Social and environmental determinants of neuropsychological development from birth to preadolescence

Joan Forns i Guzman

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Thesis director: Prof. Jordi Sunyer i Déu

Center for Research in Environmental Epidemiology (CREAL)

“Fem de viure una festa, fem-ho abans de fer-nos grans que demà serà impossible, que demà ja serà tard” (Els Amics de les Arts)

CONTENTS

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'Hablo, pero no puedo afirmar nada; buscaré siempre,
dudaré con frecuencia y desconfiaré de mí mismo.' (Cicerón)

2 ABSTRACT

Introduction

Neuropsychological development is a genetically guided process which is continuously modified by socio-environmental factors. This thesis aimed to study the main socio-environmental determinants of neuropsychological development in different time-periods, such in the first two years of life, during preschool, and during preadolescence. This thesis also aimed to summarize the work done in environmental epidemiology on neuropsychological development in a novel conceptual framework.

Methods

This thesis is based on the data of the INMA (Infancia y Medio Ambiente) Project. The main objective of this project is to evaluate the impact of environmental exposures in children's health in 7 population-based birth cohorts in different regions of Spain. The neuropsychological development of approximate total of 2,650 children was assessed at different time-periods following the same protocols.

Results

(1) Maternal cognitive capacities were positively related with child cognitive development early in life in more disadvantaged occupational social classes. (2) The levels of child cortisol were not related to child neuropsychological development during the second year of life. (3) Higher levels of long-chain polyunsaturated fatty acids in colostrum due to prolonged periods of breastfeeding improved early neuropsychological development of children, in particular in those children exposed to maternal smoking during pregnancy. (4) Prenatal exposure to PCBs (specially for PCB congener 153) impacted negatively

on psychomotor development during the second year in life and on general neuropsychological development at the age of 4 years. (5) Postnatal exposure to organochlorine compounds was associated with a delay in reaction time (speed processing) during the preadolescent period. (6) The conceptual framework proposed will improve the quality of research in this area.

Conclusions

Social and cultural determinants such as maternal intelligence, educational level or occupational social class, are configuring the proximal environment in which a child develops and determine their neuropsychological development. Current levels of some organochlorine compounds, particularly polychlorinated byphenils, measured in blood samples (from umbilical cord, mothers, or children) are impairing on neuropsychological development in the general population.

RESUM

Introducció

El desenvolupament neuropsicològic infantil és un procés guiat genèticament, el qual és contínuament influenciat per factors socials i ambientals. L'objectiu d'aquesta tesi fou l'estudi dels principals determinants socioambientals del desenvolupament neuropsicològic infantil en diferents períodes de temps. Aquesta tesi també té l'objectiu de resumir en un marc conceptual els diferents components del desenvolupament neuropsicològic pel seu ús en estudis d'epidemiologia ambiental.

Mètodes

Aquesta tesi està basada en dades del projecte INMA (Infancia y Medio Ambiente). El principal objectiu d'aquest projecte és avaluar l'impacte de les exposicions ambientals en la salut infantil en 7 cohorts de població establides en diferents regions d'Espanya. Dintre del marc d'aquest projecte, s'ha avaluat el desenvolupament neuropsicològic d'aproximadament 2.650 nens en diferents moments, seguint els mateixos protocols.

Resultats

(1) Les capacitats cognitives maternes estan positivament relacionades amb el desenvolupament cognitiu infantil en les primeres etapes, en les classes socials ocupacionals menys afavorides. (2) Els nivells de cortisol infantil no mostren associació amb el desenvolupament neuropsicològic durant el segon any de vida. (3) Nivells elevats d'àcids grassos poliinsaturats de cadena llarga en conjunció amb períodes prolongats de lactància materna afavoreixen el desenvolupament neuropsicològic a les primeres etapes, especialment en aquells nens de mares fumadores

durant l'embaràs. (4) L'exposició prenatal a PCBs (espcialment, al congener 153) impacta negativament en el desenvolupament psicomotor infantil durant el segon any de vida i en el desenvolupament neuropsicològic general als 4 anys d'edat. (5) L'exposició postnatal a compostos organocloroats està associada a una capacitat de reacció (velocitat de processament) més alentida en etapes preadolescents. (6) L'ús del marc conceptual proposat a la tesi afavorirà la qualitat de la investigació en aquesta àrea.

Conclusions

Els determinants socials, com la intel·ligència materna, el nivell educatiu o la classe social basada en l'ocupació, configuren l'entorn més proper del nen i determinen el seu desenvolupament neuropsicològic. Els nivells actuals de certs compostos organoclorats, especialment els bifenils policlorinats, mesurats en mostres de sang (de cordó umbilical, mares o nens) mostren efectes negatius sobre el desenvolupament neuropsicològic en la població general.

RESUMEN

Introducción

El desarrollo neuropsicológico infantil es un proceso guiado genéticamente, el cual está contínuamente influenciado por factores sociales y ambientales. El objetivo de esta tesis fue estudiar los principales determinantes socio-ambientales del desarrollo neuropsicológico infantil en diferentes periodos de tiempo. Esta tesis también tenía como objetivo resumir en un marco conceptual el trabajo hecho en epidemiología ambiental en el estudio del desarrollo neuropsicológico infantil.

Métodos

Esta tesis está basada en datos del proyecto INMA (Infancia y Medio Ambiente). El principal objetivo de esto proyecto es evaluar el impacto de las exposiciones ambientales en la salud infantil en 7 cohortes de población establecidas en diferentes regiones de España. El desarrollo neuropsicológico de aproximadamente 2.650 niños ha sido evaluado en diferentes momentos siguiendo los mismos protocolos.

Resultados

(1) Las capacidades cognitivas maternas están positivamente relacionadas con el desarrollo cognitivo infantil en edades tempranas en las clases sociales ocupacionales menos aventajadas. (2) Los niveles de cortisol en el niño no se asocian con el desarrollo neuropsicológico durante el segundo año de vida. (3) Niveles altos de ácidos grasos poliinsaturados de cadena larga debido a periodos largos de lactancia materna mejoraron el desarrollo neuropsicológico a edades tempranas, especialmente en esos niños cuyas madres fumaron durante el embarazo. (4) La exposición prenatal a PCBs (espcialmente para el

congener 153) impacta negativamente en el desarrollo psicomotor durante el segundo año de vida y en desarrollo neuropsicológico general a la edad de 4 años. (5) La exposición postnatal a compuestos organoclorados está asociado con una peor capacidad de tiempo de reacción (velocidad de procesamiento) durante la preadolescencia. (6) El marco conceptual propuesto mejorará la calidad de la investigación en esta área.

Conclusiones

Los determinantes sociales tales como inteligencia maternal, nivel educativo o clase social basada en la ocupación, configuran el entorno más cercano en el cual el niño se desarrolla y determinan su desarrollo neuropsicológico. Los niveles actuales de ciertos compuestos organoclorados, especialmente los bifeniles policlorinados, medidos en sangre (de cordón umbilical, madre, o niño) tienen efectos negativos sobre el desarrollo neuropsicológico de la población general.

3 PREFACE

This thesis was written at the Centre for Research in Environmental Epidemiology (CREAL) between 2008 and 2011, and it was supervised by Prof. Jordi Sunyer i Deu. This work consists of a compilation of the scientific publications co-authored by the PhD candidate according to the procedures of the Biomedicine PhD program of the Department of Experimental and Health Sciences of University Pompeu Fabra.

The thesis includes an abstract, a general introduction, a rationale, the objectives, the results (7 original articles and one brief report presenting a conceptual framework), a discussion, and final conclusions. The thesis is focused on the socio-environmental determinants of child neuropsychological development from the first years of life until preadolescence. All of the original scientific papers are based on data from the INMA-Project, a population-based birth cohort study composed by seven birth cohorts in different regions of Spain.

At the end of this book I include a report co-authored by the PhD candidate included in a European project called Enrieco (Environmental Health Risks in European Birth Cohorts) in a separate annex. The aim of this project was to advance our knowledge in specific environment and health causal relationships in pregnancy and birth cohorts by providing support to exploitation of the wealth of data generated by past or ongoing studies funded by the EC and national programmes. The report included in this thesis comprises a revision of the neuropsychological protocols used in European birth cohorts and a list of recommendations for future studies.

4 INTRODUCTION

4.1 Brain development

The development of the central nervous system (CNS) or neurodevelopment is a genetically driven process which comprises several stages, such as neurulation, proliferation, migration, myelination, and synaptic pruning (Figure 1). This process is structurally and functionally nonlinear (1). The human brain changes and adapts throughout the lifespan, although during the early stages of life (fetal development and childhood) the most dramatic changes occur (2). The successful development of the brain into a properly functioning, integrated organ requires that each area first be formed and then be correctly interrelated with the others (3).

The process of neurulation (during which the neural tube is formed) is initiated in the first two weeks of the embryonic period. The neural tube is completely formed by the fourth gestational week.

Neurons and glial cells are formed on the outside wall of the neural tube. During this period, some important areas are formed, such as the cerebral hemispheres, the olfactory bulb, and the pituitary gland. The next phases are cell proliferation and neurogenesis in which neurons are generated from neural stem and progenitor cells. From the 24th week to the time of birth neural maturation and migration take place. There is a precise timing for both maturation and migration of neurons according to the cytoarchitectonic regions to which cell belongs. At birth, most of the neurons have migrated to their target areas. Their migration along

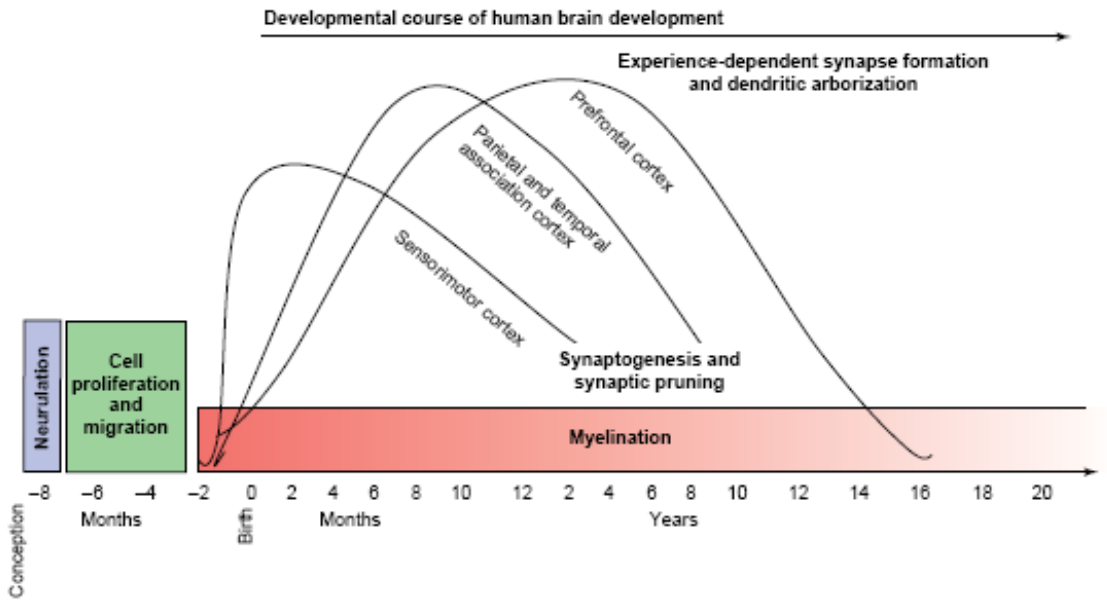

Figure 1. Development course of the human brain development (from Casey et al, 2005)(1)

radial glial fibres to their proper location is regulated by physical as well as chemical processes (4,5) .

The postnatal period is marked by increased cortical complexity (6). The brain continues to grow and specialize according to a precise genetic program which is modified by environmental influences. Most neurons have migrated to their appropriate locations within the cerebral cortex, hippocampus, cerebellum, and other regions of the brain. Nevertheless, some neurogenesis continues after birth during the first year in some important brain areas such as the hippocampus, olfactory bulb, and cerebellum (4,7).

At birth, the brain weighs about one quarter of its adult mass and the cortex and subcortical gray-matter are developed (4). However, the brain tends to increase its mass during irregular periods commonly called growth spurts (6). This increase is accounted for by the processes of dendritic growth and myelination. Myelination is a very slow process which is carried out by oligodendrocytes. The presence of myelin has been noted in the spinal cord at the end of the first trimester of gestation and proceeds caudorostrally. Myelin is present at birth in the pyramidal tract and primary sensory and motor pathways. During the postnatal process, myelination occurs in the frontal and parietal association regions and continues through the first twenty years or so of life (8). In general, myelination increases brain weight from approximately 400 grams at birth to 850 grams at 11 months, to 1,100 grams at 36 months, to 1,350 to 1,410 grams at age 15, and continues to increase to the age of 60 (6). Such an increase in cortical complexity would be expected to correlate with increased complexity in behavioural and cognitive functions during each growth spurt (9).

In addition to myelination, the dendritic branching of neurons and the number of synaptic connections greatly increase. The overproduction of brain connections is followed by dendritic pruning and synapse elimination. Synapse elimination starts early, and this continues until preschool age, by which time synaptic density has reached adult levels. The time courses of such neuronal and synaptic formation and elimination are considerably different among different cortical areas, with the prefrontal cortex generally being one of the latest (10). In the prefrontal cortex, the peak occurs at 3-4 years of age, and substantial decline does not occur until mid- to late adolescence (11). The neuroanatomical structure of the prefrontal cortex in humans undergoes considerable maturation during early childhood. In particular, it can be characterized by a reduction of synaptic and neuronal density, a growth of dendrites, and an increase in both gray and white matters. Thus, the period from early childhood to preschool age should be important in the development of the cognitive functions related to the prefrontal cortex ("executive functions") (11,12).

The relationship between cognitive-behavioural development and neuroanatomical development is relatively unknown in normal developing children. But obviously, there is a subjacent development of the cognitive functions, such as language, motor abilities, perceptual-performance, attention or executive functions according to brain development. Table 1 shows the relationship between acquisition of cognitive functions and myelination of some areas of the brain.

Table 1. The development of higher cognitive abilities (9)

Age	Visual/motor functions	Social/intellectual functions	Myelination
Birth	Sucking reflex, rooting, swallowing, Moro reflex, grasping, and blinking to light		Motor root +++; sensory root ++; superior cerebellar peduncle ++; optic tract ++
6 weeks	Neck turning and extension when prone; regards mom's face; follows objects.	Smiles when played with.	Optic tract ++; optic radiation +; middle cerebellar peduncle; pyramidal tract +
3 months	Infantile grasp; volitional sucking; holds head up; turns to objects in visual field; may respond to sound.	Watches own hands.	Sensory root +++; optic trace & radiation +++; pyramidal tract ++; cingulum +; frontopontine tract +; middle cerebellar peduncle +; corpus callosum ±; reticular formation ±
6 months	Grasps with both hands; puts weight on forearms; rolls; supports weight on legs brief periods.	Laughs and shows pleasure. Makes primitive sounds. Smiles at self in mirror.	Medial lemniscus +++; superior cerebellar peduncle ++; middle cerebellar peduncle +; pyramidal tract ++; corpus callosum +; reticular formation +; association areas ±; acoustic radiation +

Table 1. The development of higher cognitive abilities (9)

Age	Visual/motor functions	Social/intellectual functions	Myelination
12 months	Releases objects. Cruises and walks with one hand held; plantar reflex flexor in 50%.	Uses 2–4 words with meaning; understands nouns; may kiss on request.	Medial lemniscus +++; pyramidal tract +++; fornix +++; corpus callosum +; intracortical neuropil ±; association areas ±; acoustic radiation ++
24 months	Walks up and down stairs; (two feet-step); bends and picks up object; turns knob; partially dresses; plantar reflex flexor 100%.	Uses 2–3 word entences; uses I, me, and you; plays simple games; names 4–5 body parts; obeys simple commands	Acoustic radiation +++ corpus callosum ++; association areas +; nonspecific thalamic radiation ++
36 months	Goes up stairs (one foot) pedals tricycle; dresses self fully except shoelaces, belts, and buttons; visual acuity 20/20/OU.	Asks numerous questions; says nursery rhymes; copies circles; plays with others.	Middle cerebellar peduncle +++
5 years	Skips; ties shoelaces; copies triangles; gives age.	Repeats 4 digits; names 4 colors.	Nonspecific thalamic radiation +++; reticular formation ++; corpus callosum +++; intracortical neuropil & association areas ++
Adult			Intracortical neuropil & association areas ++ to +++

4.2 Socio-environmental factors and vulnerability of the brain

The development of the brain and its associated neuropsychological functions is a genetically driven process which is modified by environmental influences, both positive and negative. These influences include exposure to industrial and chemical agents (such as lead, methyl mercury, arsenic, polychlorinated biphenyls, solvents and pesticides), tobacco smoke, alcohol and certain drugs, as well as factors such as low socio-economic status, elevated maternal stress, negative parenting behaviours, or family violence that may indirectly influence this process (13–15).

The developing brain is more susceptible to damage caused by these toxic agents than the developed brain of an adult. This vulnerability is mainly due to the dramatic changes that occur in the developing brain in the first steps of its development. For these reasons, the time of the exposure to the environmental influences (and the amount of the exposure) is an important factor. Exposures during the first steps of the brain's development may cause a disruption in the cascade of the developmental processes mention in section 4.1. However, the development of the brain and the neural networks is initiated in the prenatal period and continues postnatally through adolescence. Exposure resulting in damage at any point of this process may result in an aberrant neural structure and consequently, in an impairment in neuropsychological development (15,16).

Several factors contribute to the vulnerability of the brain. During the prenatal life, the developing brain receives environmental exposures

from the mother via cord blood. The placenta is not an effective barrier against some environmental pollutants (17). The blood-brain barrier, which protects the adult brain from many toxic chemicals, is not completely formed until about 6 months after birth (18,19). The detoxification systems of children are immature, and therefore, the brain is at greater risk of damage in children than in adults. The neonatal brain consumes a large amount of nutrients due to its high metabolic activity, especially during the development of certain areas of the cortex (20). Furthermore, human milk may contain environmental contaminants with potential neurotoxic effects that can be transferred to the children via breastfeeding (15,21)

The brain receives multiple exposures throughout an individual's lifespan (Figure 2). These exposures can be differentiated into environmental and social exposures. Early exposures can lead to an increased susceptibility in adults. Moreover, multiple exposures (at low doses in most cases) may also promote disturbances or aberrant structural and/or functional consequences that can lead to significant problems later in life.

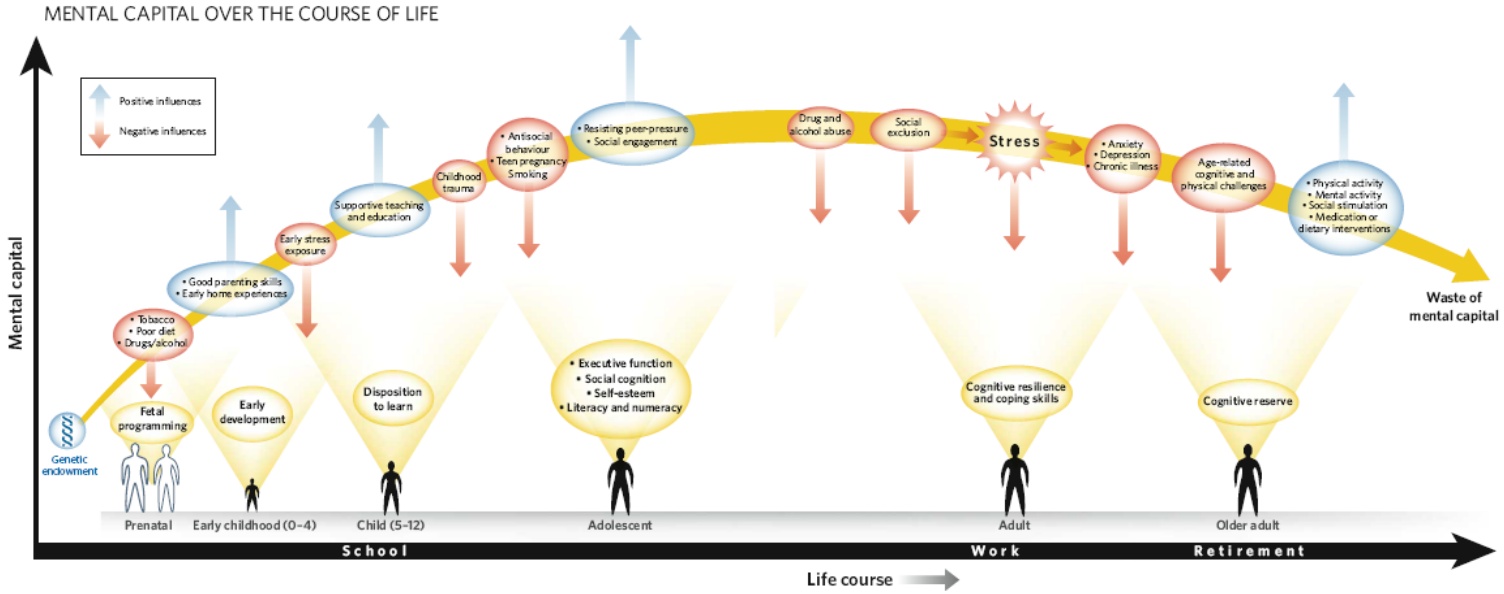

Figure 2. The mental capital over the course of life (from Beddington et al, 2008) (22)

4.2.1 Social environment

The term "social environment" refers to socio-demographic characteristics of the proximal environment in which a child develops and also to those characteristics of society which impact on neuropsychological development (23). The social environment in which children develop is composed of parental characteristics, such as intelligence, mental health, stress, and also social class and educational level. These socio-demographic characteristics will configure the experiences of the child, its learning opportunities, other important aspects of child development such as dietary patterns, physical activity, health care, and lifestyles.

Parental intelligence (or cognitive capacity) has been linked to childhood neuropsychological development through both genetic and environmental influences. The genetic endowment accounts for 50% in the variance of the cognitive capacities of an individual (24). Some authors (Chen at al, 2006) found that mothers with lower cognitive capacities than average are prone to having children with lower cognitive capacities and higher behavioural problems than their peers, supporting a genetic explanation (25). As opposed to this deterministic contribution, other authors claim that the child's neuropsychological development is configured not only by its genes, but also by the influences of the environment. Maternal cognitive abilities influence the configuration of the social environment of the child by providing a better quality of nutrition, health care, housing, as well as a cognitively-stimulating environment (13,23). The educational level and socioeconomic status (SES) are highly related to the cognitive abilities (26–28). It has been

argued the effects of parental SES are most likely mediated via dietary patterns, breastfeeding duration, living conditions, intellectual stimulation at home, access to educational materials and quality of schooling. Stimulating materials and experiences mediate the relationship between socioeconomic status and family income and children's intellectual and academic achievement, from infancy to adolescence (29). In fact, SES, maternal IQ, and the home environment (stimulating materials) have been found to positively predict children's cognitive development (30).

Some other parental characteristics may also influence the proximal environment in which children develop, such as maternal mental health or well-being. Maternity represents a period of time in which new mothers are particularly prone to develop mood disorders. A mother suffering from a mental disease will most likely establish a worse relationship as compared to a healthy woman. Severity and chronicity of postnatal depression are related to an increase in developmental problems in children (31). Several care-giving activities also appear to be compromised by postpartum depression including feeding practices, especially breastfeeding, sleep routines, and well-child visits, vaccinations and safety practices (32). The involvement of fathers when mothers are suffering mental health problems can mitigate the consequences of a compromised relationship between mother and child (33).

4.2.2 Stress hormones (cortisol)

The hypothalamic–pituitary–adrenal (HPA) axis is a system activated in reaction to environmental stressors, which culminates in production of glucocorticoid hormones (cortisol) by the adrenal glands. Cortisol is a hormone produced by the HPA axis and has multiple metabolic effects including a direct influence on the regulation of the plasticity and circuitry of many brain regions, and influences some cognitive domains, such as attention and concentration, memory or executive function (34–36). Nevertheless, prolonged exposure to elevated levels of cortisol may result in negative consequences on the structural development of the brain, and also impairments in cognitive and social-emotional development (37–39). Data obtained from animal and human studies indicate that chronic exposure to stress has enduring effects on the brain, through activation of the HPA axis and the release of glucocorticoids. Some brain areas with a role in regulating the HPA axis, such as the hippocampus, the frontal cortex, and the amygdala may be affected by elevated levels of cortisol during pregnancy (40). There is evidence that exposure to excess glucocorticoids in utero 'programs' the fetal HPA axis, permanently altering basal and stress-induced HPA axis activity and regulation in offspring (41,42).

Another possible mechanism of mother-child exposure occurs in the postnatal period, in addition to fetal programming. It has been argued that breastfeeding may be a possible mechanism to transfer cortisol from mother to child. One recent study has pointed out that breastfeed present higher levels of cortisol than non-breastfed children (43). There is evidence that during childhood, the highest impact of elevated levels of cortisol is on those areas that at the time of this exposure are

developing. This is the case of the hippocampus during childhood and the frontal lobes during adolescence (40).

4.2.3 Breastfeeding

The relationship between breastfeeding and child neuropsychological development has been extensively studied over the last two decades in environmental epidemiology. The first report of a positive association between breastfeeding and higher scores on cognitive tests was published in 1929 (44). The majority of observational studies published subsequently also reported on the benefits of prolonged periods of breastfeeding (45–48). The improvements observed in children who have been breastfed are usually explained by the positive effects of three main factors: long-chain polyunsaturated fatty acids (LC-PUFAs), certain parental characteristics (SES, education, or cognitive abilities), and contact between the mother and child during breastfeeding (49–51). In the last decade, some authors have questioned these positive associations between breastfeeding and neuropsychological development (48,52,53). The main criticisms focus on possible residual confounding; for example, when maternal intelligence is included in analyses, such positive associations are seen to disappear (52). Home environment has also been implicated as a confounder, and only a small number of high quality studies have investigated such effects (53).

Despite the apparent positive effect of breastfeeding observed in most studies, breastfeeding is also a potential source of exposure to some contaminants. For example, synthetic persistent lipophilic compounds such as organochlorine compounds (OCs) may be transferred to children

via breastfeeding (54). Although there is evidence that breastfeeding may counteract the negative effects of some compounds such as OCs (55,56), and the existing recommendations state that this practice should be maintained for a periods of >6 months (57), some authors have raised doubts about the overall benefits of breast-feeding against the risks due to exposure to persistent chemical agents in breast milk (57). .

4.2.4 Environmental determinants of neuropsychological development

A large number of chemical agents may have neurotoxic effects in humans. The number of these contaminants that have specific neurotoxic effects on brain during its development is considerable. In animal studies, it has been demonstrated that the exposure to chemicals such as Dichlorodiphenyltrichloroethane (DDT), pyrethroids, organophosphates (OPs), nicotine, paraquat and polychlorinated biphenyls (PCBs) during the 'brain growth spurt' can lead to irreversible changes in adult rat brain function (58).

A review of the evidence from population-based studies identified five chemicals as having neurotoxic effects for developing brain: arsenic, lead, methyl mercury, toluene, and PCBs (15). The first epidemiological studies of developmental neurotoxicity for any chemical were carried out in the 1970s, in the aftermath of the environmental contamination disasters in Minamata (59) and in Yusho and Yu-cheng (60). In both cases, high doses of contaminants (methylmercury and PCBs, respectively) were transferred by mother to child during pregnancy, with aberrant neurodevelopmental consequences only in children. Subsequently, environmental epidemiological studies were initiated and the number of

such studies has been increasing throughout the last few two decades. Currently, a large number of birth-cohort studies are looking at the effects of a variety of chemical agents on the developing brain.

There is now strong evidence to support the theory that neuropsychological development is impaired in children exposed in utero to high doses of Methylmercury (MeHg). Lower exposures may not be evident early in life, but the presence of MeHg in the brain (even at low doses) may have consequences when the cognitive capacities repertoire is more developed (61,62). The negative effects of lead on child neuropsychological development are well-established. Low levels of lead in blood (10 µg/dl) have been associated with reduced scores in intelligence tests and with poor academic performance (63–65). Exposure to lead has also been related to some neurodevelopmental disorders, such as attention deficit hyperactivity disorder (ADHD) and antisocial behaviour (66).

PCBs are ubiquitous environmental toxins. There is concern that at even low concentrations, PCBs may be transferred to the fetus across the placenta and, due to their lipophilic nature, may be concentrated and transferred via breast milk to the infant, thereby increasing exposure in the postnatal period. Cognitive subclinical deficits have been detected in children exposed to PCBs in several studies (67–69), and postnatal exposure to PCBs via breastfeeding has been associated with neuropsychological deficits (70). It has been suggested that PCBs injure the developing brain by interference with maternal thyroid function (71). In animal studies changes in levels of neurotransmitters in various brain areas have been observed in monkeys, rats, and mice, induced by exposure to PCBs congeners (72). Despite the relatively large body of literature on potential associations between early-life exposure to PCBs

and adverse neurodevelopmental effects, controversy still exists over whether PCBs are in fact neurotoxic, and to date, the U.S. Environmental Protection Agency has not established regulatory guidance values for PCBs based on neurotoxicity (73). There are inconsistencies among studies in exposure measurement, in terms of sampling (cord blood, maternal serum, child serum, milk), and quantification of the different congeners. In addition, there are also inconsistencies in the assessment of child neuropsychological development (ages of assessment, tests and cognitive areas assessed). Thus, two recent reviews concluded that the effects of prenatal exposure to background levels of PCBs on child cognition are still not clearly established and it is unclear which is the neuropsychological profile of those children exposed to PCBs (74,75).

Measurable concentrations of arsenic are present in ground water worldwide, and industrial contamination by this element is widespread (15). Epidemiological studies have found that the chronic exposure to arsenic is associated to impairments in language development and in long-term memory during childhood (76). In fact, the negative effects of arsenic on neuropsychological development have been isolated from those of other contaminants such as lead (77). However, a recent study in Bangladesh where increased levels of arsenic are found in groundwater in many areas, reported no associations between this contaminant and cognitive development scores (78). Toluene is an organic solvent that is widely used by industry and is ubiquitous in our environment. Epidemiological studies have identified some impairments during childhood in children exposed in utero to toluene such as developmental delays, including mental retardation, language impairment, and hyperactivity (79,80).

In addition to the previous chemical agents, negative effects on neuropsychological development due to exposure to pesticides have also been well-documented (81). Many different kinds of chemical are used as pesticides, including OCs and organophosphate compounds (OP). DDT is an insecticide used worldwide until some decades when its use was prohibited because its consequences for human health. DDT has been identified as toxic for brain development in animal studies (73). Studies conducted in humans have found negative effects of DDT and dichlorodiphenyldichloroethylene (DDE), the most stable metabolite of DDT, on cognitive development (81). Few studies have been conducted analyzing the neurotoxic effects of hexachlorobenzene (HCB) on neuropsychological development. To date, epidemiological studies have found negative associations between HCB exposure and social competence and ADHD symptoms at 4 years of age, but less strong evidence for a negative effect on sustained attention at 8 years of age (82,83). However, the current levels of these compounds in the serum samples of pregnant women are much lower than in the past and disentangling the effects at these levels on neuropsychological development is challenging (84).

An increasing number of substances with potential neurotoxic effects are being recognised. As shown in Figure 3, only five substances have been reliably demonstrated as having developmental neurotoxic effects. As the evidence base grows, many more chemicals may be demonstrated to have neurotoxic effects on the development brain.

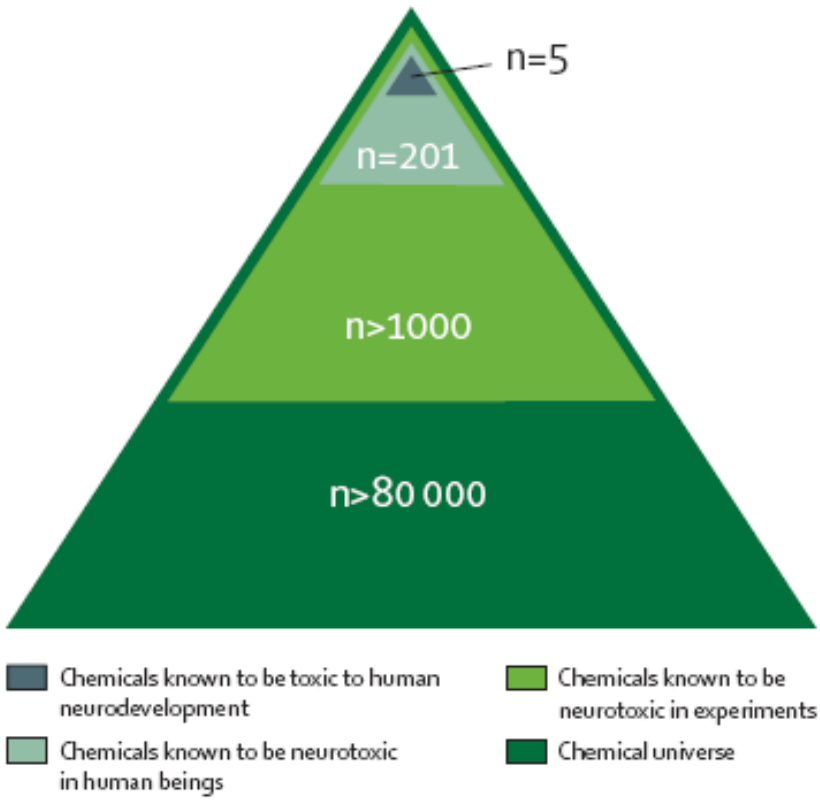

Figure 3. Diagram of the extent knowledge of neurotoxic chemicals (From Grandjean and Landrigan, 2006) (15)

In the Spanish context, the INMA project was the first population-based birth cohort study established in order to study the effects to some contaminants on child health (specifically, on neuropsychological development) (85). A birth cohort study was established in Ribera d'Ebre (Spain) with the aim to study the effects of OCs on the neuropsychological development. This study was justified by the high HCB levels found in the area and the lack of specific studies about health consequences of OC exposures in new born babies. The cohort of Ribera d'Ebre (n=102) was built with all new born of the town of Flix and 5

adjacent towns (nearby Ribera d'Ebre), between March 1997 and December 1999. Children were evaluated at the moment of birth, at 8 weeks of life, at 1 year and at 4 years using different neuropsychological tests. Two new cohorts were established in Menorca (n=530) and Granada (n=668) assessing the child neuropsychological development as one of the child health outcomes. From these data, a great number of papers and two previous thesis have been published: “Exposició a compostos organoclorats i efectes sobre la salut infantil durant el primer any de vida”; ISBN 8468857165 from Nuria Ribas-Fito; and “Early life factors influencing neurodevelopment and the study of the interrelations between different behavioural areas”; ISBN 9788469248553 from Jordi Julvez. Based on the experience from these previous cohorts, a common protocol was developed for four new birth cohorts: Sabadell, Valencia, Guipuzkoa, and Asturias.

4.3 Child Neuropsychology in environmental epidemiology

Clinical neuropsychology is an applied field of psychology the main aim of which is to describe, diagnose and treat cognitive, behavioural and emotional alterations as natural responses to structural and/or functional changes at the central nervous system level. Specifically, child neuropsychology is the study of brain function and behaviour in children and adolescents (9,86).

Neuropsychological evaluations are based on well-developed theoretical models and empirically supported principles that establish connections between observable behaviours or cognitive functions and

neuroanatomical systems. However, in evaluations with preschool children it is important not only to understand the impact of damage to these systems, but also how this damage could alter the typical development of specific neuropsychological functional domains (87). The child's brain develops continuously from prenatal period up to adolescence and therefore, the skills and cognitive abilities are changing and improving. For this reason, the neuropsychological tests used in child neuropsychology adopt a dynamic perspective adapting to these changes (88).

A number of misconceptions arise due to extensions made between the fields of adult and child neuropsychology. From a neurodevelopmental point of view, children are not ''small'' adults, and childhood disorders are not similar to adult disorders (89). The developing brain is continually changing during the first years of life. Some skills may develop at different ages in different children. Questions in child clinical neuropsychology begin to focus on the sequence in which skills are developed, the rate at which skills are developed, and the ways in which these skills change at each developmental stage. Furthermore, there is an emphasis on how disabilities interfere with—or disrupt—normal development, rather than on identifying which brain areas are deficient.

Child neuropsychologists add to their evaluations the assessment of neuropsychological domains generally not included in developmental evaluations (i.e., attention, executive functioning, or visual spatial skills), knowledge of brain–behaviour relationships, neural development, and neural recovery; knowledge of the impact of aetiology or disease course on cognitive functioning; and possibly the prognosis related to specific neuropsychological deficits. Moreover, child neuropsychology has advanced in its knowledge of brain development and functioning through

the use of medical technologies, including structural and functional magnetic resonance imaging (MRI), positron emission tomography (PET), single-photon emission computed tomography (SPECT), computerized tomography scans, and recently Diffusion Tensor Imaging (DTI) (90). However, the exact nature of brain functioning is complex and our knowledge is still incomplete, particularly concerning the developing brain.

The main function of neuropsychology within environmental epidemiology is to know the "normal" or "typical" development of the neuropsychological domains in order to detect the possible impact of environmental agents on brain function and psychological health. Given that neuropsychological development is an expression of brain development, the most cost-effective means of measuring it is the use of neuropsychological tests. Employing neuropsychological tests in environmental epidemiological research is well-suited to detecting alterations in cognitive, psychomotor, or behavioural development due to exposure to neurotoxic agents.

"The basic aim of every neuropsychological assessment - be it with adults, adolescents, or children - is to produce a reliable and valid "picture" of the relationships between the brain and behaviour."

Byron Rourke et al. 1983

5 RATIONALE

The study of brain development in environmental epidemiology has increased dramatically over the last two decades. Environmental epidemiological studies have shown detrimental effects for a range of exposures to lead compounds, and MeHg. However, for some other compounds, the evidence is not so clear. Environmental levels of contaminants with known neurotoxic effects are much lower than in previous decades in the developed world, where the majority of epidemiological research is conducted. Thus, it is important to detect if the current levels of some contaminants are still affecting neuropsychological development.

The brain, due to its particular nature, is a vulnerable organ in general but during development, this vulnerability is particularly high. The brain develops in an orchestrated sequence of processes guided by genetic heredity, and modified by environmental influences. During this process, which may extend through adolescence, the brain is an organ with a particularly high vulnerability to exogenous environmental insults.

We must take into account that any insult affecting brain may hamper optimal development of an individual's potential, and this may have consequences for their entire lifespan. Clearly, if a number of individuals are exposed to some chemical agents, it is possible that these individuals will not develop optimally. As such, this insult will impact at the population level. When we consider the large number of potentially neurotoxic chemical agents present in the environment, and combine this with the potentially huge number of socio-demographical variables

also modifying their effects on a population, we can begin to imagine the complexity of the puzzle presented to us.

The study of factors that may affect neuropsychological development should not focus only on the young. This doctoral thesis is mainly focused on studying some of the socio-environmental factors related to neuropsychological development up to 2 years of age, but efforts were also made to include work focused on factors associated to neuropsychological development in older children. There is evidence that the effects of some contaminants are not detected through infancy when the repertoire of cognitive domains is much more extensive. Finally, I believe that it is necessary to provide a summary of the work in this field of knowledge. Many cohort studies around the world are applying their own protocols and assessing different areas of neuropsychological development using different methodologies. In many cases, this work is not managed by an expertise neuropsychologist. For this reason, we considered that the elaboration of a conceptual framework summarizing the different areas of neuropsychological development was a necessary and useful complement to this thesis and novel contribution to the field.

6 OBJECTIVES

General objective:

To study the main social and environmental determinants of neuropsychological development up to two years of life, at preschool age, and during the preadolescent period.

Specific objectives:

1. Assess the effects of some maternal characteristics (intelligence, mental health) and cortisol on early neuropsychological development.
2. Assess the effects of breastfeeding (LC-PUFAs) on early neuropsychological development and the interaction with maternal smoking during pregnancy.
3. Assess the effects of OCs in neuropsychological development up to two years of life.
4. Assess the cognitive profile of PCBs exposure in preschool children.
5. Assess the effects of some early socio-environmental factors on neuropsychological development at preadolescence.
6. Elaborate a conceptual framework summarizing the different areas of neuropsychological development for environmental epidemiological studies.

7 METHODS

This thesis project is based on the data of the INMA (Infancia y Medio Ambiente)-Project (85). The main objective of this project is to evaluate the impact of environmental exposures in children's health. 7 Spanish birth cohorts participate in the project: 3 of them started on 1997-2000 (Riera d'Ebre (n=102), Menorca (n=482), Granada (n=668)), and the remaining 4 cohorts are new cohorts: Valencia (n=855), Sabadell (n=787), Asturias (n=498) and Gipuzkoa (n=637), started on 2004-2006 and following the same protocol.

Extensive assessments were carried out in pregnant women and children. The information was gathered from a variety of sources: ad hoc administered questionnaires

in face-to-face interviews by trained INMA personnel, clinical data, physical examinations, ultrasound scans, biological samples (blood, placenta, urine, saliva, hair, nails and mother's milk), biomarkers, diet determinants and environmental measurements (air pollution, water pollution and persistent and semi-persistent pollutants). Data collected at each wave varied slightly among cohorts according to internal interests, but the main common variables were included in all cohorts.

