SEVENTH FRAMEWORK PROGRAMME

HEALS

Health and Environment-wide Associations based on Large population Surveys

FP7-ENV-2013- 603946
http://www.heals-eu.eu/

15.1 Review on the application of HEALS model/methodologies

WP 15 Neurodevelopmental and neurodegenerative disorders - link with metals/metalloids and pesticides

Version 1

Lead beneficiary: ISS
Date: 09/03/2017
Nature: Report
Dissemination level: Public

HEALS FP7-ENV-2013-603946	D15.1 - Review on the application of HEALS model/methodologies		
	WP15: Neurodevelopmental and neurodegenerative disorders - link with metals/metalloids and pesticides	**Security**:	
	Author(s): G. Calamandrei, D. Sarigiannis, Tratnik, F. Chiarotti, F. Mirabella. K. Polanska. I. Annesi-Maesano	**Version**:	0.1

Document Information

Grant Agreement Number	ENV-603946	**Acronym**	HEALS
Full title	Health and Environment-wide Associations based on Large population Surveys		
Project URL	http://www.heals-eu.eu/		
EU Project Officer	Tuomo Karjalainen - Tuomo.KARJALAINEN@ec.europa.eu		

Deliverable	**Number**	15.1	**Title**	Review on the application of HEALS model/methodologies
Work Package	**Number**	15	**Title**	Neurodevelopmental and neurodegenerative disorders - link with metals/metalloids and pesticide

Delivery date	**Contractual**	M24	**Actual**	28/04/2015
Status	Draft ☐		Final X	
Nature	Demonstrator ☐	Report X	Prototype ☐	Other ☐
Dissemination level	Confidential ☐	Public X		

Author (Partners)	G. Calamandrei (ISS), D. Sarigiannis (AUTH), Tratnik I (ISS), F. Chiarotti (ISS), F. Mirabella (ISS). K. Polanska. I (UOM). Annesi-Maesano (UPMC)			
Responsible Author	Gemma Calamandrei (ISS)		**Email**	Gemma.calamandrei@iss.it
	Partner	ISS	**Phone**	

Document History

Name	Date	Version	Desciption

TABLE OF CONTENTS

Abstract **5**

INTRODUCTION **6**

1 Understanding the link between environmental stressors and neurodevelopmental disorders in the exposome perspective **8**

1.1 The external exposome: the specific and unspecific environmental stressors 9

1.2 The external exposome: mechanisms of action 10

1.3 The external exposome: the individual dimension 11

1.4 Gene – environment interaction 12

1.5 The HEALS geo-data base 14

1.6 Assessing the health effects: measuring the health outcome 14

1.7 The internal exposome: Biomarkers of effects/ Selection of omic biomarkers related to the behavioural phenotype 16

1.8 The HEALS geo-data base 16

2 The HEALS cohorts measuring neuropsychological outcomes in children **20**

2.1 The Polish Mother and Child Cohort (REPRO_PL) 21

2.1.1 Aim 21

2.1.2 Study design and population 21

2.1.3 Current stage of the study 22

2.1.4 Child psychomotor assessment 24

2.1.5 Pre- and postnatal exposure to environmental and lifestyle-related factors and child neurodevelopment 25

2.1.5.1 Socio-demographic and lifestyle factors 25

2.1.5.2 Association between maternal lifestyle factors during pregnancy and child neurodevelopment 28

2.1.5.3 Maternal stress during pregnancy and child neurodevelopment 30

2.1.5.4 Microelements during pregnancy and child neurodevelopment 31

2.1.5.5 Environmental factors during pregnancy (Pb, Hg and PAH) and child neurodevelopment 35

2.1.5.6 Phthalate exposure and child neurodevelopment: 36

2.2 The HERACLES (Waste Management) Study 39

2.2.1.1 Introduction 39

2.2.1.2 Data used in the analysis 40

2.3 The PHIME cohort Milena, Janja, ... 41

2.4 The EDEN cohort Isabella 41

3 The EWAS approach: applying the HEALS methodology to real data sets **45**

3.1 Standardization and harmonization of data in view of unraveling the HEAL paradigm 45

3.2 EWAS.application examples 48

3.2.1 Comparing the Phime and REPRO_PL cohorts to investigate the effects of the same environmental stressors on the neuropsychological outcome in the first 2 years of life in different countries 48

3.2.1.1 Methods 48

3.2.1.2 Can this approach be easily translated to other chronic diseases? 48

3.2.1.3 Comments 53

3.3 Paving the way to EWAS analysis: investigating the role of the exposome on different health outcomes 53

3.3.1.1 Comments 55

3.4 Comprehensive EWAS analysis 55

3.4.1 Application on the REPRO_PL study 56

3.4.1.1 Comments 61

3.4.2 Application on the HERACLES Greek cohort 62
3.4.2.1 Data clustering 62
3.4.2.2 Results and interpretation of EWAS analysis 65
3.4.2.3 Comments 73
3.4.3 Conclusions in the full EWAS analysis 73
References 74

Abstract

HEALS (Health and Environment-wide Associations based on Large population Surveys) brings together a comprehensive array of novel technologies, data analysis and modeling tools that support the efficient design and execution of large-scale exposome studies and environment-wide association studies (EWAS) to improve health risk assessment of environmental stressors in human populations. The integrated approach proposed by the exposome appears as the more adapted to unravel the complexity of emerging environmental health issues able to implement the development of chronic diseases. This overall approach is pursued by HEALS by d tackling various levels of environmental exposure, age windows and gender differentiation of exposure, as well as socio-economic and genetic variability. The main objective is performing environment-wide association studies (EWAS) to search for environmental factors associated with disease on a broad scale in support of EU-wide environment and health assessments and prevention.

In this Technical Report we show how the HEALS methodological framework, implemented by the activities carried out by Stream 2, and Stream 4 has been applied in WP 15 to gather and harmonise data available and reanalyse within the EWAS perspective the data available from different birth cohorts dealing with neurodevelopmental effects of environmental stressors. In Stream 5, preliminary to the harmonisation process and application of the EWAS model, a thorough revision of the available cohort data was performed. Referring to the two categories of external and internal exposome as defined by HEALS we have tried to characterize strengths and weaknesses of each data set, with specific attention to major gaps in knowledge, missing information and criticalities related to the specific health outcomes in study. The first chapter describes in the exposome perspective the key variables, the major gaps in knowledge, the key uncertainties and the research needs in the field of the environmental origin of neurodevelopmental disorders. The second chapter presents the study designs, the characteristics and the main results of the HEALS cohorts selected for the EWAS. The third and final chapter describes the procedure followed for cohort harmonisation in Stream 5, the comparison between two HEALS cohorts sharing the same evaluation tool for evaluation of the health outcome, the investigation of the environmental factors potential affecting different health outcome in children (respiratory diseases and neurodevelopmental disorders) and finally the application of EWAS approach to real data set.

Despite the differences in test batteries, type of environmental, exposure, sociodemographic data and biospecimens available the strength of the methodology allowed us to better identify associations between different exposure factors and neurodevelopment. Several gestation factors have a beneficial (e.g. the concentration of selenium in maternal blood) or a negative influence (e.g. maternal bodyweight) in child neurodevelopment. On the other hand, child exposure to phthalates itself has a stronger negative influence in child neurodevelopment than maternal exposure. Children exposure to heavy metals and proximity to waste management sites have a negative influence in child neurodevelopment, however these effects are significantly modified by sociodemographic parameters (such as children SES and parents educational level, as well as diet. Further steps of analysis in both cohorts will include the results of the toxicity pathways identified to be perturbed from the omics analysis.

INTRODUCTION

It is widely acknowledged that the majority of chronic diseases (including cancer, metabolic, neurological and neuropsychiatric diseases) result from the complex interaction between several factors, which include variations (i.e. single nucleotide polymorphisms, mutations, deletions and copy number variants) in different genes, the involvement of epigenetic mechanisms, different kinds of adverse environmental stressors (maternal infections, obstetric complication, early exposure to chemicals of therapeutics during pregnancy) and the effect of modifiers such as socioeconomic status (SES). The integrated approach proposed by the exposome appears as the more adapted to unravel the complexity of emerging environmental health issues able to implement the development of chronic diseases.

HEALS (Health and Environment-wide Associations based on Large population Surveys) brings together a comprehensive array of novel technologies, data analysis and modeling tools that support the efficient design and execution of large-scale exposome studies and exposome-wide association studies (EWAS) to improve health risk assessment of environmental stressors in human populations.

In such framework, HEALS aims at identifying genetic, socioeconomic and lifestyle factors that contribute to modulate the individual's susceptibility and vulnerability to chemical exposures. This overall approach is pursued by tackling various levels of environmental exposure, age windows and gender differentiation of exposure, as well as socio-economic and genetic variability. The main objective is performing environment-wide association studies (EWAS) to search for environmental factors associated with disease on a broad scale in support of EU-wide environment and health assessments and prevention.

The first objective of Stream 5 was that of re-analysing within the exposome perspective the data collected in pre-existing European population studies of singletons and twins across Europe. Through applying the EWAS model to existing datasets, HEALS aims at identifying a panel of reliable biomarkers of exposure, effects and susceptibility to support the causal association between environmental factors and human health at the European level. The final objective is applying the resulting model in a pilot environment and health examination survey covering ten EU Member States, which will be centered on the prenatal and perinatal periods to define the health effects (in terms of allergy/asthma, neurodevelopmental disorders, and obesity) brought about by diverse environmental factors in singleton and twins populations of children. The lessons learned will then be translated into scientific advice towards the development of protocols and guidelines for the setting up of a European environment and health examination survey, specifically aimed at protecting the health of vulnerable population sub-groups.

In the following paragraph we will illustrate how the HEALS methodological framework, implemented by the activities carried out by Stream 2 and Stream 4, has been applied to gather and harmonise data available and reanalyse within the EWAS perspective the data from 3 birth cohorts dealing with neurodevelopmental effects of environmental stressors. Preliminary to the harmonisation process and application of the EWAS model, a thorough revision of the available cohort data was performed. Referring to the two categories of

external and internal exposome as defined by HEALS, we have tried to characterize strengths and weaknesses of each data set, with specific attention to major gaps in knowledge, missing information and criticalities related to the specific health outcomes in study. These issues will be addressed in the first chapter that will describe in the exposome perspective the key variables, the major gaps in knowledge, the key uncertainties and the research needs in the field of the environmental origin of neurodevelopmental disorders. The second chapter will present the characteristics of the cohorts selected for the EWAS and the main results so far obtained when the effects of environmental stressors on the health outcome were analysed by multivariate analyses. The third and final chapter will illustrate the procedure followed for cohort harmonization in STREAM 5 and how we apply the HEALS model/methodologies to real data set including assessment of the neuropsychological outcome.

1 Understanding the link between environmental stressors and neurodevelopmental disorders in the exposome perspective

WP15 focuses in particular on a specific class of children's adverse health outcome, namely that of neurodevelopmental disorders (NDDs). NDDs encompass a group of clinical heterogeneous conditions with onset in the developmental period. These disorders typically manifest early in development and are characterized by developmental deficits that produce lifetime impairments of personal, social, academic, or occupational functioning. The range of developmental deficits varies from very specific limitations of learning or control of executive functions to global impairments of social skills or intelligence. Commonly known NDDs include ASD, ADHD, communication, speech and language disorders, and genetic disorders such as Fragile X or Rett syndrome. To date, the etiological bases of the majority of these conditions are still unknown, though a great body of data support their polygenic and multifactorial etiology.

Susceptibility and vulnerability to adverse environmental factors represent the drawback of brain plasticity. The brain has a protracted period of susceptibility to environmental inputs, which extends well beyond organogenesis up to the second decade of life [1; see also WP1 deliverable on critical periods]. Within the constraints posed by the genome, the brain receives information from the environment and uses this information to shape and refine synaptic connections. In such a way, experience adjusts the underlying brain circuitry founded on the distinctive environment in which each individual lives and grows up. Moreover, some children are more exposed to environmental hazards because of their social conditions.

The dynamic interplay between genes and environment, which forms the basis of typical neurobehavioral maturation is being also called upon to explain the etiology of complex NDDs that are characterized by abnormal brain morphology and/or functional activity, even those with a strong genetic component. Diverse environmental stressors—chemical pollutants, drugs, nutritional factors, maternal infection, stress, deprivation—may interfere with typical brain developmental trajectories, eventually increasing the risk of either subclinical neuropsychological alterations or manifest clinical conditions such as learning disabilities, autism spectrum disorders (ASD) and attention deficit/hyperactivity disorder (ADHD).

In spite of the impressive number of epidemiological data that support the inverse association between environmental exposure and child neurodevelopment [2,3,4], there are still important knowledge gaps in this field that hamper the proper evaluation of neurobehavioral effects by risk assessors. The main criticalities identified following the revision of data set made available to the HEALS consortium together with reconsideration of the most recent epidemiological literature, refer to i) availability of reliable and comparable biomarkers of exposure and effects to measure both the external and internal exposure, ii) the difficulty of identifying pathways and targets of toxicity for several established neurotoxicants, iii) the need of defining the critical periods for exposure as well as the sequence in time of all the possible adverse exposures (maternal infection, exposure to neurotoxicants, parental stress, low SES), iv) the role of confounders or effect modifiers that

often affect *per se* the neuropsychological outcome and v) the methodological tools for outcome measurement.

1.1 The external exposome: the specific and unspecific environmental stressors

A list of all the environmental stressors that can affect typical brain development is out of the scope of this report. Recently Grandjean and Landrigan (Grandjean and Landrigan, 2014) performed a systematic review on studies published from 2006 to 2012 on the neurotoxic effects of industrial chemicals in human beings. This article updates the previous one published from the same authors in 2006 (Grandjean and Landrigan, 2006), where a series of 201 chemicals among metals and inorganic compounds, organic solvents, pesticides, and other organic substances were identified as neurotoxic for adult individuals, though, at that moment, only very few of such chemicals had been classified as neurodevelopmental toxicants. In particular, in the 2006 survey, lead, methylmercury, toluene and PCB were implicated in neurobehavioral deficits in children following prenatal exposures at concentrations considered as subtoxic in the adults.

Since 2006, new data have emerged about the weakness of the developing brain and the neurotoxicity of industrial chemicals, and the recent systematic review from the same authors reports new evidence that derives from prospective epidemiological birth cohort studies. Six additional agents emerge as neurodevelopmental toxicants of concern. In particular, epidemiological data associate manganese, fluoride, chlorpyrifos, dichlorodiphenyltrichloroethane, tetrachloroethylene, and PBDEs with diminished intellectual functioning, learning disabilities, attention problems, aggressiveness, hyperactivity, ADHD and ASD (Bouchard et al., 2010; Choi et al., 2012; Engel et al., 2011; Miodovnik et al., 2011; Rauh et al., 2011). Other suspected developmental neurotoxicants are further indicated: among these, phthalates and bisphenol A, air pollution, and comparable complex emission such as the traffic-related pollution. In particular, carbon monoxide, nitrogen oxides, and polycyclic aromatic hydrocarbons are recently reported to act as neurotoxic agents that can cause cognitive and neurological impairment (Calderon-Garciduenas et al., 2008; Perera et al., 2009), as well as ASD (Volk et al., 2013), and ADHD (Froehlich et al., 2011). Further to environmental chemicals exposure, adverse maternal conditions, such as stress, infections, malnutrition can profoundly interfere with fetal brain development through a plethora of mechanisms, including abnormal activation of the hypothalamic-pituitary-adrenal axis, neuroinflammation, alteration of the hormonal milieu, and dysfunction in the immune responses.

The observation that health and chemical burden are sustained at higher level by low SES populations informs on the potential risk for human health in those population in which cumulative risk factors, such as psychological stress due to socioeconomic deprivation and chemical exposure could occur. The individual vulnerability rising from allostatic load derived by low SES and psychosocial stress can in turn influence responses to chemical exposure modulating response and resilience, with health effects which appear dependent on geographical, race and social factors (Morello-Frosch and Shenassa, 2006). Both epidemiological and experimental studies have suggested common target physiological systems and pathways for both toxicants and stress. In a very recent study conducted on the US cohort of women in reproductive age enrolled inside the National Health and Nutrition

Examination Survey (NHANES) program, chronic stress was found to modify the association between elevated lead/methylmercury exposure and race/ethnicity (Evans et al., 2014), highlighting the importance of evaluating chemical and nonchemical stressor exposures. Previous studies had showed controversial relationships between higher environmental chemicals' exposure and lower social economic status, suggesting that more epidemiological research is needed to clarify which variables (chemical and nonchemical) are really involved in the potential interaction between chronic stress and chemical hazard (Cory-Slechta, 2005; Kobrosly et al., 2012).

Another aspect that impacts on susceptibility and vulnerability to pollutants' exposure is the dietary style of the individuals/population in study. Several studies indicate that diet significantly impacts on neurodevelopmental outcome and cognitive function although this relationship is still relatively poorly understood. Research in animal models and human populations show that some nutrients are particularly important during pregnancy and the first years of life to support rapidly developing brain systems and maturation of cognitive functions. Experimental studies suggest that dietary patterns including Long-chain polyunsaturated fatty acids (LCPUFA) and micronutrients such as iron, zinc, and iodine which are abundant in a diet rich in marine fish could positively influence brain development, by promoting plasticity and maturation of cognitive functions. Marine fish is also rich in selenium whose impact on neurodevelopment is not well established. The large body of data collected in the past decade in two different birth cohorts (Faroe Islands and Seichelles; see refs) highlights a complex interaction between the well-known developmental neurotoxicity of methylmercury (MeHg) and nutrients such as LCPUFA and Selenium that could in part counteract the adverse effects of MeHg on brain development. It seems likely that the combination of LCPUFA and specific trace elements or micronutrients may produce synergistic effects that will reinforce their individual properties. On the contrary, deficiency in important micronutrients can adversely affect neurodevelopment.

1.2 The external exposome: mechanisms of action

The estimation of the dose-effect relationship is a crucial point for establishing the causative role of environmental chemicals in human diseases. Dose-effect estimation requires linking environmental exposure to the biologically effective dose of xenobiotics at the main target tissues, and then relating internal dose at target tissue with the physiological perturbation/health outcome observed [5]. This implies both identification of reliable biomarkers of exposure (i.e., peripheral indicators predictive of concentrations at target tissues) and knowledge of the mechanisms of neurotoxicity for the chemical compounds in study. Peripheral biomarkers of exposure so far available for many environmental chemicals are indeed poor predictors of effects on the CNS (Stangle et al., 2004) and estimating the concentration of a toxic compound or metabolite in the brain on the basis of concentrations found in other matrices may lead to exposure misclassification (Bellinger, 2009).

Indeed, a critical gap relates to the mechanisms by which different classes of chemicals act on behavioral development. Mechanistic studies carried out in animals and in *in vitro* models point to multiple pathways and targets of toxicity for several established neurotoxicants. Chemicals may directly interfere with formation and closure of the neural tube, cell proliferation, migration, death, or synapse formation inducing changes ranging from overt morphological alterations to subtle dysfunction in synaptic connectivity. The same chemical

compound may affect different developmental processes or different cell types, depending on the time window of exposure. Furthermore, compounds belonging to the same chemical class may have dissimilar mechanisms of action, as is the case for different organophosphate insecticides that produce disparate neurotoxic outcomes despite their shared property as cholinesterase inhibitors. To complicate the picture further, the same chemical may have multiple mechanisms of action: agents endowed with endocrine disrupting activity may affect neurobehavioral development by directly interacting with steroid receptors in brain cells and/or in periphery, and at the same time influence the density of synaptic connections in specific brain areas with mechanisms possibly independent from their hormone-like action [6]. Thus, more experimental research is needed to elucidate the causal links between eternal exposome and disease, addressing different levels of biological organization through the combined use of in silico, *in vitro* and *in vivo* models. This will support building adverse outcome pathways for neurodevelopmental effects.

1.3 The external exposome: the individual dimension

A second main issue is how to capture the individual dimension of the exposure history. Different factors may indeed contribute to variability in the measured outcome, including the temporal dimension of the exposure, the spatial dimension of exposure, the co-exposure to other contaminants or stressors, and the existence of genetic vulnerability that may render an individual more susceptible than others.

The temporal dimension of the exposure: the individual exposome is dynamic and continually changing. Indeed, all exposures and their determinants and modifiers can vary over the course of a day, not to mention over the weeks, months, and years that make up a lifetime, as our bodies, diets, risk factors and lifestyles change. The exposures can also have different effects according to the period and the length of life (window of exposure). Because sources and levels of exposure change over time and window of exposure, the exposome has to be constructed by assessing the exposures at critical life periods through representative snapshots that act as demonstrative measures of these critical periods (Sabel et al. 2009). Thus, one major challenge consists in identifying critical life stages that are informative at most as well as the snapshots reflecting the exposures and the downstream consequences at the individual level.

The literature on exposure-outcome relationships is incomplete with regard to data on age at exposure and age at which exposure effects can be detected. Systematic studies across ages of exposure, ages at which outcomes are measured and specific toxicants do not yet exist. For example, the neurotoxicology literature suggests that lead exposure in early childhood is associated with IQ changes (Needleman 1982) and that prenatal methyl mercury exposure is associated with domain-specific neuropsychological effects at the age of 7 years (Grandjean et al 1997). It is therefore difficult to pinpoint critical ages at which specific types of neuropsychological outcomes should be measured with the aim of revealing the health outcomes of specific environmental chemicals. Each developmental stage may be associated to specific effects on brain development: whereas exposure to chemicals in the early phase of brain development have a higher probability to cause structural alteration or even malformation, later exposures may induce more subtle changes (i.e. reduced or increased synaptic connectivity, reduced or increased levels of neurotrophic molecules) that can result in behavioural alteration even years or decades after the insult.

The spatial dimension of the exposure: the individual exposure can vary also due to the microenvironments where people are exposed (home, school, work, transportation…). In addition, modelling the mobility patterns of the population at risk at the individual level is challenging. There are considerable conceptual and computational difficulties involved in intersecting data on the distributions of pollutants, and/or the patterns of movements of recipient individuals or groups, reflecting the limitations of available data on environmental conditions and human distributions. All these spatial dimensions are needed for an accurate estimation of the individual exposome.

Confounders or effect modifiers: chemical exposure may be associated with other risk factors that should be precisely measured to prevent underestimation or overestimation of toxicant effects (Julvez et al., 2013). These factors are usually taken into account as confounders or effect modifiers in the result interpretation: among them, the most frequently considered are child sex and age, and SES indicators, whose association with neurocognitive outcomes (the higher the SES, the better the outcome) has been widely demonstrated (for a review on the effect of SES on the neurocognitive performance see (Hackman and Farah, 2009)). Together with their role as confounders or effect modifiers with respect to toxicant exposure evaluation, these factors can have a direct beneficial or harmful effect on neurodevelopment that should be studied *per se*. For example, SES indicators, including education and/or income of either child's parents or the community have been shown to contribute to ASD prevalence - the higher the "community" SES, the higher the probability of ASD diagnosis for children affected, especially for mild cases, and to ASD risk - the lower the family income, the higher the risk of ASD (Rai et al., 2012).

