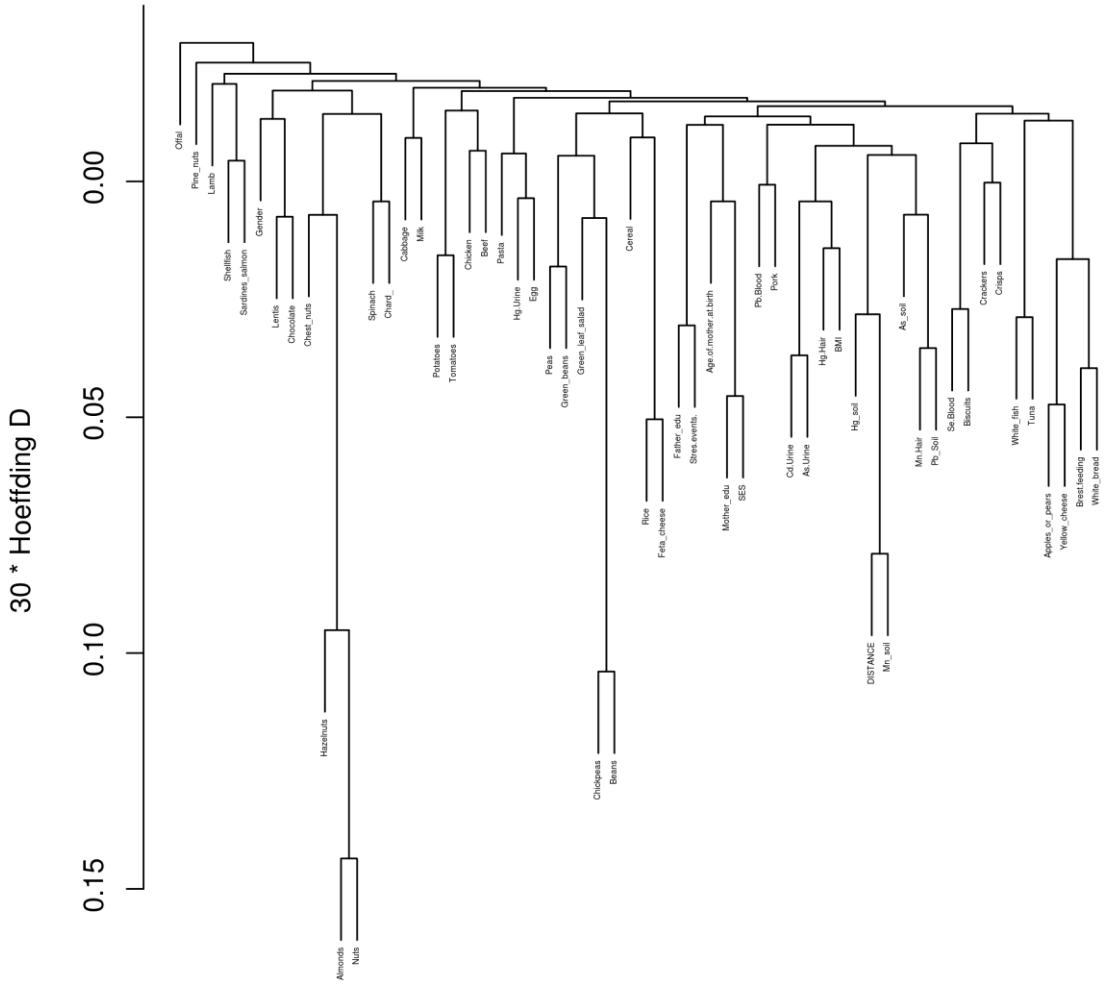


Figure 7. Hierarchical clustering using the Hoeffding D method

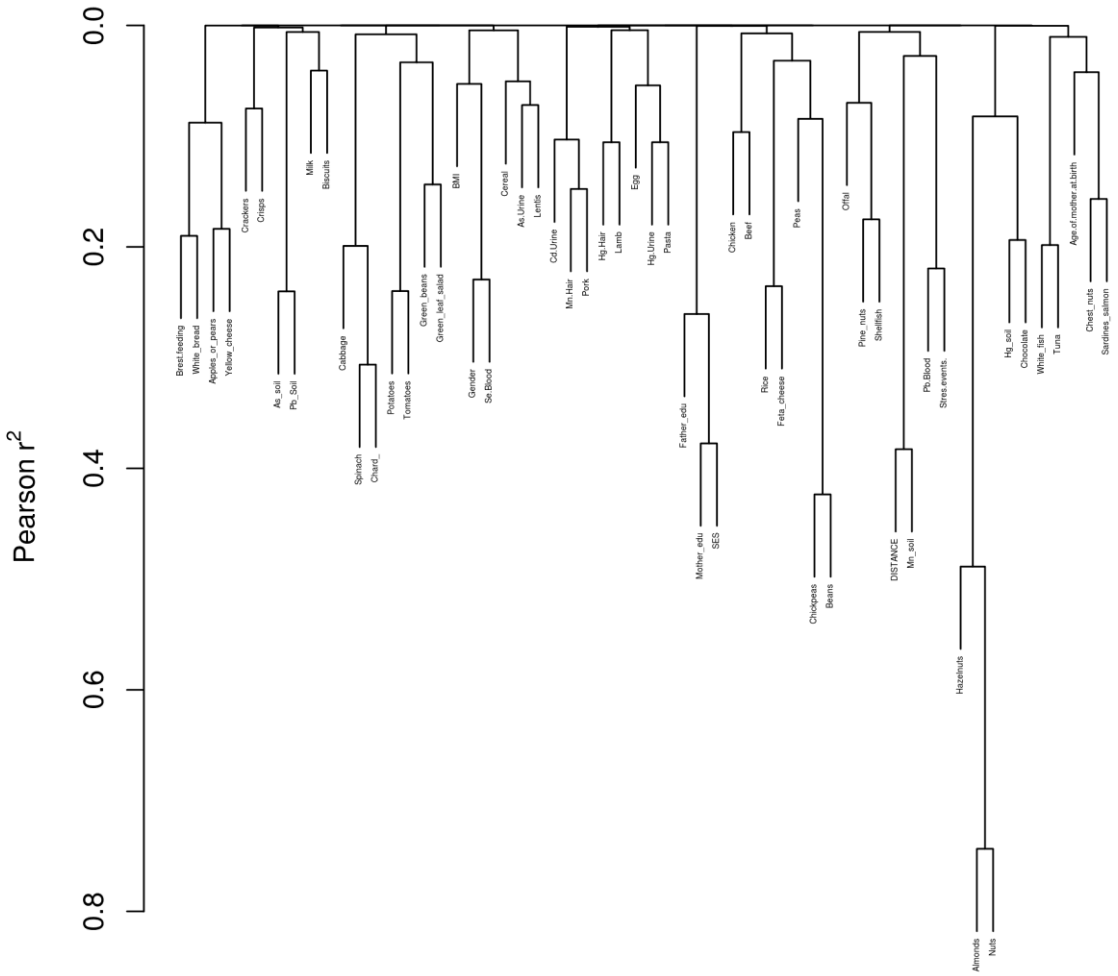


Figure 8. Hierarchical clustering using the Pearson correlation

The auto-correlations of the various parameters, are illustrated in both the heatmap (Figure 9) and the correlation globe (Figure 10).

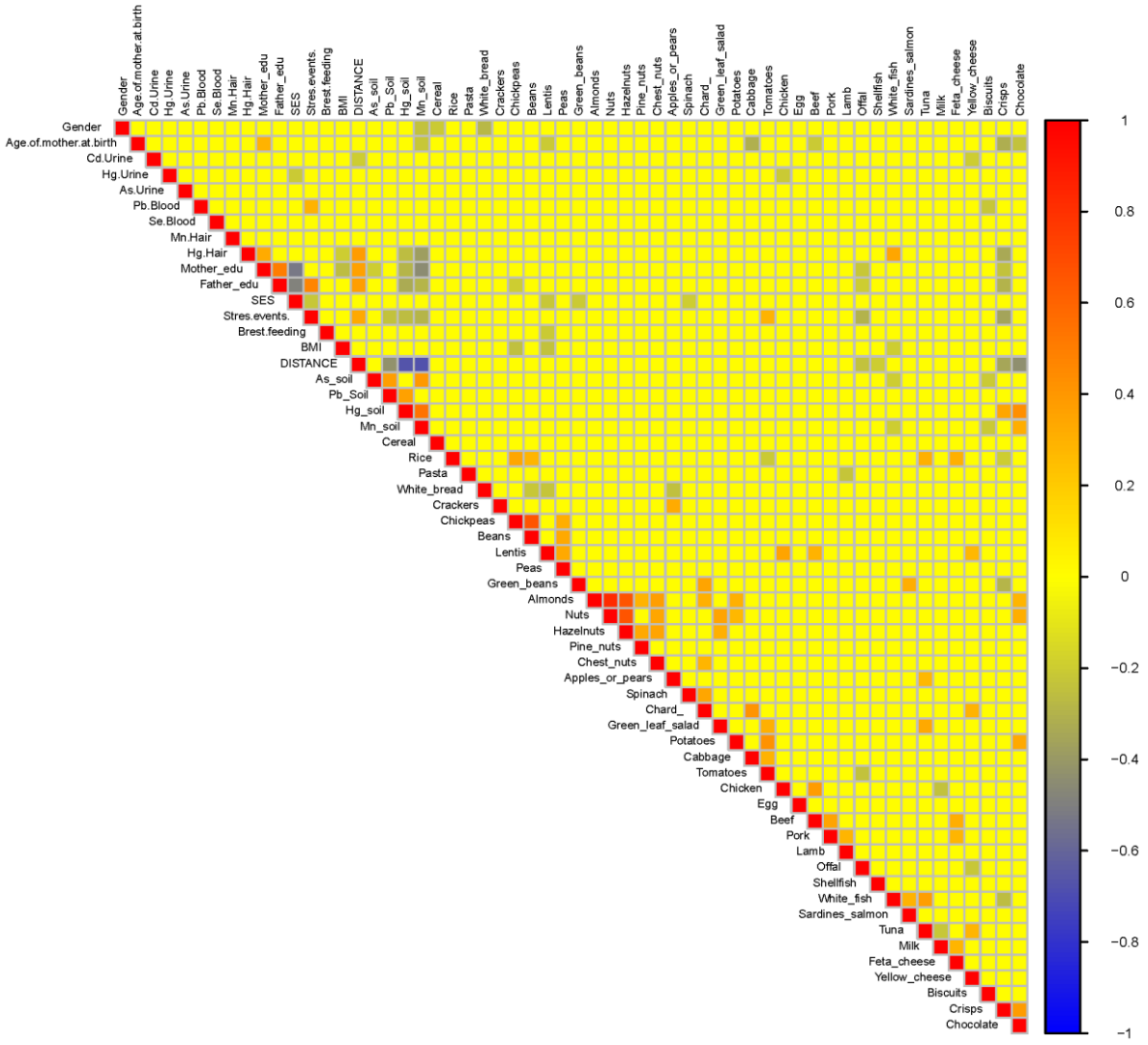


Figure 9. Heatmap of the exposure parameters of the landfill study

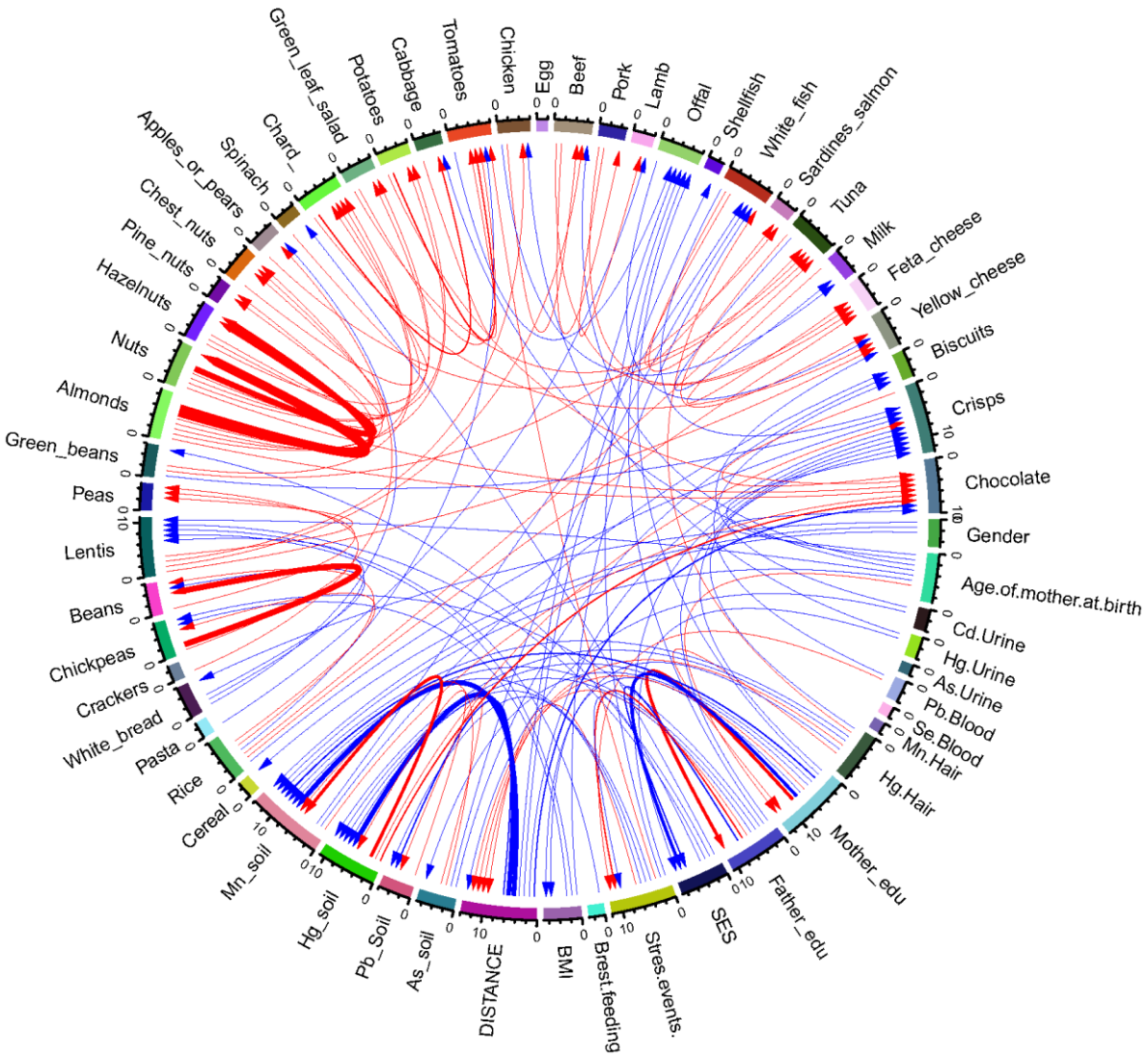


Figure 10. Correlation globe of the environmental, dietary and exposure factors of the landfill study

Results and interpretation of EWAS analysis

The outcomes of the associations among the various exposure and sociodemographic factors for one selected indicative outcome of each test battery are illustrated in the following Figures.

EWAS analysis results relevant to the **Child Behavioral CheckList** (CBCL) test battery results show that socio-cultural factors are strongly associated with children behavior. More specifically the *mother school title* and the *age of the mothers at birth* show both a robust statistical association (p-value<0.05 and in some cases p-value <0.01) with most of the CBCL indices considered. Looking at the volcano plots both parameters show a negative association with the CBCL scores indicating that lower educational level of the mothers as well as a lower age of the mother at the children birth may have negative impact on the children behavior.

The *stress index* was derived by merging the total number of stressful events detected by the mother and their average intensity is also playing an important role on the children behavior (p-value<0.05 and in some cases p-

value<0.01) showing a negative effect on both internalizing and externalizing problems indices such as anxiety and depression, withdrawal and depression and somatic complaints, aggressive and rule-breaking behavior.

The *concentration of lead in blood* shows a strong statistical significance (p-value <0.05) with most of the CBCL indices analyzed. In this case the association shows a positive direction revealing a negative impact of higher blood concentration of lead on the on cognitive functions in children. This result is confirmed by a number of research studies which indicate exposure to lead as one of the most environmental determinants of neurodevelopmental disorders in children. On this subject the National Toxicology Program (NTP) has concluded that childhood lead exposure is associated with reduced cognitive function, including lower intelligence quotient (IQ) and reduced academic achievement (National Toxicology Program, 2012). The NTP has also concluded that childhood lead exposure is associated with attention-related behavioral problems (including inattention, hyperactivity, and diagnosed attention-deficit/hyperactivity disorder (ADHD)) and increased incidence of problem behaviors including delinquent, criminal, or antisocial behavior (National Toxicology Program, 2012).

Of opposite sign but still with robust statistical significance is the association of the concentration of selenium in blood which appears to act as beneficial element especially with regard to Internalizing Problems and ADHD as measured by CBCL battery indices. These results confirm the antioxidant properties of selenium which is a well-known regulator of brain function (Dominiak et al., 2016). These positive properties that selenium possesses are attributed to its ability to be incorporated into selenoproteins as an amino acid. Several selenoproteins are expressed in the brain, in which some of them, e.g. glutathione peroxidases (GPxs), thioredoxin reductases (TrxRs) or selenoprotein P (SelP), are strongly involved in antioxidant defense and in maintaining intercellular reducing conditions. Since increased oxidative stress has been implicated in neurological disorders higher levels of selenium in blood may be among the important factors protecting against those pathologies.

Breast feeding in the first months of children life is another parameter that shows a significant statistical association (p-value <0.05) especially with the internalizing problems as measured by CBCL battery indices. Also in this case the association shows a negative sign indicating that breastfeeding and especially its duration during the first year of life results in a beneficial effect on anxiety/depression, withdrawal/depression and somatic complaints as reported by the CBCL indices.

The concentration of mercury in hair reveals a strong association (p-value <0.05) with many CBCL indices considered, however its effect appears to have a controversial behavior as witnessed by its negative sign reported in the volcano plots indicating that higher concentration levels of Hg in hair may results in potential positive effect on the problem behavior in children.

CBCL indices as measured by teachers reveal slightly different patterns. Even though the socio cultural factors such as *mother school title* still show robust associations with most of the Child Behavioral CheckList test battery outcomes, other variables appear to play an important role. Among them the *distance of the residence address from the waste management site* shows a strong association especially with the internalizing problems. The negative sign of the association corroborates the negative impact of living in areas close to the waste management site especially on anxiety/depression, withdrawal/depression and somatic complaints.

Concentration of lead in blood is yet another significant variable (p-value <0.05) associated with Attention Deficit Hyperactivity Disorder while *Breast feeding* shows a strong association with Oppositional Defiant Disorder (ODD). Among the various food items considered, some of them show significant statistical association with CBCL indices. Consumption of *pork* (e.g. pork dishes, lard, bacon, salami) appears to be inversely associated (p-value <0.05) with the CBCL indices related to externalizing problems such as aggressive and rule-breaking behavior as well as with association with Oppositional Defiant Disorder and with Conduct Problems. High consumption of *chicken* reveals a strong association with Attention Deficit Hyperactivity Disorder measured by the teachers. Consumption of cabbage and lentils appears to influence negatively Attention Deficit Hyperactivity Disorder, Oppositional Defiant Disorder and with Conduct Problems too. High consumption of *coffee* is associated with externalizing problems such as aggressive and rule-breaking behavior and with Attention Deficit Hyperactivity Disorder and Conduct Problems measured by the teachers. *Fish consumption* reveals opposite effects on the basis of the fish type: while higher consumption of

sardines and/or salmon (also canned) appears to be statistically associated with ADHD, consumption of herring, mackerel and trout (also canned) indicates a beneficial effect on the CBCL indices related to Conduct Problems. Higher consumption of *pine nuts and nuts* as well as of *white and wheat bread* indicates a beneficial effect on externalizing problems such as aggressive and rule-breaking behavior. Finally, higher consumption of eggs and of beans are also associated to beneficial effects on internalizing problems (i.e. anxiety/depression, withdrawal/depression and somatic complaints) indices.

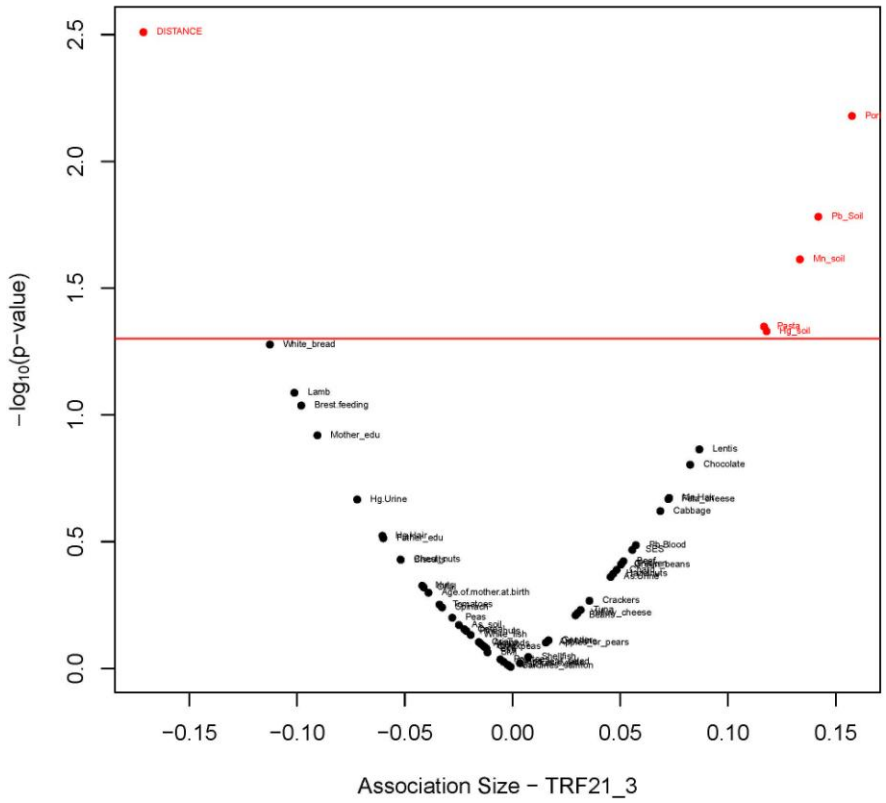


Figure 11. Associations of attention deficit / hyperactivity problems (from the CBCL test battery) with the environmental, dietary and exposure factors

EWAS analysis results relevant to the **Cambridge Neuropsychological Test Automated Battery (CANTAB)** test battery results show that concentration of Manganese in the hair is associated (p-value <0.05) with the Spatial Working Memory (SWM) with a positive sign revealing that higher Manganese levels in the hair increase error generation. Socio-cultural factors show again significant statistic association with the outcomes of the CANTAB test battery.