Figure 4. Geographical location of INMA cohorts (From Guxens et al, 2011) (85)

7.1 Neuropsychological assessment in the INMA-project

The neuropsychological assessment in the 4 new cohorts was initiated at 14 months using Bayley Scales for Infant Development (BSID) (91). BSID is divided in mental and psychomotor scales. The mental scale consisted of 163 items that assessed age-appropriate cognitive development in areas such as performance ability, memory, and first verbal learning. The psychomotor scale consisted of 81 items assessing fine and gross psychomotor development.

The next assessment was done at 4-5 years old. Most of the new cohorts are currently performing this visit. Cognitive and motor development was

assessed using the Spanish version of the McCarthy Scales of Children's Abilities (MCSA) (92). The General Cognitive Scale and 5 subscales (verbal, perceptive–performance, memory, quantitative, and motor) were examined, and in addition some items were used to construct a new summary measure to assess those cognitive tasks associated with executive function. In the same visit we also assessed other outcomes: 1) attention function using the Conners' Kiddie Continuous Performance Test (93); 2) symptomatology of ADHD using the Attention Deficit Hyperactivity Disorder Criteria of Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition form list (94); 3) social competence development using California Preschool Social Competence Scale (95); and 4) symptomatology of Asperger Syndrome using Childhood Asperger Syndrome Test (96).

To limit inter-observer variability in the neuropsychological development area, we applied a strict protocol, including training sessions where inter-observer differences were quantified and three sets of quality controls (inter-observer-reliability-tests) undertaken during the fieldwork. For each visit (at 14 months and at 4-5 years old) we performed a quality control session in which all the fieldworkers are committed and evaluated several children in order to calculate the Intra-Class Correlation. For both visits, the results were optimal.

Apart from the new 4 cohorts, in this thesis project we also used data from the Menorca Cohort. This is the oldest cohort together with Ribera d'Ebre. In Menorca cohort children have been assessed at 11 years old using The Continuous Performance Test-II (CPT-II) (97). The CPT-II is a computerized measure of vigilance/attention control and response inhibition for children aged 6 and older.

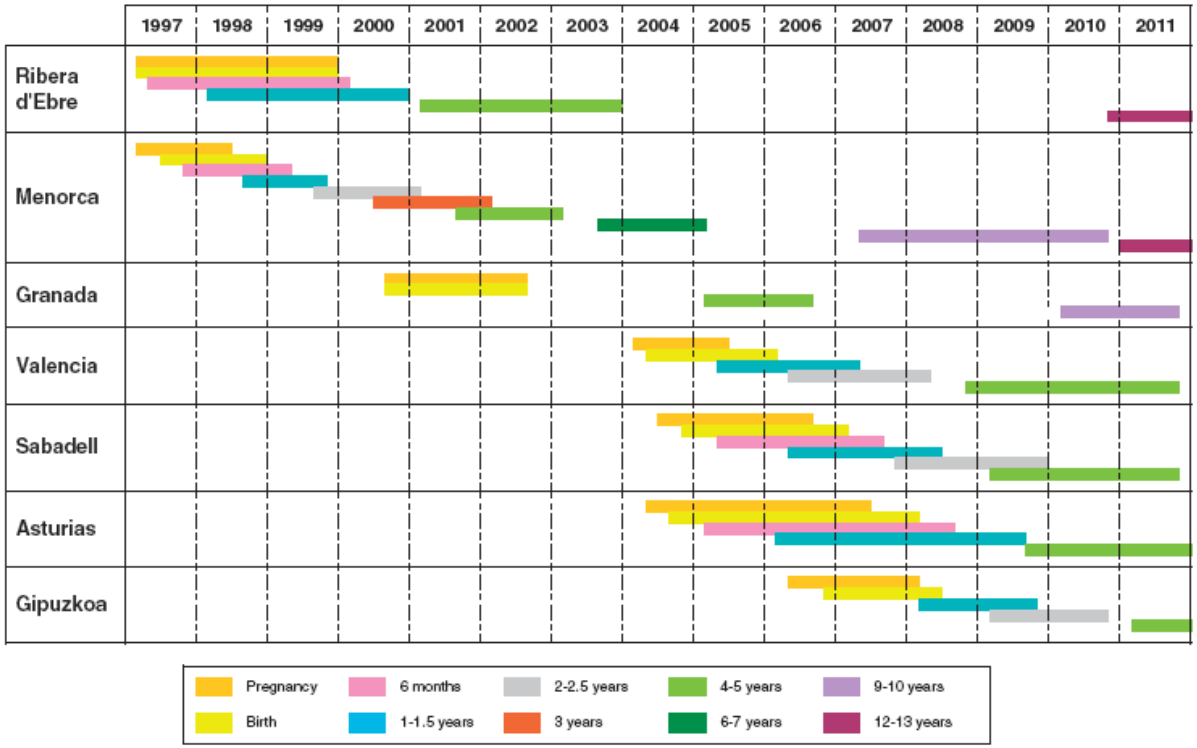

Figure 5. Follow-up of INMA cohorts (From Guxens et al, 2011) (85)

8 RESULTS

Number of paper	Title
1	Maternal intelligence - mental health and child neuropsychological development at 14 months
2	Postnatal maternal and child cortisol and neuropsychological development at 14 months
3	Breastfeeding, long-chain polyunsaturated fatty acids in colostrum, and infant mental development
4	The role of maternal smoking habits in the association between breastfeeding and cognitive development during the 2nd year of life
5	Prenatal exposure to organochlorine compounds and neuropsychological development up to two years of life
6	Prenatal exposure to Polychlorinated Biphenyls and child neuropsychological development in 4-year-olds: an analysis per each congener and specific cognitive domain
7	Longitudinal association between early life socio-environmental factors and attention function at the age 11 years
8	A Conceptual Framework in the Study of Neuropsychological Development in Epidemiological Studies

8.1 Paper 1

Maternal intelligence - mental health and child neuropsychological development at 14 months

Joan Forns, Jordi Julvez, Raquel Garcia-Esteban, Mònica Guxens, Muriel Ferrer, James Grellier, Martine Vrijheid & Jordi Sunyer.

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Maternal intelligence - mental health and child neuropsychological development at 14 months (Inteligencia y salud mental maternas y desarrollo neuropsicológico infantil a los 14 meses de edad)

Joan Forns[a,b,c]., Jordi Julvez[a,b,c,d], Raquel Garcia-Esteban[a,b,c]., Mònica Guxens[a,b,c]., Muriel Ferrer[a,b,c]., James Grellier[a,b,c,f], Martine Vrijheid[a,b,c] & Jordi Sunyer[a,b,c,e].

aCenter for Research in Environmental Epidemiology (CREAL), Doctor Aiguader 88, 08003, Barcelona, Spain

[b]Hospital del Mar Research Institute (IMIM), Doctor Aiguader 88, 08003, Barcelona, Spain.

[c]CIBER Epidemiologia y Salud Pública (CIBERESP), Doctor Aiguader 88, 08003, Spain

[d]Department of Environmental Health, Harvard School of Public Health, Boston, MA, USA.

[e]Universitat Pompeu Fabra (UPF), Barcelona, Spain

[f]Department of Epidemiology and Biostatistics, Imperial College London, UK

Corresponding Author and Requests for Reprints:

Joan Forns Guzmán

Centre for Research in Environmental Epidemiology- IMIM

C. Doctor Aiguader 88; 08003 Barcelona; Spain

Phone: +34 93 214 73 11 Fax: +34 93 214 73 02

E-mail: jforns@creal.cat

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Conflict of interest: none declared.

Authorship

J. Forns analyzed the data, wrote the manuscript, drafted the manuscript and gave final approval of the version to be submitted. J. Julvez, M. Vrijheid, M. Guxens, M. Ferrer and J. Sunyer designed the study, developed the questionnaires, supervised the survey and revised the manuscript critically. J. Grellier has revised the manuscript critically, contributed to writing parts in it, and corrected the quality of written English. R. Garcia-Esteban contributed in the statistical analyses and read the manuscript critically. All the authors have read and approved the final manuscript.

Title: Maternal intelligence - mental health and child neuropsychological development at 14 months / Título: Inteligencia y salud mental maternas y desarrollo neuropsicológico infantil a los 14 meses de edad.

Resumen

Objetivos: Este estudio examinó la relación entre inteligencia y salud mental materna y desarrollo neuropsicológico infantil a los 14 meses de edad en población normal, teniendo en cuenta la clase social basada en la ocupación y el nivel educativo maternos.

Métodos: Este es un estudio prospectivo de cohortes de nacimiento englobado dentro del proyecto INMA (Infancia y Medio Ambiente). El desarrollo cognitivo y psicomotor fue evaluado mediante la escala Bayley de Desarrollo Infantil. La inteligencia y salud mental maternas fueron evaluadas usando el Test de Cattell y Cattell y el Cuestionario de Salud General de 12 ítems respectivamente.

Resultados: Se observó una asociación cruda entre inteligencia materna y desarrollo cognitivo infantil a los 14 meses de edad. Sin embargo, esta asociación desaparecía cuando la educación materna era incluida. Las asociaciones fueron estratificadas por educación y clase social basada en la ocupación materna. En el estrato de clase social manual materna se observó una diferencia significativa en la escala mental de la escala Bayley entre esos niños cuyas madres puntuaron en el tercil más alto de inteligencia materna, comparado con el tercil más bajo. No obstante, no se observaron diferencias entre aquellos niños de clase social no-manual materna.

Conclusiones: Existe un patrón diferente en la asociación entre inteligencia materna y desarrollo cognitivo infantil según clase social materna. Mientras la asociación no es confundida por educación ni otras

variables en clases sociales manuales, el nivel educativo materno explica esta asociación en las clases sociales no-manuales.
Palabras clave: Desarrollo infantil; inteligencia; salud mental; neuropsicología.

Abstract

Objective: This study examined the relationship between maternal intelligence-mental health and neuropsychological development at age 14 months in a normal population, taking into account maternal occupational social class and education.

Methods: This was a prospective population-based birth cohort as part of the INMA (Environment and Childhood) Project. Cognitive and psychomotor development was assessed at 14 months using Bayley Scales of Infant Development. Maternal intelligence and mental health was assessed by Cattell and Cattell test and the General Health Questionnaire-12 respectively.

Results: We observed a crude association between maternal intelligence and cognitive development of children at 14 months. However, this association disappeared when maternal education was included. The associations were stratified by maternal education and occupational social class. Within the manual maternal occupational social class, there was a significant difference in cognitive development between children whose mothers scored in the highest tertile of maternal IQ, compared with the lowest tertile. In contrast, no differences were observed among children whose mothers were in the non-manual occupational social class.

Conclusions: There is a different pattern between maternal intelligence and child cognitive development by occupational social class. While this association was not confounded by education or other variables in manual occupational social class, maternal education explains this association among advantaged occupational social classes.

Keywords

Child development; intelligence; mental health; neuropsychology.

INTRODUCTION

The developing brain is exceptionally sensitive to environmental influences, such as chemical agents, nutrition, medical care, but also to education, experiences, and the social environment[1-5]. The social environment is composed of familial and cultural characteristics that exist in society and impact on neuropsychological development[6]. Early development is recognized as a policy priority since 'the early childhood period is considered to be the most important developmental phase in an individual's lifespan[7]. A review of proximal risk factors for child development in the context of developing countries identified inadequate cognitive stimulation as the most important psychosocial determinant, along with maternal depression and exposure to violence[8]. In developed countries, it has been found that poor cognitive stimulation at early ages may produce a wide range of non-adaptive neurobehavioral outcomes[9].

Early parental influences are crucial factors in the early stages of brain maturation, specifically related to their sensitivity and early cognitive stimulation[10]. Among these influences, maternal intelligence is an important determinant in the early stages of child neuropsychological development. On average, about 50% of the variance in intelligence is due to genetic differences[11]. Apart from the genetic contribution, mothers with higher scores in an intelligence test probably have better parenting skills, such as dietary patterns, health care, housing, as well as a cognitively-stimulating environment. A fundamental characteristic of brain development is that environmental experiences are as important as inherited factors[12]. Tong et al (2007)[13] found that the maternal intelligence quotient (IQ) is positively predictive of children's cognitive development. The same study also found that socioeconomic position

and the domestic environment are positively associated with child neuropsychological development. Income, education and occupation have been found to be positively associated with better parenting[14]. A cross-cultural review has found that socioeconomic indicators are strongly related to cognitive development from infancy to middle childhood[15].

On the other hand, maternal depression may be considered a risk factor for child development, including impairments in the areas of social-emotional development, behaviour, and cognitive development[16-19]. Effects of depression on parenting practices mothers may include disruption in displays of affection, inadequate attention to basic safety and care, regulation of sleep, use of harsh discipline, less utilization of primary care and more utilization of emergency care, and others[20].

The aim of this study was to disentangle the direct effects of maternal IQ and mental health on early childhood neuropsychological development. In order to reduce the residual confounding in the associations, and because of the strong relationship between maternal intelligence and mental health and socioeconomic variables, maternal education and occupational social class were treated as confounders and potential effect modifiers in the analyses. Moreover, a large number of potentially confounding variables, especially those related to child stimulation, have also been included.

METHODS

A population-based birth cohort was established in the city of Sabadell (Catalonia, Spain) as part of the INMA [Environment and Childhood] Project[21]. Between July 2004 and July 2006, 657 pregnant women who visited the public health centre of Sabadell for an ultrasound in the first

trimester were recruited. They were then followed during pregnancy. A total of 619 (94.2%) children were enrolled at birth, and 588 (89.5%) were followed until 14 months. The main analyses in this report are based on 523 children with complete information on maternal IQ-mental health and neuropsychological development assessment. Information on maternal education, socioeconomic background, parity, alcohol, dietary intake, and smoking habits during pregnancy was obtained through questionnaires administered during pregnancy (first and third trimester of gestation). Anthropometric measures, sex, and gestational age were obtained at birth from clinical records. Data on breastfeeding were based on questionnaires administered to the mothers at 6 and 14 months after delivery. The study was approved by the Clinical Research Ethical Committee of the Municipal Institute of Health Care (CEIC-IMAS), and all the participating mothers gave informed consent.

Neuropsychological development assessment

Neuropsychological development was assessed at 14 months (range 12 to 17 months) using the mental and psychomotor scales of the Bayley Scales of Infant Development (BSID)[22]. All testing was done at the primary health-care centre in the presence of the mother by 2 specially trained neuropsychologists who were unaware of any exposure information. Four-hundred and fifty-nine children were assessed by psychologist 1 and 102 children by psychologist 2. Nine children's tests were excluded because of specific pathologies in the child (immaturity (n=4), Down's syndrome, very low preterm, autistic traits (n=2) and hypotonia).

The mental scale consisted of 163 items that assessed age-appropriate cognitive development in areas such as performance ability, memory,

and first verbal learning. The psychomotor scale consisted of 81 items assessing fine and gross motor development. Index scores were computed based on the assumption of a normal distribution with a mean of 100 corresponding to the mean of the raw scores, and a standard deviation of 15.

In order to limit inter-observer variability, a strict protocol was applied. This included inter-observer-training and three sets of quality controls (inter-observer reliability-tests) done during the fieldwork. The inter-rater reliability was estimated by Intra-Class Correlation of 0.93 for mental test score, and 0.96 for psychomotor scale. Cronbach's Alpha Coefficient was used to determine the internal consistency of the mental and psychomotor scales. For the mental test score, the Alpha Coefficient was 0.78 and for the psychomotor scale was 0.73[23].

Finally, psychologists also flagged those tests that may be difficult to evaluate because of less than optimal cooperation of the child (n=24), classified as behavioural problems (e.g. bad moods, nervousness, etc.) or a particular situation (e.g. tiredness, colds, asleep, etc) in a new variable designated "quality of neuropsychological test".

Maternal IQ and mental health

Two maternal factors were considered in this study: maternal IQ and mental health, assessed at the same time as the child's neuropsychological developmental tests (14 months).

Maternal IQ was assessed by 2 psychologists using the 2 and 3 scales of Factor "G" of Cattell and Cattell[24], which is a nonverbal test of fluid intelligence[22]. The test was administered in 532 of the recruited women. Mean and standard deviation data of our sample were used to compute

index scores assuming a normal distribution. Raw scores were standardized to a mean of 100 with a standard deviation of 15.

Maternal mental health was assessed using the Spanish version of the General Health Questionnaire-12 items (GHQ-12) as a self-reported questionnaire[25, 26]. Each of the 12 items assess the severity of a mental problem over the past few weeks using a 4-point Likert-type scale (from 0 to 3). The score was used to generate a total score ranging from 0 to 36, where higher scores indicate poorer mental health.

For GHQ-12, 79 mothers were selected randomly in order to calculate the one-month test-retest reliability using the Kappa value. We obtained Kappa coefficients of agreement around 0.87 for GHQ-12. Cronbach's Alpha Coefficients showed acceptable internal congruence (0.79). Spearman coefficients were used to analyse the correlation between maternal IQ and mental health, and obtained a correlation between maternal IQ and maternal mental health of rho=-0.09 (p=0.038). Paternal IQ and mental health were also assessed using same methodologies, but IQ was available only for 104 subjects.

Other variables

Educational level was defined using three categories: primary or less, secondary school, and university. Occupational social class was coded from the longest-held job during the pregnancy, or, if the mother did not work in this time period, the last job prior to pregnancy. In the few cases that the mother never worked, the last job of the father was used (n=22). Occupations were coded using the four-digit Spanish National Classification of Occupations (Clasificación Nacional de Ocupaciones) (CNO94), which is closely related to the international ISCO88 coding system[27]. Five social class categories were then created following the

methodology proposed by the Spanish Epidemiological Society[28]. These categories were regrouped into manual and non-manual jobs.

Predominant breastfeeding was defined as an infant receiving breast milk only (but allowing supplementation of non-milk liquids): drinking water or water-based drinks (sweetened and flavoured water, teas, infusions, etc), fruit juice, oral rehydration salts solution, drops, and syrup forms of vitamins, minerals, and medicines[29]. Duration of predominant breastfeeding was categorized into four groups (<2 weeks, 2-16, 16-24, >24). Other variables were maternal work status at the time of BSID assessment (employed, unemployed), parental country of birth (Spain, foreign), child's sex (girls, boys), low birth weight (<2500 grams), preterm birth (<37 weeks), Apgar score at 1 minute (<8 considered non-optimal), number of siblings at time of birth (0, 1, 2 or more), and maternal smoking during pregnancy (never, former, current).

Statistical analysis

Univariate descriptive analysis of neuropsychological test (mental and psychomotor scales of BSID), predictive variables (maternal IQ and mental health), socioeconomic characteristics of parents, and anthropometric measures of children were carried out to describe the population.

Bivariate associations (t-test or ANOVA) between parental socio-demographic covariates and child clinical measures and outcomes (Mental and Psychomotor Scale) were analysed in order to detect potential confounders (p-value<0.25).

Multivariable linear regression models were built for mental and psychomotor test scores considering all potential confounding variables

using backward stepwise regression methods. Any covariate either with a p-value of less that 0.25 or resulting in a change in estimate of predictive variables (maternal IQ or mental health) greater or equal than 10% was retained in the model.

For each predictive variable, we fit three multiple linear regression models: 1) adjusting only for psychologist, child's age in days, and quality of neuropsychological test, 2) also adjusting for potential confounders plus maternal occupational social class; and 3) also adjusting for maternal education. Predictive variables were examined in tertiles taking the lowest one as the reference group. This categorical approach allowed us to explore the differences in infant neuropsychological development between extreme groups. To study the combined impact of both the predictive variables, we also fitted a model in which they were mutually adjusted.

Finally, maternal education and occupational social class were considered as potential effect modifiers (p-value for interaction<0.10) for the relationship between maternal IQ-mental health and child cognitive and psychomotor development. Statistical analyses were done using Stata 8.2 (Stata Corporation, College Station, Texas).

RESULTS

The mean age of the children was 14.8 months (range: 12-17 months). Four percent of children were born low birth weight, 2% with a gestational age less than 37 weeks (preterm), and 5% with a non-optimal score in the Apgar test at 1 minute (Table 1). Forty-two percent of the mothers had secondary education and 32% had university education. Fifty-three percent were classified as non-manual occupational social class. Ten percent of mothers were immigrant and 15% were current

smokers during pregnancy. The educational level in fathers was similar to mothers, but the occupational social class distribution was markedly different. More than 50% of fathers in the sample were classified in the manual occupational class. In general, mothers who participated in the psychological profile tests had higher levels of education than those who did not participate (data not shown). For occupational social class no differences were found.

Distributions of the BSID scores by maternal IQ and mental health, and parental socioeconomic characteristics were plotted (Table 2). A positive trend was observed in child mental test score related to maternal intelligence, but not with psychomotor scores. There was a positive trend in the distribution of maternal IQ related to the maternal education and occupational social class (Table 3). Mothers with a university degree or belonging to the non-manual occupational social class scored significantly higher in the IQ test.

The crude associations between parental and child characteristics and mental and psychomotor scales were plotted (Figure 1). In the multivariate regression models, the mental test score increased significantly with higher maternal IQ; after adjusting for all potential confounders except maternal education, the association remained marginally statistically significant (Table 4). However, the association disappeared when maternal education was included. No association was observed between the mental test score and maternal mental health. None of the three specific factors of mental health were associated with mental test score (p-values>0.10, not shown). No associations were found between the psychomotor tests scores and maternal profile variables. Paternal mental health did not have any effect in the models.

Finally, these associations were stratified by maternal education and occupational social class (Table 5). Within maternal manual occupational social class, there was a significant difference (coef=7.92) in mental test score between children whose mothers scored in the highest tertile of maternal IQ, compared with the lowest one. No such differences were observed, however, among children whose mothers were in the non-manual occupational social class. These differences between strata showed a significant interaction (p-value for interaction=0.044). A similar result was found among children whose mothers had primary education (coef=6.75), but it was not statistically significant (p-value=0.18), also contrasting with no differences among children whose mothers were highly educated. Maternal mental health did not show any difference between different occupational social classes. Maternal education and occupational social class did not modify the relationship between maternal IQ-mental health and infant psychomotor development (data not shown).

DISCUSSION

In this population-based birth cohort, the effect of maternal intelligence on child cognitive development at 14 months of age was stronger than the effect of maternal mental health and this relationship was found to be mostly explained by maternal education. However, in more disadvantaged occupational social classes, maternal intelligence appeared to be the best predictor of optimal child cognitive development, exceeding both maternal education and mental health.

In previous research[13], an independent association was found between maternal IQ and cognitive development of toddlers, along with SES and home environment. In our study, the observed association between

maternal IQ and child cognitive development disappeared after adjustment for maternal education, but persisted when maternal occupational social class was included. Some authors have pointed out that general intelligence is highly predictive of educational level[30]. This might imply that IQ should exceed educational level, perhaps due to a strong hereditary component, and to some extent should predict educational attainment in life. If this were the case, the scores of children in mental test scores should be strongly associated with maternal IQ and, to a lesser degree, with maternal education. However, our data does not support such a hypothesis. Other authors[31] have pointed out that the proportion of variability in a population's IQ that is attributable to genetic factors, is unclear. We found that maternal educational attainment is strongly related to child neuropsychological development. Along with SES, low maternal education has been found to be an important predictor of neuropsychological developmental problems in different contexts[10]. It has been hypothesised that women with higher IQ scores and higher educational level offer better parenting, such as life-styles, stimulating environment, health care, housing, as well as cognitively-stimulating environment as those several studies that pointed out a relationship between maternal education and dietary patterns in infancy[32]. In turn, studies suggest that mothers with low education lack the intellectual issues to stimulate their children[33].

Within manual occupation social classes, maternal IQ appears as an important predictor of child cognitive development. Children of those mothers of manual occupational social class with high scores in IQ tests scored significantly higher values on the BSID mental test score than children from less intelligent mothers from the same occupational social class. Previous studies have found that SES is an important predictor of

neuropsychological development, especially for language and executive function[30]. There is some support for the hypothesis that in deprived SES less cognitive stimulation is offered by parents and the environment, and that this mechanism may produce disparities in neuropsychological development between different social classes[34]. In our study, those mothers belonging to manual occupational social classes but with higher intelligence than the group average represent a special subgroup within the manual class. From this, we might deduce that mothers with higher intelligence have children with higher intelligence, irrespective of their educational level. By way of explanation, these mothers might offer their children a more stimulating environment within which they have a greater opportunity to develop their cognitive functions.

We did not find any association between maternal mental health and child neuropsychological development. A possible explanation could be that this study is applied in a non-clinical population in which mothers were not suffering from major depressive disorders or other serious psychiatric disorders. The distribution of scores of the GHQ-12 had low variability and did not detect any subclinical symptomatology. Nevertheless, throughout the school years, cognitive development becomes more specific and in turn, neuropsychological testing is more precise, reliable and valid than at the previous ages[34]. The follow-up of the birth cohort will allow us to explore whether an effect on cognitive development appears at older ages, and whether this affects children's social behaviour.

No associations were found between maternal IQ and mental health and child psychomotor development at 14 months. The psychomotor scale showed a stronger association with preterm birth, Apgar scores at 1 minute, maternal work status at 14 months, and maternal age. Very

preterm infants had significantly lower scores than full-term infants on the psychomotor scale of BSID but no significant differences were found between the groups on the mental test score[35]. In other studies, negative Apgar scores have been found to be related with neurological disability[36]. Maternal employment at the ninth month was found to be linked to lower scores in a cognitive test at 36 months[37].

A major strength of this study was its large sample size. We studied a population-based cohort of 559 children using field staff, interviewers, laboratory technicians, and project paediatricians, all of whom were specifically trained for the project. For the neuropsychological assessments several quality controls were introduced (inter-observer reliability-tests) and the psychologists who assessed children with the BSID questionnaire received extensive training to this end.

Our study was limited by a number of factors. Although we included a large number of covariates, we were unable to control for some factors that may affect children's cognition, such as the quality of their home environment, nutrition, maternal postpartum depression or maternal stress during pregnancy. Omitting these factors may have introduced residual confounding[38]. Moreover, we used self-reported questionnaires for the parents to assess socio-demographic characteristics and mental health. This could have led to the introduction of a response bias, but such a bias would most likely be non-differential and lead to reduced statistical power. Despite the importance of maternal characteristics during the first years of life, information on the father is also valuable. Unfortunately, it was not available and we could not assess paternal IQ and mental health across the entire sample. Finally, measurements of mental health vary with time. For the assessment of long-term effects

repeated measurements during and just after pregnancy would be necessary.

We conclude that in this general population, variations in maternal mental health were low and their effects on neuropsychological development were not detected at 14 months of age. Maternal IQ plays an important role in the first stages of cognitive development of the children in more disadvantaged occupational social classes. For the other groups, the effects of maternal IQ on cognitive development were mostly explained by maternal education. It is important for future research to assess the continuation of these findings at a later stage in development when the phenotypes will be more developed.

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FIGURE LEGEND

Figure 1. Bivariate association (Coefficient and 95% Confidence Interval) between outcomes and covariates of interest.

Table 1. Description of the sample (n=523):

Child characteristics	
Age (months), Mean (SD)	14.80 (0.67)
Sex	
Girls	260 (49.7)
Boys	263 (50.3)
Predominant breastfeeding	
<2 weeks	117 (22.5)
2-16 weeks	155 (29.8)
16-24 weeks	196 (37.7)
>24 weeks	52 (10.0)
Low birth weight (<2500 gr)	23 (4.4)
Preterm (<37 weeks)	13 (2.5)
Apgar 1 minute (≥8, optimal)	493 (95.4)
Siblings at birth	
0	306 (58.7)
1	186 (35.7)
2 or +	29 (5.6)
Maternal characteristics	
Age (years), Mean (SD)	32.8 (4.3)
Occupational social class	
Manual	242 (46.3)
Non-manual	281 (53.7)
Education	
Primary or less	130 (25.0)
Secondary school	223 (42.9)
University	167 (32.1)
Work status at child's 14 months (Employed)	368 (70.9)
Country of birth (Foreign)	51 (9.9)
Smoking during pregnancy	
Never	220 (42.5)
Former	217 (42.0)
Current	80 (15.5)
Paternal characteristics	
Age (years), Mean (SD)	34.7 (4.9)
Occupational social class	
Manual	290 (58.7)
Non-manual	204 (41.3)

Paternal characteristics	
Education	
Primary or less	170 (32.8)
Secondary school	225 (43.4)
University	123 (23.8)
Country of birth (Foreign)	58 (11.2)

SD=Standard Deviation.
Values are n (percentages) unless otherwise noted
Differences in the number of observations for some of the variables presented in the table are due to missing values.

Table 2. Distribution of the infant mental and psychomotor test scores by maternal intelligence and mental health and parental socioeconomic characteristics:

		Mental test score			Psychomotor Scale		
	n	Mean	(SD)	p	Mean	(SD)	p
Maternal Intelligence							
T1 (≤94)	194	97.79	(14.98)	0.029	99.61	(14.01)	0.769
T2 (94-107)	175	100.58	(14.33)		99.27	(15.25)	
T3 (≥107)	154	101.95	(15.68)		100.4	(15.36)	
Maternal Mental Health							
T1 (≥11)	156	100.22	(15.59)	0.302	99.75	(14.03)	0.171
T2 (8 – 11)	149	98.23	(15.20)		97.80	(15.63)	
T3 (≤8)	201	100.66	(15.18)		100.76	(14.35)	
Maternal Education							
Primary or less	130	96.28	(17.52)	0.002	97.20	(15.39)	0.062
Secondary	223	100.13	(13.65)		99.98	(14.50)	
University	167	102.43	(14.36)		101.24	(14.65)	
Maternal Occupational Social Class							
Manual	242	98.70	(14.76)	0.077	99.48	(14.47)	0.717
Non-manual	281	101.03	(14.35)		99.96	(15.24)	
Paternal Education							
Primary or less	170	99.17	(15.05)	0.165	98.37	(15.47)	0.353
Secondary	225	99.13	(14.19)		100.08	(14.28)	
University	123	102.09	(16.38)		100.72	(14.98)	
Paternal Occupational Social Class							
Manual	290	98.85	(14.92)	0.046	99.27	(15.36)	0.311
Non-manual	204	101.60	(14.92)		100.65	(14.29)	

T: Tertile.

Differences in the number of observations for some of the variables presented in the table are due to missing values.

Table 3. Distribution of the maternal IQ and mental health by maternal education and occupational social class

	Intelligence				Mental Health			
	n	Mean	(SD)	p	n	Mean	(SD)	p
Maternal Education								
Primary or less	130	89.82	(12.76)	<0.001	121	10.62	(4.34)	0.088
Secondary	223	99.39	(13.17)		220	10.37	(4.44)	
University	167	108.83	(12.26)		162	9.59	(3.78)	
Maternal Occupational Social class								
Manual	242	93.03	(13.52)		232	10.52	(4.42)	0.101
Non-manual	281	106.05	(12.73)	<0.001	274	9.90	(4.02)	

Differences in the number of observations for some of the variables presented in the table are due to missing values.

Table 4. Adjusted association between infant mental and psychomotor test scores and maternal intelligence and mental health:

	Mental test score								
	Model1			Model2			Model3		
	Coef	(S.E.)	P	Coef.	(S.E.)	P	Coef.	(S.E.)	P
Maternal IQ (Reference T1 ≤94)									
T2 (94-107)	2.69	(1.38)	0.052	1.91	(1.45)	0.188	0.88	(1.52)	0.563
T3 (≥107)	4.31	(1.43)	0.003	3.13	(1.54)	0.043	1.39	(1.69)	0.411
Maternal MH (Reference T1 ≥11)									
T2 (8 – 11)	-1.31	(1.43)	0.360	-1.33	(1.45)	0.357	-1.43	(1.44)	0.321
T3 (≤8)	0.60	(1.40)	0.672	-2.68	(1.56)	0.094	-2.58	(1.56)	0.099
Mutually adjusted									
Maternal IQ (Reference T1 ≤94)									
T2 (94-107)	2.70	(1.40)	0.054	1.82	(1.47)	0.217	0.81	(1.53)	0.598
T3 (≥107)	4.59	(1.47)	0.002	3.34	(1.58)	0.035	1.49	(1.74)	0.392
Maternal MH (Reference T1≥11)									
T2 (8 – 11)	-1.55	(1.44)	0.280	-1.34	(1.47)	0.365	-1.32	(1.47)	0.372
T3 (≤8)	0.87	(1.43)	0.542	-2.86	(1.59)	0.072	-2.65	(1.60)	0.098

	Psychomotor Scale								
	Model1			Model 4			Model5		
	Coef	(S.E.)	P	Coef	(S.E.)	P	Coef	(S.E.)	P
Maternal IQ (Reference T1 ≤94)									
T2 (94-107)	-0.38	(1.46)	0.797	-0.59	(1.49)	0.693	-1.48	(1.56)	0.343
T3 (≥107)	0.95	(1.52)	0.533	-0.13	(1.57)	0.932	-1.47	(1.74)	0.397
Maternal MH (Reference T1≥11)									
T2 (8 – 11)	0.98	(1.46)	0.503	0.97	(1.48)	0.513	-1.82	(1.52)	0.230
T3 (≤8)	-1.46	(1.58)	0.357	-0.89	(1.59)	0.575	-0.79	(1.49)	0.594
Mutually adjusted									
Maternal IQ (Reference T1 ≤94)									
T2 (94-107)	0.21	(1.47)	0.884	-0.09	(1.50)	0.953	-0.88	(1.56)	0.575
T3 (≥107)	1.37	(1.55)	0.374	0.26	(1.60)	0.871	-0.97	(1.77)	0.584
Maternal MH (Ref T1≥11)									
T2 (8 – 11)	0.75	(1.50)	0.615	0.93	(1.52)	0.539	-1.92	(1.54)	0.215
T3 (≤8)	-1.84	(1.62)	0.255	-1.12	(1.63)	0.491	-0.80	(1.52)	0.597

Coef :Coefficient ; (S.E.):Standard Error ; T: Tertile ; Maternal MH: Maternal Mental Health

Model1: adjusted for psychologist, child's age in days and quality of neuropsychological test.

Model2: adjusted for variables in Model1 plus parental occupational social class, sex of the child, parental country of birth, maternal age and maternal work status at child's 14 months.

Model3: adjusted for variables in Model2 plus maternal education

Model4: adjsuted for variables in Model1 plus Apgar score at 1 minute, preterm birth, maternal work status at child's 14 months and maternal age. Model5: adjusted for variables in Model4 plus maternal education

Table 5.1. Adjusted association between infant mental test score and maternal intelligence and mental health stratifyng by maternal education and maternal occupational social class:

	Primary†			Secondary & University			
	Coef	(S.E.)	P	Coef	(S.E.)	P	p-value for interaction
Reference*	94.86			101.74			
Maternal IQ - T2 (94-107)	1.35	(3.72)	0.689	-0.18	(1.70)	0.918	0.696
Maternal IQ - T3 (≥107)	6.75	(5.03)	0.183	0.63	(1.82)	0.729	
Maternal MH - T2 (8 – 11)	-2.98	(3.37)	0.380	-0.47	(1.60)	0.771	0.527
Maternal MH - T3 (≤8)	1.35	(3.55)	0.705	0.66	(1.56)	0.674	

Maternal MH:Maternal Mental Health; T: Tertile ; Coef : Coefficient ; (S.E.) : Standard Error

*Reference as score of children from mothers with IQ ≤94 and Mental Health ≥11

†Adjusted for psychologist, child's age in days, quality of neuropsychological test, sex of the child, paternal country of birth, maternal age, maternal occupational social class and maternal work statuts at 14 months

‡Adjusted for psychologist, child's age in days, quality of neuropsychological test, sex of the child, paternal country, maternal age and maternal education

Table 5.2. Adjusted association between infant mental test score and maternal intelligence and mental health stratifyng by maternal education and maternal occupational social class:

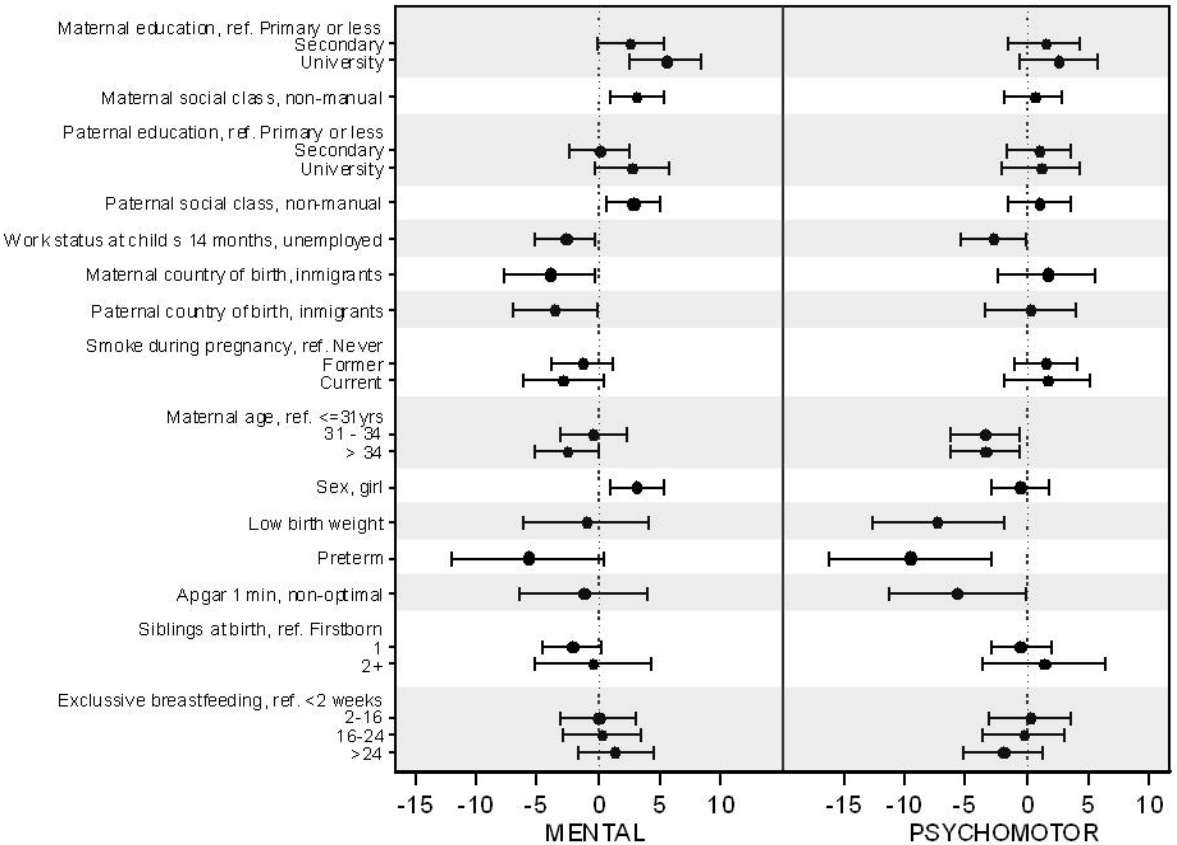
	Manual‡			Non-manual			
	Coef	(S.E.)	P	Coef	(S.E.)	P	p-value for interaction
Reference*	97.63			94.35			
Maternal IQ - T2 (94-107)	0.38	(1.98)	0.847	0.09	(2.33)	0.971	0.044
Maternal IQ - T3 (≥107)	7.92	(2.79)	0.005	-0.95	(2.31)	0.680	
Maternal MH - T2 (8 – 11)	-1.74	(2.14)	0.417	-1.54	(1.99)	0.441	0.966
Maternal MH - T3 (≤8)	1.05	(2.06)	0.609	0.72	(1.99)	0.718	

Maternal MH:Maternal Mental Health; T: Tertile ; Coef : Coefficient ; (S.E.) : Standard Error

*Reference as score of children from mothers with IQ ≤94 and Mental Health ≥11

†Adjusted for psychologist, child's age in days, quality of neuropsychological test, sex of the child, paternal country of birth, maternal age, maternal occupational social class and maternal work statuts at 14 months

‡Adjusted for psychologist, child's age in days, quality of neuropsychological test, sex of the child, paternal country, maternal age and maternal education


Maternal education, ref. Primary or less
Secondary
University
Maternal social class, non-manual
Paternal education, ref. Primary or less
Secondary
University
Paternal social class, non-manual
Work status at child s 14 months, unemployed
Maternal country of birth, inmigrants
Paternal country of birth, inmigrants
Smoke during pregnancy, ref. Never
Former
Current
Maternal age, ref. <=31yrs
31 - 34
> 34
Sex, girl
Low birth weight
Preterm
Apgar 1 min, non-optimal
Siblings at birth, ref. Firstborn
1
2+
Exclussive breastfeeding, ref. <2 weeks
2-16
16-24
>24
-15 -10 -5 0 5 10
MENTAL
-15 -10 -5 0 5 10
PSYCHOMOTOR

8.2 Paper 2

Postnatal maternal and child cortisol and neuropsychological development at 14 months

Joan Forns, Oscar Vegas, Jordi Julvez, Raquel Garcia-Esteban, Marcela Rivera, Nerea Lertxundi, Mònica Guxens, Eduardo Fano, Muriel Ferrer, Jesús Ibarluzea & Jordi Sunyer.