1.4 Gene – environment interaction

The genetic susceptibility: Among the potential sources of inter-individual variation in susceptibility to chemicals in population studies are genetic polymorphisms. Common gene variants may induce susceptibility to environmental factors by increasing or decreasing physiological responses to common effects from intrauterine infections and cytokines, or from environmental toxins, through the mother's internal or external environment (Connors et al., 2008). Single Nucleotide Polymorphisms (SNPs), namely variations at a single position in a DNA sequence among individuals, are the most common type of human genetic variation. In the past decade much research has been devoted to find SNPs that may help predict an individual's response to certain drugs, susceptibility to environmental pollutants, and risk of developing particular diseases (Christensen and Murray, 2007). In particular, it is suggested that variations in a group of so called "environmental responsive genes" may confer higher vulnerability to the adverse effects of environmental toxicants (Livingston et al., 2004). Notably, several studies have identified SNPs in genes involved in the detoxification of environmental pollutants in some individuals with ASD, and it is estimated that more than 100 such genes may contribute to ASD risk (Herbert et al., 2006). The number of studies investigating the role played by genetic polymorphisms in modifying neurotoxicity has increased rapidly in recent years, but the results of these epidemiologic studies are far from being consistent.

Mercury (Hg) is one of the most well studied environmental pollutants: notwithstanding its recognized neurotoxic effects at low doses, several epidemiological studies have yielded conflicting results as for the effects of children's neuropsychological development.

The hypothesis that genetic factors may increase susceptibility to MeHg toxicity, thus accounting for the great variability in effects' size, has been addressed by several epidemiological studies, by considering gene mutations that affect the absorption, distribution, metabolism and elimination of elemental Hg and MeHg in the body. Recently, the Avon Longitudinal Study of Parents and Children (Bristol, UK) analyzed the association between prenatal MeHg exposure and IQ scores at 8 years in 1135 children for whom data on 247 SNPs within relevant genes were available. Among 40 SNPs showing nominally significant main effects, MeHg interactions with IQ scores were detected for paraoxonase 1 (PON1), progesterone receptor, transferrin and brain-derived neurotrophic factor. Thus, heterogeneities in several relevant genes not specifically linked to Hg uptake or excretion indicate possible genetic predisposition to Hg neurotoxicity in a substantial proportion of a population with a low level of Hg exposure (Julvez et al., 2013). In addition, a very recent study carried out by Woods and coworkers (Woods et al., 2014) chose thirteen candidate genes identified as associated with various neuropsychiatric disorders and also with alterations in Hg toxicokinetics and tissue distributions (Woods et al., 2014). In particular, these genes present different variants that modify the effects of Hg exposure on a broad or limited range of neurobehavioral functions in children. Of the 13 genes evaluated, only four of them showed variants that significantly alter the effects of Hg exposure on neuropsychological domains in children: the gene encoding the heme pathway enzyme, CPOX4, the MTs with their two isoforms MT1M and MT2A and the cathecol-O-methyltransferase gene (COMT) that has been linked to diverse neuropsychiatric conditions such as schizophrenia and ADHD. In conclusion, the observations raised by different epidemiological studies seem to support the hypothesis that some genetic variants may be in part responsible for the increased susceptibility of the negative effects of Hg exposure on neurobehavior in children (see Table X). However, the social environment, the potential for MeHg to interact with other chemicals present in marine food and the protective effects of some nutrients present in fish (e.g. PUFA, selenium) might also significantly contribute to the final behavioral outcome (Rice, 2008). We must also consider that the clinical significance of a gene-environment interaction at the population level depends not only on the size of the effect of a genetic variant on toxicity, but also on the frequency of that variant in the population (Bellinger, 2007).

Genetic polimorphism (Single nucleotid Polymorphism, SNP)	Hg source	Size of the study	Reference
ABC (**A**TP **B**inding **C**assette) transporter genes	Environmental exposure (fish diet)	1651 (birth cohorts)	Llop et al. 2014
PON-1, Progesteron Receptor, Trasferrin, Brain Derived Neurotrophic Factor (BDNF)	Not known (Avon longitudinal study of parents and children)	1135 Cord blood + neuropsychological assessment at 8y	Julvez et al. 2013
Metallothionein	Dental amalgalm tooth filling	505/330 (8-12 y)	Woods et al. 2013
Glutathione-related genes	Environmental exposure (fish diet)	400 (adults)	Barcelos et al. 2013
Apolipoprotein E (APOE)	Environmental exposure (fish diet)	180 (0-2y)	Ng et al. 2013
Catechol -O- methyltransferase (COMT)	Dental amalgalm tooth filling	505/330 (8-12 y)	Woods et al. 2014

1.5 The HEALS geo-data base

Modelling the mobility patterns of the population at risk at the individual level is challenging. There are considerable conceptual and computational difficulties involved in intersecting data on the distributions of pollutants, and/or the patterns of movements of recipient individuals or groups, reflecting the limitations of available data on environmental conditions and human distributions.

1.6 Assessing the health effects: measuring the health outcome

A critical question, which is of particular significance for risk assessment, is that of the robustness of the outcome measurements. 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 (and this holds true for the cohorts examined in HEALS) 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). Whether these sub-clinical behavioral

alterations may signal increased risk to develop a frank neuropsychiatric disorder at some point in the individual's life course remains to be determined.

While consideration of specific clinical diagnoses is indeed important, it is equally critical to include in the EWAS also subclinical effects of environmental exposures on neurodevelopment or intermediate phenotypes like in the case of 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. This aspect has been carefully considered by WP15 in examining the cohort data available. 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 et al., 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. 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).

For selecting the HEALS cohorts more adapted to perform EWAS and then comparing results, selection was based on a review of the literature and on specific criteria. A subset of tests was identified: the following website includes tables that summarise the developmental neurotoxicology literature that applied standardised test outcomes (http://www.nationalchildrensstudy.gov/research/reviewsreports/Pages/Neuropsychological-Assessments-in-Children-from-a- Longitudinal-Perspective-for-the-National-Children-s-Study.pdf). Information on the different neuropsychological tests applied at different ages is also available at www.**childrenshealthfund**.org/.../dev-and-mental-**health**-primary-care-**screening**-**tools**.pdf a document prepared by the Children's Health Fund that presents a synthetic description of test and behavioural domains assessed.

In addition, structure-function relationships have been described for many of the tests, relating impaired performance on certain tests to particular structures of the CNS. 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).

Comparison among studies would also be facilitated if common endpoints were assessed at the same developmental stages, preferably using the same assessment tools and adjusting

for standard sets of covariates. Two of the cohorts selected by WP15 HEALS adopted the Bailey Scale of Infant Development (REPROPL, PHIME) though at different ages. EDEN children were tested at later ages thus with different test batteries. For age 0.5–2 years, the Bayley Scales of Infant Development- III are recommended. Although other scales exist (eg, Fagan test, Brazelton Scale), the Bayley Scale has the best standardisation and has been used extensively in previous exposure-outcome research (i.e lead,PCBs, methylmercury.

1.7 The internal exposome: Biomarkers of effects/ Selection of omic biomarkers related to the behavioural phenotype

(DENIS? Possibly we should make reference to recent studies linking metabolomic, transcriptomic or DNA methylation to the neural or behavioural outcome)

Finally yet importantly, a critical issue of importance for the exposome perspective is the identification and validation of peripheral biomarkers of effects that can inform on typical and atypical brain development, and help to establish biologically plausible links between chemical exposure and health effects. The brain, the target organ of neurotoxicants, it is not accessible if not using highly invasive or extremely costly (e.g. neuroimaging) methods. The same holds true for biomarkers of effect, which should measure early biological changes related to exposure and also predict health effects. To date no available biomarker is a clear and validated indicator of typical brain development: recent studies suggest that levels of inflammatory cytokines in amniotic fluid could predict ASD risk (Goines and Ashwood, 2013); placental miRNA expression profiles and DNA methylation of specific genes are associated with measures of neurobehavioral outcome in the infants as well as with increased risks of neurological and neurodegenerative diseases (Sheinerman and Umansky, 2013); altered amyloid-beta protein in plasma is related with neurodegenerative risk after prenatal Pb exposure (Mazumdar et al., 2012). It is widely acknowledged that much research is needed to identify omics biomarkers in peripheral tissues that can reliably inform on typical and "abnormal" brain development. Even if animal models can be extremely useful to validate peripheral biomarkers as informative measures also of CNS status (e.g. for oxidative stress biomarkers (De Felice et al., 2014; Minghetti et al., 2013)) evidence is still scant on this topic.

1.8 The HEALS geo-data base

All the data gathered in HEALS are contained in the HEALS geo-data base that include data according to spatio-temporal distributions.

References first chapter

1. Andersen, S.L. Trajectories of brain development: Point of vulnerability or window of opportunity? *Neurosci Biobehav Rev* **2003**, *27*, 3-18.

2. London, L.; Beseler, C.; Bouchard, M.F.; Bellinger, D.C.; Colosio, C.; Grandjean, P.; Harari, R.; Kootbodien, T.; Kromhout, H.; Little, F.*, et al.* Neurobehavioral and neurodevelopmental effects of pesticide exposures. *Neurotoxicology* **2012**, *33*, 887-896.

3. Grandjean, P.; Landrigan, P.J. Neurobehavioural effects of developmental toxicity. *Lancet Neurol* **2014**, *13*, 330-338.

4. Grandjean, P.; Landrigan, P.J. Developmental neurotoxicity of industrial chemicals. *Lancet* **2006**, *368*, 2167-2178.

5. Bellinger, D.C. Interpreting epidemiologic studies of developmental neurotoxicity: Conceptual and analytic issues. *Neurotoxicology and teratology* **2009**, *31*, 267-274.

6. Rubin, B.S. Bisphenol a: An endocrine disruptor with widespread exposure and multiple effects. *J Steroid Biochem Mol Biol* **2011**, *127*, 27-34.

7. Julvez, J.; Smith, G.D.; Golding, J.; Ring, S.; Pourcain, B.S.; Gonzalez, J.R.; Grandjean, P. Prenatal methylmercury exposure and genetic predisposition to cognitive deficit at age 8 years. *Epidemiology* **2013**, *24*, 643-650.

8. Hackman, D.A.; Farah, M.J. Socioeconomic status and the developing brain. *Trends Cogn Sci* **2009**, *13*, 65-73.

9. Rai, D.; Lewis, G.; Lundberg, M.; Araya, R.; Svensson, A.; Dalman, C.; Carpenter, P.; Magnusson, C. Parental socioeconomic status and risk of offspring autism spectrum disorders in a swedish population-based study. *J Am Acad Child Adolesc Psychiatry* **2012**, *51*, 467-476 e466.

10. Jurewicz, J.; Polanska, K.; Hanke, W. Chemical exposure early in life and the neurodevelopment of children--an overview of current epidemiological evidence. *Ann Agric Environ Med* **2013**, *20*, 465-486.

11. Bellinger, D.C. A strategy for comparing the contributions of environmental chemicals and other risk factors to neurodevelopment of children. *Environmental health perspectives* **2012**, *120*, 501-507.

12. Grosse, S.D.; Matte, T.D.; Schwartz, J.; Jackson, R.J. Economic gains resulting from the reduction in children's exposure to lead in the united states. *Environmental health perspectives* **2002**, *110*, 563-569.

27. Bouchard, M.F.; Bellinger, D.C.; Wright, R.O.; Weisskopf, M.G. Attention-deficit/hyperactivity disorder and urinary metabolites of organophosphate pesticides. *Pediatrics* **2010**, *125*, e1270-1277.

28. Choi, A.L.; Sun, G.; Zhang, Y.; Grandjean, P. Developmental fluoride neurotoxicity: A systematic review and meta-analysis. *Environmental health perspectives* **2012**, *120*, 1362-1368.

29. Engel, S.M.; Wetmur, J.; Chen, J.; Zhu, C.; Barr, D.B.; Canfield, R.L.; Wolff, M.S. Prenatal exposure to organophosphates, paraoxonase 1, and cognitive development in childhood. *Environmental health perspectives* **2011**, *119*, 1182-1188.

30. Miodovnik, A.; Engel, S.M.; Zhu, C.; Ye, X.; Soorya, L.V.; Silva, M.J.; Calafat, A.M.; Wolff, M.S. Endocrine disruptors and childhood social impairment. *Neurotoxicology* **2011**, *32*, 261-267.

31. Rauh, V.; Arunajadai, S.; Horton, M.; Perera, F.; Hoepner, L.; Barr, D.B.; Whyatt, R. Seven-year neurodevelopmental scores and prenatal exposure to chlorpyrifos, a common agricultural pesticide. *Environmental health perspectives* **2011**, *119*, 1196-1201.

32. Calderon-Garciduenas, L.; Mora-Tiscareno, A.; Ontiveros, E.; Gomez-Garza, G.; Barragan-Mejia, G.; Broadway, J.; Chapman, S.; Valencia-Salazar, G.; Jewells, V.; Maronpot, R.R.*, et al.* Air pollution, cognitive deficits and brain abnormalities: A pilot study with children and dogs. *Brain Cogn* **2008**, *68*, 117-127.

33. Volk, H.E.; Lurmann, F.; Penfold, B.; Hertz-Picciotto, I.; McConnell, R. Traffic-related air pollution, particulate matter, and autism. *JAMA Psychiatry* **2013**, *70*, 71-77.

34. Froehlich, T.E.; Anixt, J.S.; Loe, I.M.; Chirdkiatgumchai, V.; Kuan, L.; Gilman, R.C. Update on environmental risk factors for attention-deficit/hyperactivity disorder. *Curr Psychiatry Rep* **2011**, *13*, 333-344.

Herbstman JB1, Sjödin A, Kurzon M, Lederman SA, Jones RS, Rauh V, Needham LL, Tang D, Niedzwiecki M, Wang RY, Perera F Prenatal exposure to PBDEs and neurodevelopment. Environ Health Perspect. 2010 May;118(5):712-9. doi: 10.1289/ehp.0901340. Epub 2010 Jan 4.

Lezak M. Neuropsychological assessment. 4th ed. New York: Oxford, 2004.

.Needleman HL. The neurobehavioral consequences of low lead exposure in childhood. Neurobehav Toxicol Teratol 1982;4:729–32.

Grandjean P, Weihe P, White RF, et al. Cognitive deficit in 7-year-old children with prenatal exposure to methylmercury. Neurotoxicol Teratol 1997;19:417–28.

Bellinger DC, Needleman HL, Leviton A, et al. Early sensory-motor development and prenatal exposure to lead. Neurobehav Toxicol Teratol 1984;6:387–402.

Walkowiak J, Wiener JA, Fastabend A, et al. Environmental exposure to polychlorinated biphenyls and quality of the home environment: effects on psychodevelopment in early childhood. Lancet 2001;358:1602–7.

Winneke G, Bucholski A, Heinzow B, et al. Developmental neurotoxicity of polychlorinated biphenyls (PCBs): cognitive and psychomotor functions in 7-month old children. Toxicol Lett 1998;102–103:423–8.

Davidson PW, Myers GJ, Cox C, et al. Longitudinal neurodevelopmental study of Seychellois children following in utero exposure to methylmercury from maternal fish ingestion: outcomes at 19 and 29 months. Neurotoxicology 1995;16:677–88.

Jedrychowski W, Jankowski J, Flak E, et al. Effects of prenatal exposure to mercury on cognitive and psychomotor function in one-year-old infants: epidemiologic cohort study in Poland. Ann Epidemiol 2006;16:439–47.

2 The HEALS cohorts measuring neuropsychological outcomes in children

This chapter will describe the three birth cohorts included in HEALS where the neuropsychological outcome was measured in children with standardised test batteries. We decided to consider only those cohorts where motor, cognitive and language scores were recorded by trained neuropsychologists, excluding those studies where the assessment of the psychological wellbeing of the child was derived by parent/caregiver or teacher report. As described below, each cohort considered the exposure to both chemical (phthalates, passive smoking, PAH, heavy metals, PM10, phenols, NO2) and nutritional factors during the prenatal, perinatal or postnatal stage. Several "confounders" were also examined, including SES, maternal stress, dietary factors, maternal lifestyle. The age at testing was different for three cohorts considered, but they included at least assessment in the first two years of age. There are significant differences also in the matrices where biomarkers of exposure were measured even if all the three cohorts have comparable measures in the cord blood (see Table 2).

Table 2. Cohort description

COHORT	Exposure biomarkers	WHERE: Matrix	WHEN: Sample Collection	Outcome	Confounders
REPRO_PL (Poland)	ETS (cotinine)	Saliva Urine	Prenatal Postnatal	Bailey Scales of Infant Development 1 and 2-3 years of age	SES,
	Nutrition (Se, Zn, Cu, Mg Vit A, E)	Blood, cord blood	Prenatal and delivery		Diet and Lifestyle (Smoking, Coffee, Alcohol, Physical activity),
	PAH (1-OHP)	Urine	Prenatal		Maternal stress,
	Phthalates (various metabolites)	Urine	Prenatal and postnatal		Environmental exposures,
	Heavy metals (Pb, Hg)	Cord blood Hair	Delivery Prenatal		Genetic polymorphism (DNA repair, antioxidative enzymes -GPx1, GPx4 –genes)
PHIME (Slovenija, Croatia, Italy, Greece)	Heavy metals (Cd, Pb, As, Hg, Mn)	Hair, cord blood, milk, meconium	Delivery and postnatal	Bailey Scales of Infant Development 1.5 years of age	SES (work and level of education),
	Nutrition (Se, Cu, Zn, Fe, Mg, Ca)	Cord blood, plasma, serum, milk		(follow-up ongoing for Slovenija and Italy)	Diet and lifestyle,
					(Smoking, Coffee, Alcohol),
					Parental mental health,
					Environmental exposures,
					Genetic polymorphisms
EDEN (France)	Phenols, Phthalates	Urine	Prenatal	Language 2-6 years	SES,
	NO2, PM10 (external)		Prenatal and delivery	Brunet-Lezine cognitive and motor development (delivery-6 years of age)	Diet and lifestyle (Smoking, Coffee, Alcohol, Physical activity),
				PEG moving task (2-3 years	Maternal stress,
				Nepsy (2-6 years)	Environmental exposures
				Wipsy (4-6 years)	

Kinga, Milena, Isabella: if possible please insert here a description of the cohort and the main results with respect to the effects of environmental stressors on the neuropsychologicl outcome)

2.1 The Polish Mother and Child Cohort (REPRO_PL)

2.1.1 Aim

REPRO_PL aims at evaluation of the impact of exposure to environmental and lifestyle-related factors during pregnancy and after birth on pregnancy outcomes and children's health and neurodevelopment. The list of research objectives is not final as additional questions may be formed and answered along with obtaining results of the REPRO_PL study.

2.1.2 Study design and population

Polish Mother and Child Cohort (REPRO_PL cohort) is a multicenter prospective cohort that was established in 2007. A detailed description of the cohort methodology has been published by Polanska et al. (2009, 2011). Briefly, the women were recruited into the study if they fulfilled the following criteria: single pregnancy up to the 12th week of gestation, no assisted conception, no pregnancy complications and no chronic diseases as specified in the study protocol. The study subjects were interviewed once in each trimester of pregnancy to collect and update information about environmental, occupational and lifestyle factors, sociodemographic data, medical, and reproductive history. In addition, during each visit and after the delivery, biological samples (including saliva, urine, blood, cord blood, and hair) were collected.

One year after the child's birth an invitation letter was sent to mothers participating in the REPRO_PL cohort proposing to have the child's exposure, health status, and neurodevelopment examined. Within the next two weeks a phone call was made to schedule a (mother and child) visit at the medical center for an examination by a pediatrician as well as a psychologist/child development specialist. The same procedure was repeated when the child was 24 months old.

Current stage of the study is focusing at child examination at age of 7 years (covering exposure, health status and psychomotor assessment - the Strength and Difficulties Questionnaire and the Intelligence and Development Scales).

Figure 1.

2.1.3 Current stage of the study

The Figure below shows the structure and response/follow-up rate of the REPRO_PL cohort. We obtained all the scheduled data during pregnancy and after the delivery as well as detailed information on the newborns from 1045 women out of 1763 women who fulfilled the inclusion criteria and agreed to participate in the study (59.3%). At least one visit during pregnancy was made and data on the newborn as obtained in the case of 221 women (12.5%), and in the case of 368 women (20.9%) at least 2 visits during pregnancy took place but the outcome data was not available. The remaining mothers either experienced a miscarriage of fetal/infant death (42 women, 2.4%), refused to participate in the follow-up visits (82 women, 4.7%) or were lost in the follow-up (5 women, 0.3%).

Taking into account organizational feasibility of the study, Phase II of the REPRO_PL cohort covering child examinations at the age of 1 and 2 was realized in 2 regions of Poland (Łódź and Legnica). At that stage of the study, we examined 547 out of 876 children who were invited to participate (62.4%). Among them, 310 children had both examinations (at the age of 1 and 2), 199 were examined at the age of 1 year and 38 only at 2 years of age (Figure 2). Reduction in the follow-up numbers resulted from withdrawal, child health problems and loss-to-follow-up due to an unknown address and telephone number. At the age of 7 about 900 children are planned to be examined.

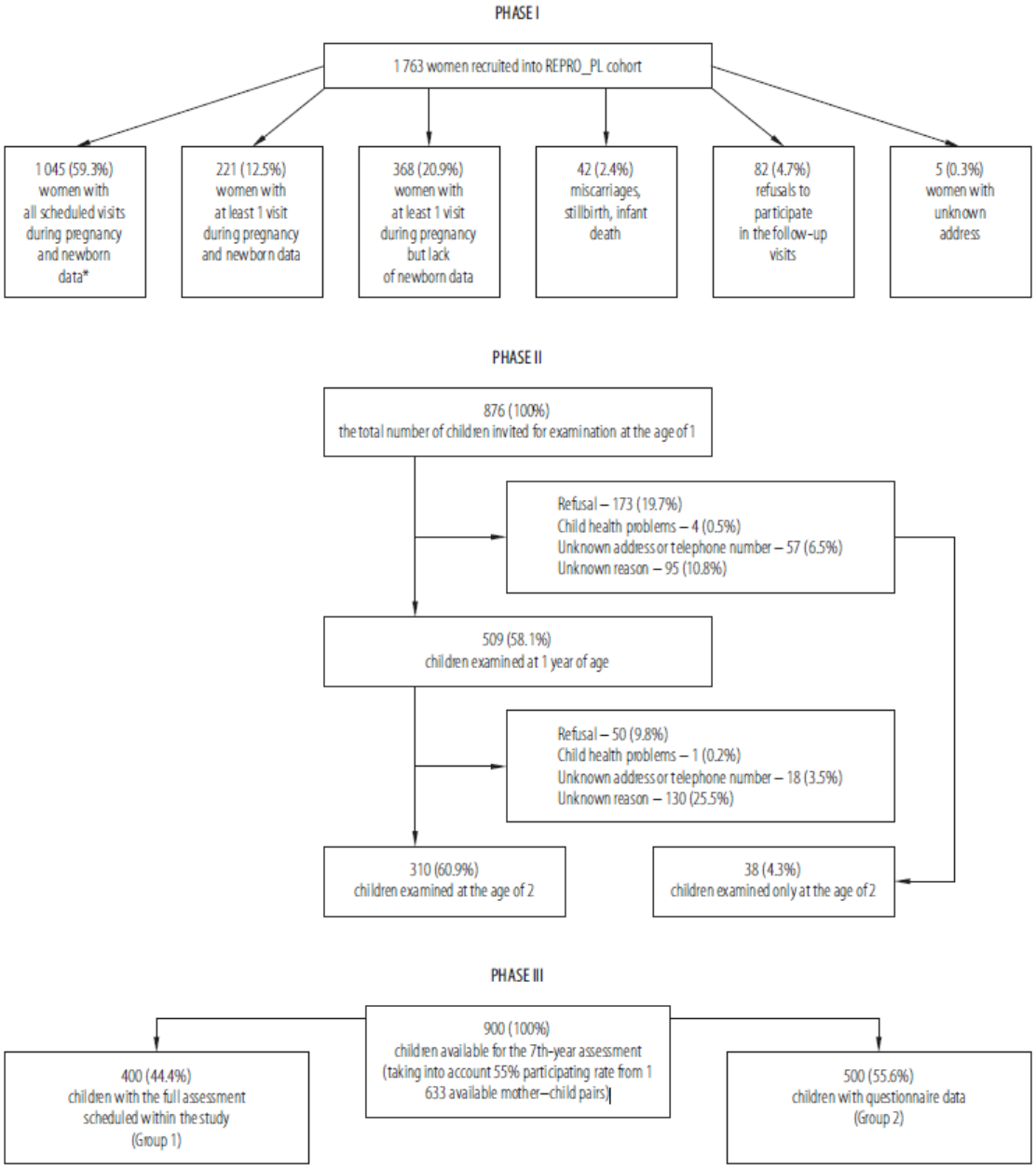

Figure 2.