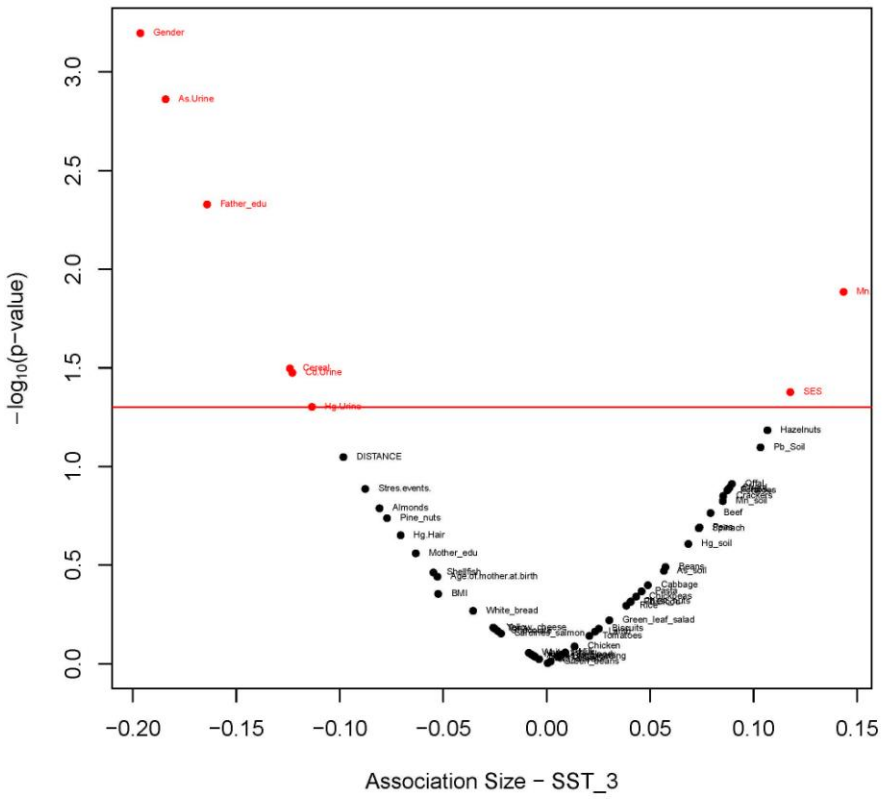


Figure 12. Associations of Stop Signal Task Mean correct RT on GO trials (from the CANTAB test battery) with the environmental, dietary and exposure factors

More in detail *Mother School title* appears to have a beneficial effect (p-value < 0.05) on the Stop Signal Task while *Father School title* on the spatial Working Memory Strategy index (p-value < 0.05). The *stress index* is also strongly associated (p-value <0.01) with the Spatial Working Memory (SWM) with a negative sign showing that higher stress levels decrease the error production.

EWAS analysis results relevant to the **Social Responsiveness Scale (SRS)** test battery show that also in this case socio-cultural factors are strongly associated with the Social Responsiveness Scale outcomes considered. *Mother school title* (p-value <0.000) and to a lower extent *Father school title* (p-value < 0.05) show both a robust statistical association the T scores of both the parents and teachers. Moreover, the associations have a negative direction demonstrating that lower educational level of the parents may have negative impact on the Autism Spectrum Disorder (ASD) impairments of children.

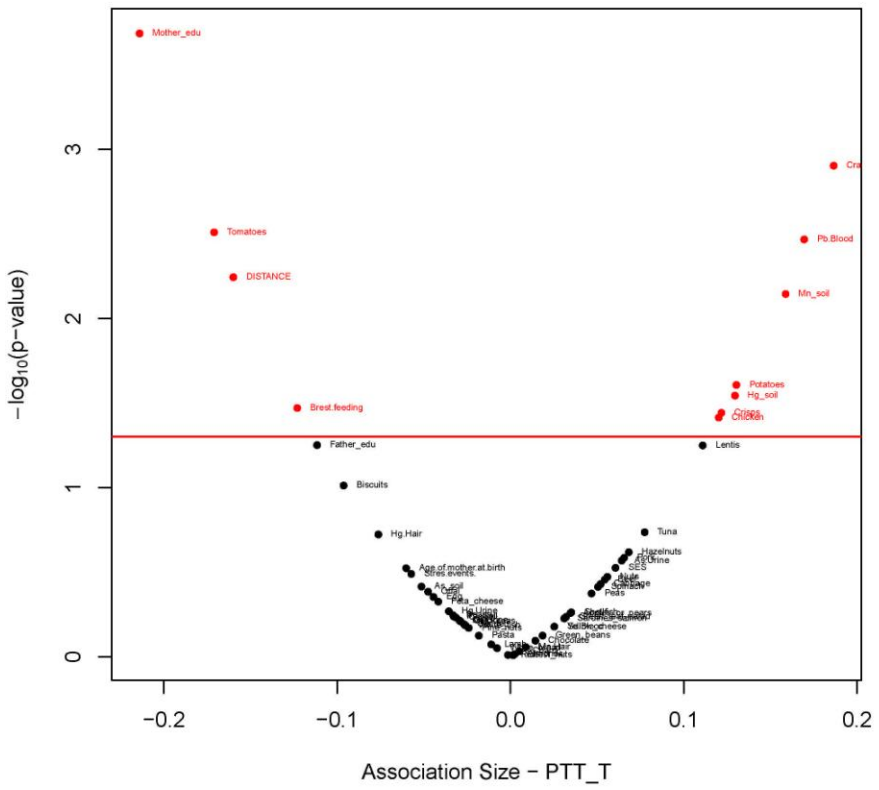


Figure 13. Associations of Total score T / Teachers (from the Social Responsiveness Scale test battery) with the environmental, dietary and exposure factors

Distance of the residence address from the waste management site shows a good association (p-value <0.001) with the T scores of the teachers (Figure 13). The negative sign of the association confirms the potential negative impact of living in the areas close to the waste management site on ASD impairments of children

Breastfeeding in the first months of children life also shows a good statistical association (p-value <0.05) with T scores as reported by teachers. The association shows a negative sign indicating the positive effect of breastfeeding, and especially its duration during the first year of life, on ASD impairments of children.

Among the biomonitoring data *selenium* in blood appears to be inversely associated (p-value <0.05) with the T scores of the parents. The negative sign of the association supports the positive impact of selenium on the neurodevelopmental disorders. Mercury concentration in hair shows a significant statistical association (p-value <0.05) with SRS battery indices and its effect appears to result in potential positive effect on ASD impairments.

Among the different food items higher consumption of pork dishes (lard, bacon, sausage) (p <0.01), coffee (p-value < 0.01), chicken (p < 0.05), breadsticks, crackers and rusks (p-value <0.05) and lentils (p-value <0.05) are associated with higher T scores of the SRS test battery indicating a potential negative effect on ASD impairments. On the contrary higher consumption of tomatoes (p-value < 0.001), white fish (e.g. codfish, rumble fish) (p-value < 0.01) and soft cheese (p-value <0.05)) are related with lower T scores of the SRS test battery signifying a potential positive effect on ASD impairment of children.

EWAS analysis results relevant to the WISC-IV test results show that the variable *distance of the residence address from the waste management site* is a key factor associated with almost all the indices of the WISC IV test. More specifically this variable shows a robust statistical association (p-value <0.001) with the Intelligence Quotient (IQ), Verbal Comprehension index, Perceptual Reasoning index, Working Memory index. Analysis of the results show a positive association with the WISC IV scores indicating that living far from the waste management site has a positive impact on the children cognitive functions. Some interesting conclusions can be drawn from the analysis of food consumption patterns. *Tomatoes* consumption appears to be statistically (p-value <0.05) associated to QI, Verbal Comprehension index and Working Memory index while cereal consumption reveals a strong association (p-value < 0.01) with the Perceptual Reasoning index. Both these food items show a positive sign meaning that their consumption has potential positive effects on the cognitive functions of the children. Epidemiological evidence suggests that consumption of lycopene, natural antioxidant presents in tomatoes, is able to reduce the risk of chronic diseases such as cancer, cardiovascular diseases as well as psychiatric syndromes (Story et al., 2010). In another study (Li and Zhang, 2007) reported that low serum levels of lycopene have been associated with increased risk of psychiatric disorders. One review of 22 studies examining the association of breakfast cereal consumption and academic performance in children and adolescents concluded that breakfast consumption may improve cognitive function related to memory, test grades, and school attendance (Rampersaud et al., 2005).

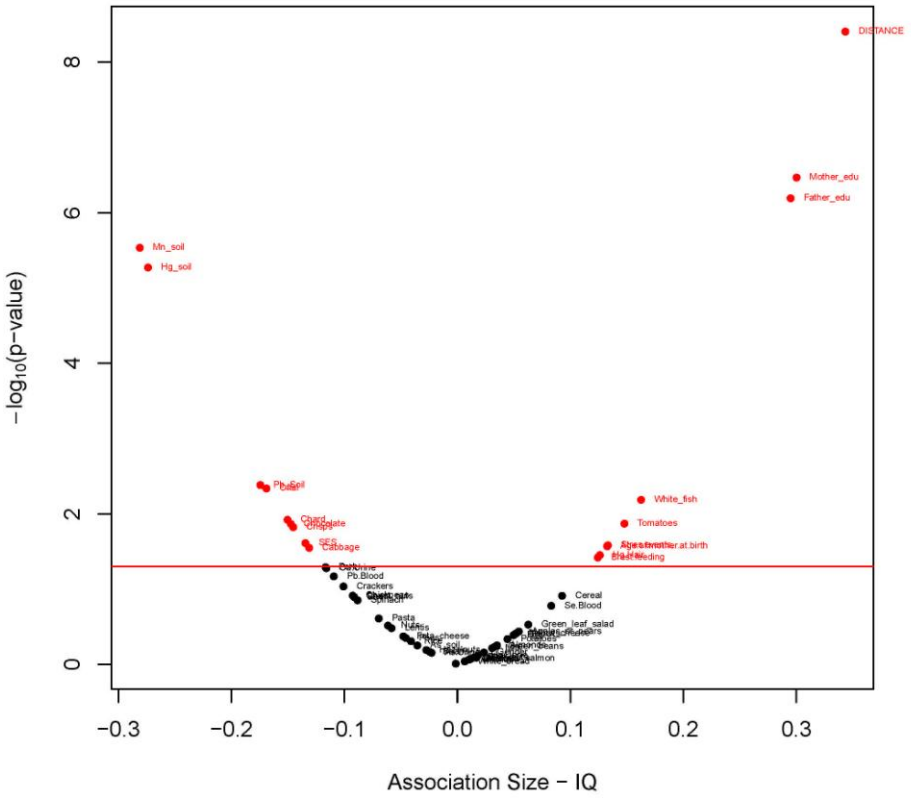


Figure 14. Association of intelligence quotient (from the WISC-IV test battery) with the environmental, dietary and exposure factors

Like for the CBCL test battery *consumption of fish* shows a dual behavior depending on the type of fish considered: higher consumption of *white fish* appears to have positive effects on the IQ and Verbal Comprehension index (p-value <0.001) while higher consumption of white fish reveals a negative effect (p-value <0.01) on Perceptual Reasoning index and Working Memory index.

Cancer

Italy

Metals exposure in Lazio and associated cancer risks

The first Italian human biomonitoring survey (PROBE – PROgramme for Biomonitoring general population Exposure) considered a reference population of adolescents, aged 13-15 years, living in urban and rural areas, for their exposure to metals. The previous study was integrated up to 453 adolescents living in the same areas of Latium Region (Italy) and blood samples were analyzed for 19 metals (As, Be, Cd, Co, Cr, Hg, Ir, Mn, Mo, Ni, Pb, Pd, Pt, Rh, Sb, Sn, Tl, V, and W) by sector field inductively coupled plasma mass spectrometry. The exposure assessment was contextualized 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 Liner Model (GLM) capturing co-exposures to pairs of key determinants.

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

In this study the exposome paradigm was used to analyze the HBM data of PROBE. This approach did not consider the existence of confounding factors, effectively taking into account all specific and non-specific exposure determinants and modifiers and all exposure biomarkers measured in the PROBE cohort adopting an unbiased (untargeted) perspective towards the development of exposome-wide associations. That allowed the exploration of a larger parameters space when it came to exposure determinants. Linkage disequilibrium was tested in this large parameter space to identify putative causal relationships between exposure determinants/modifiers and measured exposure biomarker levels in human biosamples. Linkage disequilibrium, a concept borrowed from population genetics (Slatkin, 2008), is defined in this context as the non-random association of exposure determinants and modifiers for different exposure biomarkers. Exposure determinants/modifiers are considered as being in linkage disequilibrium when the frequency of association of different determinants differs from the expected value had these determinants been independent and associated randomly.

Sample treatment and analysis

Blood samples were collected by a specific protocol and a system specifically designed for trace metal analysis (7.5 mL S-Monovette LH and Monovette needle, Sarstedt, Numbrecht, Germany). The sample were stored at -20 C and transported to laboratory in a deep-frozen state. Blood sample treatment in laboratory (mineralization and analysis) were described elsewhere (Pino et al., 2012). The analytical method used is validated by ACCREDIA (the Italian National Accreditation Body) and the following validation performances were assessed: linearity, Limit of Detection (LoD) and Limit of Quantification (LoQ), specificity, accuracy (precision and trueness) and robustness.

Study population

A population of 453 adolescents aged 13–15 years (242 females and 211 males) living in Latium Region (Italy) were enrolled. The areas were Viterbo (VT), two areas in the Rome province (Fontenuova, FN and Monterotondo, MR), and the city of Rome (RM).The adolescents` distribution in the four sampled areas was: 161 from VT, 131 from FN, 72 from MR and 89 from RM. Metals investigated were: As, Be, Cd, Co, Cr, Hg, Ir, Mn, Mo, Ni, Pb, Pd, Pt, Rh, Sb, Sn, Tl, V, and W. Non-fasting blood specimens were obtained for our purposes by ANMI during the annual screening for microcytemia in schools (2009); this was an excellent chance to ethically collect blood. The children`s parents gave their written consent and filled a questionnaire for each subject; the entire study design was approved by the Ethical Committee of the Italian National Institute for Health (ISS). The questionnaire key points included information that was used to stratify the population according to: sex, presence of dental fillings and/or braces, current use of piercings and tattoos, second hand smoke, frequency intake of fish, and milk, socio-economic status (SES) of the family that was derived merging the educational level and the occupational status of the parents. International Standard Classification of Occupations (ILO, 2012) was adopted to describe the parental occupations. From the questionnaires administered several information were obtained: 138 adolescents had dental braces and/or fillings while 49 got piercing and 93 adolescents had parents smoking at home. Relating to dietary habits, 265 adolescents consumed fish 1 time a week (1/w) and 81 twice a week or more (≥2/w) while 241 had milk every day (7/w), 63 from 4 to 6 times a week (4-6/w) and 99 from1 to 3 times a week (1-3/w) (Table 31).

Table 31. Characteristics of the population

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

Environmental data

To have a view of the exposure approaching to the exposome sense, metal concentrations were related to environmental data of air and water quality supplied by the Environmental Protection Agency of the Lazio region (ARPA). Kriging techniques were applied to derive spatially resolved concentration of chemicals in the outdoor air starting from data collected by air monitoring stations. Pollutants considered were NOx, NO, NO_2, CO, Benzene, PM10. Arsenic levels in drinking water were derived from the database managed by ARPA Latium. (Data repositories: Water samples monitoring activity. Available: http://www.arpalazio.gov.it/ambiente/salute/dati.htm#). Relating to water supply, a city map was prepared reporting water sources for the different boroughs, streets and squares. For each water source the As concentration was measured, and these values were attributed to subjects according to their home address. An example of the As concentrations in the water supplies are showed in Table 32.

Table 32. Arsenic concentrations in the water supply networks of the Viterbo municipality

Water sources	Arsenic (µg/L)
Tank of Grotticella	18*
Tank 480	18*
Tank of Monte Jugo	19
Tank of Settecannelle	9.5
Tank 3000	9*
Tank of Votamare/Chiesuola	9.5
Tank of Pratoleva/Fastello	9
Grotte S. Stefano/Tank Magugnano	10.5
Vallebona	9
Roccalvecce-S. Angelo di Roccalvecce/Tank Montesecco	13*
Tank Montecalvello	6
Castel D`Asso/Service Pidocchio	22
Tank Campo sportive	9
Tank Colonia	5
Tank Balletti	5
Service Tobia/Tank Canale	17
Service Carcarelle/Tank Carlini	36

Service Rio Trai	1

*Mean value every three months

Data treatment

The basic statistics of data relating to 453 adolescents include the 50th and 95th percentiles, geometric mean (GM) and the corresponding 95% confidence interval (95%CI); the GM represents better the central tendency of data if the distribution is asymmetric instead of the mean index. In the statistical evaluation values below the LoD were considered as LoD/2 and extreme values were excluded. This procedure was used to derive Reference Values (RVs) where the 95th percentile describes the upper value useful in health care and environmental policy. The adolescent cohort was also stratified by some characteristics including sex, residence area in turn associated to traffic intensity, presence of dental fillings and/or braces, piercings and tattoos, second hand smoke, fish and milk consumption, SES; each variable was coded per the levels applied in the questionnaires. For all comparisons and statistical analyses, the data base including the extreme values was considered. Differences for each metal concentration among subgroups based on the different variables were tested by the Mann-Whitney U test or the Kruskall-Wallis test (depending on the number of levels for each grouping variable). Mann-Whitney U test with Bonferroni`s correction was used for multiple comparisons, when appropriate. Significance level was set at p<0.05. Statistical calculations were performed on STATA 8.1 (STATA Corporation, USA, TX). The geo-statistical analyses of the subjects were carried out based on their residence address in a Geographical Information System (GIS) and stored in a Geodatabase along with human biomonitoring data, diet habits, environmental and land cover data. A Generalized Linear Model (GLM) formulation was used to investigate associations between human biomonitoring data and diet patters (fish and milk) and land cover. The form of the model was:

$$HBM\ value = i + a_1 C_f + a_2 C_m + a_3 LC + a_4 C_f \cdot LC + a_5 C_f \cdot C_m + a_6 C_m \cdot LC + a_7 C_f \cdot C_m \cdot LC$$

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

EEA 2006 (http://www.eea.europa.eu/data-and-maps/data/corine-land-cover-2006-raster-3) land cover data at high spatial resolution (100 m) were used to analyze possible spatial relationships between the type of land cover where adolescents lived and metal concentration in blood.

The data were further analysed following the Environment-Wide Association Study (EWAS) framework to discover potential associations among covariates and HMB data in an untargeted manner. A systematic sensitivity analyses was carried out, whereby validated factors were modeled under different assumptions or with additional covariates. Moreover, the correlation of dependence between the factors, revealing potential evidence for exposure or confounding route has been computed using pair-wise validation.

Results of biomonitoring data

Reference values computed excluding extreme values (see Methods for details) in the overall group of adolescents, and in male and female subgroups, are presented in Table 33.

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

around Viterbo than in other areas, and Cd, Hg, Ir, Mn, Ni, Pb, Pt, Rh, Sb and U were significantly higher ($p<0.01$) in Rome. Finally, socio-economic status of the family was positively associated with As concentration ($p<0.01$).

Table 33. RVs in blood for adolescents of Latium, Italy (μg/L)

	Overall				Males				Females			
		Percentiles				Percentiles				Percentiles		
Element	Subjects N	50th	95th	GM (95% CI)	Subjects N	50th	95th	GM (95% CI)	Subjects N	50th	95th	GM (95% CI)
As	443	0.73	2.95	0.71 (0.66-0.78)	205	0.75	2.82	0.71 (0.63-0.81)	238	0.75	3.08	0.72 (0.64-0.81)
Cd	431	0.3	0.6	0.29 (0.28-0.31)	200	0.31	0.63	0.3 (0.28-0.33)	231	0.3	0.57	0.29 (0.27-0.31)
Co	445	0.09	0.28	0.09 (0.09-0.10)	207	0.1	0.31	0.1 (0.09-0.11)	238	0.09	0.27	0.09 (0.08-0.10)
Cr	414	0.3	1.25	0.31 (0.29-0.34)	195	0.3	1.3	0.31 (0.28-0.36)	219	0.3	1.2	0.31 (0.28-0.34)
Hg	436	0.83	2.05	0.78 (0.73-0.83)	207	0.76	1.96	0.76 (0.70-0.82)	229	0.87	2.05	0.8 (0.73-0.87)
Ir *	437	6.84	15	6.71 (6.39-7.05)	201	6.84	15.96	6.94 (6.46-7.46)	236	6.84	14.5	6.52 (6.09-6.99)
Mn	449	7.46	16	7.22 (6.89-7.57)	210	7.52	15.33	7.17 (6.71-7.67)	239	7.31	16.25	7.26 (6.79-7.75)
Mo	449	1.1	2.39	1.11 (1.06-1.16)	209	1.08	2.42	1.11 (1.04-1.19)	240	1.14	2.32	1.11 (1.05-1.18)
Ni	411	1.02	2.6	0.94 (0.88-1.01)	188	1.07	2.63	1 (0.90-1.10)	223	0.98	2.5	0.9 (0.82-0.99)
Pb	440	9.55	21.6	9.6 (9.16-10.06)	205	11.1	22.11	10.7 (10.02-11.42)	235	8.73	20.59	8.73 (8.19-9.31)
Pd*	440	22.1	38.6	21.3 (20.4-22.2)	203	22.2	38.49	21.22 (19.91-22.63)	237	21.85	38.65	21.32 (20.17-22.55)
Pt*	423	10.9	23.3	10.9 (10.4-11.4)	191	10.7	22.31	10.6 (9.82-11.36)	232	10.88	25.8	11.17 (10.52-11.87)
Rh*	437	22.1	35.6	21.2 (20.5-22.0)	200	21.9	36.19	21.21 (20.11-22.37)	237	22.21	35.22	21.24 (20.27-22.27)
Sb	425	0.39	0.78	0.37 (0.35-0.38)	193	0.39	0.81	0.38 (0.35-0.41)	232	0.38	0.76	0.36 (0.33-0.38)
Sn	439	0.56	1.52	0.57 (0.53-0.60)	205	0.53	1.56	0.57 (0.52-0.62)	234	0.56	1.47	0.56 (0.52-0.61)
Tl	442	0.04	0.09	0.04 (0.038-0.042)	204	0.04	0.09	0.04 (0.037-0.043)	238	0.04	0.1	0.041 (0.038-0.044)
U*	445	4.85	14.3	5.09 (4.83-5.37)	208	5.05	15.51	5.29 (4.88-5.75)	237	4.62	13.74	4.91 (4.59-5.26)
V	445	0.08	0.17	0.07 (0.066-0.073)	207	0.07	0.18	0.072 (0.067-0.077)	238	0.07	0.16	0.068 (0.063-0.072)
W	444	0.03	0.08	0.03 (0.027-0.030)	205	0.03	0.08	0.03 (0.028-0.032)	239	0.03	0.07	0.028 (0.026-0.030)

*ng/L

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 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 man than in women. Another interesting result was the metal concentrations in function of the

land use that show as 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 a higher exposure to traffic-derived metals (Table 34).

