Effect of hormone disruptors on birth outcomes and child's anogenital distance

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To my family

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ABSTRACT

Introduction: Persistent Organic Pollutants (POPs) include a wide range of synthetic chemical substances that are ubiquitous in the environment and are identified as endocrine disrupting chemicals (EDCs) which can alter the function of endocrine systems in humans and animals. Exposure to such chemicals during critical developmental phases, such as in utero has been associated with adverse reproductive and child health outcomes.

Aims: The main aim of this thesis was to evaluate the effect of in utero exposure to hormone disruptors on birth outcomes and anogenital distance of the child in five European population-based cohort studies in Greece, Spain, England, Denmark and Norway.

Methods: We measured dioxin-like activity in maternal and cord blood plasma samples collected at delivery using the Dioxin-Responsive Chemically Activated LUciferase eXpression (DR CALUX®) bioassay. Concentrations of several PCBs and other organochlorine compounds (dichlorodiphenyl dichloroethene [DDE], dichlorodiphenyl trichloroethane [DDT] and hexachlorobenzene [HCB]), were determined in 1st trimester maternal serum by triple quadrupole mass spectrometry. Information on birth outcomes was retrieved from medical records. Anogenital distances were measured in newborn and young girls.

Results: Plasma dioxin-like activity was higher in maternal than in cord blood samples. Newborns in the highest tertile of exposure had a reduction of approximately half a week in their gestational age as compared with those in the lowest tertile. This association was stronger in boys than in girls, although the statistical evidence for interaction was weak. Birth weight was negatively associated with increasing levels of HCB and PCBs. Adjustment for maternal gestational weight gain explained only to a small extent the association between POP levels and birth weight. Furthermore, in a stratified analysis, the association between POPs and birth weight was only observed in women with inadequate or excessive gestational weight gain. Anogenital distances were sexually dimorphic, being longer in males than females. Plasma dioxin-like activity was negatively associated with AGD (anogenital distance: anus to upper penis), in male newborns. Negative but smaller and nonsignificant associations were observed for AGD in young boys. No associations were found in girls. Negative but not statistically significant associations were observed for AGD and HCB, DDE and total PCBs in young boys. In young girls, anoclitoral (ACD: anus to clitoris) and anofourchetal distance (AFD: anus to fourchette) were positively associated with all POPs.

Conclusions: Results from these international general population studies suggest an association between low-level prenatal exposure to POPs and impaired fetal growth. Moreover, our results suggest that male infants may be

susceptible to endocrine-disrupting effects of dioxins while associations with other POPs remain unclear.

RESUM

Introducció: Els contaminants orgànics persistents (COPs) inclouen una àmplia gamma de substàncies químiques sintètiques que són omnipresents en el medi ambient i són identificats com pertorbadors endocrins (EDC) que poden alterar la funció dels sistemes endocrins en els éssers humans i els animals. L'exposició a aquests productes químics durant fases crítiques de desenvolupament, com a l'úter, s'ha associat amb efectes adversos en la salut reproductiva i infantil.

Objectius: L'objectiu principal d'aquesta tesi és avaluar l'efecte de l'exposició a l'úter als disruptors hormonals sobre el pes en néixer, l'edat gestacional i la distància anogenital dels nens en cinc estudis europeus de cohorts de base poblacional a Grècia, Espanya, Anglaterra, Dinamarca i Noruega .

Mètodes: Es va mesurar l'activitat de tipus de dioxines (dioxin-like activity) en mostres de plasma de la sang materna i del cordó recollits en el part mitjançant el bioassaig Dioxin-Responsive Chemically Activated LUciferase eXpression (DR CALUX®). Les concentracions de diversos PCBs i altres compostos organoclorats (dicloroeteno diclorodifenil [DDE], diclorodifenil tricloroetà [DDT] i hexaclorobenzè [HCB]), es van determinar en sèrum matern del primer trimestre amb espectrometria de masses. La informació sobre els parts va

ser recuperada dels registres mèdics. Les distàncies anogenitals es van mesurar en nadons i nens joves .

Resultats: L'activitat de tipus de dioxines en plasma va ser més alta en les mares que en les mostres de sang de cordó. Els nounats al tercil més alt d'exposició van tenir una reducció d'aproximadament la meitat d'una setmana a l'edat gestacional, en comparació als del tercil inferior. Aquesta associació va ser més forta en els nens que en les nenes, encara que l'evidència estadística per a la interacció era feble. El pes en néixer es va associar negativament amb l'augment dels nivells de HCB i PCB. Ajustant per l'augment de pes matern gestacional explicava només una petita part de l'associació entre els nivells de COPs i el pes en néixer. A més, en una anàlisi estratificada, l'associació entre els contaminants orgànics persistents i el pes en néixer es va observar només en dones amb augment de pes gestacional inadequat o amb augment excessiu. Les distàncies anogenitals eren dimòrfiques, sent majors en els nens que en les nenes. L'activitat en plasma de tipus de dioxines es va associar negativament amb l'AGD (distància anogenital: anus al límit superior del penis) en els nadons mascles. Es van observar associacions negatives però més petites i no significatives per a l'AGD en els nens joves. No es van trobar associacions en les nenes. Es van observar associacions negatives, però no estadísticament significatives per a l'AGD i HCB, DDE i PCBs totals en els nens joves. En les nenes joves, les distàncies ACD (anus fins el clítoris) i AFD (anus a

fourchette-llavis menors) es va associar positivament amb tots els COP.

Conclusions: Els resultats d'aquests estudis internacionals de població general suggereixen una associació entre l'exposició prenatal de baix nivell als contaminants orgànics persistents i retard del creixement fetal. D'altra banda, els nostres resultats suggereixen que els nens mascles poden ser susceptibles als efectes d'alteració endocrina de dioxines mentre que les associacions amb altres COP són menys evidents.

PREFACE

Pesistent Organic Pollutants (POPs) include a wide range of synthetic chemical substances that are persistent and ubiquitous in the environment. Exposure to such chemicals during critical developmental phases, such as in utero has been associated with adverse reproductive and child outcomes. This thesis aims to assess the association between prenatal exposure to environmental contaminants and fetal growth indicators within population-based cohort studies from five European countries. The novelty of this study lies in the use of a biologically relevant bioassay for exposure assessment and also the use of anogenital distance as a marker of endocrine disruption during pregnancy.

This thesis has been written at the Centre for Research in Environmental Epidemiology (Barcelona, Spain) between 2010 and 2013 and supervised by Prof. Manolis Kogevinas and Dr Martine Vrijheid. It consists of a compilation of scientific publications in agreement with the regulation of the Doctoral Programme in Biomedicine of the Department of Experimental and Health Sciences at the Pompeu Fabra University. This thesis includes an abstract, a general introduction, a rationale, the objectives, the methods, the results (a compilation of four scientific publications), an overall discussion section and final conclusions.

CONTENTS

AKNOWLEDGEMENTS v
ABSTRACT ix
RESUM xiii
PREFACE xvii
CONTENTS xix
1 INTRODUCTION 1
1.1 Persistent organic pollutants 2
1.1.1 Dioxins and dioxin-like compounds 3
1.1.2 Non-dioxin like PCBs 8
1.1.3 Organochlorine pesticides 8
1.2 Endocrine disruption 9
1.2.1 Sources of exposure 12
1.3 Health effects 15
1.3.1 Importance of early exposures 15
1.3.2 Prenatal exposure and reproductive effects 17
1.4 Key methodological issues in research on POPs and reproductive effects 20
1.4.1 Exposure assessment – biologically relevant biomarkers 20
1.4.2 Outcome assessment – towards sensitive endpoints 21
1.4.3 Evaluation of key confounders and effect modifiers 25
2 RATIONALE 27
3 OBJECTIVES 29

3.1 General objective 29
3.2 Specific objectives 29
4 METHODS 31
4.1 Study population 31
4.2 Prenatal exposure assesment 32
4.2.1 Plasma dioxin-like activity - DR CALUX® bioassay 32
4.2.2 Serum concentrations of Persistent organic pollutants 33
4.3 Outcome assesment 34
4.3.1 Birth outcomes 34
4.3.2 Anogenital distance 35
5 RESULTS 39
5.1 Paper I 42
5.2 Paper II 89
5.3 Paper III 135
5.4 Paper IV 142
6 GENERAL DISCUSSION 173
6.1 Contribution and main strengths of this thesis 173
6.1.1 Exposure biomarkers 173
6.1.2 Novel outcomes 177
6.1.3 Confounders and effect modifiers 180
6.2 Limitations 182
6.3 Public health implications 183
6.4 Future research 185
7 CONCLUSIONS 187
8 REFERENCES 189

1 INTRODUCTION

Environmental exposures play an important role in the causation of disease (Grandjean and Landrigan 2006; Thayer et al. 2012; Schug et al. 2011). The development and use of synthetic chemicals have grown exponentially since the 1940s, and there are now more than 84000 different chemicals in commerce (Landrigan and Goldman 2011). Many of these chemicals have helped promote quality of life but, simultaneously, widespread exposure to hundreds of these chemicals among men, women, and children globally has been documented (Meeker 2012). Adults and children can be exposed to a wide variety of environmental pollutants in food, water, air, and through everyday activities. The link between some environmental toxicants and adverse health effects may have been established (Ferguson et al. 2013; Norman et al. 2013; Wright and Brunst 2013; Llop et al. 2013), however, there are many other suspected links between emerging environmental exposures and health effects that have not been characterized well enough (Philippat et al. 2012; Holtcamp 2012; Cantonwine et al. 2013; Valvi et al. 2013). Moreover, the combined impact of mixtures of low levels of contaminants is also of concern (Patel et al. 2010).

1.1 Persistent organic pollutants

Pesistent Organic Pollutants (POPs) include a wide range of synthetic chemical substances that are persistent and ubiquitous in the environment. Because of their chemical stability, resistance to degradation, long half lives and lipophilic properties these compounds persist in the environment years after their application, bioaccumulate in human and animal tissue and biomagnify through the food chain. Initially, twelve POPs have been recognized as causing adverse effects on humans and the ecosystem, also known as the “dirty Dozen”, and were targeted by the Stockholm convention. The Stockholm Convention on Persistent Organic Pollutants is an international environmental treaty, signed in 2001 and effective from May 2004, that aims to eliminate or restrict the production and use of POPs (http://chm.pops.int/).

Polychlorinated dibenzodioxins (PCDDs), dibenzofurans (PCDFs) and biphenyls (PCBs), and organochlorine pesticides such as dichlorodiphenyl dichloroethene (DDE), the major degradation product of dichlorodiphenyl trichloroethane (DDT), and hexachlorobenzene (HCB) are some of the 12 POPs originally targeted by the Stockholm convention (Figure 1).

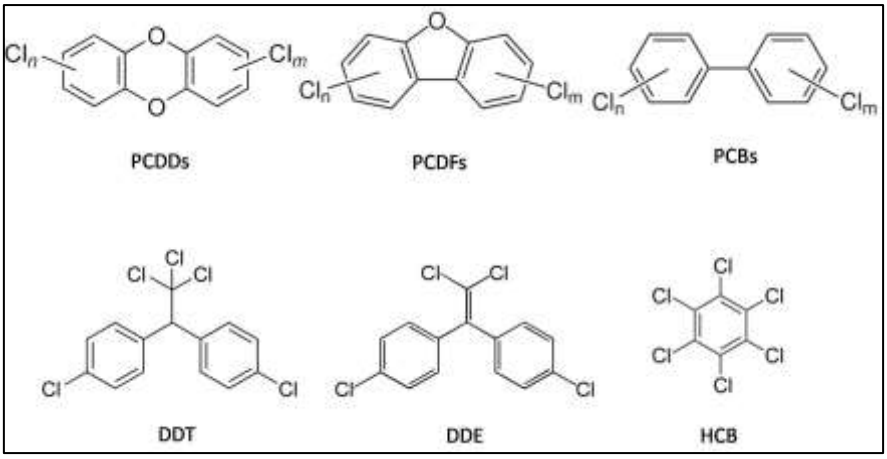

Figure 1. Chemical structures of PCDDs, PCDFs, PCBs, DDT, DDE and HCB.

In addition to the dirty dozen, increasing concerns are given to emerging pollutants, such as brominated and fluorinated compounds whose toxic effects are similar to those of the classic chlorinated POPs (Stockholm Convention 2009).

1.1.1 Dioxins and dioxin-like compounds

Dioxins and dioxin-like compounds (DLCs) represent a group of polyhalogenated aromatic hydrocarbons that includes some of the most toxic POPs. The classical dioxins and DLCs include 7 PCDDs, 10 PCDFs, and 12 dioxin-like PCBs (non-ortho coplanar PCBs: PCB 77, 81, 126, and 169 and mono-ortho-substituted PCBs: PCB 105, 114, 118, 123, 156, 157, 189). The most toxic compound (congener) of the family is 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, dioxin) and has been classified as a human carcinogen by the International Agency for Research on Cancer (IARC) (IARC 1997). In

recent years sufficient evidence has been gathered for two more members of this group, 2,3,4,7,8-pentachlorodibenzofuran and 3,3′,4,4′,5-pentachlorobiphenyl (PCB 126) to be classified as carcinogenic to humans (Group 1) by IARC (Baan et al. 2009; IARC 2012). A half life of around 7 years for 2,3,7,8-TCDD has been reported by EPA (2000) but for each congener, variation in halflife exists both among individuals and within the same individual over his or her lifetime (Milbrath et al. 2009). PCDDs/Fs have never been produced for commercial purposes and are unwanted by-products of combustion processes of chlorine containing components. In contrast, PCBs have dielectric properties and were produced for commercial purposes for approximately 50 years until the 1980s when the production was banned.

Dioxins and DLCs are grouped together because of the similarity of their physical and chemical properties and their ability to elicit comparable toxicological responses (Schecter et al. 2006). The toxic effects of PCDDs, PCDFs and dioxin-like PCBs are mediated mainly through binding to the aryl hydrocarbon receptor (AhR). The AhR is an intracellular ligand-activated transcription factor involved in regulation of the expression of a large number of genes, which is expressed in many tissues of the human body (Safe 1995). Upon binding to the ligand, the cytosolic ligand-AhR complex translocates into the nucleus, where it heterodimerizes with the AhR nuclear translocator (ARNT). This complex then

binds to its specific DNA recognition sites to activate the transcription of dioxin responsive genes (Hankinson 1995), such as the drug metabolizing enzymes CYP1A1 and CYP1A2 (Safe and Krishnan 1995) (Figure 2).

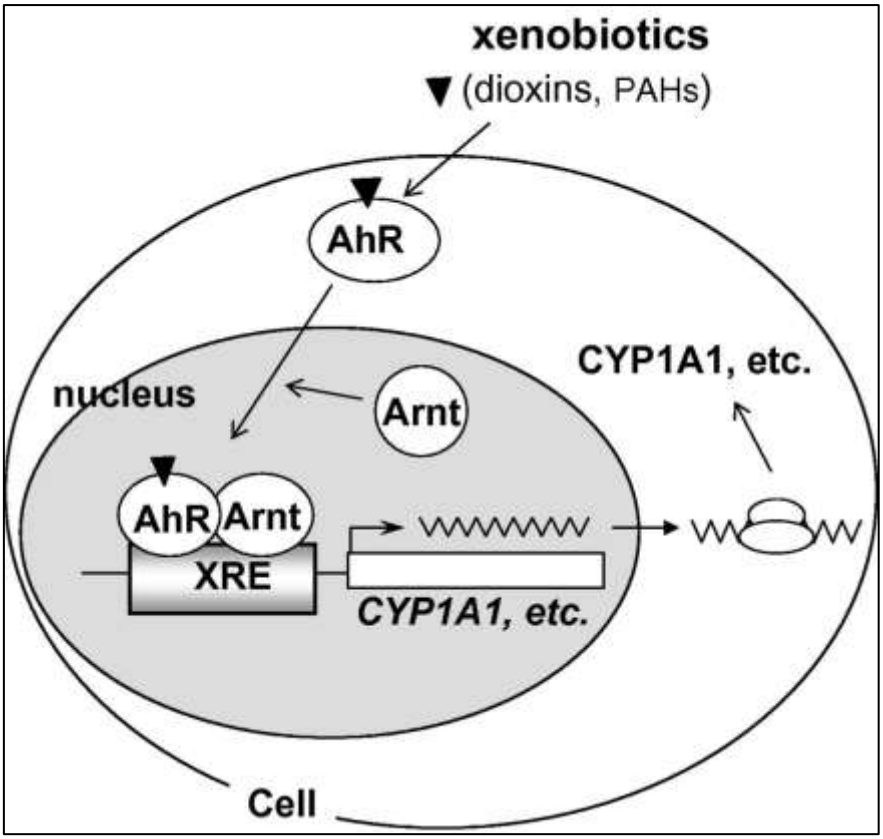

Figure 2. Cellular responses to dioxins and dioxin-like chemicals (Adapted from Kitamura and Kasai 2007).

AhR is activated by a variety of exogenous ligands and apart from its role as a mediator of the biochemical response to xenobiotics, AhR plays key endogenous regulatory roles in normal physiology and development (Denison et al. 2011).

The affinity of a specific compound with the Ah receptor determines its toxic potential, measured by the Toxic Equivalency Factor (TEF), which expresses the order of magnitude of toxicity relative to 2,3,7,8-TCDD (TEF = 1) (Van den Berg et al. 1998). The TEFs of 1998 and the re-evaluated based on scientific evidence TEFs of 2005 (Van den Berg et al. 2006) are presented at Table 1.

The toxicities of these compounds vary over more than five orders of magnitude and largely depend on the number and position of the chlorine atoms. The overall toxicity of such a complex mixture is generally expressed as a TCDD Toxic Equivalent (TEQ) concentration. The concept of TEQ has thus been introduced to simplify risk assessment and regulatory control (Van den Berg et al. 1998) and has been established for assessment of dioxins and dioxin-like compounds in biological and environmental samples. The classical TEQs are calculated by multiplying the concentration of individual PCDDs/PCDFs/PCBs by their respective TEFs.

Table 1. Summary of WHO 1998 and WHO 2005 TEF values.

Compound	WHO 1998	WHO 2005
PCDDs		
2,3,7,8-TCDD	1	1
1,2,3,7,8-PentaCDD	1	1
1,2,3,4,7,8-HexaCDD	0.1	0.1
1,2,3,6,7,8-HexaCDD	0.1	0.1
1,2,3,7,8,9-HexaCDD	0.1	0.1
1,2,3,4,6,7,8-HeptaCDD	0.01	0.01
OCDD	0.0001	0.0003
PCDFs		
2,3,7,8-TCDF	0.1	0.1
1,2,3,7,8-PentaCDF	0.05	0.03
2,3,4,7,8-PentaCDF	0.5	0.3
1,2,3,4,7,8-HexaCDF	0.1	0.1
1,2,3,6,7,8-HexaCDF	0.1	0.1
1,2,3,7,8,9-HexaCDF	0.1	0.1
2,3,4,6,7,8-HexaCDF	0.1	0.1
1,2,3,4,6,7,8-HeptaCDF	0.01	0.01
1,2,3,4,7,8,9-HeptaCDF	0.01	0.01
OCDF	0.0001	0.0003
PCBs (IUPAC number)		
3,3',4,4'-TCB (PCB 77)	0.0001	0.0001
3,4,4',5-TCB (PCB 81)	0.0001	0.0003
2,3,3',4,4'-PentaCB (PCB 105)	0.0001	0.00003
2,3,4,4',5-PentaCB (PCB 114)	0.0005	0.00003
2,3',4,4',5-PentaCB (PCB 118)	0.0001	0.00003
2',3,4,4',5-PentaCB (PCB 123)	0.0001	0.00003
3,3',4,4',5-PentaCB (PCB 126)	0.1	0.1
2,3,3',4,4',5-HexaCB (PCB 156)	0.0005	0.00003
2,3,3',4,4',5'-HexaCB (PCB 157)	0.0005	0.00003
2,3',4,4',5,5'-HexaCB (PCB 167)	0.00001	0.00003
3,3',4,4',5,5'-HexaCB (PCB 169)	0.01	0.03
2,3,3',4,4',5,5'-HeptaCB (PCB 189)	0.0001	0.00003

1.1.2 Non-dioxin like PCBs

As a class consisting of 209 congeners with various numbers of chlorine substitutions, PCBs can be distinguished according to their toxicological properties. Besides the twelve coplanar PCB congeners with no substitutions at the ortho-position (closest to the bond connecting the two phenyl rings) that show toxicological properties similar to dioxins, those with substitutions (noncoplanar) have not been found to activate the AhR, and are not considered part of the dioxin group. Non dioxin-like PCBs may act through different pathways than the dioxin-like chemicals, so their effects are not represented in the use of TEFs (Carpenter 2006).

1.1.3 Organochlorine pesticides

Organochlorine pesticides (OCPs) such as DDT/DDE and HCB are also ubiquitous contaminants in different compartments of the environments. HCB was first introduced in 1933 as a fungicide for food crops. Although HCB production and use in agriculture was banned in Europe at 1981, HCB is still being released to the environment as an unintended byproduct in chemical processes, incomplete combustion and an impurity in pesticides (Wang et al. 2010). Current emissions are estimated to be 70%–95% lower than that in 1970 because of the banning of HCB for agricultural use (Barber et al. 2005).