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POSTNATAL MATERNAL AND CHILD CORTISOL AND NEUROPSYCHOLOGICAL DEVELOPMENT AT 14 MONTHS

Joan Forns[a,b,c]., Oscar Vegas[e], Jordi Julvez[a,b,c,d], Raquel Garcia-Esteban[a,b,c], Marcela Rivera[a,b,c], Nerea Lertxundi[e], Mònica Guxens[a,b,c], Eduardo Fano[e,] Muriel Ferrer[a,b,c], Jesús Ibarluzea[c,f] & Jordi Sunyer[a,b,c,g].

(a) Centre for Research in Environmental Epidemiology (CREAL), Barcelona, Spain.

(b) Hospital del Mar Research Institute (IMIM), Barcelona, Spain.

(c) CIBER Epidemiología y Salud Pública (CIBERESP), Barcelona, Spain.

(d) Department of Environmental Health, Harvard School of Public Health, Boston, MA, USA.

(e) Faculty of Psychology, Basque Country University, San Sebastián, Spain

(f) Subdirección de Salud Pública de Gipuzkoa. The Basque Government's Health Department. San Sebastián, Spain

(g) Pompeu Fabra University. Barcelona, Spain.

Correspondence and queries to:

Joan Forns Guzmán - Centre for Research in Environmental Epidemiology- CREAL

C. Doctor Aiguader 88; 08003 Barcelona; Spain

Phone: +34 93 214 73 11 Fax: +34 93 214 73 02 E-mail: jforns@creal.cat

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Abbreviations: HPA, hypothalamic-pituitary-adrenal; BSID, Bayley Scales of Infant Development; CV, coefficient of variation; INMA: Infancia y Medio Ambiente.

ABSTRACT

Preliminary evidence suggests that children who are breastfeed have higher levels of cortisol than children who are not breastfed and that higher levels of postnatal maternal cortisol are associated with neuropsychological development. This study aimed to assess the relationship between: 1) postnatal cortisol levels in mother and in child; 2) the duration of breastfeeding and cortisol levels in children; and 3) cortisol levels in mother and child with early neuropsychological development. This is a cross-sectional at 14 months of age within a population-based birth cohort study established in Sabadell (Spain) as part of the INMA [Environment and Childhood] Project. We included 388 mother-child pairs with information on neuropsychological assessment (Bayley Scales of Infant Development) and cortisol measurements (from mothers and child). High levels of cortisol in the mother had a significant association with the cortisol levels of the child at 14 months ($p<0.01$). The duration of breastfeeding was associated with cortisol in children (an increase in 0.02 pg/ml per week). However, the levels of child cortisol were not associated with neuropsychological development.

Keywords: child development, cognition, intelligence tests, neuropsychology, cortisol.

INTRODUCTION

Activation of the hypothalamic–pituitary–adrenal (HPA) axis in reaction to environmental stressors is part of a response aimed at adapting the organism to the changes in the environment. The activation of the HPA axis produces two main types of stress hormones, glucocorticoids (cortisol), and catecholamines (adrenaline and noradrenaline) (1). Glucocorticoids interact with their receptors in multiple target tissues, including the HPA axis and several brain regions (2-4). Some authors have suggested that a deregulation of the HPA axis may represent a health risk to the organism and that the integrity of the HPA axis is therefore essential for an effective regulation of physiological and behavioural responses to different environmental demands (3-5).

Three important areas of the brain (hippocampus, amygdala, and frontal lobes) contain glucocorticoid receptors (3). Animal studies have shown that normal levels of glucocorticoids benefit brain maturation by initiating terminal maturation, re-modeling axons and dendrites and guaranteing cell survival (6). In fact, cortisol has a role in sustaining and facilitating cognitive functions in humans (4). However, prolonged exposure to elevated levels of cortisol has been linked to negative consequences in the structural development of the brain, such as neural atrophy in the hippocampus and the medial prefrontal cortex (7,8). It has also been suggested that exposure to high concentrations of cortisol is associated with impairments in cognitive and social-emotional development (4,9).

During the early stages of development, the human brain is exposed to the mother's cortisol. In the prenatal period, several possible mechanisms have been proposed to explain the exposure of the foetus to maternal cortisol: transplacental transport of cortisol to foetus,

release of placental hormones induced by maternal stress, and/or decrease in the blood flow to the placenta induced by maternal stress (10). In the postnatal period, breastfeeding has been proposed as a potential mechanism of exposure as shown in a previous study in which breastfed children have up to 40% higher levels of cortisol than children who are not breastfed (11). However, the published data are very scarce.

The aims of this study were: 1) to determine whether the levels of postnatal maternal and child cortisol were related; 2) to assess the association between child cortisol levels and the duration of breastfeeding; and 3) to assess the the association between child and maternal cortisol levels and child neuropsychological development at 14 months of age. In addition, as it is still unclear whether HPA axis activity in children is sex-specific (12), the effects of maternal and child levels of cortisol on child neuropsychological development were investigated for boys and girls independently. Finally, given the hypothesis that the main route of exposure during the postnatal period was through breastfeeding, the effect of duration of breastfed on the mentioned associations was also studied (11).

METHODS

Study design

This is a cross-sectional study within a population-based birth cohort study established in the city of Sabadell (Catalonia, Spain) as part of the INMA [Environment and Childhood] Project (13). Between July 2004 and July 2006, 657 pregnant women who visited the primary health care center of Sabadell for a routine ultrasound in the first three months of pregnancy were recruited and followed during pregnancy. A total of 619 (94.2%) children were enrolled at birth, and 588 (89.5%) were followed

until 14 months. Additional information of socio-demographic variables was collected through questionnaires administered the first and third trimester of gestation. Anthropometric measures, sex, and gestational age at birth were obtained from clinical records. The study was approved by the Clinical Research Ethical Committee of the Municipal Institute of Health Care (CEIC-IMAS), and all the participating mothers gave informed consent.

Neuropsychological testing

Cognitive and psychomotor development was assessed at 14 months (range 12 to 17 months) using the mental and psychomotor scalesof the Bayley Scales of Infant Development (BSID) (14). All testing was done at the primary health-care center in the presence of the mother by 2 specially trained neuropsychologists who were unaware of any exposure information. A total of 559 children followed through birth (95%) were evaluated. The analysis excluded 18 infants born before week 37 because it is well known that such prematurity affects neuropsychological development during infancy (15), and 27 infants difficult to evaluate according to the psychologists because of less than optimal cooperation during the test. The mental scale consisted of 163 items that assessed age-appropriate cognitive development in areas such as performance ability, memory, and first verbal learning. The psychomotor scale consisted of 81 items assessing fine and gross motor development. Tests scores were computed based on the assumption of a normal distribution with a mean of 100 corresponding to the mean of the raw tests scores, and a standard deviation of 15.

In order to limit inter-observer variability, a strict protocol was applied. This included inter-observer-training and three sets of quality controls

(inter-observer reliability-tests) done during the fieldwork. The inter-rater reliability was estimated by Intra-Class Correlation with 0.93 for mental test scores, and 0.96 for psychomotor test scores. Cronbach's Alpha Coefficient was used to determine the internal consistency of the mental and psychomotor tests scores. For the mental test scores, the Alpha Coefficient was 0.78 and for the psychomotor test scores 0.73 (16).

Cortisol assay

We measured cortisol levels in samples obtained from saliva, which facilitates patient compliance compared to venipuncture. Salivary hormonal measurement provided a reliable, non-intrusive method that has been shown to reflect accurately the free (non-protein bound) fraction of plasma (17-21).

Child saliva samples were collected at 14 months of age. All mothers were instructed to collect saliva samples at home on a regular (preferably working) day. They were given a study pack that consisted of standardized written instructions with a schedule and two different kits to extract the samples: a needleless 2 cc plastic syringe for children and a hygienic saliva collection kit for mothers (Salivette, Sarsdtedt, Nümbchet, Germany). Given that numerous substances affect cortisol levels in saliva, mothers were asked to refrain from brushing their teeth, smoking, eating, and drinking 30-min before sampling.

The target collection time was between 5:00 PM and 9:00 PM before dinner because this is a quiescent period of the circadian cycle (22). Eighty-seven percent of the samples were collected within the designated time of the day. After cortisol collection, mothers filled in a brief questionnaire assessing date, hour, changes in routines (yes/no), changes in the child behavior in the last 7 days (yes/no), and maternal

anxiety (yes/no) with the aim of collecting important variables to adjust the effects of the circadian and diurnal rhythms. The day after saliva collection, samples were handed in to the INMA fieldworkers at the time of the BSID assessment was performed.

A total of 722 saliva samples (415 from mothers and 307 from children) were frozen and stored in the laboratory at −80°C and analyzed later in the Faculty of Psychology at the University of the Basque Country. As part of the analysis, the samples were centrifuged at 3000 rpm for 15 minutes to remove mucins. All samples were assayed using an enzyme immunoassay kit (Salimetrics, State College, USA, for corticosterone). The average intra-assay coefficient of variation (CV) was 6.7% (26.3 pg/ml), and the average inter-assay CV was 9.6% (13.1 pg/ml). The sensitivity of the kit was <1.5% pg/ml.

Other variables

Additionally to the BSID assessment, we assessed the maternal intelligence quotient (IQ), mental health, and attachment. Maternal IQ was assessed by two trained neuropsychologists using the 2 and 3 scales of Factor "G" of Cattell and Cattell (23). Maternal mental health and mother-to-child attachment were assessed respectively using two self-reported questionnaires: the Spanish version of the General Health Questionnaire-12 items (GHQ-12) (24), and the Condon questionnaire (25).

Educational level was defined using three categories: primary or less, secondary school, and university. Social class was based on both the mother's occupation as defined by the longest-held job reported by the mother in the period from one month before pregnancy and the 3rd trimester of pregnancy, and, the father's principal occupation during the

last 10 years. Social class categories were based on the International Standard Classifications of Occupations (ISCO-88) (26). The categories were: I for managers and professionals (non-manual); II for technicians and associate professionals; III for other non-manual workers; IV for skilled manual workers; V for semi-skilled or unskilled manual workers; and VI for unclassifiable or unknown (also includes occupationally inactive people).

Detailed information about child feeding was gathered using interviewer-administered questionnaires given to the mothers when their children were 6 months and again at 14 months. Breastfeeding was defined as receiving breast milk and allowing food and/or liquid supplements including nonhuman milk (27). A number of variables were assessed when the child was 14 months: mother's occupational status (employed, unemployed), parental country of birth (Spain, other), child's sex (male, female), low birth weight (<2500 grams), number of siblings at birth (0, 1, 2 or more), maternal smoking during pregnancy (never, former, current), and current maternal smoking habits (yes/no). Infant weight (to the nearest gram) and length (to the nearest 0.1 cm) were also measured at 14 months by trained staff using standard protocols. The child's body mass index (BMI) was calculated using weight in kilograms divided by the square of height in meters.

Statistical analysis

Cortisol levels were log-transformed due to skewed distribution observed after univariate analyses. The residuals of the regression model on (log-) cortisol levels were adjusted by hour, day, and season of collection in order to reduce the variability in cortisol levels introduced by these factors were used for subsequent analyses. Child and maternal cortisol

levels were examined as continuous variables, and in quartiles taking the lowest one as the reference group to explore the differences between extreme groups.

Multivariate linear regression models adjusting for potential confounders were used to study: 1) the association between child and maternal cortisol levels, 2) the relationship between duration of breastfeeding and child cortisol levels, and 3) the effect of child and maternal cortisol measurements (in separated models) on mental and psychomotor test scores (dependent variables). Adjustment for the confounding variables was done using the manual backward stepwise method. Only covariates showing associations with p-value<0.10 with BSID scores or those that resulted in a change in estimate of the exposure of interest ≥ 10% were retained in the model.

In addition, to control for potential residual confounding, a series of models was run to assess the effect of additionally adjusting one by one for some parental characteristics such as maternal IQ, parental mental health, and attachment. Statistical analyses were done using Stata 10.0 (Stata Corporation, College Station, Texas).

RESULTS

A complete data set was available for 388 children with BSID assessment, and one cortisol measurement either for the mother or the child, who constituted the present study sample. The study sample was significantly different from the non-participants in terms of sex (53% females vs 41%) and paternal social class (56% manuals vs 48%).

Table 1 describes the socio-demographic variables included in the analyses. The mean age of children was 14.7 months. Twenty-six percent of mothers' highest education level was primary school and 70% were

classified as non-manual social class. Fathers had a similar educational level as mothers, but a distribution of social class markedly different (40% were classified as non-manual social class). In table 2, we show the crude association between outcomes (mental and psychomotor test scores) and those variables retained in the final multivariate models: parental education and social class, smoking habits, maternal anxiety, main childminder, and child's sex and birthweight.

We found a direct association between child and maternal cortisol levels (Coeficient (Coef) = 0.25; p-value<0.001, from linear regression model)(Figure 1). Only in children who never breastfed (n=41), this association was not significant (Coef = 0.31; p-value = 0.511). Neither sex nor duration of breastfeeding (excluding formula-fed group) did not modify the effect of maternal cortisol levels on child cortisol (p-value for interaction were 0.346 and 0.619, respectively). We also found a positive association between child cortisol levels and the duration of breastfeeding after adjusting for child's sex, maternal social class, education, child's age and body mass index (BMI) (Coef = 0.02; p-value = 0.006, from linear regression model) (Figure 1).

We found no association between mental and psychomotor test scores and child cortisol levels (Table 3). This association was not modified by child's sex. The pattern of the association between mental and psychomotor test scores and maternal cortisol levels was different for boys and girls. The inclusion of the maternal IQ, parental mental health, or parental attachment did not have any effect in these models (data not shown).

DISCUSSION

In this study it is shown that maternal and offspring cortisol levels were significantly associated at the age 14 months. A positive association between levels of child cortisol and duration of breastfeeding was also found. However, the results indicate that the levels of child cortisol were not related with neuropsychological development at this age. The effects of maternal postnatal cortisol levels on child neuropsychological development at 14 months were modified by sex.

The levels of cortisol of children and mothers were positively related, while the levels of child cortisol were also positively associated with the duration of breastfeeding. These results are consistent with a previous study suggesting that breastfeeding is a plausible mechanism to transfer cortisol from mother to child. Our data provide support the hypothesis that the maternal-fetal connection may be extended during the postnatal period through early childhood via prolonged periods of breastfeeding (11).

We did not find associations between child cortisol levels and child neuropsychological development at 14 months. One explanation could be that at 14 months, the HPA axis, which has been considered as an age-dependent system (12), is still inmature and might not determine the neuropsychological development. Further assessments at older ages will allow us to disentangle whether the child cortisol levels may have impact on child neuropsychological development. In the other hand, the fewer number of saliva samples in relation to BSID assessments (307 samples from children in comparison to 559 neuropsychological tests) could also introduce a selection bias. Participants differed from non-participants in terms of sex and paternal social class. The importance of sex (12) and socioeconomic status (22) on both variables (neuropsychological

development and the integrity of HPA-axis) might result on the acceptance of the null hypothesis.

The results of the present study suggest that the association between maternal postnatal cortisol and child neuropsychological development at 14 months was modified by sex. This is a cross-sectional association which could not assess the temporal order of the events. It is, therefore, important to consider that the possibility of reverse causation could not be rejected. Nevertheless, because of this hormone is released as a result of stress conditions (22), it can be hypothesized that greater levels of cortisol might represent the greater distress in mothers. The immaturity of boys compared to girls at earlier ages in terms of brain development (28 – 30) may explain these differences.

This study has a number of strenghts. The data collection was performed by field staff, interviewers, laboratory technicians, and project paediatricians, specifically trained for the project. For the neuropsychological assessments several quality controls were introduced (inter-observer-reliability-tests) and the psychologists who assessed children with the BSID received a proper training. However, this study is limited in that we only collected one sample of saliva to assess cortisol levels. To confirm the stability of this measure, more assessments in different time points would have needed. Nevertheless, the observed association between child and maternal cortisol levels suggests that the measurement error is weak. Despite of the population-based birth cohort design, the present study adopts a cross-sectional criterion. Both outcome and exposure were collected at the same time, and therefore, we only can hypothesize about the acute effects of this association. We could not adjust for maternal stress during pregnancy, maternal postpartum depression, paternal stress, or quality of home environment

which may be involved in the relationship between maternal postnatal stress and child neuropsychological development. However, we included some psychosocial covariates such as mother's age, parental social class, education, work status, maternal IQ, parental mental health, or parental attachment in order to reduce part of the residual confounding (31). Despite the importance of maternal characteristics during the first years of life, information on the father is also valuable. Unfortunately, it was not available and we could not assess paternal IQ across the entire sample. Another potential limitation refers to the non-response ratio which may introduce selection bias as commented before.

Overall, the results from our study suggest that breastfeeding may be an important mechanism of cortisol exposure during the postnatal period. The levels of child cortisol were not related to neuropsychological development at the age of 14 months. The longitudinal study of this cohort study will allow us to replicate these models at older ages when the phenotypes will be more developed.

Competing interests: none declared.

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FIGURE LEGEND

Figure 1. Association between maternal cortisol levels and duration of breastfeeding and child cortisol levels using Generalized Additive Models (GAMs)

Table 1.1. Description of the sample (n=388):

	Child	
Age (months), mean (SD)	14.74	(0.65)
Sex, n (%)		
Female	203	(52.44)
Male	185	(47.56)
Low birth weight , n (%)	9	(2.31)
Siblings at birth, n (%)		
0	225	(57.99)
1	142	(36.60)
2 o +	21	(5.41)
Duration of breastfeeding (weeks), mean (SD)	2.18	(20.31)
Main Childminder	129	(32.91)
Mother	184	(48.04)
Both parenths	65	(16.97)
Grandparents	18	(4.70)
Both parents +grandparents	71	(18.54)
Others	45	(11.75)

SD=Standard Deviation.

Table 1.2. Description of the sample (n=388):

	Mothers		Fathers	
Age (years), mean (SD)	32.86	(4.22)	34.68	(4.92)
Education, n (%)				
Primary or less	102	(26.42)	136	(35.32)
Secondary	159	(41.19)	159	(41.30)
University	125	(32.38)	90	(23.38)
Social class, n (%)				
Professionals, managers and technicians	91	(23.51)	102	(26.22)
Other non-manual	180	(46.51)	51	(13.10)
Skilled, semi-skilled and unskilled	81	(20.93)	217	(55.78)
Unclassifcable and unknown	35	(9.04)	19	(4.88)
Country of origin (Foreign) , n (%)	28	(7.29)	41	(10.62)
Smoke during pregnancy, n (%)				
Never	161	(41.82)	-	
Former	167	(43.38)	-	
Current	57	(14.81)	-	

SD=Standard Deviation.

Table 2. Associations between mental and psychomotor test scores and variables of interest:

	Mental test scores†			Psychomotor test scores†		
	Coef	(SE)	p	Coef	(SE)	p
Maternal characteristics						
Education, (Ref. Primary or less)	99.26			98.71		
Secondary	2.52	(1.45)	0.008	1.47	(1.55)	0.299
University	4.81	(1.54)		2.57	(1.65)	
Social Class, (Ref. Profess, manag & tech)	104.42			102.32		
Other non manual	-2.45	(1.48)	0.052	-2.37	(1.58)	0.224
Skilled, semi-skilled and unskilled	-4.64	(1.76)		-2.94	(1.88)	
Unclassificable	-4.02	(2.18)		-4.28	(2.33)	
Maternal smoking habits at 14 months (Ref. No)	101.91			2.07	(1.61)	(0.20)
Yes	-3.82	(1.53)	0.013	99.60		
Maternal anxiety (Ref. No)	101.91			100.53		
Yes	-0.32	(1.48)	0.832	-2.72	(1.59)	0.087
Paternal characteristics						
Education, (Ref. Primary or less)	101.56			99.65		
Secondary	-0.68	(1.34)	0.149	0.75	(1.43)	0.864
University	2.23	(1.57)		0.60	(1.67)	
Social Class, (Ref. Profess, manag & tech)	104.32			100.40		
Other non manual	-4.00	(1.88)	0.091	-0.02	(2.01)	0.995
Skilled, semi-skilled and unskilled	-3.16	(1.39)		-0.29	(1.49)	
Unclassificable	-2.45	(2.83)		-0.55	(3.03)	
Child characteristics						
Sex, (Ref. Male)	100.53			100.10		
Female	2.69	(1.16)	0.020	0.20	(1.24)	0.869
Birthweight (Ref. >2500 gr)	101.89			100.36		
<2500 gr	-0.12	(3.46)	0.972	-5.68	(3.67)	0.122
Duration of breastfeeding (per each week)	0.01	(0.03)	0.760	0.01	(0.03)	
Main Childminder (Ref. mother)	100.79			98.74		
Both parenths	1.61	(1.66)	0.039	1.58	(1.76)	
Grandparents	0.49	(2.84)		1.55	(3.01)	
Both parents +grandparents	6.08	(1.95)		3.10	(2.07)	
Others	0.43	(1.61)		2.24	(1.71)	
BMI at 14 months (per each kg/m2)	0.00	(0.44)	0.995	0.00	(0.44)	0.995

†Adjusted for psychologist, child's age in days, and quality of test

Table 3.1. Association between mental and psychomotor test scores and child and maternal cortisol levels (Multivariate Regression models):

	Mental test scores[a]						
	Boys			Girls			
	Coef.	(SE)	p	Coef.	(SE)	p	p-inter
Child Cortisol levels							
Per each (log) unit increase	1.74	(1.75)	0.322	1.16	(1.79)	0.519	0.577
Quartiles (Ref. Q1)							
Q2	-2.04	(3.22)	0.529	3.32	(3.43)	0.335	0.770
Q3	3.62	(3.29)	0.274	4.19	(3.40)	0.220	
Q4	2.83	(3.43)	0.411	2.64	(3.48)	0.450	
Maternal Cortisol levels							
Per each (log) unit increase	-2.19	(1.50)	0.146	2.12	(1.43)	0.140	0.050
Quartiles (Ref. Q1)							
Q2	-0.92	(2.91)	0.753	7.07	(2.79)	0.012	0.066
Q3	-2.13	(2.93)	0.468	6.62	(2.83)	0.020	
Q4	-5.98	(3.08)	0.054	5.02	(2.71)	0.065	

Models were adjusted for child's age, psychologist, child's body mass index, and duration of breastfeeding

[a]Also adjusted for main childminder, maternal education, and paternal social class

[b]Also adjusted for maternal anxiety, maternal smoking habits at 14 months, maternal social class, and birthweight

Coefficient; SE=Standard Error; p-inter=p-interaction

Table 3.2. Association between mental and psychomotor test scores and child and maternal cortisol levels (Multivariate Regression models):

	Psychomotor test scores[b]						
	Boys			Girls			
	Coef.	(SE)	P	Coef.	(SE)	p	p-inter
Child Cortisol levels							
Per each (log) unit increase	1.40	(1.86)	0.450	0.12	(1.79)	0.946	0.708
Quartiles (Ref. Q1)							
Q2	5.06	(3.43)	0.143	3.45	(3.58)	0.337	0.316
Q3	-0.90	(3.54)	0.799	2.59	(3.57)	0.469	
Q4	6.03	(3.65)	0.101	0.35	(3.51)	0.920	
Maternal Cortisol levels							
Per each (log) unit increase	-1.81	(1.53)	0.237	0.75	(1.55)	0.626	0.234
Quartiles (Ref. Q1)							
Q2	-4.06	(2.98)	0.175	8.77	(3.02)	0.004	0.019
Q3	-0.63	(2.97)	0.831	3.87	(3.07)	0.209	
Q4	-3.83	(3.07)	0.213	3.87	(2.87)	0.180	

Models were adjusted for child's age, psychologist, and duration of breastfeeding

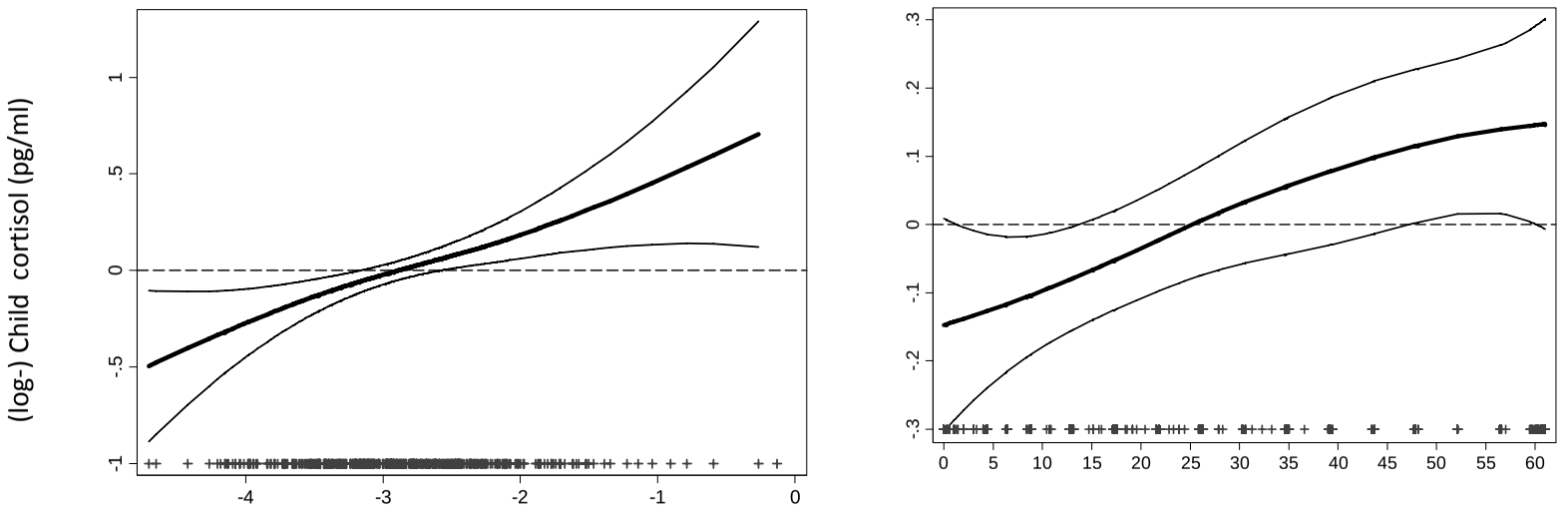
[a]Also adjusted for main childminder, maternal education, and paternal social class

[b]Also adjusted for maternal anxiety, maternal smoking habits at 14 months, maternal social class, and birth weight

[c]Models for child cortisol levels were also adjusted for child's body mass index

Coef=Coefficient; SE=Standard Error; p-inter=p-interaction

Figure 1. Association between maternal cortisol levels and duration of breastfeeding and child cortisol levels using Generalized Additive Models (GAMs):



(log-) Maternal cortisol (pg/ml)†

Duration of breastfeeding (weeks)

GAMs were adjusted for child's sex, maternal social class, education, child's age, and child's Body Mass Index.

†Also adjusted for breastfeeding

8.3 Paper 3

Breastfeeding, long-chain polyunsaturated fatty acids in colostrum, and infant mental development

Mònica Guxens, Michelle A. Mendez, Carolina Moltó-Puigmartí, Jordi Julvez, Raquel García-Esteban, Joan Forns, Muriel Ferrer, Martine Vrijheid, M. Carmen López-Sabater, Jordi Sunyer.

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Breastfeeding, long-chain polyunsaturated fatty acids in colostrum, and infant mental development

Mònica Guxens, MD, MPH, PhD[a,b,c], Michelle A. Mendez, PhD[a,b,c], Carolina Moltó-Puigmartí, DPharm, PhD[c,d], Jordi Julvez, PhD[a,b,c], Raquel García-Esteban,MSc[a,b,c], Joan Forns, BSc, MPH[a,b,c], Muriel Ferrer, BSc[a,b,c], Martine Vrijheid, PhD[a,b,c], M. Carmen López-Sabater, DPharm, PhD[c,d], Jordi Sunyer, MD, PhD[a,b,c,e]

[a]Center for Research in Environmental Epidemiology (CREAL), Dr. Aiguader, 88, 08003 Barcelona, Catalonia, Spain

[b]Hospital del Mar Research Institute (IMIM), Dr. Aiguader, 88, 08003 Barcelona, Catalonia, Spain

[c]CIBER Epidemiologia y Salud Pública (CIBERESP), Dr. Aiguader, 88, 08003 Barcelona, Catalonia, Spain

[d]Department of Nutrition and Food Science, Faculty of Pharmacy, University of Barcelona, Av. Joan XXIII, s/n - 08028 Barcelona, Catalonia, Spain

[e]Pompeu Fabra University (UPF), Dr. Aiguader, 80, 08003 Barcelona, Catalonia, Spain

Short title: Polyunsaturated fatty acids and infant mental development

Keywords: child development; cognition; breast feeding; fatty acids, unsaturated; intelligence

Figure legend.

Figure 1. Association between PUFA levels (total n-3/n-6 PUFAs ratio), tertiles of cumulative intensity of breastfeeding, and infant mental development score[1].

[1]The reference group was subjects with low tertile of cumulative breastfeeding and low PUFA levels. Beta coefficients were adjusted for psychologist, child's age in days, quality of the neuropsychological test, parental education, social class, attachment to the child, intelligence quotient, and mental health, maternal age, maternal alcohol use during pregnancy, use of a gas stove at home during pregnancy, and child's age of food introduction. Bars, 95% confidence interval (CI).

Table 1a. Child characteristics of the study population (n=504)

	n	Distribution	Number of missings
Sex (female)			0
Male	257	51.0	
Female	247	49.0	
Gestational age (weeks)	504	39.7 (1.4)	0
Birthweight	503	3257.6 (416.6)	1
Number of siblings at child's birth			2
0	300	59.8	
1 or more	202	40.2	
Main childminder at 14 months			17
Mother	229	47.0	
Both parents with/without grandparents	148	30.4	
Other combinations	110	22.6	
Nursery attendance at 14 months			12
Yes	340	69.1	
No	152	30.9	

Values are percentages for categorical variables and mean (SD) for continous variables

Table 1b. Maternal characteristics of the study population (n=504)

	n	Distribution	Number of missings
Education level			3
Primary or less	123	24.6	
Secondary	215	42.9	
University degree	163	32.5	
Social class			0
I/II Managers/Technicians	122	24.2	
III/IV Skilled manual/non-manual	245	48.6	
V/VI Semi-skilled/unskilled	137	17.2	
Age at child's birth (years)	503	31.6 (4.2)	1
Country of bith			6
Spain	447	89.8	
Foreign	51	10.2	
Family status			1
Biparental	495	98.4	
Monoparental	8	1.6	
Smoking at 3rd trimester			2
Yes	433	86.3	
No	69	13.7	
Use of gas stove during pregnancy			1
Yes	308	61.2	
No	195	38.8	
Pre-pregranacy body mass index			0
Underweight	17	3.4	
Normal weight	350	69.5	
Overweight	98	19.4	

	n	Distribution	Number of missings
Obese	39	7.7	
Maternal intelligence quotient	478	100.3 (14.7)	26
Mother-to-child attachment	475	58.3 (5.2)	29
Maternal mental health	474	10.2 (4.3)	30

Values are percentages for categorical variables and mean (SD) for continous variables

Table 1c. Parental characteristics of the study population (n=504)

	n	Distribution	Number of missings
Education level			5
Primary or less	171	34.3	
Secondary	214	42.9	
University degree	114	22.8	
Social class			0
I/II Managers/Technicians	129	25.6	
III Skilled manual/non-manual	214	42.5	
IV/V Semi-skilled/unskilled	161	31.9	
Age at child's birth (years)	502	33.4 (4.8)	2
Country of birth			3
Spain	441	88.0	
Foreign	60	12.0	
Paternal intelligence quotient	102	99.5 (15.0)	402
Father-to-child attachment	456	49.5 (4.0)	48
Paternal mental health	453	9.3 (3.7)	51

Values are percentages for categorical variables and mean (SD) for continous variables

Table 2. Mental and psychomotor development scores by breastfeeding.

		Mental score[1]		Psychomotor score[1]	
	n (%)	Mean	(95% CI)	Mean	(95% CI)
Cumulative intensity of breastfeeding					
1st tertile	168 (33.3)	100.0	(97.9 to 102.1)	100.5	(98.2 to 102.8)
2nd tertile	167 (33.3)	101.0	(98.9 to 103.1)	100.4	(98.2 to 102.7)
3rd tertile	169 (33.3)	103.4	(101.3 to 105.5)	99.3	(97.0 to 101.5)
Exclusive breastfeeding					
Never	82 (16.3)	99.4	(96.5 to 102.4)	101.2	(98.1 to 104.3)
≤ 4 months	162 (32.1)	101.7	(99.5 to 103.8)	100.6	(98.4 to 102.9)
4-6 months	206 (40.9)	101.2	(99.3 to 103.1)	99.4	(97.5 to 101.4)
> 6 months	54 (10.7)	104.9	(101.4 to 108.5)	99.0	(95.2 to 102.7)

[1]Adjusted for psychologist, child's age in days, and quality of the neuropsychological test.

Cumulative intensity of breastfeeding was also adjusted for age of food introduction.

Table 3. Association (β coefficient and 95% of Confidence Interval) between breastfeeding and infant mental development at 14 months[1] (n=504).

	Breastfeeding	
	Cumulative intensity of breastfeeding[2]	Exclusive breastfeeding (>6 months vs. never)
	β (95% CI)	β (95% CI)
Model 1[3]	0.37 (0.06 to 0.67)	5.48 (0.96 to 10.00)
Model 1[3] + Maternal social class	0.30 (0.05 to 0.61)	4.58 (0.05 to 9.12)
Model 1[3] + Maternal education	0.30 (-0.01 to 0.61)	4.80 (0.29 to 9.31)
Model 1[3] + Maternal intelligence quotient	0.34 (0.03 to 0.64)	4.89 (0.36 to 9.41)
Model 1[3] + Mother-to-child attachment	0.37 (0.06 to 0.67)	5.52 (0.96 to 10.04)
Model 1[3] + Maternal mental health	0.38 (0.07 to 0.68)	5.63 (1.10 to 10.15)
Model 1[3] + Paternal social class	0.35 (0.04 to 0.65)	5.20 (0.67 to 9.72)
Model 1[3] + Paternal education	0.35 (0.04 to 0.66)	5.21 (0.66 to 9.76)
Model 1[3] + Paternal intelligence quotient	0.36 (0.06 to 0.66)	4.31 (-0.23 to 8.85)
Model 1[3] + Father-to-child attachment	0.36 (0.06 to 0.67)	5.53 (1.01 to 10.05)
Model 1[3] + Paternal mental health	0.36 (0.06 to 0.67)	5.47 (0.94 to 9.99)
Model 1[3] + Persistent toxic compounds[4]	0.37 (0.07 to 0.68)	5.22 (0.69 to 9.75)
Adjusted model[5]	0.33 (0.02 to 0.63)	3.17 (-1.34 to 7.68)

[1]CI, Confidence Interval

[2]A one-unit increase represents the equivalent of one month of full breastfeeding, whether accumulated in a single month, or over several months of partial breastfeeding.

[3]Adjusted for psychologist, child's age in days, and quality of the neuropsychological test. Cumulative intensity of breastfeeding was also adjusted for age of food introduction.

[4]Organochlorine compounds in maternal serum at 1st trimester of pregnancy and mercury in cord blood.

[5]Adjusted for variables in model 1 plus maternal and paternal education, maternal and paternal social class, maternal and paternal attachment to the child, maternal and paternal intelligence quotient, maternal and paternal mental health, maternal and paternal age, maternal alcohol use during pregnancy, maternal height, use of a gas stove at home during pregnancy, child sex, child´s low tract respiratory infections at 6 months, and main childminder at 14 months.

Table 4. Descriptive polyunsaturated fatty acid (PUFA) levels and association (β coefficient and 95% Confidence Interval) between infant mental development score at 14 months and PUFA levels.

	PUFA levels in colostrum (weight %) (n=504) Mean (SD)	PUFA levels in colostrum (high vs. low) (n=504)[1] β	(95% CI)
n-3 PUFAs			
Alpha-linolenic acid (ALA; C18:3n-3)	0.35 (0.09)	1.02	(-1.60;3.64)
Ecosapentaenoic acid (EPA; C20:5n-3)	0.06 (0.02)	0.18	(-2.18;3.44)
Docosapentaenoic acid (DPA; C22:5n-3)	0.40 (0.13)	1.35	(-1.39;4.08)
Docosahexaenoic acid (DHA; C22:6n-3)	0.64 (0.27)	0.58	(-2.08;3.23)
Total n-3 PUFAs	1.45 (0.41)	1.76	(-0.88;4.40)
n-6 PUFAs			
Linoleic acid (LA; C18:2n-6)	12.62 (2.57)	-0.44	(-3.23;2.35)
Gamma-linolenic acid (GLA; C18:3n-6)	0.04 (0.02)	0.18	(-2.56;2.93)
Dihomo-gamma-linolenic acid (DGLA C20:3n-6)	0.85 (0.26)	0.64	(-1.87;3.15)
Arachidonic acid (AA; C20:4n-6)	1.08 (0.29)	0.70	(-1.94;3.34)
Adrenic acid (ADA; C22:4n-6)	0.64 (0.22)	-1.03	(-3.67;1.61)
Osbond acid (OA; C22:5n-6)	0.16 (0.05)	-0.48	(-3.09;2.13)
Total n-6 PUFAs	15.38 (2.74)	-0.39	(-3.17;2.38)
n-3/n-6 PUFA ratios			
ALA / LA ratio	0.03 (0.01)	1.72	(-0.94;4.39)
EPA / AA ratio	0.05 (0.02)	1.21	(-1.48;3.89)
DPA/ADA ratio	0.65 (0.16)	1.60	(-1.05;4.25)
DHA / OA ratio	4.20 (1.52)	1.04	(-1.63;3.71)
DHA / AA ratio	0.59 (0.18)	1.26	(-1.47;3.98)
Total n-3/n-6 PUFAs ratio	0.10 (0.03)	2.00	(-0.78;4.78)

1All models were adjusted for psychologist, child's age in days, quality of the neuropsychological test, maternal and paternal education, maternal and paternal social class, maternal and paternal attachment to the child, maternal and paternal intelligence quotient, maternal and paternal mental health, maternal age, maternal alcohol use during pregnancy, use of a gas stove at home during pregnancy, and child's age of food introduction.

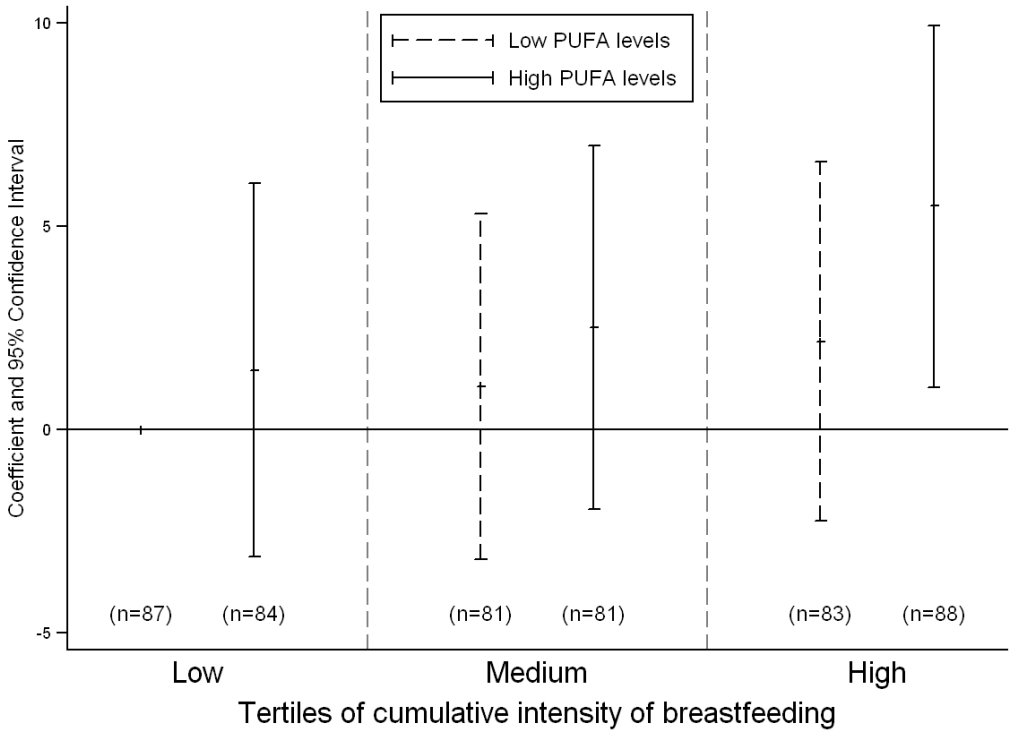
Table 5.1. Association (β coefficient and 95% Confidence Interval) between infant mental development score at 14 months and polyunsaturated fatty acid (PUFA) levels in colostrum by tertiles of cumulative intensity of breastfeeding1.