Table 34. Internal doses (in μg/L) as a function of the land use

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

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

Also by means of the GLM (Figure 15) and EWAS (Figure 16) approaches, we identified 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.

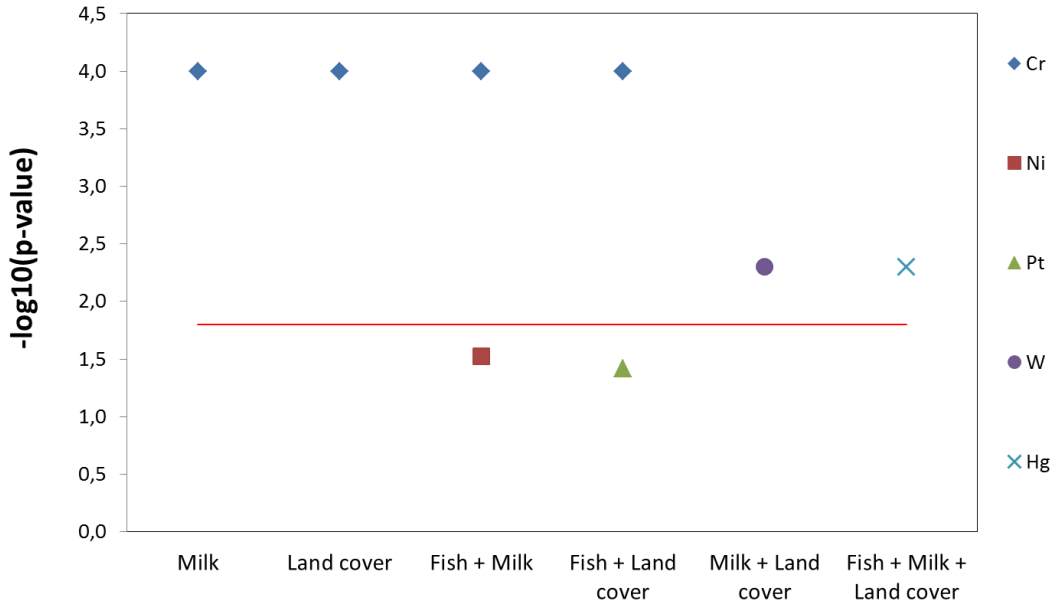


Figure 15. GLM results – Manhattan plot of between-subjects effects

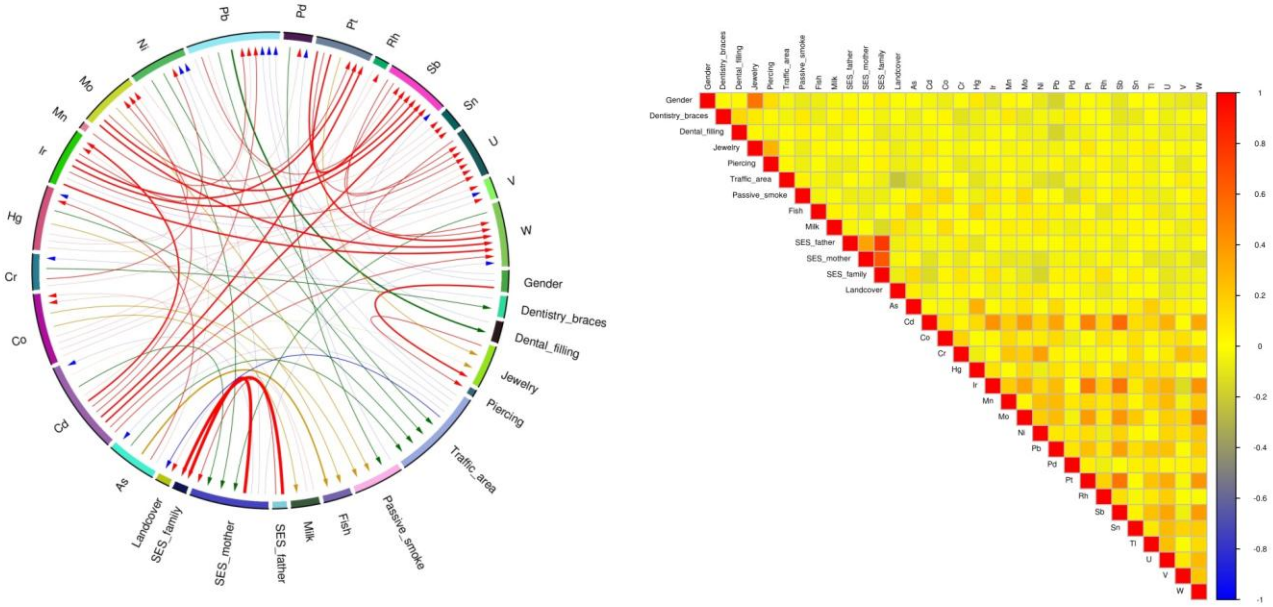


Figure 16: EWAS results – correlation globe (on the left) amd heatmap (on the right).

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.

Spain

Estimating cancer risk associated to organochlorine compounds

Complaints of odor by the population of a village of 5,003 (2,531 male) inhabitants located in the vicinity of an organochlorinated-compounds factory (Flix, Catalonia, Spain) led to the detection of unusually high atmospheric levels of hexachlorobenzene (HCB). The factory, the only one in the village, was built in 1898 and has been producing volatile chlorinated solvents over the last decades. The village is situated in a rural area, but the occupational pattern of its population is essentially related to the industrial activities of the factory. In fact, the village was developed as result of the establishment of the factory, which stabilized the population in this area. Other compounds, such as polychlorobiphenyls (PCBs), were manufactured during some periods, although their production ended in 1987. Air pollutants in the village were examined together with the concentration of organochlorinated compounds in the sera of a number of inhabitants, and the mortality and cancer incidence in the community.

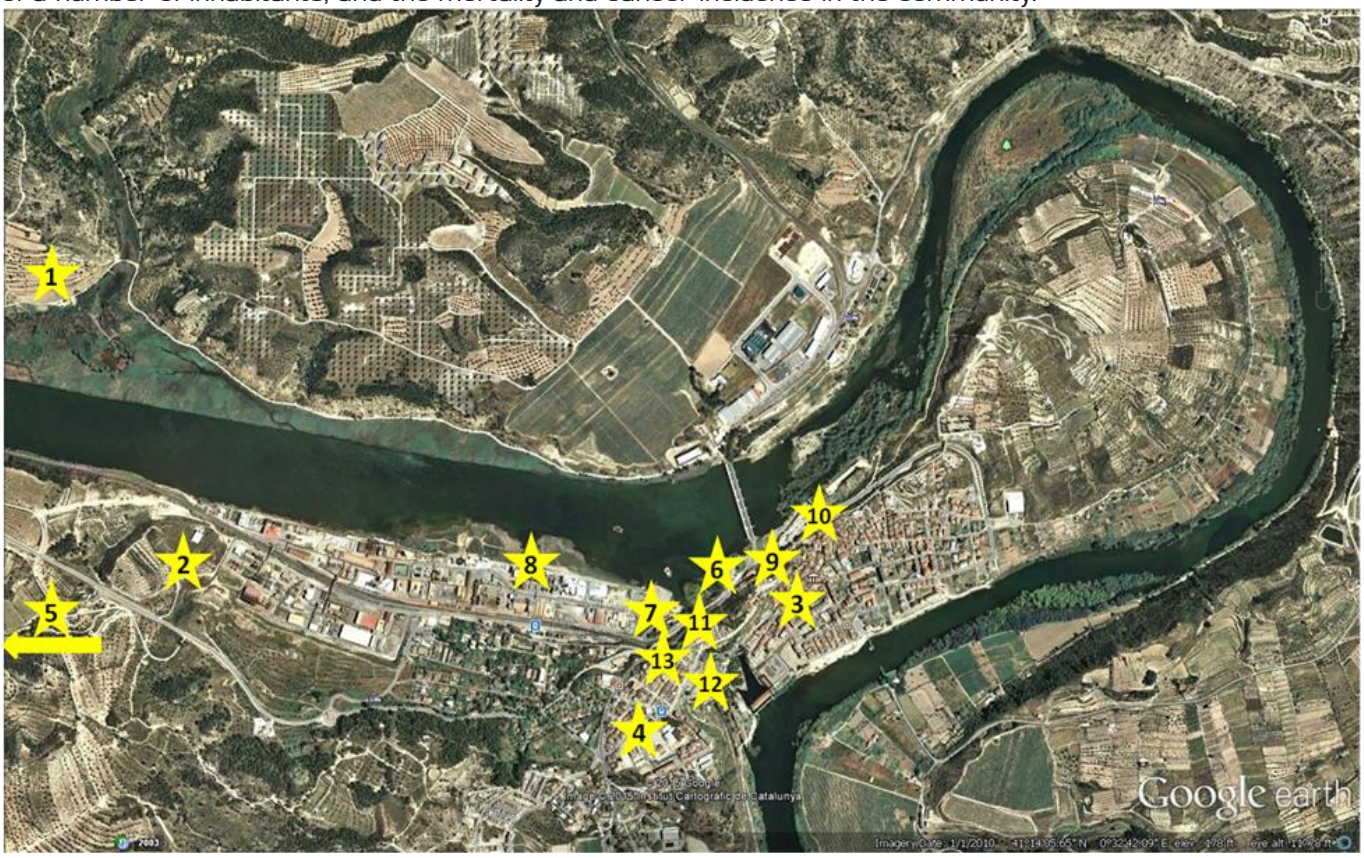


Figure 17. Sampling sites of organochlorine compounds in air

Table 35. Air samples collected in 2013.

	Site 1	Site 2	Site 3	Site 4	Site 5	Site 6	Site 7	Site 8
2 oct.	12:05-	19:35-20:05	17:10-17:40	13:35-	18:45-19:15	-	-	-

16 oct.	12:40 15:15-15:47	08:37-09:08	16:15-16:45	14:05 10:40-11:13	12:10-12:40	14:20-14:50	17:10-17:41	-
17 oct.	- 11:20-11:50	09:15-09:50 12:20-12:54	- 09:00-09:30	- 09:53-10:23	- 13:00-13:30	- 10:40-11:10	- -	- -
8 nov.	-	12:55-13:10	-	-	-	-	09:40-10:10	12:15-12:40

The VOCs present in the air were concentrated in stainless steel cartridges (89 mm long x 6.4 mm outer diameter) packed with a multiadsobent bed consisting of 180 mg of Carbotrap 180 mg, 180 mg of Carbotrap C and 180 mg of Carbotrap X. Sampling was done using a suction low flow rate pump (Universal Deluxe Pump SKC) at a flow of 40 ml/min. Cartridges packed with 200 mg of Tenax TA were used for hexachlorobenzene (HCB). The pump was calibrated with a digital meter (510L Defender, SKC) at the beginning and end of each sampling. The average flow measured was chosen to determine the air sampled volume. After each sampling the cartridges, capped on both ends, were stored at 4 C within a sealed box free of VOCs. The samples were analyzed as soon as possible.

Average gas-phase levels of organochlorinated compounds in ambient air over 24 hr were monitored by high-volume pumping through polyurethane foam (PUF) columns placed after a glass-fiber filtration stage (< 0.50 µm). Shorter-period measurements (1 to 2 hr) were made by low-volume pumping and adsorption on Tenax GC tubes after filtration through 0.50 µm. The same procedures were also used to blindly analyze the air of another community (Barcelona city) situated 190 km away from the area of study and selected to control possible laboratory artifacts.

Table 36. Samples collected in 2014.

	Site 1	Site 3	Site 4	Site 9	Site 10	Site 11	Site 12
19 ag.	-	-	09:35-10:10	-	-	-	-
20 ag.	-	-	-	-	12:20-12:39	-	-
17 set.	-	-	-	12:14-12:34	-	-	-
24 set.	-	-	-	-	-	18:12-18:55	-
3 oct.	-	09:08-10:08	-	-	-	-	-
8 oct.	-	-	12:45-13:45	-	-	-	-
16 oct.	-	-	10:38-12:31	-	-	-	-
30 oct.	09:19-10:56	-	-	-	-	-	-
6 nov.	-	12:42-14:18	-	-	-	-	-
11 nov.	-	-	13:33-15:11	-	-	-	-
10 des.	-	12:17-14:18	-	-	-	-	-
16 des.	-	16:33-18:15	-	-	-	-	-
23 des.	-	11:34-13:22	-	-	-	-	-
24 des.	-	-	-	-	-	-	18:10-21:03
29 des.	-	13:26-14:52	-	-	-	-	-

Table 37. . Samples collected in 2015

	Site 1	Site 3	Site 12	Site 13
7 gen.	-	10:00-12:12	-	-
14 gen.	-	-	12:22-14:45	-
2 feb.	-	16:27-18:32	-	-
10 feb.	11:10-13:52	-	-	-
11 feb.	-	17:00-18:24	-	09:02-10:30
25 feb.	-	09:25-10:48	-	-

The PUF columns were Soxhlet-extracted with n-hexane and the extracts were fractionated by silica/alumina column chromatography. The organochlorinated fractions were blindly analyzed with gas chromatography (GC) coupled to electroncapture detection (ECD) and GC coupled to chemical ionization negative-ion mass spectrometry (NICI-MS). The Tenax GC tubes were analyzed by thermal desorption with an automatic system coupled to GC-ECD. All

compounds were quantitated by reference to authentic standards. PCBs were quantitated by summation of congeners numbers 28, 44, 52, 101, 118, 138, 153, 180 and 187.

Organochlorinated compounds in the sera of a non-random sample of general population subjects were analyzed to provide a first body-burden estimator. The subjects were recruited among those who carried out routine clinical blood analysis. A brief standardized questionnaire was compiled, with questions on occupation and residence. As a control for possible laboratory artifacts, sera samples of 13 subjects from a hospital in Barcelona were analyzed. All sera samples were extracted with n-hexane and the extracts blindly assayed by GC-ECD.

Mortality was assessed retrospectively from the official registry of the Health Department of Catalonia. Age and sex-standardized mortality ratios were calculated in comparison with the expected number of deaths from Catalonia figures. Cancer incidence was excerpted from the Tarragona Cancer Registry, a population-based register that covers the entire Province of Tarragona, including the community under study. During this decade the Registry collected information from different sources. Clinical records were reviewed to confirm the diagnosis and the residence of the subjects in study, and to excerpt their occupational histories. Poisson regression models were used to estimate the age-standardized incidence ratios and their 95% confidence intervals, comparing the community under study with the rest of the Province of Tarragona.

A compound that showed important changes was carbon tetrachloride. A concentration range between 0.1 and 10 $\mu g/m^3$ was observed. The concentrations of this compound generally followed those of trichloroethylene and tetrachloroethylene. This meant that these three compounds had a similar origin, probably emissions from mobilization of the sludge. The highest concentrations were found in site 1 and in a second level in sites 7 and 11 (Figure 17).

In the 90s, concentrations similar to those found at present during October 1992 were observed and others much higher in the period between November 1996 and October 1997. In October 1992, concentrations of 2-7.6 $\mu g/m^3$ in site 3 (Figure 17), 4-9 $\mu g/m^3$ in site 4 and 6.1-13.2 $\mu g/m3$ in site 7 were found. In the interval between November 1996 and October 1997, 1.5-20 $\mu g/m^3$ in site 3, nd-6.6 $\mu g/m^3$ in site 4, 1.2-52 $\mu g/m^3$ in site 7 and nd-18 $\mu g/m^3$ in site 1 were observed.

The samples studied in 2013-15 (Table 35 - Table 37) show that the most abundant compounds are trichloroethylene and perchloroethylene. These compounds have been observed in a range of concentrations ranging between 0.3 and 140 $\mu g/m^3$ in the first case and between 0.1 and 100 $\mu g/m^3$ in the second (Figure 18 - Figure 27).

The concentrations of these compounds are highly variable both geographically and temporarily. This means that the pollution episodes are due to specific emissions and meteorological changes instead of continuous emissions from constant sources into the atmosphere.

In general, the highest concentrations are located in site 3. High concentrations were also observed in sites 1, 7, 9, 11 and 13. In terms sampling days, the highest concentrations were observed on 6 November 2014 (Figure 24). Other days with high concentrations were 3 October 2014 Figure 23), 30 October 2014 (Figure 23), 10 December 2014 (Figure 25), 2 February 2015 (Figure 27) and 11 February 2015 (Figure 27). According to these concentrations, assuming that the warm periods also constitute a representative number of samples, it can be concluded that cold periods in the fall and winter display the highest concentrations. Comparison of these concentrations with others measured in the past show that some of the present levels were significant. For example, in the case of trichloroethylene concentrations measured in October 1992 ranged from 0.5-2 $\mu g/m^3$ in site 3, 0.2-2.5 $\mu g/m^3$ in site 4 and 1.2-3.6 $\mu g/m^3$ in site 7. Between the months of November 1996 and October 1997 they varied between not detected (nd) and 2.5 $\mu g/m^3$ in site 3, nd and 2.7 $\mu g/m^3$ in site 4, nd and 10 $\mu g/m^3$ in site 7, nd and 4.6 $\mu g/m^3$ in site 1. In this last period of study concentrations in the range of 0 to 140 $\mu g/m^3$ were found.

Concerning tetrachloroethylene, in October 1992 the concentrations were 3.3-7 μg/m^3 in site 3, 2.2-7.5 μg/m^3 in site 4, 4.9-10.4 μg/m^3 in site 7. Between November 1996 and October 1997, they were 1.5-20 μg/m^3 in site 3, nd-6.6 μg/m^3 in site 4, 1.2-52 μg/m^3 in site 7 and nd-18 μg/m^3 in site 1. In this last sampling period the concentrations were in the interval of 0.1-100 μg/m^3.

The present concentrations of hexachlorobutadiene were found between under quantification limit and 1.8 μg/m^3 (Figure 18 - Figure 27). In most samples this compound was found under quantification limit (0.07 μg/m^3). The sample showing 1.8 μg/m^3 was located in site 2 (2 October 2013). This compound was found above 0.07 μg/m^3 in several sites, such as 3 (0.16 μg/m^3, 16 October 2013; 0.23 μg/m^3, 3 October 2014; 0.42 μg/m^3, 6 November 2014; 0.22 μg/m^3, 10 December 2014; 0.07 μg/m^3, 29 December 2014; 0.13 μg/m^3, 11 February 2015; 0.26 μg/m^3, 25 February 2015), 4 (0.08 μg/m^3, 16 October 2013; 0.14 μg/m^3, 8 October 2014; 0.24 μg/m^3, 16 October 2014; 0.22 μg/m^3, 11 November 2014), 6 (0.21 μg/m^3, 16 October 2013; 0.2 μg/m^3, 17 October 2013), 7 (0.5 μg/m^3, 16 October 2013), 13 (0.40 μg/m^3, 11 February 2015), 9 (0.14 μg/m^3, 17 Septembre 2014), 11 (0.32 μg/m^3, 24 Septembre 2014), 8 (0.43 μg/m^3, 8 November 2013), 1 (0.26 μg/m^3, 30 October 2014) and 2 (0.1 μg/m^3, 8 November 2013).

In October 1992, the concentrations of this compound ranged between nd-0.3 μg/m^3 in site 3, 0.1-0.8 μg/m^3 in site 4, 0.2-1.6 μg/m^3 in site 7. In the period between November 1996 and October 1997 the concentrations of these compounds changed between nd-11 μg/m^3 in site 3, nd-5.6 μg/m^3 in site 4, nd-16 μg/m^3 in site 7 and nd-4.9 μg/m^3 in site 1. In some cases they were higher than in the recent period.

The HCB concentrations (Figure 47 - Figure 32) showed the highest levels in an air sample collected in site 2 on 2 October 2013 (130 ng/m^3), together with the hexachlorobutadiene maximum. This compound was found above the 2 ng/m^3 threshold in the air samples collected in site 3 (5.8 ng/m^3, 2 October 2013; 14 ng/m^3, 16 October 2013; 9.4 ng/m^3, 6 November 2014; 3.4 ng/m^3, 10 December 2014; 2 ng/m^3, 16 December 2014; 0.7 ng/m^3, 23 December 2014; 2.4 ng/m^3, 29 December 2014), a Ribarroja d`Ebre (3.2 ng/m^3, 2 October 2013), also in sites 2 (5.5-13 ng/m^3, 16 October 2013; 4.4 ng/m^3, 17 October 2013; 28 ng/m^3, 8 November 2013), 6 (27 ng/m^3, 16 October 2013; 53 ng/m^3, 17 October 2013), 7 (41 ng/m^3, 16 October 2013; 3 ng/m^3, 8 November 2013), 4 (5.6 ng/m^3, 16 October 2013), 8 (100 ng/m^3, 8 November 2013) and 1 (17 ng/m^3, 30 October 2014).

In the period between July 1989 and October 1992 the concentrations of this compound ranged between 11-44 ng/m^3, and in the period between November 1996 and October 1997 the HCB concentrations ranged between 6-255 ng/m^3 in site 3, nd-64 ng/m^3 in site 4, 62-1200 ng/m^3 in site 7, nd-130 ng/m^3 in site 1. Thus, the concentrations in this last period were not low but they were much lower than those in previous dates.

In this work only the acute toxicity due to air concentrations are considered. In all cases only the concentrations corresponding to inhlalation are considered.

Carbon tetrachloride

In this solvent, the level for diseases not related to cancer is estimated at 100 μg/m^3. The threshold of one cancer per million people throughout life (70 years) is 0.17 μg/m^3. This compound is possibly carcinogenic to humans, Group 2B by IARC (IARC, 2013b) and Group B2. USEPA defines this compound as probably carcinogenic to humans. The WHO sets a reference value or tolerable concentration for air quality of 6.1 μg/m^3 (WHO, 2000).

Trichloroethylene

This compound is classified as Grup 1 (IARC Working Group on the Evaluation of Carcinogenic Risk to Humans, 2014) and in range A (IRIS, 2011) as human carcinogen. WHO sets 2.3 μg/m^3 as air threshold for the risk of development of one cancer in the lifetime per million of individuals (WHO, 2010). USEPA sets a reference value for

inhalation, RfC, of 2 μg/m^3. Concentrations below this threshold probably do not represents any health risk for the lifetime (IRIS, 2011). The reference value for neurotoxic effects is 500 μg/m^3.