Perhaps the best studied OCP is DDT. DDT is a composite term frequently used to include the active ingredient p,p′-DDT, its contaminant o,p′-DDT and primary metabolite p,p′-DDE. Agricultural use was banned or restricted in industrialised countries in 1970s. Even though it has been prohibited, it is still used in some developing countries for the control of vector-borne diseases like malaria.

1.2 Endocrine disruption

Public concern about contamination by POPs increased recently because several of these compounds are identified as endocrine disrupting chemicals (EDCs) which can alter the normal function of endocrine and reproductive systems in humans and wildlife.

> "An endocrine disruptor is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations.
> A potential endocrine disruptor is an exogenous substance or mixture that possesses properties that might be expected to lead to endocrine disruption in an intact organism, or its progeny, or (sub) populations." (IPCS, 2002).

According to the International Programme on Chemical Safety (IPCS), endocrine disruption is not considered a toxicological end point per se but a functional change that may lead to adverse effects (WHO 2002).

Endocrine disruptors act via nuclear receptors, nonnuclear steroid hormone receptors (e.g., membrane ERs), nonsteroid receptors (e.g., neurotransmitter receptors such as the serotonin receptor, dopamine receptor, norepinephrine receptor), orphan receptors [e.g., aryl hydrocarbon receptor (AhR)—an orphan receptor], enzymatic pathways involved in steroid biosynthesis and/or metabolism, and numerous other mechanisms that converge upon endocrine and reproductive systems (Diamanti-Kandarakis et al. 2009). Even low-dose exposure to chemicals that interact with hormone receptors can interfere with reproduction, development, and other hormonally mediated processes. Furthermore, since endogenous hormones are typically present in the body in relatively tiny concentrations, the theory holds that exposure to relatively small amounts of exogenous hormonally active substances can disrupt the proper functioning of the body's endocrine system. Thus, an endocrine disruptor might be able to elicit adverse effects at much lower doses than a toxicant acting through a different mechanism. Conducted studies however, have focused on narrowly defined groups of chemicals, without considering combined exposures, genetic polymorphisms or lifestyle factors and as a consequence

have failed to effectively test the hypothesis that endocrine disruption is associated with adverse health effects in humans (Olea and Fernandez 2007).

Several scientific bodies, including the Endocrine Society, now support that EDCs can affect human health (Diamanti-Kandarakis et al. 2009). The realization that exposure to many environmental EDCs is now ubiquitous, coupled with proven or suggested trends for increased rates of certain endocrine-related diseases and disorders among children, has resulted in growing concern regarding potential links between the two among scientists, governments, physicians, and patients (Meeker 2012).

In 2012, the United Nations Environment Programme (UNEP) and WHO presented an update of the IPCS (2002) document, entitled “State of the Science of Endocrine Disrupting Chemicals—2012” (WHO 2012) to provide new information about the mechanisms by which environmental chemicals can interfere with hormone actions, the degree to which our environment is contaminated with such chemicals, and the relationship between chemical exposures and health outcomes in humans and in wildlife. In this report, the need for new approaches to examine the effects of mixtures of endocrine disruptors on disease susceptibility and etiology is pointed out.

Very recently, an editorial entitled “Scientifically unfounded precaution drives European Commission’s recommendations on EDC regulation, while defying common sense, well-established science and risk assessment principles” undersigned by toxicology journal editors was published as an intervention regarding proposed European Union endocrine disruptor regulations (Dietrich et al. 2013). In response to the editorial by Dietrich and his colleagues, 41 scientists published their own commentary in Environmental Health (Bergman et al. 2013). In this commentary, they express their strong concerns about the editorial that in their opinion is inaccurate, factually incorrect and ignores scientific evidence and well-established principles of chemical risk assessment. Another editorial, signed by 104 scientists and editors, was published as a response to the editirial by Dietrich and his colleagues stating that “Policymakers in Europe and elsewhere should base their decisions upon science, not assumptions based upon principles that arose out of research on chemicals that are not EDCs. The letter by Dietrich et al does the EuropeanCommission, science - including the field of toxicology–and most importantly, public health - a profound disservice.” (Gore et al. 2013).

1.2.1 Sources of exposure

Because of effective regulations, POPs levels have declined in the last decades. The carbon–chlorine bond is very stable

toward hydrolysis and the greater the number of chlorine substitution and/or functional group, the greater the resistant to biological and photolytic degradation. Because POPs break down very slowly, they will present in the environment for long time to come, even if all new sources were immediately eliminated (El-Shahawi et al. 2010). Oceans and seas are the largest POPs reservoirs, since they accumulate POPs from river sediments, by the atmospheric deposition, by disposing wastes, and by accidents. POPs are stored in sediments on the beds of seas, oceans, and large lakes, where they can be released from after a time and then re-enter the atmosphere as shown in Fig. 2 (El-Shahawi et al. 2010).

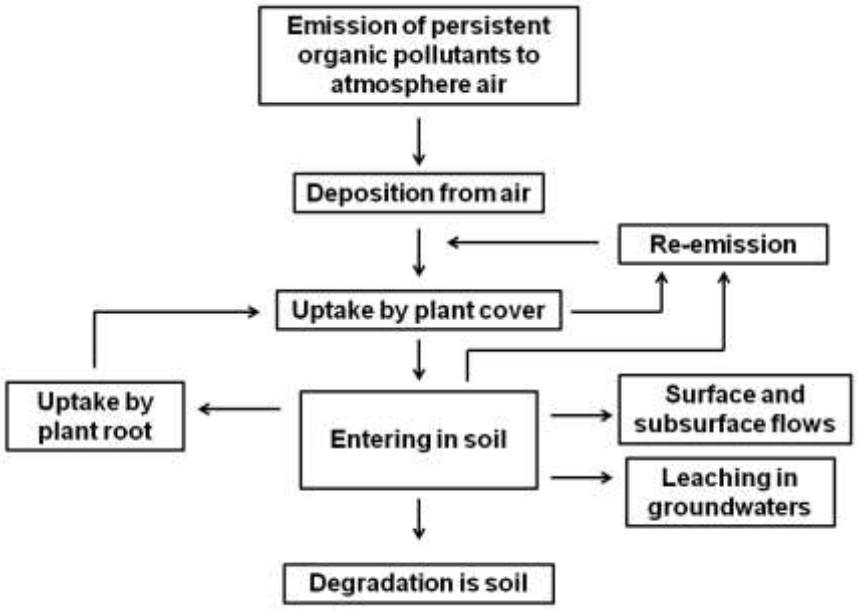

Figure 3. Conceptual model for the behavior of persistent organic pollutants in the air–plant soil system (Adapted from El-Shahawi et al. 2010).

Thus, although levels are decreasing, the general population is still exposed to these substances at low doses (Porta et al. 2008). Human and animal exposure occurs through contaminated air inhalation, soil ingestion, soil dermal contact and food consumption.

In the majority of the general population – which is not occupationally exposed to POPs – exposure occurs largely through dietary intake (Patandin et al. 1999; Gasull et al. 2011). Since POPs are highly lipophilic, they are stored in fat tissues and bio-magnify through the food chain. Hence, consumers of food of animal origin such as fish, meat, milk and dairy products end up with high levels of exposure. Human exposure to POPs begins in the uterus, since most POPs cross the placental barrier (Covaci et al. 2002; Tsukimori et al. 2013). Breast milk has a relatively high content of fat. Lipophilic compounds from the mother will be transported to the milk. Hence, newborns are exposed to POPs postnatally via breast milk (Mannetje et al. 2012; Needham et al. 2011).

The levels of these compounds vary widely among individuals and the cause of this variability has yet to be determined. In recent years, diverse studies have examined whether certain sociodemographic or lifestyle factors influence this variation and have found that country of origin, sex, parity, body mass index (BMI), age, breastfeeding, and educational level are all

associated with serum levels of various POPs (Llop et al. 2010; Cerrillo et al. 2006; Arrebola et al. 2009).

1.3 Health effects

Concerns over the detrimental effects of exposure to POPs initially arose from studies in animals that showed reproductive, developmental, endocrine and carcinogenic effects (Li et al. 2006; Colborn et al. 1993; Tanabe 2002). In humans, a number of adverse health effects such as endometriosis, infertility, immunotoxicity, neurotoxicity and spontaneous abortions, breast cancer, prostate cancer and neurodegenerative disorders have been suggested as a result of exposure to POPs (Meeker 2012; Mrema et al. 2013).

1.3.1 Importance of early exposures

Whilst the adaptation of western lifestyles leads to the increase of non-communicable diseases, there is growing recognition of the role played by developmental factors. Early in the 80's David Barker and colleagues developed a hypothesis, widely known as the "Barker hypothesis". This hypothesis emphasized the importance of early exposures for later develpoment and proposed that undernutrition during gestation is an important contributor to low birth weight and an early origin of adult cardiac and metabolic disorders due to

fetal programming in response to undernutrition that permanently shaped the body's structure, function, and metabolism (Barker and Osmond 1986; Barker et al. 1989; Barker et al. 1993). This theory stimulated interest in the fetal origins of adult disorders, and The Developmental Origins of Health and Disease (DOHaD) concept was developed over the last 25 years. The developmental-origins hypothesis proposes that an altered long-term risk of disease is initially induced through adaptive responses that the fetus or infant makes to cues from the mother about her health or physical state (Gluckman et al. 2008). For example, developmental trajectories established in early life influence the response of the individual to later exposures, such as adult lifestyle (Hanson et al. 2011). The connection between pregnancy and disease later in life appears to be in the concept of "fetal programming" whereby a stimulus or insult during a critical period of development has lasting or lifelong effects (Godfrey and Barker 2001).

According to the concept of developmental plasticity, an organism adapts epigenetically during in utero development to the anticipated external environment via cues available within the maternal–fetal microenvironment (Hochberg et al. 2011). As a consequence, the developing fetus is exquisitely sensitive to not only nutritional factors within the maternal circulation, but also to toxicants (Bruner-Tran and Osteen 2011). Furthermore, fetuses and neonates are believed to be

more vulnerable to the effects of environmental pollutants as their organs and detoxification enzymatic systems are relatively immature (Barr et al. 2007).

1.3.2 Prenatal exposure and reproductive effects

The timing of exposure is key to human disease. Findings from epidemiological and toxicological studies have shown that the risk of adverse health outcomes depends not only on the dose and potency of a given toxicant, but also on the occurrence of exposure during critical developmental time periods (Selevan et al. 2000; Wigle et al. 2008).

Fetal growth depends on the genetic potential of the fetus combined with all the environmental factors that could modulate it. Infant gestational age and birth weight are critical components of a pregnancy and reflective of developmental progression from the time of conception to birth. Low birth weight has been associated with infant mortality as well as outcomes later in life such as asthma, lower IQ, and hypertension (Wilcox 2001). Moreover, birth outcomes may be intermediate between prenatal toxic exposures and long-term health; hence the in utero effects of environmental agents on pregnancy outcomes are critical to study.

Accidental exposure of pregnant women to high levels of PCDDs/Fs and dioxin-like PCBs suggested that maternal

exposure to dioxins could affect fetal growth and infant development. In particular, on 10 July 1976, an explosion at a chemical plant near Seveso, Italy, resulted in the highest TCDD levels known in human residential populations (Mocarelli et al. 1988). Some of the effects reported are altered infant sex ratio in the offspring (Mocarelli et al. 1996) and reduction in birth weight in the most heavily exposed individuals (Eskenazi et al. 2003). During the Vietnam War, in a military campaign named the 'Operation Ranch Hand', the US Air Force repeatedly sprayed the herbicide Agent Orange which is contaminated with TCDD, in South Vietnam in an attempt to deprive the vegetation cover used by North Vietnamese forces for concealment. Results of a meta-analysis combining data from 22 studies support the hypothesis that exposure to Agent Orange is associated with a statistically significant increase in the risk of birth defects in the offspring of Vietnamese population and U.S. veterans (Ngo et al. 2006). Mass poisonings, called Yusho and Yu-cheng, occurred in western Japan in 1968 and central Taiwan in 1979, respectively. These occurrences were separately caused by ingestion of rice oils contaminated with PCBs, PCDDs and PCDFs (Masuda 1985). In these cases, exposure during pregnancy resulted in lower birth weight, retarded growth and other adverse birth outcomes (Tsukimori et al. 2012; Yamashita and Hayashi 1985; Rogan et al. 1988; Guo et al. 2004; Tsukimori et al. 2008).

Several studies of lower-level exposure to dioxins and PCBs during pregnancy also reported associations with decreased birth weight or other growth parameters (Fein et al. 1984; Patandin et al. 1998; Hertz-Picciotto et al. 2005; Tajimi et al. 2005; Sagiv et al. 2007; Nishijo et al. 2008; Sonneborn et al. 2008; Konishi et al. 2009; Tawara et al. 2009; Govarts et al. 2012; Karmaus and Zhu 2004; Kezios et al. 2012; Lamb et al. 2006; Murphy et al. 2010; Ribas-Fito et al. 2002; Rylander et al. 2000; Tan et al. 2009; Wu et al. 2011); other studies found no convincing evidence of these associations (Rogan et al. 1986; Longnecker et al. 2005; Weisskopf et al. 2005; Halldorsson et al. 2009; Berkowitz et al. 1996; Givens et al. 2007; Gladen et al. 2003; Khanjani and Sim 2006; Vartiainen et al. 1998; Wolff et al. 2007).

Results also are conflicting for organochlorine pesticides and birth outcomes, with some studies finding negative associations with HCB or DDT/DDE (Ribas-Fito et al. 2002; Weisskopf et al. 2005; Wolff et al. 2007; Longnecker et al. 2001; Wassermann et al. 1982; Al-Saleh et al. 2012; Kezios et al. 2013; Brucker-Davis et al. 2010; Eggesbo et al. 2009; Fenster et al. 2006; Lopez-Espinosa et al. 2011) and others null associations (Sagiv et al. 2007; Govarts et al. 2012; Karmaus and Zhu 2004; Tan et al. 2009; Rogan et al. 1986; Berkowitz et al. 1996; Gladen et al. 2003; Khanjani and Sim 2006; Fenster et al. 2006; Bjerregaard and Hansen 2000;

Farhang et al. 2005; Garced et al. 2012; Jusko et al. 2006; Pathak et al. 2009).

1.4 Key methodological issues in research on POPs and reproductive effects

It is clear from the above that previous reports of associations between exposure to POPs and pregnancy outcomes are inconsistent. These mixed results may reflect differences in study design, study population, and adjustment for covariates. Some methodological issues to consider in order to advance this line of research are:

1.4.1 Exposure assessment – biologically relevant biomarkers

Exposure assessment is a key aspect of environmental epidemiology. Exposure misclassification may be reduced by measurement of specific receptor activations through in vitro reporter-gene bioassays, as variation in uptake and affinity to the receptor are integrated in the toxic potency measurements. Analytical methods that are used to quantify exposure to dioxins include very sensitive and specific techniques such as high-resolution gas chromatography/high-resolution mass spectrometry (HRGC/HRMS). However, these methods are time-consuming and expensive; they require large sample volumes (frequently tens of ml of blood)

and extensive sample clean-up (Warner et al. 2005). Exposure to dioxins and dioxin-like compounds can be estimated with the DR CALUX bioassay, which is less expensive and quicker (Brouwer et al. 2004). Compared to classical analysis, this assay being cell based is more biologically relevant. The DR CALUX bioassay operates through measuring the biological response elicited following binding at the receptor in a living cell and analyses the total toxic equivalence of chemical mixtures in samples instead of determining the number of individual congeners in a complex mixture.

1.4.2 Outcome assessment – towards sensitive endpoints

Most studies evaluating the endocrine-disrupting effects of environmental chemicals have focused on birth outcomes such as birth weight. Given the current interest in the reproductive and developmental effects of endocrine-disrupting chemicals the identification of novel, endocrine-sensitive outcomes is needed.

Male sexual differentiation is androgen-dependent (and potentially estrogen-dependent), whereas female differentiation occurs largely independently of estrogens and androgens (Diamanti-Kandarakis et al. 2009). Therefore, chemical compounds with endocrine disrupting effects, that

overall mimic estrogens and/or antagonize androgens, might interfere with several pathways of the reproductive system and could have different effects on males and females. Fetal life is considered among the most vulnerable periods for the reproductive function in both humans and animals because during this period, rapid structural and functional events are taking place. Exposures to endocrine disruptors in fetal life have been proposed to affect a range of male reproductive disorders including testicular cancer, cryptorchidism and hypospadias (Wilcox and Bonde 2013; Fernandez et al. 2007) but also endometriosis, irregularities of the menstrual cycle, miscarriages in females (Crain et al. 2008). Reproductive tract malformations and shorter anogenital distance have also been reported.

Anogenital distance (AGD) is the distance from the anus to the genitalia routinely used as a developmental endpoint in animal toxicology studies as a measure of fetal androgen action. In rodents and other mammals AGD reflects the amount of androgens to which a fetus is exposed in early development. AGD is sexually dimorphic; males have a longer AGD than females due to higher in utero androgen exposure and the use of AGD to sex newborns is common (Greenham and Greenham 1977; Marty et al. 2003). AGD usually tracks through life, varies by dose of antiandrogen, and can be predictive of other androgen-responsive outcomes such as hypospadias and cryptorchidism (Gray et

al. 1999). Animal studies have clearly shown that androgen driven masculinisation of all male reproductive tract tissues is mediated during the early programming window and that deficient androgen action during this window induced decreased AGD (Welsh et al. 2008).

In utero exposure to compounds with anti-androgenic activity in rat studies resulted in shortened anogenital distance (Foster 2006; Jiang et al. 2011; Christiansen et al. 2009). Moreover, in male rodents, shortened (weight-adjusted) AGD persists into adulthood (van den Driesche et al. 2011; Hotchkiss et al. 2004) and predicts compromised reproductive function in the mature male (Macleod et al. 2010; Scott et al. 2008). Prenatal exposure of female rodents to exogenous androgens results in physiological and behavioral masculinization including longer and more masculine AGD (Hotchkiss et al. 2007; Wolf et al. 2002; Dean et al. 2012; Wu et al. 2010).

AGD has been identified as an important clinical measure to address endocrine-sensitive endpoints in the U.S. Environmental Protection Agency guidelines for reproductive toxicity studies in humans (Arbuckle et al. 2008) but has only been examined in recent years in human studies. Anogenital distance is sexual dimorphic also in humans and associated with body size (Sathyanarayana et al. 2010; Salazar-Martinez et al. 2004; Thankamony et al. 2009; Papadopoulou et al. 2013). A longitudinal study showed that neonatal anogenital

distance increases from birth to the 1st year of life when it reaches a plateau (Thankamony et al. 2009). Moreover anogenital distances of boys and girls can track through life (Papadopoulou et al. 2013) and are highly reliable anthropometric measurements (Papadopoulou et al. 2013; Romano-Riquer et al. 2007).

In utero exposure to endocrine disruptors can affect the reproductive system and AGD has been inversely associated with prenatal exposure to environmental endocrine disruptors, namely phthalates, dichlorodiphenyldichloroethylene (DDE), and bisphenol A (BPA) (Miao et al. 2011; Swan et al. 2005; Torres-Sanchez et al. 2008). It has been suggested that human hypospadias and cryptorchidism may be associated with reduced AGD as a result of endocrine disruption (Hsieh et al. 2012; Hsieh et al. 2008; Jain and Singal 2013). Prenatal stress was associated with significantly longer AGD in female infants suggesting that prenatal stress may masculinize some aspects of female reproductive development in humans (Barrett et al. 2013). Findings of recent studies have linked AGD length and reproductive parameters in adulthood. Decreased AGD was associated with poorer semen quality (Eisenberg et al. 2012a; Mendiola et al. 2011), fertility (Eisenberg et al. 2011), hypogonadal testosterone levels (Eisenberg et al. 2012b), while prostate cancer patients found to have shorter distances than non-patients (Castano-Vinyals et al. 2012). Only one study has examined the relationships

between AGD and female reproductive system characteristics in adult women and they reported that AGD was positively and strongly associated with the presence of greater ovarian follicular number (Mendiola et al. 2012).

1.4.3 Evaluation of key confounders and effect modifiers

A key challenge in epidemiological studies is the concern that the observed effect is indeed related to the exposure of interest and not confounded by correlated factors. If confounding is not addressed in the epidemiologic study design or the data analysis, the effect of the contaminant exposure will be underestimated. For example, a recent paper (Verner et al. 2013) reporting the use of pharmacokinetic models has questioned the validity of the association between PCBs and other POPs with lower birth weight and has suggested that the observed association may be due to uncontrolled confounding by maternal gestational weight gain (GWG). This hypothesis suggests that GWG is associated negatively with PCB levels in maternal and cord blood and positively with birth weight, hence, could substantially confound the association of PCBs and birth weight.