	Low PUFA levels				
	Low tertile of breast-feeding	Medium tertile of breastfeeding		High tertile of breastfeeding	
		β	(95% CI)	β	(95% CI)
n-3 PUFAs					
Alpha-linolenic acid (ALA; C18:3n-3)	Ref.	0.70	(-3.84;5.24)	2.22	(-2.17;6.62)
Ecosapentaenoic acid (EPA; C20:5n-3)	Ref.	0.56	(-4.62;5.75)	1.39	(-3.75;6.53)
Docosapentaenoic acid (DPA; C22:5n-3)	Ref.	-0.07	(-4.45;4.32)	2.05	(-2.34;6.44)
Docosahexaenoic acid (DHA; C22:6n-3)	Ref.	0.04	(-4.49;4.56)	2.19	(-1.97;6.35)
Total n-3 PUFAs	Ref.	0.47	(-4.03;4.98)	2.56	(-1.72;6.84)
n-6 PUFAs					
Linoleic acid (LA; C18:2n-6)	Ref.	0.82	(-3.68;5.31)	2.99	(-1.22;7.20)
Gamma-linolenic acid (GLA; C18:3n-6)	Ref.	0.50	(-5.12;6.13)	2.03	(-3.45;7.51)
Dihomo-gamma-linolenic acid (DGLA C20:3n-6)	Ref.	-1.24	(-5.73;3.26)	1.95	(-2.52;6.43)
Arachidonic acid (AA; C20:4n-6)	Ref.	-0.02	(-4.43;4.39)	1.45	(-2.87;5.77)
Adrenic acid (ADA; C22:4n-6)	Ref.	0.60	(-3.86;5.06)	3.37	(-1.01;7.74)
Osbond acid (OA; C22:5n-6)	Ref.	1.52	(-3.27;6.30)	3.81	(-0.66;8.28)
Total n-6 PUFAs	Ref.	-0.60	(-5.23;4.02)	1.26	(-3.03;5.56)
n-3/n-6 PUFA ratios					
ALA / LA ratio	Ref.	2.86	(-1.47;7.19)	2.99	(-1.38;7.36)
EPA / AA ratio	Ref.	1.63	(-2.74;6.00)	3.36	(-0.90;7.62)
DPA/ADA ratio	Ref.	0.60	(-3.73;4.94)	3.04	(-1.25;7.34)
DHA / OA ratio	Ref.	0.49	(-3.83;4.80)	2.59	(-1.64;6.83)
DHA / AA ratio	Ref.	0.72	(-3.53;4.98)	2.16	(-2.02;6.34)
Total n-3/n-6 PUFAs ratio	Ref.	1.06	(-3.19;5.31)	2.17	(-2.24;6.58)

[1]All models were adjusted for psychologist, child's age in days, quality of the neuropsychological test, maternal and paternal education, maternal and paternal social class, maternal and paternal attachment to the child, maternal and paternal intelligence quotient, maternal and paternal mental health, maternal age, maternal alcohol use during pregnancy, use of a gas stove at home during pregnancy, and child's age of food introduction.

Table 5.2. Association (β coefficient and 95% Confidence Interval) between infant mental development score at 14 months and polyunsaturated fatty acid (PUFA) levels in colostrum by tertiles of cumulative intensity of breastfeeding1.

	High PUFA levels				
	Low tertile of breast-feeding	Medium tertile of breastfeeding		High tertile of breastfeeding	
		β	(95% CI)	β	(95% CI)
n-3 PUFAs					
Alpha-linolenic acid (ALA; C18:3n-3)	Ref.	0.62	(-3.83;5.08)	1.80	(-2.59;6.18)
Ecosapentaenoic acid (EPA; C20:5n-3)	Ref.	-0.39	(-5.08;4.30)	0.82	(-3.70;5.34)
Docosapentaenoic acid (DPA; C22:5n-3)	Ref.	0.04	(-4.46;4.54)	2.02	(-2.38;6.43)
Docosahexaenoic acid (DHA; C22:6n-3)	Ref.	-0.53	(-4.92;3.85)	1.26	(-2.88;5.40)
Total n-3 PUFAs	Ref.	1.22	(-3.23;5.67)	2.56	(-1.73;6.85)
n-6 PUFAs					
Linoleic acid (LA; C18:2n-6)	Ref.	-0.50	(-5.04;4.05)	0.63	(-3.71;4.96)
Gamma-linolenic acid (GLA; C18:3n-6)	Ref.	-0.39	(-5.44;4.67)	0.79	(-4.14;5.72)
Dihomo-gamma-linolenic acid (DGLA C20:3n-6)	Ref.	-1.21	(-5.65;3.22)	1.72	(-2.72;6.16)
Arachidonic acid (AA; C20:4n-6)	Ref.	-0.87	(-5.40;3.67)	1.02	(-3.35;5.39)
Adrenic acid (ADA; C22:4n-6)	Ref.	-0.93	(-5.46;3.61)	0.31	(-3.99;4.61)
Osbond acid (OA; C22:5n-6)	Ref.	0.59	(-3.96;5.13)	1.10	(-3.23;5.44)
Total n-6 PUFAs	Ref.	-2.27	(-7.00;2.45)	-0.05	(-4.59;4.49)
n-3/n-6 PUFA ratios					
ALA / LA ratio	Ref.	2.90	(-1.61;7.41)	2.07	(-2.38;6.52)
EPA / AA ratio	Ref.	1.95	(-2.63;6.53)	2.13	(-2.06;6.33)
DPA/ADA ratio	Ref.	1.42	(-3.12;5.96)	2.52	(-1.67;6.71)
DHA / OA ratio	Ref.	0.43	(-4.12;4.98)	1.76	(-2.40;5.91)
DHA / AA ratio	Ref.	0.50	(-4.05;5.05)	1.60	(-2.45;5.64)
Total n-3/n-6 PUFAs ratio	Ref.	1.47	(-3.12;6.05)	2.52	(-1.95;6.99)


Low PUFA levels
High PUFA levels
Coefficient and 95% Confidence Interval
10
5
0
-5
(n=87)
(n=84)
(n=81)
(n=81)
(n=83)
(n=88)
Low
Medium
High
Tertiles of cumulative intensity of breastfeeding

8.4 Paper 4

The role of maternal smoking habits in the association between breastfeeding and cognitive development during the 2nd year of life

Forns J, Garcia-Esteban R, Julvez J, Guxens M, Grellier J, Ferrer, M, Lertxundi N, Ezama E, Murcia M, Riaño I, Ibarluzea J, Rebagliato M, & Sunyer J.

Submitted to Pediatric Research (under review)

The role of maternal smoking habits in the association between breastfeeding and cognitive development during the 2nd year of life

Forns J[1,2,3]., Garcia-Esteban R[1,2,3], Julvez J[1,2,3,4], Guxens M[1,2,3], Grellier J[1,2,3,5], Ferrer, M[1,2,3,] Lertxundi N[6], Ezama E[7], Murcia M[3,8], Riaño I[10], Ibarluzea J[3,9], Rebagliato M[3], & Sunyer J[1,2,3,11].

(1) Centre for Research in Environmental Epidemiology (CREAL), Doctor Aiguader 88, 08003 Barcelona, Spain.

(2) Hospital del Mar Research Institute (IMIM), Doctor Aiguader 88, 08003 Barcelona, Spain.

(3) CIBER Epidemiologia y Salud Pública (CIBERESP), Doctor Aiguader 88, 08003 Barcelona, Spain.

(4) Department of Environmental Health, Harvard School of Public Health, Boston, MA, USA.

(5) Department of Epidemiology and Biostatistics, Imperial College London, UK

(6) Faculty of Psychology, Basque Country University, San Sebastián, Spain.

(7) Centro de Investigaciones Comunicacionales (CICOM), Oviedo, Spain.

(8) Center for Public Health Research (CSISP), Valencia, Spain

(9) Subdirección de Salud Pública de Gipuzkoa. The Basque Government's Health Department, San Sebastián, Spain

(10) Department of Pediatrics. Hospital San Agustin, Aviles, Spain

(11) Pompeu Fabra University, Barcelona, Spain.

Word count: Abstract: 242 words; Text: 2901 words; Tables: 3; Figures: 1; Supplementary tables: 5; References: 47.

Keywords: child development, breastfeeding, smoking, neuropsychology.

ABSTRACT

A positive association between longer periods of breastfeeding and neuropsychological development has been reported previously. However, breastfeeding may be reduced by some factors such as maternal smoking during pregnancy. This study aimed to assess the effect of maternal smoking habits on the association between breastfeeding and cognitive development. A population-based birth cohort design was established in four regions of Spain as part of the NMA-INfancia y Medio Ambiente Project. We included 1,911 mother-child pairs with information on breastfeeding and neuropsychological assessment (Bayley Scales of Infant Development) in our analysis. The association between breastfeeding and mental test score was modified by four maternal smoking-related variables such as smoking during pregnancy, intensity of smoking during pregnancy, and postnatal smoking. We observed that infants which were exposed to smoking during pregnancy (Coefficient = 0.40, 95%CI= 0.004, 0.75) increased their cognitive scores from longer periods of breastfeeding than the non-exposed group (Coefficient = 0.16, 95%CI = 0.001, 0.32). Moreover, mothers which smoked postnatally exhibited a reduced period of breastfeeding. It is strongly recommended that breastfeeding for extended periods be promoted given its beneficial effects on cognitive development, especially amongst those mothers who smoked during and after pregnancy, whoappear to be prone to reducing it.

INTRODUCTION

Over the past 20 years, the association between breastfeeding practices and child cognitive development has been widely studied. Most of the observational studies have reported benefits in terms of cognitive development for children who are breastfed[1-6]. Various mechanisms have been proposed in explanation of these benefits, including the role played by parental socio-demographic characteristics, factors associated with the feeding environment (e.g. physical and psychological contact between mother, father, and child), and the effects of long-chain polyunsaturated fatty acids (LC-PUFAs) present in breast-milk[7-10].

Despite the benefits observed in neuropsychological development for prolonged periods of breastfeeding, some factors may negatively interact with this association, including maternal smoking habits [11]. Several potentially neurotoxic compounds present in tobacco smoke[12-15] may be transferred to children via breast milk[14]. In addition, the duration of breastfeeding tends to be reduced among mothers who have smoked during their pregnancy[16-18], and possibly in those exposed to environmental tobacco smoke (ETS) during pregnancy[19]. Such a reduction in duration of breastfeeding might be expected to exacerbate the impact of potential negative effects of active maternal smoking during pregnancy on child neuropsychological development[20].

Thus, the goal of this study was to disentangle the possible effect modification that maternal smoking habits may play on the association between breastfeeding and child cognitive development in a population-based birth cohort study. We also aimed to assess which of the variables related to maternal smoking status, if any, may be associated with a reduction in breastfeeding.

METHODS

Study design and participants

Population-based birth cohorts were established as part of the INMA – INfancia y Medio Ambiente [Environment and Childhood] project in several regions of Spain using a common protocol[21]. This analysis uses the INMA cohorts of Valencia, Sabadell, Asturias, and Gipuzkoa established between 2003 and 2008. A total of 2,644 eligible women (≥16 years, intention to deliver at the reference hospital, ability to communicate in Spanish or regional languages, singleton pregnancy, unassisted conception) were recruited during prenatal visits in the first trimester of pregnancy. Women were followed through their pregnancies, and their children were subsequently followed from birth through to their second year of life. Participants provided informed consent and the study was approved by the hospitals and institutional ethics committees in each region.

Child cognitive development test

Cognitive development of 2,213 children was assessed at around 14 months (range 11-23 months) using the Bayley Scales of Infant Development (BSID)[22]. The Bayley mental scale comprises 163 items that assess age-appropriate mental development, including performance abilities, memory, and early language skills. All testing was done by twelve specially trained psychologists in health care centres with the mother present. Psychologists were not aware of any exposure information. To limit inter-observer variability, we applied a strict protocol, including training sessions where inter-observer differences were quantified and three sets of quality controls (inter-observer-

reliability-tests) were undertaken during the fieldwork. The inter-rater reliability, estimated by intra-class correlation, was 0.90.

Fourteen children were excluded owing to specific pathologies, and 114 were excluded because their test results were of uncertain quality due to less than optimal cooperation. Raw scores were standardized for child's age in days at administration of the test using a parametric method for the estimation of age-specific reference intervals[23]. The parameters of the distribution were modelled as a fractional polynomial function of age and estimated by maximum likelihood. Residuals were then standardized to a mean of 100 points with a standard deviation of 15 points to homogenize the mental test scores[24].

Breastfeeding definition

Detailed information about child feeding practices was obtained by interviewer-administered questionnaires with the mother at 6 months and through the 2nd year of life. We used two definitions of breastfeeding: exclusive breastfeeding was used to describe the practice of feeding infants breast milk as their predominant source of nourishment (including milk expressed). Any breastfeeding was used to describe the practice of supplementing breastfeeding (direct from the breast or expressed) with other drinks, formula, or infant food[25,26]. This distinction was made as some infants may receive liquids in the form of water and water-based drinks, fruit juices, drops or syrups (e.g. vitamins, minerals and medicines). Exclusive and any were defined as continuous and as categorical variables (0-0.5 months, >0.5-3 months, >3-6 months, and >6 months).

Definition of tobacco smoke exposure

Interviewer-administered questionnaires were used to obtain information from mothers relating to their own smoking habits (including daily number of cigarettes and passive exposure to second hand smoke (SHS) during pregnancy) and those of their partners at the 3rd trimester of pregnancy, and also in a subsequent interview held during child's 2nd year of life. The number of cigarettes smoked by mothers per day was transformed into two different variables: 1) any active smoking of the mother during pregnancy was coded as maternal smoking during pregnancy; and 2) the number of cigarettes smoked per day during pregnancy (0, <5, ≥5). SHS at home during pregnancy was coded as a binary variable (yes, no). In addition, data on maternal postnatal smoking habits were collected, and this was treated as a binary variable (yes, no).

We collected urine samples from mothers (n=2,229) at the third trimester of pregnancy in order to analyze the concentrations of cotinine (ng/mL). Samples were analyzed using competitive immunoassay cotinine micro-plate enzyme immunoassay (EIA) (Ora Sure Technologies, Inc.). Maternal levels of cotinine were coded as "non-smokers" (<100 ng/mL) and "smokers" (≥100 ng/mL)[27].

Other parental and child variables

Information on paternal education, social class, use of a gas cooker at home during pregnancy, country of birth (Spain, foreign), age, parity, maternal alcohol intake, and marital status was obtained through questionnaires administered during the 1st and 3rd trimesters of pregnancy. Parental educational level was defined using three categories: primary school or less, secondary school, and university. Maternal social class based on the occupation was derived from the

longest-held job reported during the pregnancy for the mothers or, if the mother did not work during the pregnancy, the last job before the pregnancy. When social class could not be derived, the last job of the father was used. Nine social class categories were created according to the Spanish national occupational code (the "Código Nacional de Ocupaciones-94" (CNO-94)) and subsequently regrouped into three categories: I+II for managers, technicians, and associate professionals (non-manual), III for other non-manual workers, and IV+V for skilled, semi-skilled and unskilled manual workers[28,29]. Information related to the child's gestational age, sex, type of delivery (caesarean, other), anthropometric measures, and Apgar score at birth was obtained from clinical records. In a subsequent interview at 14 months, data on the main caregiver, caregiver employment status (employed/unemployed), nursery attendance, and infections during the two first years of life were collected. All questionnaires were administered face-to-face by trained interviewers.

Statistical analysis

Multiple imputation of missing values for the smoking status variables (<2%) and socio-demographical variables was performed using chained equations on those 1,911 subjects for which complete information relating to neuropsychological assessment and breastfeeding practices was available[30]. Ten completed data sets were generated and analyzed separately, and the results were combined using the standard Rubin's rules (Supplementary Tables 1 and 2)[31]. Results did not differ meaningfully from complete case analysis (Supplementary Table 3).

Multivariate linear regression models were built for mental test scores and AB and EB considering potential confounders using backward

selection procedure. Covariates showing associations with p-value <0.05 with mental test score or those that resulted in a change in regression coefficient by ≥ 10%, were retained in the model. The potential effect modifier of maternal smoking habits in the relationship between breastfeeding and mental test score was evaluated using the product between breastfeeding and maternal smoking habits variables to assess the interaction, and separated models for smoking variables were presented.

Finally, survival analysis was used to examine the duration of any and exclusive up to the child reaching 6 months. This cut-off was selected from the fact that it is widely recommended to maintain breastfeeding for the first 6 months of a child's life (57). The outcome of interest was breastfeeding cessation. Children who did not cease to breastfeed at 6 months and those lost of follow-up were censored either at 6 months or at the last time of contact. Kaplan-Meier curves were plotted and the differences in the any and exclusive breastfeeding survival functions according to pre- and post-natal tobacco exposures were assessed using the log-rank test. Hazard Ratios (HR) and 95% Confidence intervals (95% CI) were estimated using multivariate Cox regression models. All statistical analyses were done using Stata 10.1 (Stata Corporation, College Station, Texas).

RESULTS

Overall, 2,644 pregnant women were recruited during their 1st trimester of pregnancy. A total of 2,506 (94.8%) children were enrolled at birth, and 2,360 (89.3%) were assessed in their 2nd year of life. After excluding for preterm births, children with unknown gestational age, children with specific pathologies, and children with low quality neuropsychological

tests, our analyses were based on 1,911 (72.3%) mother-child pairs with complete information on breastfeeding, maternal smoking status and mental test scores.

Of the 1,911 children included in the analysis, 49.8% were males and 2.8% were low birth weight (<2,500 grams) (Table 1). Distributions of the two types of breastfeeding practices were markedly different. 44.8% and 10.4% of women maintained any and exclusive breastfeeding practices for more than 6 months, respectively. Forty-two percent of the women had secondary education and 35% had university education. Fifty percent were classified in lower social classes. Seventeen percent were classified as active smokers during pregnancy and 32.4% had levels of cotinine >100 ng/dl at the third trimester of pregnancy and were considered active smokers.

Infants with longer any breastfeeding whose mothers smoked during pregnancy using self-reported questionnaire exhibited bigger change in mental test score (Coefficient (Coef) = 0.40, 95% confidence interval (CI) = 0.004, 0.75) points in mental test score per each breastfeeding month] than those whose mothers did not smoke (Coef = 0.16, 95% CI = 0.01, 0.32), being the interaction marginally significant (p-value<0.10) (Table 2). The same pattern of associations was observed for exclusive, number of cigarettes per day during pregnancy (p-value for interaction (inter) = 0.060), and maternal postnatal smoking (p-inter = 0.052). When analyzing breastfeeding as categorical variable, a positive trend was observed for those children exposed to tobacco smoke. They were benefited for prolonged periods of breastfeeding. (Supplementary Tables 4 and 5)

Forty-three percent of non-smoker mothers during pregnancy maintained any breastfeeding at 6 months, whereas only 5% maintained

exclusive at this time. Among smoker mothers during pregnancy, 32% and 3% maintained any and exclusive practices more than 6 months, respectively. A similar pattern was observed for maternal postnatal smoking. By Kaplan-Meier analysis, we observed that the duration of any and exclusive up to age 6 months in maternal smoking group (both during and after pregnancy) was shorter than in the non-smoking group (Log-rank test p-values<0.001; Figure 1).

The hazard ratios of the maternal smoking variables associated with any and exclusive are shown in Table 3. Maternal postnatal smoking was a risk factor for stopping any breastfeeding (Hazard ratio (HR) = 1.24, 95% CI = 1.06, 1.45) and stopping exclusive (HR = 1.23, 95% CI = 1.08, 1.40) before 6 months compared with the non-smoker group. Infants of mothers who had smoked during pregnancy had a higher risk of discontinuing exclusive before 6 months (HR = 1.20, 95% CI = 1.03, 1.39); although this association turned non-significant when adjusting for SHS at home exposure during pregnancy, and maternal postnatal smoking. The inclusion of paternal postnatal smoking in any model discussed above did not change any result.

DISCUSSION

This study demonstrates that the association between breastfeeding practices and early cognitive development is modified by different maternal smoking status variables, such as smoking during pregnancy, number of cigarettes per day during pregnancy, and postnatal maternal smoking. Children exposed to active smoking benefited more from prolonged periods of either any or exclusive breastfeeding than the non-exposed. Additionally, the group of active smoking mothers after

pregnancy reduced their duration of any breastfeeding and exclusive breastfeeding.

A similar study[20] found that negative effects of smoking during pregnancy on neuropsychological development were only present in those children who were never breastfed. Our findings go beyond those of the previous studies because we observed that children exposed to potentially neurotoxic compounds in tobacco smoke during pregnancy[32-36], benefited in particular from longer periods irrespective of the type of breastfeeding practice. We also found that postnatal maternal smoking modifies the relationship between breastfeeding and early cognitive development, especially in the effects of exclusive breastfeeding. In our data, 87% of mothers who smoked during pregnancy also smoked after birth reflecting a group of persistent smokers. Despite the possibility of transfer of compounds in tobacco smoke to the infant through breast milk[14], our results suggest that prolonged periods of exclusive breastfeeding improve the early cognitive development in those children with a prolonged exposure to smoking.

It does not seem plausible that socio-demographic characteristics or factors associated with the feeding environment explain the benefits observed for prolonged periods of breastfeeding in those children exposed to smoking. A recent published paper on data derived from only one of the INMA cohorts (Sabadell), showed that maternal education, social class, IQ, mental health, and attachment to the child did not fully explain the association between breastfeeding and cognitive development, suggesting a greater effect of LC-PUFAs[37]. Nevertheless, we adjusted the final models for a large number of variables, such as maternal social class, maternal age, parental country of birth, and main caregiver.

We hypothesized that the effects of the LC-PUFAs may be one of the most plausible mechanisms that may explain the benefits of breastfeeding for children exposed to tobacco. LC-PUFAs are important during pre and postnatal periods of life, and are essential for the development of the brain and retina[38]. LC-PUFAs are also important structural elements of cell membranes and, therefore, essential in the formation of new tissue, including neurons and glial cells[39,40]. They also may play a neuroprotective role, making nervous tissue less susceptible to damage[41], or acting as second messengers and also induce release of acetylcholine and noradrenaline, which are involved in learning and memory[42].

Accordingly with the previous literature[16-18], active smoker mothers during pregnancy reduced their breastfeed duration. In our data, postnatal maternal smoking was the main risk factor to stop breastfeeding before than 6 months. There are several mechanisms that may explain these facts. Firstly, it is thought that nicotine has a negative effect on breast milk supply by suppressing prolactin levels[43]. Secondly, smoking may act as a proxy for behavioural characteristics, in which smoking women may show lower motivation to breastfeed and less likely to initiate breastfeeding[44]. In our sample, we hypothesize that these results are greater due to the psychosocial factors. As described before, a great proportion of women who smoked postnatally were also active smokers during pregnancy. These mothers were younger, showed lower prevalence of bachelor degrees and belonged to manual social classes (data not shown). These socio-demographic characteristics which have been related with parenting (living-styles, cognitively-stimulating environment, or health care, or dietary patterns[45,46], may be related with smoking habits and with the fact to stop breastfeeding prematurely.

Moreover, we did not find increase of the risk for stopping breastfeeding in those women exposed to SHS[19].

The present study has several important strengths, including the sample size, longitudinal design and relatively long follow-up. We assessed 2213 children with BSID and we collected information of 1911 mothers about their breastfeeding practices and tobacco habits. The measurement of cotinine levels in urine samples give strength to the results derived from smoking data reported during pregnancy. We collected all these data using field staff, interviewers, laboratory technicians, and project paediatricians, specifically trained for the project. Regarding the neuropsychological assessments several quality controls were introduced (inter-observer-reliability-tests) and the psychologists who assessed children with the BSID received a strict formation.

Although we included a large number of covariates, we were unable to control the multivariate models, including the 4 cohorts, for maternal IQ, mental health, or quality of home environment which may be involved in the relationship between breastfeeding and child cognitive development. Inclusion of psychosocial covariates such as mother's age, parental social class, education, and employment status reduced part of the residual confounding. But similar pattern was observed in the cohort of Sabadell (n=650), when we controlled the final models by maternal IQ, mental health and attachment to the child (data not shown). Moreover, non-participants in this study (observations without BSID assessment and/or breastfeeding practices) differed to participants in important variables. Non-participant mothers breastfed less time and there were more prevalence of active smokers during pregnancy (data not shown). These differences could underestimate our findings.

In conclusion, children exposed to active maternal smoking during pregnancy and after birth were significantly benefited for prolonged periods of breastfeeding practices. The implications of these findings are great for public health. Despite the possibility of harm from tobacco products in breast milk of smoking mothers, breastfeeding can be strongly recommended in order to balance the potential impact of these exposures. Obviously, the main attempt is to help mothers with active smoking habits to stop this consumption. But, a second challenge is to encourage to the persistent smoking mothers to breastfeed for more prolonged periods, because they are more prone to reduce the breastfeeding period and breast milk can be especially beneficial for the cognitive development of their children.

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FIGURE LEGEND

Figure 1. Kaplan-Meier breastfeeding survival functions according to maternal prenatal (a), and postnatal (b) smoking

Table 1. Description of the INMA cohort (n=1911):

Child	n	(%)
Age (months), mean (SD)	14.73	(2.56)
Male	946	(49.84)
Low birth weight	53	(2.81)
Siblings at birth (0)	1102	(58.12)
1	689	(36.34)
2 o +	105	(5.54)
Any breastfeeding (0-0.5 months)	341	(17.97)
>0.5-3 months	273	(14.38)
>3-6 months	433	(22.81)
>6 months	851	(44.84)
Exclusive breastfeeding (0-0.5 months)	473	(25.93)
>0.5-3 months	396	(21.71)
>3-6 months	766	(42.00)
>6 months	189	(10.36)
Mothers		
Age (years), mean (SD)	30.71	(4.15)
Education (Primary or less)	426	(22.49)
Secondary	798	(42.13)
University	670	(35.37)
Social class (CS I+II)	417	(21.98)
CS III	526	(27.73)
CS IV+V	954	(50.29)
Country of birth (foreign)	144	(7.61)
Smoking during pregnancy (yes)	326	(17.40)
Cotinine levels (>100 ng/ml)	337	(32.44)
SHS at home during pregnancy (exposed)	714	(38.10)
Postnatal maternal smoking (yes)	483	(25.90)
Use of gas stove during pregnancy (yes)	813	(44.07)
Fathers		
Age (years), mean (SD)	32.80	(4.88)
Education (Primary or less)	634	(33.65)
Secondary	835	(44.32)
University	415	(22.03)

Child	n	(%)
Social class (CSI+II)	387	(20.87)
CS III	335	(18.07)
CS IV+V	1132	(61.06)
Country of birth (foreign)	158	(8.34)

Values are n (%) unless specified.
SD=Standard Deviation

Table 2. Mental test scores in relation to duration (months) of breastfeeding:

	Any breastfeeding				Exclusive breastfeeding			
	Coef	95% CI		p-inter	Coef	95% CI		p-inter
Crude	0.17	0.03 ,	0.31		0.18	-0.14 ,	0.51	
Adjusted†	0.20	0.06 ,	0.34		0.18	-0.14 ,	0.50	
Stratified by‡:								
Maternal smoking during pregnancy								
No	0.16	0.01 ,	0.32	0.075	0.05	-0.29 ,	0.41	0.019
Yes	0.40	0.04 ,	0.75		0.86	0.02 ,	1.71	
Cigarettes during pregnancy								
0	0.16	0.01 ,	0.32	0.149	0.05	-0.29 ,	0.41	0.060
>0-5	0.58	0.10 ,	1.06		1.20	0.06 ,	2.33	
>5	0.25	-0.30 ,	0.80		0.51	-0.85 ,	1.88	
Cotinine levels at third trimester of pregnancy								
≤100 ng/dl	0.19	-0.05 ,	0.44	0.283	0.17	-0.36 ,	0.70	0.224
>100 ng/dl	0.36	0.02 ,	0.70		0.69	-0.15 ,	1.54	
SHS at home during pregnancy								
Non-exposed	0.19	0.02 ,	0.36	0.59	0.18	-0.21 ,	0.56	0.866
Exposed at home	0.25	0.00 ,	0.51		0.15	-0.43 ,	0.74	

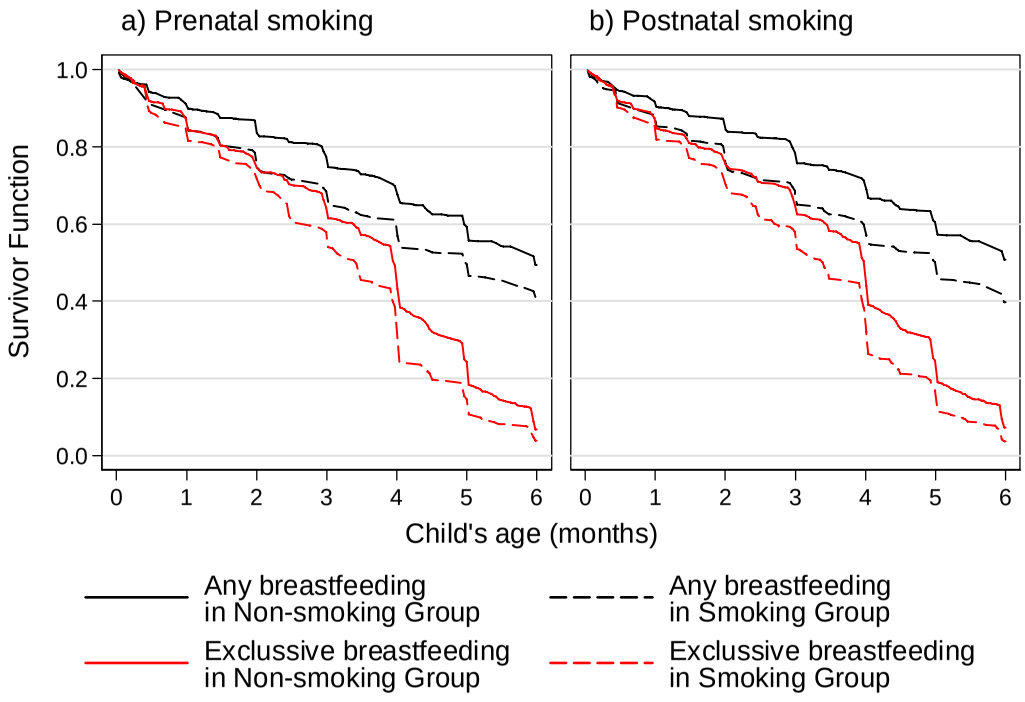
	Any breastfeeding				Exclusive breastfeeding			
	Coef	95% CI		p-inter	Coef	95% CI		p-inter
Postnatal maternal smoking								
No	0.14	-0.02 ,	0.31	0.122	0.02	-0.34 ,	0.40	0.053
Yes	0.35	0.06 ,	0.65		0.58	-0.10 ,	1.26	

Abbreviations: SHS, second hand smoke; Coef, coefficient; CI, confidence interval; p-inter, p-value for interaction.
Reference=Intercept
† Adjusted for psychologist, child's sex, main caregiver, gestational age, paternal country of birth, maternal social class, maternal age, maternal smoking during pregnancy, SHS at home during pregnancy, and postnatal maternal smoking.
‡ Stratified models were adjusted for the same covariates as in †.

Table 3. Multivariate Cox regression for the risk for discontinuing any and exclusive breastfeeding before 6 months:

	Any breastfeeding				Exclusive breastfeeding			
	Adjusted†		Mutually adjusted		Adjusted†		Mutually adjusted	
	HR	95% CI	HR	95% CI	HR	95% CI	HR	95% CI
Smoking during pregnancy	1.12	0.94 , 1.35	0.97	0.78 , 1.22	1.20	1.03 , 1.39	1.08	0.90 , 1.30
SHS at home during pregnancy	1.06	0.82 , 1.17	1.01	0.86 , 1.18	1.04	0.93 , 1.18	0.98	0.87 , 1.12
Postnatal maternal smoking	1.24	1.06 , 1.45	1.26	1.05 , 1.53	1.23	1.08 , 1.40	1.19	1.01 , 1.39
Cotinine levels at 32 weeks of pregnancy*	1.01	0.83 , 1.23	0.80	0.62 , 1.04	1.18	1.01 , 1.38	0.98	0.80 , 1.21

Abreviations: SHS, second hand smoke; HR, hazard ratio; CI, confidence interval; p-inter, p-value for interaction.
*Cotinine levels at third trimester of pregnancy were included in the models instead of smoking during pregnancy
†Adjusted for maternal education, age, and country of birth


a) Prenatal smoking
b) Postnatal smoking
Survivor Function
1.0
0.8
0.6
0.4
0.2
0.0
0
1
2
3
4
5
6
Child's age (months)
Any breastfeeding in Non-smoking Group
Any breastfeeding in Smoking Group
Exclussive breastfeeding in Non-smoking Group
Exclussive breastfeeding in Smoking Group

Supplementary Table 1. Description of the imputation procedure.

Software used and key setting: STATA 10.1 software (Stata Corporation, College Station, Texas) – ice command (with 10 cycles)
Number of imputed datasets created: 10
Variables included in the imputation procedure: Variables used in the main analyses (outcome, exposure and potential confounders/effect modifier): Bayley mental score, duration of breastfeeding (months), maternal smoking (no/yes) and nº of cigarettes/day during pregnancy, maternal cotinine levels at third trimester of gestation (ng/dl), SHS at home during pregnancy (no/yes), maternal postnatal smoking (no/yes), child's sex (male/female), gestational age (weeks), maternal age at delivery (years), maternal social class (I+II, III, IV+V), paternal country of birth (Spanish/foreigner), and. main caregiver (mother/both parents/grandparents/parents and grandparents/others). Variables used only for imputation models: Maternal characteristics: maternal education (≤primary, secondary, university), country of birth (Spanish/foreigner), alcohol intake during pregnancy (gr/day), SHS exposure i) at home during pregnancy (nº of cigarettes/day), ii) at work (yes/no), iii) at public places (no/yes); employment status at age 1 year (employed/unemployed). Paternal characteristics: age at delivery (years), education ((≤primary, secondary, university), employment status at age 1 year (not/working), social class (I+II, III, IV+V), postnatal smoking and nº of cigarettes/week, nº of cigarettes/week had smoked after birth, Child characteristics: birth weight (grams), birth length (cm), birth head circumference (cm), type of delivery (caesarean/other), amniorrexis (spontaneous/artificial), amniotic fluid (normal/other), Apgar score at 5 min, nº of siblings at birth (0, 1, 2+), day care attendance (no/yes). Other variables: type of residential area (rural/urban), other people smoking at home at child's age 1 year and gas stove used during pregnancy (no / yes).

Treatment of non-normally distributed variables: Nº of cigarettes were right-censored (lower bound = 0) and conditionally imputed, i.e. nº of cigarettes/day were imputed only if smoker and 0 code was otherwise Interval regression used for imputation of for log-transformed cotinine levels at third trimester of gestation.
Treatment of binary/categorical variables: logistic, ordinal, and multinomial models. Low birth weight (birth weight < 2500) was passively imputed from birth weight.
Statistical interactions included in imputation models: imputations were done separately for each cohort
No missing values in the imputation sample: Bayley scales, child's age at Bayley examination, sex, gestational age, duration of any breastfeeding,, and maternal alcohol intake during pregnancy.

Supplementary Table 2. Distribution of sociodemographic characteristics and smoking status variables in the imputed and the observed datasets. N eligible= 1911.

Imputed variable	Percent (categorical variables) or Mean (SE) (continuous variables)		
	% data imputed	Imputed dataset	Observed data
Parental characteristics			
Maternal age (yrs)	0.05	30.7	30.7
Maternal education (%)	0.21		
≤Primary		22.6	22.9
Secondary		42.0	42.1
University		35.4	35.4
Maternal Social class (%)	0.05		
CSI+II		22.0	22.0
CSIII		28.0	27.7
CSIV+V		50.0	50.3
Paternal education (%)	0.73		
≤Primary		33.8	33.6
Secondary		44.2	44.3
University		22.0	22.1
Paternal Social class (%)	2.35		
CSI+II		20.8	20.9
CSIII		18.1	18.1
CSIV+V		61.0	61.0
Child characteristics			
Siblings at birth (%)	0.10		
0		58.0	58.2
1		36.3	36.3
2 or +		5.7	5.5
Birth weight (gr)	0.52	3297	3296
Smoking status variables			
Smoking during pregnancy, yes (%)	1.31	17.3	17.4

Imputed variable	Percent (categorical variables) or Mean (SE) (continuous variables)		
	% data imputed	Imputed dataset	Observed data
Nº of cigarettes/day during pregnancy	1.31		
0		82.6	82.6
>0-5		9.0	9.0
>5		8.4	8.4
Postnatal smoking, yes (%)	1.78	25.7	25.8
SHS at home during pregnancy, exposed (%)	1.31	38.1	38.0
Cotinine at third trimester of gestation(ng/ml)	45.4		
≤100 ng/dl		67.4	67.4
>100 ng/dl		32.6	32.6

† Geometric mean shown. The % imputed includes the proportion of the observed data which had values below the limits of detection (LOD); in the imputed data, a distribution for values below the LOD was imputed. N(%) <LOD in the original data was =719 (37.62).

Supplementary Table 3. Mental test scores in relation to duration (months) of breastfeeding (case-complete):

	Any breastfeeding				Exclusive breastfeeding			
	Coef	95% CI		p-inter	Coef	95% CI		p-inter
Crude	0.17	0.03 ,	0.31		0.18	-0.14 ,	0.51	
Adjusted†	0.19	0.05 ,	0.34		0.16	-0.18 ,	0.49	
Stratified by‡:								
Maternal smoking during pregnancy								
No	0.16	-0.01 ,	0.32	0.099	0.02	-0.35 ,	0.38	0.015
Yes	0.35	-0.03 ,	0.72		0.80	-0.10 ,	1.68	
Cigarettes during pregnancy								
0	0.16	-0.01 ,	0.32	0.245	0.02	-0.35 ,	0.38	0.060
>0-5	0.55	0.05 ,	1.03		1.08	-0.07 ,	2.25	
>5	0.22	-0.42 ,	0.79		0.46	-1.09 ,	1.88	
Cotinine levels at third trimester of pregnancy								
≤100 ng/dl	0.19	-0.07 ,	0.44	0.42	0.16	-0.44 ,	0.69	0.275
>100 ng/dl	0.31	-0.06 ,	0.68		0.62	-0.27 ,	1.51	
SHS at home during pregnancy								
Non-exposed	0.19	0.01 ,	0.37	0.632	0.15	-0.26 ,	0.56	0.774
Exposed at home	0.22	-0.04 ,	0.48		0.13	-0.47 ,	0.73	

	Any breastfeeding				Exclusive breastfeeding			
	Coef	95% CI		p-inter	Coef	95% CI		p-inter
Postnatal maternal smoking								
No	0.14	-0.04 ,	0.30	0.156	-0.01	-0.42 ,	0.35	0.052
Yes	0.35	0.04 ,	0.66		0.58	-0.13 ,	1.30	

Abbreviations: SHS, second hand smoke; Coef, coefficient; CI, confidence interval; p-inter, p-value for interaction.
Reference=Intercept

† Adjusted for psychologist, child's sex, main caregiver, gestational age, paternal country of birth, maternal social class, maternal age, maternal smoking during pregnancy, SHS at home during pregnancy, and postnatal maternal smoking.

‡ Stratified models were adjusted for the same covariates as in †.

Supplementary Table 4. Mental test scores in relation to any breastfeeding:

	Duration of any breastfeeding (months)									
	0-0.5	>0.5 -3			>3-6			>6		
	Ref.	Coef	95% CI		Coef	95% CI		Coef	95% CI	p-trend
Crude	107.49	-0.88	-3.20 ,	1.63	-1.17	-3.10 ,	1.22	1.49	-0.73 , 3.14	0.042
Adjusted†	102.32	-0.52	-3.45 ,	1.28	-0.83	-3.40 ,	0.86	1.31	-0.74 , 3.11	0.085
Stratified by‡:										
Maternal smoking during pregnancy										
No	108.46	-0.80	-3.49 ,	1.89	-1.88	-4.26 ,	0.51	0.57	-1.59 , 2.74	0.272
Yes	103.31	-1.56	-6.79 ,	3.66	1.20	-3.83 ,	6.23	3.93	-0.63 , 8.48	0.023
Cigarettes during pregnancy										
0	108.49	-0.80	-3.49 ,	1.89	-1.88	-4.26 ,	0.51	0.57	-1.59 , 2.74	0.272
>0-5	104.71	0.62	-6.72 ,	7.97	1.55	-5.69 ,	8.78	6.50	0.02 , 12.98	0.022
>5	105.41	-5.36	-14.03 ,	3.31	-0.42	-8.91 ,	8.08	0.41	-6.62 , 7.43	0.500
Cotinine levels at third trimester of pregnancy										
≤100 ng/dl	108.87	-0.36	-4.19 ,	3.48	-0.75	-4.32 ,	2.82	1.70	-1.54 , 4.94	0.148
>100 ng/dl	101.48	-2.67	-7.93 ,	2.58	0.45	-4.65 ,	5.55	2.21	-2.24 , 6.65	0.145
SHS at home during pregnancy										
Non-exposed	106.43	-0.79	-3.77 ,	2.20	-0.80	-3.46 ,	1.86	1.43	-0.96 , 3.83	0.463
Exposed at home	107.34	-2.27	-6.24 ,	1.71	-2.66	-6.29 ,	0.97	0.63	-2.66 , 3.92	0.037

	Duration of any breastfeeding (months)									
	0-0.5	>0.5 -3			>3-6			>6			
	Ref.	Coef	95% CI		Coef	95% CI		Coef	95% CI		p-trend
Postnatal maternal smoking											
No	107.91	-0.34	-3.24 ,	2.55	-1.68	-4.22 ,	0.86	0.85	-1.44 ,	3.15	0.212
Yes	108.78	-2.12	-6.37 ,	2.14	-0.31	-4.39 ,	3.78	1.93	-1.77 ,	5.62	0.118

Abbreviations: SHS, second hand smoke; Coef, coefficient; CI, confidence interval; p-inter, p-value for interaction.
Reference=Intercept / p-trend= p-value for linear trend.
† Adjusted for psychologist, child's sex, main caregiver, gestational age, paternal country of birth, maternal social class, maternal age, maternal smoking during pregnancy, SHS at home during pregnancy, and postnatal maternal smoking.
‡ Stratified models were adjusted for the same covariates as in †.