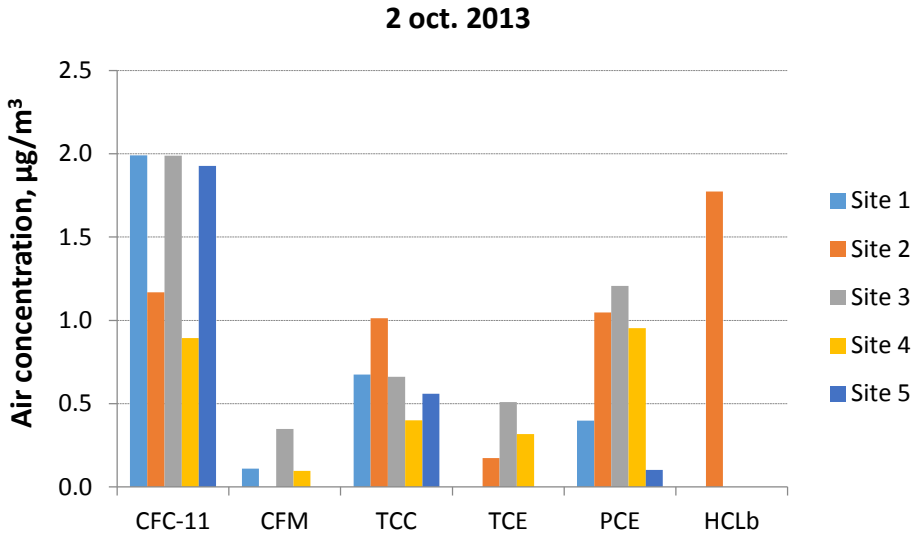


Figure 18

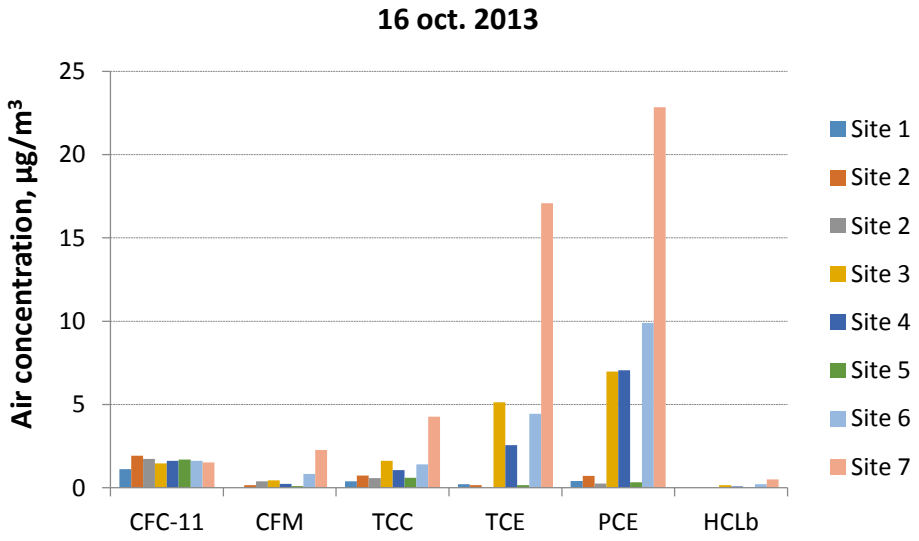


Figure 19

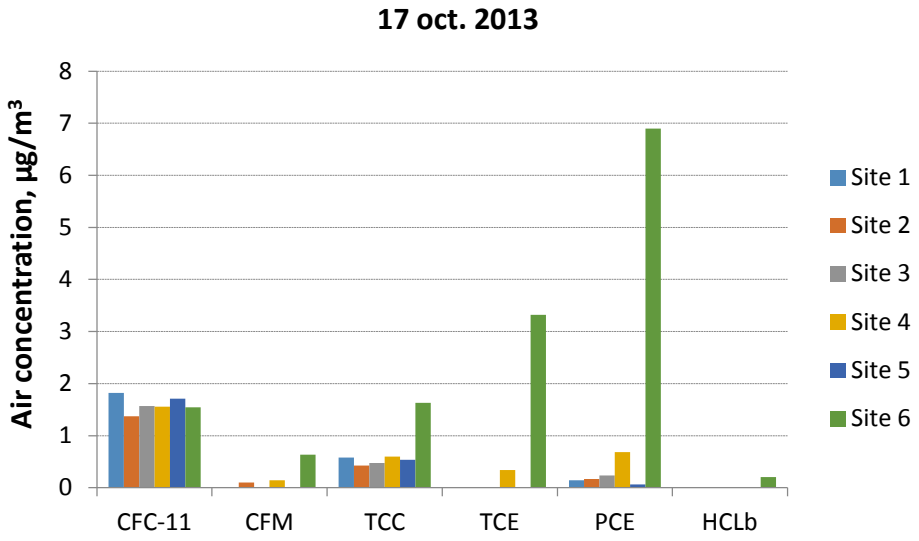
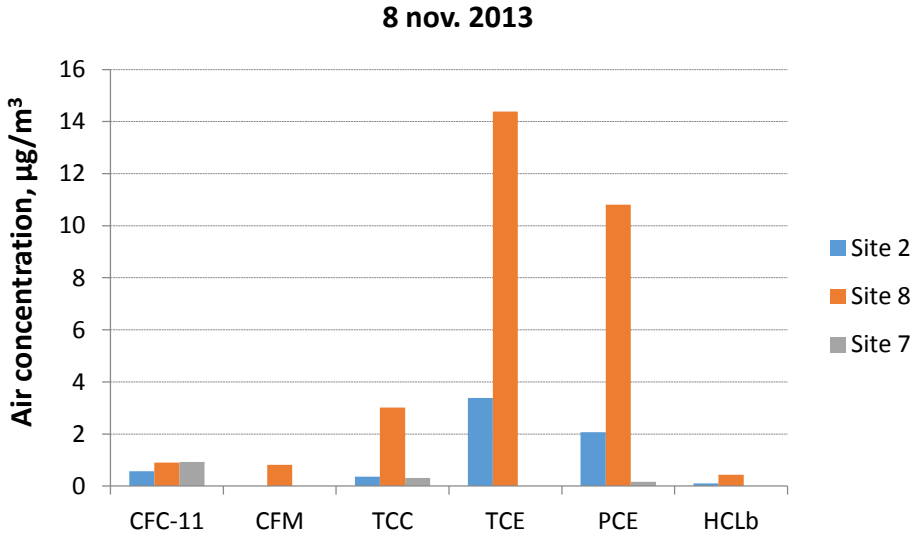


Figure 20



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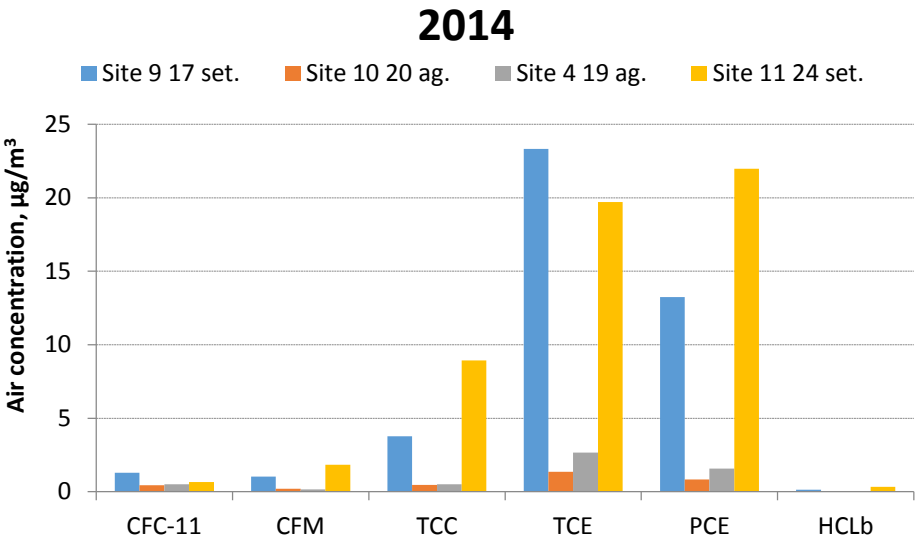

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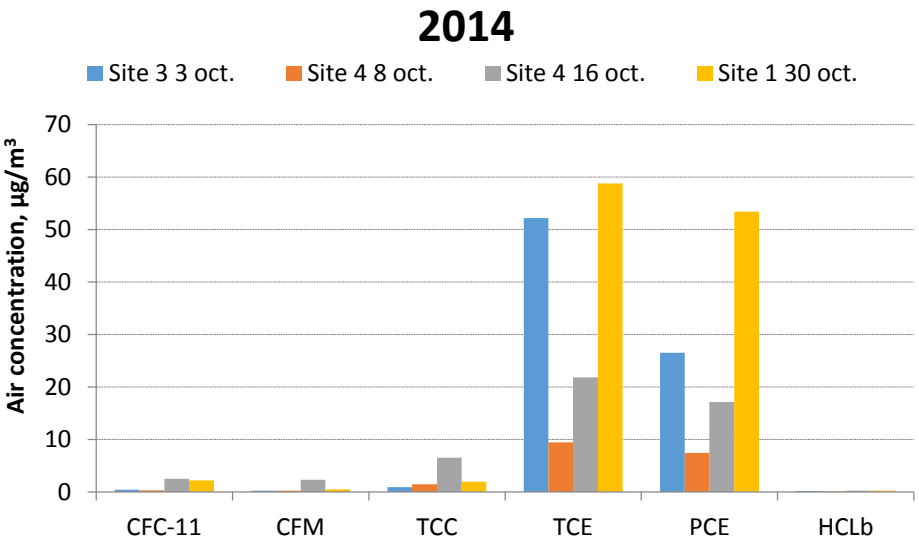

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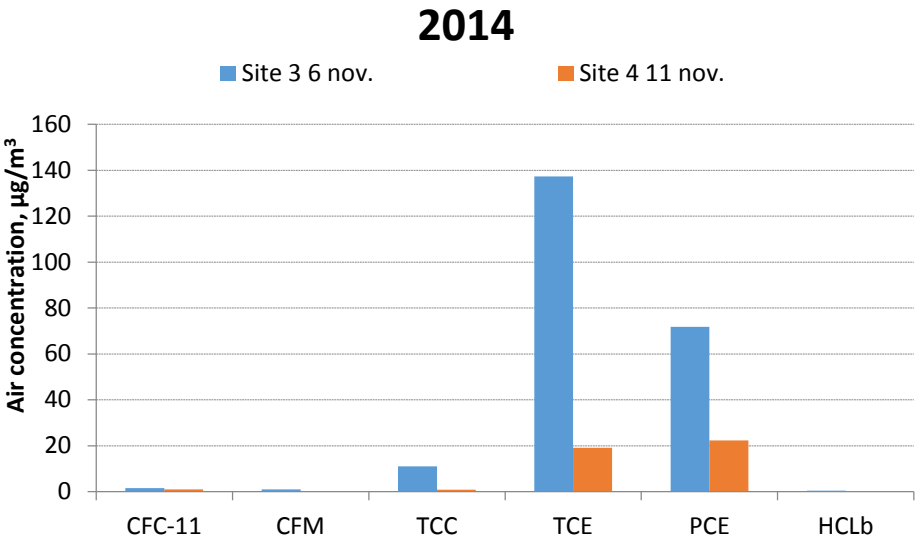

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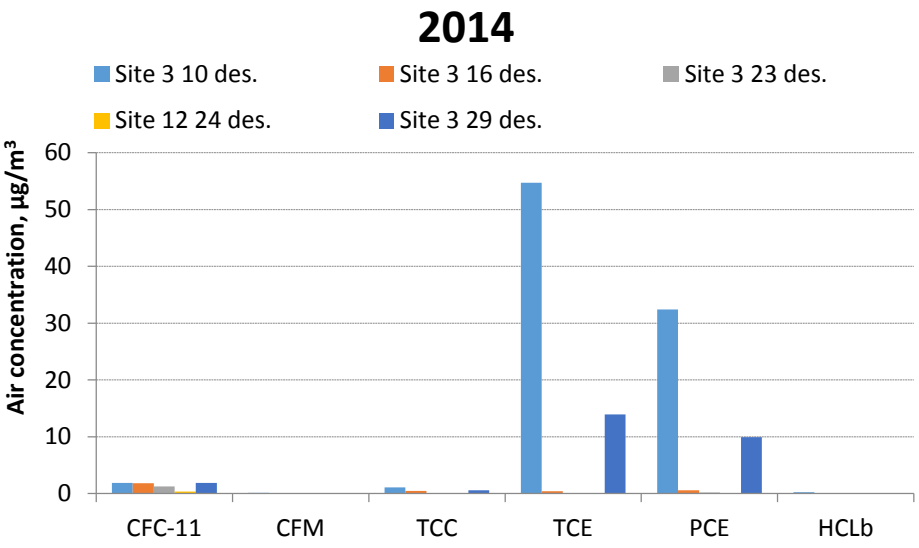

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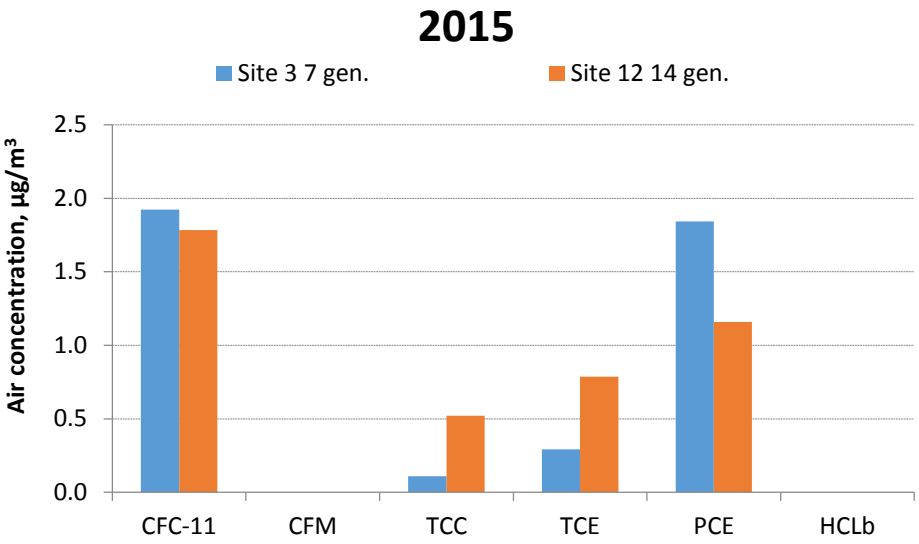

Figure 26

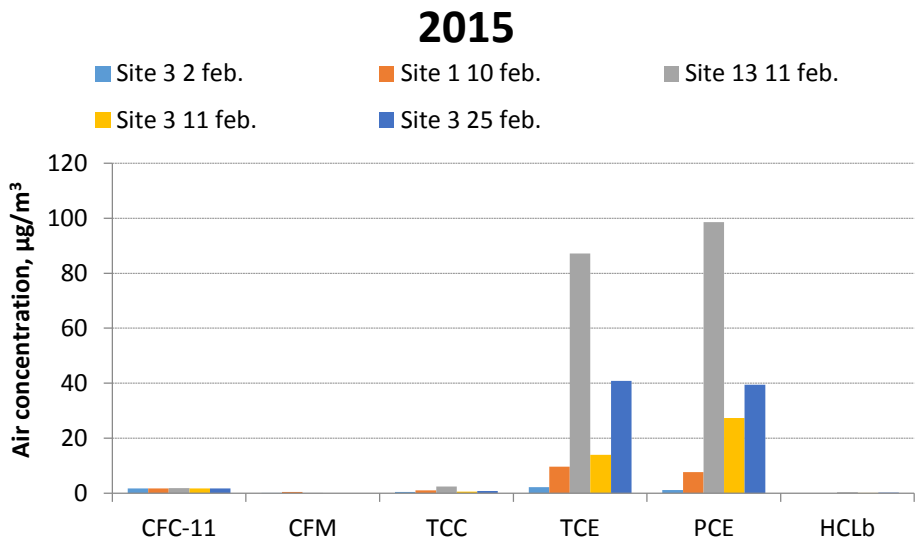

Figure 27

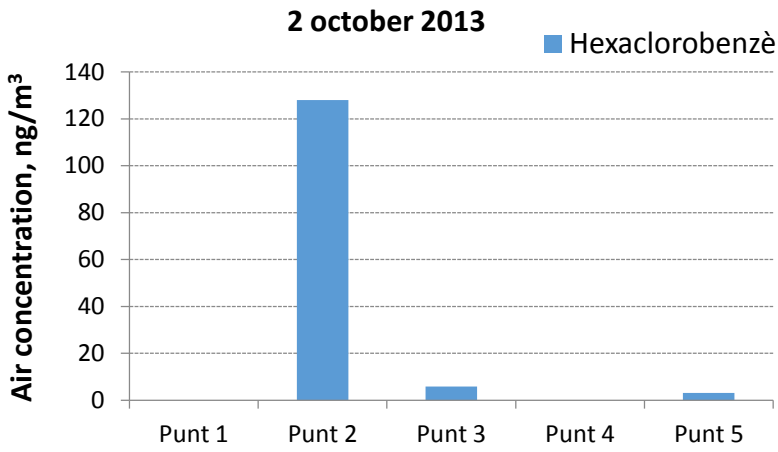

Figure 28

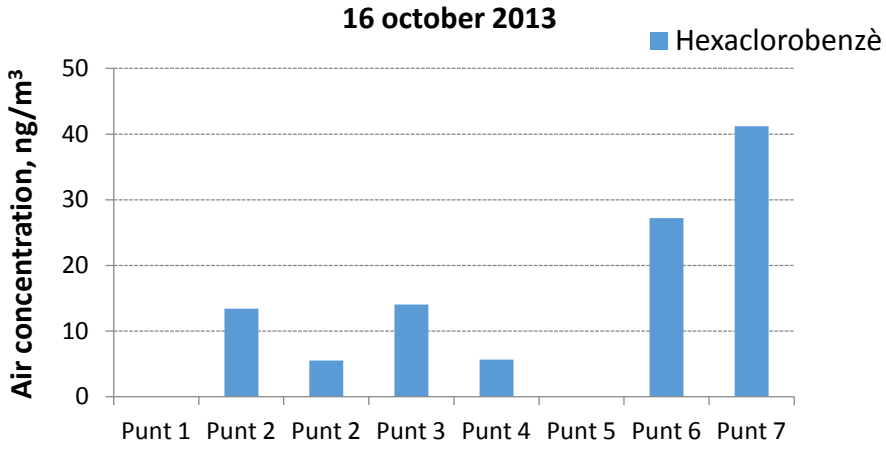

Figure 29

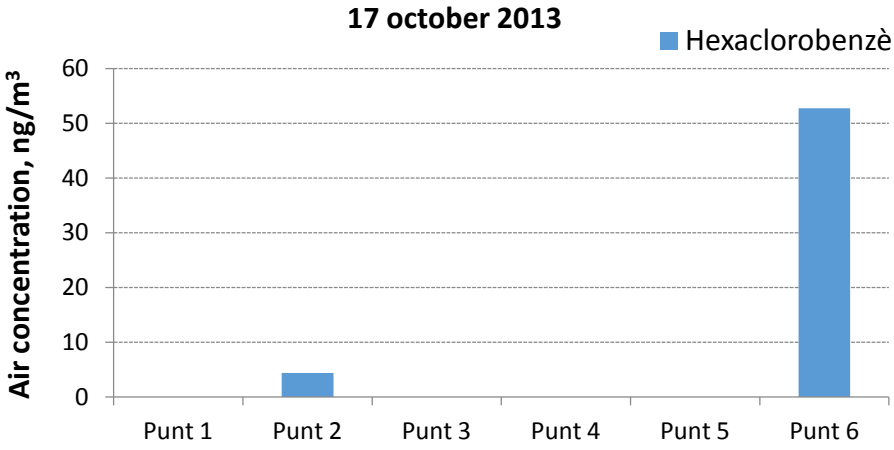

Figure 30

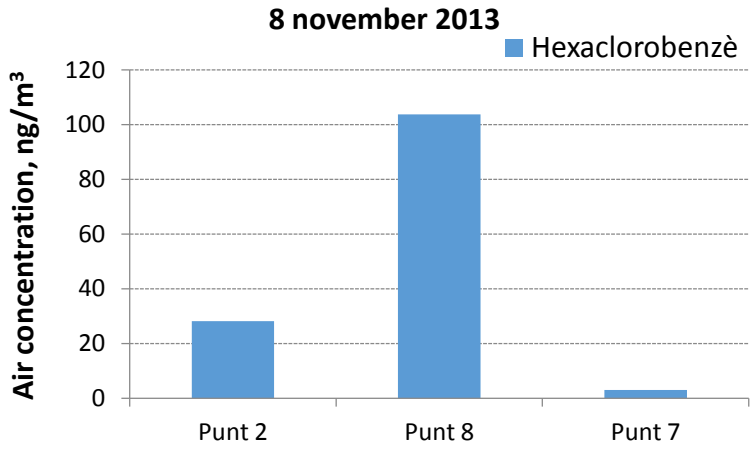

Figure 31

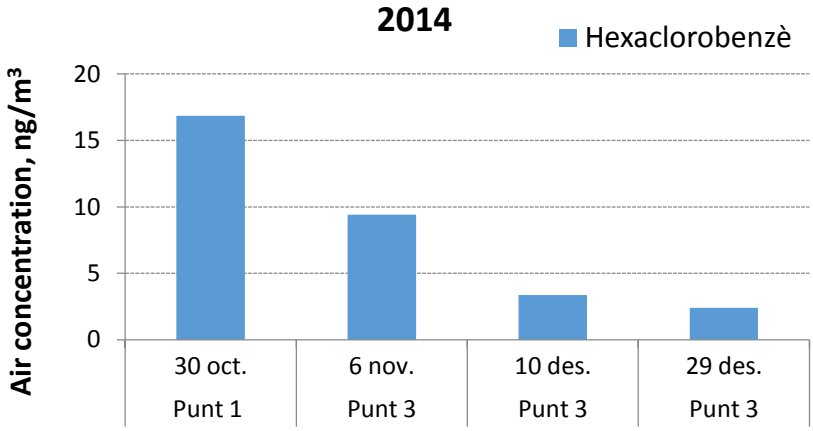

Figure 32

Tetrachloroethylene

This compound is probably carcinogen in humans, Grup 2A (IARC, 2013a) and Grup B1 (USEPA IRIS, 2012). Accoding to USEPA, the reference threshold for chronic inhalation (RfC) of tetrachloroethylene is 40 μg/m^3. Under this value no neurotòxic effects are observed (USEPA IRIS, 2012). The air quality guide of WHO sets an anual average concentration of 250 μg/m^3 for the general health risks (WHO, 2010). The toxicity threshold for cancer is 4 μg/m^3. Consistently with this threshold 40 μg/m^3 correspond to one cancer every 100000 inhabitants.