2 RATIONALE

Every pregnant woman is exposed to many and varied environmental chemicals, including POPs, that are persistent and ubiquitous in the environment. Preconception and prenatal exposure to environmental chemicals are of particular importance.

The fetus and young infant appear to be susceptible to endocrine-disrupting effects of environmental chemicals. Exposure during critical developmental phases, such as in utero and in the early postnatal period, may have a profound and lasting impact on health across the life course. Striking evidence of adverse health outcomes was found in association with high-level in utero exposure to chemicals like dioxins, PCBs and organochlorine pesticides. However, the scientific evidence on low-level prenatal exposure to such chemicals in relation to reproductive and developmental health is inconclusive, and the inconsistencies and limitations numerous.

Many of these chemicals are endrocrine disruptors that can interfere with normal functions of endocrine and reproductive systems. Anogenital distance is considered an important clinical measure to address endocrine-sensitive endpoints. In human studies anogenital distance has been examined only in recent years and the results for the effects of prenatal

exposure to environmental chemicals on childrens anogenital distance are iconsistent.

3 OBJECTIVES

3.1 General objective

The main aim of this thesis is to evaluate the effect of in-utero exposure to endocrine disrupting chemicals on birth outcomes and anogenital distance of the child within five European population-based cohort studies in Greece, Spain, England, Denmark and Norway.

3.2 Specific objectives

- To evaluate the association between in utero dioxin-like activity and birth outcomes in 5 European mother-child studies, within the NewGeneris project.
- To examine whether in utero exposure to current low levels of different POPs is associated with fetal growth and gestational age in a mother-child cohort in Crete, Greece (Rhea study) and to evaluate specifically whether maternal gestational weight gain may affect this association.
- To assess whether in utero exposure to dioxins and dioxin-like compounds adversely influences anogenital distance in newborns and young children from the Rhea study in Crete, Greece and the Hmar study in Barcelona, Spain.
- To evaluate the association between in utero exposure to current low levels of different POPs and anogenital distance in newborns and young children from the Rhea study in Crete, Greece.

4 METHODS

This section provides an overall view of the study population and the exposure assesment methods used for the different papers included in this thesis. Further methodological details regarding each analysis can be found in the results section.

4.1 Study population

This thesis is based on data from mother-child birth cohorts/biobanks in five European countries; Greece, Spain, England, Denmark and Norway that are part of NewGeneris (Newborns and Genotoxic exposure risks) project (Figure 4).

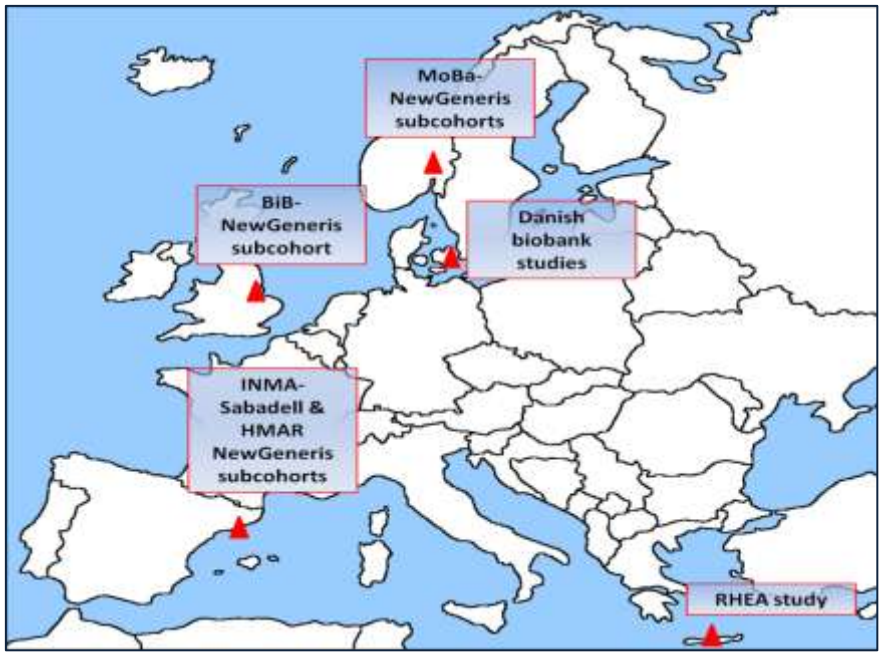

Figure 4. European studies contributing to this thesis.

The NewGeneris is a European project funded by the European 6th Framework Program that started in 2006 and finished in 2011. This project investigated the role of fetal environmental exposures in childhood disease causation and focused on a series of dietary and environmental chemicals (Merlo et al. 2009). Pregnant women enrolled during 2006–2010 in 11 maternity units located in Copenhagen, Denmark; Heraklion, Greece; Oslo and Akershus, Norway; Barcelona and Sabadell, Spain; and Bradford, England (Pedersen et al. 2012). Detailed information on recruitment periods and inclusion criteria for this study are provided in paper I and in table 2 at the end of this chapter.

4.2 Prenatal exposure assesment

4.2.1 Plasma dioxin-like activity - DR CALUX® bioassay

Within the NewGeneris project, peripheral blood samples from the mothers and umbilical cord blood samples from the children were collected in heparinized tubes immediately after delivery. The blood was centrifuged and the plasma was stored at −20 °C. Detailed procedures for blood collection, processing, storage, and distribution were developed for the study (Merlo et al. 2009). Dioxin-like activity in maternal and cord plasma samples was determined through the Dioxin-Responsive Chemically Activated LUciferase eXpression (DR CALUX®) assay at Biodetection Systems B.V., Amsterdam,

the Netherlands. The CALUX® assay is based on a genetically modified H4IIE rat hepatoma cell line which contains the firefly luciferase reporter gene under the transcriptional control of the aryl hydrocarbon receptor. Upon exposure of the cells to dioxins or dioxin-like chemicals, through binding to the aryl hydrocarbon receptor, the cells express luciferase as well as proteins and enzymes associated with dioxin-responsive elements. With addition of the substrate luciferine for the luciferase enzyme, light is emitted in proportion to the strength of the receptor binding. The luminance is calibrated with respect to 2,3,7,8-TCDD in units of toxic equivalency quantity (TEQs), and results are expressed as picograms CALUX®–TEQ/g lipid or as pg CALUX®- TEQ/ml plasma. The protocol for sample processing has been presented elsewhere (Pedersen et al. 2010; Murk et al. 1997) and detailed information can be found in papers I and III.

4.2.2 Serum concentrations of Persistent organic pollutants

Within the Rhea study, maternal serum samples were collected at the first prenatal visit around the 3rd-4th month of pregnancy, centrifuged, and were then stored in aliquots at -80 °C until assayed. The samples were analyzed gravimetrically by triple quadrupole mass spectrometry at the National Institute for Health and Welfare, Chemical Exposure

Unit, Kuopio, Finland. Serum concentrations of six individual PCB congeners (IUPAC numbers: 118, 138, 153, 156, 170 and 180), HCB, DDT and DDE, and BDE47 were determined and expressed as pg/ml serum. Due to high percentages of samples below the limit of quantification (LOQ), DDT and BDE-47 were not used in the analyses. The protocol for sample processing has been presented elsewhere (Koponen et al. 2013) and detailed information can be found in papers II and IV.

4.3 Outcome assesment

4.3.1 Birth outcomes

Information on birth weight, length, and head circumference was gathered from clinical records by each center. Gestational age for participants from Denmark, Greece, Spain and England was based on last menstrual period, and corrected by ultrasound measurement if there was a difference of seven days or more between the two estimates. Ultrasound-based estimation was provided for the majority of participants from Norway. Detailed information on methodology can be found at papers I and II.

4.3.2 Anogenital distance

Anogenital distance measurements were collected within the Rhea and HMAR studies by well trained examiners using a digital caliper. In the Rhea study anogenital distances were measured in newborns and young children while in HMAR study only in newborns. Protocols from published studies (Swan et al. 2005) were modified to include additional measurements (Salazar-Martinez et al. 2004; Callegari et al. 1987) and a standardized analytical protocol develped. Anogenital distance (AGD; anus to upper penis), anoscrotal distance (ASD; anus to scrotum), and penis width (PW) were measured in boys; anoclitoral (ACD; anus to clitoris) and anofourchetal distance (AFD; anus to fourchette) were measured in girls (Figure 5).

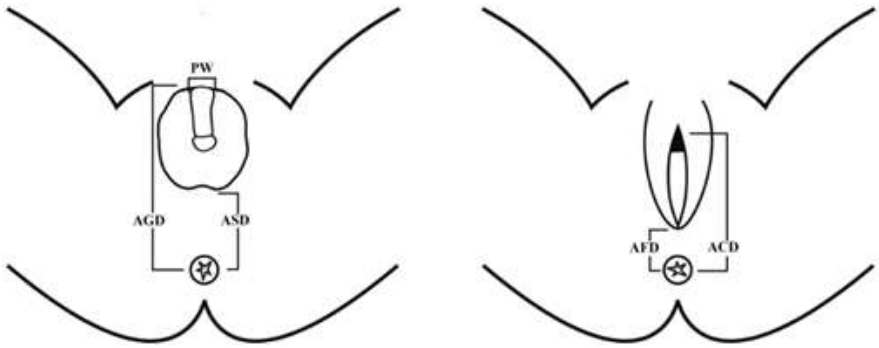

Figure 5. Schematic diagram of anogenital distance measurements in boys and girls.

Each measurement was repeated three times and the average of the three measurements was recorded. Weight,

length, and head circumference were also collected, and average values were used for analysis. Further details on the anogenital distance measurements can be found in paper III and IV.

Similar to other anthropometric measurements, the reliability of anogenital distances is influenced by measurement error. For that reason, within the Rhea study, we conducted a reliability study for the genitalia measurements. Thirteen males and 17 females (mean age: 23 months) participated and 1460 measurements were done in total, by two examiners (EP and MV). They were singleton births, randomly selected among the youngest children of the birth cohort. Each child was measured by both examiners, at two scheduled home visits, one visit for each examiner. Each examiner did 10 repeated blind measurements per visit, resulting in two sets of 10 measurements for each distance. Thus we collected 40 measurements for each girl (for ACD and AFD) and 60 measurements for each boy (for AGD, ASD and PW). To ensure that the examiner was not biased, the instrument's screen was covered and the measurement was read and recorded by the assistant. Examiners were therefore blind concerning their own measurements. (Papadopoulou et al. 2013).

Table 2. Description of study population included in this thesis and availability of data on exposures and outcomes.

Study		Location	Period of Recruitment	Biomarker availability	Anthropometry at birth	Anogenital distance
Rhea study		Heraklion, Crete, Greece	February 2007 to February 2008	Maternal and cord blood DR CALUX®, maternal blood POPs	yes	yes
INMA (INfancia y Medio Ambiente) - NewGeneris subcohorts	INMA-Sabadell cohort	Sabadell, Catalonia, Spain	May 2007 to June 2007	Maternal and cord blood DR CALUX®	yes	no
	HMAR study	Barcelona, Catalonia, Spain	October 2008 to March 2010	Maternal and cord blood DR CALUX®	yes	yes
BiB (Born in Bradford) - NewGeneris subcohort		Bradford, England	January 2008 to December 2009	Maternal and cord blood DR CALUX®	yes	no
Danish Biobanks	Danish biobank '07	Copenhagen, Denmark	December 2006 to December 2007	Maternal and cord blood DR CALUX®	yes	no
	Danish biobank '09		September 2009 to December 2009	Maternal and cord blood DR CALUX®	yes	no
MoBa (Norwegian Mother & Child Cohort Study) - NewGeneris subcohorts		Oslo & Akershus, Norway	April 2007 to July 2008	Maternal and cord blood DR CALUX®	yes	no

5 RESULTS

Paper I: In utero exposure to compounds with dioxin-like activity and birth outcomes.

Main findings

- Plasma dioxin-like activity was higher in maternal than cord samples and levels differed among countries.
- Findings from this international study of in utero low-level dioxin-like activity show an association with shorter gestational age mainly in boys.
- Weaker associations were detected for birth weight, and no association was found for head circumference.
- No associations were observed between maternal serum dioxin-like activity and any of the birth outcomes.

Paper II: Persistent organic pollutants exposure during pregnancy, maternal gestational weight gain, and birth outcomes in the mother-child cohort in Crete, Greece (RHEA study).

Main findings

- Increasing maternal serum levels of HCB and PCBs were associated with decreased birth weight.
- Mothers with excessive gestational weight gain tended to have lower POP levels.

- Adjustment for maternal gestational weight gain explained only to a small extent the association between POP levels and birth weight.
- Small reductions were observed for head circumference, while associations were close to the null for gestational age.

<u>Paper III</u>: In Utero Exposure to Dioxins and Dioxin-like Compounds and Anogenital Distance in Newborns and Infants.

Main findings

- Anogenital distances were sexually dimorphic, being longer in males than females.
- Plasma dioxin-like activity was negatively associated with AGD in male newborns.
- Negative but smaller and nonsignificant associations were observed for AGD in young boys while no associations were found in girls.
- Male infants may be susceptible to endocrine-disrupting effects of dioxins. Our findings are consistent with the experimental animal evidence used by the Food and Agriculture Organization/World Health Organization to set recommendations for human dioxin intake.

<u>Paper IV</u>: Prenatal exposure to persistent organo-chlorines and anogenital distance in the mother-child cohort in Crete, Greece (RHEA study).

Main findings

- Negative but not statistically significant associations were observed for AGD and HCB, DDE and total PCBs in young boys.
- In young girls, ACD and AFD were positively but not significantly associated with all organochlorine compounds.
- Our results provided some evidence of an endocrine disruptive effect of organochlorine compounds, expressed as phenotypic alterations of the reproductive system of young boys and girls.

5.1 Paper I

In utero exposure to compounds with dioxin-like activity and birth outcomes[1]

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In utero **exposure to compounds with dioxin-like activity and birth outcomes.**

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Abstract

Background: Maternal exposure to dioxins and dioxin-like compounds may affect fetal growth and development. We evaluated the association between in utero dioxin-like activity and birth outcomes in a prospective European mother-child study.

Methods: We measured dioxin-like activity in maternal and cord-blood plasma samples collected at delivery using the Dioxin-Responsive Chemically Activated LUciferase eXpression (DR CALUX®) bioassay in 967 mother-child pairs, in Denmark, Greece, Norway, Spain and England. Multiple linear regression models were used to investigate the associations with birth weight, gestational age, and head circumference.

Results: Plasma dioxin-like activity was higher in maternal sample than in cord samples. Birth weight was lower with medium (-58 g 95% Confidence Interval (CI)= -176 to 62) and high (-82 g 95% CI: [-216 to 53]) tertiles of exposure (cord-blood) compared with the lowest tertile. Gestational age was shorter by approximately half a week in the highest compared with the lowest (-0.4 weeks [95% CI= -0.8 to -0.1]). This association was stronger in boys than in girls, although the statistical evidence for interaction was weak (p for interaction = 0.22). Analysis based on CALUX®-toxic equivalents expressed per ml of plasma showed similar trends. We found no association between dioxin-like activity in maternal plasma and birth outcomes.

Conclusions: Results from this international general population study suggest an association between low-level prenatal dioxin-

like activity and shorter gestational age, particularly in boys, with weaker associations for birth weight.

Introduction

Polychlorinated dibenzodioxins (PCDDs), dibenzofurans (PCDFs) and biphenyls (PCBs) represent a group of polyhalogenated aromatic hydrocarbons that are widespread and persistent organic pollutants. 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), the most toxic member of this group, has been classified as a known human carcinogen by the International Agency for Research on Cancer (IARC) and has been shown to disrupt multiple endocrine pathways.[1-3] In recent years two more members of this group, 2,3,4,7,8-pentachlorodibenzofuran and 3,3′,4,4′,5-pentachlorobiphenyl (PCB 126) have been classified as carcinogenic to humans (Group 1) by IARC.[4] PCDDs, PCDFs and dioxin-like PCBs are lipophilic, have long half-lives, and therefore accumulate in the body. Food, of animal origin is the main source of dioxin exposure for humans, contributing over 95% of total exposure for a non-occupationally exposed person.[5] Accumulated dioxins can be transferred from mother to fetus prenatally through the placenta,[6] and from mother to child postnatally via breast milk.[7] The toxic effects of PCDDs, PCDFs and dioxin-like PCBs are mediated mainly through binding to the aryl hydrocarbon receptor, which is expressed in many tissues of the human body.[8] Upon exposure to TCDD, the aryl hydrocarbon receptor translocates into the nucleus, where it heterodimerizes with the aryl hydrocarbon receptor nuclear translocator. This complex then binds to its

specific DNA recognition sites to activate the transcription of dioxin responsive genes,[9] such as the drug-metabolizing enzymes CYP1A1 and CYP1A2.[10] Aryl hydrocarbon receptor is activated by a variety of exogenous ligands and apart, from its role as a mediator of the biochemical response to xenobiotics, aryl hydrocarbon receptor plays key endogenous regulatory roles in normal physiology and development.[11]

Dioxins and dioxin-like compounds are found in environmental and biological samples as complex mixtures of various congeners. Therefore, a calculation of total TCDD toxic-equivalent concentration simplifies risk assessment and regulatory control, and is the most relevant exposure measure in studies of health effects of dioxins and dioxin-like compounds.[12,13] The Dioxin-Responsive Chemically Activated LUciferase eXpression (DR CALUX®) assay measures the ability of such mixtures to activate the aryl hydrocarbon receptor-dependent gene expression of the firefly luciferase gene in genetically modified cell lines, requiring for this purpose only small amounts of blood plasma.[14] Moreover because assay includes an extensive clean-up procedure, to remove other contaminations or chemicals, only stable compounds (e.g. dioxins, furans and dioxin-like PCBs) are measured.

Studies of pregnant women who were accidentally exposed to high levels of PCDDs/DFs and dioxin-like PCBs have suggested that maternal exposure to dioxins can affect fetal growth and infant development. In particular, a chemical plant explosion near Seveso, Italy in 1976 led to the highest known exposure of

residential populations to TCDD. Some of the effects reported are altered infant sex ratio in the offspring[15] and reduction in birth weight in infants born to the most heavily exposed mothers.[16] The prevalence of birth defects was reported higher in the offspring of Vietnamese population and U.S. veterans exposed to the herbicide Agent Orange contaminated with TCDD,[17] and birth weight was reduced among infants exposed in utero to PCBs.[18-20] Several studies have examined exposure to dioxins and PCBs during pregnancy in relation to adverse birth outcomes in infants.[21-30] Only three studies have evaluated birth outcomes and dioxin-like compounds in cord blood[21,22,26] (which is probably the most direct measure of contaminants passed to the fetus during pregnancy); however, these studies measured only a limited selection of PCBs. Five studies reported sex-specific results,[19,23,24,28,29] and these indicated a stronger deficit in boys. However, these associations have not been demonstrated in other studies.[31-34]Among the epidemiologic studies mentioned above, some examined possible effects of various mixtures of PCB congeners[21,22,24,28,31-33] and some focused on dioxin-like effects.[23,25-27,29,30,34] In the present study of newborn children from five European countries, the DR CALUX® bioassay was used to measure dioxin-like activity in maternal and cord plasma in association with birth weight, birth head circumference and gestational age, including sex- specific results.

Methods

Study population

Data come from extensions of four existing European birth cohorts and two newly established biobanks: the Mother-Child cohort in Heraklion, Crete, Greece; the Spanish Mother-Child cohort in Sabadell; Spain, sub-cohorts to the Norwegian Mother-Child cohort study in Oslo and Akershus; the Born in Bradford cohort study in Bradford, England; the Hospital del Mar study in Barcelona, Spain; and the Danish NewGeneris biobanks in Copenhagen, Denmark, all participants in the Newborns and Genotoxic exposure risks (NewGeneris) EU-funded project.[35] Pregnant women were enrolled from 2006 to 2010 in eleven maternity units located in Copenhagen, Denmark; Heraklion, Greece; Oslo and Akershus, Norway; Barcelona and Sabadell, Spain; and Bradford, England.[36-41] All research procedures were approved by the local ethical committees, and written informed consent was obtained from all women participating in the studies. Rules for ethical conduct are stated in the Technical Annex to the contract with the EU Commission (Contract No 016320-2) and in the Ethical Review documents submitted to the European Commission. Additional information can be found on the NewGeneris website.[42] The present analysis included 967 mother-child pairs (singletons only with information on birth outcomes and at least one measure of DR CALUX® [cord, maternal, DR CALUX® pg TEQ/g lipid, or DR CALUX® pg TEQ/ml plasma]).

Data collection

Information on birth weight, length, and head circumference was gathered from clinical records by each center. Gestational age for participants from Denmark, Greece, Spain and England was based on last menstrual period, and corrected by ultrasound measurement if there was a difference of seven days or more between the two estimates. Ultrasound-based estimation was provided for the majority of participants from Norway. Questionnaires and medical records were used to obtain information on nutrition, maternal health, occupational and environmental exposures, lifestyle and socioeconomic factors during pregnancy in each study.