1. *Polańska K., Hanke W., Gromadzińska J., Ligocka D., Gulczyńska E., Sobala W., Wąsowicz W.: Polish mother and child cohort study - defining the problem, the aim of the study and methodological assumptions. International Journal of Occupational Medicine and Environmental Health 2009;22(4):383-391.*

2. *Polańska K., Hanke W., Jurewicz J., Sobala W., Madsen C., Nafstad P., Magnus P.: Polish Mother and Child Cohort Study (REPRO_PL) - methodology of follow-up of the*

children. International Journal of Occupational Medicine and Environmental Health 2011; 24(4): 391-398

2.1.4 Child psychomotor assessment

Children's neurodevelopment was assessed at around 12 months (range, 10 to 18 months) and repeated at around 24 months of age (range, 23 to 30 months) using **Bayley Scales of Infant and Toddler Development (third edition—Bayley-III)**. The testing was done in the presence of the mother or relative by trained psychologist or child development specialist. Bayley III is an individually applied examination that assesses the developmental functioning of children up to 42 months. The test presents children with situations and tasks designed to produce an observable set of behavioral responses. It assesses five developmental areas: cognitive, motor, language, social-emotional, and adaptive behavior. The cognitive development was measured using age-appropriate activities such as puzzle completion, matching and recognizing pictures, and pretending to play. Motor scale evaluates fine motor skills, such as visual tracking, reaching and grasping, as well as gross motor skills, such as sitting, crawling, standing, jumping, and walking up and down the stairs. In the area of language, Bayley-III assesses the development of communication skills including recognizing and naming objects. The child psychomotor development measured by row score/chronologic age was yielded with each subtests, and composite scores for language, motor scales, and composite score equivalent for cognitive scale were generated based on data.

The mean composite scores for cognitive, language and motor development of the children from REPRO_PL were on average or high average level in the assessments carried out at one and two years of age.

Characteristics of child neurodevelopment

Variables	N	Mean	SD	Median	Min	Max
Composite score for 1-year old children; N = 500						
Cognitive		107.2	10.4	109.0	80.0	145.0
Language		108.1	13.8	108.0	68.0	141.0
Motor		104.8	15.0	106.0	73.0	151.0
Composite score for 2-year old children; N = 340						
Cognitive		110.0	16.3	109.0	80.0	145.0
Language		102.1	13.0	100.0	74.0	144.0
Motor		110.2	14.3	110.0	73.0	154.0

2.1.5 Pre- and postnatal exposure to environmental and lifestyle-related factors and child neurodevelopment

2.1.5.1 Socio-demographic and lifestyle factors

About 53% of the children were girls. On average, the children were born at the 39th week of gestation (±1.4 week) with the mean birth weight of 3336 g (±482 g). About 54% of the children were breastfed at least for 6 months after their birth. Almost the same percentage (57%) of the children did not have siblings and 7% and 22% of the mothers indicated child day care attendance at one and two years of age, respectively. The mean age of mothers and fathers was above 30 years. Most of the mothers (59%) and 40% of the fathers had a university degree. High proportion of the women were married (75%).

Variables	n or mean	% or SD
Child characteristics		
Gender of the child (N= 538)	252	46.8
Boys	286	53.2
Girls		
Gestational age (weeks); mean; SD (N=522)	39.2	1.4
Birth weight (g); mean, SD (N=522)	3336,2	481.6
Length (cm); mean, SD (N=522)	55.0	3.3
Head circumference (cm); mean, SD (N=465)	34.2	1.6
Type of delivery (N=500)		
Cesarean	180	36.0
Vaginal	320	64.0
Breastfeeding (months) (N=535)		
No	49	9.2
<6	198	37.0
≥6	288	53.8
Age during Bayley test administration (months)		
For one-year olds; mean, SD (N=500)	12,3	1.5
For two-year olds; mean, SD (N=340)	24,4	2.4
Daycare attendance at 12 months (N=516)		
Yes	36	7.0
No	480	93.0
Daycare attendance at 24 months (N=320)		
Yes	69	21.6
No	251	78.4
Number of siblings (N=516)		
0	294	57.0
≥1	222	43.0
Parental characteristics		
Maternal age (years); mean, SD (N=512)	30.5	4.5
Paternal age (years); mean, SD (N=505)	32.5	5.5
Maternal education (N= 538)		
Primary/vocational	46	8.6
Secondary	175	32.5
University	317	58.9
Paternal education (N= 528)		
Primary/vocational	103	19.5
Secondary	216	40.9
University	209	39.6
Marital status (N=535)		
Married	400	74.8
Unmarried	135	25.2
Maternal employment in pregnancy (N= 537)		
Employed	457	85.1
Unemployed	80	14.9

Socio-economic status (N=531)		
Low	7	1.3
Medium	418	78.7
High	106	20.0
Major pregnancy complications (N=500)		
Yes	25	5.0
No	475	95.0
Lifestyle variables		
Maternal pre-pregnancy BMI (kg/m^2); (N=532)		
< 18,5	49	9.2
18,5-24,9	384	72.2
25-29.9	73	13.7
≥30	26	4.9
LTPA (MET indicator) (N=519)		
< 3 or ≥ 3 and < 2,5 h/week	434	83.6
≥ 3 and ≥ 2,5 h/week	85	16.4
Folic acid supplementation before or during pregnancy (N=534)	325	60.9
Yes	209	39.1
No		
Saliva cotinine level in the first trimester of pregnancy (ng/ml); mean, SD (N = 490)	17,5	52.6
Alcohol consumption during pregnancy (N=484)		
Yes	48	9.9
No	436	90.1

About 10% of the women indicated alcohol consumption at least once per month during pregnancy period (Polanska 2015). The mean cotinine level in saliva collected during the first trimester of pregnancy was 18 ng/mL (±53) and about 14% of the study participants were classified as smokers and 50% as exposed to ETS during the pregnancy period. About 9% of the mothers were categorized as underweight and 19% as overweight or obese, based on the pre-pregnancy BMI. Only 16% of the women followed recommendations for recreational physical activity during pregnancy (2.5 h of moderate exercise per week). Sixty one percent of the women indicated folic acid supplementation before and/or within the First trimester of pregnancy at least in the amount recommended by Polish Gynaecological Society. Smoking was negatively correlated with LTPA and folic acid supplementation ($r = -0.3$ and $r = -0.2$; $p<0.05$, respectively).

Polychoric correlation between maternal lifestyle factors during pregnancy.

	Recommended level of LTPA	Folic acid	Smoking	Alcohol	BMI not in normal range
Recommended level of LTPA	1.0				
Folic acid	0.2*	1.0			
Smoking	−0.3*	−0.2*	1.0		
Alcohol	−0.01	−0.09	0.1	1.0	
BMI not in normal range	−0.08	−0.03	−0.1	−0.09	1.0

Smoking during pregnancy – saliva cotinine level > 10 ng/mL.
* $p < 0.05$.

2.1.5.2 Association between maternal lifestyle factors during pregnancy and child neurodevelopment

The analysis with adjustment for a variety of confounders indicated statistically significant negative associations between cotinine level in maternal saliva and child motor development at both assessment points and cognitive development at 24 months of age ($p<0.05$). Child language scores at 12 months ($\beta=-5.7$; $p<0.001$) and cognitive ($\beta=-4.7$; $p = 0.04$) as well as motor scores ($\beta=-5.2$; $p = 0.04$) at 24 months of age were lower for the kids of the mothers who were underweight before pregnancy compared to the kids of the mothers with normal BMI. The recommended level of LTPA during the first trimester of pregnancy was beneficial for child language functions at 24 months of age ($\beta=4.1$;$p=0.04$). Similar results were obtained for the impact of LTPA during the second and third trimesters of pregnancy on language development at 2 years of age ($\beta=4.0$;$p=0.05$ and $\beta=4.2$;$p=0.06$ respectively). In multivariable analysis with inclusion of LTPA in three trimesters and confounding factors the results are not statistically significant. This can result from the correlations of LTPA between trimesters. The final multivariable model with inclusion of all the lifestyle factors and a variety of confounding variables confirmed most of the results observed in adjusted model. Adverse effects of prenatal exposure to tobacco constituents on motor development at one and two years of age ($\beta=-0.8$; $p = 0.01$ and $\beta=-1.4$; $p<0.001$) and maternal pre-pregnancy underweight and child language abilities at one year of age ($\beta=-5.2$; $p = 0.01$); as well as borderline significance ($p= 0.06$) of the associations between underweight and decreased cognitive as well as motor functions at two years of age were reported. The recommended level of physical activity during pregnancy had positive impact on child language abilities at 24 months of age ($\beta=4.8$; $p=0.02$).

Lifestyle factors during pregnancy and child cognitive, language and motor development — model adjusted for the examiner, parental age, parental education, child gender, and for cognitive development additionally marital status and child day care attendance.

Variable	1-year old children β (95% CI)			2-year old children β (95% CI)		
	Cognitive	Language	Motor	Cognitive	Language	Motor
Saliva cotinine level (ng/mL)[a,b]	−0.3 (−0.9 to 0.3)	−0.5 (−1.1 to 0.1)	−0.6 (−1.2 to −0.01)*	−0.8 (−1.6 to −0.02)*	−0.5 (−1.3 to 0.2)	−1.5 (−2.3 to −0.7)**
Alcohol consumption[c,d]						
Yes	−1.5 (−5.2 to 2.3)	−0.9 (−4.6 to 2.9)	−1.9 (−5.9 to 2.1)	−1.6 (−6.2 to 3.0)	−1.4 (−5.9 to 3.1)	−0.2 (−1.7 to 8.1)
No	ref.	ref.	ref.	ref.	ref.	ref.
Pre-pregnancy BMI (kg/m^2)[e,f]						
<18.5	−1.4 (−5.2 to 2.5)	−5.7 (−9.5 to −1.8)**	−1.9 (−6.0 to 2.3)	−4.7 (−9.5 to −0.1)*	−2.7 (−7.4 to 2.1)	−5.2 (−10.3 to −0.1)*
18.5–24.9	ref.	ref.	ref.	ref.	ref.	ref.
≥25	2.8 (−0.04 to 5.7)	1.6 (−1.2 to 4.5)	0.8 (−2.2 to 3.9)	2.2 (−1.4 to 5.7)	1.4 (−2.1 to 4.9)	−0.04 (−3.8 to 3.7)
LTPA (MET indicator)[g,h]						
≥3 and ≥2.5 h/week	−1.9 (−4.9 to 1.1)	−1.4 (−4.4 to 1.6)	−1.3 (−4.6 to 1.9)	0.4 (−3.4 to 4.3)	4.1 (0.2 to 8.0)*	0.9 (−3.3 to 5.0)
<3 or ≥3 and <2.5 h/week	ref.	ref.	ref.	ref.	ref.	ref.
Folic acid supplements[i,j]						
Yes	0.3 (−4.0 to 0.9)	1.1 (−3.5 to 1.3)	0.09 (−2.5 to 2.7)	1.8 (−1.2 to 4.9)	1.3 (−1.7 to 4.3)	1.5 (−1.7 to 4.8)
No	ref.	ref.	ref.	ref.	ref.	ref.

BMI — body mass index; LTPA — leisure-time physical activity; MET — Metabolic Equivalent Task.
β — beta coefficient; p — p value; 95% CI — 95% confidence interval.
For one year old assessment [a]N = 439; [c]N = 408; [e]N = 437; [g]N = 424; [i]N = 438; for two year old assessment [b]N = 295; [d]N = 267; [f]N = 279; [h]N = 269; [j]N = 281.
* p < 0.05.
** p < 0.001.

Lifestyle factors during pregnancy and child cognitive, language and motor development — a multivariable analysis.

Outcomes	β (95% CI)	p
1-year old children[a]		
Language development		
BMI < 18.5 kg/m^2	−5.2 (−9.1 to −1.3)	0.01
Motor development		
Saliva cotinine level (ng/mL)	−0.8 (−1.5 to −0.2)	0.01
2-year old children[b]		
Cognitive development		
Saliva cotinine level (ng/mL)	−0.5 (−1.3 to 0.3)	0.2
BMI < 18.5 kg/m^2	−4.4 (−9.2 to 0.1)	0.06
Language development		
LTPA (MET indicator) ≥3 and ≥ 2.5 h/week	4.8 (0.8 to 8.8)	0.02
Motor development		
Saliva cotinine level (ng/mL)	−1.4 (−2.2 to −0.5)	<0.001
BMI < 18.5 kg/m^2	−4.5 (−9.8 to 0.1)	0.06

Adjusted for: examiner, maternal age, maternal education, child gender, and for cognitive development additionally marital status and child day care attendance.
β — beta coefficient; p — p value; 95% CI — 95% confidence interval.
BMI: body mass index; LTPA — leisure-time physical activity; MET — Metabolic Equivalent Task.
BMI 18.5–24.9 kg/m^2 — ref.
LTPA (MET indicator) <3 or ≥3 and <2.5 h/week — ref.
[a] N = 421.
[b] N = 261.

Polańska K, Muszyński P, Sobala W, Dziewirska E, Merecz-Kot D, Hanke W. Maternal lifestyle during pregnancy and child psychomotor development - Polish Mother and Child Cohort study. Early Hum Dev. 2015 May;91(5):317-25.

2.1.5.3 Maternal stress during pregnancy and child neurodevelopment

Occupational stress measured by the Subjective Work Characteristics Questionnaire (SWCQ) with the mean of 93 points (range 57-180) and the mean number of stressful psychosocial factors at work equal 22 (range 2–50) can be interpreted as moderate. The level of stress measured by Perceived Stress Scale (PSS) (mean 18, range 2-34) was also medium. The mean for the Social Readjustment Rating Scale (SRRS), which was 101 (range 40-435), indicated a low level of stress in life and low probability of developing a stress-related disorder. The mean APGAR Family score was 10 points (range 7-19), which indicates moderate satisfaction with family support and functioning.

Variables	N	Mean	SD	Median	Min	Max
Subjective Work Characteristics Questionnaire						
Sum in points (potential range: 55-275)	372	93.1	23.9	89.0	57.0	180.0
Number of psychosocial factors at work (potential range: 0-55)	372	21.6	9.4	21.0	2.0	50.0
APGAR Family Scale						
Sum in points (potential range 7-21)	372	9.7	2.4	9.0	7.0	19.0
Perceived Stress Scale						
Sum in points (potential range: 0-40)	372	17.5	5.6	17.0	2.0	34.0
Social Readjustment Rating Scale						
Sum of wages (potential range: 40-1398)	367	100.7	78.8	78.0	40	435.0
Subjective stress related to events (sum of scores) (potential range: 0-120)	367	4	5	2	0	26

Negative impact on child cognitive development at the age of two was observed for PSS (β=-0.8; p=0.01) and SRRS (β=-0.4; p=0.03). No association was observed between occupational stress and the analyzed domains of child development. Mother's satisfaction with family functioning and support during pregnancy was not significantly associated with the child's psychomotor development.

	One year old children β (95% CI)			Two years old children β (95% CI)		
	Cognitive	Language	Motor	Cognitive	Language	Motor
Subjective Work Characteristics Questionnaire						
Sum in points	0.006 (-0.05 to 0.06)	-0.02 (-0.08 to 0.04)	-0.01 (-0.07 to 0.05)	-0.06 (-0.1 to 0.02)	-0.002 (-0.08 to 0.07)	0.04 (-0.04 to 0.1)
Number of psychosocial factors at work	0.01 (-0.2 to 0.2)	-0.04 (-0.2 to 0.09)	-0.05 (-0.2 to 0.08)	-0.01 (-0.3 to 0.3)	-0.03 (-0.2 to 0.1)	0.08 (-0.1 to 0.2)
APGAR Family Scale						
Sum in points	-0.6 (-1.5 to 0.3)	-0.4 (-0.9 to 0.2)	-0.3 (-0.9 to 0.3)	-1.4 (-3.3 to 0.4)	-0.3 (-1.1 to 0.4)	-0.6 (-1.3 to 0.2)
Perceived Stress Scale						
Sum in points	-0.2 (-0.6 to 0.2)	-0.02 (-0.9 to 0.8)	-0.01 (-0.8 to 0.8)	**-0.8 (-1.3 to -0.2)***	-0.3 (-0.6 to 0.05)	0.1 (-0.5 to 0.2)
Social Readjustment Rating Scale						
Sum of impact scores	-0.02 (-0.05 to 0.02)	0.001 (-0.02 to 0.02)	-0.003 (-0.02 to 0.02)	-0.03 (-0.08 to 0.03)	-0.01 (-0.04 to 0.02)	-0.02 (-0.04 to 0.01)
Subjective stress related to events	-0.3 (-0.9 to 0.3)	0.02 (-0.3 to 0.3)	0.03 (-0.3 to 0.3)	**-0.4 (-0.8 to -0.05)***	-0.2 (-0.6 to 0.2)	-0.2 (-0.6 to 0.2)

Model adjusted for: examiner, child gender, maternal age, maternal education, cotinine level in maternal saliva during pregnancy, cotinine level in child urine, child health status and for cognitive development additionally marital status, changes in marital status during the study period, and child day care attendance
*p≤0.05

K. Polanska, A. Krol, D. Merecz-Kot, J. Jurewicz, Teresa Makowiec-Dabrowska, F. Chiarotti, G. Calamandrei, W. Hanke Maternal stress during pregnancy and neurodevelopmental outcomes of children during the first two years of life. Journal of Paediatrics and Child Health, in press ***– publication under HEALS project***

2.1.5.4 Microelements during pregnancy and child neurodevelopment

The mean Se level decreased through pregnancy period and at delivery (from 48.3±10.6 µg/l in the first trimester to 38.4±11.8 µg/l at delivery) ($p < 0.05$). A moderate correlation was observed between Se levels in blood collected in the first and the second and between the first and the third trimester of pregnancy ($r = 0.4$) as well as between the second and the third trimester of pregnancy, and between the third trimester of pregnancy and delivery ($r=0.5$). The weakest correlation was observed between Se levels in the three trimesters of pregnancy and cord blood (the lowest between Se levels in the second trimester and cord blood $r=0.08$). The correlation between Se level in maternal blood collected at delivery and in cord blood was 0.4.

Se concentrations during pregnancy at delivery, and in cord blood

Variables	Mean	SD	Min	Max
Se levels (µg/l)				
in the first trimester of pregnancy ($N = 410$)	48.3	10.6	16.1	91.4
in the second trimester of pregnancy ($N = 151$)	42.3	9.1	14.8	69.7
in the third trimester of pregnancy ($N = 130$)	37.3	9.8	14.2	61.7
in maternal blood at delivery ($N = 310$)	38.4	11.8	13.8	86.3
in cord blood ($N = 311$)	31.1	8.2	13.8	56.3

Pearson correlation between the levels of Se in each trimester of pregnancy,

Se levels	Se (first trimester)	Se (second trimester)	Se (third trimester)	Se (maternal blood at delivery)	Se (cord blood)
Se (first trimester)	1				
Se (second trimester)	0.4	1			
Se (third trimester)	0.4	0.5	1		
Se (maternal blood at delivery)	0.3	0.3	0.5	1	
Se (cord blood)	0.1	0.08	0.2	0.4	1

The Zn concentrations in maternal blood slightly decreased through pregnancy period but in cord blood it reached the highest level. In the case of Cu – the lowest level was observed in cord blood (0.6mg/l comparing to about 2mg/ml in maternal blood). The microelements levels in maternal blood were moderately correlated. The weakest correlation was observed between Cu levels in the three trimesters of pregnancy and cord blood).

Zn and Cu concentrations during pregnancy at delivery, and in cord blood

Pearson correlation between Zn and Cu in each trimester of pregnancy (A-first, B-second, C-third trimester) at delivery (D) and in cord blood (E)

	ZnA	ZnB	ZnC	ZnD	ZnE
ZnA	1				
ZnB	0.31	1			
ZnC	0.46	0.49	1		
ZnD	0.18	0.23	0.29	1	
ZnE	0.18	0.22	0.26	0.28	1
CuA	1				
CuB	0.35	1			
CuC	0.17	0.27	1		
CuD	0.18	0.18	0.44	1	
CuE	-0.06	0.07	0.13	0.15	1

The analysis with adjustment for a variety of confounders indicated a statistically significant positive association between Se levels in blood collected in the first trimester of pregnancy and motor functions at 1 (β=0.2, p= 0.002) and language functions at 2 y of age (β=0.2, p=0.03). For cognitive abilities at 2 y of age, the association was of borderline significance (β=0.2, p= 0.05).

Variables (mg/l)	Mean	SD	Min	Max
Zn (first trimester) (N= 392)	0.91	0.27	0.37	2.41
Zn (second trimester) (301)	0.77	0.27	0.23	1.96
Zn (third trimester) (296)	0.74	0.21	0.32	1.45
Zn (maternal blood at delivery) (283)	0.77	0.30	0.26	3.20
Zn (cord blood) (289)	1.10	0.30	0.59	1.41
Cu (first trimester) (N= 392)	1.98	0.57	0.55	4.88
Cu (second trimester) (304)	2.36	0.59	0.81	4.52
Cu (third trimester) (296)	2.55	0.54	0.37	5.0
Cu (maternal blood at delivery) (287)	2.39	0.65	0.66	4.59
Cu (cord blood) (289)	0.59	0.28	0.14	1.72

Selenium level in different pregnancy periods	1-y-old children β (95% CI)			2-y-old children β (95% CI)		
	Cognitive	Language	Motor	Cognitive	Language	Motor
Model 1						
Se (first trimester)	0.01 (-0.1 to 0.1)	0.04 (-0.07 to 0.2)	0.2 (0.1 to 0.4)**	0.2 (0.02 to 0.4)*	0.2 (-0.001 to 0.3)***	0.2 (0.003 to 0.3)*
Se (maternal blood at delivery)	-0.07 (-0.2 to 0.03)	-0.1 (-0.2 to 0.001)	0.1 (-0.1 to 0.2)	0.1 (-0.07 to 0.3)	0.1 (-0.07 to 0.3)	0.1 (-0.1 to 0.3)
Se (cord blood)	0.01 (-0.1 to 0.2)	-0.04 (-0.2 to 0.1)	0.2 (-0.02 to 0.4)	0.1 (-0.1 to 0.3)	0.3 (0.05 to 0.5)*	0.1 (-0.2 to 0.3)
Model 2						
Se (first trimester)	0.04 (-0.1 to 0.1)	0.04 (-0.1 to 0.1)	0.2 (0.1 to 0.4)*	0.2 (-0.003 to 0.4)***	0.2 (0.02 to 0.4)*	0.2 (-0.02 to 0.4)
Se (maternal blood at delivery)	-0.05 (-0.2 to 0.1)	-0.1 (-0.2 to 0.03)	0.1 (-0.1 to 0.2)	0.1 (-0.1 to 0.3)	0.1 (-0.1 to 0.3)	0.1 (-0.1 to 0.3)
Se (cord blood)	0.003 (-0.2 to 0.2)	-0.1 (-0.2 to 0.1)	0.2 (-0.02 to 0.4)	0.1 (-0.2 to 0.4)	0.2 (-0.1 to 0.4)	0.1 (-0.2 to 0.4)

Model 1 is adjusted for the examiner; for 1-y-old assessments: Se (first trimester): $N = 373$, Se (maternal blood at delivery): $N = 301$, Se (cord blood): $N = 301$; for 2-y-old assessments: Se (first trimester): $N = 231$, Se (maternal blood at delivery): $N = 231$, Se (cord blood): $N = 231$. Model 2 is adjusted for the examiner, mother age, mother education, child gender, marital status, and cotinine level; for 1-y-old assessments: Se (first trimester): $N = 368$, Se (maternal blood at delivery): $N = 278$, Se (cord blood): $N = 278$; for 2-y-old assessments: Se (first trimester): $N = 191$, Se (maternal blood at delivery): $N = 158$, Se (cord blood): $N = 157$.
β, beta coefficient; CI, confidence interval.
*$P < 0.05$; **$P = 0.001$; ***$P = 0.05$.