Supplementary Table 5. Mental test scores in relation to exclusive breastfeeding:

	Duration of exclusive breastfeeding (months)									
	0-0.5	>0.5 -3			>3-6			>6		
	Ref.	Coef	95% CI		Coef	95% CI		Coef	95% CI	p-trend
Crude	102.02	0.55	-1.50 ,	2.51	0.75	-1.03 ,	2.45	1.88	-0.74 , 4.42	0.186
Adjusted†	107.01	-0.03	-1.99 ,	1.94	0.58	-1.25 ,	2.20	1.98	-0.74 , 4.36	0.167
Stratified by‡:										
Maternal smoking during pregnancy										
No	107.77	0.17	-2.03 ,	2.37	-0.04	-1.94 ,	1.87	1.40	-1.34 , 4.14	0.582
Yes	105.00	-0.68	-5.20 ,	3.84	2.91	-1.30 ,	7.13	4.47	-3.20 , 12.14	0.038
Cigarettes during pregnancy										
0	107.77	0.17	-2.03 ,	2.37	-0.04	-1.94 ,	1.87	1.40	-1.34 , 4.14	0.582
>0-5	105.57	0.89	-5.27 ,	7.05	6.30	0.56 ,	12.05	3.65	-6.83 , 14.13	0.028
>5	106.81	-4.50	-12.23 ,	3.22	-0.48	-7.26 ,	6.29	3.08	-9.92 , 16.09	0.508
Cotinine levels at third trimester of pregnancy										
≤100 ng/dl	109.28	0.24	-3.05 ,	3.53	0.74	-2.12 ,	3.60	1.60	-2.54 , 5.74	0.348
>100 ng/dl	101.22	2.66	-7.15 ,	1.84	2.12	-2.04 ,	6.28	3.88	-3.51 , 11.27	0.105
SHS at home during pregnancy										
Non-exposed	105.89	1.04	-1.41 ,	3.49	0.49	-1.41 ,	3.49	2.37	-0.66 , 5.39	0.844
Exposed at home	106.94	-2.26	-5.61 ,	1.10	0.29	-2.70 ,	3.28	0.47	-4.39 , 5.33	0.074

	Duration of exclusive breastfeeding (months)									
	0-0.5	>0.5 -3			>3-6			>6		
	Ref.	Coef	95% CI		Coef	95% CI		Coef	95% CI	p-trend
Postnatal maternal smoking										
No	107.55	0.12	-2.26 ,	2.49	-0.05	-2.07 ,	1.98	0.99	-1.88 , 3.85	0.702
Yes	107.55	-0.33	-3.99 ,	3.33	1.55	-1.82 ,	4.93	3.96	-2.06 , 9.98	0.087

Abbreviations: SHS, second hand smoke; Coef, coefficient; CI, confidence interval; p-inter, p-value for interaction.

Reference=Intercept / p-trend= p-value for linear trend.

† Adjusted for psychologist, child's sex, main caregiver, gestational age, paternal country of birth, maternal social class, maternal age, maternal smoking during pregnancy, SHS at home during pregnancy, and postnatal maternal smoking.

‡ Stratified models were adjusted for the same covariates as in †.

8.5 Paper 5

Prenatal exposure to organochlorine compounds and neuropsychological development up to two years of life

**Forns J, **Lertxundi N, Aranbarri A, Murcia M, Gascon M, Martinez D, Lertxundi A, Julvez J, Fano E, Goñi F, Grimalt JO, Ballester F, Sunyer J & Ibarluzea J.

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Prenatal exposure to organochlorine compounds and neuropsychological development up to two years of life

**Forns J[1,2,3], **Lertxundi N[4,5], Aranbarri A[4,5], Murcia M[3,6], Gascon M[1,2,3], Martinez D[1,2,3], Lertxundi A[5,7], Julvez J[1,2,3,8], Fano E[4,5], Goñi F[3,9], Grimalt JO[10], Ballester F[3,6,11], Sunyer J[1,2,3,12] & Ibarluzea J[2,5,13].

(1) Centre for Research in Environmental Epidemiology (CREAL), Barcelona, Spain.

(2) Hospital del Mar Research Institute (IMIM), Barcelona, Spain.

(3) CIBER Epidemiologia y Salud Pública (CIBERESP), Barcelona, Spain.

(4) Faculty of Psychology, University of the Basque Country UPV/EHU, 20018, Donostia, Spain.

(5) BIODONOSTIA Health Research Institute, Donostia, Spain.

(6) Center for Public Health Research (CSISP), Valencia, Spain

(7) Faculty of Medicine and Dentistry, Basque Country University, San Sebastián, Spain.

(8) Department of Environmental Health, Harvard School of Public Health, Boston, MA, USA

(9) Laboratorio de Salud Pública de Guipúzcoa, San Sebastián, Spain

(10) Department of Environmental Chemistry, Institute of Environmental Assessment and Water Research (IDAEA-CSIC), Barcelona, Spain.

(11) University of Valencia, Valencia, Spain,

(12) Pompeu Fabra University, Barcelona, Spain.

(13) Subdirección de Salud Pública de Gipuzkoa. The Basque Government's Health Department, San Sebastián, Spain

**Shared autorship

Correspondence and queries to:

Joan Forns Guzmán; Centre for Research in Environmental Epidemiology

C. Doctor Aiguader 88; 08003 Barcelona; Spain

Phone: +34 93 214 73 11 Fax: +34 93 214 73 02

E-mail: jforns@creal.cat

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ABSTRACT

Polychlorinated biphenyls (PCB), hexachlorobenzene (HCB), and dichlorodiphenyl dichloroethylene (pp'DDE) are persistent, bioaccumulative, and toxic pollutants with a potential neurotoxic effect. Despite the growing body of studies analyzing these compounds, the specific effects of them on early neuropsychological development is still unclear. This study is based in a population-based birth cohort design established in 3 regions of Spain (Sabadell, Gipuzkoa, and Valencia) as part of the INMA [Environment and Childhood] Project. The main analyses in this report were based on 1391 mother-child pairs with complete information on Organochlorine Compounds and neuropsychological assessment (Bayley Scales of Infant Development) at age 14 months. We found that prenatal PCBs exposure, particularly congeners 138 and 153, resulted in psychomotor development impairment (Coefficient = -1.24, 95% confidence interval = -2.41, -0.07), while no effects were reported on cognitive development. Prenatal exposure to pp'DDE or HCB was not associated to early neuropsychological development. The negative effects of PCBs on early psychomotor development have been reported previously suggesting that the potential neurotoxic effect of these compounds may be evident even at low doses.

Keywords: child development, Organochlorine Compounds, Polychlorinated Biphenyls, Hexachlorobenzene, Dichlorodiphenyl Dichloroethylene, neuropsychology.

INTRODUCTION

Organochlorine Compounds (OCs), an important sort of Persistent Organic Pollutants (POPs), include polychlorinated biphenyls (PCBs), hexachlorobenzene (HCB), dichlorodiphenyltrichloroethane (pp'DDT) or dichlorodiphenyl dichloroethylene (pp'DDE), which is the metabolite of DDT. These compounds are highly widespread in the environment and in human tissues. Newborns are exposed to PCBs and other OCs across the placenta and through breastfeeding. (1,2) With the exception of DDT, still used in countries with endemic malaria, the use of OCs has been forbidden and therefore their concentrations have decreased over the past 30 years. However, these compounds are still detectable in blood of current generations (3).

PCBs are mixtures of synthetic organic compounds that were widely used as insulators, coolants, and lubricants in electrical transformers, capacitors, and hydraulic equipment and as plasticizers in plastic and rubber products. The negative effects of PCBs on early cognitive and psychomotor development in children ranging from 7 to 30 months have been observed in a number of birth cohort studies (4–6). Nevertheless, these negative results were not replicated in other studies with children of similar ages (7–9)

DDT is a potent insecticide that was used worldwide for agricultural and public health purposes from the 1940s until the 1970s. Because of its toxicity on wildlife and humans, its environmental persistence, and its concentration in the food supply, its use was prohibited in many countries, leading to a decrease of its concentrations in current generations worldwide (10). Human studies in children confirm that pp'DDT, and its most stable metabolite pp'DDE (11), are neurodevelopmental toxicants that may disrupt the central nervous

system (12–15). Regarding HCB, the evidence is less consistent; in a study with Spanish children, effects on children's behaviour at age 4 years was found, however, this same study could not detect any effects on motor or cognitive development (5,16).

The aim of the present study is to assess the early neuropsychological effects of the main OCs in a current newborn cohort with a lower exposure to these OCs than previous generations (17–19).

METHODS

Study design and participants

Population-based birth cohorts were established as part of the INMA – INfancia y Medio Ambiente [Environment and Childhood] Project in several regions of Spain following a common protocol (20). This analysis uses the INMA cohorts of Valencia, Sabadell, and Gipuzkoa established between 2003 and 2008. A total of 2150 eligible women (≥16 years, intention to deliver at the reference hospital, ability to communicate in Spanish or regional languages, singleton pregnancy, no assisted conception) were recruited during prenatal visits in the first trimester of pregnancy. Women were followed through pregnancy and children from birth through the second year of life. Participants provided informed consent and the study was approved by hospital and institutional ethics committees in each region.

Neuropsychological testing

Cognitive development of 1801 children was assessed at around age 14 months (range 11-21 months) using the Bayley Scales of Infant Development (BSID) (21). The mental scale consisted of 163 items that assessed age-appropriate cognitive development in areas such as

performance ability, memory, and first verbal learning. The psychomotor scale consisted of 81 items assessing fine and gross motor development. All testing was done in the health care centre in the presence of the mother, by eight specially trained psychologists who were not aware of any exposure information. To limit inter-observer variability, we applied a strict protocol, including training sessions where inter-observer differences were quantified and three sets of quality controls (inter-observer-reliability-tests) undertaken during the fieldwork. The inter-rater reliability estimated by intra-class correlation was 0.90 for mental test scores, and 0.91 for psychomotor test scores.

Raw scores were standardized for child's age in days at test administration using a parametric method for the estimation of age-specific reference intervals. The parameters of the distribution were modelled as a fractional polynomial function of age and estimated by maximum likelihood. Residuals were then standardized to a mean of 100 points with a standard deviation of 15 points to homogenize the scales (22).

OCs Exposure Measurement

Concentrations of OCs (HCB, pp'DDE, and PCB congeners 28, 118, 138, 153 and 180) were measured in maternal serum (n=1811) extracted between the 7th week and the 26th week of pregnancy (median=12.9 weeks) from peripheral veins. Sera samples were stored in crystal tubes at -80ºC and analyzed with gas chromatograph using methods described elsewhere (23–25). Samples collected in Gipuzkoa and Sabadell were analyzed on Gipuzkoa Basque Government Laboratory by a method that required 500 µl of serum per sample (24). Initial extraction was performed using 96-well solid-phase extraction disk plates and was

followed by a clean-up with silica gel/sulphuric acid. Quantification was carried out by gas chromatography with electron capture detector (GC-ECD). Gas chromatography coupled to a mass spectrometer detector (GC-MSD) was used for quantitative and qualitative confirmation. Relative standard deviation (precision) was <15% for OCPs and PCBs. Samples collected in Valencia were analyzed on Centro Superior de Investigación (Barcelona) using a method (24)where compounds were liquid-liquid extracted with hexane from 1 ml of serum and extracts cleaned up with sulphuric acid prior to quantification by GC-ECD (23). Quantitative and qualitative confirmation was performed by gas chromatography coupled to negative ion chemical ionization mass spectrometry (GC-NICI-MS). Precision, measured as relative standard deviation, was <14% for all the compounds. Both laboratories were in compliance with the Arctic Monitoring and Assessment Program (AMAP) Ring Test Proficiency Program for persistent organic pollutants in human serum (Centre de Toxicologie, Institut National de Santé Publique du Québec). Limits of detection (LOD) were 0.071 ng/ml for Sabadell and Gipuzkoa samples and between 0.010 - 0.071 ng/ml for Valencia sera. For comparison purposes, values in Valencia below 0.071 ng/ml were set as non-detected. Samples with non-detectable levels were then set at a value of half the LOD. As PCB28 and 118 were detectable in less than 1% and 25% of samples, respectively, the sum of PCBs (ΣPCBs) was calculated by summing the concentrations of predominant congeners: PCB138, 153 and 180. All exposures are expressed on a lipid basis in ng/g lipid using the method described elsewhere (26). Correlations between lipid adjusted and not adjusted values were high (0.97 for pp'DDE and 0.95 for ΣPCBs). Concentrations of Mercury were also assessed. The analytical procedure has been described elsewhere (27).

Other parental and child variables

Information on parental education, social class, use of gas cooking at home during pregnancy, country of birth (Spain, foreign), age, parity, maternal alcohol intake, and marital status was obtained through questionnaires administered during the 1st and 3rd trimesters of pregnancy. Parental educational level was defined using three categories: primary or less, secondary school, and university. Maternal social class based on the occupation was derived from the longest-held job reported during the pregnancy for the mothers or if the mother did not work during pregnancy, the last job before the pregnancy. When social class could not be derived, the last job of the father was used. Nine social class categories were created according to "Occupational National Code-94" and regrouped in three categories: I+II for managers, technicians, and associate professionals (non-manual), III for other non-manual workers, and IV+V for skilled, semi-skilled and unskilled manual workers (28). Information related to the child's gestational age, sex, type of delivery (caesarean, other), anthropometric measures, and Apgar score at birth was obtained from clinical records. In a subsequent interview at 14 months, data on the main caregiver of the children, parental employment status (employed/unemployed), nursery attendance, and infections during previous months were collected. All questionnaires were administered face-to-face by trained interviewers.

Statistical analysis

Multiple imputations of missing values for the socio-demographical variables were performed using chained equations on the 1391 subjects, for which complete information of neuropsychological assessment and OCs was available (29). Ten imputed data sets were generated and

analyzed separately, and the results were combined using the standard Rubin's rules (Supplementary Tables 1 and 2) (30). Results did not differ meaningfully from complete case analysis (Supplementary Table 3).

OCs levels were transformed to the log10 scale. Multivariate linear regression models were built for mental and psychomotor test scores and OCs considering all potential confounders using a backward selection procedure. Covariates showing associations with p-value<0.05 with mental or psychomotor test scores or those that resulted in a change in estimate of breastfeeding ≥ 10%, were retained in the model. Breastfeeding and sex (31) were assessed as potential effect modifiers by studying the interaction between these variables and the different OCs. Statistical analyses were done using Stata 10.1 (Stata Corporation, College Station, Texas).

RESULTS

Overall, 2150 pregnant women were recruited during the 1st trimester of pregnancy. A total of 2020 (94%) children were enrolled at birth, and 1801 (83%) were assessed in the second year of life using BSID. We excluded 72 preterm births (<37 weeks), 12 because of unknown gestational age, 17 infants with pathologies including plagiocephaly, and 108 infants flagged by psychologists as difficult to evaluate because of suboptimal cooperation classified as having neurodevelopment tests of uncertain quality. After these exclusions, our analysis was based on 1391 (64%) mother-child pairs with complete information on neuropsychological development assessment and OCs levels. Differences between participants and non-participants were studied. Children not included had lower maternal education, higher maternal smoking use, and shorter breastfeeding duration.

Because of the high proportion of samples below the limit of detection (>80%), levels of pp'DDT were not considered in the analysis. In general, concentrations of HCB were much lower (43.4 ng/g lipid) than pp'DDE (119.1 ng/g lipid) or ΣPCBs (102.7 ng/g lipid) (Table 1). Correlation coefficients between different OCs were: 0.28 (pp'DDE and ΣPCBs), 0.39 (pp'DDE and HCB) and 0.31 (ΣPCBs and HCB), all with $p<0.001$ (Table 2).

Maternal levels of PCBs were higher in mothers of higher social classes, higher levels of education, Spanish, and whose children never breastfed (Table 3). On the other hand, lower social classes, lower levels of education, or non-Spanish were related to higher maternal pp'DDE levels. The levels of HCB were higher in those mothers who reported active smoking during pregnancy, never breastfed, and in Spanish mothers.

Table 4 presents the change in mental and psychomotor test scores associated with a 10-fold increase in OCs serum levels. After adjusting for covariates, we did not detect any association between maternal serum OCs levels and mental test scores. We found 1.24-point decreases in psychomotor test scores during the second year of life associated with 10-fold increases in serum levels of sum of PCBs (Coefficient (Coef) = -1.24, 95% confidence interval (CI) = -2.41, -0.07). The analysis at individual PCB congener level revealed that all the coefficients were negative being marginally significant for PCB138 and 153.

The association between pp'DDE and psychomotor test scores was not linear (p-gain for linearity <0.10) and showed a U-shape. For this reason, pp'DDE levels were included in the models as a dichotomic variable with the median as a cut-off point. No associations were found between DDE or HCB levels and psychomotor test scores. In the multipollutant model, we observed that the association between PCBs levels and psychomotor

test scores was maintained with a marginal significance (Coef = -1.22, 95%CI = -2.63, 0.19).

There was no heterogeneity among cohorts because the relationship between OCs and mental and psychomotor tests scores did not vary among them. The associations were not modified neither by duration of breastfeeding nor sex (data not shown). Hg levels assessed in cord blood were not included in the multivariate models because did not satisfy the criteria to be a confounder (data not shown).

DISCUSSION

This study provides further evidence that in utero exposure to relatively lower levels of PCBs than in previous studies is associated with early psychomotor but not with cognitive development impairment. Prenatal exposure to DDE and HCB did not have any effect on neuropsychological development at the second year of life.

Although levels of PCBs were much lower than in the preceding cohorts (9,17,18), the results of the present study are in accordance with previous literature. As observed previously, the mental scale of BSID to assess cognitive assessment was not able to detect adverse effects of exposure to PCBs (9). Conversely, and as already reported by several other birth cohort studies (4,32–34) psychomotor scale was enough sensitive to detect adverse effects of these compounds on early psychomotor development.

One possible mechanism to explain this early neuropsychological pattern observed in PCB exposure may imply the cerebellum (35,36). The cerebellum is an important brain area which has a an essential role in motor function, including balance and coordination (37). Animal studies have revealed that PCBs could alter intracellular calcium signalling, being

the cerebellum the more sensitive brain area to these alterations in comparison with others such as the prefrontal cortex (38,39). Similar PCB levels were found in the cerebellum as the frontal cortex in rats exposed to PCBs (39), while in other brain areas the PCB accumulation was lower. Another mechanism by which PCBs are affecting the psychomotor development of the children could be through thyroid hormones (37). The thyroid hormone is essential for growth and development of brain, and in particular for the cerebellum (40). Deficiency of this hormone during the perinatal period results in abnormal cerebellar development, which is well documented in rodent animal models (41). However, the link between PCB-induced thyroid dysfunction and PCB-related neuropsychological developmental deficits still remains to be established (42).

In this cohort study, we did not find any adverse effects of prenatal exposure to PCBs on early cognitive development. Recent hypothesis postulate that the more clearly negative effects of PCBs on cognitive development could be focused on executive functions (9,17,18). The executive functions broadly encompass a set of cognitive skills that are responsible for the planning, initiation, sequencing, and monitoring of complex goal directed behavior (43). Animal studies have reported disturbance in dopamine levels of the prefrontal cortex in rats exposed to PCB prenatally (44). If there is specific effect of PCBs on executive functions, this could not be detected at early ages, like in the present study, because the prefrontal cortex which is involved to these complex processes is still immature until mid-to-late adolescence (45).

The results of this study, in which children were assessed up to the age of 2 years, show no effects of the pp'DDE and HCB prenatal levels on early neuropsychological development. pp'DDE may exert its negative effects

by disrupting the nervous system causing chemical changes into adulthood (46). The potential mechanism of HCB could interfere with myelination during development alter regional brain concentrations of serotonin, dopamine, and norepinephrine and produce oxidative stress (47–49). Because of the negative effects of these compounds on neuropsychological development reported in previous studies, specially for pp'DDE, further studies at older ages when abilities are wider and more differentiated, will be needed to clarify the tendency observed in our study.

OCs concentrations reported in this study are not directly comparable with those of previous studies because of differences in biospecimens, laboratory methods, and the congeners contributing to the summary estimate of OCs concentrations. However, we used the three higher chlorinated compounds as indicators of the internal PCB exposure which is in accordance with previous recommendations (50). The levels of one of the PCB congeners (PCB153) are within the range of those found previously by Longnecker et al (51). The median concentration of PCB 153 in ten studies also including children revised ranged from 30 to 450 ng/g serum lipid. The median concentration of maternal serum PCB153 in our study was 44.8 ng/g serum lipid. In the present study, Hg levels were measured in the same maternal serum samples, because an interactive effect of PCBs and MeHg has been previously reported (52). However, we did not find variations in the coefficients when the Hg levels were included in the final regression models, nor an interaction was found between Hg and PCBs.

A major strength of this study was its large sample size. We studied a population-based cohort of 1391 children using field staff, interviewers, laboratory technicians, and project paediatricians, all of whom were

specifically trained for the project. For the neuropsychological assessments several quality controls were introduced (inter-observer reliability-tests) and the psychologists who assessed children with the BSID test received extensive training to this end. On the other hand, our study was limited by a number of factors. Although we included a large number of covariates, we were unable to control for some factors that may affect children's cognition, such as the maternal intelligence, quality of their home environment, nutrition, maternal postpartum depression or maternal stress during pregnancy. However, it is very unlikely that these variables were related with the exposure and play a residual confounding role (53). Measurements of OCs were only done in prenatal period. Further measures in cord blood and in breast milk would be very useful in order to disentangle the effects of postnatal exposure to these compounds.

In conclusion, results of the present study suggest that even at low levels of exposure to PBCs, infants may be at risk of neurodevelopment impairment at early ages. Persistence of the effects of PCBs, but also the potential effects of other neurotoxicants, such as DDE and HCB, should be further assessed at later stages of development, when the phenotypes (cognitive and psychomotor abilities, including executive function) are fully developed.

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Table 1. Percentage of samples above the limit of detection (LOD) and concentrations (ng/g lipid) of PCBs, pp'DDT, pp'DDE, and HCB in the three INMA cohorts (n=1391):

	% >lod	Median	(p25-p75)
PCB118	23.22	6.36	(5.55 - 7.77)
PCB138	86.28	26.10	(16.26 - 38.44)
PCB153	95.18	43.43	(28.58 - 61.51)
PCB180	90.65	31.07	(19.86 - 45.76)
ΣPCB*	90.70	102.72	(66.17 - 145.29)
pp'DDT	18.90	6.21	(5.46 - 7.28)
pp'DDE	99.35	119.06	(74.44 - 200.26)
HCB	90.87	43.42	(23.76 - 74.84)

pp'DDE= dichlorodiphenyldichloroethylene, HCB= hexachlorobenzene, PCBs= polychlorinated biphenyls.

*ΣPCB is computed by the sum of congeners 138, 153, and 180 (congener 118 was excluded by its low detectability)

Table 2. Correlation between different OCs:

	PCB138	PCB153	PCB180	ΣPCBs	pp'DDE	HCB
PCB138	1					
PCB153	0.84*	1				
PCB180	0.71*	0.88*	1			
ΣPCBs	0.94*	0.93*	0.88*	1		
pp'DDE	0.35*	0.29*	0.18*	0.28*	1	
HCB	0.30*	0.32*	0.27*	0.31*	0.39*	1

ΣPCB is computed by the sum of congeners 138, 153, and 180 (congener 118 was excluded by its low detectability)
pp'DDE= dichlorodiphenyldichloroethylene, HCB= hexachlorobenzene, PCBs= polychlorinated biphenyls.
* p-value<0.01

Table 3.1. Geometric mean (GM) and 95% Confidence Interval (95%CI)) of the concentrations of pp'DDE, HCB and ΣPCBs (ng/g lipids) by characteristics of the study population (N=1391).

		ΣPCBs		
	%	GM (95%CI)		p
Child's sex				
Girls	50.9	98.65	93.82 , 103.74	0.976
Boys	49.0	98.54	93.20 , 104.18	
Region				
Gipuzkoa	34.4	119.30	113.05 , 125.89	<0.001
Sabadell	35.0	69.76	66.03 , 73.71	
Valencia	30.0	104.27	97.47 , 111.56	
Duration of any breastfeeding				
Formula fed	11.0	108.37	98.28 , 119.50	<0.001
<6 months	39.1	88.48	82.87 , 94.47	
≥6 months	50.0	105.11	99.97 , 110.51	
Maternal education				
Primary or less	22.6	87.36	80.53 , 94.76	<0.001
Secondary	40.0	93.79	88.00 , 99.96	
University	37.4	112.71	106.99 , 118.74	
Maternal Social Class				
CSI+II	23.9	115.16	108.10 , 122.68	<0.001
CS III	28.8	103.34	96.91 , 110.19	
CS IV+V	47.3	88.61	83.47 , 94.06	
Maternal smoking during pregnancy				
No	70.0	99.25	94.78 , 103.93	0.590
Yes	30.0	97.05	91.00 , 103.49	
Maternal country of birth				
Spain	95.0	102.33	98.67 , 106.13	<0.001
Foreign	5.0	51.88	40.95 , 65.72	

pp'DDE= dichlorodiphenyldichloroethylene, HCB= hexachlorobenzene, PCBs= polychlorinated biphenyls.

Table 3.2. Geometric mean (GM) and 95% Confidence Interval (95%CI)) of the concentrations of pp'DDE, HCB and ΣPCBs (ng/g lipids) by characteristics of the study population (N=1391).

	pp'DDE		
	GM (95%CI)		p
Child's sex			
Girls	129.36	121.73 , 137.47	0.677
Boys	131.87	123.29 , 141.04	
Region			
Gipuzkoa	96.39	89.87 , 103.38	0.002
Sabadell	126.17	116.97 , 136.10	
Valencia	185.94	170.05 , 203.32	
Duration of any breastfeeding			
Formula fed	126.65	112.41 , 142.70	0.398
<6 months	127.21	118.66 , 136.38	
≥6 months	135.41	126.39 , 145.07	
Maternal education			
Primary or less	143.95	130.29 , 159.04	0.038
Secondary	130.81	121.45 , 140.89	
University	123.08	114.95 , 131.79	
Maternal Social Class			
CSI+II	127.67	117.46 , 138.77	0.015
CS III	119.14	110.78 , 128.13	
CS IV+V	139.21	129.46 , 149.70	
Maternal smoking during pregnancy			
No	131.57	124.48 , 139.07	0.812
Yes	129.99	120.14 , 140.65	
Maternal country of birth			
Spain	120.84	116.05 , 125.82	<0.001
Foreign	323.66	245.27 , 427.11	

Table 3.3. Geometric mean (GM) and 95% Confidence Interval (95%CI)) of the concentrations of pp'DDE, HCB and ΣPCBs (ng/g lipids) by characteristics of the study population (N=1391).

	HCB		
	GM (95%CI)		p
Child's sex			
Girls	48.35	45.70 , 51.16	0.625
Boys	49.33	46.62 , 52.19	
Region			
Gipuzkoa	32.90	30.71 , 35.25	<0.001
Sabadell	37.16	34.56 , 39.96	
Valencia	57.86	52.00 , 64.39	
Duration of any breastfeeding			
Formula fed	60.74	53.84 , 68.52	<0.001
<6 months	50.10	46.97 , 53.44	
≥6 months	46.28	43.73 , 48.98	
Maternal education			
Primary or less	50.92	46.64 , 55.61	0.436
Secondary	48.96	45.97 , 52.15	
University	47.49	44.56 , 50.61	
Maternal Social Class			
CSI+II	47.86	44.10 , 51.94	0.417
CS III	50.93	47.54 , 54.56	
CS IV+V	48.08	45.26 , 51.08	
Maternal smoking during pregnancy			
No	47.67	45.48 , 49.98	0.049
Yes	52.08	48.26 , 56.20	
Maternal country of birth			
Spain	49.69	47.73 , 51.73	<0.001
Foreign	32.70	25.76 , 41.51	

Table 4. Association between mental and psychomotor test scores and OCs:

	Mental scale†			Psychomtor scale‡		
	Coef	95% CI	p	Coef	95% CI	p
ΣPCBs	-0.11	-1.39 , 1.17	0.868	-1.24	-2.41 , -0.07	0.038
PCB138	0.08	-1.03 , 1.19	0.885	-1.00	-2.03 , 0.04	0.059
PCB153	-0.28	-1.45 , 0.90	0.646	-0.99	-2.07 , 0.09	0.072
PCB180	-0.21	-1.29 , 0.87	0.705	-0.65	-1.64 , 0.34	0.200
pp'DDE	0.21	-0.80 , 1.21	0.685	0.70*	-0.94 , 2.34	0.404
HCB	0.51	-0.45 , 1.46	0.296	-0.55	-1.39 , 0.28	0.190
Multipollutant model						
PCB138	-0.44	-1.92 , 1.05	0.565	-1.22	-2.63 , 0.19	0.090
PCB153						
PCB180						
pp'DDE	0.14	-0.94 , 1.22	0.802	1.26*	-0.44 , 2.97	0.146
HCB	0.58	-0.51 , 1.67	0.293	-0.30	-1.28 , 0.69	0.554

pp'DDE= dichlorodiphenyldichloroethylene, HCB= hexachlorobenzene, PCBs= polychlorinated biphenyls.

ΣPCB: sum of PCB congeners #138, #153, and #180

†Models were adjusted for cohort, sex, main caregiver, maternal country of birth, maternal social class, birth height, gestational age, and duration of any breastfeeding.

‡Models were adjusted for cohort, paternal social class, and gestational age.

Coefficients represented change in mental and psychomotor test scores per natural logarithm (log_{10}) of exposure, unless specified.

*Coefficient associated to pp'DDE levels> 120.42 ng/gr lipid (Reference: ≤ 120.42 ng/gr lipid = Median)

Supplementary Table 1. Description of the imputation procedure.

Software used and key setting: STATA 10.1 software (Stata Corporation, College Station, Texas) – ice command (with 10 cycles)
Number of imputed datasets created: 10
Variables included in the imputation procedure: Variables used in the main analyses (outcome, exposure, and potential confounders) Bayley mental and psychomotor score, psychologist, child age at bayley's test (month), duration of breastfeeding (months), predominant breastfeeding (months), number of siblings (0, 1, 2 or more) , type of delivery (vaginal, instrumental, cesarean), maternal smoking (no/yes) during pregnancy, child's, sex (male/female), gestational age (weeks), birth height (cm), low birth (no, yes), birth cranial perimeter (cm), birth weight, maternal age at delivery (years), maternal social class (I+II, III, IV+V), maternal education (primary, secondary, universitary) paternal age at delivery, paternal social class (I+II, III, IV+V), main caregiver (mother/both parents/grandparents/parents and grandparents/others), gas stove used during pregnancy (no / yes), mercury, lipids , and concentrations of OCs (HCB, DDE, and PCB congeners 118, 138, 153 and 180) (ng/ml) in maternal serum.
Treatment of non-normally distributed variables: log-transformed
Treatment of binary/categorical variables: logistic, ordinal, and multinomial models
Statistical interactions included in imputation models: imputations were done separately by each region (Valencia, Sabadell, and Gipuzkoa)

Supplementary Table 2. Distribution of sociodemographic characteristics in the imputed and the observed datasets. N eligible= 1391.

Imputed variable	Percent (categorical variables) or Mean (SE) (continuous variables)		
	% data imputed	Imputed Dataset	Observed data
Parental characteristics			
Maternal education (%)	0.4		
≤Primary		23.1	23.2
Secondary		40.6	40.4
University		36.4	37.4
Maternal country of birth (%)	0.4		
Spain		91.8	91.5
Foreign		8.1	8.1
Paternal education (%)	4.1		
≤Primary		34.2	32.8
Secondary		43.4	41.6
University		22.4	21.5
Paternal Social class (%)	2.7		
CSI+II		22.3	21.6
CSIII		17.1	16.5
CSIV+V		60.6	59.2
Paternal country of birth (%)	0.2		
Spain		91.3	91.2
Foreign		8.5	8.6
Child characteristics			
Siblings at birth (%)	0.1		
0		56.2	56.2
1		38.0	38.0
2 or +		5.6	5.6
Birthweight (gr)	0.2	3296.6	3296.1
Any breastfeeding (weeks)	2.37	33.6	33.4
Smoking during pregnancy, yes (%)	1.0	30.1	29.9

Imputed variable	Percent (categorical variables) or Mean (SE) (continuous variables)		
	% data imputed	Imputed Dataset	Observed data
Main caregiver (%)	3.74		
Both parents		18.8	18.2
Grandparents		5.5	5.2
Both + grandparents		7.0	6.6
Others		13.1	12.6

Supplementary Table 3. Association between mental and psychomotor test scores and OCs (case-complete):

	Mental scale†			Psychomtor scale‡		
	Coef	95% CI	P	Coef	95% CI	P
ΣPCBs	-0.60	-2.08 ,0.88	0.427	-1.36	-2.65 ,-0.06	0.040
PCB138	-0.16	-1.43 ,1.11	0.808	-1.26	-2.32 ,-0.20	0.020
PCB153	-0.39	-1.80 ,1.03	0.593	-1.50	-2.69 ,-0.30	0.014
PCB180	-0.76	-2.13 ,0.62	0.279	-0.75	-1.93 ,0.43	0.212
pp'DDE	-0.21	-1.26 ,0.83	0.687	0.49*	-1.18 ,2.17	0.563
HCB	0.50	-0.52 ,1.53	0.337	-0.80	-1.73,0.12	0.088
Multipollutant model						
ΣPCBs	-0.96	-2.63 ,0.71	0.259	-1.18	-2.73 ,0.36	0.134
pp'DDE	-0.23	-1.36 ,0.91	0.694	1.19*	-0.57 ,2.95	0.185
HCB	0.83	-0.30 ,1.97	0.151	-0.60	-1.67 ,0.48	0.278

ΣPCB: sum of PCB congeners #138, #153, and #180

†Models were adjusted for cohort, sex, main caregiver, maternal country of birth, maternal social class, birth height, gestational age, and duration of any breastfeeding.

‡Models were adjusted for cohort, paternal social class, and gestational age.

Coefficients represented change in mental and psychomotor test scores per natural logarithm (log_{10}) of exposure, unless specified.

*Coefficient associated to pp'DDE levels> 120.42 ng/gr lipid (Reference: ≤ 120.42 ng/gr lipid = Median)

8.6 Paper 6

Prenatal exposure to Polychlorinated Biphenyls and child neuropsychological development: an anaylsis per each congener and specific cognitive domain.

Forns J, Torrent M., Garcia-Esteban R, Gascon M, Julvez J, Guxens M, Grimalt JO, & Sunyer J.

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Prenatal exposure to Polychlorinated Biphenyls and child neuropsychological development in 4-year-olds: an anaylsis per each congener and specific cognitive domain.

Forns J[1,2,3], Torrent M[4]., Garcia-Esteban R[1,2,3], Gascon M[1,2,3], Julvez J[1,2,3,5], Guxens M[1,2,3], Grimalt JO[6], & Sunyer J[1,2,3,7]

(1) Centre for Research in Environmental Epidemiology (CREAL), Doctor Aiguader 88, 08003 Barcelona, Spain.

(2) Hospital del Mar Research Institute (IMIM), Doctor Aiguader 88, 08003 Barcelona, Spain.

(3) CIBER Epidemiologia y Salud Pública (CIBERESP), Doctor Aiguader 88, 08003 Barcelona, Spain.

(4) Area de Salud de Menorca, IB-SALUT, Menorca, Spain.

(5) Department of Environmental Health, Harvard School of Public Health, Boston, MA, USA.

(6) Department of Environmental Chemistry, Institute of Environmental Assessment and Water Research (IDAEA-CSIC), Barcelona, Spain.

(7) Pompeu Fabra University, Barcelona, Spain.

Correspondence and queries to:

Joan Forns Guzmán

Centre for Research in Environmental Epidemiology- IMIM

C. Doctor Aiguader 88; 08003 Barcelona; Spain

Phone: +34 93 214 73 11

Fax: +34 93 214 73 02

E-mail: jforns@creal.cat

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ABSTRACT

Polychlorinated biphenyls (PCB) are synthetic organochlorine compounds with a potential neurotoxic effect. Despite the negative effects observed in previous studies on neuropsychological development due to the PCBs exposure, there are inconsistencies in these effects at current levels of these compounds which are much lower than in previous generations. This study aimed to disentangle the effects of prenatal and postnatal PCBs exposure on neuropsychological development at the age of 4 years. This study is based in a population-based birth cohort design established in Menorca (Spain) as part of the INMA [Environment and Childhood] Project. We assessed the general neuropsychological development using the McCarthy Scales of Children Abilities (MCSA). A total of 422 4-year old children were assessed with the MCSA. The levels of PCBs were measured in cord blood (n=405) and at 4 years (n=285). We found no statistically significant effects of the sum of prenatal PCBs on MCSA scores. Nevertheless, the analyses per each congener yielded a significant detrimental effects of prenatal PCB153 on the majority of MCSA scores (Global cognitive score = -3.05, p-value = 0.019), while no effects were reported for the other congeners. The levels of PCBs at 4 years old were not associated with neuropsycholgical development. Thus, prenatal exposure to low-level concentrations of PCBs, particularly PCB153, was associated with a global detrimental effect on neuropsychological development at the age of 4 years.

Keywords: child development, Polichlorinated Biphenyls, Hexachlorobenzene, Dichlorodiphenyl Dichloroethylene, neuropsychology.

INTRODUCTION

Polychlorinated biphenyls (PCBs) are syn¬thetic organochlorine compounds that were widely used as insulators, coolants, and lubricants in electrical transformers, capacitors, and hydraulic equipment and as plasticizers in plastic and rubber products from the early 1930s.

Despite of PCBs were banned since 1970s in most industrialized countries, their presence is still detectable because of their high biostability and lipophilicity and because they are resistant to both chemical and biological degradation (1–3).

Two recent systematic reviews (3,4) and one literature review (5), have pointed out the existing evidence about the effects of PCBs on neuropsychological development. The conclusions of the literature review done by Boucher et al (2009) suggest that there are negative effects of prenatal exposure to PCBs on neuropsychological development in children, with specific impairments in executive functions (5). Since poisoning episodes in which developmental neurotoxicity was recognized (6,7), birth cohort studies were established between 1976 and 2010 in several countries to study the effects of prenatal, perinatal, and postnatal exposures of these compounds on child neuropsychological development. Differences across studies in the effects of PCBs on neuropsychological development were reported. Four of these studies showed a decrement in global intelligence quotient score during childhood (8–11) while the other two did not find any association (12,13). Nevertheless, despite the relatively large body of literature on neurotoxic effects of early-life exposure to PCBs on neuropsychological development, controversy still exists over whether PCBs are in fact neurotoxicants at current levels of exposure (4).

We had previously studied the effects of DDE/DDT and HCB on neuropsychological development in the Menorca cohort, due their high values in Spain (14,15). However we did not asses the role of PCBs. Thus, the aim of this study was to assess the potential detrimental effects of prenatal and postnatal exposure to current levels of PCBs, which are lower than in previous generations (1,2), on general neuropsychological development and specific cognitive domains. We assessed children at the age of 4 with the McCarthy Scales of Children Abilities (MCSA) following the criteria set by current reviews (4,5).

METHODS

Study design and participants

This study is based on a population-based birth cohort design established in Menorca (Spain) as part of the INMA [Environment and Childhood] Project, which focuses on environmental exposures and growth, development and health in children (16). All women presenting for antenatal care in a 12 months' period starting in mid-1997, were eligible and invited to participate. 482 mothers (94% of those eligible) were finally enrolled into the cohort. At age 4 years 470 mother–child pairs (98% of those enrolled) remained in the follow-up. All families signed a consent form to participate in the study.