Blood sample collection, processing, storage, and distribution

Detailed procedures for blood collection, processing, storage, and distribution were developed for the study.[35] Peripheral blood samples from the mothers and umbilical cord blood samples from the children were collected in heparinized tubes immediately after delivery. To prevent clotting, 0.5 mL extra heparin was added to the tubes used to collect umbilical cord blood. The blood was centrifuged and the plasma was stored at −20 °C until shipment to the Netherlands on dry ice.

DR CALUX® bioassay

Dioxin-like activity in maternal and cord plasma samples was determined through the Dioxin-Responsive Chemically Activated LUciferase eXpression (DR CALUX®) assay at Biodetection

Systems B.V., Amsterdam, the Netherlands. The CALUX® assay is based on a genetically modified H4IIE rat hepatoma cell line which contains the firefly luciferase reporter gene under the transcriptional control of the aryl hydrocarbon receptor. Upon exposure of the cells to dioxins or dioxin-like chemicals, through binding to the aryl hydrocarbon receptor, the cells express luciferase as well as proteins and enzymes associated with dioxin-responsive elements. With addition of the substrate luciferine for the luciferase enzyme, light is emitted in proportion to the strength of the receptor binding. The luminance is calibrated with respect to TCDD toxic equivalents and results are expressed as picogram (pg) CALUX®-toxic equivalent per gram of lipid. In situations where the fat content of the samples is very low (as is frequently the case for cord blood), results of the DR CALUX® bioassay can be alternatively normalized against the amount of plasma used for the analysis and expressed as pg CALUX®-toxic equivalent/mL plasma. The DR CALUX® bioassay has shown a high agreement with congener-specific methods and has been used in many epidemiologic and biomonitoring studies.[14,34,37,43-49] The protocol for sample processing has been presented in further details previously,[44,50] and additional details are provided in the eAppendix. The assay analysis was performed on 791 mother and 269 cord blood samples with enough fat to express the bioassay results per gram fat. Dioxin-like activity expressed by ml of plasma (819 cord and 896 maternal samples) was also calculated.

Statistical analysis

Due to non-normality, maternal and cord plasma dioxin-like activity levels were log_{10}-transformed when analyzed as continuous variables. We used multivariate linear regression models to examine how maternal and cord dioxin-like activity varies by categories of selected maternal and fetal characteristics and to calculate unadjusted and country adjusted geometric means and 95% confidence intervals (95% CIs).

For the main analysis based on CALUX®-TEQ expressed per gram fat, samples below the limit of detection (LOD) for DR CALUX® bioassay were assigned a value equal to 0.5×LOD. The limit of detection was 12 pg CALUX®-toxic equivalent/g lipid for the maternal and 20 pg CALUX®- toxic equivalent/g lipid for the cord samples. The percent of samples below the LOD was 23% in the cord and 19% in the maternal samples. For the analysis based on CALUX®-toxic equivalent expressed per ml of plasma irrespective of fat content, 360 cord blood samples (44%) were below the limit of detection that was 0.11 pg CALUX®-toxic equivalent/ml plasma. Because this proportion is high, no value was assigned to these samples and analyses were limited to the 459 samples above the limit of detection.

Generalized additive models (GAMs) were applied to explore the shape of the relationships between dioxin-like activity in maternal and cord plasma and birth outcomes. Multivariate linear regression models were used to examine the association of log_{10}-

transformedcord and maternal dioxin-like plasma activity as continuous variables and as tertiles with birth outcomes.

Country of enrolment (Greece, Spain, England, Denmark and Norway), gestational age (completed weeks) and gestational age squared were always included in the regression models for birth weight and head circumference, while country alone was included in the models for gestational age. Covariates evaluated as potential confounders, selected a priori to be comparable with previous studies, included: smoking during pregnancy (no/yes), maternal age (years), parity (primiparous/multiparous), pre-pregnancy body mass index (BMI) (kg/m^2), maternal educational level (low/medium/high), maternal ethnicity (non white/white), delivery type (vaginal delivery/Cesarean section) and infant sex.

We followed two adjustment strategies. First, we ran fully adjusted models that included all potential confounders that predicted the outcome with $p < 0.2$ when added to the basic model. Second, we used causal diagrams (eFigure 1 for birth weight) to select variables. Results were similar following both adjustment methods, and we present those from the models constructed with the first approach. In addition to estimating associations adjusted for country, we estimated country-specific associations between dioxin-like activity and birth weight and gestational age, and performed a meta-analysis to derive pooled estimates of effect. Heterogeneity of effects across studies was assessed by the Cochran's Q test and the coefficient of inconsistency (I^2). Only results from fixed-effects models are shown because

heterogeneity of effects between countries was not statistically significant.

Effect modification of dioxin-like activity on birth outcomes by child's sex, smoking status and maternal pre-pregnancy BMI (Normal 18.5-25 kg/m^2, Overweight/Obese ≥25 kg/m^2) was assessed through inclusion of interaction terms in the models and stratified analyses. We also performed sensitivity analyses to test robustness of the results. Analyses were conducted using STATA software, version 10.0 (Statacorp, College Station, TX).

Results

Participant's characteristics

In the study population with cord dioxin-like activity measurements, 48% of the infants were boys and 55% were vaginally delivered (Table 1). Maternal mean age at childbirth (standard deviation [SD] =31 (5) years. 92% of the mothers were white, and two-thirds were primiparous. The median pre-pregnancy BMI was 23 kg/m^2 (interquartile range=5.6), with 33% percent of the mothers being overweight or obese (BMI≥25 kg/m^2) before pregnancy. One-quarter had what was considered to be a low educational level. Smoking during pregnancy was reported by 17% of the women.

The study population with measurements of maternal dioxin-like activity had similar characteristics with 50% of the children being boys, 58% vaginally delivered and 35% firstborn (Table 1). Mean maternal age was 31 (SD = 5.0) years.BMI had a median value of 23 kg/m^2 (interquartile range=5.4), with 31 percent of the mothers being overweight or obese before pregnancy. Again, one-quarter

had a low educational level, and smoking during pregnancy was reported by 17%.

Dioxin and dioxin-like compounds plasma activity levels and birth outcomes

Dioxin-like activity levels in cord and maternal plasma differed among countries (Table 1). The highest levels of cord dioxin-like activity were found in England (geometric mean=59.7 pg CALUX®-TEQ/g lipid) and the lowest in Spain (16.9 pg).The highest levels of dioxin-like activity in maternal plasma were found in Spain (46.4 pg) and the lowest in Greece (15.5 pg). Moreover, cord dioxin-like activity differed between infants delivered vaginally (25.3 pg) and by caesarean (32.5 pg) – largely due to different distributions of the type of delivery by country and gestational age.

Cord dioxin-like activity increased with maternal dioxin-like activity, although the overall correlation was modest (Spearman Rank Coefficient=0.18) and differences were observed among countries (Figure 1). Plasma dioxin-like activity was higher in maternal than cord samples with median (IQR) values of 38.3 (29.3) and 34.2 (28.9) pg, respectively.

Mean birth weight in the study population with cord dioxin-like activity measurements was 3476 (SD = 450) g, mean head circumference was 352 (15) mm, and average gestation was 39 (±1) weeks (Table 2). Among those children with information on maternal dioxin-like activity, mean birth weight was 3447 (456) g, mean head circumference was 349 (2) mm, and mean gestational age was 39 (±1) weeks.

Association between dioxin-like activity and birth outcomes

GAMs examining the shape of the relationship between dioxin-like activity in maternal and cord plasma and birth outcomes indicated linear relationships for all birth outcomes (test for linearity in cord models, for birth weight, p=0.648; for head circumference, p=0.449; for gestational age, p=0.417; in maternal models: for birth weight, p=0.749, for head circumference, p=0.741; for gestational age, p=0.398). Table 3 shows the unadjusted and adjusted regression results for dioxin-like activity in relation to gestational age and fetal growth outcomes. Because models were run with CALUX-TEQ on the $\log_{10}$ scale, a unit of increase would mean a 10-fold increase in the dioxin-like activity. Increasing levels of cord dioxin-like activity were associated with a 76 g-decrease in birth weight (95% CI =-251 to 100) in the adjusted model (Table 3). A lower birth weight was observed comparing infants with cord plasma in the medium (-58 g, [95% CI = -176 to 62]) and high (-82 g [-216 to 53]) tertiles to those in the low levels tertile. A shorter gestational age of approximately half a week was observed comparing the highest exposure with the lowest (-0.4 weeks [-0.8 to -0.1]).

No association was detected between cord dioxin-like activity and head circumference, and no meaningful associations were found between maternal dioxin-like activity and any of the birth outcomes.

Analysis limited to subjects above the limits of detection showed a similar pattern for both birth weight and gestational age as for the

whole population. We obtained very similar results when we included in every model covariates selected through the use of causal diagrams (eFigure 1: maternal age, parity, pre-pregnancy BMI, maternal educational level and maternal ethnicity). When maternal models were adjusted for cord dioxin-like activity and vice versa, and when analysis was restricted to the sample that had both maternal and cord CALUX-TEQ levels, no change was observed in the results regarding the association between cord dioxin-like activity and birth outcomes (eTable 2 and 3). Similarly stratified analysis by maternal pre-pregnancy BMI (normal 18.5-25g/m^2/overweight/obese ≥25 kg/m^2) did not change our results (not shown). Cord dioxin-like activity had a stronger association with reduced birth weight in boys (-124 g [95% CI =-391 to 144]) than in girls (-57 g [-300 to 185]) although there was no clear indication for effect modification by sex (p-test for interaction=0.22). No interaction by sex was observed for gestational age (p=0.97). Stronger associations were also observed between cord dioxin-like activity and birth weight among smokers compared with nonsmokers, but again the interaction terms were not significant (p=0.80). Negative associations between cord dioxin-like activity and birth weight were observed for 4 out of 5 countries (Greece [n=43]:β= -189 g [95% CI =-585 to 208]; Spain (n=24):-57 g [-756 to 641]; Norway [n=81]:182 g [-127 to 492]; England (n= 11):-1002 g [-23323 to 21319]; and Denmark (n=88):-83 g [-440 to 275]), while a decrease in gestational age was found in all countries (Greece [n=43] :β = -0.4 weeks, [-1.7 to

0.8]; Spain (n=32):-0.3 weeks, [-2.6 to 2.1]; Norway (n=79): -0.4 weeks [-1.3 to 0.6]; England (n= 18):-0.2 weeks [-1.6 to 1.2]; and Denmark (n=88);-0,5 weeks [-1,3 to 0.3]). A meta-analysis of country-specific estimates showed that a 10-fold increase in cord CALUX®-TEQ/g lipid was associated with a 2 g (95% CI =-196 to 191)-decrease in birth weight, and a -0.4 week (-0.9 to 0.1)-decrease in gestational age, with little heterogeneity between countries for birth weight, (I^2=0.0%,Q = 2.4 on 4 degrees of freedom, p=0.66;for gestational age, I^2=0.0%,Q=0.2 on 4 degrees of freedom, p=0.996).

When we expressed dioxin-like activity per volumetric unit rather than per gram of fat, the median was 0.1 (IQR 0.2, n=819) pg CALUX®-TEQ/mL plasma with 44% <LOD for cord blood samples (Table 2). Results for the analyses based on volume (ml plasma) for all subjects did not show a consistent pattern, while those based on subjects above limits of detection had patterns for birth weight and gestational age that were similar to those based on gram of lipid. These estimates had very wide confidence intervals. For example the adjusted estimate for birth weight with each 10-fold increase in CALUX®-TEQ/mL for subjects above the LOD, was -54 grams (95% CI =-227 to 170; n=404). The corresponding estimates for gestational age were -0.5 weeks (95% CI= -1 to 0.2; n=441).

Discussion

Findings from this international study of in utero low-level dioxin-like activity show an association with shorter gestational age.

Newborns in the highest tertile of exposure had a reduction of approximately half a week in their gestational age as compared with those in the lowest tertile. This association was observed in boys and was the only sex-related association found. Weaker associations were detected for birth weight, and no association was found for head circumference. No associations were observed between maternal serum dioxin-like activity and any of the birth outcomes.

Our findings on the association of dioxin-like activity in cord plasma and length of gestation are consistent with a study in Taiwan that reported small associations of incinerator-generated dioxin exposure and gestational age.[51] However, Longnecker et al[32] found that maternal levels of PCBs during pregnancy were unrelated to length of gestation. We found more pronounced effect estimates in boys, unlike Hertz-Picciotto et al,[24] who reported that maternally mediated exposure to PCBs was associated with shorter gestations in girls only. These studies, however, examined the possible effects of mixtures of PCB congeners, rather than focusing on the dioxin-like effects.

Accidental exposure of pregnant women to high levels of PCDDs/DFs and dioxin-like PCBs, has suggested that maternal exposure to dioxins can affect fetal growth and infant development.[15-18,20,52] There is still controversy over whether low-level exposure to these compounds is associated with decreased birth weight in humans. Some studies have associated low-level dioxin and dioxin-like exposure during pregnancy with decreased

birth weight,[23,25,29] but not all,[26,27,34] including a small study in Denmark using the DR CALUX® bioassay,[34]some studies reported associations with other birth outcomes but not birth weight.[27,30]

Few studies have reported a sex difference in the effects of dioxins on birth weight. One Finnish study that reported a negative correlation between birth weight and total dioxin levels in breast milk saw a stronger effect in boys,[23] as did one Japanese study investigating dioxins in maternal blood.[29] Another Japanese study found inverse associations with birth weight among boys, but not girls, suggesting that male infants are more susceptible than females to dioxins; however these findings are derived from a unique cohort of women highly exposed to PCBs, PCDDs and PCDFs in the Yusho incident (accidental human exposure to contaminated rice oil, Japan 1968).[19] In our study, in utero exposure to dioxins did not show any sex-specific effect on birth weight.

Sex-specific results in our study and others suggest greater susceptibility of male infants to the effects of environmental contaminants with dioxin-like activity.[37,53] Some experimental animal studies support to this hypothesis, including sex-specific transgenerational effects on the reproductive system.[54] The mechanisms, through which growth restriction induced by in utero dioxin exposure might be more pronounced in boys remain unclear.

One possible explanation for inconsistent findings in the literature on dioxins and dioxin-like compounds in relation to birth outcomes

may relate to differences in exposure assessment. Previous epidemiologic studies on birth outcomes have relied on surrogate indicators of exposure (eg, fish consumption,[30] residence near waste sites[28] or in areas with high consumption of contaminated fish,[21,33] etc). Some studies have focused only on PCBs[21,22,24,26,28,31,32] and others on PCDDs/PCDFs,[27,30] while others have measured the congeners in milk[23,25,27,30] and others in maternal[24,28,29,32-34] and cord plasma.[21,26] Exposure misclassification may be reduced by measurement of specific receptor activations through in vitro reporter-gene bioassays, as variation in uptake and affinity to the receptor are integrated in the toxic potency measurements. In this study, exposure to dioxins and dioxin-like compounds was estimated in plasma with the DR CALUX® bioassay. Use of such a biomarker to measure exposure, in both cord plasma and maternal plasma, is a notable strength of this study. Methods for the quantification of dioxin exposure include sensitive and specific techniques such as high-resolution gas chromatography/mass spectrometry that measure the concentrations of specific compounds. However, these methods are time-consuming and expensive, and they require large sample volumes.[12] While the DR CALUX® does not quantify specific compounds, it provides an overall biologic response/potency of the mixture and takes into account possible interactions (synergistic, additive and antagonistic interaction) among congeners.[55]

Dioxin-like activity was higher in maternal samples compared with fetal samples. Although cord blood dioxin-like activity increased

with maternal plasma dioxin-like activity, the correlation was weak and differed among countries. Many studies have reported lower levels of dioxins and dioxin-like compounds in cord blood than in maternal samples,[7,22,44,56,57] but the reported correlations between maternal and cord blood levels are generally stronger. However the only study that used DR CALUX® to estimate exposure and examined the correlation between maternal and fetal dioxin-like activity reported results similar to ours (Spearman Rank Coefficient= 0.3.[44] Populations may differ in the mixture of dioxins, furans, and PCBs to which they were exposed, which could contribute to differences in correlations by country. Moreover, this lack of correlation may be due to the characteristics of the assay evaluating exposure through aryl hydrocarbon receptor binding that may differ between mother and offspring and could reflect the rate of transfer of dioxins through the placenta. The placenta not only connects the developing fetus to the mother's blood supply but also functions as a selective maternal-fetal barrier against transfer of several molecules. Experimental models of ex vivo human placenta perfusion systems have reported increased dioxin-like activity in the perfused placenta tissue after ex vivo TCDD perfusions.[44] These findings suggest that accumulation in the placenta might delay transplacental transfer of TCDD. Elevated levels of dioxins and dioxin-like compounds have previously been detected in placentas, compared to corresponding maternal and fetal blood samples.[58-60] Moreover, data from three ex vivo placental perfusion laboratories within the NewGeneris

project on the placental transport of thirteen immunotoxic and genotoxic agents were recently meta-analyzed, and the results showed that TCDD had the lowest transfer rate.[6] Moreover, we found that maternal (in contrast to fetal) dioxin-like activity was not associated with any of the birth outcomes evaluated. All the above suggest that the mechanisms by which dioxins are transferred from mother to fetus through the placenta are complex and not yet fully understood.

In conclusion, our data from the NewGeneris project provide evidence supporting an association of low-level prenatal dioxin-like activity with shorter gestational age, particularly in boys, while weaker associations were found for birth weight.

Table 1. Distribution of cord and maternal dioxin-like activity in plasma by the characteristics of study participants.[a]

	DR CALUX®pg TEQ/g lipid			
	Cord plasma		Maternal plasma	
	No.(%)[a]	Geometric mean[b] (95%CI)	No.(%)[a]	Geometric mean[b] (95%CI)
Characteristics	Mother			
Country of residence				
Greece	44 (16)	22.5(17.3 to 29.2)	149 (19)	15.5(13.3 to 18.1)
Spain	35 (13)	16.9(13.3 to 21.4)	157 (20)	46.4(43.1 to 50.1)
Norway	81 (30)	25.9(22.7 to 29.5)	184 (23)	24.6(21.7 to 27.9)
England	20 (8)	59.7(43.3 to 82.4)	111 (14)	26.0(22.1 to 30.7)
Denmark	89 (33)	41.0(36.4 to 46.2)	190 (24)	45.7(42.9 to 48.5)
Maternal age (years); mean[c](SD)	31.4(4.6)		31.1(5.0)	
<25	18 (7)	28.2(20.7 to 38.6)	77 (10)	31.2(26.4 to 37.0)
25-35	188 (70)	28.4(25.7 to 31.4)	531 (67)	28.8(27.1 to30.7)
>35	63 (23)	27.6(23.3 to 32.7)	180 (23)	30.4(27.3 to 33.9)
Parity				
Primiparous	87 (33)	27.0(23.4,31.2)	268 (35)	30.6(28.0,33.5)
Multiparous	178 (67)	29.4(26.6,32.5)	497 (65)	29.5(27.6,31.5)
Pre-pregnancy BMI (kg/m^2)	22.9(5.6)		22.9(5.4)	
Underweight (<18.5)	9 (4)	21.1(13.8,32.3)	27 (4)	29.7(22.5,39.1)
Normal (18.5-25)	160 (63)	26.6(23.9,29.7)	465 (66)	30.0(28.0,32.1)
Overweight (≥25-30)	56 (22)	26.7(22.4,31.8)	144 (20)	29.4(26.1,33.2)
Obese (≥30)	28 (11)	28.2(22.10,35.9)	74 (10)	30.1(25.3,35.9)
Maternal education				
High	89 (40)	31.8(25.6,39.4)	255 (38)	30.4(27.7,33.3)
Medium	81 (37)	29.4(23.2,37.1)	254 (38)	30.1(27.6,33.0)
Low	50 (23)	31.9(24.7,41.1)	162 (24)	32.0(28.6,35.9)
Smoking during pregnancy				
No	221 (83)	28.2(25.5,31.2)	642 (83)	29.5(27.8,31.3)
Yes	44 (17)	28.3(22.9,34.9)	132 (17)	29.4(25.7,33.6)
Maternal ethnicity				
Nonwhite	21 (8)	27.9(20.5,38.0)	122 (15)	30.8(26.4,35.9)
White	246 (92)	28.5(25.9,31.4)	663 (85)	29.1(27.5,30.9)
Type of delivery				
Vaginal	148 (55)	25.3(22.4,28.5)	454 (58)	30.0(27.8,32.4)
Caesarean	120 (45)	32.5(28.5,37.1)	335 (42)	28.5(25.9,31.3)
	Child			
Sex				
Boys	128 (48)	27.5(24.4,30.9)	392 (50)	29.4(27.3,31.7)
Girls	141 (52)	29.0(25.8,32.5)	399 (50)	29.4(27.3,31.6)

[a] Except where indicated [b]Country-adjusted geometric mean and 95% confidence intervals
[c]For cord plasma, m = 269; for maternal plasma, n = 788
[d]For cord plasma, n = 253; for maternal plasma, n = 710.

Table 2. Distribution of birth outcomes and dioxin-like plasma activity levels compounds in the study populations with cord and maternal samples.