There was positive association between Zn level in maternal blood at delivery and child language abilities (p<0.05) but negative between Zn level in maternal blood collected in first trimester of pregnancy and child motor functions at age 1 (p<0.05). There were no statistically significant associations between Cu levels and any domains of child neurodevelopment.

	1-year old children β (95% CI)			2-year old children β (95% CI)		
Microelements	Cognitive	Language	Motor	Cognitive	Language	Motor
ZnA	-0.7 (-7.5 to 6.1)	-4.2 (-11.2 to 2.8)	**-10.4 (-19.2 to -1.6)***	5.8 (-6.1 to 17.6)	4.3 (-6.1 to 14.6)	-3.5 (-14.7 to 7.6)
ZnD	-1.6 (-7.2 to 3.9)	**7.2 (1.4 to 13.1)***	3.5 (-3.7 to 10.8)	1.2 (-7.8 to 10.2)	5.4 (-2.8 to 13.5)	-1.6 (-10.0 to 6.8)
ZnE	-1.3 (-6.5 to 3.9)	-4.2 (-9.6 to 1.1)	-3.6 (-10.3 to 3.1)	1.2 (-7.7 to 10.1)	-0.01 (-7.7 to 7.7)	-1.1 (-9.3 to 7.1)
CuA	-0.6 (-3.2 to 2.0)	2.2 (-0.5 to 5.0)	1.8 (-1.5 to 5.2)	-2.4 (-7.1 to 2.2)	3.2 (-0.7 to 7.2)	0.4 (-4.0 to 4.7)
CuD	1.3 (-0.9 to 3.5)	-0.1 (-2.4 to 2.2)	-0.4 (-3.3 to 2.5)	1.5 (-2.4 to 5.4)	0.04 (-3.2 to 3.3)	-2.9 (-6.7 to 0.8)
CuE	-0.3 (-5.4 to 4.7)	1.4 (-4.1 to 7.0)	1.9 (-4.7 to 8.5)	6.6 (-2.4 to 15.5)	2.4 (-5.4 to 10.2)	0.6 (-7.8 to 8.9)

model adjusted for examiner, mother age, mother education, child gender, marital status and cotinine level; (A-first trimester, D –maternal blood at delivery, E –cord blood

Polanska K, Krol A, Sobala W, Gromadzinska J, Brodzka R, Calamandrei G, Chiarotti F, Wasowicz W, Hanke W. Selenium status during pregnancy and child psychomotor development-Polish Mother and Child Cohort study. Pediatr Res. 2016 Feb 17. doi: 10.1038/pr.2016.32. ***– publication under HEALS project***

Publications regarding Zn and Cu and child neurodevelopment are under preparation (the work is within HEALS project)

2.1.5.5 Environmental factors during pregnancy (Pb, Hg and PAH) and child neurodevelopment

The geometric mean cord blood lead level was 1 µg/dL with the range 0.4-5.7 µg/dL and the geometric mean hair mercury level was 0.2 µg/g (range 0.02-1.5 µg/g). The range of 1-HP in urine of pregnant women was from 0.01 to 8.5 µg/g creatinine with geometric mean 0.4 µg/g creatinine. There were no statistically significant effects of prenatal exposure to mercury or 1-HP on child psychomotor development. The adverse effect of prenatal lead exposure on cognitive score was of borderline significance (β=-6.2; p=0.06).

Variables	Geometric mean	Mean	SD	Median	Min	Max
Cord blood lead level (µg/dL); N=233	1.0	1.1	0.7	1.0	0.4	5.7
Hair mercury (µg/g); N=227	0.2	0.3	0.2	0.2	0.02	1.5
1-hydroxypyrene level in urine of pregnant women (µg/g creatinine); N=384	0.4	0.5	0.6	0.4	0.01	8.5

Association between exposure to environmental factors and child cognitive, language and motor development

Biomarkers of exposure	Model 1 β (95% CI)			Model 2 β (95% CI)		
	Cognitive	Language	Motor	Cognitive	Language	Motor
Cord blood lead level (log transformed)						
Psychomotor score at 12 and 24 months combined (n=326)	-2.2 (-5.0 to 0.7)*	-3.2 (-6.2 to -0.2)	-2.3 (-5.6 to 0.9)		-2.6 (-5.6 to 0.4)[e]	
Psychomotor score at 12 months (n=233)	-0.3 (-3.1 to 2.5)					
Psychomotor score at 24 months (n=93)	-6.7 (-13.3 to -0.1)			-6.2 (-12.8 to 0.5)[b]		
Hair mercury						
Psychomotor score at 12 and 24 months combined (n=303)	1.6 (-4.4 to 7.6)	2.7 (-3.9 to 9.4)	5.3 (-2.2 to 12.9)			
1-hydroxypyrene level in pregnant women urine						
Psychomotor score at 12 and 24 months combined (n=576)	-0.7 (-2.3 to 1.0)	-0.8 (-2.5 to 0.9)	-1.3 (-3.1 to 0.6)			

*Interaction between age and biomarker of exposure p≤0.05
[a] cumulative value for prenatal period
[b] N= 92; [c] N=576; [d] N=325; [e] N=319; [f] N=189; [g] N=104
Model 1 – adjusted for examiner and age at assessment (one or two years of age)
Model 2 – adjusted for examiner, age at assessment for combined analysis (one or two years of age), parental age, parental education, child gender and for cognitive development additionally marital status and child nursery attendance

Polańska K., Hanke W., Sobala W., Trzcinka-Ochocka M., Ligocka D., Brzeźnicki S., Strugała-Stawik H., Magnus P.: Developmental effects of exposures to environmental factors: The Polish Mother and Child Cohort Study. BioMed Research International (Journal of Biomedicine and Biotechnology) 2013; art. 629716.

2.1.5.6 Phthalate exposure and child neurodevelopment:

The following phthalate metabolites: MEP, MnBP, 3OH-MnBP, and OH-MiNP were detected in the levels above LOD in at least 90% of the urine samples from the mothers and children. In only 14% of the urine samples from children MEHP was above LOD (for mothers this metabolite was detected in 66% of the samples). For prenatal exposure the phthalate metabolites with the highest median concentration were: MEP followed by MiBP and 3OH-MnBP. For children's postnatal exposure MEP, 3OH-MnBP and MnBP were detected with the highest median concentration. For 4 phthalate metabolites, namely MEP, MiBP, MEHP, and MnOP, median levels were higher and for two metabolites (MBzP and OH-MiNP) median levels were lower in the mother's than in the children's urine samples. Correlation analysis was captured for 7 phthalates in the mother and children's urine samples. The levels of the majority of analytes were positively correlated within the same urine sample (with correlation coefficients ranging from $r = 0.2$; $p<0.05$ to $r = 0.8$; $p<0.01$). In the children's urine MnOP was poorly correlated with other phthalates except for DiBP. Mother–child statistically significant correlations were observed for metabolites of DEHP ($r = 0.3$; $p<0.01$) and High-MWP ($r = 0.2$, $p<0.05$). Low-MWP levels in the mothers were poorly correlated with the levels in their children ($r = 0.1$; $p>0.05$).

Urinary phthalate metabolites in Polish mother–child pairs

Phthalate	Phthalate metabolite	LOD	Mothers (N = 165)[a]						Children (N = 148)					
			% >LOD	Mean	SD	Median	P95	Range	% >LOD	Mean	SD	Median	P95	Range
DEP	MEP (μg/l)	0.1	100	81.3	274.0	18.7	336.4	1.5–2922	100	34.3	119.5	9.8	119.6	0.05–1331
	MEP (μg/g creatinine)			105.8	377.7	22.7	394.7	1.7–4235						
DiBP	MiBP (μg/l)	0.03	86	73.8	141.9	10.3	359.1	0.02–812	97	5.8	8.8	2.5	22.9	0.02–57
	MiBP (μg/g creatinine)			106.8	218.1	11.1	563.2	0.02–1239						
DnBP	MnBP (μg/l)	0.1	95	29.4	140.2	3.6	129.5	0.05–1692	98	8.3	11.9	4.0	32.3	0.05–78
	MnBP (μg/g creatinine)			23.0	82.5	4.6	73.8	0.02–846						
	3OH-MnBP (μg/l)	0.1	96	7.4	9.7	4	25.2	0.05–63	90	15.0	33.7	4.4	57.2	0.05–337
	3OH-MnBP (μg/g creatinine)			10.6	16.0	5.0	34.7	0.02–138						
BBzP	MBzP (μg/l)	0.03	49	0.2	0.7	0.03	0.5	0.02–8.8	85	0.9	2.3	0.3	2.9	0.02–24.7
	MBzP (μg/g creatinine)			0.2	0.6	0.05	0.5	0.02–6.7						
DEHP	MEHP (μg/l)	0.03	66	0.4	0.5	0.2	1.3	0.02–3.5	14	1.7	14.7	0.02	1.8	0.02–176
	MEHP (μg/g creatinine)			0.5	0.6	0.2	1.6	0.02–4.3						
	5OH-MEHP (μg/l)	0.1	70	15.4	34.4	2.0	75.1	0.05–256	97	7.2	17.6	2.1	32.0	0.05–190
	5OH-MEHP (μg/g creatinine)			24.1	59.6	2.73	97.0	0.02–431						
	5oxo-MEHP (μg/l)	0.1	92	8.7	20.2	1.3	55.4	0.05–132	79	4.4	23.4	1.2	10.0	0.05–283
	5oxo-MEHP (μg/g creatinine)			10.6	25.2	1.6	72.6	0.04–140						
DiNP	OH-MiNP (μg/l)	0.1	90	4.5	12.0	0.8	21.3	0.05–98.6	99	9.3	20.0	3.0	38.5	0.09–202
	OH-MiNP (μg/g creatinine)			4.8	12.7	1.1	20.3	0.03–112						
	oxo-MiNP (μg/l)	0.03	62	0.4	0.4	0.5	1.1	0.02–3.6	74	0.5	1.4	0.3	1.4	0.02–13.3
	oxo-MiNP (μg/g creatinine)			0.5	0.7	0.4	1.7	0.02–4.2						
DnOP	MnOP (μg/l)	0.03	63	0.4	1	0.2	1.3	0.02–11.2	41	0.9	7.8	0.02	1.0	0.02–95
	MnOP (μg/g creatinine)			0.4	1.1	0.2	1.3	0.02–12.3						
DnBP	∑MnBP and 3OH-MnBP (μmol/l)			0.2	0.7	0.04	0.6	0.0003–7.7		0.1	0.2	0.04	0.4	0.0004–1.8
DnBP	∑MnBP and 3OH-MnBP (μmol/g creatinine)			0.2	0.4	0.06	0.5	0.0002–3.8						
DEHP	∑MEHP, 5OH-MEHP and 5oxo-MEHP (μmol/l)			0.08	0.1	0.02	0.3	0.0004–0.9		0.05	0.2	0.01	0.1	0.0004–2.2
DEHP	∑MEHP, 5OH-MEHP and 5oxo-MEHP (μmol/g creatinine)			0.1	0.2	0.02	0.5	0.0004–1.5						
DiNP	∑OH-MiNP and oxo-MiNP (μmol/l)			0.02	0.04	0.004	0.07	0.00005–0.3		0.03	0.07	0.01	0.1	0.0003–0.7
DiNP	∑OH-MiNP and oxo-MiNP (μmol/g creatinine)			0.02	0.04	0.006	0.07	0.00004–0.4						
∑Low-MWP (μmol/l)				0.9	1.9	0.3	3.6	0.01–15.1		0.3	0.7	0.1	1.1	0.0009–9.0
∑Low-MWP (μmol/g creatinine)				1.2	2.3	0.4	4.5	0.02–21.9						
∑High-MWP (μmol/l)				0.1	0.2	0.03	0.4	0.003–0.9		0.08	0.3	0.02	0.3	0.0008–3.3
∑High-MWP (μmol/g creatinine)				0.1	0.2	0.04	0.6	0.003–1.5						

LOD — limit of detection.
SD — standard deviation.
P95 — 95th percentile.
DEP — diethyl phthalate, MEP — monoethyl phthalate, DiBP — di-iso-butyl phthalate, MiBP — mono-iso-butyl phthalate, DnBP — di-n-butyl phthalate, MnBP — mono-n-butyl phthalate, 3OH-MnBP — 3OH-mono-n-butyl phthalate, BBzP — butyl-benzyl phthalate, MBzP — monobenzyl phthalate, DEHP — di(2-ethylhexyl) phthalate, MEHP — mono(2-ethylhexyl) phthalate, 5OH-MEHP — 5OH-mono(2-ethylhexyl) phthalate, 5oxo-MEHP — 5oxo-mono(2-ethylhexyl) phthalate, DiNP — di-iso-nonyl phthalate, OH-MiNP — 7OH-mono-methyloctyl phthalate, oxo-MiNP — 7oxo-mono-methyloctyl phthalate, DnOP — di-n-octyl phthalate, MnOP — mono-n-octyl phthalate, ∑Low-MWP — the sum of low molecular weight phthalate metabolites (MEP, MiBP, MnBP, 3OH-MnBP and MBzP), ∑High-MWP the sum of high molecular weight phthalate metabolites (MEHP, 5OH-MEHP, 5oxo-MEHP, OH-MiNP, oxo-MiNP and MnOP).
[a] For creatinine-corrected concentrations N = 150.

Correlations (Spearman's rho) between phthalates within the same urine sample. Concentrationsbetween different compounds are shown for maternal urine in the upper right part of the table (creatinine-corrected concentrations, N = 150) and for the urine samples of the children in the lower left half of the table (creatinine-uncorrected concentrations, N = 148).

		Mother (upper right half)						
		MEP	MiBP	DnBP	MBzP	DEHP	DiNP	MnOP
Children (lower left half)	MEP		0.20*	0.35**	0.03	0.20*	0.12	0.13
	MiBP	0.40**		0.61**	0.33**	0.68**	0.14	0.46**
	DnBP	0.45**	0.78**		0.43**	0.57**	0.27**	0.45**
	MBzP	0.33**	0.31**	0.46**		0.29**	0.30**	0.34**
	DEHP	0.29**	0.44**	0.59**	0.35**		0.15	0.50**
	DiNP	0.26**	0.43**	0.53**	0.17*	0.50**		0.12
	MnOP	0.02	0.25**	0.13	0.05	0.12	0.10	

DnBP (∑MnBP and 3OH-MnBP), DEHP (∑MEHP, 5OH-MEHP and 5oxo-MEHP), DiNP (∑OH-MiNP and oxo-MiNP).
Correlations (Spearman's rho) between mother and children (N = 148) for DEHP r = 0.3, p < 0.01; for ∑High-MWP r = 0.2; p < 0.05, for Low-MWP r = 0.1, p > 0.05.
* p < 0.05.
** p < 0.01.

Child motor development was inversely associated with natural log concentrations (μg/g creatinine) of 3OH-MnBP (β=−2.3; 95% CI−4.0 to−0.6), 5OH-MEHP (β=−1.2; 95% CI−2.2 to−0.3), 5oxo-MEHP (β=−1.8; 95% CI−3.3 to−0.2) and sum of DEHP metabolites (β=−2.2; 95% CI−3.6 to−0.8), DnBP metabolites (β=−1.9; 95% CI−3.4 to−0.4), and high molecular weight phthalates (β=−2.5; 95% CI−4.1 to−0.9) in the urine collected from mothers during pregnancy after adjustment for a variety of potential confounders. Additional adjustment for postnatal phthalate exposure did not change the results. Postnatal child exposure to phthalates was not associated with any of the measured scores of child psychomotor development.

Association between creatinine-corrected prenatal urinary concentrations of phthalate metabolites and child cognitive, language, and motor development based on Bayley-III (N = 150)

Creatinine-corrected phthalate metabolites[a]	Model 1 β (95% CI)			Model 2 β (95% CI)		
	Cognitive	Language	Motor	Cognitive	Language	Motor
MEP	−0.3 (−1.8 to 1.2)	−0.2 (−1.6 to 1.3)	−0.5 (−2.2 to 1.3)	0.3 (−1.3 to 1.8)	0.2 (−1.2 to 1.7)	0.1 (−1.5 to 1.8)
MiBP	−0.6 (−1.3 to −0.01)*	−0.5 (−1.1 to 0.1)	−1.2 (−1.9 to −0.5)**	−0.3 (−1.0 to 0.4)	−0.3 (−1.0 to 0.3)	−0.6 (−1.3 to 0.2)
MnBP	−1.1 (−2.3 to 0.1)	−1.0 (−2.1 to 0.2)	−2.2 (−3.5 to −1.0)**	−0.5 (−1.8 to 0.7)	−0.6 (−1.8 to 0.6)	−1.2 (−2.5 to 0.1)
3OH-MnBP	−1.9 (−3.3 to −0.5)*	−1.6 (−2.9 to −0.2)*	−3.8 (−5.3 to −2.3)**	−0.5 (−2.2 to 1.2)	0.4 (−1.1 to 2.0)	−2.3 (−4.0 to −0.6)*
DnBP	−1.7 (−3.1 to −0.4)*	−1.4 (−2.7 to −0.1)*	−3.2 (−4.7 to −1.8)**	−0.8 (−2.3 to 0.7)	−0.4 (−1.9 to 1.0)	−1.9 (−3.4 to −0.4)*
MBzP	−1.5 (−3.0 to −0.01)*	−0.8 (−2.2 to 0.6)	−1.3 (−3.0 to 0.3)	−1.0 (−2.6 to 0.6)	−0.6 (−2.1 to 0.9)	−0.4 (−2.1 to 1.3)
MEHP	−0.1 (−1.4 to 1.3)	−0.7 (−2.0 to 0.6)	0.4 (−1.1 to 2.0)	0.1 (−1.3 to 1.6)	−0.7 (−2.1 to 0.6)	0.2 (−1.3 to 1.7)
5OH-MEHP	−1.0 (−1.8 to −0.2)*	−0.5 (−1.3 to 0.2)	−1.9 (−2.8 to −1.1)**	−0.5 (−1.4 to 0.4)	−0.3 (−1.1 to 0.6)	−1.2 (−2.2 to −0.3)*
5oxo-MEHP	−1.8 (−3.0 to −0.6)*	−0.9 (−2.0 to 0.2)	−2.8 (−4.1 to −1.5)**	−1.1 (−2.6 to 0.3)	−0.1 (−1.5 to 1.2)	−1.8 (−3.3 to −0.2)*
DEHP	−1.8 (−3.0 to −0.6)*	−1.0 (−2.2 to 0.1)	−3.2 (−4.4 to −2.0)**	−0.8 (−2.2 to 0.6)	−0.4 (−1.7 to 0.9)	−2.2 (−3.6 to −0.8)*
OH-MiNP	−0.5 (−2.0 to 0.9)	−0.4 (−1.8 to 0.9)	−0.1 (−1.7 to 1.6)	−0.6 (−2.0 to 0.8)	−0.6 (−1.9 to 0.8)	−1.0 (−2.5 to 0.5)
oxo-MiNP	1.0 (−0.2 to 2.2)	0.4 (−0.8 to 1.6)	2.1 (0.7 to 3.5)*	0.4 (−0.9 to 1.7)	−0.1 (−1.3 to 1.1)	0.6 (−0.8 to 1.9)
DiNP	−0.5 (−2.0 to 0.9)	−0.4 (−1.8 to 0.9)	−0.1 (−1.7 to 1.6)	−0.6 (−2.0 to 0.8)	−0.6 (−1.9 to 0.8)	−1.0 (−2.5 to 0.5)
MnOP	−0.8 (−2.2 to 0.5)	−0.6 (−1.9 to 0.7)	−2.2 (−3.6 to -0.7)*	−0.7 (−2.1 to 0.8)	−0.6 (−2.0 to 0.8)	−1.1 (−2.6 to 0.4)
∑Low-MWP	−1.1 (−2.5 to 0.4)	−1.0 (−2.4 to 0.4)	−2.1 (−3.7 to −0.5)*	0.1 (−1.5 to 1.7)	0.1 (−1.5 to 1.6)	−0.5 (−2.2 to 1.3)
∑High-MWP	−2.1 (−3.5 to −0.7)*	−0.9 (−2.2 to 0.4)	−3.3 (−4.8 to −1.9)**	−1.3 (−2.8 to 0.3)	−0.6 (−2.0 to 0.9)	−2.5 (−4.1 to −0.9)*

DnBP (∑MnBP and 3OH-MnBP), DEHP (∑MEHP, 5OH-MEHP and 5oxo-MEHP), DiNP (∑OH-MiNP and oxo-MiNP), ∑Low-MWP (MEP, MiBP, MnBP, 3OH-MnBP and MBzP), ∑High-MWP (MEHP, 5OH-MEHP, 5oxo-MEHP, OH-MiNP, oxo-MiNP and MnOP).
Model 1 – adjusted for examiner.
Model 2 – adjusted for examiner, parental age, parental education, child gender, pre and postnatal ETS exposure and for cognitive development additionally marital status and child nursery attendance.
[a] Prenatal maternal urinary concentrations were natural log-transformed, urine samples with creatinine values > 0.2 g/l were used, scores of Bayley-III per natural log changes in μg/g creatinine.
* p < 0.05.
** p < 0.001.