Neuropsychological testing

At age 4 years, 422 children (88% of the original cohort) in Menorca were assessed with a standardized version of the McCarthy Scales of Children's Abilities (MSCA) adapted to the Spanish population (17). The global cognitive scale and five subscales (verbal, perceptive-performance, memory, quantitative and motor) were examined. In addition, we

included the new measures created by Julvez et al (18), in which study the authors reorganized the MCSA subtests into new sub-area scores (executive functions, working memory, visual and verbal span, verbal memory, gross motor skills, and cognitive functions of posterior cortex) according to those tasks highly associated with specific neurocognitive function based on neuropsychological assessment knowledge. Two neuropsychologists were trained to administer and interpret the MCSA. A strict protocol was applied to avoid inter-observer variability, including inter-observer trainings and three sets of quality controls. Continuous MSCA scales were standardized to a mean score of 100 with a standard deviation of 15 to homogenize all the scales. The child's academic year was also collected during the visit.

OCs Expossure Measurement

Organochlorine Compounds (OCs) in cord serum (n=405) and in blood at 4 years old (n=285) were measured by gas chromatography with electron capture detection and gas chromatography coupled to chemical ionization negative-ion mass spectrometry as described elsewhere (19). The sum of PCBs (ΣPCBs) was calculated by summing the concentrations of the most common individual congeners #118, #138, #153, and #180. Individual congeners #28, #52, and #101 were not considered because of they were detectable in less than 21%, 25%, and 40%, respectively. The levels of dichlorodiphenyl dichloroethylene (DDE), hexachlorobenzene (HCB) in cord blood and at 4 years old were also analyzed. All analyses were carried out in the Department of Environmental Chemistry (IIQAB-CSIC) in Barcelona, Spain.

Other parental and child variables

Information on maternal and paternal education (primary, secondary, and university), maternal and paternal social class (non-manual, manual, and housewifes)(using the United Kingdom Registrar General's 1990 classification according to parental occupation, by 1988 International Standard Classification of Occupations code (http://www.ilo.org/public/english/bureau/stat/isco/isco88/index.htm)), maternal cigarretes during pregnancy, alcohol use during pregnancy, number of siblings at child's birth, and child's sex was collected after delivery. In subsequent interviews, data were collected on type and duration of any breastfeeding, marital status, maternal tobacco consumption, and child's diet at ages 4 years and 6 years. All questionnaires were administered face to face by trained interviewers. Additionally, information relating to the child's prematurity (<37 weeks of gestational age), and low birth weight (<2.500 grams) taken at birth was collected from clinical records. Finally, in a posterior visit, we assessed maternal intelligence quotient (IQ) and mental health. We assessed maternal IQ using the 2 and 3 scales of Factor "G" of Cattell and Cattell (Cattell and Cattell, 1977).

Statistical analysis

The MCSA scores followed a normal distribution, while serum levels of PCBs were skewed to the right and were normalized by base 10 logarithmic transformations (log10). The MCSA scores were examined in relation to levels of prenatal and postnatal PCBs, for ?PCBs and for each PCB congener, and the MCSA scores using linear regression models. When adjustment for an additional variable altered the organochlorine compound coefficient by 10 percent or more, that variable was

considered a confounder. Sex, academic year, psychologist, maternal social class, maternal IQ, acid folic and vitamines supplementation during pregnancy, duration of breastfeeding, paternal education, and maternal cigarretes during pregnancy were the variables that met the criteria for confounding.

The potential effect modifier of duration of breastfeeding and child's fish intake (20) were evaluated by including the interaction terms between these variables and different PCBs in the regression model. Statistical analyses were done using Stata 10.1 (Stata Corporation, College Station, Texas).

RESULTS

Our analysis was based on 331 (69%) mother-child pairs with complete information on neuropsychological development assessment and OCs levels. The characteristics of the study population are described in table 1. There were 49% of females, and 49% of children were first born. Fifty-eight percent of the women had primary education and 13% had university education. Forty-four percent were classified as non-manual social class, 34% as manual social class, and 20% as housewifes. There were no differences in global cognitive score of MCSA between children with organochlorine measurements (mean = 100.40; Standard Deviation (SD) = 15.16) and those without (mean = 97.90; SD = 13.96) (p = 0.244). Detectable concentrations of prenatal and postnatal PCB congeners 118, 138, 153, and 180 were quantified between 80 and 98 percent of the children (table 2).

We did not find any statistically significant association between prenatal ΣPCBs and MCSA scores at 4 years old (Table 3). However, all the coefficients were negative (Coefficient (Coef) global cognitive score = -

2.07; p-value = 0.179). The analysis per each congener yielded different results. We did not find any association with PCB congeners 118, and 180. PCB138 was marginally associated with general cognitive score (Coef = -2.04; p-value = 0.90) and the only significant association was established with perceptual-performance scale (Coef = -3.10 p-value = 0.10). PCB153 showed a statistically significant negative association for global cognitive score (Coef = -3.33; p-value = 0.010), verbal, quantitative, perceptual-performance, and memory scales (Table 3). Afterwards, a multipollutant model was performed to ensure the association between PCB153 and MCSA scores, including the levels of p,p'-dichlorodiphenyltrichloroethane (DDT) and hexachlorobenzene (HCB). We found that the associations between PCB153 and MCSA scores were maintained (Coef = -4.57; p-value=0.029). The association between PCB153 and MCSA new sub-areas was also studied (Figure 1). We found that executive function, particularly those involving verbal abilities, working memory, and cognitive functions associated to the posterior cortex were impaired by prenatal exposure to PCB153. None of the associations observed were modified neither by duration of breastfeeding nor fish intake at 4 years (data not shown). Finally, we did not find association between PCBs at 4 years old and MCSA scores (data not shown).

DISCUSSION

Our study suggests that the exposure to low-levels of PCBs, particularly for PCB congener 153, in utero is associated with a decrease in global neuropsychological development at the age of 4 years. We performed this study following the recommendations of previous reviews (4,5) in terms of age of assessment and neuropsychological test. The negative

association between prenatal PCB153 exposure and neuropsychological development at 4 years old was observed not only on global cognitive score, but also on executive function, verbal functions, and visuospatial abilities. The observed associations were maintained after controlling for socioeconomic factors, and they were not modified neither by breastfeeding nor by fish consumption. The levels of PCBs at 4 years old were not associated with neuropsycholgical development at the same age.

Previous evidence about the effects of prenatal exposure to PCBs on neuropsychological development at the preschool period is inconclusive. Only the Oswego (at the age of 3) (21), Dutch (22), and German (23) cohorts found effects on general cognition at similar ages, while North Carolina (13), Michigan (11), collaborative perinatal project (12), and the Oswego (21) (at the age of 4.5) cohorts did not find consistent evidence. In the present study, the negative effects of prenatal exposure to PCBs were stronger than in these previous studies. These effects were not restricted to some specific area as observed in the previous cited studies. We found that all the sub-scales from which MCSA is composed (verbal, quantitative, and perceptual-performance) were impaired by prenatal exposure to PCB153 (17).

The literature review done by Boucher et al (2009) (5) conluded that the detrimental effects of prenatal exposure to PCBs on neuropsychological functioning in children were specifically remarkable on executive functions. These review also pointed out that there are evidences about slower information processing and impairments in verbal abilities and visual memory function due to the PCBs exposure. The specific effects on executive functions found in some of those studies (21,24) may indicate an alteration of dopamine levels of the prefrontal cortex (25) which is the

brain area involved in these complex cognitive processes (26). However, our results did not suggest any specific cognitive profile caused by the exposure to PCBs in utero. We found impairments on working memory and executive function (particularly for those related to the verbal abilities), but also on cognitive functions associated with posterior cortex (such as temporal, parietal and occipital lobes(18)). These results seem to be consistent in multipollutant model adjusting for all OCs compounds and socioeconomic factors. These findings may indicate that the negative effects of PCBs are not restricted to the prefrontal cortex.

Despite we found adverse effects for the sum of PCBs (118, 138, 153, and 180) on neuropsychological development, these results were not statistically significant. The neurotoxic effects were restricted to PCB congener 153, but not in relation with the congeners. There are several possible explanations to justify our findings. Firstly, the PCB153 is among the PCB congeners present at the highest concentration in our samples as well as in some previous studies (27). Secondly, the correlations between the concentration of PCB153 and the sum of quantitated PCBs reported were 0.84 (p-value<0.001), which is consistent with previous studies analyzing the correlation between different PCBs congeners (28,29). Lastly, the cognitive consequences of PCB exposure are due to neurochemical responses. These responses are, to a large extent, dependent on the structure of the PCB congener (whether the congener is coplanar or non-coplanar) (30). PCB congener 153 is a non-coplanar congener, which is identified that alters neurotransmitter function and regulation of intracellular signaling systems, including calcium, in both experimental animals and tissue culture (31). In animal studies, the developmental exposure to PCB153 has been related with a decreased brain dopamine and serotonin concentrations (32), as well as, e.g.

decreased cholinergic muscarinic receptor density in cerebellum and cerebral cortex (33) and impairment of the glutamate–nitric oxide-cGMP pathway in cerebellum (34). Apoptosis has been demonstrated in primary neuronal cultures exposed to PCB 153 (35).

The levels of PCBs at 4 years old were not related with MCSA scores, neither for the total of PCBs nor per each congener. A fewer number of samples were collected at 4 years than at birth (285 and 405, respectively). To ensure that the differences in effects were not due to a difference in sampling, we rerun the models for prenatal PCBs restricting it to 4 year samples, and the results were similar for PCB congener 153 (Coef. Global cognitive score = -4.38 ; p-value = 0.004). Thus, it seems that the effects of prenatal PCBs exposure on neuropsychological development at the age of 4 exceeded to those of the postnatal exposure.

It is worth noting that in a large population-based sample of children; we standardized neuropsychological measurements of cognitive development at age 4, and collected data on a variety of potential socio-demographical factors including parental education, social class, duration of breastfeeding and smoking during pregnancy. For the neuropsychological assessments several quality controls were introduced (inter-observer reliability-tests) and the psychologists who assessed children with the MCSA test received extensive training to this end. This study, however, was limited by a number of factors. Although we included a large number of covariates, we were unable to control for some factors that may affect children's cognition, such as the quality of their home environment, maternal depression or maternal stress during pregnancy. However, it is very unlikely that these variables were related with the exposure and play a residual confounding role (36). In regard to

the exposure, we only measured a small set of congeners in cord blood. In our study, we observed that the specific effects of PCB153 congener exceeded the effects of the sum of the total PCBs measured. Thus, the extension of the number of PCB congeners or classes of cogneners would be necessary to disentangle which ones are the most developmentally neurotoxic. We neither have lipid measurements and so, we cannot compare our PCB levels with previous studies. Lastly, we only collected 285 samples of blood at 4 years old. Even though we demonstrated that the differences in the effects between prenatal and postnatal samples were not due to this issue, we lost statistical power to further explore the postnatal effects.

Overall, we found that prenatal PCB exposure, particularly for PCB congener 153, was adversely associated with global neuropsychological development at the age of 4 years, even at low exposure levels. The results of the present study did not suggest a specific vulnerability of the prefrontal cortex due to prenatal exposure to PCBs. Postnatal exposure to PCBs was not associated with neuropsychological development at the preschool period. Despite of the relatively large body of knowledge on this area, more efforts are needed to measure a major number of PCB congeners. The findings suggest that the analysis per congener seems to be a more accurate method to disentangle the specific effects on neuropsychological development.

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Table 1. Demographic characteristics (n=482):

	Value	
Child's characteristics		
Sex (% females)	48.55	
Age at MacCarthy examination (years) (Mean (SD))	4.36	(0.15)
Pre-term (% <37 gestation weeks)	4.78	
First child (%)	49.17	
Breastfeeding (% formula fed)	17.63	
Fish consumption (times/week) at 4 years (Mean (SD))	1.94	(1.13)
Mother's characteristics		
Age at delivery (years) (Mean (SD))	29.90	(4.60)
Social class (%)		
Non-manual	44.85	
Manual	34.33	
Housewife	20.82	
Education (%)		
Primary or less	58.37	
Secondary	28.54	
University	13.09	
Smoking during pregnancy (%)	21.16	
Fish consumption (times/week) during pregnancy (Mean (SD))	1.63	(1.45)
Supplemented with folic acid during pregnancy (%)	42.83	

SD=Standar Deviation.

Table 2. Percentage of samples above the limit of detection (LOD) and concentrations (ng/ml) of different PCB congeners:

	Cord serum				
	n	% >lod	Median	(p25-p75)	
PCB28	405	20.12	-	-	-
PCB52	405	24.90	-	-	-
PCB101	405	39.63	0.05	(0.02 -	0.06)
PCB118	405	79.25	0.07	(0.04 -	0.10)
PCB138	405	95.64	0.14	(0.09 -	0.20)
PCB153	405	97.93	0.19	(0.13 -	0.26)
PCB180	405	95.23	0.13	(0.09 -	0.19)
ΣPCBs	405	-	0.70	(0.53 -	0.96)

	At 4 years (blood samples)				
	n	% >lod	Median	(p25-p75)	
PCB28	285	43.36	-	-	-
PCB52	285	47.10	-	-	-
PCB101	285	51.24	0.05	(0.05 -	0.11)
PCB118	285	82.16	0.09	(0.06 -	0.13)
PCB138	285	85.89	0.18	(0.11 -	0.28)
PCB153	285	98.76	0.25	(0.14 -	0.41)
PCB180	285	98.55	0.11	(0.06 -	0.21)
ΣPCBs	285	-	0.80	(0.56 -	1.20)

Table 3.1. Adjusted associations (Coefficient (standard error)) between concentrations of PCBs in cord serum (ng/ml) and MCSA scores at age 4 years:

	ΣPCBs			PCB118			PCB138		
	Coef	(SE)	p	Coef	(SE)	P	Coef	(SE)	p
McCarthy General Scores									
General Cognitive	-2.44	(1.53)	0.113	-0.11	(0.99)	0.910	-2.04	(1.20)	0.090
Verbal	-1.77	(1.53)	0.249	0.44	(1.00)	0.663	-1.09	(1.22)	0.371
Quantitative	-2.14	(1.57)	0.174	0.05	(1.03)	0.958	-1.15	(1.25)	0.360
Perceptual-performance	-2.43	(1.53)	0.112	-1.09	(0.99)	0.272	-3.10	(1.19)	0.010
Memory	-2.52	(1.55)	0.106	0.09	(1.02)	0.933	-1.91	(1.24)	0.123
Motor	-1.53	(1.52)	0.315	-0.55	(0.99)	0.582	-1.73	(1.19)	0.148
McCarthy Cognitive areas	-2.44	(1.53)	0.113	-0.11	(0.99)	0.910	-2.04	(1.20)	0.090
Executive function	-1.77	(1.53)	0.249	0.44	(1.00)	0.663	-1.09	(1.22)	0.371

Coef=Coefficient ; (SE)=Standar Error ; p=p-value.

All models were adjusted for psychologist, child's age, maternal social class, folic acid supplementation during pregnancy, paternal education, child's sex, and duration of breastfeeding.

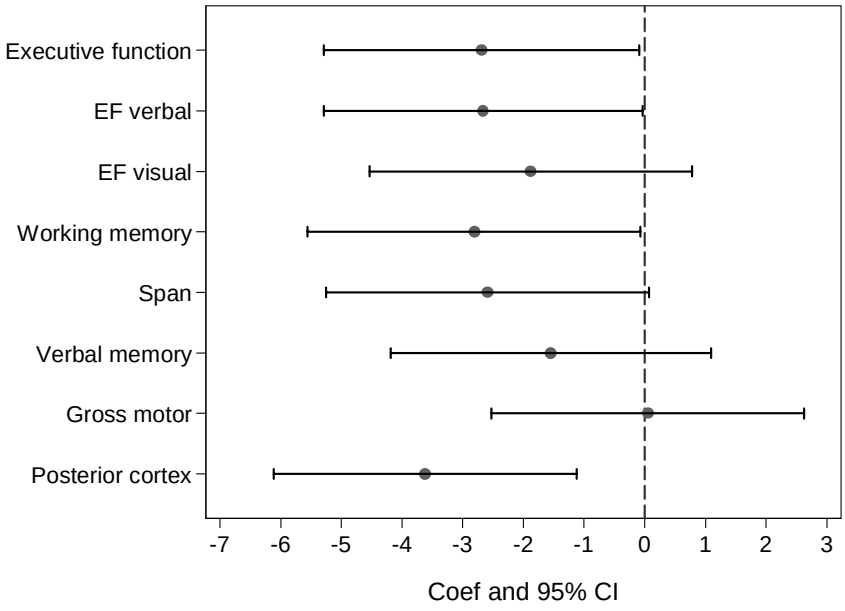
Table 3.2. Adjusted associations (Coefficient (standard error)) between concentrations of PCBs in cord serum (ng/ml) and MCSA scores at age 4 years:

	PCB153			PCB180		
	Coef	(SE)	p	Coef	(SE)	p
McCarthy General Scores						
General Cognitive	-3.33	(1.29)	0.010	-0.40	(0.78)	0.606
Verbal	-2.71	(1.31)	0.040	-0.07	(0.78)	0.925
Quantitative	-2.75	(1.35)	0.042	-0.82	(0.80)	0.307
Perceptual-performance	-3.02	(1.29)	0.020	-0.52	(0.78)	0.504
Memory	-2.82	(1.34)	0.035	-0.18	(0.79)	0.818
Motor	-2.26	(1.29)	0.080	-0.89	(0.77)	0.252
McCarthy Cognitive areas	-3.33	(1.29)	0.010	-0.40	(0.78)	0.606
Executive function	-2.71	(1.31)	0.040	-0.07	(0.78)	0.925

Coef=Coefficient ; (SE)=Standar Error ; p=p-value.

All models were adjusted for psychologist, child's age, maternal social class, folic acid supplementation during pregnancy, paternal education, child's sex, and duration of breastfeeding.

Figure 1. Adjusted associations (Coefficient (95% Confidence Interval)) between concentrations of PCBs in cord serum (ng/ml) and new MCSA sub-areas at the age of 4:



EF = Executive Function

8.7 Paper 7

Longitudinal association between early life socio-environmental factors and attention function at the age 11 years

Joan Forns, Maties Torrent, Raquel Garcia-Esteban, Alejandro Cáceres, María Pilar Gomila, David Martinez, Eva Morales, Jordi Julvez, Joan Oriol Grimalt, & Jordi Sunyer

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Longitudinal association between early life socio-environmental factors and attention function at the age 11 years

Joan Forns[1,2,3] Maties Torrent[4] Raquel Garcia-Esteban[1,2,3] Alejandro Cáceres[1,2,3], María Pilar Gomila[4] David Martinez[1,2,3] Eva Morales[1,2,3] Jordi Julvez[1,2,3,5] Joan Oriol Grimalt[6], Jordi Sunyer[1,2,3,7]

(1) Centre for Research in Environmental Epidemiology (CREAL), Barcelona, Spain.
(2) Hospital del Mar Research Institute (IMIM), Barcelona, Spain.
(3) CIBER Epidemiologia y Salud Pública (CIBERESP), 08003 Barcelona, Spain.
(4) Area de Salud de Menorca, IB-SALUT, Menorca, Spain.
(5) Department of Environmental Health, Harvard School of Public Health, Boston, MA, USA.
(6) Department of Environmental Chemistry, Institute of Environmental Assessment and Water Research (IDAEA-CSIC), Barcelona, Spain.
(7) Pompeu Fabra University, Barcelona, Spain.

Correspondence and queries to:

Joan Forns Guzmán

Centre for Research in Environmental Epidemiology- CREAL

C. Doctor Aiguader 88; 08003 Barcelona; Spain

Phone: +34 93 214 73 11 Fax: +34 93 214 73 02

E-mail: jforns@creal.cat

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ABSTRACT

Prenatal and early-life exposures can affect the course of children´s neuropsychological development, given the vulnerability of the developing brain. However, it is unkown whether life socio-environmental factors at early childhood may influence specific cognitive processes like attention at pre-adolescent age. In this study we aimed to determine which, if any, social and environmental factors early in life are associated with attention function of 11-year-olds.

We measured attention performance with the computerized test-II (CPT-II), and selected our subjects from the Menorca´s birth-cohort within the INMA-project (Spain). A list of socio-environmental factors associated with neuropsychological outcomes at younger ages was selected. A total of 393 11-year old children were assessed with the CPT-II. We found that earlier socio-environmental characteristics, such as parental social class or educational level, and maternal mental health are associated with later inattentive and impulsive symptomatology though a higher rate of omission and commission errors. In addition, omission errors were higher in children with atopy, and lower in those with dietary supplementation with folic acid and vitamines. Breastfeeding played a protective role against commissions errors, and higher DDE and PCBs levels at age 4 impacted in slower speed processing. Our findings suggest that a number of life socio-environmental factors during prenatal life and at early childhood, such as socio-demographic characteristics, breastfeeding, maternal nutritional supplementation with acid folic and vitamines, and some organochlorine compounds may influence inattentive and hyperactive/impulsive symptomatology in preadolescence.

Keywords: child development, attention, neuropsychology, epidemiology, environmental exposure.

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1. INTRODUCTION

The development of the central nervous system involves a complex sequence of processes that is influenced by both genetic and environmental factors (Eskenazi et al., 2008; Grandjean and Landrigan, 2006; Julvez and Grandjean, 2009). In that context, a recent epidemiological study has shown that a number of socio-environmental factors are associated with cognitive development at early childhood (Sunyer et al., 2010). There, Sunyer et al (2010) showed that social class, smoking during pregnancy, parity, breastfeeding, cord blood concentrations of 4,4'- dichlorodiphenyltrichloroethane (DDT), indoor levels of air pollutants, and child atopic status are determinants in cognitive development at the age 4. In addition and prospectively, Landrigan et al. (2008) have also argued that environmental exposures in utero and early-life may lead to a permanent change in the body's structure, physiology, and metabolism, influencing the risk of diseases in later life.

Currently, it is unclear whether early environmental factors are related to a specific cognitive function like attention in the general population at preadolescence age. Recently, a number of epidemiological studies have assessed the effects of some early environmental exposures on attention using Continuous Performance Test (CPT). This is a neuropsychological test paradigm used in both clinical and research fields to assess attention function (Conners, 2004). These studies have found that early life exposures to lead, methylmercury, or polychlorinated biphenyls (PCBs) impacted negatively on different measures of the CPT in children ranging from 8 to 14 years old (Julvez et al., 2010; Kim et al., 2010; Stewart et al., 2005). Another study also reported negative associations between dialkyl phospathes exposures and CPT in younger children (Marks et al., 2010).

While these studies find associations with exposure to some pollutants, it is, however, still unknown whether a number of socio-environmental factors, which have been associated with cognitive development in early stages, also contribute to later CPT performance. Thus, the aim of this study is to find which social and environmental factors are associated with the CPT performance of a large group of 11-year-olds. While there is controversy in the interpretation of the CPT outcomes, we used errors omissions, commissions, and hit reaction time (HRT) as measures of inattention, impulsivity and speed of visual processing respectively (Epstein et al., 2003; Julvez et al., 2010).

2. METHODS

2.1. Design and study population

This study was based on a population-based birth cohort established in the island of Menorca (Spain) as a part of the INMA – INfancia y Medio Ambiente [Environment and Childhood] Project (Guxens et al., 2011). All women presented for antenatal care (within 12 weeks of gestation) were recruited over a 12-month period starting in mid-1997. Children were enrolled (94%of those eligible) and, of them, 422 (87%) took neuropsychological tests up to the fourth year visit. Characteristics of the population have been described elsewhere (Julvez et al., 2007). This study was approved by the Ethics Committee of the Institut Municipal d'Investigació Mèdica, Barcelona, and all the mothers provided asigned informed consent.

2.2. Neuropsychological assessment

We assessed the attention function of 393 11-year children (84.5% of those followed since birth) using the Continuous Performance Test –

Second Edition (CPT-II) (Conners, 2000, 2004). The CPT-II is a computerized measure of vigilance/attention control and response inhibition for children aged 6 and older. Participants were shown succesive letters on a computer screen. Then they were required to press the space bar or click the mouse button when any letter except "X" appears on the screen. Stimuli are presented in six blocks with three sub-blocks, each containing 20 trials (i.e., letter presentations). Inter-stimulus intervals (ISIs) vary between 1, 2, and 4 seconds, while the display time is held constant at 250 milliseconds. During all the CPT-II test, there is 360 trials (324 targets and 36 non-targets). The CPT-II takes 14 minutes to complete.

Three main outcomes of the CPT-II were used in our analyses; 1) Omissions defined as the number of targets to which the individual did not respond; 2) Commissions defined as the number of times that the individual responded to a non-target; and 3) HRT defined as the mean response time (expressed in miliseconds) for all correct target hits over the complete CPT-II. In our data, HRT showed a right-skewed distribution, and therefore, we applied a negative inverse transformation of the variable (-1/HRT) to normalize its distribution and then multiplied by 1000 to avoid rounding problems associated with small numbers (Fazzio 1990, Ratcliff 1983). The resulting variable named "tHRT" was described and discussed in the manuscript in terms of speed (slower or faster responses).

All tests were administered to each child in a single session of 20 min at the Health Care Center, in a separate room free from distraction. All sessions were supervised by a medical doctor.

2.3. Socio-environmental factors

All variables are presented in Table 1. We considered a significant number of socio-environmental factors which have been related to neuropsychological development at the age 4 in a previous study (Sunyer et al., 2010). We assessed some contaminants measured in cord blood and in serum samples at 4 years old. Some of these contaminants were the persistent organic pollutants: DDT, dichlorodiphenyl dichloroethylene (DDE), hexachlorobenzene (HCB), and the sum of PCB congeners #118, #138, #153, and #180 (PCBs). We also considered other potential environmental exposures such as indoor NO_2, gas appliances at home, indoor allergens, duration of breastfeeding, maternal fish intake during pregnancy, and parental smoking. Furthermore, we included socio-environmental determinants such as parental education, social class, or age. Extensive information about collection, and measurement of these variables is discussed elsewhere (Julvez et al., 2007, 2009; Mendez et al., 2008; Morales et al., 2009; Ribas-Fito et al., 2007[a], 2007[b]; Torrent et al., 2007). We also collected disease information in a brief questionnaire filled out by mothers, when their children were 9 years old. Finally, during the same visit of the CPT-II assessment, we assessed maternal intelligence quotient (IQ) and mental health. We assessed maternal IQ using the 2 and 3 scales of Factor "G" of Cattell and Cattell (Cattell and Cattell, 1977), and maternal Mental Health using the Spanish version of the General Health Questionnaire-12 items (GHQ-12) (Goldberg et al., 1997).

2.4. Statistical analyses

Multiple imputations of missing values for the early life determinants was performed using chained equations on the 393 subjects, for which complete information of neuropsychological assessment was available

(Van Buuren et al., 1999). Fifty completed data sets were generated and analyzed separately, and the results were combined using the standard Rubin's rules (Supplementary Table 1a) (Royston, 2004). Assuming that the data were missing at random (MAR), we checked that the imputed values are reasonable: i) graphically by comparing the distributions of the observed and imputed values of each variable through density plots and quantile-quantile plots (Supplementary Figures 1 and 2, respectively) ii) numerically by summarizing (mean and percentages) observed and imputed values (Supplementary Table 1b). The distribution of all variables was similar for observed and imputed data, indicating no obvious problems with the imputation process. Results did not differ meaningfully from complete case analysis, making the MAR assumption plausible (Supplementary Table 1c).

We selected the variables a-priori (table 1) based on those related with neuropsychological outcomes at younger ages in previous studies (Sunyer et al, 2010). We used negative binomial regression (model for count data with overdispersion) for omissions and commissions, while tHRT was analyzed using linear regression models. We performed one fully adjusted model for each of the CPT-II outcomes including all the variables showing a p-value < 0.2 in the bivariate analyses.Furthermore, we forced the inclusion of sex and age due to the important a-priori rule on CPT-II outcomes (Conners et al., 2003). Given that breastfeeding is the pathway of intake of organochlorinated pollutants (Carrizo et al, 2006), to asses the association of the pollutants we fitted the models with and without breastfeeding.

In addition, sensitivity analyses of the final multivariate models were performed. We repeated the same models but excluding those children with a clinical diagnose of Attentional Deficit Hyperactivity Disorder

(ADHD) (n=23). Finally, the tHRT was divided in three different blocks in relation to the time duration of the CPT-II, following the same methodology of a previous paper (Julvez et al., 2010). We used Generalized Estimating Equations (GEE) model, which accounts for within-subject correlation across tHRTs measured on each third of the test. We selected the unstructured working correlation matrix based on quasilikelihood under the independence model criterion (QIC) (Pan, 2001). We studied if the effects of those environmental exposure variables associated with the tHRT were different among the three different blocks including the interaction term between the block and the exposure variables. Statistical analyses were done using Stata 10 (Stata Corporation, College Station, Texas).

3. RESULTS

Table 2 describes the univariate distribution of the CPT-II outcomes and the socio-environmental factors. There were 51% of females, 49% of children were first born. Forty-seven percent of women were classified as non-manual social class, 34% as manual social class, and 19% as housewifes.

Table 3 presents the bivariate associations between the three CPT-II outcomes and the early life socio-environmental factors. Lower paternal education, lower maternal social class, worse maternal mental health and lower IQ, not being first born, not folic acid and vitamin supplementation during pregnancy, and being atopic at age 4 increased the error rate of omissions. Paternal social class, duration of breastfeeding, number of cigarettes per day during pregnancy, atopic status, and DDE and PCBs levels at the age of 4 years were associated with the error rate of commissions. Finally, tHRT showed association with

maternal social class, duration of any breastfeeding, birthweight, siblings at birth, and DDE and PCBs levels at the age of 4.

In fully adjusted models, we found that higher paternal educational level, being first born, and supplementation with folic acid and vitamins during pregnancy reduced the Incidence Rate Ratio (IRR) of performing errors of omission (Table 4). As opposed, worse maternal mental health increased the error ratio. To assess whether the association between omissions and being atopic at the age of 4 was confounded by other variables related to allergic outcomes, we further adjusted the final model for child asthma, wheeze, and maternal atopy. The association became stronger, and marginally significant, after this adjustment (IRR = 1.37 [0.98 – 1.92]; p-value = 0.063). In addition, we adjusted these models for child medication because of the potential loosing of alertness associated to some of them (i.e. anti-histamines). After this adjustment, the association became statistically significant (IRR = 1.47 [1.05 – 2.08]; p-value = 0.026).

More disadvantaged social classes increased the error rate of commissions, while longer duration of breastfeeding reduced it (Table 4). Breastfeeding was highly correlated with PCBs (Spearman rho = 0.74) and DDE (Spearman rho = 0.78). However, the OCs did not show any significant association neither in the fully adjusted models nor after exclusion of breastfeeding from the model (p>0.2), while breastfeeding showed a protective effect both with or without the pollutants in the model.

HRT was reduced with increases in birthweight. DDE and PCBs levels at the age of 4 did not show any association when included in the model together with breastfeeding (p>0.2 for the coefficients of either PCBs,

DDE and breastfeeding). However, after excluding breastfeeding from the model these pollutants increased the HRT (Table 4).

We performed sensitivity analyses excluding those children with a clinical diagnosis of ADHD (data not shown). The associations between omissions and maternal mental health, folic acid and vitamin supplementations, and child's age were diminished. The rest of the associations did not change with the exclusion of these children. Finally, we did not observe different effect of DDE or PCBs levels at the age of 4 between the three different blocks of HRT (data not shown).

4. DISCUSSION

We have shown that a range of early life socio-environmental factors differently influenced three main outcomes of the CPT-II at age of 11. Socio-demographic determinants such as parental social class, education, maternal mental health, and siblings at birth were associated with inattention and impulsivity, as measured by omissions and commissions errors. We also found that the number of errors of commissions was decreased by longer periods of breastfeeding. Finally, children with higher environmental exposure levels of DDE and PCBs at age 4 showed slower responses.

Our observation that social class, educational level, maternal mental health, and children being firstborn were strongly related to both omissions and commissions at the age of 11 confirms the importance of social environment on child cognitive development (Bradley and Corwyn, 2002). The mechanism for the observed effects may be explained by the quality of nutrition, health care, housing, as well as parents who may offer a cognitively-stimulating environment (Francis et al., 2002; Rosales

et al., 2009). Our results also suggest that speed processing, reflected on HRT, is not related to socio-demographic characteristics.

Apart from the socio-demographical variables, we observed a tendency by which being atopic at age 4 was related with an increase of the incidence rate ratio of performing errors of omission. A previous study in the same cohort showed a negative association between atopy at age 6 and general cognition and social competence at age 4 (Julvez et al., 2009). Deregulation of the hypothalamic–pituitary–adrenal axis (HPA-axis) could be the underlying mechanism on the basis of the atopy as well as the inattentive and impulsive symptomatology maintained over time (Chida et al., 2008). This may be supported by the evidence that the HPA-axis of inattentive and combined subtypes of ADHD react differently under stress (Van West et al., 2009). The association between socio-environmental determinants and omissions may indicate the importance of psychosocial factors associated to the onset of atopy and atopic disorders (Chida et al., 2008).

The protective effect of maternal supplementation with folic acid and vitamins during pregnancy on cognitive development of preadolescents is a novel finding. Previous studies have found benefits for cognitive development during childhood in children whose mothers have been supplemented during pregnancy (Julvez et al., 2009; Wehby and Murray, 2008), while other study found no association (Dobó and Czeizel, 1998). Our results suggest that maternal supplementation during pregnancy may have a persistent effect on cognitive development of children.

Our finding that commission errors were decreased by longer duration of breastfeeding is largely supported by earlier studies. A number of these studies have shown a positive association between breastfeeding and neuropsychological development during the first years of life (Anderson

et al., 1999; Golding et al., 1997). In Horwood et al (1998), they reported positive associations between breastfeeding and cognitive development in school and adolescent children, despite weaknesses in the association when socio-demographic variables were included. In fact, a recent review claims that most of the observed associations between breastfeeding and cognitive development are confounded by maternal IQ and the other socioeconomic factors (Der et al., 2006). In this study, however, we find evidence that supports the positive effects of longer periods of breastfeeding to the development of a specific phenotype, such as commissions, after controlling for a number of socio-demographic variables, including maternal IQ and mental health. These results confirm the findings from a study in the same cohort that reported a positive breastfeeding association for similar cognitive domains at the age 4 (Julvez et al., 2007).

We observed associations between levels of contaminants (DDE/PCBs) in blood samples at the age 4 and HRT at the age of 11. This is a very relevant finding because, in this cohort, we have never found associations between OCs at 4 years and neuropsychological outcomes. Previously, negative associations have been found in Menorca and Ribera d'Ebre cohorts between prenatal DDE and DDT levels and neuropsychological development at age 1 (Ribas-Fito et al., 2003), and at age 4 (Sunyer et al., 2010). Inversely, in our study the levels of OCs in cord blood were not associated to HRT. DDE and PCBs may be incorporated into children in utero, through breastfeeding and via diet (Grimalt et al., 2009). In spite of the high association between duration of breastfeeding and OCs levels at the age 4 (Carrizo et al, 2006), we did not observe negative consistent effects of breastfeeding on HRT in multivariate models nor an interaction between breastfeeding and OCs.

DDE and PCB are known to have neurotoxic effects (Grandjean and Landrigan, 2006). In regard to the reaction time, slower speed processing as a result of prenatal PCBs exposure was detected in Michigan, Netherlands, and Faroe Islands cohorts in children ranging from 7 to 11 years old (Boucher et al, 2009). Nevertheless, we found the effects only for OCs measured in child blood at the age of 4.

This is in line with the hypothesis of Grandjean and Landrigan (2006) that the vulnerability of the developing brain is restricted not only to the prenatal period, but extends through infancy into early childhood. After birth, some processes, such as growth of glial cells, myelinisation of axons and wiring continues for several years (Rodier, 1995), and therefore the postnatal exposure to some compounds with neurotoxic effects, such as DDE or PCBs can still have an effect in cognitive function later in life. The observed association persisted in similar strength on the three separated blocks of the HRT outcome, in relation to time duration of the test.

It is worth noting that in a large sample of children; we had standardized neuropsychological measurements of cognitive development at age 11, and had collected data on a variety of potential socio-environmental factors including parental education and social class, maternal IQ, mental health, and several contaminants with potential neurotoxic effects. This study, however, is far from a complete survey of all possible variables that may be related to the CPT-II performance. We did not have, for instance, information about visual acuity of children, computer experience, or computer game experience. In addition, information about the concrete time in which children were tested could be informative on possible tiredness.

5. CONCLUSSIONS

In conclusion, the results of the present study suggest that a range of different socio-environmental factors during prenatal and early in life are associated with inattentive and hyperactive/impulsive symptoms, in a Spanish sample at preadolescence period. Specifically, we found DDE and PCBs levels at age 4 were associated with slower speed processing capacities. These data provide support to confirm that the neurotoxic effect of the DDE and PCBs may not only be restricted to prenatal period, but also it can extend through childhood, and have an impact on child neuropsychological development at older ages.

Competing interests:

The author(s) declare that they have no competing interests.