	Subjects with cord sample						Subjects with maternal sample					
	Birth Outcomes											
				Percentile						Percentile		
Characteristics	No.		Mean (SD)	25th	50th	75th	No.		Mean (SD)	25th	50th	75th
Birth weight (g)	269		3476 (450)	3170	3452	3782	791		3447 (456)	3130	3430	3750
Birth head circumference (mm)	211		352 (15)	340	350	360	697		349 (2)	340	350	360
Gestational age (weeks)	269		39 (1)	38	39	40	791		39 (1)	38	39	40
	Dioxin-like activity in plasma											
				Percentile						Percentile		
	n	Geometric Mean (95% CI)	Mean (SD)	25th	50th	75th	n	Geometric Mean (95% CI)	Mean (SD)	25th	50th	75th
pg TEQ/g lipid	269	29.6 (27.1 to 32.4)	38.0 (26.0)	20.1	34.2	49	791	29.9 (28.2 to 31.7)	39.0 (22.7)	24.26	38.3	53.5
pg TEQ/ml plasma	819	0.1 (0.1 to 0.1)	0.2 (0.1)	0.1	0.1	0.2	896	0.3 (0.2 to 0.3)	0.3 (0.2)	0.2	0.3	0.4

Table 3. Association between cord and maternal plasma dioxin-like activity DR CALUX® and birth outcomes.

	Change in birth weight (g)		Change in head circumference (mm)		Change in gestational age (weeks)	
	n=247		n=205		n=260	
	Crude[a]	Adjusted[b]	Crude[a]	Adjusted[c]	Crude[d]	Adjusted[e]
Cord DR CALUX®	β (95%CI)	β (95%CI)	β (95%CI)	β (95%CI)	β (95%CI)	β (95%CI)
Log_{10} pg TEQ/g lipid	-82 (-264 to 100)	-76 (-251 to 100)	-0.4 (-7 to 7)	-1 (-7 to 6)	-0.8 (-1.4 to -0.2)	-0.5 (-1.0 to 0.04)
Low[i]	0	0	0	0	0	0
Middle	-58 (-182 to 66)	-58 (-176 to 62)	0.2 (-5 to 5)	0.2 (-4 to 5)	-0.2 (-0.6 to 0.2)	-0.1 (-0.5 to 0.3)
High	-80 (-219 to 59)	-82 (-216 to 53)	-2 (-7 to 4)	-2 (-7 to 3)	-0.6 (-1.1 to -0.2)	-0.4 (-0.8 to -0.1)
Trend test	0.250	0.225	0.510	0.410	0.007	0.029
Maternal DR CALUX®	n=701		n=632		n=763	
	Crude[a]	Adjusted[f]	Crude[a]	Adjusted[g]	Crude[d]	Adjusted[h]
Log_{10} pg TEQ/g lipid	7 (-89 to 102)	5 (-88 to 97)	2 (-2 to 5)	1 (-2 to 5)	0.3 (-0.003 to 0.6)	0.2 (-0.04 to 0.5)
Low[i]	0	0	0	0	0	0
Middle	0.9 (-77 to 78)	-11 (-87 to 65)	1 (-2 to 3)	-1 (-3 to 2)	0.2 (-0.1 to 0.4)	0.2 (-0.03 to 0.4)
High	7 (-76 to 89)	-2 (-83 to 79)	1 (-2 to 4)	1 (-2 to 4)	0.3 (0.1 to 0.6)	0.2 (-0.1 to 0.4)
Trend test	0.875	0.971	0.641	0.641	0.019	0.121

[a] Model includes country, gestational age and gestational age squared.
[b] Model includes country, gestational age, gestational age squared, maternal age, parity, maternal pre pregnancy BMI, maternal ethnicity, type of delivery and gender.
[c] Model includes country, gestational age, gestational age squared , parity, maternal pre pregnancy BMI, type of delivery and gender.
[d] Model includes country.
[e] Model includes country, smoking during pregnancy, parity and type of delivery.
[f] Model includes country, gestational age, gestational age squared, parity, maternal pre pregnancy BMI and gender.
[g] Model includes country, gestational age, gestational age squared, maternal pre pregnancy BMI, type of delivery and child gender.
[h] Model includes country, parity, type of delivery and child gender.
[i] Reference category.
Cord DR CALUX® tertiles: Low (10-24 pg TEQ/g lipid), Middle (25-43.1 pg TEQ/g lipid), High (43.3-156 pg TEQ/g lipid).
Maternal DR CALUX® tertiles: Low (6-30 pg TEQ/g lipid), Middle (30.1-47.6 pg TEQ/g lipid), High (47.9-129.1) pg TEQ/g lipid).

References

1. Birnbaum LS. Developmental effects of dioxins. *Environ Health Perspect* 1995;**103 Suppl 7**:89-94.
2. Birnbaum LS. Developmental effects of dioxins and related endocrine disrupting chemicals. *Toxicol Lett* 1995;**82-83**:743-50.
3. IARC. IARC Working Group on the Evaluation of Carcinogenic Risks to Humans: Polychlorinated Dibenzo-Para-Dioxins and Polychlorinated Dibenzofurans. Lyon, France, 4-11 February 1997. *IARC Monogr Eval Carcinog Risks Hum* 1997;**69**:1-631.
4. Baan R, Grosse Y, Straif K, Secretan B, El Ghissassi F, Bouvard V, Benbrahim-Tallaa L, Guha N, Freeman C, Galichet L, Cogliano V. A review of human carcinogens--Part F: chemical agents and related occupations. *Lancet Oncol* 2009;**10**(12):1143-4.
5. Parzefall W. Risk assessment of dioxin contamination in human food. *Food Chem Toxicol* 2002;**40**(8):1185-9.
6. Mose T, Mathiesen L, Karttunen V, Nielsen JK, Sieppi E, Kummu M, Morck TA, Myohanen K, Partanen H, Vahakangas K, Knudsen LE, Myllynen P. Meta-analysis of data from human ex vivo placental perfusion studies on genotoxic and immunotoxic agents within the integrated European project NewGeneris. *Placenta* 2012;**33**(5):433-9.
7. Nakano S, Noguchi T, Takekoshi H, Suzuki G, Nakano M. Maternal-fetal distribution and transfer of dioxins in pregnant women in Japan, and attempts to reduce maternal transfer with

Chlorella (Chlorella pyrenoidosa) supplements. *Chemosphere* 2005;**61**(9):1244-55.

8. Safe SH. Modulation of gene expression and endocrine response pathways by 2,3,7,8-tetrachlorodibenzo-p-dioxin and related compounds. *Pharmacol Ther* 1995;**67**(2):247-81.
9. Hankinson O. The aryl hydrocarbon receptor complex. *Annu Rev Pharmacol Toxicol* 1995;**35**:307-40.
10. Safe S, Krishnan V. Chlorinated hydrocarbons: estrogens and antiestrogens. *Toxicol Lett* 1995;**82-83**:731-6.
11. Denison MS, Soshilov AA, He G, DeGroot DE, Zhao B. Exactly the same but different: promiscuity and diversity in the molecular mechanisms of action of the aryl hydrocarbon (dioxin) receptor. *Toxicol Sci* 2011;**124**(1):1-22.
12. Warner M, Eskenazi B, Patterson DG, Clark G, Turner WE, Bonsignore L, Mocarelli P, Gerthoux PM. Dioxin-Like TEQ of women from the Seveso, Italy area by ID-HRGC/HRMS and CALUX. *J Expo Anal Environ Epidemiol* 2005;**15**(4):310-8.
13. Van den Berg M, Birnbaum L, Bosveld AT, Brunstrom B, Cook P, Feeley M, Giesy JP, Hanberg A, Hasegawa R, Kennedy SW, Kubiak T, Larsen JC, van Leeuwen FX, Liem AK, Nolt C, Peterson RE, Poellinger L, Safe S, Schrenk D, Tillitt D, Tysklind M, Younes M, Waern F, Zacharewski T. Toxic equivalency factors (TEFs) for PCBs, PCDDs, PCDFs for humans and wildlife. *Environ Health Perspect* 1998;**106**(12):775-92.

14. Brouwer A, Sonneveld E, Botschuijver S, Besselink H, Burg van der B. A low volume method for the analysis of dioxins and dioxin-like compounds in serum and whole blood using BDS' DR CALUX® bioassay. *Organohalog Compd* 2004;**66**:687–90.
15. Mocarelli P, Brambilla P, Gerthoux PM, Patterson DG, Jr., Needham LL. Change in sex ratio with exposure to dioxin. *Lancet* 1996;**348**(9024):409.
16. Eskenazi B, Mocarelli P, Warner M, Chee WY, Gerthoux PM, Samuels S, Needham LL, Patterson DG, Jr. Maternal serum dioxin levels and birth outcomes in women of Seveso, Italy. *Environ Health Perspect* 2003;**111**(7):947-53.
17. Ngo AD, Taylor R, Roberts CL, Nguyen TV. Association between Agent Orange and birth defects: systematic review and meta-analysis. *Int J Epidemiol* 2006;**35**(5):1220-30.
18. Yamashita F, Hayashi M. Fetal PCB syndrome: clinical features, intrauterine growth retardation and possible alteration in calcium metabolism. *Environ Health Perspect* 1985;**59**:41-5.
19. Tsukimori K, Uchi H, Mitoma C, Yasukawa F, Chiba T, Todaka T, Kajiwara J, Yoshimura T, Hirata T, Fukushima K, Wake N, Furue M. Maternal exposure to high levels of dioxins in relation to birth weight in women affected by Yusho disease. *Environ Int* 2012;**38**(1):79-86.
20. Rogan WJ, Gladen BC, Hung KL, Koong SL, Shih LY, Taylor JS, Wu YC, Yang D, Ragan NB, Hsu CC. Congenital poisoning by polychlorinated biphenyls and their contaminants in Taiwan. *Science* 1988;**241**(4863):334-6.

21. Fein GG, Jacobson JL, Jacobson SW, Schwartz PM, Dowler JK. Prenatal exposure to polychlorinated biphenyls: effects on birth size and gestational age. *J Pediatr* 1984;**105**(2):315-20.

22. Patandin S, Koopman-Esseboom C, de Ridder MA, Weisglas-Kuperus N, Sauer PJ. Effects of environmental exposure to polychlorinated biphenyls and dioxins on birth size and growth in Dutch children. *Pediatr Res* 1998;**44**(4):538-45.

23. Vartiainen T, Jaakkola JJ, Saarikoski S, Tuomisto J. Birth weight and sex of children and the correlation to the body burden of PCDDs/PCDFs and PCBs of the mother. *Environ Health Perspect* 1998;**106**(2):61-6.

24. Hertz-Picciotto I, Charles MJ, James RA, Keller JA, Willman E, Teplin S. In utero polychlorinated biphenyl exposures in relation to fetal and early childhood growth. *Epidemiology* 2005;**16**(5):648-56.

25. Tajimi M, Uehara R, Watanabe M, Oki I, Ojima T, Nakamura Y. Relationship of PCDD/F and Co-PCB concentrations in breast milk with infant birthweights in Tokyo, Japan. *Chemosphere* 2005;**61**(3):383-8.

26. Sagiv SK, Tolbert PE, Altshul LM, Korrick SA. Organochlorine exposures during pregnancy and infant size at birth. *Epidemiology* 2007;**18**(1):120-9.

27. Nishijo M, Tawara K, Nakagawa H, Honda R, Kido T, Nishijo H, Saito S. 2,3,7,8-Tetrachlorodibenzo-p-dioxin in

maternal breast milk and newborn head circumference. *J Expo Sci Environ Epidemiol* 2008;**18**(3):246-51.

28. Sonneborn D, Park HY, Petrik J, Kocan A, Palkovicova L, Trnovec T, Nguyen D, Hertz-Picciotto I. Prenatal polychlorinated biphenyl exposures in eastern Slovakia modify effects of social factors on birthweight. *Paediatr Perinat Epidemiol* 2008;**22**(3):202-13.
29. Konishi K, Sasaki S, Kato S, Ban S, Washino N, Kajiwara J, Todaka T, Hirakawa H, Hori T, Yasutake D, Kishi R. Prenatal exposure to PCDDs/PCDFs and dioxin-like PCBs in relation to birth weight. *Environ Res* 2009;**109**(7):906-13.
30. Tawara K, Nishijo M, Honda R, Maruzeni S, Seto T, Kido T, Saito S, Nakagawa H. Effects of maternal dioxin exposure on newborn size at birth among Japanese mother-infant pairs. *Environ Health Prev Med* 2009;**14**(2):88-95.
31. Rogan WJ, Gladen BC, McKinney JD, Carreras N, Hardy P, Thullen J, Tinglestad J, Tully M. Neonatal effects of transplacental exposure to PCBs and DDE. *J Pediatr* 1986;**109**(2):335-41.
32. Longnecker MP, Klebanoff MA, Brock JW, Guo X. Maternal levels of polychlorinated biphenyls in relation to preterm and small-for-gestational-age birth. *Epidemiology* 2005;**16**(5):641-7.
33. Weisskopf MG, Anderson HA, Hanrahan LP, Kanarek MS, Falk CM, Steenport DM, Draheim LA. Maternal exposure to Great Lakes sport-caught fish and dichlorodiphenyl dichloroethylene, but not polychlorinated biphenyls, is

associated with reduced birth weight. *Environ Res* 2005;**97**(2):149-62.

34. Halldorsson TI, Thorsdottir I, Meltzer HM, Strom M, Olsen SF. Dioxin-like activity in plasma among Danish pregnant women: dietary predictors, birth weight and infant development. *Environ Res* 2009;**109**(1):22-8.

35. Merlo DF, Wild CP, Kogevinas M, Kyrtopoulos S, Kleinjans J. NewGeneris: a European study on maternal diet during pregnancy and child health. *Cancer Epidemiol Biomarkers Prev* 2009;**18**(1):5-10.

36. von Stedingk H, Vikstrom AC, Rydberg P, Pedersen M, Nielsen JK, Segerback D, Knudsen LE, Tornqvist M. Analysis of hemoglobin adducts from acrylamide, glycidamide, and ethylene oxide in paired mother/cord blood samples from Denmark. *Chem Res Toxicol* 2011;**24**(11):1957-65.

37. Vafeiadi M, Agramunt S, Papadopoulou E, Besselink H, Mathianaki K, Karakosta P, Spanaki A, Koutis A, Chatzi L, Vrijheid M, Kogevinas M. In utero exposure to dioxins and dioxin-like compounds and anogenital distance in newborns and infants. *Environ Health Perspect* 2013;**121**(1):125-30.

38. Chatzi L, Mendez M, Garcia R, Roumeliotaki T, Ibarluzea J, Tardon A, Amiano P, Lertxundi A, Iniguez C, Vioque J, Kogevinas M, Sunyer J. Mediterranean diet adherence during pregnancy and fetal growth: INMA (Spain) and RHEA (Greece) mother-child cohort studies. *Br J Nutr* 2012;**107**(1):135-45.

39. Magnus P, Irgens LM, Haug K, Nystad W, Skjaerven R, Stoltenberg C. Cohort profile: the Norwegian Mother and Child Cohort Study (MoBa). *Int J Epidemiol* 2006;**35**(5):1146-50.

40. Pedersen M, Wichmann J, Autrup H, Dang DA, Decordier I, Hvidberg M, Bossi R, Jakobsen J, Loft S, Knudsen LE. Increased micronuclei and bulky DNA adducts in cord blood after maternal exposures to traffic-related air pollution. *Environ Res* 2009;**109**(8):1012-20.

41. Raynor P. Born in Bradford, a cohort study of babies born in Bradford, and their parents: protocol for the recruitment phase. *BMC Public Health* 2008;**8**:327.

42. NewGeneris. NewGeneris Project: Newborns and Genotoxic exposure risks http://www.newgeneris.org/ Accessed 14/03/2012, 2012.

43. Koppen G, Den Hond E, Nelen V, Van De Mieroop E, Bruckers L, Bilau M, Keune H, Van Larebeke N, Covaci A, Van De Weghe H, Schroijen C, Desager K, Stalpaert M, Baeyens W, Schoeters G. Organochlorine and heavy metals in newborns: results from the Flemish Environment and Health Survey (FLEHS 2002-2006). *Environ Int* 2009;**35**(7):1015-22.

44. Pedersen M, Halldorsson TI, Mathiesen L, Mose T, Brouwer A, Hedegaard M, Loft S, Kleinjans JC, Besselink H, Knudsen LE. Dioxin-like exposures and effects on estrogenic and androgenic exposures and micronuclei frequency in mother-newborn pairs. *Environ Int* 2010;**36**(4):344-51.

45. Pedersen M, Halldorsson TI, Autrup H, Brouwer A, Besselink H, Loft S, Knudsen LE. Maternal diet and dioxin-like activity, bulky DNA adducts and micronuclei in mother-newborns. *Mutat Res* 2012;**734**(1-2):12-9.

46. Hochstenbach K, van Leeuwen DM, Gmuender H, Gottschalk RW, Lovik M, Granum B, Nygaard U, Namork E, Kirsch-Volders M, Decordier I, Vande Loock K, Besselink H, Tornqvist M, von Stedingk H, Rydberg P, Kleinjans JC, van Loveren H, van Delft JH. Global gene expression analysis in cord blood reveals gender-specific differences in response to carcinogenic exposure in utero. *Cancer Epidemiol Biomarkers Prev* 2012;**21**(10):1756-67.

47. Croes K, Van Langenhove K, Den Hond E, Bruckers L, Colles A, Koppen G, Loots I, Nelen V, Schoeters G, Nawrot T, Van Larebeke N, Denison MS, Vandermarken T, Elskens M, Baeyens W. Quantification of PCDD/Fs and dioxin-like PCBs in small amounts of human serum using the sensitive H1L7.5c1 mouse hepatoma cell line: optimization and analysis of human serum samples from adolescents of the Flemish human biomonitoring program FLEHS II. *Talanta* 2011;**85**(5):2484-91.

48. Van Wouwe N, Windal I, Vanderperren H, Eppe G, Xhrouet C, Massart AC, Debacker N, Sasse A, Baeyens W, De Pauw E, Sartor F, Van Oyen H, Goeyens L. Validation of the CALUX bioassay for PCDD/F analyses in human blood plasma and comparison with GC-HRMS. *Talanta* 2004;**63**(5):1157-67.

49. Koppen G, Covaci A, Van Cleuvenbergen R, Schepens P, Winneke G, Nelen V, Schoeters G. Comparison of CALUX-TEQ values with PCB and PCDD/F measurements in human serum of the Flanders Environmental and Health Study (FLEHS). *Toxicol Lett* 2001;**123**(1):59-67.

50. Murk AJ, Leonards PE, van Hattum B, Luit R, van der Weiden ME, Mit M. The CALUX assay adapted and validated for measuring TCDD equivalents in blood plasma. *Environ Toxicol Chem* 1997;**16**:1583–1589.

51. Lin CM, Li CY, Mao IF. Birth outcomes of infants born in areas with elevated ambient exposure to incinerator generated PCDD/Fs. *Environ Int* 2006;**32**(5):624-9.

52. Yen YY, Lan SJ, Yang CY, Wang HH, Chen CN, Hsieh CC. Follow-up study of intrauterine growth of transplacental Yu-Cheng babies in Taiwan. *Bull Environ Contam Toxicol* 1994;**53**(5):633-41.

53. Main KM, Skakkebaek NE, Virtanen HE, Toppari J. Genital anomalies in boys and the environment. *Best Pract Res Clin Endocrinol Metab* 2010;**24**(2):279-89.

54. Manikkam M, Tracey R, Guerrero-Bosagna C, Skinner MK. Dioxin (TCDD) induces epigenetic transgenerational inheritance of adult onset disease and sperm epimutations. *PLoS One* 2012;**7**(9):e46249.

55. Long M, Deutch B, Bonefeld-Jorgensen EC. AhR transcriptional activity in serum of Inuits across Greenlandic districts. *Environ Health* 2007;**6**:32.

56. Nakamura T, Nakai K, Matsumura T, Suzuki S, Saito Y, Satoh H. Determination of dioxins and polychlorinated biphenyls in breast milk, maternal blood and cord blood from residents of Tohoku, Japan. *Sci Total Environ* 2008;**394**(1):39-51.