Association between postnatal urinary concentrations of phthalate metabolites and child cognitive, language, and motor development based on Bayley-III test (N = 148)

Phthalate metabolites[a]	Model 1 β (95% CI)			Model 2 β (95% CI)		
	Cognitive	Language	Motor	Cognitive	Language	Motor
MEP	−0.1 (−1.6 to 1.3)	−0.1 (−1.5 to 1.3)	−0.6 (−2.4 to 1.2)	0.3 (−1.2 to 1.8)	−0.0 (−1.5 to 1.5)	0.2 (−1.6 to 2.0)
MiBP	0.1 (−1.3 to 1.4)	0.0 (−1.3 to 1.3)	0.5 (−1.1 to 2.1)	0.3 (−1.2 to 1.8)	0.2 (−1.2 to 1.7)	0.4 (−1.3 to 2.2)
MnBP	−0.9 (−2.7 to 0.8)	−0.5 (−2.2 to 1.2)	0.4 (−1.6 to 2.5)	−0.8 (−2.6 to 1.0)	−0.2 (−2.0 to 1.6)	0.9 (−1.2 to 3.0)
3OH-MnBP	−0.8 (−1.9 to 0.2)	−0.4 (−1.4 to 0.7)	−1.5 (−2.7 to −0.3)*	−0.6 (−1.7 to 0.5)	−0.4 (−1.5 to 0.7)	−1.2 (−2.4 to 0.0)
DnBP	−1.0 (−2.7 to 0.7)	−0.6 (−2.2 to 1.1)	−1.6 (−3.5 to 0.4)	−0.3 (−2.1 to 1.4)	−0.2 (−2.0 to 1.5)	−1.0 (−3.0 to 1.0)
MBzP	−2.5 (−3.8 to −1.1)**	−1.0 (−2.3 to 0.2)	−0.8 (−2.3 to 0.8)	−0.8 (−2.3 to 0.7)	0.4 (−1.0 to 1.8)	−0.2 (−1.8 to 1.5)
MEHP	−0.4 (−1.5 to 0.8)	−0.0 (−1.2 to 1.1)	−1.1 (−2.5 to 0.3)	−0.4 (−1.6 to 0.8)	−0.1 (−1.3 to 1.0)	−0.2 (−1.6 to 1.2)
5OH-MEHP	−0.3 (−1.8 to 1.1)	−0.4 (−1.8 to 1.1)	−0.9 (−2.6 to 0.8)	−0.3 (−2.0 to 1.4)	−0.5 (−2.1 to 1.1)	0.2 (−1.6 to 2.1)
5oxo-MEHP	−1.5 (−2.7 to −0.3)*	−1.5 (−2.7 to −0.4)*	−2.2 (−3.6 to −0.8)*	−0.8 (−2.3 to 0.7)	−0.9 (−2.3 to 0.6)	−0.5 (−2.2 to 1.2)
DEHP	−1.1 (−2.7 to 0.4)	−1.2 (−2.7 to 0.3)	−1.9 (−3.7 to −0.2)*	−0.7 (−2.5 to 1.0)	−0.9 (−2.6 to 0.8)	−0.1 (−2.1 to 1.8)
OH-MiNP	0.1 (−1.3 to 1.4)	−0.4 (−1.7 to 0.9)	−0.5 (−2.1 to 1.1)	0.0 (−1.4 to 1.4)	−0.6 (−1.9 to 0.8)	−0.3 (−1.9 to 1.3)
oxo-MiNP	0.5 (−0.8 to 1.8)	0.1 (−1.1 to 1.4)	0.4 (−1.2 to 1.9)	0.2 (−1.1 to 1.5)	0.5 (−0.8 to 1.7)	0.2 (−1.3 to 1.7)
DiNP	0.1 (−1.3 to 1.4)	−0.4 (−1.7 to 0.9)	−0.5 (−2.1 to 1.1)	0.0 (−1.4 to 1.4)	−0.6 (−1.9 to 0.8)	−0.3 (−1.9 to 1.3)
MnOP	1.2 (−0.1 to 2.4)	0.6 (−0.6 to 1.8)	0.3 (−1.2 to 1.7)	0.2 (−1.1 to 1.4)	−0.2 (−1.5 to 1.0)	0.0 (−1.4 to 1.5)
∑Low-MWP	−0.6 (−2.4 to 1.3)	−0.3 (−2.1 to 1.5)	−1.0 (−3.1 to 1.2)	0.0 (−1.9 to 2.0)	−0.1 (−2.0 to 1.9)	0.3 (−2.1 to 2.6)
∑High-MWP	−0.9 (−2.7 to 0.8)	−1.4 (−3.0 to 0.3)	−1.6 (−3.6 to 0.4)	−0.5 (−2.4 to 1.3)	−1.2 (−3.1 to 0.6)	−0.2 (−2.3 to 1.9)

Model 1 – adjusted for examiner.
Model 2 – adjusted for examiner, parental age, parental education, child gender, pre and postnatal ETS exposure and for cognitive development additionally marital status and child nursery attendance.
[a] Postnatal urinary concentrations were natural log-transformed, scores of Bayley-III per natural log changes in μg/l.
* $p < 0.05$.
** $p < 0.001$.

Association between pre and postnatal phthalate exposure and child cognitive, language, and motor development based on Bayley-III (N = 148) — multivariable model.

Phthalate metabolites	Model 3 β (95% CI)		
	Cognitive	Language	Motor
Prenatal exposure			
∑Low-MWP	−0.3 (−2.1 to 1.5)	0.2 (−1.5 to 1.9)	−0.8 (−2.7 to 1.2)
∑High-MWP	−1.2 (−2.9 to 0.5)	0.0 (−1.5 to 1.6)	−2.5 (−4.3 to −0.7)*
Postnatal exposure			
∑Low-MWP	0.1 (−2.0 to 2.2)	−0.1 (−2.2 to 1.9)	−0.3 (−2.7 to 2.2)
∑High-MWP	−0.5 (−2.5 to 1.6)	−1.3 (−3.2 to 0.6)	0.4 (−1.7 to 2.5)

Model 3 – adjusted for confounders as in models 1 and 2 + phthalate metabolites level in child urine (in analysis of the impact of prenatal phthalate on child neurodevelopment) and + phthalate metabolite level in maternal urine (in analysis of the impact of postnatal phthalate on child neurodevelopment).
For prenatal exposure – creatinine-corrected phthalate metabolites.
* $p < 0.05$.

Polanska K, Ligocka D, Sobala W, Hanke W. Phthalate exposure and child development: the Polish Mother and Child Cohort Study. Early Hum Dev. 2014 Sep;90(9):477-85

2.2 The HERACLES (Waste Management) Study

2.2.1.1 Introduction

The HERACLES (Waste Management) Greek cohort, is a cohort study aiming at assessing the contribution of heavy metals environmental contamination due to waste management practices in the urban and peri urban environment associated to children neurodevelopment. The study has been established in 2012. Around 350 children aged 3 to 8 living in the proximity between 0.5 to 12 km were enroled.

2.2.1.2 Data used in the analysis

2.2.1.2.1 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
  - Concentration of heavy metals in the soil of the child address

2.2.1.2.2 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)

2.2.1.2.3 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.

2.3 The PHIME cohort Milena, Janja,

2.4 The EDEN cohort Isabella

Heterogeneous results on NDDs were published using longitudinal data drawn from the French population based birth cohort study (EDEN; N = 1311 mother-child pairs) followed

from the pregnancy onwards. They reported on different stressors, different period of life and different NDDS.

A low family socioeconomic status before pregnancy was the main environmental risk factor for inattention-hyperactivity symptoms at 3 years assessed with the Strengths and Difficulties Questionnaire, and its effect occurred via two pathways (Foulon). The first was a risk pathway, where lower SES was associated with higher maternal depression and anxiety during pregnancy; then to higher maternal and child distress and dysregulation in infancy; and in turn to higher levels of inattention-hyperactivity at 3 years. The second was a protective pathway, where higher SES was associated with longer duration of breastfeeding during infancy; then to better child neurodevelopmental status in toddlerhood; and in turn to lower levels of inattention-hyperactivity at 3 years.

An association between caffeine intake during pregnancy and impaired cognitive development in offspring, a result in line with animal data was also found (Galera).

Air pollution exposure during pregnancy, particularly NO_2 (for which motorized traffic is a major source), was associated with delayed psychomotor development during childhood. 1.25 to -0.11] per increase of 10 µg/m in NO2) (Guxens). Similar trends were observed in most regions. No associations were found between any air pollutant and cognitive development. Due to the widespread nature of air pollution exposure, the public health impact of the small changes observed at an individual level could be considerable.

Higher scores on social withdrawal behaviour as assessed with the Alarm Distress BaBy (ADBB) scale were associated with delays in reaching language milestones, and to a lesser extent with lower motor ability scores (Guedeney). Taking the contribution of social withdrawal behaviour into account may help understand the unfolding of developmental difficulties in children.

More recently, chronicity of maternal depression predicted children's cognitive development at school entry age, particularly in boys. Compared to children of mothers who were never depressed, children of mothers with persistent high levels of depressive symptoms since pregnancy had reduced Verbal IQ, Performance IQ, and Full-scale IQ scores (van der Waerden). This association was moderated by the child's sex, boys appearing especially vulnerable in case of persistent maternal depression. As maternal mental health is an early modifiable influence on child development, addressing the treatment needs of depressed mothers may help reduce the associated burden on the next generation.

EDEN REFERENCES

van der Waerden J, Bernard JY, De Agostini M, Saurel-Cubizolles MJ, Peyre H,

Heude B, Melchior M; EDEN Mother-Child Cohort Study Group.. Persistent maternal

depressive symptoms trajectories influence children's IQ: The EDEN mother-child

cohort. Depress Anxiety. 2016 Sep 7. doi: 10.1002/da.22552. PubMed PMID:

27603172.

Guedeney A, Forhan A, Larroque B, de Agostini M, Pingault JB, Heude B; EDEN mother-child cohort study group.. Social Withdrawal Behaviour at One Year of Age Is Associated with Delays in Reaching Language Milestones in the EDEN Mother-Child Cohort Study. PLoS One. 2016 Jul 8;11(7):e0158426. doi: 10.1371/journal.pone.0158426. PubMed PMID: 27391482; PubMed Central PMCID: PMC4938506.

Galéra C, Bernard JY, van der Waerden J, Bouvard MP, Lioret S, Forhan A, De Agostini M, Melchior M, Heude B; EDEN Mother-Child Cohort Study Group.. Prenatal Caffeine Exposure and Child IQ at Age 5.5 Years: The EDEN Mother-Child Cohort. Biol Psychiatry. 2016 Nov 1;80(9):720-726. doi: 10.1016/j.biopsych.2015.08.034. PubMed PMID: 26444074.

Foulon S, Pingault JB, Larroque B, Melchior M, Falissard B, Côté SM. Developmental predictors of inattention-hyperactivity from pregnancy to early childhood. PLoS One. 2015 May 4;10(5):e0125996. doi: 10.1371/journal.pone.0125996. PubMed PMID: 25938453; PubMed Central PMCID: PMC4418828.

7: Guxens M, Garcia-Esteban R, Giorgis-Allemand L, Forns J, Badaloni C, Ballester F, Beelen R, Cesaroni G, Chatzi L, de Agostini M, de Nazelle A, Eeftens M, Fernandez MF, Fernández-Somoano A, Forastiere F, Gehring U, Ghassabian A, Heude B, Jaddoe VW, Klümper C, Kogevinas M, Krämer U, Larroque B, Lertxundi A, Lertxuni N, Murcia M, Navel V, Nieuwenhuijsen M, Porta D, Ramos R, Roumeliotaki T, Slama R, Sørensen M, Stephanou EG, Sugiri D, Tardón A, Tiemeier H, Tiesler CM, Verhulst

FC, Vrijkotte T, Wilhelm M, Brunekreef B, Pershagen G, Sunyer J. Air pollution during pregnancy and childhood cognitive and psychomotor development: six European birth cohorts. Epidemiology. 2014 Sep;25(5):636-47. doi: 10.1097/EDE.0000000000000133. PubMed PMID: 25036432.

8: Peyre H, Bernard JY, Forhan A, Charles MA, De Agostini M, Heude B, Ramus F. Predicting changes in language skills between 2 and 3 years in the EDEN mother-child cohort. PeerJ. 2014 Apr 1;2:e335. doi: 10.7717/peerj.335. PubMed PMID: 24749012; PubMed Central PMCID: PMC3976115.

3 The EWAS approach: applying the HEALS methodology to real data sets

3.1 Standardization and harmonization of data in view of unraveling the HEAL paradigm

Standardized and harmonized data are needed to allow comparisons among data coming from different studies and to get overall results. The purpose of data standardization is to make disparate sets of data consistent and clear, allowing for an overall analysis, for example a meta-analysis, of multiple studies on the basis of comparable data. Consistency is ensuring that the output is reliable so that related data can be identified using a common terminology and format. Clarity is to ensure that the data can be easily understood by those who are not involved with the data maintenance process.

Data standardization is the critical process of bringing data from different studies into a common format (see figure) that allows for collaborative research, large-scale analysis, and sharing of sophisticated tools and methodologies. This approach is important because d**ata can vary greatly from one study to the other as they** are collected for different purposes. In addition, data may be stored in different formats using different database systems and information models. Lastly, despite the growing use of standard terminologies in healthcare, the same concept (e.g., blood cholesterol) may be represented in a variety of ways from one setting to the next.

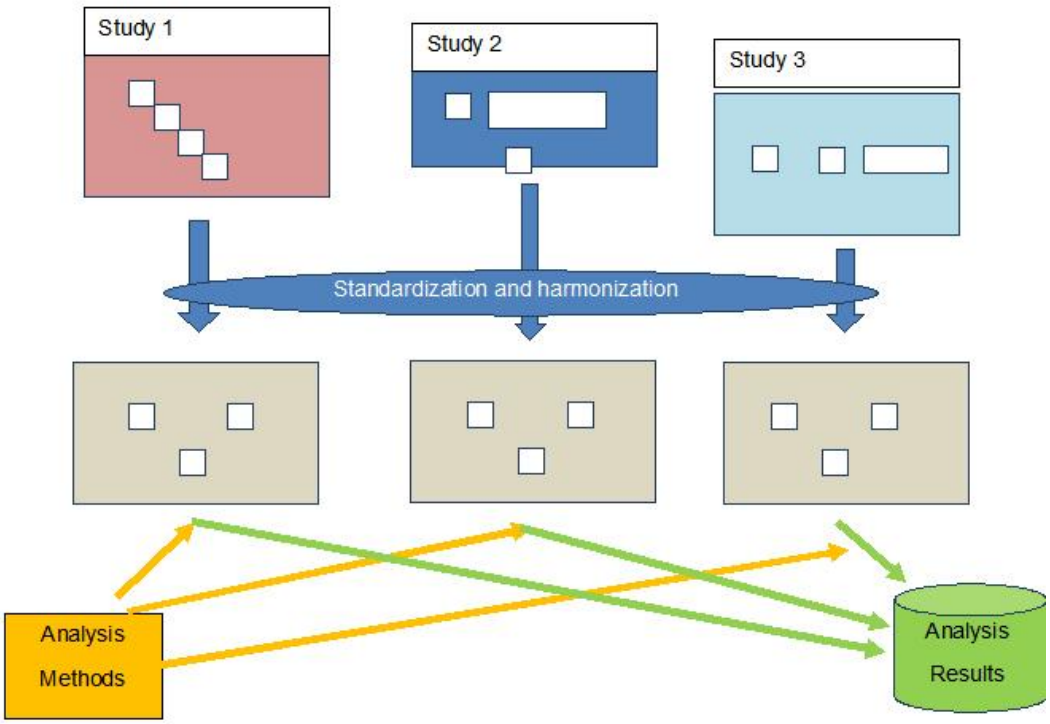

Figure 3.

Data harmonization is the adjustment of differences and inconsistencies between different measurements, methods, procedures, schedules, specifications or systems to make them uniform or mutually compatible. Harmonization also aims to avoid duplication of effort or place an undue burden on informants. The main difference between the harmonization and standardization processes lies in the degrees of strictness of the standards. Standardization entails moving towards the eradication of any variation among variables and data, whereas harmonization involves a reduction in variations.

The HEALS project is deeply involved in the adoption of a stardardized and harmonized common data model in order to provide a unique dataset to build the exposome of various health outcomes.

In HEALS, we have converted a wide variety of datasets from pre-existing population-based studies of singletons and twins into a unique database. Data from each study were combined into a common format as well and variables were converted to a common representation (terminologies, vocabularies, coding schemes). This enables systematic analyses using a library of standard analytic routines that have been written based on the common format.

In HEALS, in order to harmonize the data sets from different cohort studies, a standardized format of the variables were created in an excel file. Altogether, 396 variables were included

in the final file. The individual worksheets consists of 'Identification and individual characteristics', 'Health data by questionnaire and medications used', 'Factors and exposures', 'Exposure assessments', 'Neurodevelopment and neuropsychological outcome', 'Additional Data' and 'Comments regarding the preparation of the data'. For each of the variables, a description, a common variable name, the format of the variables (character/numeric categorical/numeric binary) with the maximum number of digits or letters; along with its domain, core and role are included in the file. This file was sent to the different partners who are responsible for the individual studies and twins registries, such that they can retrieve the information on the required variables from their studies using the standardized format. The partners were contacted via email, with the request for providing their data in the specific standardized format. In addition to the data harmonization file, a 'Data Request and Transfer Agreement Form' was also prepared and attached to the email. For the purpose of data protection and confidentiality, the individual partners were requested to send their data sets encrypted by email to the members responsible for the mentioned work-.

Singleton studies data come from countries are Croatia, France, Italy, Norway, Poland, Portugal, Slovenia, Spain, United Kingdom. Presently, the data obtained from the different singleton studies sources includes information from almost 25000 singleton individuals.

The same approach is being applied in the case of twins. General data from various twins registries across Europe, as well as datasets obtained within the context of specific studies, are being collected and harmonized for the purpose of aiding the health-exposome analyses conducted within HEALS and to provide insight into the role genetics and epigenetics plays in various health outcomes. The twins data is coming from registries of Denmark, Finland, Italy, Netherlands, Norway, Sweden, and the UK. Many of these registries have been collecting data over the course of decades, which makes combining and harmonizing a challenge. Although the detail for each set of twins varies, most include basic health data such as asthma status and some include highly detailed data from biospecimens such as selenium, zinc, arsenic, cadmium, mercury and lead levels within the blood. When completed, we expect to have data on at least 30,000 twins across Europe.

The combined dataset would be used for statistical analysis including EWAS (Environment-Wide Association Studies) relating to the investigation of the exposome impinging on the development and aggravation of chronic diseases. The focus of these analyses in HEALS is asthma and allergies, metabolic and neurodegenerative disorders caused by exposure to several internal and external environmental stressors. The data obtained from the birth-cohorts especially emphasizes on the risk factors associated with early life environmental exposures in case of mother (or both parents) and their offspring.

Altogether, the two datasets, one for singletons and one for twins, combined with internal and external environments data, will be invaluable in terms of exploring the effects of the exposome on health on a population level.

3.2 EWAS.application examples

3.2.1 Comparing the Phime and REPRO_PL cohorts to investigate the effects of the same environmental stressors on the neuropsychological outcome in the first 2 years of life in different countries

When single environmental factors were examined in separate regression analyses (see above in cohort description, CHAPTER 3)

- **PHIME**: significant effects of both SES and maternal IQ, with a complete mediation effect of home environment in affecting cognitive and language domains. Hg prenatal exposure affects neuropsychological outcome in children carrying specific gene polymorphisms.
- **REPRO_PL**: significant effects of phthalates, selenium intake, prenatal stress, physical activity on the neuropsychological outcome

3.2.1.1 Methods

Firstly, we reviewed recent studies applying the EWAS to chronic diseases. A good example of EWAS approach comes from the work by Patel et al. (PLOS One 2010) who studied Type 2 Diabetes (T2D) that is known to result from environmental and genetic factors. Patel and coworkers conducted a pilot Environmental-Wide Association Study (EWAS), in which epidemiological data were comprehensively and systematically interpreted in a manner analogous to a Genome Wide Association Study (GWAS). Logistic regression models were adjusted for age, sex, body mass index (BMI), ethnicity, and an estimate of socioeconomic status (SES). As in GWAS, multiple comparisons were controlled and significant findings were validated with other cohorts. This type of study was made possible by the use of a large number of cross sectional epidemiological data, the National Health and Nutrition Examination Survey (NHANES), a nationally representative, biannual health survey conducted by the Centers For Disease Control and Prevention (CDC). The EWAS consists of two methodological steps that have analogs in a GWAS. First, the authors considered a panel of 266 unique environmental assays, or environmental ‘‘loci’’, measured across cases of diabetics and controls, yielding several environmental factors with significantly high association with T2D while controlling for multiple hypotheses. Second, they validated the associations by taking advantage of data from other cohorts in NHANES. Using a method analogous to GWAS they found five environmental factors associated with the disease, and with a particular SNP that may increases T2D risk when combined with serum levels of nutrients.

3.2.1.2 Can this approach be easily translated to other chronic diseases?

Altogether, the application of the EWAS to neuropsychiatric and neurodegenerative diseases is at its very early beginning. One of the main problems compared with other chronic/metabolic diseases is that related to the nature of the outcome measured, that very often (especially in the case of neurodevelopmental disorders in children) is in the subclinical range and is made up of several diverse variables in different functional domains (i.e. language, motoric competence, attention, cognition etc.). It is very complicated to reconstruct an index representative of strengths and weaknesses of the individual child at a

particular developmental stage. Applying the EWAS analysis to this field is thus a challenging task.

As a first step, the two databases were merged to select the common variables (harmonisation)

For each cohort, we identified the variables found significantly associated with at least one scale (cognition, language, motricity) of the Bayley test in the univariate analysis.

These variables were used in a linear regression model to identify what environmental factors more likely affect neuropsychological functions.

PHIME cohort

Table xxx Relationships between the Bayley scale at 18 months and environmental stressors

	Independent variables														
Dependent variables	**Cu**	**Zn**	**Pb**	**Hg**	**As**	**Mn**	**Se**	**Sibling**	**Coffee**	**Edulevel**	**Passivesmke**	**Diabetespreg**	**BMI delivery**	**Asthresppreg**	**Sex**
Cognitive scale	- 0.312 *0.001*	-	-	-	-		-	-	-	-	-	-	-	-	-
Language scale	-	-	-	-	-	-	-	- 0.181 *0.002*	-	-	-	- 0.143 *0.015*	-	-	- 0.300 *0.000*
Motor scale	-	-	-	-	-	-	-	-	0.101 *0.038*	-	-	-	-	-	-
Fine-Motor subtest	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-0.187 *0.038*
Gross-Motor subtest	-	-	-	-	-	-	-	- 0.112 *0.029*	0.225 *0.000*	-	-	-0.114 *0.024*	-	0.111 *0.029*	-

Legend: Beta values and *statistical significance* of the variables in the linear regression analyses.
ng/g, concentration in cord blood of Cu, Zn, Pb, Hg, As, Mn, Se; Sibling=Number of brothers/sisters,
Coffee= Coffee intake during pregnancy, Edulevel= Level of education of mother ,
Passivesmke=Passive smoke exposure during pregnancy , Diabetespreg=Maternal diabetes during pregnancy,
BMI delivery= Maternal BMI at delivery, Asthresppreg= Maternal asthma or respiratory disease during pregnancy,
negative positive

REPRO_PL cohort

Table xxx Relationships between the Bayley scales at 12 and 24 months

		Independent variables								
Dependent variables		**Zn**	**Pb**	**Se**	**Birth Weight**	**MatWePreg**	**Passive Smoke**	**Smoke Preg**	**Diabetes preg**	**Sex**
Cognitive scale	12 months	--	--	--	--	--	-0.177 *0.000*	--	--	--
	24 months	--	--	--	--	0.112 *0.042*	--	--	--	-0.132 *0.017*
Language scale	12 months	--	--	--	--	--	--	-0.090 *0.047*	--	-0.141 *0.002*
	24 months	--	--	--	--	--	--	--	0.148 *0.045*	-0.291 *0.000*
Motor scale	12 months	--	--	0.118 *0.049*	--	--	--	--	--	--
	24 months	--	--	--	--	--	--	--	--	--

Legend: Beta values and *statistical significance* of the variables in the linear regression analyses.
ng/g, concentration in cord blood of Zn, Pb,Se; Birth Weight = weight of the child at birth,

MatWePreg = Maternal weight before pregnancy, Passive smoke = Passive smoke exposure during pregnancy, Smoke Preg = smoking during pregnancy, Diabetes preg = Maternal diabetes during pregnancy, Sex (0=female, 1=male).

3.2.1.3 Comments

- The comparison of the two cohorts shows differences in 1) matrices where chemical exposure was measured, 2) age of outcome assessment, 3) lifestyle variables collected.
- At this stage harmonisation implies huge reduction of cohort sizes and loss of important information
- The study of the separate cohorts by multivariate regression models allows one to identify the more promising variables on which attention should be focused for building a comprehensive prospective study. However, this is contrary to the untargeted approach we have planned in HEALS.