Hexachlorobutadiene

The estimated threshold for one càncer through lifetime is 0.05 μg/m^3 (USEPA IRIS, 1987). No reference level is established for chronic inhalation by USEPA IRIS and no reference guide for air quality is available from the WHO. IARC classifies this compound in Grup 3 as non carcinogen in humans (IARC, 1987) and USEPA IRIS in Grup C, as possible carcinogen (IARC, 1987).

Hexachlorobenzene

No reference concentration for chronic inhalation is indicated by USEPA IRIS and WHO. The threshold for one cancer every million people is 0.002 μg/m^3 (EPA, 2000). This compound is classified as Grup 2B as possible carcinogen in humans (IARC, 2015).

Risk assessment

As the above mentioned threshold values correspond to chronic exposure, the median concentrations of the analytical determinations for the period 2013-15 have been used for the assessment of the toxicity risks.

Comparison of these median values with the measurements in 1996-97 is performed in Table 38. The median vàlues of carbon tetrachloride and HCB in this previous period, 2.2 μg/m^3 and 0.045 μg/m^3, respectively, are higher than those in 2013-15, 0.63 μg/m^3 and 0.002 μg/m^3, respectively. In contrast, the medians of perchloroethylene and hexachlorobutadiene are aproximately the same in both periods (Table 38). Trichloroethylene has higher medians in this last period, 2.4 μg/m^3, than in the 1996-97 period, 0.37 μg/m^3.

Given that these toxicology reference values correspond to toxic exposure, a period of 8 h has been assumed for life in the exterior for the total daily time of 24 h. These calculated concentrations of exposure are compared in Table 4 with the toxicity thresholds.

Table 38. Median concentrations observed in all samples collected in 2013-15 compared with the concentrations measured in the same sites in 1992, in the period 1996-97 and the toxicity thresholds

Compound	Median 2013-15	Exposure concentration	Median 1992	Median 1996-97	Threshold for non carcinogenic effects (Rfc)	Threshold for carcinogenic effects [b]
Trichloroethylene	2.4[a]	0.8	1.5	0.37	2	0.2
Tetrachloroethylene	1.7	0.6	6.5	1.6	40	3.3
Carbon tetrachloride	0.63	0.21	6.7	2.2	100	0.17
Hexachlorobutadiene	<LD	<LD	0.3	<LD	-[c]	0.05
Hexachlorobenzene	0.006	0.0007	0.002	0.045	-	0.002

[a]Units in μg/m^3. [b]One cancer in one million of individuals through lifetime (70 years). [c]Not reported.

The reference toxicicty values published on 12 September 2014 by EPA IRIS (www.epa.gov/iris) have been taken as reference.

In all cases the median concentrations measured in Flix are lower than the EPA IRIS reference values for non carcinogen risk (trichloroethylene, perchloroethylene and carbon tetrachloride) (Table 38). In the absence of equivalent reference values for hexachlorobutadiene and HCB the reference toxicology thresholds for carcinogenic risk may be considered. These thresholds are generally lower than those of non carcinogenic effects (Table 38). The median exposure concentrations of these two compounds are also lower than these reference values.

Concening to carcinogenic threshold concentrations, perchloroethylene, HCB and hexachlorobutadiene show median concentrations that are lower tan the level of 1/1000000 whereas those of trichloroethylene and carbon tetrachloride show a threshold below 1/100000.

Hexachlorobenzene exposure and internal dose

The HCB air levels in the community studied are described in Table 39. These samples encompass data from the cold and warm seasons. The values reported in this Table correspond to site 4, about 100 m higher and 1 km away from the factory. The differences in HCB concentration between samples corresponding to short (1 to 2 hr) and long (approx. 24 hr) periods were not significant. The average value, 35 ng/m^3, was more than 100 times higher than those found in the control city. The levels of polychlorobiphenyls (PCBs) were in the order of 1 ng/m^3 in both areas. Total dioxin equivalents in the community under study were below 1 pg/m^3

The levels of HCB in sera were much higher in inhabitants from the community under study (mean 26 μg/L; range 7.5 to 69) than in the reference population (4.8, 1.5 to 15) even after age, sex and occupation had been taken into account ($p < 0.001$). The subjects from the community under study who had never worked in the chemical factory showed an average HCB level of 22 μg/L. The 9 males

showed a higher level (mean 32 μg/L; range 19 to 69) than the 12 female (mean 22 μg/L; range 7.5 to 34). Neither time of residence in the town (the minimum registered time among the 8 subjects not born in this place was 27 years and the average time of residence was 46 years) nor working in agriculture were associated with HCB levels. pp'DDE was also found in the sera samples of both communities. The average concentration in the community under study (12 μg/L) was much higher than in the reference group (1.6 μg/L), even after adjusting for age and sex ($p < 0.001$). No other organochlorinated compounds were detected in sera of both populations.

Table 39. Average 24-hr air levels (in ng/m3) of organochlorinated compounds in the study and the reference communities

Compound	Study community		Reference community	
	average	range	average	range
HCB	35	11-44	0.3	0.25-0.4
PCBs	1	0.1-2	0.7	0.1-1
4,4`-DDE	0.2	0.02-0.5	0.1	0.06-0.3

Mortality for all causes was lower than expected (age and sex-standardized mortality ratio, 0.89, 0.78 to 1.00; number of observed deaths, 300) as well as mortality for all cancers (0.78, 0.60 to 1.00; n, 67). A significant increase for specific causes of death was obtained only for neoplasms of unknown primary origin (2.9, 0.95 to 5.0; n, 13).

Table 40 shows the number of primary incident cases, the age standardized incidence ratio and its 95% confidence interval. Thyroid neoplasms, soft-tissue sarcomas and brain neoplasms showed a higher incidence in males than expected. Females did not show such excess. The highest incidence-rate ratio was found for thyroid (6.7, 1.6 to 28; n = 2), with a male/female ratio of 3.25. This ratio is in contrast with the incidence data from the reference area, where it was 0.36. Soft-tissue sarcomas presented the second highest ratio (5.5, 1.7 to 17.5; n = 3). The brain neoplasm excess (2.7, 0.99 to 7.2; n = 4) was near the limit of the statistical significance. Neoplasms of unknown primary site showed a statistically significant ratio in males (2.35, 1.25 to 4.4; n = 10). Although the review of the corresponding clinical records did not allow their reclassification, the probability is very low that any of these cases were primarily of the thyroid, the brain or even of soft tissue. Rectum and bladder neoplasms showed a small excess in males, not statistically significant.

A specific case of pollution for atmospheric emissions of HCB is observed in the community under study. The airborne HCB levels are generally 1,000 times higher than the levels reported in other urban locations. The HCB measurements indicated in Table 39 correspond to situations in which total chlorine concentrations in the atmo-sphere of the village were moderate, mean 30 μg/m^3, and, in one case, a maximum 24-hr average of 64 μg/m^3 was observed. However, the records of these total chlorine concentrations in previous years, show higher monthly average values, and, particularly, in many occasions (about 20% of the measurement days) the 24-hr average concentrations were higher than 100 μg/m^3. This difference suggests that the atmospheric HCB levels in the past were probably higher than the concentrations reported in Table 39.

This excess is consistent with the high HCB concentrations found in sera of inhabitants from this community, which are higher than the levels in other communities in Spain (Camps et al., 1989) or other countries (Greve and Van Zoonen, 1990).

Table 40. Cancer incidence by detailed cause and sex in the community under study

		Male		Female
Cause (ICD-9)[1]	Number[2]	SIR[3] (95% CI)	Number[2]	SIR[3] (95% CI)
Thyroid (193)	2	6.7 (1.6-28)	1	1.0 (0.14-7.4)
Soft-tissue (171)	3	5.5 (1.7-17.5)	1	2.2 (0.3-16)
Brain (191)	4	2.7 (0.90-7.2)	1	0.93 (0.13-6.7)
Unknown (199)	10	2.35 (1.25-4.4)	4	1.2 (0.45-3.2)

Bladder (188)	12	1.3 (0.75-2.3)	2	1.1 (0.28-4.6)
Breast (174)	0		19	1.3 (0.84-2.1)
Rectum (154)	6	1.3 (0.60-3.0)	2	0.60 (0.15-2.4)
Colo-rectum (153-4)	10	1.0 (0.54-1.9)	7	0.82 (0.39-1.7)
Colon (153)	4	0.74 (0.28-2.0)	5	0.96 (0.40-2.3)
Lung (162)	11	0.76 (0.42-1.4)	2	1.6 (0.39-1.7)
Lymphomas (196)	1[4]	0.56 (0.08-4.02)	1	0.67 (0.09-4.8)
Liver (155)	0	-	1	0.82 (0.11-5.8)
Total (140-208)	74	0.96 (0.76-1.2)	55	0.93 (0.72-1.2)

[1]ICD-9, international classification of diseases code, 9th revision. [2]Obs, number of observed cases. [3]SJR, age-standardized incidence ratio. [4]Lymphoma of small intestine.

This HCB pollution probably represents a cause of chronic human exposure, since it is generated as a sub-product in the synthesis of chlorinated solvents, an activity carried out in the factory during the last decades. Airborne HCB levels in the sixties and the seventies were not measured, partly because no analytical techniques were available to perform these determi-nations. However, accounts from the population consistently indicate that the complaints related to the emissions of organo-chlorine compounds were considerably higher in these previous decades. This information is in agreement with the above-described difference in terms of total chlorine content in the atmosphere and with the fact that many technical improve-ments were introduced in the factory during the eighties, in order to diminish environmental emissions. Thus, themeasure-ments reported in Table 39 probably under-estimate past exposure.

The concentrations of the other airborne organochlorinated compounds were of the same order both in the study and in the reference communities. The total dioxin equivalents in the area of study fulfill the ambient-air quality standard for no significant health effects. The atmospheric pp'DDE concentrations are also of the same order in the study and in the reference towns, which do not correspond with the higher pp'DDE levels in sera of the inhabitants in the community of study. However, these levels are not significantly higher than those observed in other populations. Thus, the annual averages of 90% values between 1978 and 1982 in The Netherlands were 3.9 to 22 μg/L (Greve and Van Zoonen, 1990), whereas in the community of study they were 12 μg/L.

A cluster of unusual cancers such as thyroid and soft-tissue sarcomas and brain cancer has been observed in males from the community under study. The incidence-rate ratios corre-sponding to the villages close to Flix do not show such high values for these cancers. Environmental data in these villages did not show any significant organochlorinated contamination. The small size of the population in the community of study makes it difficult to exclude that the cluster could be the result of chance. However, in the case of soft-tissue sarcoma, the probability of chance to explain such excess is very unlikely, even when both sexes are taken together ($p < 0.01$). In addition, soft-tissue sarcoma has previously been related to exposure to organochlorinated compounds in human populations (Fingerhut et al., 1991; Saracci et al., 1991).

In the case of thyroid cancer, the numbers are very small, but the antecedents of thyroid cancer in animals fed with HCB and in workers exposed to organochlorinated compounds suggest that this excess might be related to environmental exposures. In principle, the hypothesis for an excess of soft-tissue sarcoma and thyroid cancer was defined a priori, based on previous animal and occupational data, and was not due to ex-post facto analysis. This reinforces the likelihood of the cluster resulting from these specific causes.

The male pattern observed could be explained by higher exposure in the workplace, but could also be attributed to sex differences in the excretion of HCB derivatives. However, the occupational histories of all males with cancer (available in about 50% of the clinical records) reveal that all them had worked in the factory. Thus, the observed male-female differences in HCB sera concentrations may be due to this occupational pattern, since data provided by questionnaires showed that no females had worked in the factory, whereas all the males except one had done so.

Brain cancer was a less specific outcome of environmental exposure. However, it has been related with PCBs in studies with human populations (Sinks et al., 1992). PCBs were made in the chemical factory of

the community under study, but they have been found only in small concentrations in the air and water of the village, and were not found in the sera of the inhabitants.

Errors in diagnosis of these thyroid, soft-tissue and brain cancers are unlikely, since the diagnoses were made before the present descriptive study was undertaken and before this specific problem of HCB pollution was known. In addition, the clinical records of the cases under study have been reviewed and the diagnoses confirmed.

Furthermore, changes in the size of the population cannot explain these excesses, since the population (22% younger than 14 years and 14% older than 64 years) has been stable, with a very low rate of migration. In 1986, about 65% of the current inhabitants were born in the village and most of the foreign population (approx. 80%) has residence times of at least 20 years.

Conclusions

The concentrations of the chlorinated volatile organic compounds (VOC) measured in the interval between 2013 and 2015 likely correspond to the extraction of the polluted mud accumulated in the river and the ulterior treatment of these residues. Estimation of the chronic exposure to these compounds from the measurements shows median concentrations of trichloroethylene, perchloroethylene and carbon tetrachloride that are under the threshold values for non carcinogen effects. The median concentrations of hexachlorobutadiene and HCB are also lower than the thresholds for carcinogen risk that have been considered in the absence of recent values for non carcinogen risk from EPA IRIS. Concerning carcinogenic risks, perchloroethylene, HCB and hexachlorobutadiene show median concentrations thresholds below 1/1000000 and those of trichloroethylene and carbon tetrachloride are below 1/100000.

The present findings add new information on the possible relation between exposure to organochlorinated compounds, particularly HCB, and cancer. However, further research is needed for proper assessment of a direct association between HCB and the above-described neoplasms. This example of a general population chronically exposed to high levels of HCB is important, since this chlorinated hydrocarbon is one of the most common organochlorinated products found in human tissues.

Greece

Estimating cancer risk associated to biomass emitted PAHs

Introduction and study design

Several epidemiological studies have shown the adverse health effects of airborne particulate matter deposited in the human respiratory tract (HRT) (Pope and Dockery, 2006). HRT deposition of a particular particle depends on its aerodynamic diameter (d_p). Particulate matter can be divided to coarse particles (d_p>2.5 μm), which are mainly deposited in the upper respiratory system, fine particles (0.1<d_p<2.5 μm), which are deposited in the tracheobronchial region of the human respiratory tract, and ultrafine particles (d_p<0.1 μm) which are deposited in the pulmonary/alveolar region (Lin et al., 2008). As a result, xenobiotics contained in ultrafine particles can be easily translocated in the human body via systemic circulation.

Genotoxic effects of inhaled particulate matter are mainly attributed to adsorbed polycyclic aromatic hydrocarbons (PAHs). PAHs include a variety of semi-volatile organic compounds of low vapor pressure that can be transferred in long distances as they are mostly adsorbed in fine and ultrafine particles (Dvorská et al., 2012; Venkataraman et al., 1994). Such compounds may be retained for long in human tissue due to their high lipophilicity. About 90% of PAHs are emitted by vehicles (Nielsen, 1996). Other sources include industry, biomass combustion, coke and tar production, as well as tobacco smoke (Freeman and Cattell, 1990; Masclet et al., 1987). Including benzo[a]pyrene (B[a]P), the only PAH classified as known carcinogen to humans by IARC, the most hazardous PAHs are mainly distributed in the particulate phase (IARC, 2010). After human exposure to particulate-bound PAHs, the compounds are distributed in alveolar (80%) and tracheobronchial region (20%) of the HRT. However, the ultimate

dose of more toxic substances and their carcinogenic metabolites is much greater in the latter region due to the lower rate of diffusion through the bronchial epithelium. Ultimate PAH metabolites may alter the replication and transcription mechanisms of DNA and induce tumors (Armstrong et al., 1994; Boström et al., 2002). Taking as basis the toxicity of benzo[a]pyrene and using Toxic Equivalent Factors (TEFs), it is possible to calculate the overall toxicity (Toxic Equivalent Concentration, TEQ) of the PAHs mixture assuming that the TEF of benzo[a]pyrene is equal to 1 (Nisbet and LaGoy, 1992). The majority of studies related to carcinogenic potential of PAHs (e.g. (Wiriya et al., 2013; Yu et al., 2008) apply the EPA equation or calculate the Incremental Life Cancer Risk (ILCR) without any differentiation between age groups of the individuals exposed. HRT deposition modeling has been applied only by Chiang and Liao (2006 to determine the PM mass lung/indoor ratio after exposure to heavy incense burning in a Taiwanese temple and the PM size distribution in different HRT regions (these results are not linked with PM-bound PAHs), and by (Zhang et al., 2012) to estimate the distribution of PAHs to particles of different diameter and calculating Lifetime Cancer Risk (LCR).

An increasing number of studies has been dedicated to the analysis of PAHs in the ambient air in the last two decades. Concerning Greece, Manoli et al. (2002 and Chrysikou et al. (2009 report concentrations of B[a]P as high as 2.6 and 0.61 ng/m^3 in PM_{10} and $PM_{2.5}$ respectively in Thessaloniki, while in Heraklion a B[a]P concentration of 1.07 ng/m^3 is observed by Tsapakis and Stephanou (2005. Similar findings have been reported for other Mediterranean cities, such as Istanbul (Hanedar et al., 2011), Florence (Martellini et al., 2012) and Algiers (Ladji et al., 2009).

In recent years in Greece, solid biomass combustion is being increasingly used for space heating, resulting in increased levels of PM pollution during the wintertime (Sarigiannis et al., 2014). Biomass burning has been associated to emissions of smaller fractions of PM (Sarigiannis et al., 2014). PAHs of higher molecular weight, hence more toxic, are mostly adsorbed to finer PM (Shen et al., 2013). In this context, analysis of PAHs in an area significantly polluted by PM emitted from biomass burning is of particular interest. This analysis includes the estimation of the toxicity of PM attributed to biomass combustion, juxtaposed to PM emitted from other sources. In order to incorporate the different characteristics of particles emitted from different sources, a refined exposure-risk characterization model is introduced. This takes into account exposure and HRT deposition parameters, focusing on the actual dose deposited on the target tissue, which, for our purposes, is lung epithelium.

This study aimed at identifying the potential cancer risks associated to PM emitted from biomass burning. Towards this aim, several methodological elements have to be integrated, including:

- PM measurements for the three main fractions (PM10. PM2.5 and PM1). This is essential, since smaller PM fractions have been associated to biomass burning. Measurements include two different sampling sites, so as to be able to differentiate the amount and the composition of PM attributed to contribution of different sources.
- PM chemical analysis, aiming at:
    - identifying their carcinogenic potency, through PAHs composition analysis.
    - identifying their origin, through levoglucosan and black carbon (BC) analysis. Levoglucosan is considered as the most specific biomass burning tracer, while BC is considered as product of internal combustion sources.
- Refining the exposure and associated risk assessment methodology. It is known that PM of different size interact differently with the Human Respiratory Tract (HRT). PM from biomass burning has specific characteristics, which have to be taken into account when estimating the associated risk. According to our methodology, this information is incorporated by employing an HRT deposition model. The latter allows us to estimate for a PM fraction of given size the actual amount of PAHs coming in contact with the lower respiratory tract. Using this refined exposure assessment approach, differences in physiology among different age groups/susceptible populations are taken into account in the corresponding cancer risk estimates.

Sample collection and chemical analysis

An extensive campaign was carried out from January to April 2013 at two locations in the urban area of Thessaloniki to determine the chemical composition of urban aerosols and to correlate their toxicity with biomass combustion as a way of residential heating. The urban background site is located in the Ilioupoli district of western Thessaloniki (40º40` N, 22º55` E), a densely populated area where road construction and elevated buildings do not favor pollutants dispersion. Samplers were placed at the roof of a building at a height of approximately 9 m from the ground. No significant traffic sources were close to the site. The traffic site is located in the campus area of the Aristotle University of Thessaloniki, at a balcony of the School of Engineering, Building D (40º37` N, 22º57` E). The site is crossed by the main highway of Thessaloniki, Egnatia Street, at a distance of approximately 50 m and its distance from residential buildings is as far as 500 m. Samplers were placed at a height of 6 m from the ground. For the collection of aerosols, low volume samplers were used (TCR-Tecora). PM1.0, PM2.5 and PM10 samples were collected on PTFE filters (Pall Corporation, 47 mm diameter) for 24 h. Volumetric flow rate of the pump was set at 2.3 m^3/h. Mean ambient temperature was 8.9±3.6 ºC while average relative humidity was 68%. PM mass concentrations were calculated by weighing the filters before and after sampling.

The concentrations of the PAHs were determined according to the following method. Half of each of the 47mm filter was cut and spiked with known amount of surrogate standards (fluorene-d10 and pyrene-d10, Cambridge Isotope Laboratories). The spiked filter part was extracted with 5 ml of dichloromethane (Merck, 99.8%) in an ultrasonic bath for 20 min. Five ml of hexane (Merck 99.9%) were added and the sample was filtered through a 0.2 mm PTFE filter (Membrane Solutions) using a 20 mL syringe into a conical flask vial. Then, it was evaporated under a gentle stream of nitrogen to 0.5 ml. Two ml of hexane and known amount of deuterated internal standards (acenaphthene-d10, phenanthrene-d10, chrysene-d12, perylene-d12, Cambridge Isotope Laboratories) were added before a final stage of evaporation to 0.5 ml. Samples were stored in 2 ml vials at -20ºC.

Analysis was performed by a 7890A Agilent gas chromatographer coupled with a 5975C Agilent inert MSD mass spectrometer operated in the SIM mode. Two μl of each sample were injected into the GC in splitless mode where the inlet temperature was kept at 280 ºC. A fused silica capillary column (30 m 250 μm 0.25 μm i.d., HP-5MS Agilent) was used for the separation of the fifteen PAHs with helium as carrier gas (flow of 1 ml/min). The GC oven temperature was 60 ºC for 1 min, increased with a rate of 10 ºC /min to 120 ºC, then increased with a rate of 5 ºC /min to 240 ºC and then increased to 300 ºC (rate of 6 ºC /min) and held for 20 min. Total run time was 60 min. For the estimation of the calibration curves, the internal standard calibration method was used. The analysed PAHs include fluorene (Flu), phenanthrene (Phe), anthracene (Ant), fluoranthene (Fla), pyrene (Pyr), benzo[a]anthracene (BaA), chrysene (Chr), benzo[b]fluoranthene (BbF), benzo[k]fluoranthene (BkF), benzo[e]pyrene (BeP), benzo[a]pyrene (BaP), perylene (Per), indeno[1,2,3-cd]pyrene (Ind), dibenzo[a,h]anthracene (DbA) and benzo[g,h,i]perylene (BgP). For the quality assurance and control, laboratory blanks and field blanks were extracted and analyzed in the same way as the samples. The method detection limit (MDL) was estimated as three times the standard deviation of the mean blank concentration. In case of PAHs not detected in blanks, the MDL was calculated as three times the instrumental detection limit (IDL). The method was validated by analyzing a standard reference material of urban particulate matter (NIST, SRM 1649b). The results obtained were in good agreement with certified values with PAH recoveries above 80%.