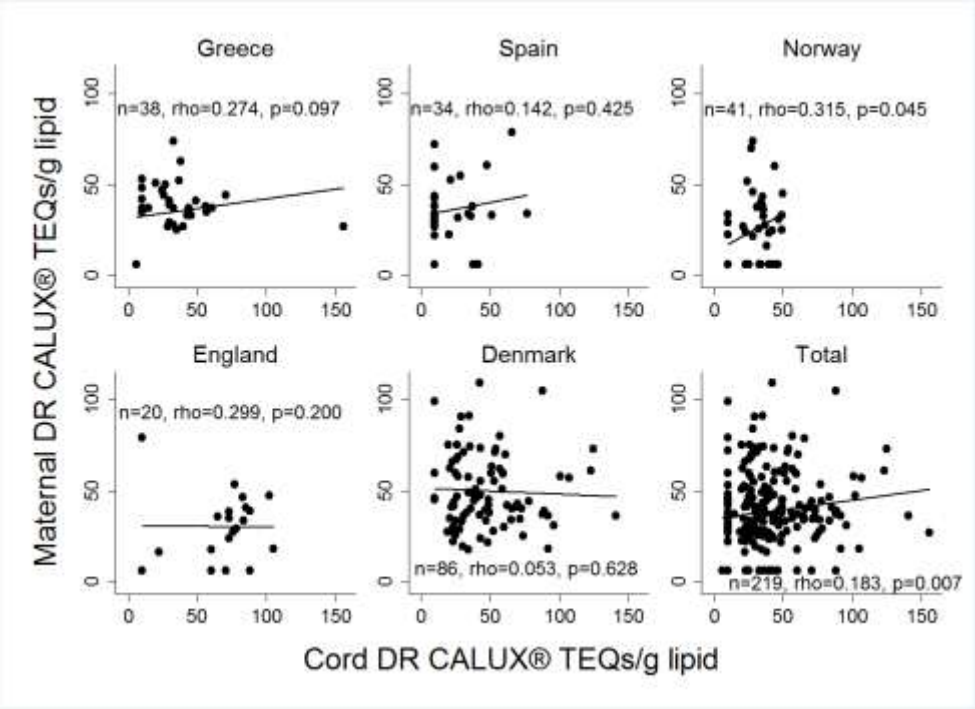
57. Tsukimori K, Uchi H, Mitoma C, Yasukawa F, Fukushima K, Todaka T, Kajiwara J, Yoshimura T, Hirata T, Wake N, Furue M. Comparison of the concentrations of polychlorinated biphenyls and dioxins in mothers affected by the Yusho incident and their children. *Chemosphere* 2011;**84**(7):928-35.

58. Wang SL, Lin CY, Guo YL, Lin LY, Chou WL, Chang LW. Infant exposure to polychlorinated dibenzo-p-dioxins, dibenzofurans and biphenyls (PCDD/Fs, PCBs)--correlation between prenatal and postnatal exposure. *Chemosphere* 2004;**54**(10):1459-73.

59. Suzuki G, Nakano M, Nakano S. Distribution of PCDDs/PCDFs and Co-PCBs in human maternal blood, cord blood, placenta, milk, and adipose tissue: dioxins showing high toxic equivalency factor accumulate in the placenta. *Biosci Biotechnol Biochem* 2005;**69**(10):1836-47.

60. Tsukimori K, Morokuma S, Hori T, Takahashi K, Hirata T, Otera Y, Fukushima K, Kawamoto T, Wake N. Characterization of placental transfer of polychlorinated dibenzo-p-dioxins, dibenzofurans and polychlorinated biphenyls in normal pregnancy. *J Obstet Gynaecol Res* 2012.

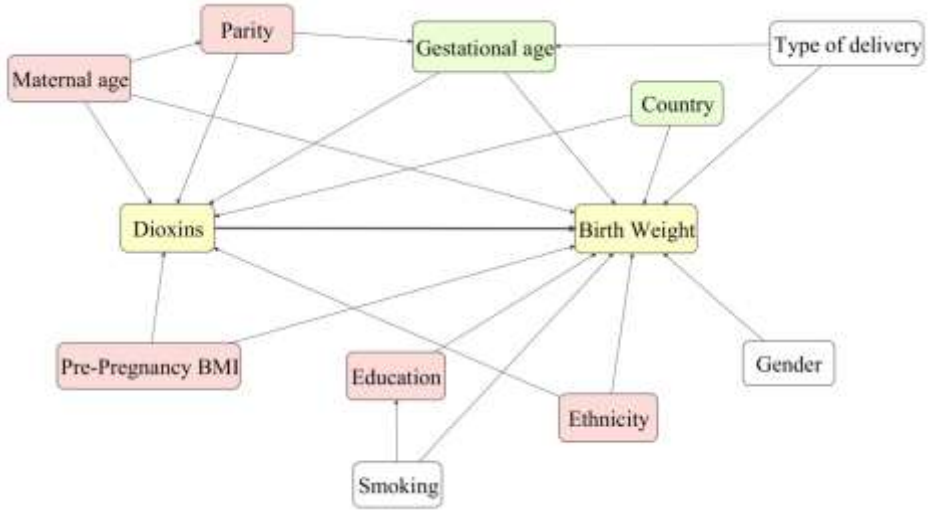
Figure. Correlations between maternal and fetal dioxin-like activity, overall and by country



Online Supplemental Material

Protocol for the DR CALUX® bioassay

The total fat from approximately 1mL and 3mL of plasma from maternal and cord blood respectively was extracted in 97% hexane 3% diethyl ether solution by shake-solvent extraction. The extracted fat was then passed through two acid silica columns topped with sodium sulfate (first 20% and then 30% H2SO4) to remove matrix components. The purified extracts were evaporated under nitrogen and re-dissolved in 8µL of dimethyl sulfoxide (DMSO). The CALUX® cells were cultured in alpha-minimum essential (α-MEM) culture medium supplemented with 10% (v/v) fetal calf serum (FCS) under standard conditions (37°C, 5% CO2, 100% humidity) and were exposed in triplicate to cleaned extracts for 24 hours in 96-well microtiter plates. After incubation, the cells were lysed. A luciferine containing solution was added and the luciferase activity was measured using a luminometer. Each 96-well microtiter plate contained a TCDD calibration range (0–3 pM TCDD per well), a DMSO control, a procedure blank and an internal reference material. Total DR CALUX®-TEQ in the samples was determined by interpolation from the fitted 2,3,7,8-TCDD calibration curve and corrected for procedure blank. Lipid content of the plasma was determined gravimetrically. The LOD was calculated as the signal measured from the DMSO control on each plate plus three times its standard deviation.



eFigure 1. Diagram of the potential influences on the relationship between dioxins and birth weight. In green are the variables already adjusted for in the crude model, in white the variables considered but discarded in the final model and in pink are the variables that were kept in the final model. Smoking is associated with birth weight but only indirectly with with dioxin levels through education. Type of delivery is associated with birth weight but potentially only indirectly with dioxin levels trough gestational age. Finally dioxins have been associated to child gender in conditions of very high exposure to TCDD in the Seveso population, given that we have low general population exposure we have discarded this association.

eTable 1. Distribution of the total 967 samples analysed with DR CALUX® according to whether they were cord or maternal, lipid or plasma, and number (%) of samples above LOD.

Type of Sample available	Number of samples	Number with DR CALUX® measurements above LOD(%)
Cord lipid	269	207 (77)
Cord plasma	819	459 (56)
Cord lipid and plasma	255	128 (50.2)
Maternal lipid	791	644 (81.4)
Maternal plasma	896	771 (86)
Maternal lipid and plasma	775	637 (82.2)
Cord and maternal lipid	219	156 (71.2)
Cord and maternal plasma	754	430 (57)
Cord and maternal, lipid and plasma	208	105 (50.5)

eTable 2. Characteristics of study participants in the different study centers.

	Subjects with cord DR CALUX® pg TEQ/g lipid						
Characteristics	Rhea	Inma	MoBa Bramat	MoBa Bramiljoe	BiB	Danish biobank '07	Danish biobank '09
Maternal age (years)	44(29.3±5.6)	35(31.5±4.1)	73(31.3±4.2)	8(31.0±2.6)	20(30.9±5.9)	46(33.2±4.2)	43(32.0±3.6)
Parity							
Primiparous	5(11.6)	13(38.2)	29(39.7)	5(62.5)	5(26.3)	4(8.9)	26(60.5)
Multiparous	38(88.4)	21(61.8)	44(60.3)	3(37.5)	14(73.7)	41(91.1)	17(39.5)
Pre-pregnancy BMI (kg/m2)	44(25.6±4.8)	28(24.6±4.7)	73(23.8±4.4)	8(22.4±4.0)	11(24.9±6.8)	46(24.2±4.3)	43(22.5±3.5)
Maternal education							
High	5(12.8)	0	21(30.9)	4(50.0)	5(35.7)	25(54.3)	27(62.8)
Medium	28(71.8)	2(100.0)	23(33.8)	4(50.0)	3(21.4)	12(26.1)	11(25.6)
Low	6(15.4)	0	24(35.3)	0	6(42.7)	9(19.6)	5(11.6)
Smoking during pregnancy							
No	22(50.0)	22(64.7)	67(95.4)	8(100.0)	17(89.5)	44(95.7)	41(95.3)
Yes	22(50.0)	12(35.3)	4(5.6)	0	2(10.5)	2(4.2)	2(4.7)
Maternal ethnicity							
Caucasian	44(100.0)	23(69.7)	73(100.0)	8(100.0)	12(60.0)	43(93.5)	43(100.0)
Non Caucasian	0	10(30.3)	0	0	8(40.0)	3(6.5)	0
Type of delivery							
Vaginal delivery	17(38.6)	26(76.5)	64(87.7)	7(87.5)	1(5.0)	0	33(76.7)
Caesarean section	27(61.4)	8(23.5)	9(12.3)	1(12.5)	19(95.0)	46(100.0)	10(23.3)

	Subjects with maternal DR CALUX® pg TEQ/g lipid						
Maternal age (years)	146(29.4±5.2)	157(30.0±5.4)	68(31.5±4.6)	116(31.5±4.0)	111(30.6±5.2)	91(33.2±4.4)	99(32.5±4.2)
Parity							
Primiparous	38(27.5)	64(41.0)	31(45.6)	55(47.4)	10(9.5)	16(18.8)	54(55.7)
Multiparous	100(72.5)	92(59.0)	37(54.4)	61(52.6)	95(90.5)	69(81.2)	43(44.3)
Pre-pregnancy BMI (kg/m2)	136(25.0±5.0)	140(24.1±4.9)	67(23.3±4.1)	116(22.7±3.4)	67(28.9±7.3)	88(23.5±4.3)	96(22.1±3.6)
Maternal education							
High	21(18.6)	31(25.6)	19(29.7)	52(44.8)	27(36.5)	45(51.1)	60(62.5)
Medium	74(66.1)	47(38.8)	22(34.4)	46(39.7)	17(23.0)	20(22.7)	28(29.2)
Low	17(15.2)	43(35.5)	23(35.9)	18(15.5)	30(40.5)	23(26.1)	8(8.3)
Smoking during pregnancy							
No	86(59.3)	113(73.4)	64(97.0)	108(95.6)	94(85.5)	84(94.4)	93(95.9)
Yes	59(40.7)	41(26.6)	2(3.0)	5(4.4)	16(14.5)	5(5.6)	4(4.1)
Maternal ethnicity							
Caucasian	149(100.0)	82(53.2)	68(100.0)	116(100.0)	67(60.4)	86(95.6)	95(97.9)
Non Caucasian	0	72(46.8)	0	0	44(39.6)	4(4.4)	2(2.1)
Type of delivery							
Vaginal delivery	65(43.6)	136(87.7)	62(91.2)	108(93.1)	1(0.9)	0	81(81.8)
Caesarean section	84(56.4)	19(12.3)	6(8.8)	8(6.9)	110(99.1)	91(100.0)	18(18.2)

Values are n [% or (Mean±SD)]

eTable 3. Association between fetal and maternal dioxin-like plasma activity and birth outcomes with mutual adjustments for maternal and fetal dioxin-like activity.

	Change in birth weight (g)		Change in head circumference (cm)		Change in gestational age (weeks)	
	n=197		n=160		n=211	
	Crude[a]	Adjusted[b]	Crude[a]	Adjusted[c]	Crude[d]	Adjusted[e]
Cord DR CALUX®	β(95%CI)	β(95%CI)	β(95%CI)	β(95%CI)	β(95%CI)	β(95%CI)
Log10 pg TEQ/g lipid	-97.0 (-297.4 to 103.3)	-86.0 (-283.2 to 111.3)	-0.1 (-0.9 to 0.7)	-0.1 (-0.9 to 0.7)	-1.0 (-1.6 to -0.3)	-0.6 (-1.2 to -0.03)
Low[k]	0	0	0	0	0	0
Middle	-115.1 (-253.1 to 23.0)	-113.7 (-247.2 to 19.7)	-0.04 (-0.6 to 0.5)	-0.05 (-0.6 to 0.5)	-0.3 (-0.7 to 0.2)	-0.1 (-0.6 to 0.3)
High	-127.4 (-280.0 to 25.1)	-127.6 (-277.4 to 22.2)	-0.2 (-0.8 to 0.4)	-0.2 (-0.8 to 0.3)	-0.8 (-1.3 to -0.3)	-0.6 (-1.0 to -0.1)
Trend test	0.099	0.092	0.449	0.411	0.003	0.011
Maternal DR CALUX®	n=200		n=162		n=214	
	Crude[f]	Adjusted[g]	Crude[f]	Adjusted[h]	Crude[i]	Adjusted[j]
Log10 pg TEQ/g lipid	68.6 (-138.7 to 275.9)	51.5 (-152.9 to 255.8)	0.2 (-0.7 to 1.0)	-0.01 (-0.8 to 0.8)	0.6 (-0.1 to 1.3)	0.6 (-0.02 to 1.2)
Low[k]	0	0	0	0	0	0
Middle	-85.2 (-297.3 to 126.9)	-85.5 (-290.3 to 119.2)	-0.2 (-1.0 to 0.5)	-0.3 (-1.0 to 0.4)	0.1 (-0.6 to 0.8)	-0.1 (-0.7 to 0.5)
High	-120.5 (-425.4 to 184.5)	-133.7 (-428.2 to 160.8)	-0.6 (-1.7 to 0.5)	-0.7 (-1.7 to 0.4)	-0.2 (-1.2 to 0.8)	-0.4 (-1.3 to 0.5)
Trend test	0.489	0.403	0.240	0.181	0.473	0.238

[a]Model includes country gestational age, gestational age squared and maternal dioxin-like activity.
[b]Model includes country, gestational age, gestational age squared, maternal age, parity, maternal pre pregnancy BMI, maternal ethnicity, type of delivery, gender and maternal dioxin-like activity.
[c]Model includes country, gestational age, gestational age squared, parity, maternal pre pregnancy BMI, type of delivery, gender and maternal dioxin-like activity.

[d]Model includes country and maternal dioxin-like activity.

[e]Model includes country, smoking during pregnancy, parity, type of delivery and maternal dioxin-like activity.

[f]Model includes country gestational age, gestational age squared and fetal dioxin-like activity.
[g]Model includes country, gestational age, gestational age squared, parity, maternal pre pregnancy BMI, gender and fetal dioxin-like activity.
[h]Model includes country, gestational age, gestational age squared, maternal pre pregnancy BMI, type of delivery, gender and fetal dioxin-like activity.

[i]Model includes country and fetal dioxin-like activity.

[j]Model includes country, parity, type of delivery and gender.

[k]Reference category.

eTable 4. Association between fetal and maternal dioxin-like plasma activity and birth outcomes in the subsample with both maternal and cord dioxin-like plasma activity.

	Change in birth weight (g)		Change in head circumference (cm)		Change in gestational age (weeks)	
	n=197		n=160		n=211	
	Crude[a]	Adjusted[b]	Crude[a]	Adjusted[c]	Crude[d]	Adjusted[e]
Cord DR CALUX®	β (95%CI)	β (95%CI)	β (95%CI)	β (95%CI)	β (95%CI)	β (95%CI)
Log_{10} pg TEQ/g lipid	-79.3 (-272.7 to 114.1)	-70.5 (-261.0 to 120.0)	-0.02 (-0.8 to 0.7)	-0.1 (-0.8 to 0.6)	-0.8 (-1.4 to -0.2)	-0.5 (-1.1 to 0.1)
Low[i]	0	0	0	0	0	0
Middle	-99.6 (-233.5 to 34.2)	-99.6 (-229.1 to 29.8)	0.001 (-0.5 to 0.5)	-0.3 (-0.5 to 0.5)	-0.2 (-0.6 to 0.3)	-0.05 (-0.5 to 0.4)
High	-112.9 (-262.1 to 36.3)	-114.3 (-260.9 to 32.4)	-0.2 (-0.8 to 0.4)	-0.1 (-0.8 to 0.3)	-0.7 (-1.2 to -0.2)	-0.5 (-1.0 to -0.1)
Trend test	0.128	0.117	0.513	0.440	0.007	0.026
Maternal DR CALUX®	n=200		n=162		n=214	
	Crude[a]	Adjusted[f]	Crude[a]	Adjusted[g]	Crude[d]	Adjusted[h]
Log_{10} pg TEQ/g lipid	40.7 (-159.8 to 241.2)	21.8 (-175.2 to 218.7)	0.1 (-0.6 to 0.9)	-0.02 (-0.8 to 0.7)	0.4 (-0.2 to 1.1)	0.5 (-0.1 to 1.1)
Low[i]	0	0	0	0	0	0
Middle	6.8 (-129.2 to 142.8)	3.9 (-138.5 to 130.6)	0.1 (-0.4 to 0.7)	-0.03 (-0.5 to 0.5)	0.02 (-0.4 to 0.5)	0.2 (-0.3 to 0.6)
High	79.9 (-77.4 to 237.2)	66.0 (-89.8 to 221.8)	0.1 (-0.5 to 0.7)	0.2 (-0.4 to 0.7)	0.3 (-0.2 to 0.8)	0.1 (-0.4 to 0.6)

Trend test	0.323	0.407	0.653	0.592	0.461	0.587

[a] Model includes country, gestational age and gestational age squared.

[b] Model includes country, gestational age, gestational age squared, maternal age, parity, maternal pre pregnancy BMI, maternal ethnicity, type of delivery and gender.

[c] Model includes country, gestational age, gestational age squared , parity, maternal pre pregnancy BMI, type of delivery and gender.

[d] Model includes country.

[e] Model includes country, smoking during pregnancy, parity and type of delivery.

[f] Model includes country, gestational age, gestational age squared, parity, maternal pre pregnancy BMI and gender.

[g] Model includes country, gestational age, gestational age squared, maternal pre pregnancy BMI, type of delivery and child gender.

[h] Model includes country to, parity, type of delivery and child gender.

[i] Reference category.

5.2 Paper II

Persistent organic pollutants exposure during pregnancy, maternal gestational weight gain, and birth outcomes in the mother-child cohort in Crete, Greece (RHEA study)[2]

Marina Vafeiadi, Martine Vrijheid, Eleni Fthenou, Georgia Chalkiadaki, Panu Rantakokko, Hannu Kiviranta, Soterios A. Kyrtopoulos, Leda Chatzi and Manolis Kogevinas

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5.3 Paper III

In Utero Exposure to Dioxins and Dioxin-like Compounds and Anogenital Distance in Newborns and Infants[3]

Marina Vafeiadi, Silvia Agramunt, Eleni Papadopoulou, Harrie Besselink, Kleopatra Mathianaki, Polyxeni Karakosta, Ariana Spanaki, Antonis Koutis, Leda Chatzi, Martine Vrijheid, Manolis Kogevinas

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In Utero Exposure to Dioxins and Dioxin-like Compounds and Anogenital Distance in Newborns and Infants

Marina Vafeiadi,[1,2,3,4] Silvia Agramunt,[1,2,5] Eleni Papadopoulou,[1,2,3,4,6] Harrie Besselink,[7] Kleopatra Mathianaki,[8] Polyxeni Karakosta,[8] Ariana Spanaki,[9] Antonis Koutis,[8] Leda Chatzi,[8] Martine Vrijheid,[1,2,3] and Manolis Kogevinas[1,2,3,6]

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Background: Anogenital distance in animals is used as a measure of fetal androgen action. Prenatal exposure to dioxins and dioxin-like compounds in rodents causes reproductive changes in male offspring and decreases anogenital distance.

Objective: We assessed whether *in utero* exposure to dioxins and dioxin-like compounds adversely influences anogenital distance in newborns and young children (median age, 16 months; range, 1–31 months).

Methods: We measured anogenital distance among participants of the "Rhea" mother–child cohort study in Crete and the Hospital del Mar (HMAR) cohort in Barcelona. Anogenital distance (AGD; anus to upper penis), anoscrotal distance (ASD; anus to scrotum), and penis width (PW) were measured in 119 newborn and 239 young boys; anoclitoral (ACD; anus to clitoris) and anofourchetal distance (AFD; anus to fourchette) were measured in 118 newborn and 223 young girls. We estimated plasma dioxin-like activity in maternal blood samples collected at delivery with the Dioxin-Responsive Chemically Activated LUciferase eXpression (DR CALUX®) bioassay.

Results: Anogenital distances were sexually dimorphic, being longer in males than females. Plasma dioxin-like activity was negatively associated with AGD in male newborns. The estimated change in AGD per 10 pg CALUX®–toxic equivalent/g lipid increase was −0.44 mm (95% CI: −0.80, −0.08) after adjusting for confounders. Negative but smaller and nonsignificant associations were observed for AGD in young boys. No associations were found in girls.

Conclusions: Male infants may be susceptible to endocrine-disrupting effects of dioxins. Our findings are consistent with the experimental animal evidence used by the Food and Agriculture Organization/World Health Organization to set recommendations for human dioxin intake.