3.3 Paving the way to EWAS analysis: investigating the role of the exposome on different health outcomes

We then applied multivariate regression models to a subset of the EDEN cohort (n= 100) where two different health outcomes were considered, namely asthma (1-10 years of age), language (2 years) and general IQ (5 years), in relation to exposure to phthalates, and considering many "confounders" indicative of maternal lifestyle. We show here that different health outcomes are affected by different environmental factors, even in the absence of a significant effect of chemical exposure.

Following univariate analyses, we found significant correlation between “asthma_ever” and geographic localization and breastfeeding

		asthma_ever
Latitude	Pearson correlation	-0.250*
	Significance level (two-tailed)	0.012
	N	100
Longitude	Pearson correlation	-0.241*
	Significance level (two-tailed)	0.016
	N	100
Breasfeeding	Pearson correlation	-0.240*
	Significance level (two-tailed)	0.016
	N	100

After stepwise logistic regression (yellow), only breastfeeding has an independent protective effects on asthma development

Correlation between neuropsychological scores and several environmental factors

		Language 24 months	IQ score 5 years
ab_use	Pearson correlation	-,124	**-,212***
	Significance level (two-tailed)	,241	,038
	N	91	96
weight_m	Pearson correlation	**-,255***	-,080
	Significance level (two-tailed)	,015	,441
	N	91	96
height_m	Pearson correlation	,099	**,311****
	Significance level (two-tailed)	,351	,002
	N	91	96
income	Pearson correlation	,059	**,348****
	Significance level (two-tailed)	,576	,001
	N	91	96
bfexpt	Pearson correlation	-,011	**,201***
	Significance level (two-tailed)	,914	,050
	N	91	96
breastfed	Pearson correlation	,155	**,256***
	Significance level (two-tailed)	,143	,012
	N	91	96
gestage_b	Pearson correlation	,019	**-,205***
	Significance level (two-tailed)	,861	,045
	N	91	96

daycare2nd	Pearson correlation	**,220***	,173
	Significance level (two-tailed)	,036	,091
	N	91	96
edu_m	Pearson correlation	**,226***	**,528****
	Significance level (two-tailed)	,031	,000
	N	91	96

Stepwise linear regression showed that:

- language development at 2 years is negatively affected by maternal weight at delivery
- both language development at 2 years and IQ at 5 years

are influenced by the education level of the mother, the former more strongly than the latter. With increasing age the "educational factor" becomes more prominent, incorporating other sociodemographic and lifestyle characteristics

- latitude and longitude are also positively correlated to the IQ level at 5 years of age: the higher the latitude and the longitude, the higher the IQ. Reasons for this correlation must be still elucidated (sociodemografic factors?)

3.3.1.1 Comments

To sum up, the application of classical statistical analysis to two different health outcomes simultaneously showed how they share the exposome.

3.4 Comprehensive EWAS analysis

To further investigate the origins of neurovelopmental disorders, a more comprehensive EWAS analysis took place using the selected datasets of the REPRO_PL and the HERACLES studies for which the number of exposome factors is elevated. The EWAS framework and analysis approach applied herein, is based on the GWAS and the framework of EWAS were introduced by Patel et al. (2010). Firstly, in the proposed framework an initial scan for environmental factors associated with the observed effects/variables through general linear modeling (e.g logistic regression) is carried out. The model takes into consideration the factors(?) 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.

3.4.1 Application on the REPRO_PL study

Investigation of the associations between the various neurodevelopment indices evaluated by the Bailey test revealed that different factors might have a negative, or a beneficial contribution.

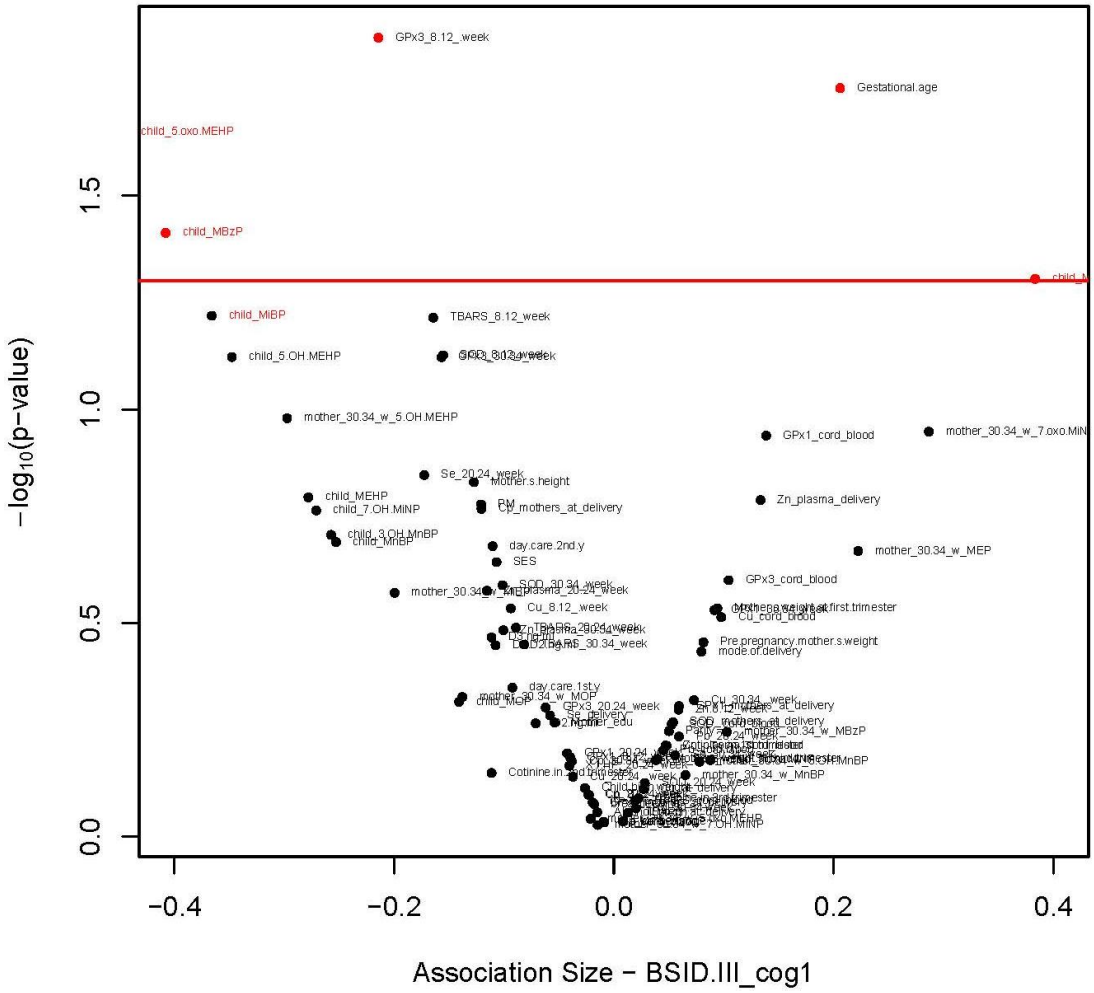

Figure 4. Association of the cognitive development at one year of age with exposure and modifiers in the Repro-Pl birth cohort

With regard to cognitive development at one year of life (Figure 4), it was found that child exposure to DEHP and BBzP result in lower cognitive scores, while the same occurs for the levels of glutathione peroxidase 3 in the mother plasma during pregnancy. On the other hand, the gestational age of the mother has beneficial effect, however the causality (socio-developmental or physiology) has to be further investigated.

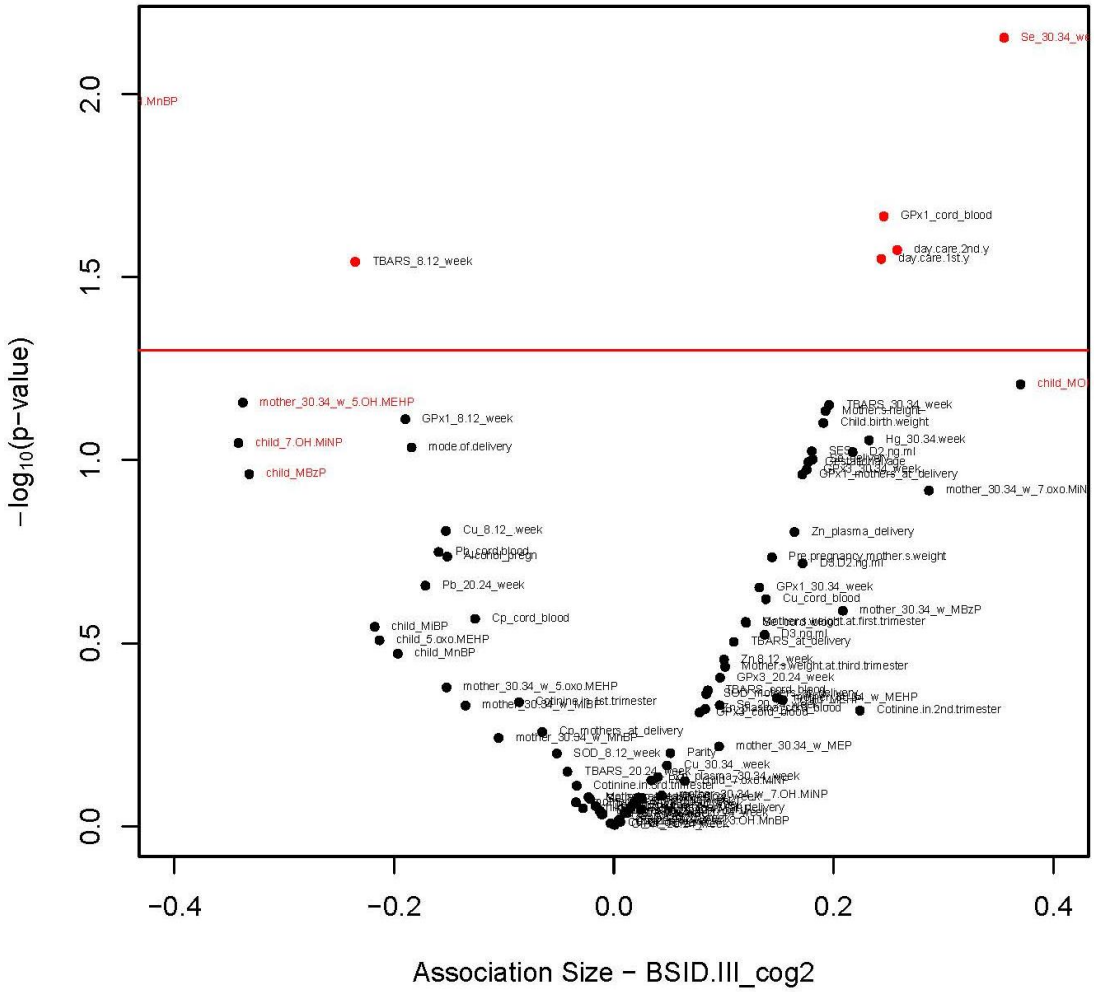

Figure 5. Association of the cognitive development at two years of age with exposure and modifiers in the Repro-PI birth cohort

In the second year of age (Figure 5), cognitive development is negatively affected by child exposure to DnBP and the presence of thiobarbituric acid reactive substances in the mother plasma during pregnancy. On the contrary, the presence of selenium during the third semester of pregnancy seem to be a significant beneficial parameter, followed by glutathione

peroxidase 1 in the mother plasma during pregnancy. It is also very important to highlight that cognitive development is strongly depends on the attendance of day care center by the child; children hosted in day care centers present higher cognitive skills.

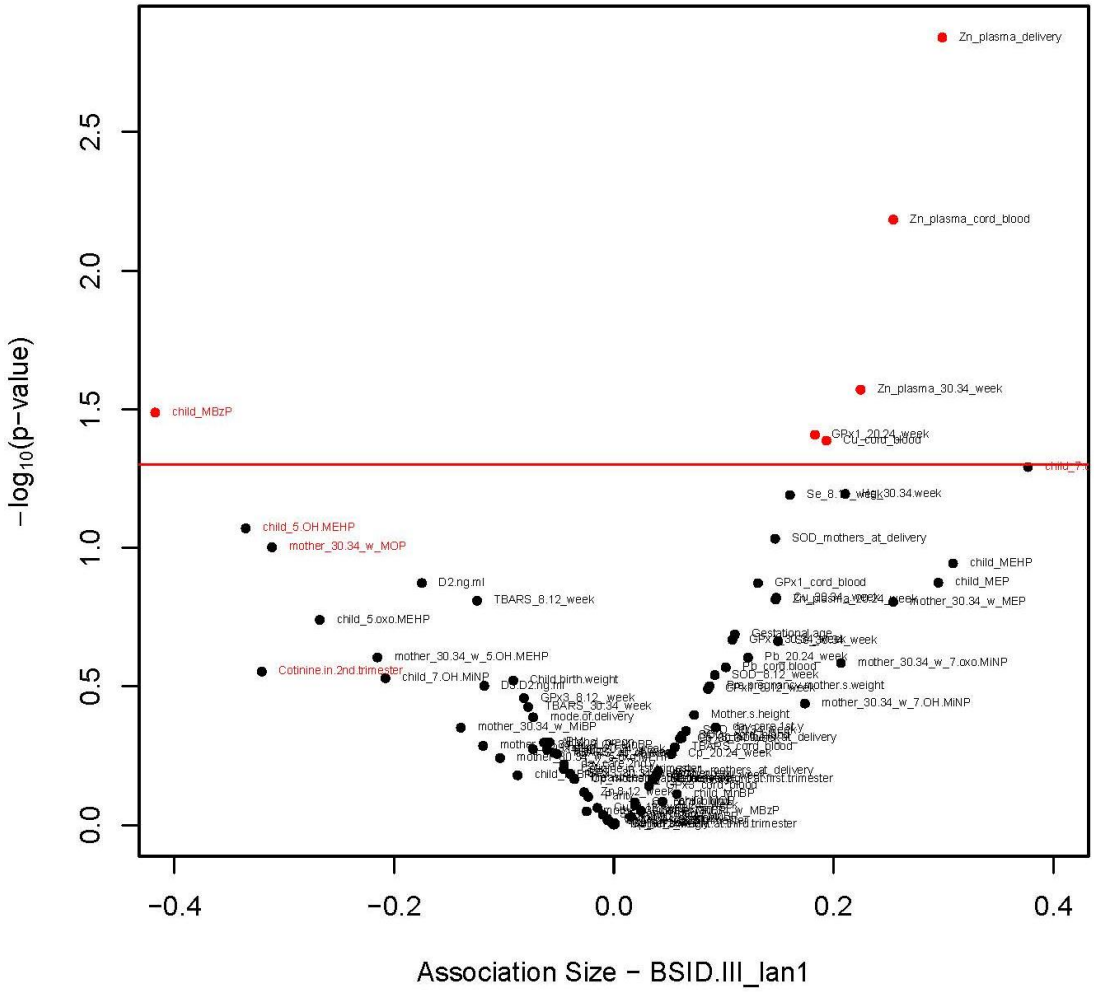

Figure 6. Association of the language development at one year of age with exposure and modifiers in the Repro-PI birth cohort

With regard to language development at one year of age (Figure 6), child exposure to phthalates (BBzP) seem to affect negatively. Interestingly, the presence in maternal plasma of Zn (during all the trimesters of pregnancy), glutathione peroxidase 1 (during the second trimester) and Cu act beneficially in the linguistic development.

Language development at two years of age is affected negatively by child exposure to DEP, DnBP and DinP, indicating once more the detrimental effects of children exposure to both low and high molecular phthalates. However, prenatal exposure to thiobarbituric acid reactive substances, glutathione peroxidase 3, glutathione peroxidase 1 and selenium during the last trimester of pregnancy, indicate the importance of this developmental period for the future language development of the child.

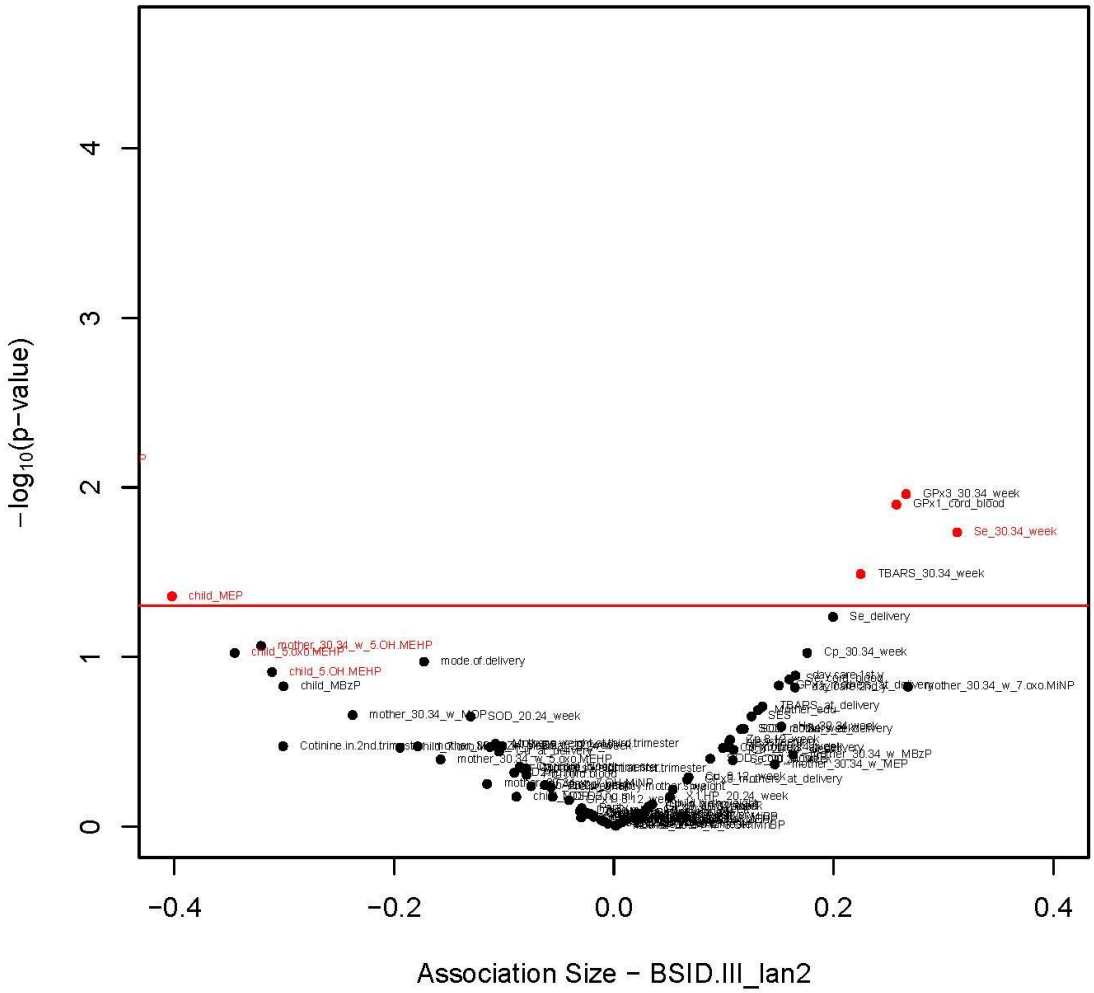

Figure 7. Association of the language development at two years of age with exposure and modifiers

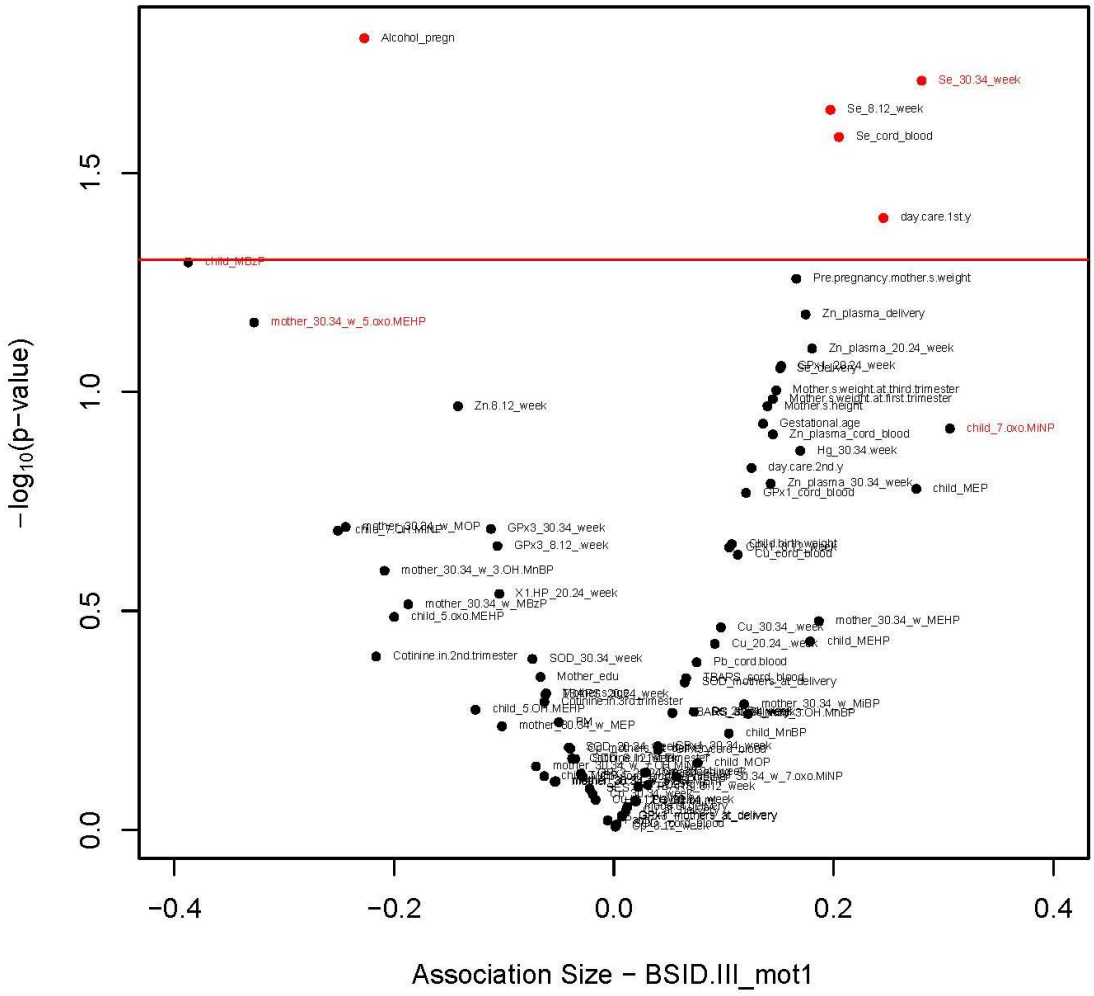

Figure 8. Association of the motor development at one year of age with exposure and modifiers in the Repro-PI birth cohort

With regard to motor development, none of the environmental exposure factors seems to hold a key role. At year one (Figure 8), maternal alcohol consumption during pregnancy results in lower scores of motor development, while the presence of selenium (during the 1st and the 3rd trimester), contributes beneficially. In addition, child attendance in a day care center contributes beneficially as well.