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Table 1. List of variables included in the study:

Socio-demographical	Child at birth	Gender, number of siblings, gestational age, and Weight
	Maternal	Education, social class, mental health, IQ, and alcohol, smoking, fish intake, and folic acid and vitamins supplementation during pregnancy
	Paternal	Education, social class, and smoking during pregnancy
	Child at 4 years	Breastfeeding, fish intake, and atopy
Chemical environment	At birth	DDE, HCB, PCBs, indoor NO_2, and gas appliances at home
	At age 4	DDE, HCB, and PCBs

Table 2. Descriptive of CPT-II outcomes at 11 years of age (Omissions, Commissions, and HRT) and socio-demographical characteristics:

	P50	(p25-p75)
CPT-II outcomes		
Omissions (count)	4.00	(1.00 - 9.00)
Commissions (count)	22.00	(16.00 - 27.00)
Hit Reaction Time (miliseconds)	381.01	(347.41 - 416.32)
Age at examination (years)	11.66	(11.25 - 12.05)
Sociodemographic characteristics		
Sex, female (%)	50.64	
Birthweight (gr)	3200	(2920 - 3550)
Gestational age (weeks)	39	(38 - 40)
Atopy at age 4, yes (%)	11.96	
Siblings at birth, firstborn (%)	48.60	
Maternal social class (%)		
Non-manual	47.14	
Manual	33.85	
Housewifes	19.01	
Maternal education (%)		
Primary	55.41	
Secondary	29.82	
University	14.78	
Paternal social class (%)		
Non-manual	35.66	
Manual	64.36	
Paternal education (%)		
Primary	64.77	
Secondary	26.94	
University	8.29	
Duration of anybreastfeeding (months)	4.13	(1.38 - 6.44)
Folic acid and vitamines supplementation, yes (%)	66.84	
Maternal smoking during pregnancy, yes (%)	18.44	
Cigs/day among smokers	5.00	(3.25 - 7.75)
Maternal Mental Health (GHQ-12)	10	(8.00 - 13.00)
Maternal IQ (Factor G Cattell and Catell)	10	(8.00 - 12.00)
Fish consumption during pregnancy (times/week)	1.5	(1.00 - 2.00)
Fish consumption at 4 years (times/week)	2	(1.00 - 2.50)

	P50	(p25-p75)
Chemical environmental exposures		
Number of gas appliances at home during pregnancy (%)		
No gas cooking or gas fire	25.45	
Gas cooking or gas fire	54.20	
Gas cooking and gas fire	20.36	
Indoor measured NO_2 concentration (µg/m3)	10.99	(5.38 - 20.00)
DDE levels in cord serum (ng/ml)	1.08	(0.56 - 1.85)
PCBs levels in cord serum (ng/ml)	0.54	(0.41 - 0.76)
HCB levels in cord serum (ng/ml)	0.68	(0.40 - 1.01)
DDE levels at age 4 (ng/ml)	0.85	(0.46 - 1.81)
PCBs levels at age 4 (ng/ml)	0.68	(0.43 - 1.09)
HCB levels at age 4 (ng/ml)	0.32	(0.20 - 0.52)

Table 3.1. Crude associations between CPT-II outcomes (omissions, commissions, and HRT) and early life factors:

	Omissions (counts)		Commissions (counts)	
	IRR	CI95%	IRR	CI95%
Maternal education (Ref. Primary)				
Secondary	0.93	0.72 , 1.20	1.02	0.94 , 1.11
University	0.79	0.57 , 1.10	0.97	0.87 , 1.07
Paternal education (Ref. Primary)				
Secondary	0.65	0.50 , 0.83	0.95	0.87 , 1.03
University	0.55	0.37 , 0.82	0.95	0.83 , 1.08
Paternal Social Class (Ref. Non-manual)				
Manual	1.13	0.90 , 1.42	1.09	1.01 , 1.18
Maternal Social Class (Ref. Non-manual)				
Manual	1.22	0.96 , 1.56	1.00	0.92 , 1.08
Housewifes	1.47	1.10 , 1.96	0.99	0.90 , 1.09
Maternal Mental Health (GHQ-12) (per unit increase)	1.03	1.00 , 1.05	1.00	0.99 , 1.01
Maternal IQ (Factor G) (per unit increase)	0.98	0.94 , 1.01	1.00	0.99 , 1.01
Siblings at birth, ≥1	1.49	1.20 , 1.84	1.03	0.96 , 1.10
Duration of breastfeeding (per each month)	0.99	0.96, 1.01	0.99	0.98 , 1.00
Maternal smoking during pregnancy (per each cigarretes/day)	1.01	0.97 , 1.04	1.01	1.00 , 1.02
Folic acid and vitamines suplementation (Ref. No)	0.74	0.59 , 0.93	0.99	0.92 , 1.07
Birthweight (per each 100 gr)	0.99	0.97 , 1.02	1.01	0.99 , 1.01
Gestational age (per each week)	0.99	0.93 , 1.05	1.01	0.99 , 1.03
Atopy at age 4 (Ref. No)	1.41	1.01 , 1.97	1.09	0.97 , 1.23
Maternal fish consumption during pregnancy (per times/week increase)	1.04	0.95 , 1.12	0.99	0.97 , 1.02
Child's fish consumption at 4 years (time/week increase)	0.98	0.90 , 1.06	0.99	0.96 , 1.02
Number of gas appliances at home (Ref: No gas cooking or gas fire)				
Gas cooking or gas fire	0.97	0.75 , 1.26	0.96	0.88 , 1.04
Gas cooking and gas fire	1.15	0.84 , 1.59	0.97	0.88 , 1.08
Indoor measured NO_2 concentration (increase per 1 ppb)	1.00	0.99 , 1.01	1.00	1.00 , 1.00
DDE levels in cord serum (ng/ml)‡	0.96	0.85 , 1.09	0.98	0.94 , 1.02
PCBs levels in cord serum (ng/ml)‡	0.94	0.78 , 1.13	0.99	0.93 , 1.05
HCB levels in cord serum (ng/ml)‡	1.02	0.85 , 1.22	1.00	0.94 , 1.06
DDE levels at age 4 (ng/ml)‡	0.99	0.89 , 1.10	0.97	0.93 , 1.00
PCBs levels at age 4 (ng/ml)‡	1.02	0.86 , 1.22	0.95	0.89 , 1.01
HCB levels at age 4 (ng/ml)‡	0.98	0.83 , 1.16	0.97	0.92 , 1.03

IRR=Incidence Rate Ratio ; Coef=Coefficient ; CI=Confidence interval; ‡ The estimate represents the change in outcome per each log-unit increase

Table 3.2. Crude associations between CPT-II outcomes (omissions, commissions, and HRT) and early life factors:

	tHRT (-1.000/HRT)	
	Coef	CI95%
Maternal education (Ref. Primary)		
Secondary	-0.047	-0.129 , 0.036
University	-0.067	-0.173 , 0.039
Paternal education (Ref. Primary)		
Secondary	-0.052	-0.134 , 0.030
University	-0.034	-0.166 , 0.098
Paternal Social Class (Ref. Non-manual)		
Manual	-0.007	-0.082 , 0.068
Maternal Social Class (Ref. Non-manual)		
Manual	0.064	-0.016 , 0.145
Housewifes	0.081	-0.015 , 0.178
Maternal Mental Health (GHQ-12) (per unit increase)	0.002	-0.006 , 0.010
Maternal IQ (Factor G) (per unit increase)	-0.005	-0.016 , 0.005
Siblings at birth, ≥1	0.064	-0.007 , 0.136
Duration of breastfeeding (per each month)	0.01	0.001 , 0.020
Maternal smoking during pregnancy (per each cigarretes/day)	-0.007	-0.018 , 0.005
Folic acid and vitamines suplementation (Ref. No)	-0.021	-0.096 , 0.055
Birthweight (per each 100 gr)	-0.009	-0.016 , -0.002
Gestational age (per each week)	-0.012	-0.031 , 0.007
Atopy at age 4 (Ref. No)	-0.055	-0.172 , 0.063
Maternal fish consumption during pregnancy (per times/week increase)	-0.001	-0.025 , 0.023
Child's fish consumption at 4 years (time/week increase)	0.003	-0.029 , 0.034
Number of gas appliances at home (Ref: No gas cooking or gas fire)		
Gas cooking or gas fire	0.042	-0.044 , 0.127
Gas cooking and gas fire	0.000	-0.106 , 0.106
Indoor measured NO_2 concentration (increase per 1 ppb)	-0.001	-0.003 , 0.001
DDE levels in cord serum (ng/ml)‡	0.019	-0.023 , 0.061
PCBs levels in cord serum (ng/ml)‡	-0.023	-0.089 , 0.042
HCB levels in cord serum (ng/ml)‡	0.007	-0.054 , 0.069
DDE levels at age 4 (ng/ml)‡	0.043	0.007 , 0.079
PCBs levels at age 4 (ng/ml)‡	0.065	0.005 , 0.125
HCB levels at age 4 (ng/ml)‡	0.028	-0.026 , 0.083

IRR=Incidence Rate Ratio ; Coef=Coefficient ; CI=Confidence interval; ‡ The estimate represents the change in outcome per each log-unit increase

Table 4.1. Fully adjusted associations between CPT-II outcomes (omissions, commissions, and HRT) and early life factors:

	Omissions (counts)			
	IRR	CI95%		p
Paternal education (Ref. Primary)				
Secondary	0.70	0.55 ,	0.90	0.005
University	0.53	0.35 ,	0.80	0.002
Paternal Social Class (Ref. Non-manual)				
Manual	-			
Maternal Social Class (Ref. Non-manual)	-			
Manual	1.06	0.83 ,	1.36	0.643
Housewifes	1.15	0.86 ,	1.55	0.346
Maternal IQ (Factor G Cattell and Catell) (per unit increase)	0.99	0.96 ,	1.02	0.620
Maternal Mental Health (GHQ-12) (per unit increase)	1.03	1.00 ,	1.05	0.027
Siblings at birth, ≥1	1.36	1.09 ,	1.69	0.006
Duration of breastfeeding (per each month)	-			
Maternal smoking during pregnancy (per cig/day)	-			
Folic acid and vitamines suplementation (Ref. No)	0.80	0.64 ,	1.00	0.046
Birthweight (per 100 gr)	-			
Atopy at age 4 (Ref. No)	1.30	0.94 ,	1.81	0.110
DDE levels at age 4 (ng/ml)† ‡	-			
PCBs levels at age 4 (ng/ml)‡	-			

†Included in multivariate model alternatively to the PCBs levels at age 4.
IRR=Incidence Rate Ratio
Coef=Coefficient
CI=Confidence interval
‡ Coefficient represent change in outcome per each log-unit increase

Table 4.2. Fully adjusted associations between CPT-II outcomes (omissions, commissions, and HRT) and early life factors:

	Commissions (counts)			
	IRR	CI95%		p
Paternal education (Ref. Primary)				
Secondary	-			
University	-			
Paternal Social Class (Ref. Non-manual)				
Manual	1.08	1.00 ,	1.16	0.041
Maternal Social Class (Ref. Non-manual)				
Manual	-			
Housewifes	-			
Maternal IQ (Factor G Cattell and Catell) (per unit increase)	-			
Maternal Mental Health (GHQ-12) (per unit increase)	-			
Siblings at birth, ≥1	-			
Duration of breastfeeding (per each month)	0.99	0.98 ,	0.99	0.001
Maternal smoking during pregnancy (per cig/day)	1.01	1.00 ,	1.02	0.089
Folic acid and vitamines suplementation (Ref. No)	-			
Birthweight (per 100 gr)	1.01	1.00 ,	1.01	0.065
Atopy at age 4 (Ref. No)	1.08	0.96 ,	1.21	0.191
DDE levels at age 4 (ng/ml)† ‡	-			
PCBs levels at age 4 (ng/ml)‡	-			

†Included in multivariate model alternatively to the PCBs levels at age 4.
IRR=Incidence Rate Ratio
Coef=Coefficient
CI=Confidence interval
‡ Coefficient represent change in outcome per each log-unit increase

Table 4.3. Fully adjusted associations between CPT-II outcomes (omissions, commissions, and HRT) and early life factors:

	tHRT (-1.000/HRT)			
	Coef	CI95%		p
Paternal education (Ref. Primary)				
Secondary	-			
University	-			
Paternal Social Class (Ref. Non-manual)				
Manual	-			
Maternal Social Class (Ref. Non-manual)	-			
Manual	0.065	-0.014 ,	0.145	0.108
Housewifes	0.078	-0.021 ,	0.177	0.123
Maternal IQ (Factor G Cattell and Catell) (per unit increase)	-			
Maternal Mental Health (GHQ-12) (per unit increase)	-			
Siblings at birth, ≥1	0.063	-0.009 ,	0.136	0.088
Duration of breastfeeding (per each month)	-			
Maternal smoking during pregnancy (per cig/day)	-			
Folic acid and vitamines suplementation (Ref. No)	-			
	-		-	
Birthweight (per 100 gr)	0.011	-0.018 ,	0.004	0.002
Atopy at age 4 (Ref. No)	-			
DDE levels at age 4 (ng/ml)† ‡	0.051	0.014 ,	0.088	0.007
PCBs levels at age 4 (ng/ml)‡	0.083	0.022 ,	0.144	0.008

†Included in multivariate model alternatively to the PCBs levels at age 4.
IRR=Incidence Rate Ratio
Coef=Coefficient
CI=Confidence interval
‡ Coefficient represent change in outcome per each log-unit increase

8.8 Paper 8

A Conceptual Framework in the Study of Neuropsychological Development in Epidemiological Studies

Forns J, Aranbarri A, Grellier J, Julvez J, Vrijheid M, & Sunyer J.

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Forns J,[1,2,3], Aranbarri A,[4,5] Grellier J,[1,2,3,6] Julvez J,[1,2,3,6] Vrijheid M,[1,2,3] & Sunyer J,[1,2,3,8]

(1) Centre for Research in Environmental Epidemiology (CREAL), Doctor Aiguader 88, 08003 Barcelona, Spain.
(2) Hospital del Mar Research Institute (IMIM), Doctor Aiguader 88, 08003 Barcelona, Spain.
(3) CIBER Epidemiologia y Salud Pública (CIBERESP), Doctor Aiguader 88, 08003 Barcelona, Spain.
(4) Psychobiology area, Basic Psychological Processes and Development Department, Faculty of Psychology, University of The Basque Country, Tolosa Avenue 70, 20018, Donostia, Spain.
(5) BioDonostia Health Research Institute, Doctor Begiristain s/n, 20014, Donostia, Spain.
(6) Department of Epidemiology and Biostatistics, Imperial College London, UK.
(7) Department of Environmental Health, Harvard School of Public Health, Boston, MA, USA.
(8) Pompeu Fabra University, Barcelona, Spain.

Correspondence and queries to:

Joan Forns Guzmán

Centre for Research in Environmental Epidemiology- IMIM

C. Doctor Aiguader 88; 08003 Barcelona; Spain

Phone: +34 93 214 73 11 Fax: +34 93 214 73 02

E-mail: jforns@creal.cat

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ABSTRACT

Background and Methods: A wide range of neuropsychological development outcomes in children are currently measured in a large number of birth cohort and child cohort studies. We summarized neuropsychological development assessment protocols from a number of birth cohort studies, reviews and specific books on child neuropsychology, into a unifying conceptual framework.

Results: We suggest that neuropsychological development can be differentiated into two levels: functional and clinical. The functional level includes the skills, abilities, capacities, and knowledge acquired during maturation of the brain as a result of development of neural. It can be further divided into cognitive, psychomotor, and social-emotional development subdomains. The clinical level includes the assessment of neurodevelopmental disorders, or to the presence of symptoms (subclinical symptomatology) of these disorders in populations under investigation in environmental epidemiology studies.

Conclussions: Through explicit recognition of these levels of outcomes—and in using this framework—epidemiologists will be better able to design research through the informed selection of individual levels of outcomes. The framework also serves to standardize disparate terminologies across this field, and allows for pooling of epidemiological data on neuropsychological endpoints where the essentially similar levels of outcomes have been analyzed using different tests.

Keywords: cognitive assessment; cohort studies; epimdeiological studies; neuropsychological assessment.

Introduction and Methods:

The developing human brain is extremely sensitive to some environmental factors such as certain industrial chemicals, tobacco smoke, alcohol and certain drugs, as well as low socio-economic status, elevated maternal stress, negative parenting behaviours, or family violence [1]. This vulnerability is particularly important during early development, but it extends through infancy and childhood [2]. Both the prenatal period and the first year of life represent critical phases in the early development of neural networks and their associated cognitive and psychomotor functions. During the postnatal period, the brain requires a particularly large complement of nutrients due to its high metabolic activity, especially for the development of certain areas of the cortex [3]. The susceptibility of infants and children to many exogenous compounds is accounted for by their low capacity to detoxify them [2,4]. For these reasons, the developing central nervous system represents the bodily system most commonly disrupted by environmental teratogenic agents [5]. The developing brain is not only exposed, however, to environmental agents, but is also affected by a number of social factors that play a crucial role in the neuropsychological development process. These social influences chiefly comprise parental characteristics, such as cognitive capacities, social class, or mental health. Such parental and social characteristics modify some important aspects of child development, such as quality of nutrition, health care, housing, and the provision of a cognitively stimulating environment [6,7].

The developing brain is a highly complex organ and its development is a genetically driven process modulated by social and environmental factors [8]. Successful brain development requires that each area first be formed and then be correctly interrelated with the others [9]. Thus, a highly

structured and complex approach is needed to accurately measure this process. An optimal assessment of the neuropsychological development process is crucial to the detection of subtle or more obvious effects of the environment on this process because the integrity of the whole system may be compromised if a sole specific domain is affected. The long-term consequences of these alterations may be important at the individual and population levels. For this reason, it is important to understand normal brain development to identify any abnormal differences.

The first attempts at studying associations between exposure to chemical agents and child neuropsychological development were reported in the 1970s [10,11] Since that time, research in environmental epidemiology has increasingly turned its attention towards the developing human brain. As a result, a wide range of neuropsychological development outcomes in children are now measured in many birth and child cohort studies. The aim of this work was to synthesize information on neuropsychological assessment protocols from a number of environmental epidemiological studies into a single practical and conceptual framework. Firstly, as part of the ENRIECO Project (Environmental Health Risks in European Birth Cohorts – www.enrieco.org) we reviewed the assessment protocols on neuropsychological development in all of the European longitudinal birth cohorts involved in this project that currently collect data on environmental exposures and child health. Twenty-five cohorts were identified which assess child neuropsychological development prospectively from birth to later adolescence (depending on the starting point of each cohort). These European birth cohorts were not designed according to a common protocol and therefore the ages of assessment,

and the neuropsychological developmental areas evaluated differ among cohorts. However, in all cohorts children were assessed at least once in the first two years of life, in the preschool period, and before adolescence. The most common used tests in the ENRIECO cohorts at these different ages were the Bayley Scales of Infant Development [12], the McCarthy Scales of Children Abilities [13]/ Wechsler Preschool and Primary Scale of Intelligence [14] and the Wechsler Intelligence Scale for Children [15], respectively. All of them are tests assessing general neuropsychological development, albeit covering different neuropsychological domains.

Secondly, we reviewed the neuropsychological development assessment protocol of the National Children's Study from United States [16–20]. This protocol was elaborated by a panel of experts in this area and was designed to assess the children once every year between the ages of 6 months and 20 years. Thirdly, we reviewed relevant reviews of the neuropsychological developmental literature within the epidemiological field. We used sev¬eral electronic databases [PubMed (http://www.ncbi.nlm.nih.gov/pubmed), PsycINFO (http://www.apa.org/pubs/databases/psycinfo/index.aspx), and Web-of-Knowledge (http://apps.isiknowledge.com)] to conduct the initial literature search. Using a combination of the key¬words "birth cohort studies" and one of the following "neuropsychology", "child development", "cognitive assessment", "neurodevel¬opmental" and "neurobehavioral", we then selected relevant reviews that summarized the whole or some specific areas of neuropsychological development [3,21–28]. Lastly, we reviewed some recently published books dedicated to child neuropsychological development [29–31].

Results

Figure 1 represents the conceptual framework of child neuropsychological development that we assembled as a result of our review. Two levels of outcomes can be differentiated: functional and clinical (Table 1). The functional level refers to the skills, abilities, capacities, and/or knowledge acquisition acquired during maturation of the brain and its interaction with the social and educational environment. These abilities increase their complexity over time as a result of the development of neural networks in the cortex, which allow the individual to adapt to the increasing demands of the environment. There are three domains at this functional level: cognitive, psychomotor and social-emotional. These three domains are highly overlapped and interrelated. Their development is dependent on one another, and a non-optimal (or pathological) development of one of them may have implications for the rest of the domains.

Cognitive function can be conceptualised as a hierarchic model, where specific cognitive domains such as attention, language, executive functions etc, are posited beneath the overarching domain of general cognition (Table 1). Such cognitive domains should be assessed by trained neuropsychologists through the use of age-appropriate standardised neuropsychological tests. A trained neuropsychologist is also required to accurately interpret child neuropsychological assessment data. Each one of these cognitive domains can also be divided into several sub-domains. This is especially critical in the case of executive functions because they are understood as a set of cognitive skills that are responsible for the planning, initiation, sequencing, and monitoring of complex goal-directed behavior. The psychomotor domain can be divided into fine and gross motor skills; these are usually assessed by

neuropsychological tests or by way of questionnaires. Social-emotional domain refers to the ability to regulate the emotions appropriately, and to the ability to relate to others. This level has typically received the least attention, even though it encompasses several aspects of adaptive behavioral development; we argue that this area merits considerably more attention in birth cohorts because the potential long-term consequences of poor social development in terms of unemployment, mental health issues, marital difficulties, unadaptive behaviours, delinquency, or violence. The social-emotional domain is typically assessed using psychological questionnaires filled in through interviews with parents or teachers, which provide information on various characteristics of child behavior that occur in the ecological environment in which child is developing (i.e. at home and at school).

Clinical phenotypes refer to some neurodevelopmental disorders, or the presence of symptoms of these disorders in the population scrutinized in environmental epidemiology studies (subclinical symptomatology). The term neurodevelopmental disorder is usually used in one of two ways, describing either those conditions affecting neuropsychological development in children with a known genetic etiology (i.e. fragile X syndrome) or those conditions ascribed to presumed multifactorial etiologies in which certain domains of neuropsychological development are selectively impaired (i.e. attention deficit hyperactivity disorder (ADHD)) [32]. The process of selecting disorders for inclusion in Figure 1 was not straightforward. Based on publication rates, the most extensively studied neurodevelopmental disorders are ADHD and autistic spectrum disorders (ASD). However, it is misleading to focus only on certain disorders. In Figure 1 we include the eight most prevalent

neurodevelopmental disorders as reported by Bishop (2010) [33]. In order of decreasing prevalence, these disorders are: speech sound disorder, specific language impairment, developmental coordination disorder, developmental dyslexia, intellectual and learning disability, ADHD, developmental dyscalculia, and ASD. Environmental epidemiology has the potential to shed light on many such high prevalence neurodevelopmental disorders in the general population which currently garner little attention. These disorders may have associated consequences such as low achievement in school, behavioral adaptation (school, professional, and personal), diminished economic productivity, and possibly an increased risk of antisocial and criminal behavior. As such, they may contribute to the so-called “silent pandemic” proposed by Grandjean and Landrigan.[2] Neurodevelopmental disorders are usually assessed not only by psychological tests based on diagnostic criteria of mental disorders, but also by structured interviews and questionnaires. However, in environmental epidemiology, it would be preferable to assess the continuum of symptoms (subclinical symptomatology) associated with such disorders, rather than assessing the presence of these diagnoses as defined by clinical cut-offs.

Discussion

This framework serves as a starting point for the standardization of the relevant terminology, and thereby facilitates the choice of phenotypes in future epidemiological studies. The lack of a common framework for the study of neuropsychological development in environmental epidemiology studies, and a concomitant lack of consistency in the associated terminology, currently hinders research collaboration and the setting of targets. We have summarized the work carried out so far in

this increasingly relevant area in order to meet several objectives. This framework will allow epidemiologists with little expertise in this topic to better understand the area of neuropsychological development assessed in a specific study. Moreover, it will enable better design of future research, and foster better informed selection of the outcomes of interest. The development of this conceptual framework may also serve as a starting point towards standardizing the terminology used in the neuropsychological development field and for specific outcomes encountered in environmental epidemiology literature.

It is notable that very few neuropsychologists work in the field of environmental epidemiology. The presence of these professionals with a background in both neurodevelopment and neuropsychological development is critical to the elaboration and application of assessment protocols (based on their knowledge of the brain development and neuropsychological testing), to quality control in data collection and analysis, and to the interpretation of study findings. Their inclusion in multidisciplinary research teams may improve the quality of research in this important field.

The conceptual framework presented in this commentary also provides a theoretical justification for the conduct of pooled or meta-analyses of cohort studies that use different tests in assessing the same phenotypes. The majority of functional domains may be divided into a set of specific sub-domains. Clearly, a cautious and robust approach is needed in order to combine the data in a meaningful way, particularly in pooled analyses, where a priori theoretical background and statistical modelling are employed. Sensible combination of data originating from different neuropsychological tests is highly dependent on the specificity of the effects of particularly environmental agents on neuropsychological

development. Again, the importance of involving neuropsychologists is paramount since it is only with their understanding of the relevant tests and cognitive functions that we may advance in this field.

The publication of this framework marks a synthesis of the highly complex processes of neuropsychological development in a unified practical and conceptual framework.

Competing interests

The authors declare that they have no competing interests.

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Table 1. Definition of different outcomes:

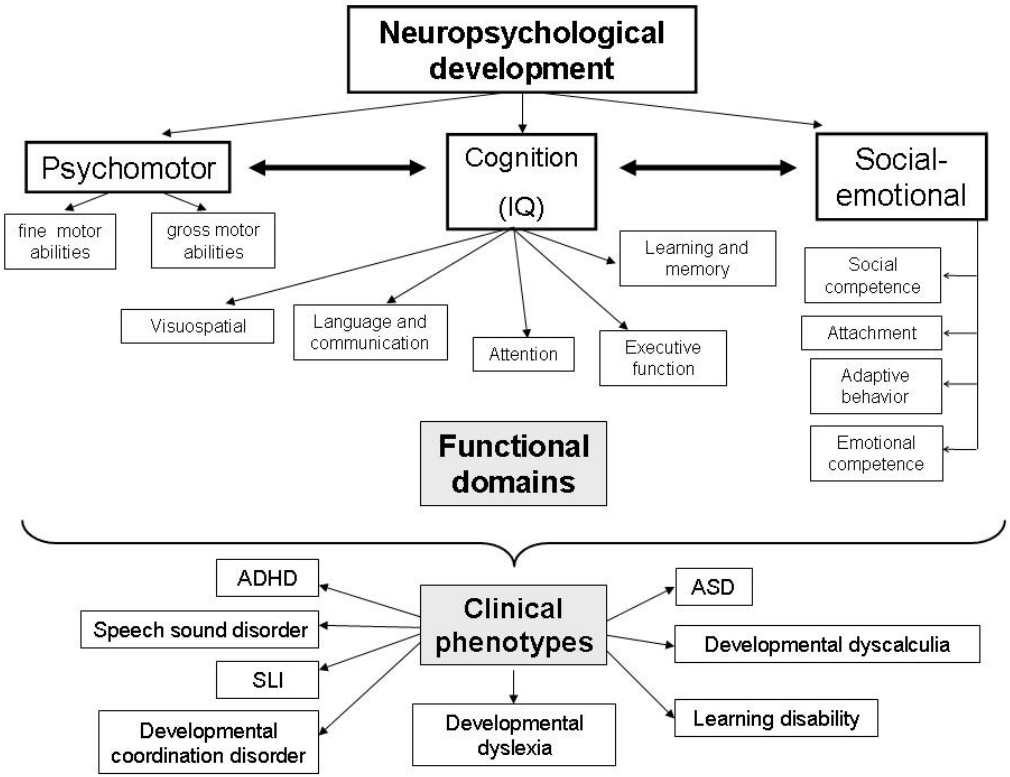
Outcome	Sub-level	Specific domains	Definition	Reference
Functional	Cognitive	Attention	"This domain encompasses several processes including the capacity to focus on and attend to stimuli over a period of time and the capacity to take in and report back stimuli immediately after presentation".	White [11]
		Language	"This domain includes basic linguistic abilities such as the capacity to produce phonemes, lexical development, production of words and language structure development (grammar), speech comprehension and linguistic aspects of writing and reading. Language skills are often divided into expressive and receptive components".	White [11]
		Executive function	"Metacognitive capacities that allow an individual to perceive stimuli from his or her environment, respond adaptively, flexibly change direction, anticipate future goals, consider consequences, and respond in an integrated of common-sense way , utilizing all these capacities to serve a common purposive goal".	Baron [8]
		Learning and memory	"Memory terms are classified in a number of ways. Among these are reference to whether there is conscious awareness of recall (explicit or declarative	Baron [8]

		memory vs. implicit or procedural memory); central stages or features (encoding, consolidation, storage, retrieval); consideration of a time-interval span (immediate, short-term, or long-term, the latter including recent and remote memory); specific memor imapirment (anterograde amnesia, retrograde amnesia); or by characteristics related to the recall (prospective memory, source memory)."	
	Visuospatial abilities	"These non-verbal abilities generally invoke the processing and manipulation of visual designs, the spatial or physical aspects of environmental objects or constructional skills".	White [11]
Psychomotor		"Psychomotor functions cover a broad range of morphologically and functionally different phenomena. Functions range from highly automised gross motor activities like walking to highly skilled fine motor skills like knitting or running a computer program by highly skilled, precisely located mouse clicks".	Kallus [26]
Social-Emotional	Social competence	"Effectiveness in developmentally appropriate social interactions".	Denham [17]
	Attachment	"Attachment begins as the deep and enduring connection established between a child and his/her caregiver in the first several years of life".	Denham [17]

		Adaptive behavior	"The development of the adaptative behavior involves the regulation of the other behaviors to the social rules and to the demands of the context surrounding the child".	Saarni [27]
		Emotional competence	"The multifaceted ability strategically to be aware of one's own and others' emotions and to act on this awareness, to negotiate interpersonal exchanges and regulate emotional experience".	Denham [17]
Clinical	ADHD		"ADHD is defined by problems with inattention and/or hyperactivity/impulsivity, with onset before the age of 7 years and resultant impairment in two or more settings".	McClellan [18]
	Speech sound disorder		"Children with SSD are delayed in the acquisition of developmentally appropriate speech sounds, resulting in reduced speech intelligibility. Idiopathic SSD is not due to known etiological factors such as cleft palate or hearing loss and is limited to disorders of speech sound production (i.e. not stuttering)".	Raitano [28]
	SLI		"Selective failure to develop language at a normal rate in the absence of frank neurological and psychiatric disease and adequate educational opportunity".	Tranel [20]
	Developmental coordination disorder		"Is characterized by motor impairment that interferes with the child's activities of daily living and academic achievement".	Dewey [29]

Developmental dyslexia	"Developmental dyslexia, or specific reading disability, is defined as an unexpected, specific, and persistent failure to acquire efficient reading skills despite conventional instruction, adequate intelligence, and sociocultural opportunity."	Démonet [30]
Intellectual disability/Learning disability	"This can be both a symptom of a known disorder, and a nonsyndromal condition of unknown etiology. Furthermore, in the UK, the term 'learning disability' is used to refer to intellectual disability, whereas elsewhere 'learning disability' is used for specific difficulties in a child of normal IQ".	Bishop [25]
Developmental dyscalculia	"Is defined by difficulty in learning and remembering arithmetic facts and in executing calculation procedures, with immature problemsolving strategies, long solution times and high error rates".	Geary [31]
ASD	"Autism spectrum disorders (ASDs) encompass the diagnoses of autism disorder, PDD-NOS and Asperger's syndrome. Children with these disorders often have life-long difficulties with their ability to communicate and socially relate to others".	McClellan [18]

Figure legend. Conceptual framework of the Neuropsychological Developmental Process.



SLI: Specific Language Impairment; ASD: Autistic Spectrum Disorder; ADHD: Attention Deficit Hyperactivity Disorder

9 GENERAL DISCUSSION

This section is comprises an overarching synthesis and discussion of the results presented in the previous sections of the thesis. Thus, as an elaboration and expansion of previous discussions, the layout of this chapter is based on the following questions:

1) What do our findings add to current understanding of the role of social determinants in neuropsychological development?
2) What do our findings add to current understanding of the role of environmental determinants in neuropsychological development?
3) What has been the contribution of child neuropsychology to the field of environmental epidemiology?
4) What are the main strengths and limitations of the work presented in this thesis?
5) What are the implications of the findings of this thesis from the point of view of public health?
6) Future investigation.

9.1 What do our findings add to the current understanding of the role of social determinants in neuropsychological development?

The first paper presented in this thesis was conducted in order to identify the effects of selected maternal characteristics on early neuropsychological development. Our results indicated that the effect of maternal education exceeded to maternal IQ in the association with early

neuropsychological development. However, maternal cognitive capacities play an important role during the early stages of cognitive development in more disadvantaged occupational social classes. This could extend the debate about the heritability of cognitive capacities. Cognitive abilities are characterized by a high level of heritability but known genetic effects can account for very little of this and it has been suggested that the effect of individual genes may be much smaller than previously assumed (98–100). Parental cognitive capacities have both a direct effect on child cognitive outcomes (by genetics) and an indirect effect through parental influence on the child's proximal environment (101). In this regard, the quality of the domestic environment, not only in terms of materials and toys at home, but also in terms of parental cognitive skills and stimulation of the child is intrinsically related to child neuropsychological development. Insufficient or inadequate cognitive stimulation during early childhood has been identified as one of the main risk factors for neuropsychological development both in developing and developed countries (102,103). In fact, there is a clear relationship between impoverished conditions and poor performance on neuropsychological tests (104).

In the same line, we presented in the seventh paper that some early in life social determinants are still related with neuropsychological development at preadolescence period. Interestingly, social class, educational level, or number of siblings are related to the attention function at 11 years old. This is a very relevant finding indicating that social conditions in which children develop from birth may configure the high-level cognitive capacities at older ages and reinforcing the importance influence of an optimal social environment in the early stages of development.

In spite of the association between maternal and child cortisol levels during the second year of life, the levels of child cortisol were not associated with the child neuropsychological development at this age as discussed in the second paper. Breastfeeding appears as a plausible via to transfer these hormone from mother to child during the postnatal period. Higher levels of maternal postnatal cortisol, representing those mothers with higher levels of distress, may affect the neuropsychological development of boys, but not girls. Because of the cross-sectional design of this study, the possibility of reverse causation could not be rejected.

Finally, to shed light to the question if the positive observed effects of breastfeeding on neuropsychological development are confounded by maternal intelligence, two papers were conducted in this thesis. Firstly, higher levels of LC-PUFAs in colostrum joined to a prolonged duration of breastfeeding were related to an increase in cognitive development during the second year of life. These results were not confounded by maternal IQ, mental health, or attachment. Secondly, children exposed to the tobacco compounds during and after pregnancy were specially benefited for a longer periods of breastfeeding. This suggests a specific effect of LC-PUFAs, probably because of their antioxidant effect. All of these findings are very relevant because they increase the evidence of a direct effect of breastfeeding on child neuropsychological development. Obviously, sociodemographic characteristics play a role in this association, but in relation of feeding election and in maintenance of these practices. But, the mechanism by which breastfeeding improves brain development is explained by the effects of LC-PUFAs.

9.2 What do our findings add to the current understanding of the role of environmental determinants in neuropsychological development?

The evaluation of the relationships between OCs and neuropsychological development comprised an important part of this thesis. It is important to remark that it has been reported that the levels of OCs in maternal serum of pregnant women from the cohorts of INMA study were at the lower end of the range reported in Spain and other countries (84). In this context, our results provide evidence that current levels of OCs found in serum samples are still impacting negatively in all the phases of the neuropsychological development of this population. Firstly, prenatal exposure to PCBs was related with a delay in early psychomotor development. The negative effects have been reported previously (69). As discussed in paper 5, finding no effects on the cognitive area may be due to the immaturity of the brain during the early stages of maturation. Secondly, prenatal exposure to PCB153 was negatively associated with general neuropsychological development at the age of 4 years. The results of the paper 6 did not indicate a vulnerability of the prefrontal cortex due to PCBs exposure as suggested previously (69). Paper 7 contributed novel data to the literature about the effects of OCs on neuropsychological development. Levels of both DDE and PCB in child's serum samples at 4 years of age were strongly related to the reaction time at the age of 11. Exposure to OCs may have persistent negative effects in important neuropsychological functions, such as reaction time. This capacity is crucial for the cognitive processing, especially in tasks characterised by time-pressure.

9.3 What has been the contribution of child neuropsychology to the field of environmental epidemiology?

As mentioned previously, the present thesis studies the role of different socio-environmental factors on the neuropsychological development. The main outcomes of the present thesis have been obtained thorough neuropsychological tests. The findings discussed above using the Bayley Scales of Infant Development demonstrated that there are some factors in the environment which are affecting the brain development since the first stages. The detailed neuropsychological assessment during the preschool period (applying the McCarthy Scales for Children Abilities and analyzing it in a specific way) also has been useful to determine how a specific chemical agent may impair the brain development and functioning. In addition, the application of a computerized assessment (of a specific paradigm based on continuous performance) during preadolescence may help to identify the precise effects of some factors on a high-order cognitive function, such as attention function.

As a final aim of this thesis, a revision process as a part of ENRIECO Project (Environmental Health Risks in European Birth Cohorts – www.enrieco.org) was performed. This revision work, presented in the Annex 1, consisted in a wide review of the protocols used in European birth cohort studies in the area of the neuropsychological development. As result of this process joined to a more wide revision including US birth cohort studies, a conceptual framework under the neuropsychological background was created. As discussed in the paper 8, the neuropsychological development can be divided into functional domains and in clinical phenotypes. Each of these parts can be divided in specific

outcomes. The main aim of this framework was to synthesize a complex background in a practical and reliable conceptual framework. As a next step, we made some recommendations such as harmonization the terminology used or use the similar protocols of assessment in order to combine the data.

9.4 What are the main strengths and limitations of the work presented in this thesis?

9.4.1 Strengths

This thesis has a number of strenghts. Firstly, the main strength of this thesis is the design of the study. The prospective nature of the INMA-project joined to the cohort design allowed us to establish longitudinal associations between socio-environmental factors and child neuropsychological development. Secondly, it is also important to remark the sample size on the one hand and longitudinal design and long follow-up on the other. Within the INMA-project, the neuropsychological development of approximately 2200 children was done at 14 months. Moreover, the Menorca cohort assessed around 450 children at 4 and at 11 years. Thus, this thesis contains data about 2650 children of five different regions of Spain. Thirdly, these 2650 children studied come from general population, and therefore, the external validity is greater than in clinical populations. Fourthly, a laborious fieldwork containing several follow-ups done by field staff, interviewers, laboratory technicians, and project paediatricians specifically trained for the project was performed. I also want to remark the thorough fieldwork done in the neuropsychological area. To reduce the variability between the different

psychologists involved in the project, three different quality controls were introduced. All the neuropsychologists were trained by an expert neuropsychologist. Afterwards, I coordinated all the fieldworkers of the different cohorts put in contact each other in meetings or conference calls. All the neuropsychologists were called for a face-to-face meeting in which the inter-rater reliability was evaluated, thereby obtaining optimal results of accordance among them. Fifthly, this thesis has covered a great number of environmental exposures and social factors that may affect the child neuropsychological development. Lastly, the INMA-project also allows the inclusion of a huge amount of important covariates for these studies. We also collected anthropometry measures, or environmental variables.

9.4.2 Limitations

I have discussed previously one of the main limitations of the study: despite of the huge amount of covariates collected, we did not collect some important variables for all cohorts, such as parental IQ, mental health, or quality of home environment. It is worth noting that we neither have information about pre- and postpartum depression nor stress during pregnancy. Moreover, we also failed in collecting these important variables for fathers. In regard to the cortisol measurement, we only collect one sample of saliva (one for each, mothers and children) by the participants. To obtain a reliable and valid measure of the normal cortisol values, more than one measurement would be needed, especially after awakening. Despite of the low rate of loss to follow-up in

the INMA-project, this could be another potential limitation of this thesis.

Regardless to the neuropsychological development area, one potential limitation was the election of the test. We used the first version of the Bayley Scales of Infant Development (BSID)(91). BSID is a widely used test to assess early cognitive and psychomotor development. Unfortunately, the version used in the INMA project was published in 1977. Two further versions of this have been published with improved materials and improved scales (cognitive, receptive and expressive language, fine and gross motor, behavioural) (107,108). Probably, the use of the new versions of this test would improve the validity and reliability of our measurement. Moreover, BSID is a test with a poorer predictive validity. In one hand, BSID is intended to assess current developmental status for mental processes like perceptual acuities, memory, learning, and elementary problem solving, as well as sensory-motor development. But in the other hand the authors of the test have emphasized that the BSID should not be interpreted as measuring 'intelligence', predominantly because of the poor predictive validity of infancy scales for later cognitive abilities (107).

Finally, another limitation of our study in which the 4 cohorts of INMA were pooled, was the variability in the child's age when the neuropsychological assessment was done. The range of age goes from 11 to 29 months being 14.8 months the mean of age. In order to limit this variability, we limited our analyses to those children ranging from 11 to 23 months. The raw scores in mental and psychomotor scales were standardized as explained in paper 3 and 4.

9.5 What are the implications of the findings of this thesis from the point of view of public health?

Firstly, we have demonstrated that the effects of maternal cognitive capacities on neuropsychological development were mediated by the occupational social class. In the more advantaged social classes the importance of the maternal intelligence was minor, probably because of the equality of conditions. However, in more disadvantaged classes the environmental inequalities must be major and then the importance of maternal capacities (and consequently of maternal stimulation) was higher. It is obvious that a great portion of the association between maternal intelligence and child neuropsychological development was explained by gens. But, it is also observed in previous studies that maternal intelligence is also important in configuring the proximal environment in which child develops. This is confirmed in our studies because of the long-term association between SES and education with attention function at the age of 11 years. This thesis provides evidence about the importance of the social environment in the neuropsychological development early in life and at preadolescence period, specially in more disadvantaged social classes.

Secondly, the results of the present thesis indicate that the levels of child cortisol are not related with neuropsychological development during the second year of life. In spite of increasing the levels of cortisol for a prolonged breastfeeding, this does not seem to impair the child neuropsychological development. However, the scientific literature suggests that higher levels of this hormone may have detrimental effects on several brain regions. Thus, the effects of this hormone on neuropsychological development will be examined at older ages.

Thirdly, the results of the papers 3 and 4 reinforce the importance to maintain a prolonged breastfeeding period. We observe that the high levels of LC-PUFAs in maternal milk promote a better cognitive development during the second year in life, and also protect to hyperactivity symptomatology at the age of 11. In addition, it seems that breastfeeding may be important in children whose mothers smoke during pregnancy. These children were more benefited than the children non-exposed to smoking during pregnancy. For these reasons, in terms of public health, the findings of this thesis reinforce the idea to maintain breastfeeding (any or predominant breastfeeding) for a prolonged periods (>6 months is recommended for the WHO). Due to the labour legislation in Spain, where the maternity leave do not extend the first 4 months after birth, it is very difficult to maintain an exclussive method of breastfeeding for 6 months. But, we observe that the effects were also observed for the any breastfeeding method (any breastfeeding was used to describe the practice of supplementing breastfeeding (direct from the breast or expressed) with other drinks, formula, or infant food).

Lastly, the levels of organochlorine compounds (specially for PCBs) presents in serum samples (from pregnant women and children at 4 years old) which are low compared to other studies in Spain, are still negatively related with child neuropsychological development from early stages to preadolescent period. Thus, more efforts are needed to reduce the production of these compounds because of their neurotoxic effect.

9.6 Future investigation

The papers included in this thesis represent a sample of the results of INMA-project. However, there are still some objectives that I will analyse and publish in the near future. Children of the 4 new cohorts of the INMA-project (Asturias, Guipuzkoa, Sabadell, and Valencia) have been assessed at 4-5 years of age. We have assessed a wide range of outcomes, such as general cognitive development, attention function, ADHD symptomatology, and social competence development. Moreover, the levels of some chemical agents (such as metals, OCs, etc) and indoor and outdoor air pollution have been assessed in child's serum samples at the age of 4. Thus, the role of some socio-environmental exposures on neuropsychological development among preschoolers will be analyzed.

Another remaining objective will be to review all the work done up to now in each of the European birth cohort studies. Within the CHICOS project (Developing a Child Cohort Research Strategy for Europe-http://www.chicosproject.eu/) we will perform a review of the protocols used, the main exposures measured, and the main results obtained in all the studies performed in European birth cohort studies during the last 20 years. This review process will provide the information for making recommendations to the future work in European birth cohorts in terms of spefic issues, such as protocols of neuropsychological assessment, ages of assessment, and cognitive areas of interest.

10 Conclusions

1. Maternal cognitive capacities play an important role in the first stages of cognitive development of the children in more disadvantaged occupational social classes exceeding the effects of maternal mental health.

· However, in advantaged occupational social class the effects of maternal IQ on early cognitive development are mostly explained by educational level of mothers.

2. Child cortisol levels do not have any effect on neuropsychological development during the second year of life.

· Child and maternal cortisol are positively associated.