Key words: anogenital distance, dioxin-like compounds, dioxins, DR CALUX®, persistent organic pollutants. *Environ Health Perspect* 121:125–130 (2013). http://dx.doi.org/10.1289/ehp.1205221 [Online 19 November 2012]

Polychlorinated dibenzodioxins (PCDDs), dibenzofurans (PCDFs), and biphenyls (PCBs) constitute a group of widespread and persistent organic pollutants with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, dioxin) being the most toxic member of this group (Birnbaum 1994b, 1995a, 1995b). PCDDs, PCDFs, and dioxin-like PCBs have long half-lives and therefore accumulate in the body. Food is the main source of dioxin exposure for humans, estimated at > 95% of the total intake for non-occupationally exposed persons (Parzefall 2002). Infant exposure starts *in utero*, through the placenta, and continues postnatally through breastfeeding.

The toxicity of PCDDs, PCDFs, and dioxin-like PCBs is traced mostly to their binding to the aryl hydrocarbon receptor (AhR). The AhR, on exposure to TCDD, translocates into the nucleus, where it heterodimerizes with the AhR nuclear translocator (ARNT). This complex then binds to its specific DNA recognition sites to activate the transcription of dioxin responsive genes (Hankinson 1995). The AhR induces expression of direct target genes such as the drug metabolizing enzyme genes *CYP1A1* and *CYP1A2* (Safe and Krishnan 1995). Furthermore, the ligand-activated AhR associates with estrogen or androgen receptors (ERα or AR) to regulate transcription as a functional unit (Ohtake et al. 2003, 2008). Although early studies focused on the AhR as mediating the biochemical response to xenobiotics, recent studies have revealed that, triggered by natural and endogenous ligands, AhR plays key endogenous regulatory roles in normal physiology and development (Abel and Haarmann-Stemmann 2010; Denison et al. 2011).

Because TCDD and other dioxin-like compounds exist as complex mixtures of various congeners throughout the environment, calculating total TCDD toxic equivalent (TEQ) concentration is the most relevant exposure measure in studies of health effects of dioxins and dioxin-like compounds (Warner et al. 2005). The Dioxin-Responsive Chemically Activated LUciferase eXpression (DR CALUX®) assay measures the ability of a chemical mixture to activate AhR-dependent gene expression of the firefly luciferase gene in genetically modified cell lines, and only small amounts of blood plasma are required for these measurements (Brouwer et al. 2004).

Anogenital distance (AGD), the distance from the anus to the genitalia, is a sensitive marker used by reproductive toxicologists in animal experiments as a measure of fetal androgen action. In rodents, perineal growth is dihydrotestosterone-dependent, males have a greater AGD than females, and use of AGD to sex newborns is common (Greenham and Greenham 1977; Marty et al. 2003). AGD usually tracks through life, varies by dose of antiandrogen, and can be predictive of other androgen-responsive outcomes such as hypospadias and cryptorchidism (Gray et al. 1999). Animal studies reviewed by the Joint FAO/WHO (Food and Agriculture Organization/World Health Organization) Expert Committee on Food Additives showed reduction in AGD as well as feminized sexual behavior in male offspring associated with TCDD exposure (FAO/WHO 2002).

In human studies AGD has been examined only in recent years (Huang et al. 2009; Longnecker et al. 2007; Miao et al. 2011; Ozkan et al. 2011; Romano-Riquer et al. 2007; Salazar-Martinez et al. 2004; Sathyanarayana et al. 2010; Suzuki et al. 2011; Swan et al. 2005; Thankamony et al. 2009; Torres-Sanchez et al. 2008). No human studies have reported on the relationship between *in utero* dioxin exposure and AGD in offspring, whereas AGD has been inversely associated with prenatal exposure to other environmental endocrine disruptors, namely phthalates,

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dichlorodiphenyldichloroethylene (DDE), and bisphenol A (BPA) (Miao et al. 2011; Swan et al. 2005; Torres-Sanchez et al. 2008).

In the present study of two mother–child cohorts in Greece and Spain, the DR CALUX® bioassay was used to measure dioxin-like activity in maternal plasma. We hypothesized that *in utero* exposure to dioxins would decrease AGD in newborns and children.

Materials and Methods

Study population. The present study was based on data from the "Rhea" mother–child cohort study in Crete, Greece, and the Hospital del Mar (HMAR) cohort in Barcelona, Spain. Both studies are part of the Newborns and Genotoxic exposure risks (NewGeneris) project.

The Rhea study prospectively examines a population-based sample of pregnant women and their children at the prefecture of Heraklion, Crete, Greece. Methods are described in detail elsewhere (Chatzi et al. 2009). Women were identified from February 2007 through February 2008 at the time of the first ultrasound examination at the 10th–13th week of gestation, were residents in the study area, were > 16 years of age, and had no communication handicap. Face-to-face structured interviews along with self-administered questionnaires and medical records were used to obtain information on nutrition, occupational, and environmental exposures and lifestyle, socioeconomic, and psychological factors during pregnancy and birth.

A total of 1,765 eligible women were approached during the enrollment period. 1,610 (91%) agreed to participate, and 1,317 (82%) were followed up until delivery. Seven hundred blood samples, provided by the study participants at delivery, were analyzed for dioxin-like activity. Anthropometric measurements were conducted for 165 newborns (84 boys and 81 girls) and 732 young children (374 boys and 358 girls). The present analysis included 121 newborns (62 boys and 59 girls) and 462 young children (median age, 16.0 months, range, 1–31 months; 239 boys and 223 girls), all of whom were singletons with information on anthropometry and *in utero* dioxin-like activity measurements.

In the HMAR study women were informed by their midwife at the delivery room about the NewGeneris project. The inclusion criteria were age ≥ 18 years, singleton pregnancy, HIV and hepatitis B/C negative, non-excessive postpartum hemorrhage, and non-urgent cesarean section. A similar questionnaire to that of the Rhea study was administered to the mothers within the first 48 hr after delivery by a trained nurse. We analyzed 205 blood samples for dioxin-like activity, and conducted anthropometric measurements for 187 newborns (95 boys and 92 girls). One hundred sixteen newborns (57 boys and 59 girls) with information on anthropometry and *in utero* dioxin-like activity were included in this analysis.

All procedures involving human subjects were approved by the ethical committee of the University Hospital in Heraklion, Crete, and by the Clinical Research Ethical Committee at Hospital del Mar. All study participants provided written informed consent for themselves and their children.

Physical examination. Examiners of both cohorts received a common and extensive training before conducting the measurements. All AGD measurements were performed using a standardized analytical protocol based on the protocol used in a previous study (Swan SH, personal communication; Swan et al. 2005), which was modified to include additional measurements (Callegari et al. 1987; Salazar-Martinez et al. 2004). Minor changes, mostly regarding the child's position during measurement, were made to adapt the protocol for young children.

In male participants we recorded AGD, the distance from the anterior base of the penis to the center of the anus; anoscrotal distance (ASD), the distance from the posterior base of the scrotum to the center of the anus; and penis width (PW), the diameter of the penis in its base. In girls we recorded, anoclitoral distance (ACD), the distance between the clitoris and the center of the anus; and anofourchettal distance (AFD), the distance from the posterior convergence of the fourchette to the center of the anus. Each measurement was repeated three times and the average of the three measurements was recorded. Weight, length, and head circumference were measured twice, and average values were used for analysis.

AGDs were measured with a Vernier digital calliper in increments of 0.01 mm (Cal C/PROOF 150MM IP67; TESA Technology, Renens, Switzerland). An electronic scale readable to increments of 0.001 kg was used to measure weight (model 354; Seca Corporation, Hamburg, Germany), a measuring mat was used to measure length (model 210; Seca Corporation), and a non-stretchable measuring tape was used to measure head circumference.

In the Rhea study three examiners conducted the measurements of newborns at the clinics and four examiners the measurements of young children at their homes. In the HMAR cohort, all measurements were conducted by a single examiner within the first 48 hr after delivery.

Blood sample collection. Maternal peripheral blood samples were collected in heparinized tubes (BD Vacutainer, Plymouth, UK) immediately after the delivery. The blood was centrifuged and the plasma was stored at –80°C until shipment to the Netherlands on dry ice.

DR CALUX® bioassay. Dioxin-like activity in maternal plasma samples was determined through the DR CALUX® assay at Biodetection Systems B.V., Amsterdam (www.bds.nl). The CALUX® assay is based on a genetically modified H4IIE rat hepatoma cell line, which contains the firefly luciferase reporter gene under the transcriptional control of AhR. When cells are exposed to dioxins or dioxin-like chemicals, through binding to the AhR, they express luciferase as well as proteins and enzymes associated with dioxin-responsive elements. With addition of the substrate luciferine for the luciferase enzyme, light is emitted. The amount of light emitted is proportional to the strength of the AhR binding. The luminance is calibrated with respect to 2,3,7,8-TCDD in units of toxic equivalency quantity (TEQs), and results are expressed as picograms CALUX®–TEQ/gram lipid. The DR CALUX® bioassay has previously been validated and used in human biomonitoring studies (Brouwer et al. 2004; Halldorsson et al. 2009; Koppen et al. 2001, 2009; Pauwels et al. 2000; Pedersen et al. 2010; Porpora et al. 2009; Van Den Heuvel et al. 2002). The protocol for sample processing has been presented elsewhere (Murk et al. 1997) and is described in detail in Supplemental Material, p. 2 (http://dx.doi.org/10.1289/ehp.1205221).

Statistical analysis. We used linear regression models to explore the associations between dioxin-like activity in maternal plasma and anogenital parameters. Samples below the limit of detection (LOD) were assigned a value equal to 0.5 × LOD before analyses for associations. Body dimensions have been found to be major predictors of AGD. All models included birth weight and weight at the time of examination for newborns and children respectively. We did not adjust for length because it was not a significant predictor of any of the outcomes in our study ($p > 0.05$). In addition to birth weight, each basic model for newborns included gestational age and cohort. In addition to weight at the time of examination, basic models for young children included age at examination and a variable indicating the examiner. We also ran fully adjusted models that included all potential confounders that predicted the outcome with $p < 0.2$ when added to the basic model for each age group.

In addition we modeled associations using weight-standardized *z*-scores of AGD as the outcome. In alternative analyses we adjusted for body size using weight percentiles for age based on WHO tables (WHO 2006). Generalized additive models (GAMs) were applied to explore the shape of the relationships between dioxin-like activity and AGDs and test departures from linearity. These models indicated linear relationships for all AGDs in newborns and young children. Analyses were conducted using STATA software, version 10.0 (StataCorp, College Station, TX, USA). The level of significance was set at $p < 0.05$ (two-sided).

Results

Participants' characteristics. Mothers of newborns and young children had a mean (± SD) age of 29.8 ± 5.4 years and 30.1 ± 4.7 years, respectively, and had a prepregnancy body mass index (BMI) within the normal range with median [interquartile range (IQR)] values of 23.4 (5.3) and 23.4 (5.1) kg/m^2 respectively (Table 1). Most of the newborns (73.7%) and half (50.8%) of the young children were vaginally delivered, with a mean (± SD) birth weight of 3,277 ± 429.2 g and 3,167 ± 441.6 g respectively. Participants were mainly white European, multiparous nonsmokers living in urban areas. Percentages of boys and girls were similar in newborns (50.2% boys and 49.8% girls) and young children (51.7% boys and 48.3% girls).

Dioxin-like compounds and anogenital parameters. Mean (± SD) AGDs were longer in male newborns (AGD = 48.8 ± 5.1 mm, ASD = 25.5 ± 4.8 mm) than in female (ACD = 35.0 ± 3.3 mm, AFD = 14.3 ± 3.0 mm) (Table 2). Similarly, in young children mean AGDs were longer in males (AGD = 80.7 ± 7.3 mm, ASD = 39.9 ± 6.9 mm) than females (ACD = 49.1 ± 6.0 mm, AFD = 21.7 ± 3.9 mm). The mean of PW was 10.7 ± 1.1 mm in newborns and 14.0 ± 1.7 mm in young boys. The mean of the samples was 52.3 ± 20.7 pg CALUX*–TEQ/g lipid in mothers of newborns and 49.7 ± 26.7 pg CALUX*–TEQ/g lipid in mothers of young children.

Mean weight at examination of newborns was lower among newborn male and female children whose mothers had CALUX*–TEQs above the median (> 53.6 pg CALUX*–TEQ/g lipid) compared with children whose mothers had values below the median, although differences were small and not statistically significant (Table 3). In young children, weight at examination was higher in children whose mothers had dioxin-like activity above the median (> 50.3 pg CALUX*–TEQ/g lipid), with a significant difference in young males. Compared with newborn children whose mothers had low dioxin-like activity, newborn children of mothers with high activity had small nonsignificant decreases in AGD in males (48.4 mm vs. 49.1 mm, $p = 0.617$) and ACD and AFD in females (34.9 mm vs. 35.1 mm, $p = 0.592$ and 14.1 mm vs. 14.4 mm, $p = 0.892$ respectively).

Relationship between plasma dioxin-like activity and anogenital parameters. Plasma dioxin-like activity was negatively associated with AGD in male newborns (Table 4). The estimated change in newborn AGD per 10 pg CALUX*–TEQ/g lipid was –0.41 mm (95% CI: –0.77, –0.06) according to the basic model (adjusted for birth weight, gestational age and cohort), with a similar estimate based on the fully-adjusted model (Table 4). Analyses by country also were similar (–0.43 mm; 95% CI: –0.88, 0.02 for Rhea and –0.39 mm; 95% CI: –1.02, 0.25 for HMAR in fully-adjusted models). Negative but not statistically significant associations were observed for ASD in male newborns (–0.14 mm; 95% CI: –0.51, 0.23 and –0.25 mm; 95% CI: –0.61, 0.11 for the basic and fully adjusted models, respectively). Small nonsignificant negative associations were observed for AGD and the weight standardized z-score of AGD in young boys. All associations were close to the null for girls, except for small positive but nonsignificant

Table 1. Maternal and child characteristics in newborns ($n = 237$) and young children ($n = 462$).[a]

	Newborns		Young children	
Characteristic	*n*	Value	*n*	Value
Maternal characteristics				
Country of residence (%)				
Greece	121	49.0	456	100.0
Spain	116	51.0	0	0
Maternal age [years [mean ± SD]]	237	29.8 ± 5.4	456	30.1 ± 4.7
Missing			6	
Prepregnancy BMI, kg/cm^2 [median (IQR)]	224	23.4 (5.3)	456	23.4 (5.1)
Missing	13		6	
Weight gain during pregnancy, kg [median (IQR)]		NA[b]	378	13.0 (7.0)
Missing			84	
Maternal ethnicity (%)				
Nonwhite European	59	25	0	0
White European	177	75	462	100
Missing	1			
Parity (%)				
Primiparous	95	42.0	155	34.7
Multiparous	131	58.0	292	65.3
Missing	11		15	
Residence (%)				
Urban	201	87.0	335	80.7
Rural	30	13.0	80	19.3
Missing	6		47	
Maternal education (%)				
Low	67	28.8	89	19.6
Medium	100	42.9	223	49.0
High	66	28.3	143	31.4
Missing	4		7	
Delivery hospital (%)				
Private	52	21.9	175	38.1
Public	185	78.1	284	61.9
Missing			3	
Smoking during pregnancy (%)				
No	154	66.7	358	78.5
Yes	77	33.3	98	21.5
Missing	6		6	
Type of delivery (%)				
Vaginal delivery	174	73.7	232	50.8
Cesarean section	62	26.3	225	49.2
Missing	1		5	
Child characteristics				
Sex (%)				
Males	119	50.2	239	51.7
Females	118	49.8	223	48.3
Birth weight [g [mean ± SD]]	237	3,277 ± 429.2	454	3,167 ± 441.6
Missing			8	
Birth length, cm [median (IQR)]	234	50.0 (2.0)	447	50.0 (3.0)
Missing	3		15	
Gestational age, weeks [median (IQR)]	237	39.0 (2.0)	449	38.0 (1.0)
Missing			13	
Weight at examination [g (mean ± SD)]	236	3,229 ± 446.7	456	11,224 ± 2066.3
Missing	1		6	
Length at examination, cm [median (IQR)]	236	50.0 (2.0)	457	82.5 (11.0)
Missing	1		5	
Age at examination, months [median (IQR)]			462	16.0 (11)
Head circumference at examination, cm [median (IQR)]	236	34.5 (1.8)	462	47.5 (3.0)
Missing	1			
Breastfeeding (%)				
Never			64	14.3
Ever			384	85.7
Missing			14	

[a]Median age, 16 months; range, 1–31 months. [b]Data not available (NA) for the HMAR cohort.

associations with ACD in young girls [see Supplemental material, Table S1 (http://dx.doi.org/10.1289/ehp.1205221)]. All estimates were similar to those reported when models were adjusted for weight percentile according to age instead of weight (data not shown).

GAMs examining the shape of the relationships between dioxin-like activity in maternal plasma expressed in picograms CALUX®–TEQ per gram lipid and AGD (millimeters) (Figure 1) showed no significant departures from linearity, both for newborn (p-gain = 0.367) and young boys (p-gain = 0.382).

Discussion

In the present study of two population-based mother–child cohorts in Greece and Spain, prenatal exposure to dioxins and dioxin-like compounds was negatively associated with AGD in males in the context of overall low-level exposures in the general population. We found no evidence that exposure was related to reduced AGDs in girls. Our results are consistent with animal studies as reviewed by the Joint FAO/WHO Expert Committee on Food Additives that noted that the most sensitive adverse effects were on the development of male offspring of rats after prenatal exposure to TCDD (FAO/WHO 2002).

To our knowledge, this is the first study to estimate the effect of dioxins on the development of the human genital system. Our findings are supported by several animal studies where prenatal and lactational exposure to TCDD was associated with a reduced AGD (Gray et al. 1995; Jin et al. 2008, 2010; Ohsako et al. 2001, 2002). Mocarelli et al. (2011) showed that semen quality and sperm counts were reduced in young men with *in utero* and lactational exposure to dioxin in the Seveso accident.

Although the health effect of *in utero* exposure to dioxins on the development of the human reproductive organs is largely unknown, the effect of other endocrine disruptors has been explored. Two studies have found that *in utero* exposure to phthalates was associated with shortened AGD (Suzuki et al. 2011; Swan et al. 2005). However, a third study reported no statistical association between phthalates and male newborns' AGI (AGD/weight) (Huang et al. 2009). No effect of prenatal exposure to DDE on AGD at birth was reported by two studies in Mexico (Longnecker et al. 2007; Torres-Sanchez et al. 2008) although the smaller study reported a significant reduction in one of the indices measured (Anal Position Index) which is a non-age-dependent measurement of AGD (Torres-Sanchez et al. 2008). A recent study reported that *in utero* exposure to BPA was associated with decreased AGD (Miao et al. 2011).

It has been suggested that human hypospadias and cryptorchidism may be associated with reduced AGDs as a result of endocrine disruption (Hsieh et al. 2008). Moreover, findings of recent studies have linked shorter AGD to reproductive parameters in adulthood. Decreased AGD predicted poorer semen quality (Mendiola et al. 2011), and men who had fathered a child had a longer AGD than infertile urology clinic patients (Eisenberg et al. 2011). Men with hypogonadal testosterone levels (< 300 ng/dL) had a significantly shorter AGD compared with men with higher testosterone levels (Eisenberg et al. 2012). In children of the Rhea cohort, neonatal AGD predicted the corresponding genitalia measure at early childhood (Papadopoulou et al. 2013).

In the present study we measured two genital distances, AGD and ASD, but found significant associations with dioxin exposure only for AGD, as did Swan et al. (2005) with exposure to phthalates. On the other hand Mendiola et al. (2011) saw significant associations with sperm parameters only for ASD. These findings suggest that different genitalia measurements may reflect androgen exposures at different stages of life.

Table 2. Distribution of dioxin-like compounds in maternal plasma, AGDs, and penis width in newborns and young children.

	Newborns (n = 237)					Young children (n = 462)[a]				
			Percentile					Percentile		
Variables	n	Mean ± SD	25th	50th	75th	n	Mean ± SD	25th	50th	75th
Anogenital distances										
Males										
AGD (mm)	119	48.8 ± 5.1	45.5	48.2	51.9	237	80.7 ± 7.3	75.2	80.7	86.5
ASD (mm)	119	25.5 ± 4.8	22.4	25.2	28.8	239	39.9 ± 6.9	34.3	39.8	44.9
PW (mm)	117	10.7 ± 1.1	10.0	10.6	11.3	235	14.0 ± 1.7	12.8	14.1	14.9
Females										
ACD (mm)	118	35.0 ± 3.3	32.7	34.8	37.1	223	49.1 ± 6.0	45.4	48.6	53.3
AFD (mm)	118	14.3 ± 3.0	12.4	14.2	15.7	223	21.7 ± 3.9	18.5	21.5	24.1
Dioxin-like compounds in maternal plasma										
pg TEQ/g lipid	237	52.3 ± 20.7	42.5	53.6	66.0	462	49.7 ± 26.7	34.7	50.3	63.5
Percent < LOD	7.6					10.6				

[a]Median age, 16 months; range, 1–31 months.