Regarding the motor development in the second year (Figure 9), a very interesting finding is that increased maternal bodyweight during the 1st and the 3rd trimester result in lower motor development. On the other hand, the presence of thiobarbituric acid reactive substances and Hg in the mother plasma during the 3rd trimester result in higher motor development scores. It

has to be noted that the presence of Hg does not act (in terms of physiology) positively in motor development; it is rather an indicator of high consumption of food items (e.g. fish), rich in Hg, but also in other nutrients (e.g. omega 3 fatty acids) that are beneficial for child development.

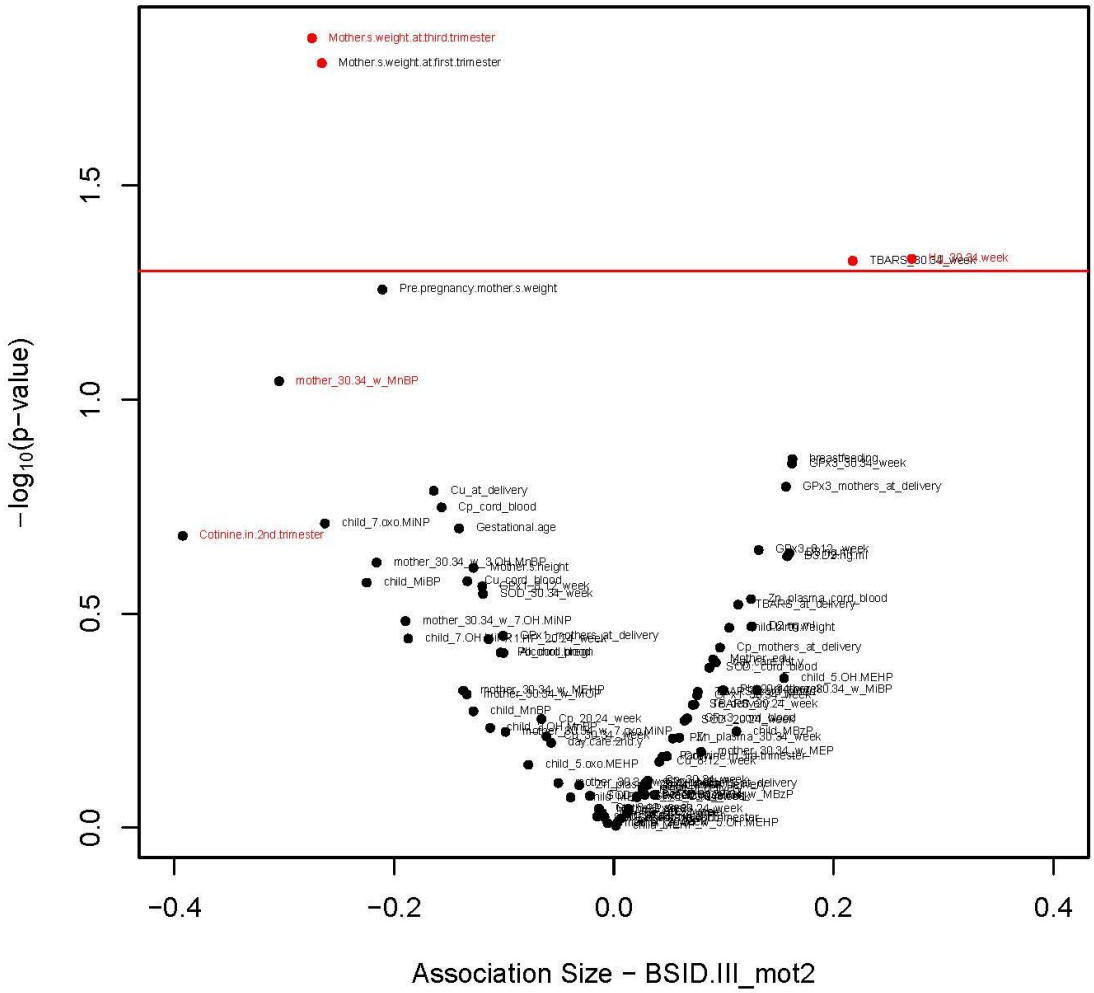

Figure 9. Association of the motor development at two years of age with exposure and modifiers in the Repro-Pl birth cohort

3.4.1.1 Comments

To sum up, the application of EWAS to Repro –PL shows …..

3.4.2 Application on the HERACLES Greek cohort

3.4.2.1 Data clustering

For clustering the various exposure related data, the two different clustering techniques described in Chapter 4 were used. The results are graphically illustrated in Figure 10 and Figure 11 respectively.

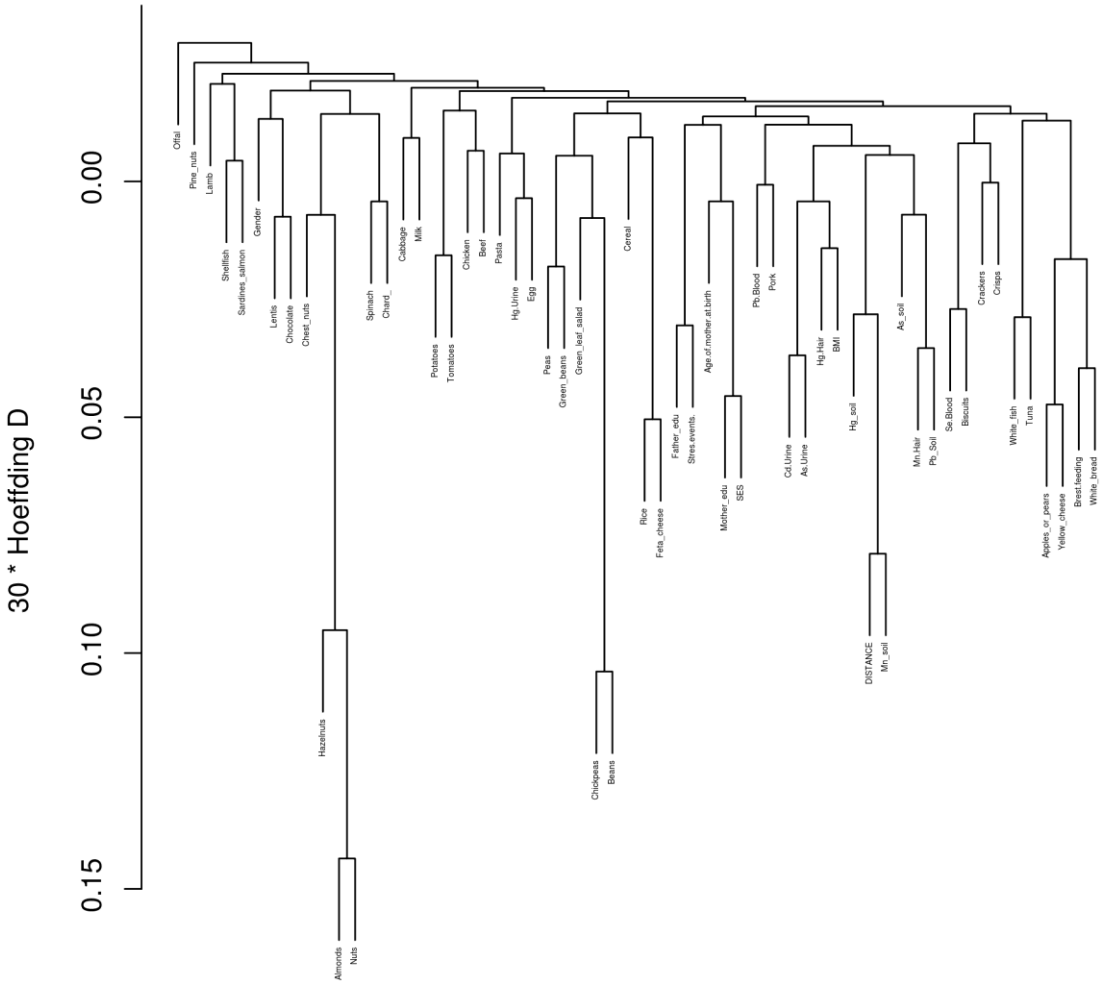

Figure 10. Hierarchical clustering using the Hoeffding D method

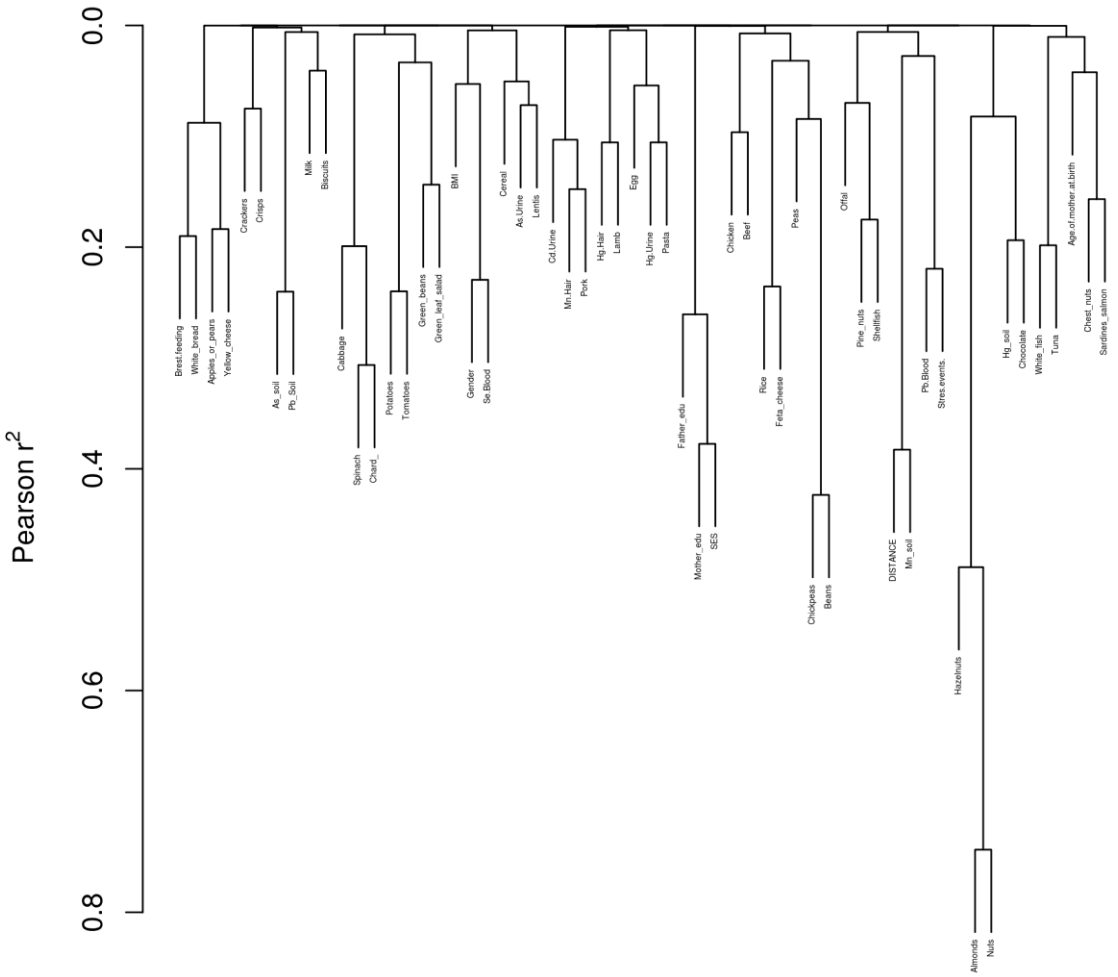

Figure 11. Hierarchical clustering using the Pearson correlation

The auto-correlations of the various parameters, are illustrated in both the heatmap (Figure 12) and the correlation globe (Figure 13).

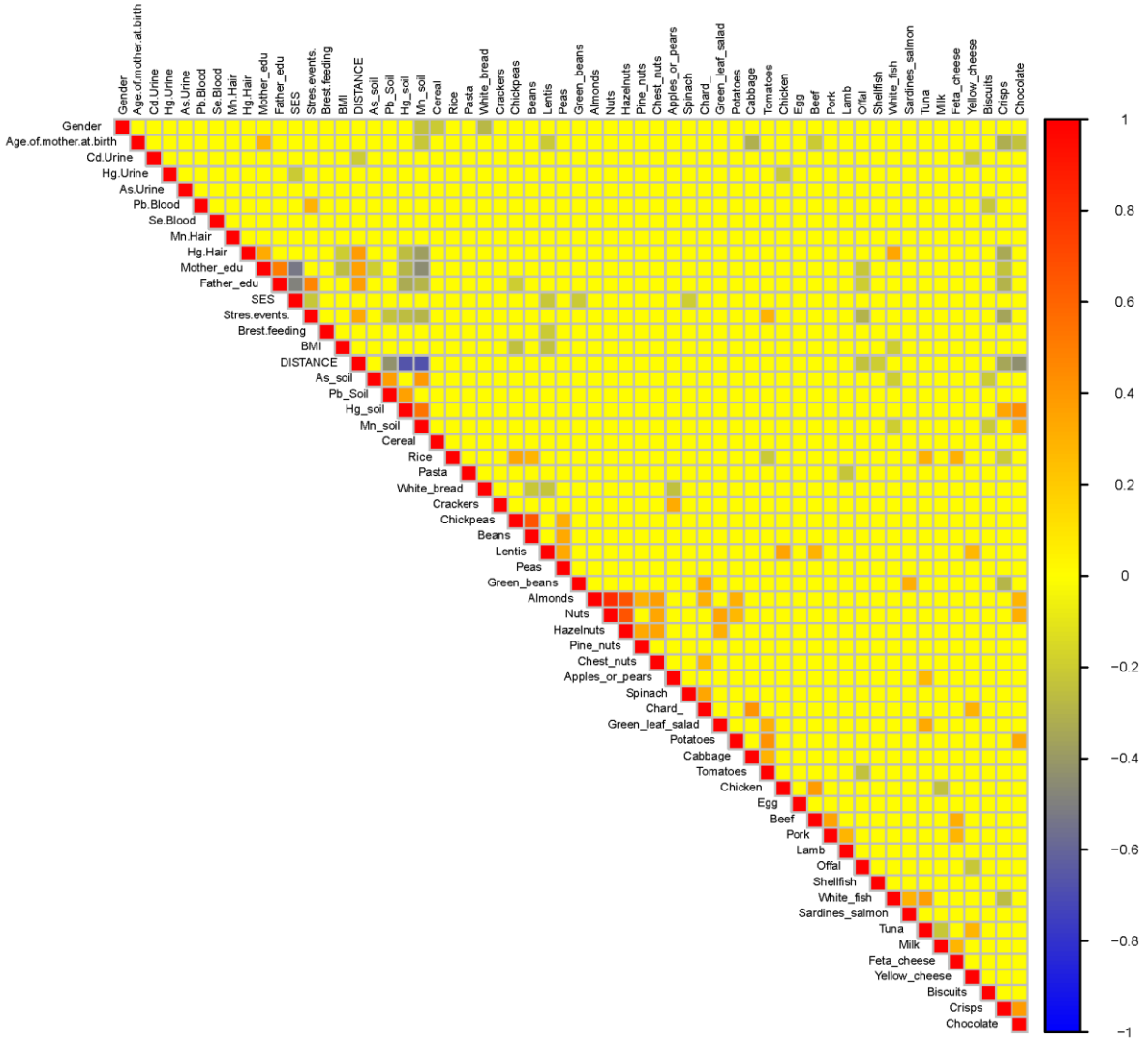

Figure 12. Heatmap of the exposure parameters of the HERACLES study

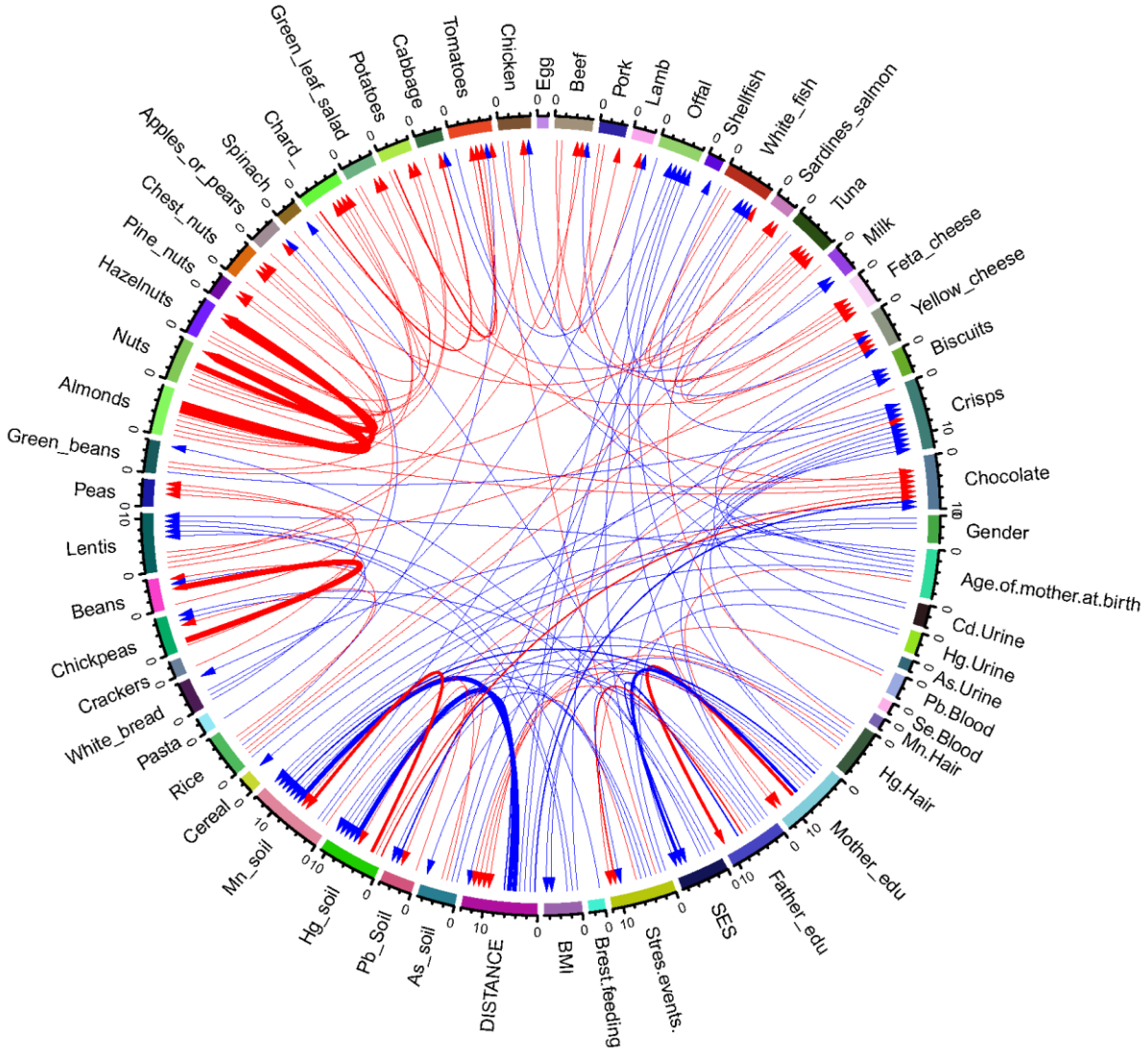

Figure 13. Correlation globe of the environmental, dietary and exposure factors of the HERACLES study

3.4.2.2 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* 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. 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.

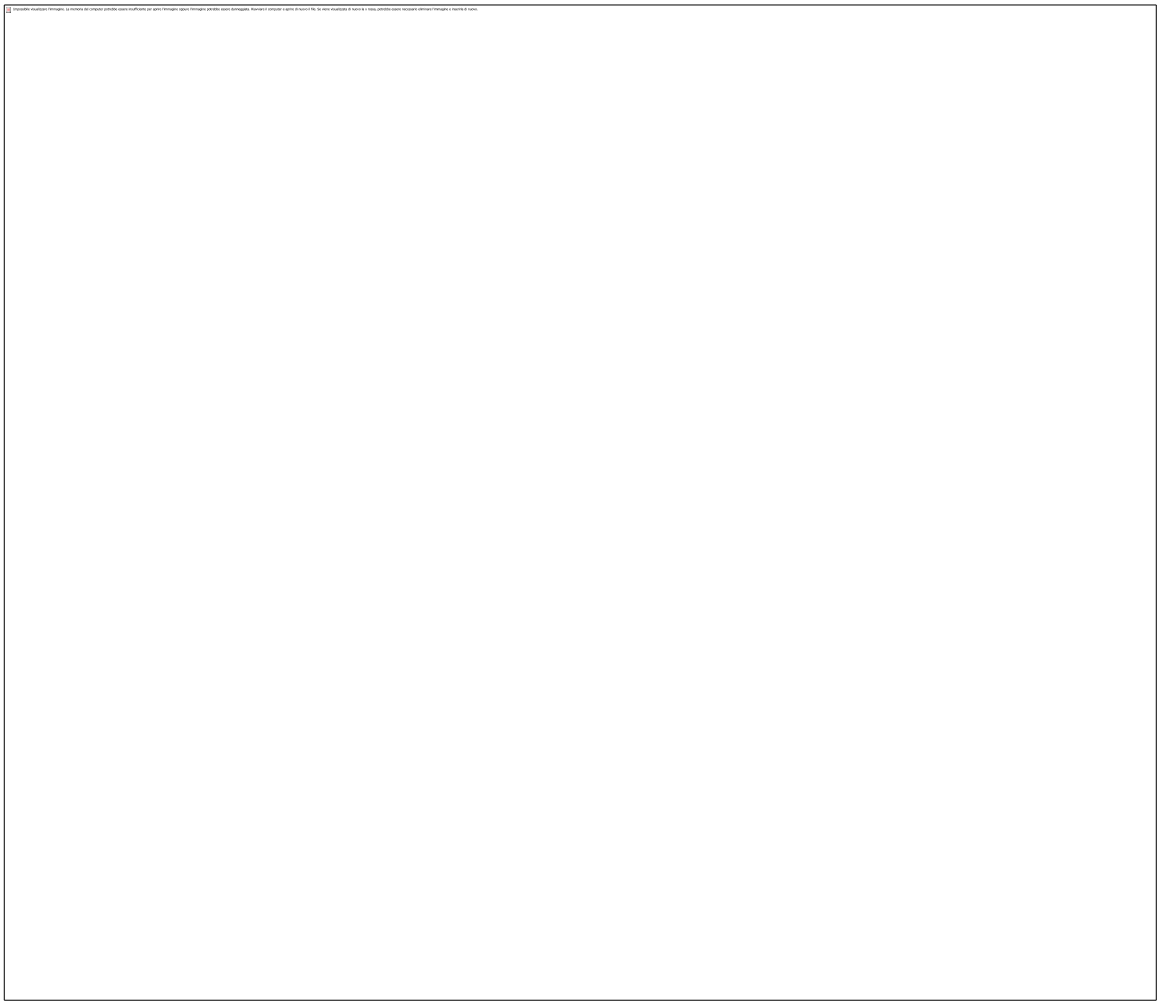

Figure 14. Associations of attention deficit / hyperactivity problems (from the CBCL test battery) with the environmental, dietary and exposure factors

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.