· The most plausible via to exposure of cortisol in the postnatal period is breastfeeding.

3. Higher levels of long-chain polyunsaturated fatty acids in colostrum joined to a prolonged duration of breastfeeding benefit early neuropsychological development, especially in those children exposed to active maternal smoking.

· Longer periods of breastfeeding also reduce the hyperactive and impulsive symptomatology at preadolescent period.

4. Prenatal exposure to PCB is negative associated with psychomotor development during the second year of life.

· The current levels of these compounds are lower than in previous generations.

· The effects of PCBs on cognitive development need to be replicated at older ages.

5. Prenatal exposure to PCB153 is associated with a delay in general neuropsychological development at preschool period.

· The prefrontal cortex is not more vulnerable than the other brain regions.

6. Postnatal exposure to OCs (DDE and PCBs) impact on neuropsychological development at preadolescence period.

· More efforts in epidemiological studies must be focused in obtaining blood samples postnatally and several neuropsychological assessments at different developmental points to elucidate the real longitudinal effects of these compounds.

7. The use of a common conceptual framework will improve the quality of research in this area.

· More efforts are necessary to harmonize protocols, terminology, and periods of assessment.

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12 Annexes

Other contributions (as first author) on Neuropsychological development:

Annex 1

Assessment of neurodevelopment in ENRIECO birth cohorts

Joan Forns (CREAL-INMA), Sylvaine Cordier (Pélagie); Henning Tiemeier (Generation R); Kinga Polanska (REPRO_PL); Cynthia Hohmann (MAS); Manon Van Eijsden (ABCD); Viaene Mineke (FLEHS); Jordi Sunyer (CREAL-INMA)

ENRIECO Work Package 3

NEUROBEHAVIOR ASSESSMENT Working Group

Report – vesion 3:

Assessment of neurodevelopment in ENRIECO birth cohorts

Participants: Joan Forns (CREAL-INMA), Sylvaine Cordier (Pélagie); Henning Tiemeier (Generation R); Kinga Polanska (REPRO_PL); Cynthia Hohmann (MAS); Manon Van Eijsden (ABCD); Viaene Mineke (FLEHS); Jordi Sunyer (CREAL-INMA)

Summary

Neurodevelopment is a genetically driven process which has several phases and it occurs since prenatal until post-adolescence years. Neurodevelopment can be divided in cognitive and social-emotional development, whereas neurobehaviour is the consequence of the maturation of these previous areas. Due to the great amount of outcomes and tools assessed in this area in ENRIECO birth cohorts, there is an urgent need to perform a review in order to identify the main neuropsychological tools and methodologies used. Therefore, the main objective of this work was to provide recommendations for future neurodevelopment assessment in birth cohorts. This will facilitate the evaluation of effects of environmental exposures on neurodevelopment. In the present report, 33 tests assessing cognitive development and 12 assessing neurobehavioral development have been reviewed. We have also made recommendations for future studies on neurodevelopmental area and specifically for two outcomes: global IQ and attention deficit and hyperactivity disorder (ADHD). We also made some recommendations, among them, to take one assessment of all the areas of neurodevelopment, assess cognitive development at different time points during childhood, and use validated and published neuropsychological tests assessed by psychologists.

Background and context

Introduction about neurodevelopment

The study of brain development and therefore, cognition and behaviour, is one of the most fascinating topics in human research. Neurodevelopment is a genetically driven process which is sequenced in several stages, such as neurulation, cell proliferation and migration, specialization, synaptogenesis, synaptic pruning, and myelination. This process occurs concurrently during prenatal life, childhood and adolescence (1, 2, 3).

The development of the central nervous system (CNS) involves a high sequence of processes influenced by both genetic and environmental factors, making this organ extremely vulnerable to environmental influences (4, 5). The developing brain incorporates environmental influences in its architecture, which can be positives or negatives (6). There are several environmental exposures that can be neurotoxic for the developing brain. These hazards have been well documented in the scientific literature. Among them, there are industrial and chemical agents (i.e. lead, methylmercury, arsenic, polychlorinated biphenyls, solvents, and pesticides) and factors related to lifestyle such as tobacco, alcohol, certain drugs, and maternal stress (7, 8, 9).

Numerous neurodevelopmental impairments or disorders have been described, such as low intelligence quotient (IQ), learning disabilities, sensory deficits, autism, attention deficit and hyperactive disease (ADHD), cerebral palsy, etc (8). Data from United States reported that one in every six children has a developmental disability and in most cases these disabilities affect the nervous system (10).

Attention deficit hyperactivity disorder (ADHD) is one of the most common childhood disorders with an estimated prevalence ranging from

3 to 8% (11, 12). ADHD is characterized as a spectrum of symptomatology characterized by inattention, impulsivity/hyperactivity or both, compromising daily functioning (13). Comorbidity is another important characteristic of ADHD. Children with ADHD are at increased risk for conduct disorder, antisocial behavior, and drug abuse later in life. Although the mechanisms for the development of ADHD remain unclear, both genetic and environmental factors have been implicated.

Autism spectrum disorders (ASDs) are a group of neurodevelopmental disorders characterized by core deficits in three domains: social interaction, communication, and repetitive or stereotypic behaviour. The degree of impairment among individuals with ASD is variable, but the impact on affected individuals and their families is universally life-altering (14).

Therefore, there is a need to improve the understanding of normal and abnormal cognitive and behavioural development in the early years of life. This knowledge will allow identifying which environmental exposures are neurotoxic and make policy recommendations to avoid neurodevelopmental delays.

Components of neurodevelopment

From birth to teenage years, there is a fourfold increase in the volume of human brain. This increasing volume is related to acquisition of huge diversity of cognitive and socio-emotional abilities. A first differentiation could be done in neurodevelopmental process: cognitive development and socio-emotional development (figure 1).

Cognitive development includes several specific domains such as attention, language, executive function, visuospatial abilities, learning memory, and psychomotor development. Social-emotional development

refers to how people function in social and familial relationships. Social-emotional development includes social competence, attachment, emotional competence, personality, and self-perceived competence.

The consequence of cognitive and social-emotional development is reflected in neurobehaviour. There are several measures of neurobehaviour: disorders such as ADHD and autism; mental health state or several desaptative conducts such as violence, delinquence or drug conssumption.

Neurodevelopmental assessment

All of neurodevelopment areas can be assessed using psychological tests. Specifically, cognitive development is assessed by neuropsychological tests.

Neuropsychological tests are standardized measuring devices designed to give quantitative information about cognitive functioning. Neuropsychological tests allow: to quantify the cognitive functioning of individuals, such as global intelligence quotient (IQ), attention, executive function, etc; to define the position of individuals within an adequate reference group; and, to identify differences between individuals in terms of cognitive function.

Usually, neuropsychological tests can be divided in IQ tests or domain-specific tests (15). First of all, an IQ approximation is computed based on administration of standardised tests. These tests are composed of subtests assessing various cognitive domains. Subtest scores are summed in order to obtain overarching measures such as IQ often accompanied by omnibus measures of verbal abilities, visuospatial abilities, attention, executive functions, memory or speed processing. On the other hand, the domain-specific tests assess specific cognitive domains such as attention, language, executive function or visuospatial abilities.

Neuropsychological tests are generally applied by neuropsychologists. These tools are paper and pencil tests but specific neuropsychological computerized tests also exist. Cognitive development can be also assessed using self-reported questionnaires (reported by informants).

Social-emotional development and behaviour development are usually assessed by psychological tests. In some cases, the psychologist can rate these areas even though in most cases these tests are presented in a self-reported way. In a recent review published by Denham et al. (16) a specific protocol to assess social-emotional development in children from infancy to early adulthood was defined.

Finally, to assess behavioural outcomes, self-reported questionnaires are the most common way to assess it. It is important to determine who the best informant is: children themselves, parents or teachers.

Current work in the European birth cohorts

Each ENRIECO cohort has used their own protocol of neurodevelopment assessment including a huge diversity of neuropsychological tools. There is an urgent need, therefore, to perform a systematic review in order to identify the main neuropsychological tools used. The main objective of this work is to provide some recommendations for existing data and for future studies on neurodevelopment area.

Cognitive assessment (description of the tests)

A complete list of neuropsychological tools used in ENRIECO cohorts to assess cognitive development has been shown in table I. To include the test in the present review it must be published, normalized and validated. As it has been mentioned previously, neuropsychological tools can be applied by a psychologist, computerized or as a self-reported

questionnaire. First of all, the tests which assess general intelligence have been listed. Usually, in this kind of tests, scores in different subtests (such as attention, language, executive function, etc) are summed in order to obtain overarching measures of IQ. In this review, there will be listed all the tests used, despite only some subtests have been assessed in some cohorts:

- The Neurological Optimality Score (NOS) is based on the Hempel examination. The items of the neurological examination have a predefined optimal range. The total number of items scored within the optimal range determines the NOS. The NOS is able to evaluate subtle differences in neurological outcomes (17).
- The Griffiths Mental Development Scales (GMDS) is used to assess cognitive and motor development of the participants from 2 to 8 years of age. This test is designed to measure the developmental progression in six areas: locomotors, personal-social, hearing and speech, eye and hand coordination, performance, and practical reasoning (18).
- The Bayley Scales of Infant Development (BSID) is a widely accepted test for the assessment of mental, motor and behaviour development in young children. The age range is from about 5 to 42 months. The mental scale assesses the child's level of cognitive functioning, language development and personal/social development. The motor scale assesses fine and gross motor functioning (19).
- The Fagan Test of Infant Intelligence (FTII) is based on the principle of novelty preference and of recognition memory. A human face is initially presented to the child for visual exploration (habituation phase), and subsequently this one is

presented together with a new unfamiliar face. Typically the child will exhibit novelty preference by visually fixating the unfamiliar face longer than the familiar one. This fixation preference score in percent observation time and usually based on 10 trials is taken as a proxy measure of intelligence (20).

- The Weschler Intelligence Scale for Children (WISC-IV) ranges from 6 years to 16 years 11 months. WISC-IV is normed and is considered to be a reliable estimate of intelligence. This test provides a total of 4 indexes: verbal comprehension, perceptual reasoning, working memory and, processing speed. In addition to the previous test, it also provides a global IQ (21).
- The Weschler Preschool and Primary Scale of Intelligence (WPPSI) ranges 2 years 6 months to 7 years 3 months. The WPPSI provides a measure of verbal IQ, performance IQ, processing speed quotient, general language composite and a full scale IQ (22).
- The McCarthy Scales of Children's Abilities (MCSA) ranges from 2 years 6 months to 8 years 6 months old. The 18 subtests are organized into 6 scales, which are especially suited for differential diagnosis: verbal, perceptual performance, quantitative, general cognitive, memory, and motor (23).
- The Batelle Developmental Inventory (BDI) comprises 96 items assessing the following five domains: personal-social skills, adaptive behavior, psychomotor ability, communication, and cognition. Sub-domain scores can be computed for psychomotor ability (fine motor skills, gross motor skills), and communication (expressive and receptive communication). The range of age goes from birth to 8 years old (24).

- The Stanford-Binet Intelligence Scale is based on a hierarchical model of a general ability factor, g, and second-order factors of crystallized abilities, abstract-visual reasoning, and short term memory. The Stanford Bient replaced the age-scale format with a point-scale format. It groups 15 subtests into four areas: verbal reasoning, abstract/visual reasoning, quantitative reasoning, and short-term memory. These scales can be applied since 2 years until more than 85 years old (25).
- The Revised Brunet-Lézine test is intended to enable 4 developmental age subscores to be calculated for children who are aged 2 to 30 months. These subscores cover 4 domains: movement and posture, coordination, language, and socialization. The evaluation is based on the child's performance during the test and on questions to the mother about behavior that can be evaluated objectively but are extremely important for judging development. These subscores allow the calculation of 4 separate Developmental Quotients (DQ) that, combined, yield a global DQ (26).

Afterwards, the tests assessing specific cognitive domains have been also listed:

- The Denver Developmental Screening Test (Denver) is a widely used, readily administered screening tool for early identification of developmental delays in children from birth to 6 years of age. It covers four areas of development: personal/social, fine-motor/adaptive, gross motor, and language development (27).

- The Age and Stages Questionnaire (ASQ) is a self-reported questionnaire, used as a measure of child development. The range of age goes from 6 to 60 months. The ASQ screens for five areas: personal-social, fine motor, gross motor, communication, and problem solving. At the end of each questionnaire, seven additional questions relate to parents' overall perception of and concerns about their children's development (28).
- The original Snijders-Oomen Nonverbal Intelligence Test (SON-R 2.5-7) is devised to assess the spatial abilities, abstract reasoning, concrete reasoning, and memory functioning of deaf children. Subsequent revisions expanded the test, provided parallel norms for hearing and deaf children, and developed separate tests for preschool and school-age children (29).
- The Reynell Developmental Language Scales (Reynell) is a standardized measure of language development for everyday clinical use from 1 to 6 years. Now in its third version the measure includes two scales: the comprehension scale and the expressive scale. It assesses the structural aspects of language and how they are adopted to acquire and use language and helps in the identification of language disorders and language delay (30).
- The MacArthur-Bates Communicative Development Inventory (CDI) is a recognized parent report instrument used to assess the early language development of children. The CDI comes in two scales: the infant scale (covering the period from 8 to 16 months) and the toddler scale (from 16 to 30 months). The infant scale looks at comprehension, word production and aspects of symbolic and communicative gesture (31).

- The Boston Naming Test (BNT) consists of a visual picture naming task in which 60 outline drawings of objects and 24 animals are presented. The items are presented in order of word frequency and difficulty. The test has shown to be a highly sensitive tool to identify naming deficits and impaired word-retrieval capacities in adults and children. This type of picture-naming vocabulary test is useful in the examination of children with learning disabilities and the evaluation of brain-injured adults (32).
- The NEPSY is widely used by school psychologists, neuropsychologists, and research psychologists to assess children ages 3-12 with developmental disabilities and to develop effective intervention strategies. It provides comprehensive assessment over five functional domains: attention/executive functions, language, sensorimotor functions, visuospatial processing, and memory and learning (33).
- The Neurobehavioral Evaluation System (NES) is a computerized test. NES evaluates memory, visual/motor function, vocabulary ability, and mood. NES was firstly designed for field testing of adults, assuming a minimum education level of 5th grade. However, many NES tests can be performed by children as young as 7 or 8 years of age and a few tests (e.g. Finger Tapping, Simple Reaction Time, and Pattern Comparison) can be performed by 6-year-old preschool children. A new version of the NES continuous performance test with pictorial stimuli suitable for preschool children has recently been implemented (34).
- The Conners' Continuous Performance Test – Second Edition (CPT-II) is a computerized neuropsychological test. This test assesses inhibitory control, sustained attention, vigilance,

reaction time, and response variability for respondents aged 6 or older (35).

- The Conners' Kiddie Continuous Performance Test Version 5 (K–CPT™ V.5) is a computerized test that ranges from 4 to 5 years old. K-CPT offers an assessment of attention problems in preschool-age children, is built on the respected and reliable foundation of the Conners' Continuous Performance Test II (36).
- The Amsterdam Neuropsychological Tasks (ANT) is a computer-aided assessment battery of response time tasks that allows for the systematic evaluation of information processing capacities. It is a good tool to assess several cognitive domains such as speed and accuracy of visual and auditory information processing, executive function, visuo-motor coordination, mental arithmetic, face recognition, and the processing of human facial emotions. More specifically, ANT offers the possibility to assess sustained, focused and divided attention, attention flexibility, inhibition and impulsiveness (37).
- The Children's Test of Nonword Repetition (CN REP) is suitable for use with children between 4 and 8 years who are attending mainstream schools and can also be used for older children with language related learning difficulties. CN REP provides a reliable indicator of short-term memory which correlates well with language and other difficulties (38).
- The Children's Communication Checklist (CCC) is a 70-item questionnaire completed by a caregiver screens for communication problems in children aged 4 to 16 years. CCC has different uses: screen for children who are likely to have language impairment, identify pragmatic impairment in children

with communication problems or assist in identifying children who may merit from further assessment for an autistic spectrum disorder (39).

- The Diagnostic Analysis of Nonverbal Accuracy (DANVA) consisted of seven subtests, four of which measured nonverbal decoding, or receptive abilities, and three of which measured nonverbal encoding, or expressive abilities. In each of its subtests, the original DANVA targeted the four basic emotions considered to be the most frequently encountered in human interactions: happiness, sadness, anger, and fear (40).
- The California Verbal Learning Test for Children (CVLT-C) assesses verbal learning through an everyday memory task in which the child is asked to recall a list for respondents aged 5 years to 16 years 11 months. An interference task is given, followed by short-delay free recall and cued recall trials. Free recall, cued recall and a word recognition trial are also administered after a 20-minute delay (41).
- The Finger Tapping Test (FTT) measures self-directed manual motor speed. The range of age is from 5 to 85 years old. FTT measures are included in neuropsychological examinations in order to assess subtle motor and other cognitive impairment (42).
- The Tactual Performance Test (TPT) involves several different abilities, including tactile perceptual skills, visuospatial ability, tactile/spatial memory, and visual constructional skills (43).
- The Contingency Naming Test (CNT) was used as a measure of executive function including working memory and reactive

flexibility. This test provides a second measure of the capacity to appropriately maintain and shift cognitive set (44).

- The Purdue Pegboard Test (PPT) has demonstrated the utility as an indicator of the presence and laterality of brain damage in adult patients. Other investigators have successfully used this measure of fine sequential motor movements to discriminate brain-damaged, retarded, and learning disabled (45).
- The Raven's Progressive Matrices (RPM) are widely used non-verbal intelligence tests. In each test item, one is asked to find the missing pattern in a series. Each set of items gets progressively harder, requiring greater cognitive capacity to encode and analyze. They are offered in three different forms for different ability levels, and for age ranges from five through adult: Coloured Progressed Matrices (younger children and special groups); Standard Progressive Matrices (average 6 to 80 year olds) and Advanced Progressive Matrices (above average adolescents & adults) (46).
- The Weschler Memory Scale (WMS) is a neuropsychological test designed to measure different memory functions in a person. It can be used with people from age 16 through 90 (47).
- The Wechsler Abbreviated Scale of Intelligence (WASI) is a reliable, brief measure of intellectual ability and is suitable for 6-89 year-old in a variety of settings. It is conceptually linked to the WISC and the WAIS so it is possible to extrapolate from the verbal, performance and full scale IQ scores on this test to likely scores on the comprehensive batteries (48).
- The Children's categories test (CTT) measures complex aspects of intellectual functioning that incorporates concept formation,

memory and learning from experience. As such, it is an ideal measure of higher order nonverbal abilities. The CCT is fast and easy to administer. Children are asked to determine the principle in each subtest using examiner feedback (49).

Behavioral assessment (description of the tests)

In Table II, a complete list of neuropsychological tools used in ENRIECO cohorts to assess behavioral development have been presented. Unfortunately, in ENRIECO cohorts a few measures of social-emotional development were performed. Most of the tests were created in the same cohort study and these questionnaires which has not published, are not satisfying published criteria of this review. In this review, questionnaires assessing general behaviour, autism, ADHD, school achievements or personality have been described together, although each of these tests measure different areas:

- The Strengths and Difficulties Questionnaire (SDQ) is a brief behavioural screening questionnaire about 3-16 year olds. It exists in several versions to meet the needs of researchers, clinicians and educationalists. All versions of the SDQ ask about 25 attributes, some positive and other negative. These 25 items are divided between 5 scales: 1) emotional symptoms, 2) conduct problems, 3) hyperactivity/inattention, 4) peer relationship problems (5 items), and, 5) prosocial behaviour (50).
- The Development and Well-Being Assessment (DAWBA) is a package of interviews, questionnaires and rating techniques designed to generate ICD-10 and DSM-IV psychiatric diagnoses on 5-17 year olds. The DAWBA primarily focuses on the common emotional, behavioural and hyperactivity disorders, even though

it covers less common disorders. A list of diagnoses covered is available: separation anxiety, specific phobia, social phobia, panic disorder/agoraphobia, post-traumatic stress disorder, obsessive compulsive disorder, generalised anxiety disorder, major depression, ADHD/hyperkinesis, etc (51).

- The Child Behaviour Checklist (CBCL) is a device by which parents or other individuals who know the child well rate a child's problem behaviours and competencies. This instrument can either be self-administered or administered through an interview. The CBCL can also be used to measure a child's change in behaviour over time or following a treatment. The first section of this questionnaire consists of 20 competence items and the second section consists of 120 items on behaviour or emotional problems during the past 6 months. Teacher Report Forms, Youth Self-Reports and Direct Observation Forms are also available for the Child Behaviour Checklist. Two versions of this instrument exist: one for children ages 1 1/2 - 5 and another for ages 6–18 ages. Information below pertains to the CBCL for 6-18 year olds. Outcomes measured are aggression, hyperactivity, bullying, conduct problems, defiance, and violence (52).
- The California Preschool Social Competence Scale (CPSCS) was designed for use by teachers within the context of a preschool program. The CPSCS measures the adequacy of preschool children's interpersonal behaviour and their degree social responsibility in children aged 2 years-6 months through 5 years-6 months. The concept of independence, understood as interpersonal autonomy, is part of its definition. The original scale was designed to be unidimentional, but further analyses

have described 5 factors: considerateness, task orientation, extraversion, verbal facility and response to unfamiliar which are unique to the scale (53).

- The Attention-Deficit Hyperactivity Disorder Criteria of Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (ADHD-DSM-IV) is an internationally recognized questionnaire comprising 18 items designed to evaluate attention-deficit [1-9], hyperactivity and impulsivity [10-18] symptoms in children (54).
- The Childhood Asperger Syndrome Test (CAST) has proved useful in early identification of children aged between 4 and 11 years, whose behaviour suggests a high risk of occurrence of Asperger's syndrome. Test is based on a variety of behavioural descriptions of the ICD-10 and DSM- IV core features of the autism spectrum (social impairments, communication impairments and repetitive or stereotyped behaviours) (55).
- The Child Sexual Behaviour Inventory (CSBI) is a psychological tool with the purpose to obtain caregiver’s report of a wide range of sexual behaviours for use in the evaluation of children who have been sexually abused or who are suspected of having been sexually abused (56).
- The Illness Behaviour Questionnaire (IBQ) assesses 6 domains of infant temperament (activity level, soothability, fear, distress to limitations, smiling and laughter, and duration of orienting). The items on the IBQ ask parents to rate the frequency of specific temperament-related behaviours observed over the past week (or sometimes 2 weeks) (57).
- The Pre-School Activities Inventory (PSAI) is a reliable and valid psychometric questionnaire for the assessment of gender role

behaviour in preschool children. Unlike existing tests, it has been designed to discriminate both within and between the sexes so that variation among as well as between boys and girls can be assessed (58).

- The Mood and Feelings Questionnaire (MFQ) was designed to detect clinical depression in children and adolescents (59).
- The Profile of Mood States (POMS) is a test designed to measure certain psychological traits. Six mood states are used in POMS: tension, depression, anger, vigour, fatigue, and confusion (60).
- The CITO-test consists of 240 multiple-choice items assessing four different intellectual skills: Language, Mathematics, Information Processing, and World Orientation (61).
- The Woodcock Johnson III - Tests of Achievement (WJ III ACH) is designed to identify and describe an individual's current strengths and weaknesses. Three oral tests, a diagnostic spelling test and a measure of phonological awareness have been added to evaluate fluency in reading and in math (62).

General recommendations

Recommendations based on existing cohorts

Based on existing data in ENRIECO cohorts, two outcomes were considered because their relevance and for the great amount of data collected. For cognitive development the outcome considered is global IQ, whereas for behavioral development is ADHD (Table III). As we have mentioned previously, there was little information collected about social-

emotional development in ENRIECO cohorts, and thus, this outcome was not considered in this section.

There are few tests assessing global IQ in ENRIECO (GMDS, BSID, FTII, WPPSI, WISC, MCSA, BDI, Stanford-Binet, and Brunete-Lezine). Inverserly, there are a huge diversity of neuropsychological tests assessing cognitive domains. Moreover, most of the global IQ tests are validated, normed and published in all countries of ENRIECO cohorts. For cognitive domains (i.e. attention), more than 15 tests have been used, and these tests were methodogically different. Lastly, global IQ is the outcome with more information (n=7.751).

For ADHD, the reasons are similars to the previous. In ENRIECO cohorts, the outcome with more assessesments is ADHD (n=22.500), compared with ASD (n=7.138), School performance (n=9370), or Mental Health (n=5.558). Also, tests used to assess ADHD are validated, normed and published in all countries (DSM-IV, CBCL, SDQ, and DAWBA). However, the other outcomes (ASD, School performance, mental health, or personality) can be studied and the recommendations would be similar to those of ADHD.

The first outcome analyzed was the Global IQ. Neuropsychological tests assessing general IQ are composed by several subtests assessing specific cognitive domains (such as attention, language, executive function, etc). These subtests are summed in order to obtain overarching measures of IQ (i.e. WISC, Griffiths, McCarthy, Stanford-Binet or Batelle). All of these tests can be compared for theoretical and psychometric reasons. But, a first consideration is that when analysing pooled data it could only be included those tests which assess that have been assessed completed, including all the subscales. There are some cohorts that only assess some subscales of these tests. These subscales can be used as a proxy of

intelligence, but in order to fit joint analyses of different cohorts about global IQ, only tests including all the subtests could be considered. In table III, we presented a list of cohorts assessing full scales as well as the tests used.

For the pooled analysis of global IQ the age of child assessment is also important. In older children, a huge diversity of cognitive functions can be examined. Then, scores on psychometric measures are more precise and reliable. For this reason, in the case of repeated exams the proposal is to use the test assessed the oldest.

In terms of statistics, whenever is possible the raw scores will be used. All the published neuropsychological tests have their reference population to transform the raw scores in standardised scores. But, this process can introduce a bias for the joint analyses due that each test has been normed in different population (different distribution of socio-economic status (SES), educational level, regions, etc). Consequently, we recommend using raw scores. Subsequently, these scores should be adjusted for child's age and psychologists. After this, the sample must be normalized to a mean of 100 and standard deviation of 15 to harmonize the final distribution. If there is no possibility to use the raw scores in all the cohorts of the analyses, the standardised scores will be used.

Lastly, there is the point concerning the residual confounding in studies involving child neurodevelopment. In order to reduce it, we must adjust all the statistical models for some parental socioeconomic variables such as, social class, education, country of origin, work status, or age. Moreover, it is also important adjust for some parental characteristics (intelligence, mental health, and quality of home environment), environmental determinants or dietary patterns. In table III, we present a

complete list of potential confounders which must include it in the analyses.

The second outcome analyzed in this section was the ADHD. The diagnostic criteria mainly used for ADHD are presented in Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) (13). In ENRIECO cohorts, only one cohort (INMA) has used DSM-IV as a questionnaire which has 18 questions about the ADHD symptomatology. The rest of the tests used in ENRIECO cohorts (SDQ, CBCL & DAWA) assess a wide range of neurobehavioral outcomes, ADHD symptomatology among them. In these questionnaires, questions assessing ADHD are based on DSM-IV criteria.

DSM-IV criteria have three main outcomes: global score of ADHD, inattention and hyperactivity/impulsivity (components of the ADHD's symptomatology). In the other scales, we could choose the specific questions concerning these three main outcomes. Thus, we could create three latent variables: global ADHD (sum of all questions concerning ADHD in each test), inattention (sum of all the questions concerning disattention symptoms in each test), and hyperactivity/impulsivity (sum of all the questions concerning hyperactivity/impulsivity symptoms in each test). After this, it will be needed for each new variable, a study of reliability (internal consistency using Chronbach's Alpha Coefficient) and validity (construct validity using Confirmatory Factor Analyses).

Apart from this, another important question refers to response bias that can be introduced using self-reported questionnaires. It is important to collect who is the informant of these questionnaires (both parents, only mothers, teachers or clinicians). The age of child assessment is another important point. ADHD symptomatology appears before seven years of

age, but in most cases can be detected at 4-5 years old. Due to the variability in the detection of symptoms, we recommend to separate the analyses in two periods: preschool period (1.5 – 5 years) and school age period (6 – 10 years).

Recommendations for future cohort studies

Neurodevelopment is a specific area that recently has received much attention. Nowadays, the impact of some environmental exposures in development of CNS has become a major topic in European public health. Consequently, the number of studies that try to shed light in these effects have grown exponentially. However, the study of neurodevelopment should be approached with extreme caution and the best tools to study this area are the neuropsychological tests.

Due to the great amount of existing tests, some reviews have been published recently. One of them (63) provides a strategy for the assessment of brain function in longitudinal cohort studies of children. We can also find a large compendium of existing neuropsychological tools (15), separating global IQ and specific cognitive domains tests. Regarding to the social-emotional development, we can find another specific paper (16) providing an overview of methodological challenges related to the epidemiological assessment of social-emotional development in children.

Regardless to the protocol of the tests, a set of recommendations for more integrated coherent European efforts in this area has been done in the present review:

- Use of prospective birth cohort methodology (unselected sample). A main advantage of this methodology is to avoid a possible selection bias. Moreover, if birth cohort is followed prenatally, we can

consider exposures present at the time of conception (genetics), during pregnancy (chemical hazards, smoking, alcohol, maternal stress, etc.), at birth (asphyxia, trauma, etc.) and during the postnatal period (infection, environmental exposures, diet and breastfeeding, social environment, etc). Prospective study design allows for the identification of any changes in exposure level. Relationships between exposures, outcomes, and other influential factors can be considered in a temporal context avoiding a recall bias. The major problems of this methodology are the risk of differential attrition over time and the costs, in terms of time, budget or personal.

- To take at least one assessment of all the areas of neurodevelopment. This means one assessment of cognitive development, social-emotional development, and behavioural outcomes.
- In cognitive development is recommended to do at least one measure in early steps of neurodevelopment, another in preschool ages and another during school years. This approach allows establishing neurodevelopmental trajectories and temporal relationships with exposures. For example in ENRIECO cohorts have applied this method INMA, RHEA, ALSPAC or C. Faroes.
- To have at least one measure assessed by psychologist (neuropsychological test applied by a psychologist). Although computerized tasks and self-reported questionnaires are valid measures, a neuropsychological test assessing cognitive development is the best approximation to study the effects of environmental exposures in neurodevelopment studies.

- The neuropsychological tests must be validated and published in population in which will be used. If not, a previous validation must be applied.
- The use of self-reported questionnaires includes a controversial decision. A response bias can be introduced using this kind of questionnaires. Moreover, in some cases as ADHD, information from parents and/or teachers can improve the assessment in this neurodevelopmental area.
- In terms of logistics, the place where the assessment is conducted must be necessarily a peaceful, quiet and undisturbed room.
- To elaborate a strict protocol of assessment. This protocol allows us to reduce the subjectivity of interviewers.
- To apply a previous training of the interviewers. Even when a psychologist applies the test, they will require training to enable a wide knowledge of selected tests.
- To apply several quality controls during fieldwork. To study the reliability and validity in our study of each test used it is an important point that must be taken into account.
- To use a standard questionnaire to assess behavioral problems, such as SDQ or CBCL, in order to homogenize the studies in this area.
- In analysing the relationships between different exposures and child neurodevelopment it is crucial to collect several confounding factors.

NEUROBEHAVIORAL/COGNITIVE FUNCTION

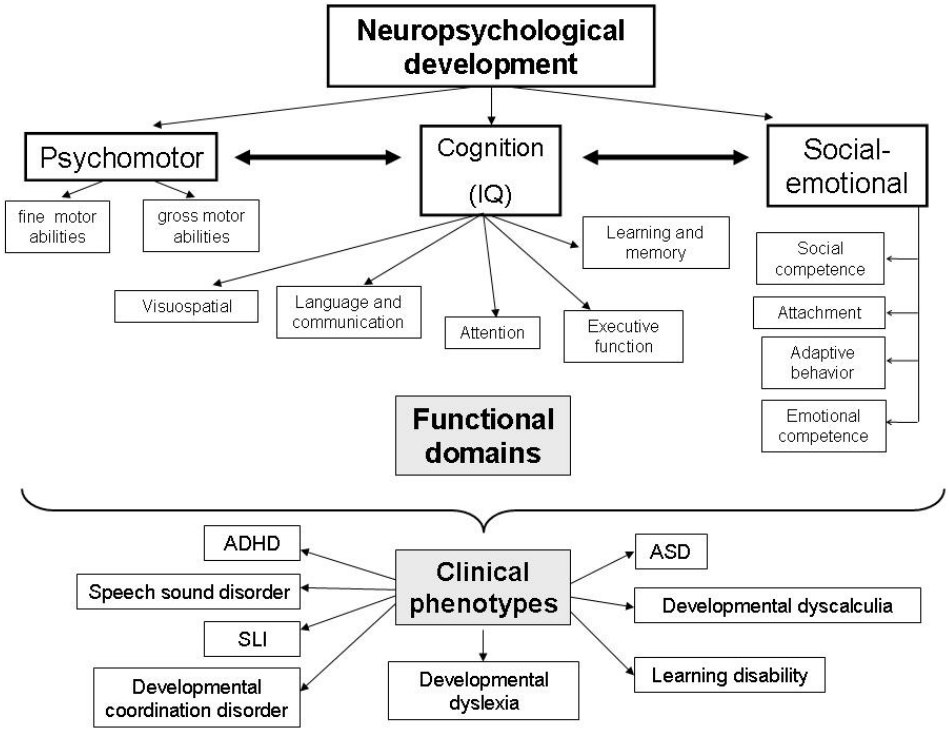

Table I. Assessment of cognitive development:

	0m-6m	7m-18m	1.5y-5y	5y-10y	10y+
NOS	C. Faroes (III, V), Duisburg				
Denver	ALSPAC, GASPII, NINFEA	ALSPAC, GASPII	ALSPAC, GASPII, NINFEA		
ASQ	MoBa	MoBa	EDEN, MoBa	MoBa	
Griffiths	INMA	ALSPAC, INMA			
Bayley	C.Faroes II	C.Faroes II, Duisburg, EDEN, INMA, PCB Cohort, REPRO_PL, RHEA	FLEHS, INMA, REPRO_PL		
CDI		ALSPAC, Generation R	EDEN, ALSPAC, GASPII		
FTII		Duisburg			
SON-R 2.5-7			FLEHS, Generation R		
Reynell			ALSPAC	ALSPAC	

	0m-6m	7m-18m	1.5y-5y	5y-10y	10y+
WISC			ALSPAC,	Duisburg, ALSPAC, C.Faroes (I, II, III), PELAGIE	INMA, ALSPAC, C.Faroes I
WPPSI			ALSPAC, C.Faroes II, EDEN, Generation R, PCB Cohort	C.Faroes II, EFESE/ELFE,	
BNT			C.Faroes III	C.Faroes (I, II, III)	C.Faroes I
Stanford-Binet			C.Faroes II	C.Faroes II	C.Faroes I
NEPSY			EDEN	C.Faroes I, PELAGIE	
NES			FLEHS	C.Faroes (I, II, III)	C.Faroes I
MCSA			INMA, RHEA		
ELOLA			EDEN		
Batelle			INMA		
K-CPT			INMA		
Brunet-Lézine			PELAGIE		

	0m-6m	7m-18m	1.5y-5y	5y-10y	10y+
CPT				C.Faroes I	C.Faroes I, INMA,
ANT				ABCD	
CCC				ALSPAC	
CN Rep				ALSPAC	
DANVA				ALSPAC	ALSPAC
Non-word reading task				ALSPAC	
CVLT-C				C.Faroes (I, II, III)	C.Faroes I
FTT				C.Faroes I	C.Faroes I
TPT				C.Faroes I	
CNT				C.Faroes III	
Purdue PegBoard				C.Faroes III	
Raven CPM				C.Faroes III	
Raven SPM Plus				C.Faroes III	C.Faroes I, INMA
WMS				C.Faroes III	C.Faroes I

	0m-6m	7m-18m	1.5y-5y	5y-10y	10y+
WASI					ALSPAC
CCT					C.Faroes I
PARCA			GenerationR		
LDS			GenerationR		
IDI	GenerationR	GenerationR	GenerationR		
SON			Generation R		
LTC			GenerationR		

NOS: Neurological Optimality Score; Denver: Denver Developmental; ASQScreening Test (Denver); Griffiths: Griffiths Mental Development Scales; ASQ: Age and Stages Questionnaire; Bayley: Bayley Scales for Infant Development; CDI: The MacArthur-Bates Communicative Development Inventory; FTII: Fagan Test of Infant Intelligence; SON-R 2.5-7: Snijders-Oomen non-verbal intelligence test; Reynell: Reynell Development Language Scale; WISC: Wechsler Intelligence Scale for Children; WPPSI: Wechsler Preschool and Primary Scale of Intelligence; BNT: Boston Naming Test; NES: Neurobehavioral Evaluation System (NES); McCarthy: McCarthy Scales of Children Abilites (McCarthy); Batelle: Batelle Development Inventory; K-CPT: Kiddie Continuos Performance Test; CPT: Continuous Performance Test; ANT: Amsterdam Neuropsychological Task; CCC: Children's Communication Checklist; CN Rep: The Children's Test of Nonword Repetition (CN REP); DANVA: Diagnostic Analysis of

Nonverbal Accuracy; CVLT-C: California verbal learning test-children version; FTT: Finger Taping Test; TPT: Tactual Performance Test; CNT: Contingency Naming Test; WMS: Weschler Memory Scale; WASI: Weschler abreviated Scale of Intelligence; CCT: children's categories test; PARCA: Parent Report Children's Abilities; LDS: Language Development Survey; IDI: Infant/child Development Inventory; LTC: Language Test for Children; DAVA: Diagnostic Analysis of Nonverbal Accuracy; SON: Non-verbal intelligence test.

Table II. Assessment of behaviour (including ADHD, autism, mental health, and school achievements):

	0m-6m	7m-18m	1.5y-5y	5y-10y	10y+
SDQ	C. Faroes (III, V), Duisburg, MoBa	MoBa	ABCD, ALSPAC, EDEN, MoBa	ALSPAC, DNBC, GINIplus, LISA, MoBa, PELAGIE	ALSPAC
DAWBA				ALSPAC	ALSPAC, DNBC
CBCL	MoBa	MoBa	FLEHS, Generation R, Koala, MoBa, PCB Cohort	C.Faroes I, Generation R, MoBa, PCB Cohort	INMA
CSBI			FLEHS		
IBQ		FLEHS	FLEHS		
CPSCS			INMA		
ADHD-DSM-IV			INMA, NINFEA		
CAST			INMA		
MFQ				ALSPAC	ALSPAC
POMS				C.Faroes I	
CITO-test				ABCD	PIAMA

SDQ: Strenghts and Difficulties Questionnaire; DAWBA: Development and well-being assessment; CBCL: Children behavior checklist; CSBI: Child sexual behavior inventory; IBQ: Illness behavior questionnaire; CPSCS: California Preschool Social Competence Scale; ADHD-DSM-IV: Attention-Deficit Hyperactivity Disorder Criteria of Diagnostic and Statistical; CAST: Childhood asperger sindrome test; MFQ: The mood and feelings questionnaire; POMS: Profile of mood states; WJIII-ACH: the Woodcock Johnson III - Tests of Achievement.

Table III.1. Assessment of two grouped outcomes (Global IQ and ADHD)

Outcome	Study design (e.g., timing of recruitment)	Technique used to assess	Tools used in ENRIECO
Global IQ	If possible, prospective birth cohort study and recruitment during pregnancy	Neuropsychological assessment performed by psychologist The full scale must be completed	GMSD BSID FTII WISC WPPSI MCSA Brunet-Lézine Stanford-Binet
ADHD	Ídem	Self-reported questionnaires (by parents or teachers)	DSM-IV CBCL SDQ DAWA

[1]Quality of assessment, it is a variable used to control possible external factors in the assessment (e.g. poor interaction psychologist-child, fever, tiredness, behavior's problems…).

Table III.2. Assessment of two grouped outcomes (Global IQ and ADHD)

Outcome	ENRIECO cohorts	Confounders	Bias	Recommendations
Global IQ	Only these cohorts that they assess the full scale: ALSPAC C.Faroes II Duisburg EDEN EFESE/ELFE FLEHS Generation R INMA PCB_Cohort REPRO_PL RHEA	Psychologist Child's age Quality of assessment[1] Academic year and trimester Birth weight Prematurity Type of delivery Congenital malformations Diseases Breastfeeding Maternal smoking during pregnancy Parental: · SES · Educational level · Country of	1) Inter-rater variability 2) Observer-expectancy effect (observer bias) 3) Different reference populations (standardised scores) in neuropsychological tests	1) Few psychologists participating Previous training Strict protocol Quality control during fieldworl ICC report 2) Psychologist not aware of the mother or child's exposure information 3) Raw scores Latent variables Adjusting for cohort, psychologist and child' age (in months)

		origin		
Outcome	ENRIECO cohorts	Confounders	Bias	Recommendations
ADHD	C.Faroes I, FLEHS, Generation R, INMA KOALA, MoBa PCB_Cohort, NINFEA, ALSPAC DNBC, LISA, ABCD GINIplus, and EDEN	- Idem, except psychologist and quality of assessment	Response bias Social desirability Recall bias	Test-retest reliability Course tutor or teacher as informant (more objectivity)

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