Levoglucosan content was determined according to the following procedure. A quarter of each 47mm filter was cut and spiked with a known amount of a surrogate standard (sedoheptulosan, Chem. Service). The spiked filter parts were extracted for 40 min with 10 ml of dichloromethane:methanol 2:1 (Merck 99.9%) in an ultrasonic bath. The extract was filtered through a 0.2 mm PTFE filter (Membrane Solutions) using a 20 mL syringe into a conical flask vial using a 5 mL syringe through a 0.2 mm teflon filter into a conical flask vial and evaporated until dryness under a gentle stream of nitrogen. Then 400 μL of dichloromethane, 20 μL of pyridine (Sigma-Aldrich) and 100 mL bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (Supelco) were added. The vials were sealed and the reaction was conducted at 70 ºC for 3 h in a heater. Then, the solution was allowed to cool down and a known amount of an internal standard (2-fluorobiphenyl, Sigma-Aldrich) was added prior to gas

chromatography-mass spectrometry (GC-MS) analysis. The latter was performed on a 7890A Agilent gas chromatographer coupled with a 5975C Agilent inert MSD mass spectrometer operated in the SCAN mode. One μl of each sample was injected into the GC in splitless mode where the inlet temperature was kept at 270 ^{o}C. A fused silica capillary column (30 m 250 μm 0.25 μm i.d., HP-5MS Agilent) was used with helium as carrier gas (flow of 1 ml/min). The GC oven started with an initial temperature of 100 ^{o}C, which was held for 2 min and then ramped up to 300 ^{o}C with a temperature increase rate of 20^{o}C /min. For quality assurance and control, laboratory blanks and field blanks were extracted and analyzed in the same way as the samples. The Method Detection Limit was determined as in PAHs analysis.

Particulate matter samples collected on filter media were analysed gravimetrically to determine the mass and subsequently black carbon concentration levels determined by a Magee Scientific SootScan™ Model OT21 Optical Transmissometer (Model OT21, Magee Scientific Company, Berkeley, California). It is possible to measure the optical absorption of the deposit of particles using a wavelength light source operating at 880nm (IR). The 880nm wavelength absorption measurement provides a quantitative measurement of BC. The Model OT21 instrument can be used to measure BC on both freshly collected filters as well as archived samples, BC does not decay or diminish during storage. The results are calculated in units of optical absorption, which can then be used for post processing and further interpretation. The analysis is non-destructive and can be completed in less than 30 seconds

PM levels and correlation to biomass combustion

The levels of the different PM size fractions (as well as main meteorological parameters) are illustrated in Figure 33 and Figure 34 for the urban background and the traffic site respectively.

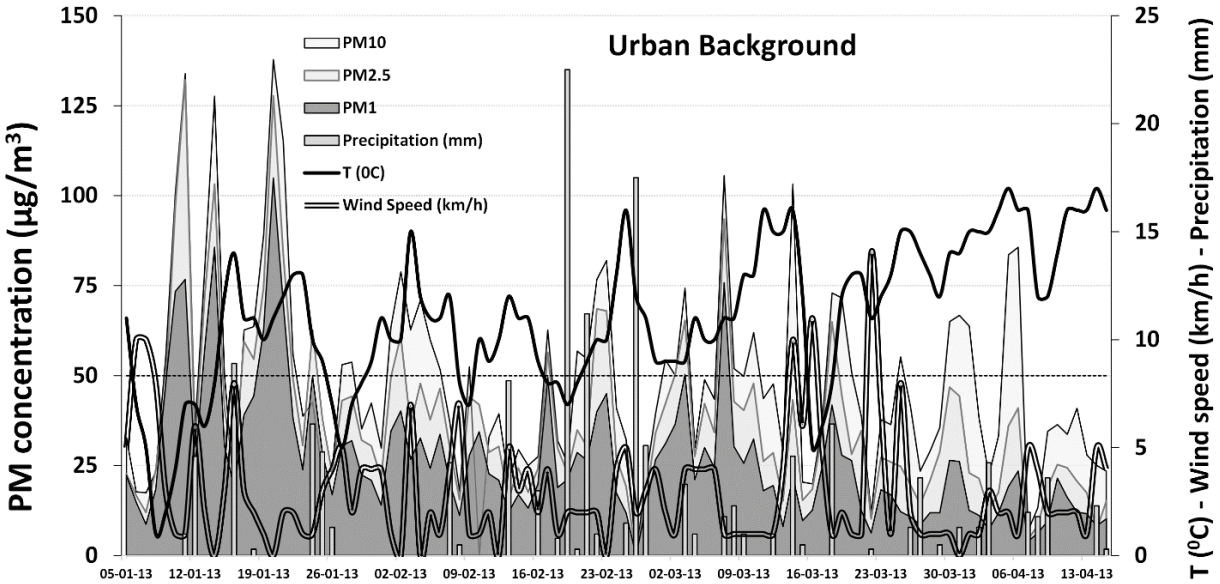

Figure 33. Inter-day variability of the ambient air PM levels at the urban background site

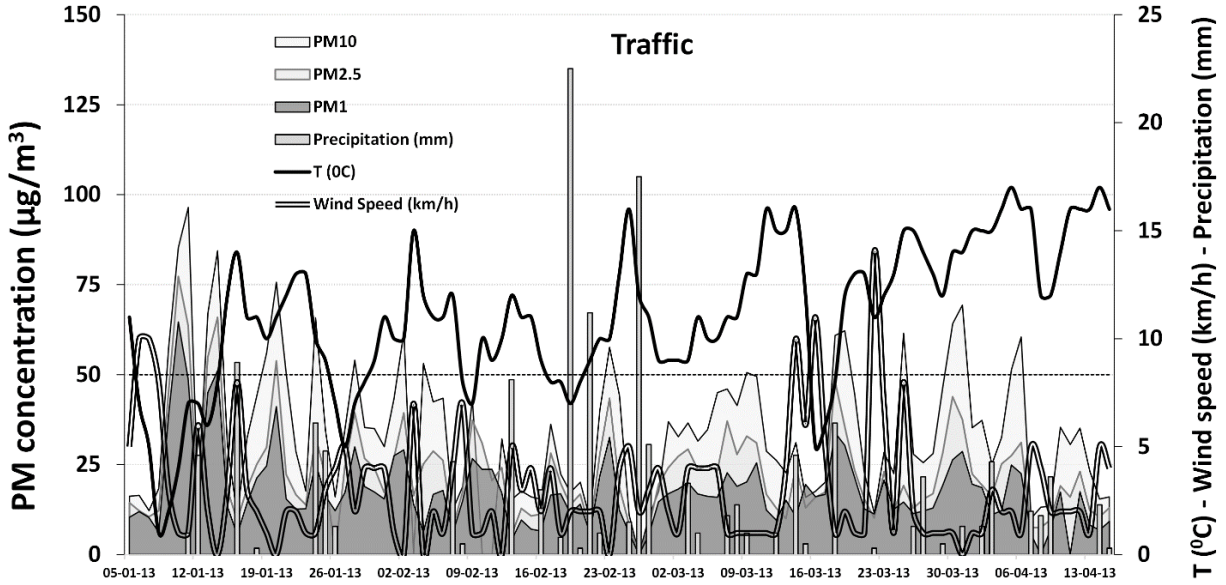

Figure 34. Inter-day variability of the ambient air PM levels at the traffic site

PM10 levels exceed the threshold of 50 µg/m^3 (as a 24 hour mean, not to be exceeded more than 35 times a year) for the majority of the measurements period. The concentrations start to decline after the end of March as result of lower demand for space heating, since ambient temperature starts to increase.

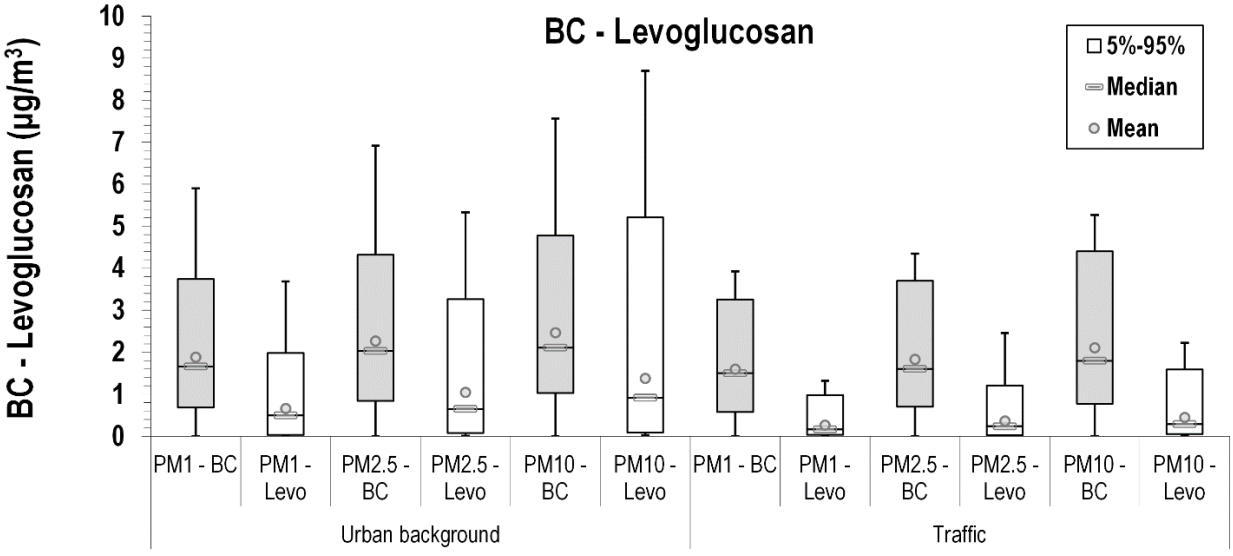

Figure 35. Variability of black carbon and levoglucosan levels for the different size fractions for the two sampling sites

The results of levoglucosan and BC analysis shed light on the relative contribution of the different sources at the two sampling sites; although BC levels (associated to internal combustion sources) did not differ significantly between the two traffic sites, the respective levoglucosan levels were significantly higher at the urban background site (*Figure 35*).

Levoglucosan is considered the most specific tracer of biomass burning (Belis et al., 2013; Perrone et al., 2012; Zhang et al., 2008). The contribution of biomass burning to PM10 levels was determined using the empirical formula proposed by Caseiro et al. (2009), according to which:

Wood smoke PM (in μg/m^3) = levoglucosan (in μg/m^3) 10.7

Based on the formula described above, the respective biomass contribution for the PM10 levels were calculated and the results are given in *Figure 36* and *Figure 37* for the urban background and the traffic site respectively. For the overall period of measurements, biomass burning accounted for an average of 23.1% of the measured PM10, while the respective contribution for the traffic site was almost 10.5%. However, biomass burning contribution during the coldest days, often accounted for about 60% at the urban background site, considered as the dominant source of PM10 in the respective area.

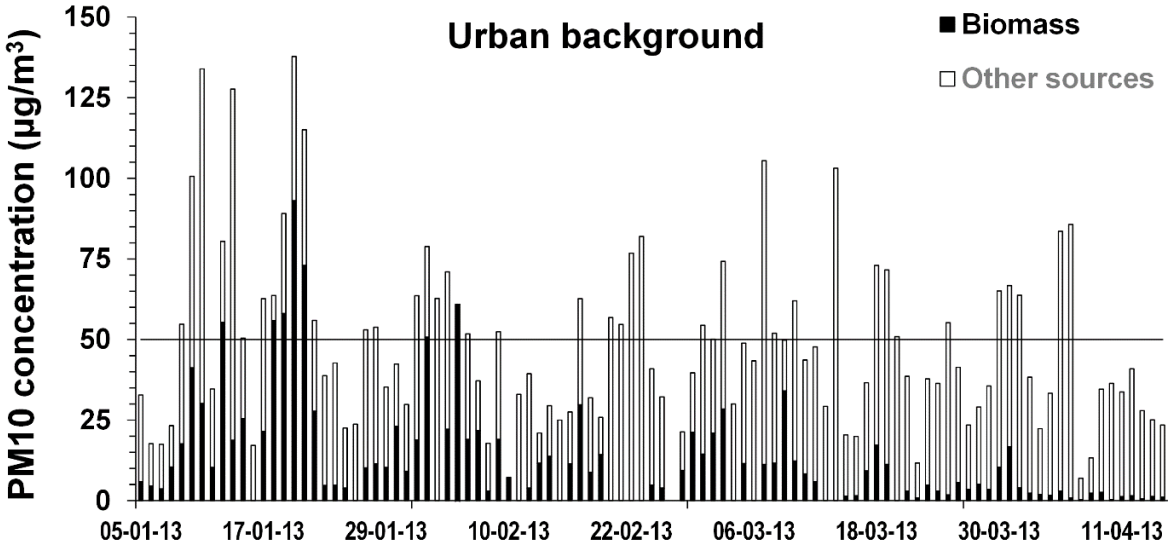

Figure 36. PM10 attributed to biomass burning/other sources for the urban background site

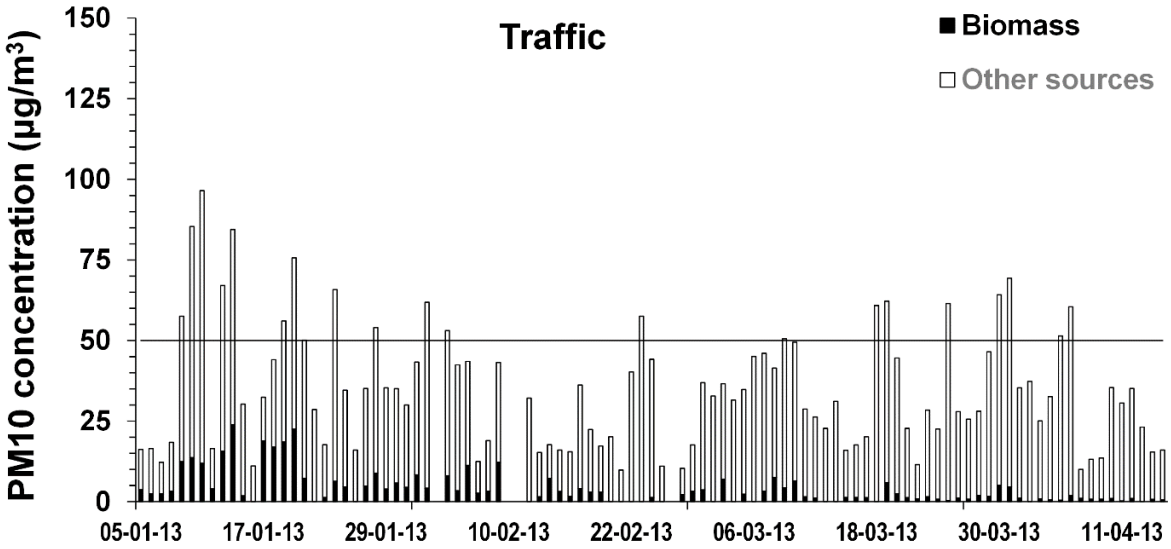

Figure 37. PM10 attributed to biomass burning/other sources for the traffic site

PAH concentrations and TEQ levels

The mean ∑PAHs levels at the urban background site are 14.8, 18.1 and 18.6 ng/m^3 for the PM1.0, PM2.5 and PM10 fraction respectively. At the traffic station, the corresponding levels are 5.3, 7.2 and 7.9 ng/m^3 (*Figure 38*). Therefore, practically, most of the PAHs are adsorbed in fine particles (PM2.5 and finer). At the urban background site mean TEQs are 3.3, 4.3 and 4.5 ng/m^3 for PM1.0, PM2.5 and PM10; the corresponding values at the traffic site are 1.2, 1.5 and 1.7 respectively (Figure 38). The TEQ at the urban background monitoring station is 3 times greater than the equivalent value found at the traffic station. TEQ/PM ratios at the urban background site are 0.091, 0.083 and 0.066 ng/μg PM for PM1, PM2.5 and PM10 respectively. At the traffic site, the respective ratios are 0.045, 0.44 and 0.032 ng/μg PM. HRT deposition.

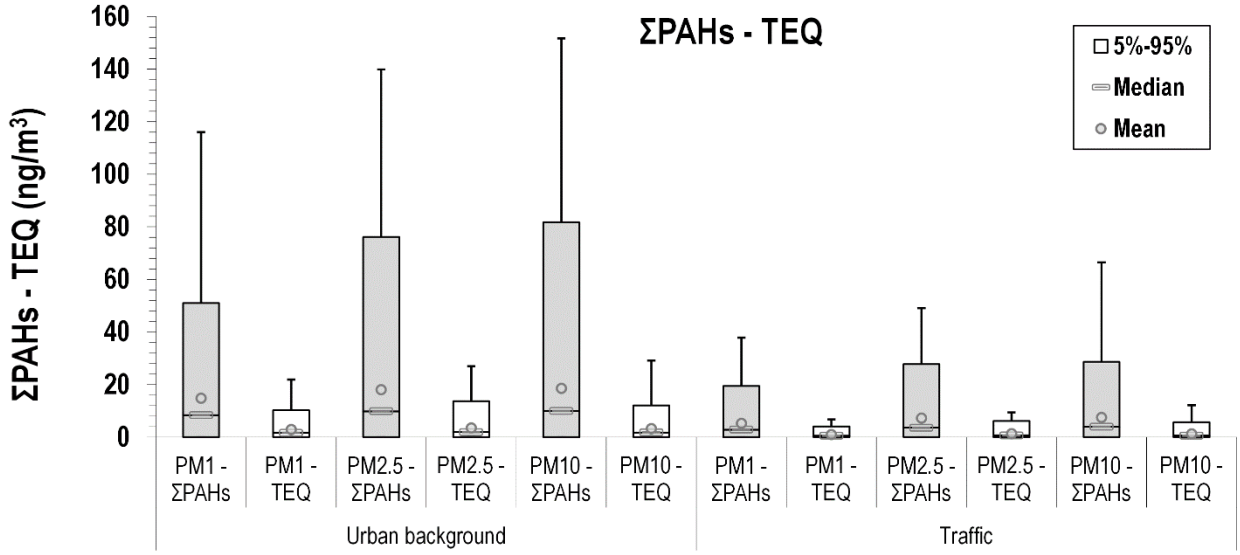

Figure 38. Total PAHs and TEQ per PM size fraction for the two sampling points

At the urban background site mean TEQs are 3.3, 4.3 and 4.5 ng/m^3 for PM1.0, PM2.5 and PM10; the corresponding values at the traffic site are 1.2, 1.5 and 1.7 respectively (Figure 38). The TEQ at the urban background monitoring station is 3 times greater than the equivalent value found at the traffic station. TEQ/PM ratios at the urban background site are 0.091, 0.083 and 0.066 ng/μg PM for PM1, PM2.5 and PM10 respectively. At the traffic site, the respective ratios are 0.045, 0.44 and 0.032 ng/μg PM. HRT deposition.

Different size-distributed particle fractions are deposited across the different HRT regions. This is the result of the different deposition processes occurring in the different regions of the HRT, which are related to the physiology/morphology of the respiratory system and the PM size distribution. This has been verified by a variety of lung deposition computational and experimental efforts (Hofmann, 1996). HRT deposition results indicate that the lower respiratory tract of infants and children (up to 14 years old) can retain up to 74% higher mass fraction of PM1 particles than that of adults (*Figure 39*).

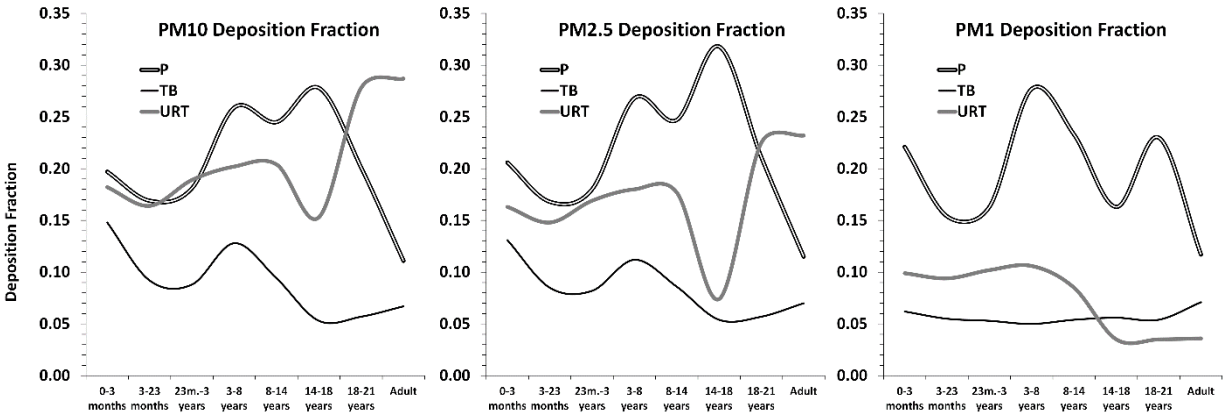

Figure 39. Deposition Fractions (DFs) for the different PM size fractions differentiated by the age of the exposed individuals. P: pulmonary region, TB: tracheobronchial region and URT: upper respiratory tract.

The maximum difference in the thoracic deposition between adults and children (referring to children between 3 and 8 years old) is that of 68% and 230% for the PM2.5-1 and PM10-2.5 fractions respectively. Thus, the PM2.5 and PM1 fractions rather than PM10 contribute to a greater extent to the absorption of PAHs by the respiratory tract in younger individuals than in adults. Although particle deposition in infants and children has been poorly studied (Patterson, 2014) and a high variability between individuals has been observed (Salem, 2005), key factors that influence the thoracic deposition of particles in children are: (a) lower ventilation rates (l/min), which favor deposition by sedimentation, a process that governs the deposition of larger inhalable particle diameters resulting in increased overall particle deposition profiles; and (b) lower FRC volumes (*Table 1*).

Risk assessment

Based on all the methodological elements described above, individual lung cancer risk was estimated for the different age groups. The results are illustrated in Figure 40, where different risk estimates are derived for people living close to the traffic site and for the population living close to the urban background site. The latter experience a higher risk (above 10^{-6}), resulting from the combination of higher PAHs/TEQ levels and higher amount of fine particles deposited at the lower HRT. It has to be noted that lung cancer incidence rate in Greece is 74/100,000 of population for males and 13/100,000 for females respectively.