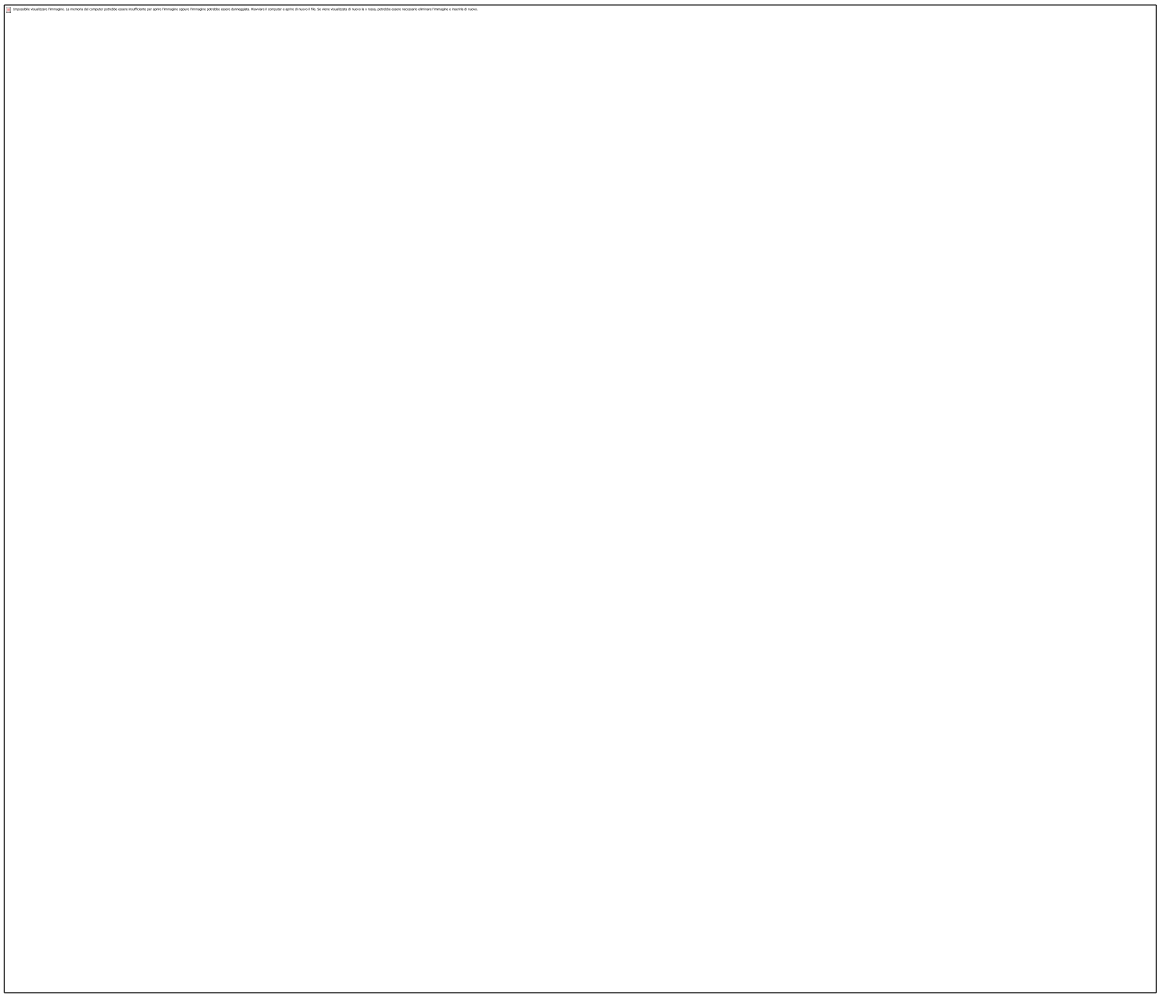

Figure 15. Associations of Stop Signal Task Mean correct RT on GO trials (from the CANTAB test battery) with the environmental, dietary and exposure factors

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.

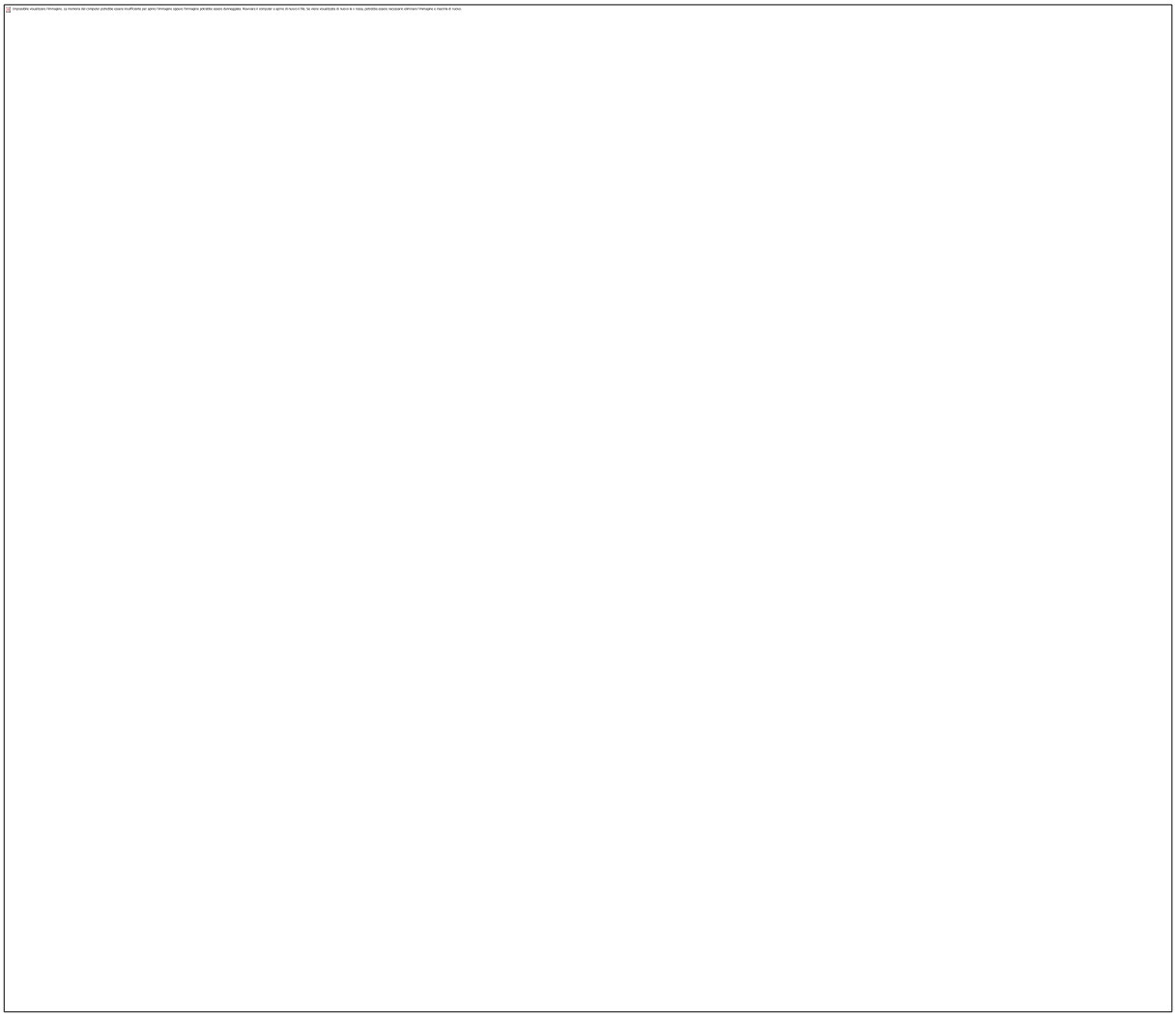

Figure 16. Associations of Total score T / Teachers (from the Social Responsiveness Scale test battery) with the environmental, dietary and exposure factors

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 16). 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 (p <0.01), coffee (p-value < 0.01), chicken (p < 0.05), crackers (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), fish (p-value < 0.01) and white 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).

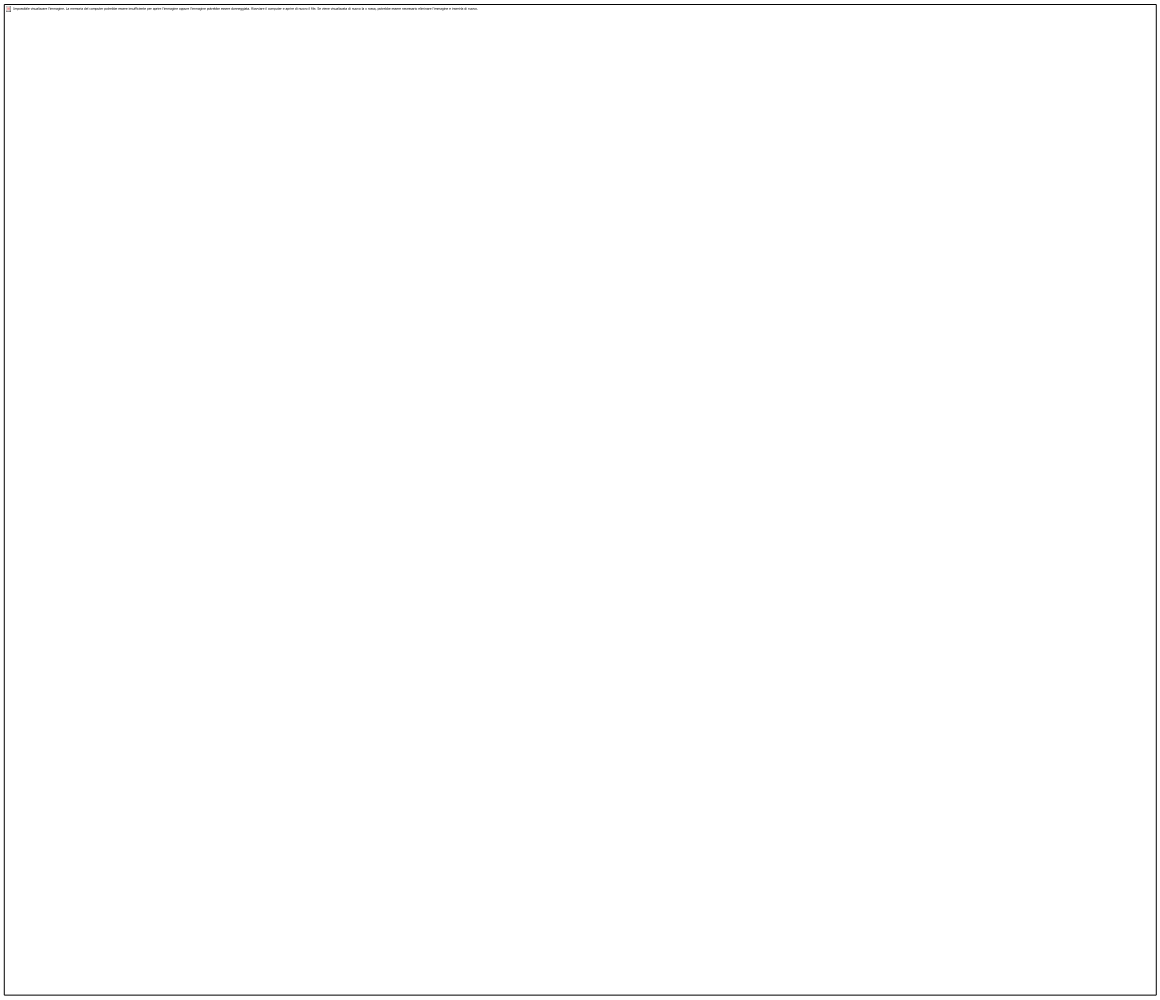

Figure 17. Association of intelligence quotient (from the WISC-IV test battery) with the environmental, dietary and exposure factors

Like for the CBCL test battery *consumption of white fish* appears to have positive effects on the IQ and Verbal Comprehension index (p-value <0.001).

3.4.2.3 Comments

To sum up, the application of classical statistical analysis to HERACLES…..

3.4.3 Conclusions in the full EWAS analysis

Among the various statistical techniques, the Environment Wide Associations Study (EWAS) was found to serve in the best way the aims of exposome analysis, that requires (a) an unbiased stance towards initial hypothesis between exposure and disease and (b) the use of multiple type of data, that all of them are considered as potential contributors to overall health or eventually pre-clinical or disease states. In practice, HEALS introduces a novel approach towards defining causal associations between health status and environmental stressors through the integrated use of EWAS. Environmental factors that are correlated are not considered confounders; rather they are co-variates, which are in “linkage disequilibrium” with each other. EWAS findings could then be used to identify further factors that may be in “disequilibrium”, for further detailed measurement and causal identification. This was clearly illustrated in the application of the methodology developed in HEALS in two different pre-existing cohorts (presented in Chapter 5), carried out in Poland (REPRO_PL study) and Greece (HERACLES study). Although these studies aimed at the associations among environmental pressures and neurodevelopmental effects, different type of environmental, exposure sociodemographic data and biospecimens were available, while evaluation of neurodevelopment was carried out using different test batteries. However, despite the differences in the availability of the data, the strength of the methodology allowed us to better identify associations between different exposure factors and neurodevelopment. In the case of Poland, it was clearly illustrated that several gestation factors have a beneficial (e.g. the concentration of selenium in maternal blood) or a negative influence (e.g. maternal bodyweight) in child neurodevelopment. On the other hand, child exposure to phthalates itself has a stronger negative influence in child neurodevelopment than maternal exposure. From the HERACLES study, it was found that children exposure to heavy metals and proximity to waste management sites have a negative influence in child neurodevelopment, however these effects are significantly modified by sociodemographic parameters (such as children SES and parents educational level, as well as diet. Further steps of analysis in both cohorts will include the results of the toxicity pathways identified to be perturbed from the omics analysis.

References

Achenbach TM, Rescorla LA. Manual for the ASEBA school-age forms & profiles: an integrated system of mult-informant assessment. University of Vermont, Research Center for Children, Youth & Families, Burlington, 2001.

American Psychiatric Association. Diagnostic and statistical manual of mental disorders. American Psychiatric Association, Washington, 2000.

Bellinger DC. Interpretation of small effect sizes in occupational and environmental neurotoxicology: individual versus population risk. Neurotoxicology 2007; 28: 245-51.

Bellinger DC. Interpreting epidemiologic studies of developmental neurotoxicity: conceptual and analytic issues. Neurotoxicol Teratol 2009; 31: 267-74.

Bouchard MF, Bellinger DC, Wright RO, Weisskopf MG. Attention-deficit/hyperactivity disorder and urinary metabolites of organophosphate pesticides. Pediatrics 2010; 125: e1270-7.

Calderon-Garciduenas L, Mora-Tiscareno A, Ontiveros E, Gomez-Garza G, Barragan-Mejia G, Broadway J, Chapman S, Valencia-Salazar G, Jewells V, Maronpot RR, Henriquez-Roldan C, Perez-Guille B, Torres-Jardon R, Herrit L, Brooks D, Osnaya-Brizuela N, Monroy ME, Gonzalez-Maciel A, Reynoso-Robles R, Villarreal-Calderon R, Solt AC, Engle RW. Air pollution, cognitive deficits and brain abnormalities: a pilot study with children and dogs. Brain Cogn 2008; 68: 117-27.

Capuron L, Ravaud A, Dantzer R. Timing and specificity of the cognitive changes induced by interleukin-2 and interferon-alpha treatments in cancer patients. Psychosom Med 2001; 63: 376-86.

Choi AL, Sun G, Zhang Y, Grandjean P. Developmental fluoride neurotoxicity: a systematic review and meta-analysis. Environ Health Perspect 2012; 120: 1362-8.

Christensen K, Murray JC. What genome-wide association studies can do for medicine. N Engl J Med 2007; 356: 1094-7.

Connors SL, Levitt P, Matthews SG, Slotkin TA, Johnston MV, Kinney HC, Johnson WG, Dailey RM, Zimmerman AW. Fetal mechanisms in neurodevelopmental disorders. Pediatr Neurol 2008; 38: 163-76.

Constantino JN, Przybeck T, Friesen D, Todd RD. Reciprocal social behavior in children with and without pervasive developmental disorders. J Dev Behav Pediatr 2000; 21: 2-11.

Cory-Slechta DA. Studying toxicants as single chemicals: does this strategy adequately identify neurotoxic risk? Neurotoxicology 2005; 26: 491-510.

De Felice C, Della Ragione F, Signorini C, Leoncini S, Pecorelli A, Ciccoli L, Scalabri F, Marracino F, Madonna M, Belmonte G, Ricceri L, De Filippis B, Laviola G, Valacchi G, Durand T, Galano JM, Oger C, Guy A, Bultel-Ponce V, Guy J, Filosa S, Hayek J, D'Esposito M. Oxidative brain damage in Mecp2-mutant murine models of Rett syndrome. Neurobiol Dis 2014; 68: 66-77.

Dominiak A, Wilkaniec A, Wroczynski P, Adamczyk A. Selenium in the Therapy of Neurological Diseases. Where is it Going? Curr Neuropharmacol 2016; 14: 282-99.

Engel SM, Wetmur J, Chen J, Zhu C, Barr DB, Canfield RL, Wolff MS. Prenatal exposure to organophosphates, paraoxonase 1, and cognitive development in childhood. Environ Health Perspect 2011; 119: 1182-8.

Evans AM, Rice GE, Teuschler LK, Wright JM. Joint exposure to chemical and nonchemical neurodevelopmental stressors in U.S. women of reproductive age in NHANES. Int J Environ Res Public Health 2014; 11: 4384-401.

Froehlich TE, Anixt JS, Loe IM, Chirdkiatgumchai V, Kuan L, Gilman RC. Update on environmental risk factors for attention-deficit/hyperactivity disorder. Curr Psychiatry Rep 2011; 13: 333-44.

Goines PE, Ashwood P. Cytokine dysregulation in autism spectrum disorders (ASD): possible role of the environment. Neurotoxicol Teratol 2013; 36: 67-81.

Grandjean P, Landrigan PJ. Developmental neurotoxicity of industrial chemicals. Lancet 2006; 368: 2167-78.

Grandjean P, Landrigan PJ. Neurobehavioural effects of developmental toxicity. Lancet Neurol 2014; 13: 330-8.

Grosse SD, Matte TD, Schwartz J, Jackson RJ. Economic gains resulting from the reduction in children's exposure to lead in the United States. Environ Health Perspect 2002; 110: 563-9.

Grzadzinski R, Di Martino A, Brady E, Mairena MA, O'Neale M, Petkova E, Lord C, Castellanos FX. Examining autistic traits in children with ADHD: does the autism spectrum extend to ADHD? J Autism Dev Disord 2011; 41: 1178-91.

Hackman DA, Farah MJ. Socioeconomic status and the developing brain. Trends Cogn Sci 2009; 13: 65-73.

Herbert MR, Russo JP, Yang S, Roohi J, Blaxill M, Kahler SG, Cremer L, Hatchwell E. Autism and environmental genomics. Neurotoxicology 2006; 27: 671-84.

Julvez J, Smith GD, Golding J, Ring S, Pourcain BS, Gonzalez JR, Grandjean P. Prenatal methylmercury exposure and genetic predisposition to cognitive deficit at age 8 years. Epidemiology 2013; 24: 643-650.

Jurewicz J, Polanska K, Hanke W. Chemical exposure early in life and the neurodevelopment of children--an overview of current epidemiological evidence. Ann Agric Environ Med 2013; 20: 465-86.

Kobrosly RW, Parlett LE, Stahlhut RW, Barrett ES, Swan SH. Socioeconomic factors and phthalate metabolite concentrations among United States women of reproductive age. Environ Res 2012; 115: 11-7.

Li Y, Zhang J. Serum concentrations of antioxidant vitamins and carotenoids are low in individuals with a history of attempted suicide. Nutr Neurosci 2007; 10: 51-8.

Livingston RJ, von Niederhausern A, Jegga AG, Crawford DC, Carlson CS, Rieder MJ, Gowrisankar S, Aronow BJ, Weiss RB, Nickerson DA. Pattern of sequence variation across 213 environmental response genes. Genome Res 2004; 14: 1821-31.

Mazumdar M, Xia W, Hofmann O, Gregas M, Ho Sui S, Hide W, Yang T, Needleman HL, Bellinger DC. Prenatal lead levels, plasma amyloid beta levels, and gene expression in young adulthood. Environ Health Perspect 2012; 120: 702-7.

Minghetti L, Greco A, Zanardo V, Suppiej A. Early-life sex-dependent vulnerability to oxidative stress: the natural twining model. J Matern Fetal Neonatal Med 2013; 26: 259-62.

Miodovnik A, Engel SM, Zhu C, Ye X, Soorya LV, Silva MJ, Calafat AM, Wolff MS. Endocrine disruptors and childhood social impairment. Neurotoxicology 2011; 32: 261-7.

Morello-Frosch R, Shenassa ED. The environmental "riskscape" and social inequality: implications for explaining maternal and child health disparities. Environ Health Perspect 2006; 114: 1150-3.

National Toxicology Program. NTP Monograph on Health Effects of Low-Level Lead. National Institute of Environmental Health Sciences, Research Triangle Park, NC, 2012.

Patel CJ, Bhattacharya J, Butte AJ. An environment-wide association study (EWAS) on type 2 diabetes mellitus. PloS one 2010; 5: e10746.

Perera FP, Li Z, Whyatt R, Hoepner L, Wang S, Camann D, Rauh V. Prenatal airborne polycyclic aromatic hydrocarbon exposure and child IQ at age 5 years. Pediatrics 2009; 124: e195-202.

Rai D, Lewis G, Lundberg M, Araya R, Svensson A, Dalman C, Carpenter P, Magnusson C. Parental socioeconomic status and risk of offspring autism spectrum disorders in a Swedish population-based study. J Am Acad Child Adolesc Psychiatry 2012; 51: 467-476 e6.

Rampersaud GC, Pereira MA, Girard BL, Adams J, Metzl JD. Breakfast habits, nutritional status, body weight, and academic performance in children and adolescents. J Am Diet Assoc 2005; 105: 743-60; quiz 761-2.

Rauh V, Arunajadai S, Horton M, Perera F, Hoepner L, Barr DB, Whyatt R. Seven-year neurodevelopmental scores and prenatal exposure to chlorpyrifos, a common agricultural pesticide. Environ Health Perspect 2011; 119: 1196-201.

Rice DC. Overview of modifiers of methylmercury neurotoxicity: chemicals, nutrients, and the social environment. Neurotoxicology 2008; 29: 761-6.

Robbins TW, Sahakian BJ. Computer Methods of Assessment of Cognitive Function. Principles and Practice of Geriatric Psychiatry. John Wiley & Sons, Ltd, 2002, pp. 147-151.

Sheinerman KS, Umansky SR. Circulating cell-free microRNA as biomarkers for screening, diagnosis and monitoring of neurodegenerative diseases and other neurologic pathologies. Front Cell Neurosci 2013; 7: 150.

Stangle DE, Strawderman MS, Smith D, Kuypers M, Strupp BJ. Reductions in blood lead overestimate reductions in brain lead following repeated succimer regimens in a rodent model of childhood lead exposure. Environ Health Perspect 2004; 112: 302-8.

Story EN, Kopec RE, Schwartz SJ, Harris GK. An update on the health effects of tomato lycopene. Annu Rev Food Sci Technol 2010; 1: 189-210.

Volk HE, Lurmann F, Penfold B, Hertz-Picciotto I, McConnell R. Traffic-related air pollution, particulate matter, and autism. JAMA Psychiatry 2013; 70: 71-7.

Warnick EM, Bracken MB, Kasl S. Screening Efficiency of the Child Behavior Checklist and Strengths and Difficulties Questionnaire: A Systematic Review. Child and Adolescent Mental Health 2008; 13: 140-147.

Wechsler D. Wechsler Intelligence Scale for Children. Psychological Corporation, San Antonio TX, 2003.

Woods JS, Heyer NJ, Russo JE, Martin MD, Farin FM. Genetic polymorphisms affecting susceptibility to mercury neurotoxicity in children: Summary findings from the Casa Pia Children's Amalgam Clinical Trial. Neurotoxicology 2014; 44C: 288-302.