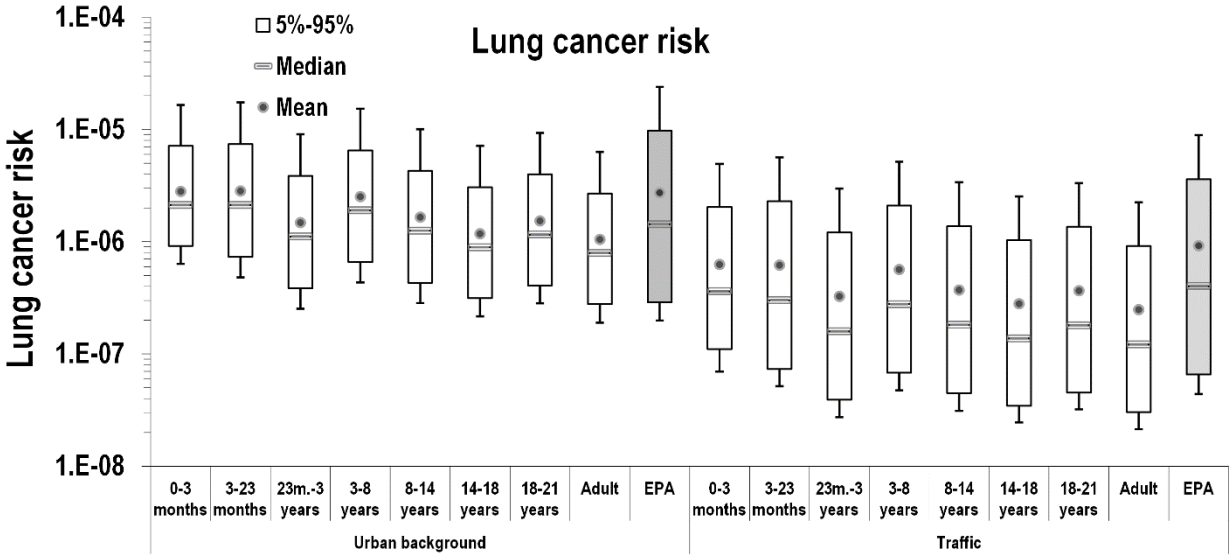

Figure 40. Estimated cancer risk distribution plots

Based on the measured data and the high spatial resolution population data for the city of Thessaloniki obtained from Karteco, high spatial cancer risk estimates were derived. This methodological step aloud us to estimate in more detail the potential health impact associated to lung cancer induced by exposure to PM Figure 41.

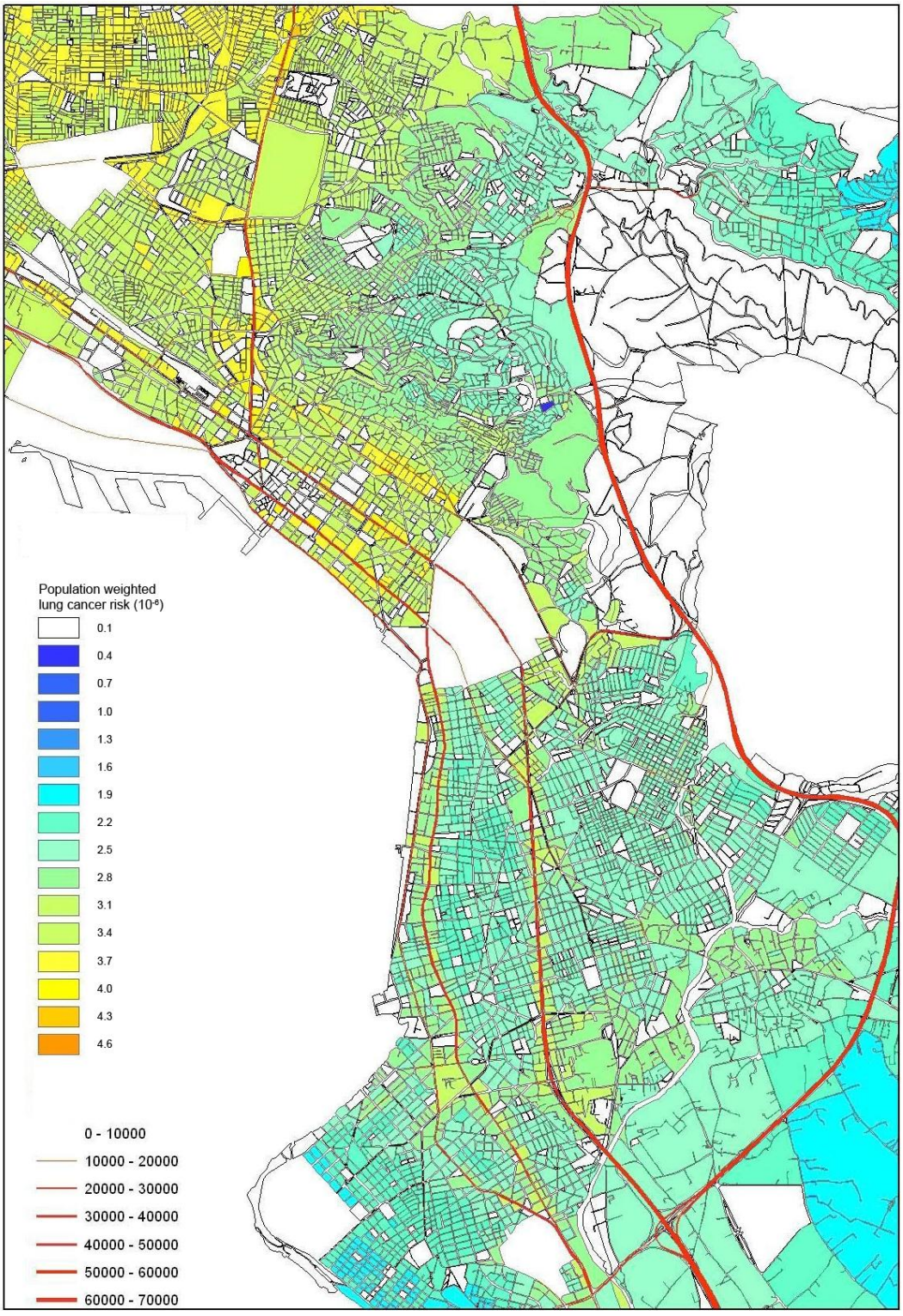

Figure 41. Spatial distribution of population weighted lung cancer risk

Individual lifetime risk is higher for children, due to the higher bodyweight normalised dose of PMs and the amount of PAHs deposited at lower HRT regions. A higher fraction of smaller particles is deposited across the children respiratory tract, resulting in higher PAH burden due to the higher TEQ per PM mass of smaller PMs. In all cases, the methodology proposed by EPA using the Inhalation Unit Risk and the ambient air TEQ concentrations, seems to overestimate the respective cancer risk, without being able to significantly differentiate risk estimates among the different age groups or population groups exposed to PM of different size distribution and PAH content (as identified in the two distinct sampling sites).

Additional health impacts of exposure to biomass emitted PM

Given the importance of the biomass emitted PM in Greece, additional health impacts related to increased mortality and morbidity have been estimated. To estimate the associated health effects of PM exposure, we used the widely established epidemiological concentrations-response functions for outdoor PM, as determined by the HRAPIE team under WHO coordination (2013). For the PM attributed mortality and morbidity (respiratory and cardiovascular hospital admissions) health endpoints used in this study; differences in toxicity depending on PM composition were not taken into account (WHO, 2007). To better capture the marginal change in mortality and morbidity associated to biomass burning originated PM we used fine particles (PM2.5) as the best exposure metric (Sarigiannis et al., 2014). The concentration-response functions (CRFs) for the health endpoints of interest are given in Table 2. The PM10 CRF estimates were converted to estimates of PM2.5 using an established methodology developed in the frame of the HEIMTSA project (IOM, 2011), based on the initial concept described by WHO (2004), since original PM2.5 functions are not available for these endpoints. Relative risk was calculated for the average concentration for the period of interest. Then the attributable fraction was derived, and finally health impact was estimated by multiplying this with the background rate of disease and the respective population of interest. The estimated mortality and morbidity are shown in Figure 42. Almost 170 additional deaths are attributed to PM during the cold period compared to the cold period, although this refers to a period half of duration (4 months compared to 8 months). Similarly, an additional number of 100 respiratory and cardiovascular hospital admissions is expected during the cold period. Among the health endpoints of interest, the highest increase is related to the lung cancer estimated risk; although the overall incidence rate remains low (lifetime individual risk around 10^{-6}), this was actually increased up to almost 6 times compared to the warm period.

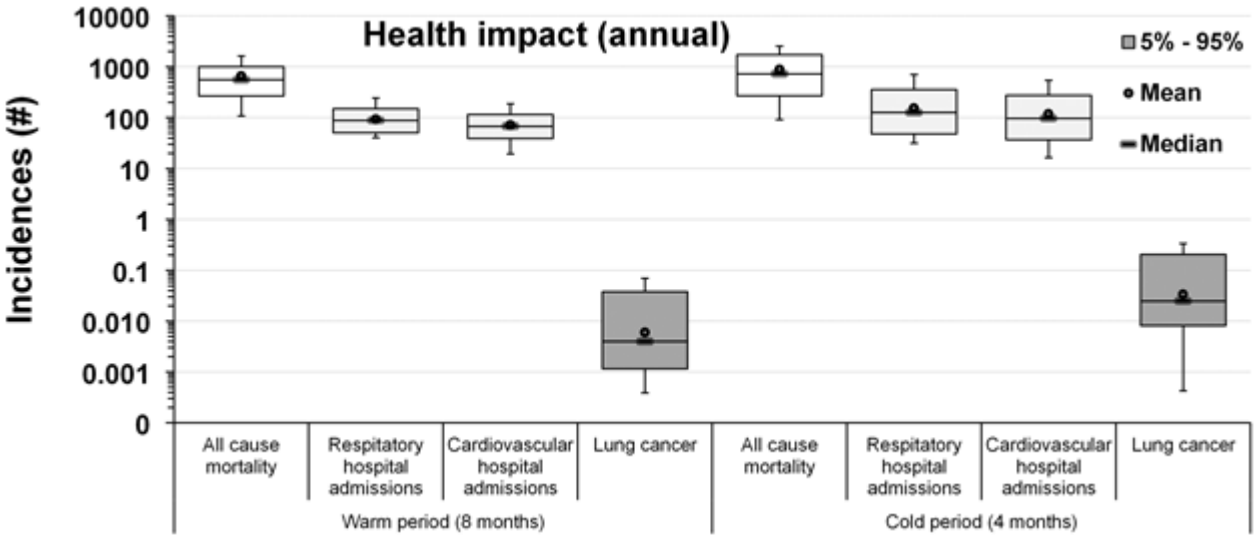

Figure 42. Annual health impact assessment in the city of Thessaloniki

Cancer risk from exposure to dioxins and furans after accidental fire in an urban waste recycling facility

Introduction

Waste recycling is one of the main cornerstones of the EU waste management strategy (Farmer et al., 2015). It offers many advantages contributing to the circular economy and the sustainable and efficient use of natural and man-made resources. However, waste recycling facilities may be associated to adverse health outcomes in the aftermath of industrial accidents involving the inadvertent generation of toxic chemicals and their release into the environment. One of the major concerns associated to accident in plastic recycling plants are the emissions of dioxins and furans (PCDDs/PCDFs). These compounds are characterized by a high carcinogenic potency (Cole et al., 2003). Because PCDDs/PCDFs appears to be acting like a potent and persistent hormone agonist, it appears reasonable to incorporate mechanistic information on receptor-mediated events in risk assessments for TCDD. This information may be obtained from steroid receptor action and from molecular data on the Ah receptor (Lucier et al., 1993). This receptor based toxicity, results in sex-dependent sensitivities, as a result of a set of sex-specific PCDDs/PCDFs -responsive genes. However, the estimation of the additional probability of cancer due to the additional exposure burden is quite difficult (Dong et al., 2016). A major obstacle is that an elevated short term external exposure associated to the accidental event, has to be translated into long term risk estimates. Considering the significant persistency and bioaccumulation of PCDDs/PCDFs in human body, the use of biokinetic models for assessing the actual internal dosimetry of this complex mixture is of particular importance. The pharmacokinetics of TCDD are relatively well understood in adult humans (Kerger et al., 2006; Michalek and Tripathi, 1999; Milbrath et al., 2009). However, the impact of pregnancy and lactation on the elimination of TCDD and other dioxins is not clear (Emond et al., 2016).

Accidental fires in plastic industry comprise one of the major events resulting in contamination of various environmental media to PCDDs/PCDFs of the surrounding area; air samples collected in the 5th day of the event were found to contain over 1000 pg/m^3 TEQ (toxic equivalent quantity) of dioxin, exceeding background levels by 2,500–25,000 fold (Fernando et al., 2014). Based on the above, the current study aimed at calculating the health burden (in terms of cancer risk) of the population living in the Aspropyrgos area (close to Athens, Greece) due to increased exposure to dioxins and furans (PCDDs/PCDFs), emitted by an accidental fire in a plastics recycling plant in June 6, 2015. The fire resulted in significant particle and gaseous emissions of several compounds related to plastic industry. In addition, release of dioxins and furans was a major concern, due to their persistence in environmental and biological matrices, as well as to their carcinogenic potency. In order to face the methodological problem mentioned above, a comprehensive methodology involving both measured data and complex internal exposure modelling was employed.

Overall study design

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.

- HBM data of PCDDs/PCDFs in the blood for the population (50 individuals) living in the area after the accidental event, for verifying the increased internal exposure levels. These data have been purchased by the NCSR Demokritos which is the national reference laboratory for PCDDs/PCDFs analysis in Greece.

- Metabolomics analysis (using an aliquot of the blood samples of the 50 individuals) for pathway analysis.

Environmental, biomonitoring data and associated risk

Analysis of ambient air samples (both particle and gaseous phase) showed that the levels of PCDDs/PCDFs in the surrounding area were 1.8 pg/m^3 TEQ WHO (toxicity equivalent concentration in accordance with the methodology of the World Health Organization). These levels are significantly higher than the ones reported in previous studies, where atmospheric background concentration of a typical industrial site in the wider area of Athens was found to be equal to 0.1 pg/m^3 TEQ WHO, but in the same order of magnitude to the levels of landfill fires (*Figure 43*).

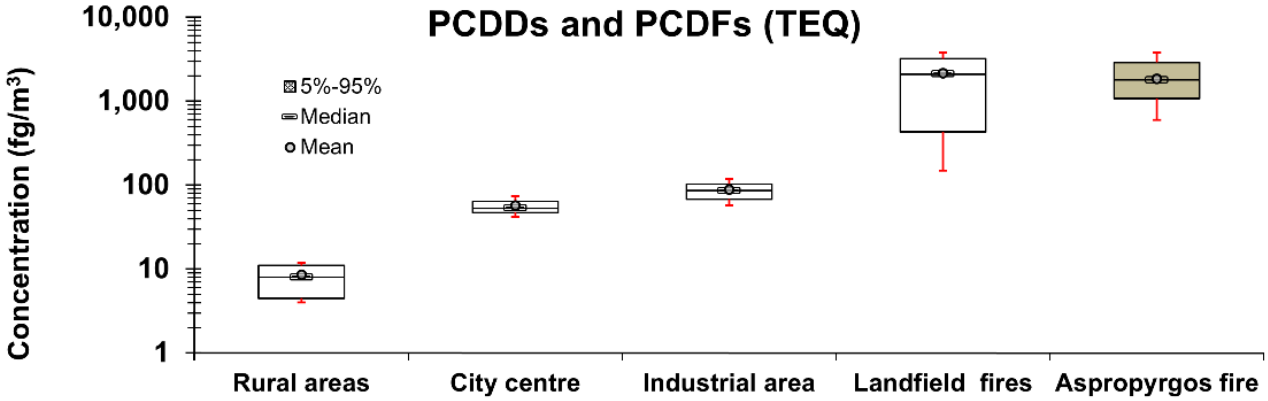

Figure 43. Levels of PCDDs/PCDFs (TEQ) at various Athens sub-areas, as well as during accidental fire events

To calculate the additional burden of exposure for the living population due to the accidental event, it was critical to estimate the actual background exposure to dioxins/furans. For this purpose, the data of a biomonitoring study held in Athens in 2006 (Costopoulou et al., 2006) were used, according to which the average PCDDs/PCDFs TEQ concentration in blood serum was equal to 7.3 pg/g lipids. Exposure reconstruction using biomonitoring data provided a comprehensive overview of the actual daily uptake from all potential pathways (e.g. ambient air and food) and routes (inhalation and oral). According to the biokinetic model, this blood concentration level corresponds to an equivalent daily intake of PCDDs/PCDFs of about 0.002 pg_TEQ/kg_bw/d. By using these uptake levels, the respective time course of the concentration of PCDDs/PCDFs in the blood for a period of 30 years is described with the blue solid line of *Figure 44*.

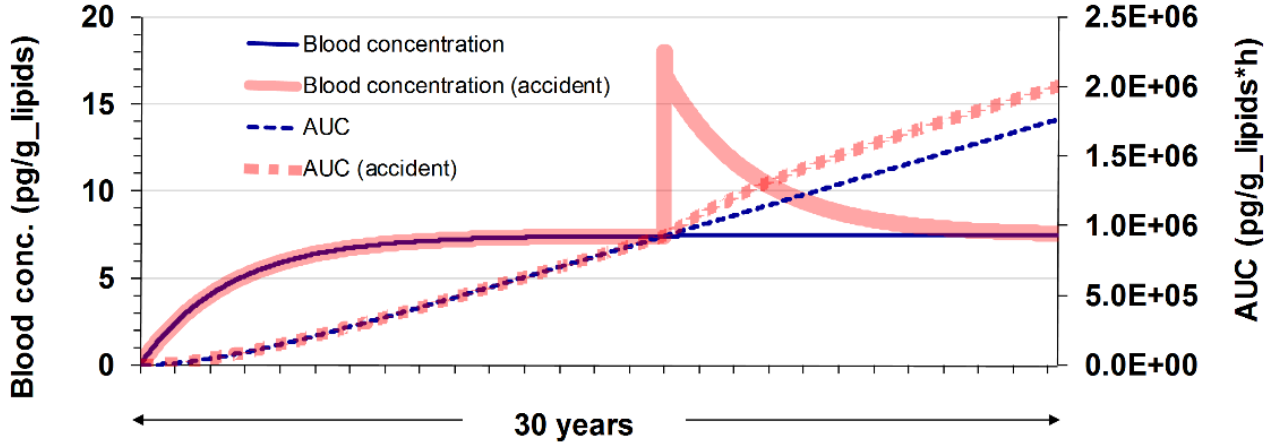

Figure 44. Internal exposure to dioxins/furans under (a) usual conditions (continuous line) and (b) under accidental release (dotted line)

As shown in *Figure 44*, exposure to the accidental fire fumes results in a significant increase of dioxin levels in the blood, up to 18 pg/g lipids (red thick line). Due to the high level of PCDDs/PCDFs persistence, the concentrations in the blood will remain higher than the ones of the background for several years. The elevated exposure levels in adult mothers, are very important for the developing fetus. The constantly elevated blood levels in mother, result in similar levels of PCDDs/PCDFs in the fetus blood. Considering that fetus has a higher amount of adipose tissue, thus a higher capacity of bioaccumulation of lipophilic compounds, this additional burden of PCDDs/PCDFs at that early life stage, results in continuously higher internal exposure (AUC is almost 20% higher) during the entire lifespan. Similarly, breast milk of exposed mothers is expected to have a concentration of PCDDs/PCDFs of almost 10 pg/g lipids, resulting in a significant exposure burden during the breast-feeding period, which in turn results in an increase of the entire lifespan AUC of almost 30%.

It has also to be noted that both the elevated internal exposure to PCDDs/PCDFs, as well as the activation of molecular pathways related to cancer have been verified by biomonitoring of the population leaving nearby the plastic recycling plant. For this purpose, human blood was sampled from 50 individuals, including both adults and children. The results of the analysis indicated that the levels of PCDDs/PCDFs were higher (~12.4 pg/g_lipid) to the ones of the background (~7.4 pg/g_lipid), indicating that the accidental event resulted in increased internal exposure, as already was predicted by the exposure assessment and the PBPK model.

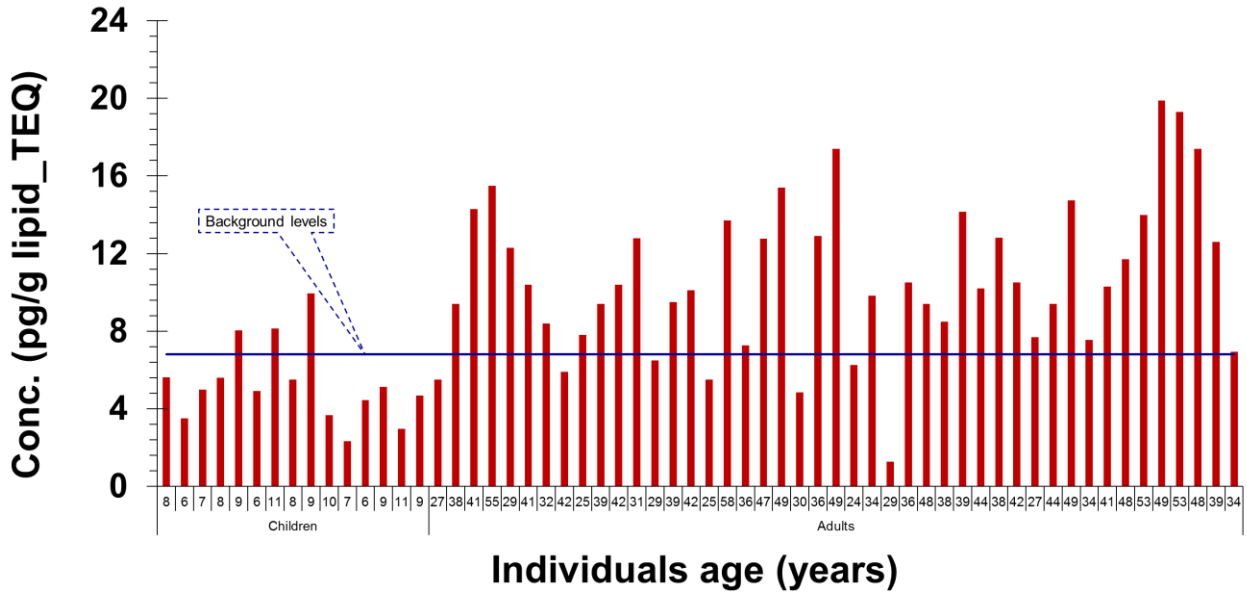

Figure 45. PCDDs/PCDFs (in TEQ levels in blood of the exposed individuals after the accidental event

In addition, untargeted metabolomics analysis indicated that increased levels of unsaturated vs saturated fatty acids were identified compared to controls (population non-exposed to the fumes). This finding could indicate perturbation of cholesterol homeostasis; the latter, is highly related to AhR deregulation. The aryl hydrocarbon receptor does not only act as a transcription factor binding the dioxin responsive element (DRE) and activating metabolizing enzymes such as CYP1A1 and CYP1B1. AhR is also involved in the regulation of other pathways in a DRE-independent manner. As an example, AhR regulates cholesterol biosynthesis by interacting with the sterol element-binding protein 2 (SREPB2) transcription factor. Furthermore, cross-talks between AhR and some nuclear receptors such as the estrogen receptor (ER) and the androgen receptor (AR) are well described (Beischlag et al., 2008). AR itself influences cholesterol homeostasis by interacting with the liver X receptor (LXR), which can regulate sulfotransferases and thereby androgens activity (Jeanneret et al., 2014). Exposure to dioxins eventually leads to alterations in several metabolic pathways, including hepatic lipogenesis, perturbed TCA cycle, disrupted carbohydrate and amino acid metabolism as well as inhibition of de novo fatty acid biosynthesis. The results of this analysis, indicated the activation of pathways associated to cancer, thus verifying the hypothesis of increased cancer risk due to exposure to dioxines and furans.

This additional internal exposure burden which is also illustrated as the difference of the area under the curve (AUC) between the blue and the red dotted lines, will result in an increased risk of cancer associated with exposure to PCDDs/PCDFs. The obtained AUC, was translated into a new equivalent chronic uptake dose, equal to 0.00226 pg_TEQ/kg_bw/d. Uptake estimates were translated into cancer risk estimates, using the slope factor proposed by the US Environmental Protection Agency US EPA (1985.

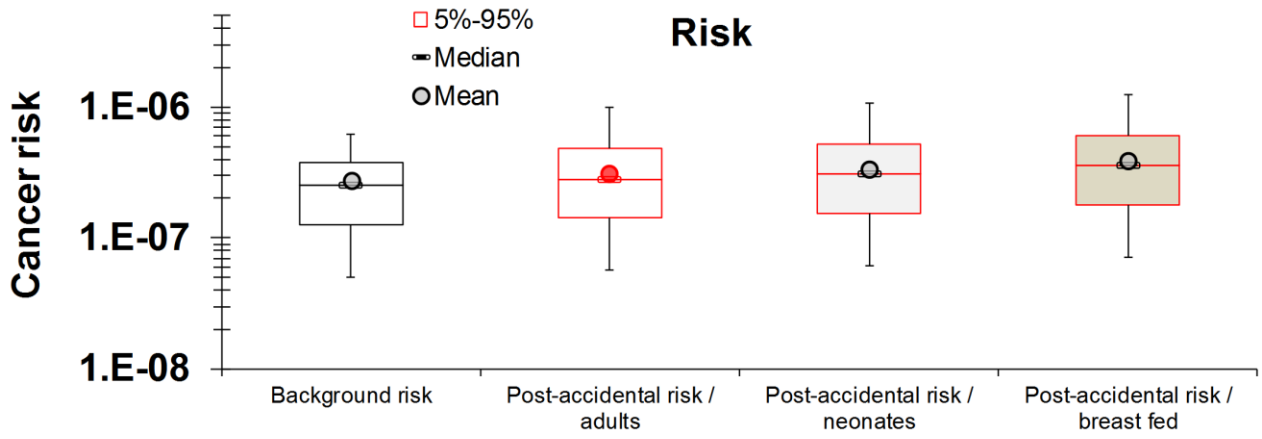

Figure 46. Estimated cancer risk for the exposed population before and after the accidental fire

Accounting for variability in exposure estimates, fat and blood lipids content, distributions of exposure estimates were derived. Based on the background level of exposure to PCDDs/PCDFs of the general population the risk of chronic exposure was estimated (mean value) equal to 2.57 10^{-7}. For the population exposed over 6 days to the PCDDs/PCDFs fumes emitted from the recycling plant fire the respective risk (mean value) was up to 2.91 10^{-7}, indicating an increase of 13% in the 30-year cancer risk. It has to be noted that for the upper bound of the post-accidental risk estimates are close to 10^{-6} (*Figure 46*).

The respective risk is expected to be higher for neonates, whose mothers were exposed to the fumes of the accidental fire during pregnancy. The higher levels of PCDDs/PCDFs in the blood during pregnancy will result in continuously higher levels during their entire life span, resulting in an increase of the estimated lifetime risk of almost 20%. The estimated lifetime risk is expected to be even higher for neonates that are also breast fed; breast feeding is considered a major source of lifetime exposure to dioxins and the increased levels of exposure to PCDDs/PCDFs are considered to contribute to up to 32%.

Cancer risk associated to arsenic and hexavalent chromium in the area of Asopos

Introduction and study design

A major local environmental issue in Greece is related to the presence of hexavalent chromium Cr(VI) in drinking water of the Oinofyta municipality, within the wider area of Asopos basin and the related cancer mortality. The Oinofyta municipality (*Figure 47*) is situated 50 km North of Athens, Greece, and it includes four villages that were initially rural but transformed into industrial areas in the early 1970s. In 1969, a ministerial decision gave permission for depositing processed industrial waste in the Asopos river, which runs through Oinofyta. This decision, furthered by a presidential decree in 1979, permitted free disposal of processed liquid industrial waste into the river. According to the Technical Chamber of Greece (TCOG 2009), in the 80s there were about 700 industries operating in the Oinofyta area, of which 500 generated liquid industrial waste. After protests from citizens who complained about the discoloration and turbidity of their drinking water in 2007 the Ministry of Environment, Regional Planning and Public Works of Greece imposed fines on 20 industries for disposing industrial waste with high levels of

hexavalent chromium into the Asopos river. Official limits on total chromium have been set by both the United States Environmental Protection Agency (US-EPA), equal to 100 μg/l, and the European Union (Council directive 98/83/EC), equal to 50 μg/l. However, as of yet, there are no limits set by any international body for Cr(VI). In 2009, the California Environmental Protection Agency proposed a public health goal level of 0.06 μg/l for Cr(VI) in drinking water (OEHHA 2009).

Figure 47: Asopos Basin and Oinofyta municipality

Because areas characterized by high Cr(VI) concentrations in drinking water are relatively uncommon, human epidemiologic studies are scant; the study carried out by Zhang and Li (1987 is one of the most cited and controversial studies analyzing the effects of oral exposure to Cr(VI) on population cancer mortality rates conducted near a chromium smelting plant in the Liaoning Province, China.

In order to examine the potential effects of elevated oral exposure to hexavalent chromium, an ecological mortality study (Linos et al., 2011) was performed in an industrial area of Greece where the water consumed by the population was contaminated with hexavalent chromium (maximum levels ranging between 41 and 156 μg/l in 2007-2009, and presumed exposure for at least 20 years). The goal of the study was to examine the cancer mortality in an area of Greece, historically satisfying its potable needs with a Cr(VI)-contaminated aquifer.

Environmental data, collected biomonitoring data and cancer risk assessment

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) 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;

b) a study conducted by the faculty of the Geology and Geo-environment department of the University of Athens (Ch. Vasilatos 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

c) 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 μg/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.

Considering the continuously growing awareness about the impact of environmental exposure to Cr(VI), the water sypply to the area in the latest years has changed, resulting in tap water concentrations around 1.53 μg/l. This has resulted in lower biomonitored Cr(VI) levels detected in human blood. The results of the biomonitoring data for a large number of heavy metals in the blood of the population leaving in the area are given in *Figure 48*. The results of Cr(VI) in blood are consisted with the Cr(VI) in hair and urine, as previously reported in the respective CROME study.

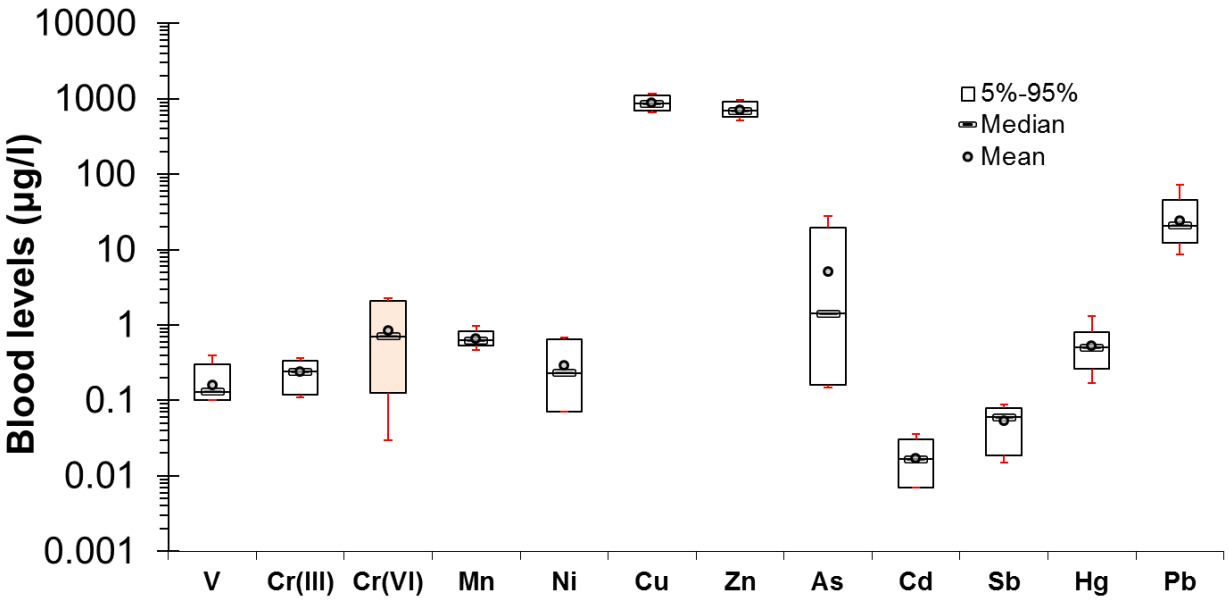

Figure 48. Results of the measured biomonitoring data in the area of Asopos.

Based on the observed biomonitored data, the respective exposure levels for As and Cr(VI) were estimated by using the generic PBPK model developed in CROME. The exposure reconstruction results indicated that intake of As was equal to 0.03 μg/kg bw/d, while the respective intake of Cr(VI) is equal to 0.028. By using the respective exposure estimates and the methodologies described in the previous sections, the associated cancer risks to As, to Cr(VI) and the respective cumulative cancer rik are presented in Figure 49.

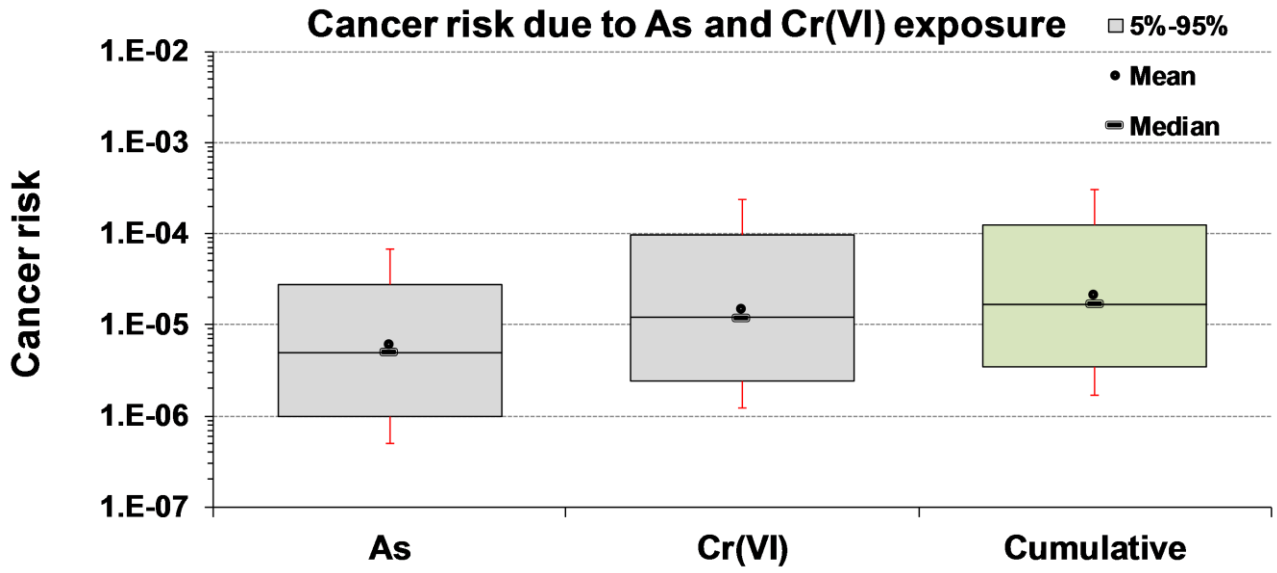

Figure 49. Cancer risk associated to As and Cr(VI) in the Asopos area.

It has also to be noted that for a better understanding the significance of the estimating cancer risk, these estimates were compared to the background of cancer incidence rate in Greece (data purchased from the Hellenic Health Fundation).

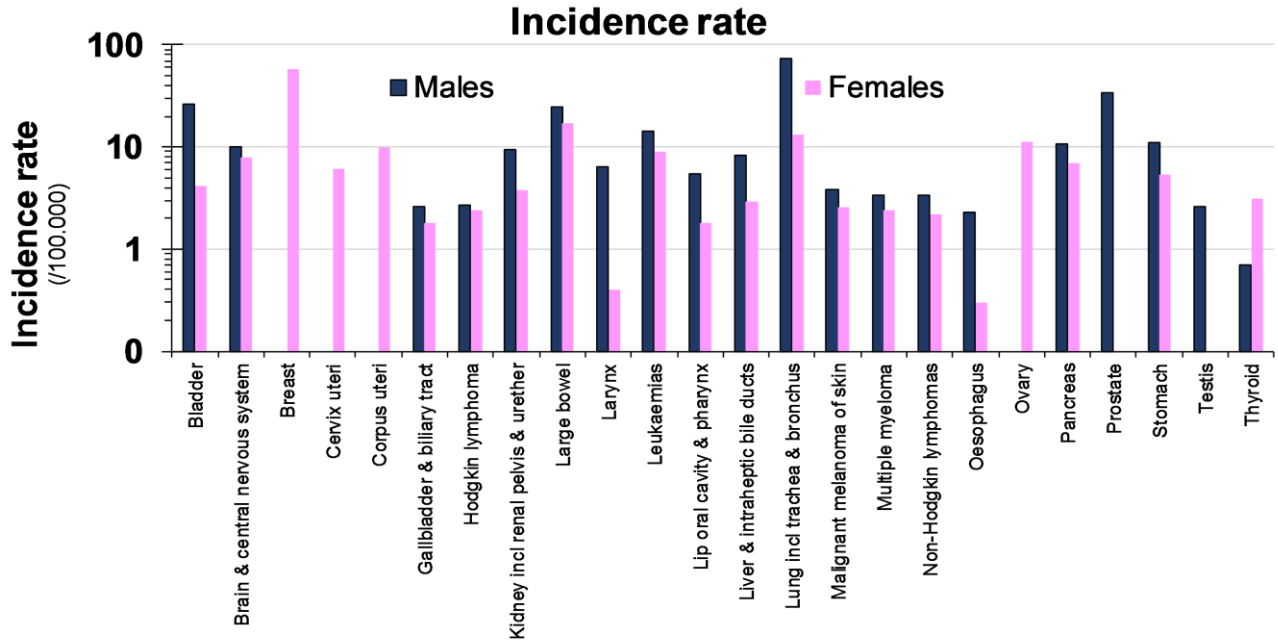

Figure 50. Incidence rate of most common cancer types in Greece

According to the above, the incidence rate of cancer types (kindney, lung and skin) is much higher (one to two orders of magnitude) compared to the one associated to arsenic exposure induced cancer, while the same occurs for the cancer types (gastrointestinal tract cancers) associated to Cr(VI) exposure. As a result, although the risks associated to Cr(VI) and As exposure are above the accepted risk of 10^{-6} for environmental streesors (Hrudey and Krewski, 1995), overall these risks are below the actual incidence rate in Greece.

Conclusions

Neurodevelopmental disorders associated to heavy metals exposure represents a major concern and although there is ample of evidence worldwide, the overall neurodevelopment is always a complex interaction between genes, environmental, socioeconomic and dietary factors of the region under study.

In Italy, a combination of post natal exposure factors such as the high levels of As. Hg, Cd and Pb (mostly related to fish consumption) were found to affect children neurodevelopment. It has to be highlighted that the mildly positive effect of Hg concentration in child`s hair on some neuropsychological test at 7 years, is explained by the autocorrelation with the beneficiary effect of fish consumption and the respective omega-3 fatty acids intake. On the other hand, Hg exposure itself, resulted in worst externalizing (anxiety) and internalizing (depression) behaviours. Another key finding is that 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. In addition, 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.

Further exploring the effects of exposure to Hg in Slovenia, it was indicated that even low-to-moderate Hg exposure in children with normal neurodevelopmental outcome can be associated with lower cognitive and fine motor scores at 18 months of age. Despite the relatively low number of subjects carrying the Apoe 4 allele, the study provided firm evidence of Hg-associated decrease in cognitive performance among Apoe 4 allele carriers, while the decrease in fine motor scores was independent of the genotype. Gene-environment interaction was indicated for the cognitive score. It was also demonstrated that accounting for beneficial factors like Se and other potentially neurotoxic substances like Pb is crucial in assessing such associations. Re-evaluation of the obtained results is needed with higher predictive values of neuropsychological test used at later age and with consideration of other polymorphisms identified that can influence neurodevelopmental performance.

With regard to heavy metals exposure, one of the key studies in the frame of course was the identification of the contribution of landfilling in child neurodevelopment. From the study, it was found that child neurodevelopmental performance was always inversely correlated with the residence distance from the landfill site. This highly confirmed the hypothesis that landfills are major pollution sources for the population living nearby, eventually with additional pollutants than heavy metals. On the other hand, it was very interesting to identify that socio-economic-cultural conditions of the family are key determinants of children neurodevelopment. The positive effect of these factors on the neurodevelopmental cognitive functions is associated to the better quality of life, translated as nutrition of higher quality, lower exposure to environmental contamination and better educational activities and opportunities.

Beyond heavy metals, persistent organic compounds like PCBs have been associated to neurodevelopmental disorders in Spain. Although it is evident that breastfeeding increases children`s blood POPs levels during postnatal life, it is suggested that effects of POPs on early brain development, particularly on psychomotor development, could mainly be attributable to prenatal exposure. In addition, the combined exposure to POPs and Hg was found in accordance with previous animal experiments. We also found that Hg, PCB138 and β-HCH inhibit glutamate transport in human placentas and this is detrimental for child neurodevelopment.

Cancer is also a major health problem affecting a larg part of the population in global scale, and similarly to neurodevelopment, identification of the contribution of environmental factors is of key importance. Cancer effects are mainly attributed to long term exposure to carcinogenic compounds, howevr, we need to keep in mind that short term exposure to persistent compounds with long biological half-life is somehow similar. Exposure to PCDDs/PCDFs is a typical case. In the respective CROME study, a comprehensive methodological framework was applied, for addressing one of the major problems related to accidental events and the release of bioaccumulative, persistent and toxic compounds, which is the translation of a short-term exposure event into a long term exposure estimate. Although waste recycling is considered one of the most environment friendly and sustainable waste management option,

accidental events might result in significant contamination of the surrounding area, affecting adversely the population leaving nearby. Towards this aim, the developed modelling framework allowed the assimilation of different type of both environmental and HBM data, for estimating the chronic internal exposure to PCDDs/PCDFs. For estimating the associating risks, the concept of TEQ for the mixture of PCDDs/PCDFs was employed, using as a TEF reference compound 2,3,7,8-TCDD, which has a well-established slope factor. Based on the above, an average increase of 13% of cancer risk attributed to PCDDs/PCDFs was estimated. A key finding of the study was that neonates and breast fed infants face even higher lifetime risks, and also that these risks were quantified (20% and 32% respectively).

With regard to long term exposure to As and Cr(VI) in the Asopos basin in Greece, the respective cumulative cancer risk is in the range of 10^{-5}. Although a higher cancer risk might be expected, due to the measures undertaken for improving quality of drinking water in the area, this is now limited to levels similar to the ones identifyied in other areas of Greece. However, due to the continuous environmental releases of this industrially contaminated area, there is a need for continuous environmental monitoring, as well as for human biomonitoring. Similarly, in Spain, the CROME findings add new information on the possible relation between exposure to organochlorinated compounds, particularly HCB, and cancer. However, further research is needed for proper assessment of a direct association between HCB and the above-described neoplasms.

With regard to biomass emitted PM during winter time and the related PAHs exposure induced lung cancer, a key finding was that biomass emitted particles are i) more toxic in terms of PAH content compared to the ones related to other sources and ii) of lower aerodynamic diameter; as a result, more refined exposure and risk characterization methods are needed so as to properly account for their potential health effects. More importantly, it was found that biomass-related PM seem to have a higher PAH content than the PM emitted from other sources, as shown by rigorous chemical analysis. Use of refined exposure metrics that take into account the spatial variability of PM concentrations, differences in exposure patterns and inhalation rates, PM deposition across HRT and PM chemical composition, allows us to significantly differentiate the actual health risk due to exposure to PM between different urban sites as well as between different age groups. This has been demonstrated in the case of lung cancer risk estimation. Incorporating internal dose assessment metrics accounts for the increased children susceptibility to chronic exposure to airborne toxicants such as particle-adsorbed PAHs. Besides the fact that body weight normalised dose of PM is higher in young children, the physiology of the human respiratory tract results in higher deposition fractions of smaller diameter PM in the lower respiratory tract, with multiple implications in the expected adverse health outcomes. It has also to be noted, that beyond lung cancer, the increased PM due to biomass burning for space heating in Greece, is accompanied by increased overall mortality accounting for ca. 200 (expressed on an annual basis) only in Thessaloniki, which is a significant number, considering that we refer to a city of almost 900.000 people.

In a more holistic overview, it was found that different waste management options pose significant risks to human and more importantly to child health after chronic exposure. Most important risks are related to exposure to metal contamination linked to neurodevelopmental disorders associated to landfill. However, it has also to be noted that although recycling is considered as the most benign waste management strategy, yet, short term exposure following the aftermath of accidents in waste recycling plants associated to dioxins, furans and other carcinogens, are linked to increased risk of later life cancer. As a result, a more comprehensive waste managemet plan has to be implemented across EU, that will eventually result in abatement of long-term associated risks.

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