Table 3. Mean (± SD) of physiological variables in newborns (n = 237) and young boys (n = 462) categorized by median levels of dioxin-like activity in maternal plasma expressed in pg CALUX®–TEQ/g lipid.

	pg CALUX®–TEQ/g lipid[a,b]				
	n	Low	n	High	p-Value[c]
Newborns					
Males					
Gestational age (weeks)	62	38.8 ± 1.7	57	38.7 ± 1.4	0.374
Weight at examination (g)	61	3,305 ± 441.6	57	3,282 ± 386.9	0.867
AGD (mm)	62	49.1 ± 5.3	57	48.4 ± 4.9	0.617
ASD (mm)	62	25.3 ± 5.3	57	25.7 ± 4.2	0.374
PW (mm)	62	10.6 ± 1.1	55	10.9 ± 1.0	0.163
Females					
Gestational age (weeks)	57	38.8 ± 1.3	61	38.5 ± 1.8	0.338
Weight at examination (g)	57	3,190 ± 506.0	61	3,140 ± 469.7	0.615
ACD (mm)	57	35.1 ± 3.8	61	34.9 ± 2.8	0.592
AFD (mm)	57	14.4 ± 3.1	61	14.1 ± 2.6	0.892
Young children					
Males					
Weight at examination (g)	108	11,239 ± 2128.8	126	11,843 ± 1967.0	0.048*
Age at examination (months)	110	17.2 ± 7.2	129	18.5 ± 5.7	0.207
AGD (mm)	110	80.0 ± 7.8	127	81.2 ± 6.9	0.142
ASD (mm)	110	39.6 ± 7.1	129	40.2 ± 6.7	0.471
PW (mm)	110	14.0 ± 1.7	125	13.9 ± 1.7	0.914
Females					
Weight at examination (g)	120	10,763 ± 2175.6	102	10,984 ± 1810.9	0.645
Age at examination (months)	121	16.9 ± 7.3	102	18.2 ± 6.3	0.162
ACD (mm)	121	48.7 ± 6.1	102	49.5 ± 6.0	0.460
AFD (mm)	121	21.8 ± 3.8	102	21.6 ± 4.0	0.760

[a]Median levels of plasma dioxin-like compounds in the low-level and high-level newborn groups were 42.5 (IQR = 21.2; range, 6–53.6) and 66.1 (IQR = 12.5; range, 53.7–106.3) pg CALUX®–TEQ/g lipid, respectively; median levels of plasma dioxin-like compounds in the low-level and high-level young children groups were 34.7 (IQR = 22.0; range, 6–50.2) and 63.5 (IQR = 14.4; range, 50.4–225.7) pg CALUX®–TEQ/g lipid, respectively. [b]Median value of dioxin-like compounds in the newborn group was 53.6pg CALUX®–TEQ/g lipid; median value of dioxin-like compounds in the young children group was 50.3 pg CALUX®–TEQ/g lipid. Values above median were categorized as high whereas values below median were categorized as low in both age groups. [c]Kruskal–Wallis test. *$p < 0.05$.

We found no evidence that *in utero* exposure to dioxins and dioxin-like compounds is associated with female AGDs. Of the two other studies that examined AGD in females, one reported no associations with prenatal DDE exposure (Torres-Sanchez et al. 2008) and the other found prenatal phthalates exposure to be associated with shorter AGI (Huang et al. 2009). Animal studies suggest that some effects of environmental chemicals, including TCDD, may not be detected until puberty or even later in life (Birnbaum 1994a; Gray and Ostby 1995; Heimler et al. 1998; Wolf et al. 1999). In humans there is some evidence that higher exposure to dioxins and dioxin-like compounds is associated with delayed breast development (Den Hond et al. 2002; Leijs et al. 2008). Hence, further follow-up of the girls in our study is needed to evaluate possible effects of dioxins on their reproductive health.

Our results provided some evidence of an adverse effect of dioxins on AGDs of young boys, although estimated effects were small and not statistically significant. The few epidemiological studies which have explored the relationship between AGDs and prenatal exposures have collected their measurements at birth, and only Swan et al. (2005) explored phthalate exposure in relation to AGD in a study of young boys 2–36 months of age. Child's body size is positively associated with AGD (Ozkan et al. 2011; Romano-Riquer et al. 2007; Salazar-Martinez et al. 2004; Sathyanarayana et al. 2010; Thankamony et al. 2009), so a possible reduction due to prenatal exposure might be masked by growth during the first years of life. Moreover, AGDs in childhood could also be affected by early life exposures. Breastfeeding is the main source of exposure to dioxins in early life, but in our study population the duration of breastfeeding was short (median, 2 months) and not associated with AGD (data not shown). Although it would have been ideal to measure the same children at birth and in early childhood, this was not possible due to the designs of the two studies.

Table 4. Associations between a 10-pg increase in maternal DR CALUX®–TEQ/g lipid and anogenital distances and penis width in newborn and young boys [β (95% CI)].

Outcomes	n	Change per 10-pg increase in DR CALUX®–TEQ/g lipid	
		Basic model[a]	Fully adjusted model
Newborns			
AGD (mm)	115	−0.41 (−0.77, −0.06)	−0.44[b] (−0.80, −0.08)
ASD (mm)	112	−0.14 (−0.51, 0.23)	−0.25[c] (−0.61, 0.11)
PW (mm)	116	0.03 (−0.05, 0.11)	0.02[d] (−0.06, 0.09)
Young boys			
AGD (mm)	207	−0.13 (−0.44, 0.18)	−0.07[e] (−0.39, 0.24)
ASD (mm)	218	0.06 (−0.25, 0.38)	0.08[f] (−0.23, 0.39)
PW (mm)	215	−0.05 (−0.11, 0.02)	−0.04[g] (−0.10, 0.03)
Weight standardized *z*-scores of anogenital distances[h]			
AGD *z*-score	207	−0.01 (−0.06, 0.03)	−0.01 (−0.05, 0.04)
ASD *z*-score	218	0.01 (−0.03, 0.06)	0.01 (−0.03, 0.06)
PW *z*-score	215	−0.03 (−0.07, 0.01)	−0.02 (−0.07, 0.02)

[a]Basic model adjusted for birth weight, gestational age and cohort in newborns, and for weight and age at examination and examiner in young boys. [b]Basic model plus maternal ethnicity and maternal education. [c]Basic model plus maternal ethnicity, smoking during pregnancy, and type of delivery. [d]Basic model plus maternal age and delivery hospital. [e]Basic model plus delivery hospital, maternal education, smoking during pregnancy, and residence. [f]Basic model plus maternal age, parity, prepregnancy BMI, and maternal education. [g]Basic model plus maternal age, parity, delivery hospital, and maternal education. [h]All models for weight standardized *z*-scores of anogenital distances are adjusted for the same variables as in the models for the crude measurements of anogenital distances in young boys without weight at the time of measurement.

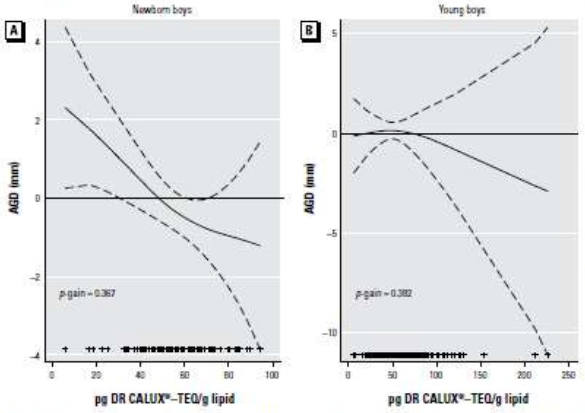

Figure 1. GAMS; adjusted associations (95% CIs) between dioxin-like activity and AGD in newborn (*A*) and young (*B*) boys. (*A*) Adjusted for birth weight, gestational age, cohort, maternal ethnicity, and maternal education. (*B*) Adjusted for weight and age at examination, examiner, delivery hospital, maternal education, smoking during pregnancy, and residence. ++, observations.

In this study, exposure to dioxins and dioxin-like compounds was estimated with the DR CALUX® bioassay. Methods for quantification of dioxin exposure include sensitive and specific techniques such as high-resolution gas chromatography/mass spectrometry. However, these methods are time-consuming and expensive and require large sample volumes (Warner et al. 2005). Although the DR CALUX® does not quantify specific compounds, it provides an overall biological response/potency of mixture that will reflect the effects of possible interactions (synergistic, additive and/or antagonistic interaction) between congeners (Long et al. 2007).

Mean plasma levels of our study (52.3 ± 20.7 and 49.7 ± 26.7 pg CALUX®–TEQ/g lipid in newborns and young children, respectively) were similar to those in other published European studies, except for a Dutch study (mean, 103.7 pg TEQ/g lipid) conducted in the early 1990s (Koopman-Esseboom et al. 1994). Mean levels in two studies of pregnant women in Denmark were 46.0 and 37.0 pg CALUX®–TEQ/g lipid respectively (Halldorsson et al. 2009; Pedersen et al. 2010). A case–control study on endometriosis in Rome reported 18.6 and 20.0 pg TEQ/g lipid in cases and controls (Porpora et al. 2009). Moreover, Belgian studies in young and middle aged women, adolescents, and newborns have reported 46.8 (Pauwels et al. 2000), 36.0 (Koppen et al. 2001), 28.6 and 34.9 (in girls and boys respectively) (Van Den Heuvel et al. 2002), and 23.0 pg CALUX®–TEQ/g lipid (Koppen et al. 2009).

Conclusions

Our results suggest that male infants may be susceptible to endocrine-disrupting effects of dioxins even in the context of overall low-level exposure in the general population. Our findings are consistent with the experimental animal evidence used by FAO/WHO to set recommendations for human dioxin intake.

References

Abel J, Haarmann-Stemmann T. 2010. An introduction to the molecular basics of aryl hydrocarbon receptor biology. Biol Chem 391:1235–1248.

Birnbaum LS. 1994a. Endocrine effects of prenatal exposure to PCBs, dioxins, and other xenobiotics: implications for policy and future research. Environ Health Perspect 102:676–679.

Birnbaum LS. 1994b. The mechanism of dioxin toxicity: relationship to risk assessment. Environ Health Perspect 102(suppl 9):157–167.

Birnbaum LS. 1995a. Developmental effects of dioxins. Environ Health Perspect 103(suppl 7):89–94.

Birnbaum LS. 1995b. Developmental effects of dioxins and related endocrine disrupting chemicals. Toxicol Lett 82–83:743–750.

Brouwer A, Sonneveld E, Botschuijver S, Bassolink H, van der Burg B. 2004. A low volume method for the analysis of dioxins and dioxin-like compounds in serum and whole blood using BDS' DR CALUX® bioassay. Organohalogen Compounds 66:687–690.

Calleganl C, Everett S, Ross M, Brasel JA. 1997. Anogenital ratio: measure of fetal virilization in premature and full-term newborn infants. J Pediatr 111:240–243.

Chatzi L, Plana E, Daraki V, Karakosta P, Alegkakis D, Tsatsanis C, et al. 2009. Metabolic syndrome in early pregnancy and risk of preterm birth. Am J Epidemiol 170:829–836.

Den Hond E, Roels HA, Hoppenbrouwers K, Nawrot T, Thijs L, Vandermeulen C, et al. 2002. Sexual maturation in relation to polychlorinated aromatic hydrocarbons: Sharpe and Skakkebaek's hypothesis revisited. Environ Health Perspect 110:771–776.

Donison MS, Soshilov AA, He G, DeGroot DE, Zhao B. 2011. Exactly the same but different: promiscuity and diversity in the molecular mechanisms of action of the aryl hydrocarbon (dioxin) receptor. Toxicol Sci 124:1–22.

Eisenberg ML, Hsieh MH, Walters RC, Krasnow R, Lipshultz LI. 2011. The relationship between anogenital distance, fatherhood, and fertility in adult men. PLoS One 6:e18973; doi:10.1371/journal.pone.0018973 [Online 11 May 2011].

Eisenberg ML, Jensen TK, Walters RC, Skakkebaek NE, Lipshultz LI. 2012. The relationship between anogenital distance and reproductive hormone levels in adult men. J Urol 187:594–598.

FAO/WHO (Food and Agriculture Organization/World Health Organization). 2002. Evaluation of Certain Food Additives and Contaminants: Fifty-seventh Report of the Joint FAO/WHO Expert Committee on Food Additives. Rome:FAO/WHO.

Gray LE Jr, Kelce WR, Monosson E, Ostby JS, Birnbaum LS. 1995. Exposure to TCDD during development permanently alters reproductive function in male Long Evans rats and hamsters: reduced ejaculated and epididymal sperm numbers and sex accessory gland weights in offspring with normal androgenic status. Toxicol Appl Pharmacol 131:108–118.

Gray LE Jr, Ostby JS. 1995. *In utero* 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) alters reproductive morphology and function in female rat offspring. Toxicol Appl Pharmacol 133:285–294.

Gray LE Jr, Wolf C, Lambright C, Mann P, Price M, Cooper RL, et al. 1999. Administration of potentially antiandrogenic pesticides (procymidone, linuron, iprodione, chlozolinate, *p,p'*-DDE, and ketoconazole) and toxic substances (dibutyl- and diethylhexyl phthalate, PCB 169, and ethane dimethane sulphonate) during sexual differentiation produces diverse profiles of reproductive malformations in the male rat. Toxicol Ind Health 15:94–118.

Greenham LW, Greenham V. 1977. Sexing mouse pups. Lab Anim 11:181–184.

Halldorsson TI, Thorsdottir I, Meltzer HM, Strøm M, Olsen SF. 2009. Dioxin-like activity in plasma among Danish pregnant women: dietary predictors, birth weight and infant development. Environ Res 109:22–29.

Hankinson O. 1995. The aryl hydrocarbon receptor complex. Annu Rev Pharmacol Toxicol 35:307–340.

Heimler I, Trewin AL, Chaffin CL, Rawlins RG, Hutz RJ. 1998. Modulation of ovarian follicle maturation and effects on apoptotic cell death in holtzman rats exposed to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) *in utero* and lactationally. Reprod Toxicol 12:69–73.

Hsieh MH, Breyer BN, Eisenberg ML, Baskin LS. 2008. Associations among hypospadias, cryptorchidism, anogenital distance, and endocrine disruption. Curr Urol Rep 9:137–142.

Huang PC, Kuo PL, Chou YY, Lin SJ, Lee CC. 2009. Association between prenatal exposure to phthalates and the health of newborns. Environ Int 35:14–20.

Jin MH, Hong CH, Lee HY, Kang HJ, Han SW. 2010. Toxic effects of lactational exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on development of male reproductive system: involvement of antioxidants, oxidants, and p53 protein. Environ Toxicol 25:1–8.

Jin MH, Ko HK, Hong CH, Han SW. 2008. *In utero* exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin affects the development of reproductive system in mouse. Yonsei Med J 49:843–850.

Koopman-Esseboom C, Huisman M, Weisglas-Kuperus N, Boersma ER, de Ridder MAJ, Van der Paauw CG, et al. 1994. Dioxin and PCB levels in blood and human milk in relation to living areas in the Netherlands. Chemosphere 29:2327–2338.

Koppen G, Covaci A, Van Cleuvenbergen R, Schepens P, Winneke G, Nelen V, et al. 2001. Comparison of CALUX-TEQ values with PCB and PCDD/F measurements in human serum of the Flanders environmental and health study (FLEHS). Toxicol Lett 123:59–67.

Koppen G, Den Hond E, Nelen V, Van De Mieroop E, Bruckers L, Bilau M, et al. 2009. Organochlorine and heavy metals in newborns: results from the Flemish Environment and Health Survey (FLEHS 2002–2006). Environ Int 35:1015–1022.

Leijs MM, Koppe JG, Olie K, van Aalderen WM, Voogt P, Vulsma T, et al. 2008. Delayed initiation of breast development in girls with higher prenatal dioxin exposure; a longitudinal cohort study. Chemosphere 73:999–1004.

Long M, Deutch B, Bonefeld-Jorgensen EC. 2007. AhR transcriptional activity in serum of Inuits across Greenlandic districts. Environ Health 6:32; doi:10.1186/1476-069X-6-32 [Online 23 October 2007].

Longnecker MP, Gladen BC, Cupul-Uicab LA, Romano-Riquer SP, Weber JP, Chapin RE, et al. 2007. *In utero* exposure to the antiandrogen 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (DDE) in relation to anogenital distance in male newborns from Chiapas, Mexico. Am J Epidemiol 165:1015–1022.

Marty MS, Chapin RE, Parks LG, Thorsrud BA. 2003. Development and maturation of the male reproductive system. Birth Defects Res B Dev Reprod Toxicol 68:125–136.

Mendiola J, Stahlhut RW, Jorgensen N, Liu F, Swan SH. 2011. Shorter anogenital distance predicts poorer semen quality in young men in Rochester, New York. Environ Health Perspect 119:958–963.

Miao M, Yuan W, He Y, Zhou Z, Wang J, Gao E, et al. 2011. *In utero* exposure to bisphenol-A and anogenital distance of male offspring. Birth Defects Res A Clin Mol Teratol 91:867–872.

Mocarelli P, Gerthoux PM, Needham LL, Patterson DG Jr, Limonta G, Falbo R, et al. 2011. Perinatal exposure to low doses of dioxin can permanently impair human semen quality. Environ Health Perspect 119:713–718.

Murk AJ, Leonards PE, van Hattum B, Luit R, van der Weiden ME, Min M. 1997. The CALUX (chemical-activated luciferase expression) assay adapted and validated for measuring TCDD equivalents in blood plasma. Environ Toxicol Chem 16:1583–1589.

Ohsako S, Miyabara Y, Nishimura N, Kurosawa S, Sakaue M, Ishimura R, et al. 2001. Maternal exposure to a low dose of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) suppressed the development of reproductive organs of male rats: dose-dependent increase of mRNA levels of 5α-reductase type 2 in contrast to decrease of androgen receptor in the pubertal ventral prostate. Toxicol Sci 60:132–143.

Ohsako S, Miyabara Y, Sakaue M, Ishimura R, Kakeyama M, Izumi H, et al. 2002. Developmental stage-specific effects of perinatal 2,3,7,8-tetrachlorodibenzo-*p*-dioxin exposure on reproductive organs of male rat offspring. Toxicol Sci 66:293–292.

Ohtake F, Baba A, Fujii-Kuriyama Y, Kato S. 2008. Intrinsic AhR function underlies cross-talk of dioxins with sex hormone signalings. Biochem Biophys Res Commun 370:541–546.

Ohtake F, Takeyama K, Matsumoto T, Kitagawa H, Yamamoto Y, Nohara K, et al. 2003. Modulation of oestrogen receptor signalling by association with the activated dioxin receptor. Nature 423:545–550.

Ozkan B, Konak B, Cayir A, Konak M. 2011. Anogenital distance in Turkish newborns. J Clin Res Pediatr Endocrinol 3:122–125.

Papadopoulou E, Vafeiadi M, Agramunt S, Basagaña X, Mathianaki K, Karakosta P, et al. 2013. Anogenital distances in newborns and children from Spain and Greece: predictors, tracking and reliability. Paediatr Perinat Epidemiol 27(1):89–99.

Parrefall W. 2002. Risk assessment of dioxin contamination in human food. Food Chem Toxicol 40:1185–1189.

Pauwels A, Comijn PH, Schepens PJ, Brouwer A. 2000. Comparison of chemical-activated luciferase gene expression bioassay and gas chromatography for PCB determination in human serum and follicular fluid. Environ Health Perspect 108:553–557.

Pedersen M, Halldorsson TI, Mathiesen L, Mose T, Brouwer A, Hedegaard M, et al. 2010. Dioxin-like exposures and effects on estrogenic and androgenic exposures and micronuclei frequency in mother–newborn pairs. Environ Int 36:344–351.

Perpora MG, Madda E, Abballe A, Balli S, De Angelis I, di Domenico A, et al. 2009. Endometriosis and organochlorinated environmental pollutants: a case–control study on Italian women of reproductive age. Environ Health Perspect 117:1070–1075.

Romano-Riquer SP, Hernandez-Avila M, Gladen BC, Cupul-Uicab LA, Longnecker MP. 2007. Reliability and determinants of anogenital distance and penis dimensions in male newborns from Chiapas, Mexico. Paediatr Perinat Epidemiol 21:219–228.

Safe S, Krishnan V. 1995. Chlorinated hydrocarbons: estrogens and antiestrogens. Toxicol Lett 82–83:731–736.

Salazar-Martinez E, Romano-Riquer P, Yanez-Marquez E, Longnecker MP, Hernandez-Avila M. 2004. Anogenital distance in human male and female newborns: a descriptive, cross-sectional study. Environ Health 3:8; doi: 10.1186/1476-069X-3-8 [Online 13 September 2004].

Sathyanarayana S, Beard L, Zhou C, Grady R. 2010. Measurement and correlates of ano-genital distance in healthy, newborn infants. Int J Androl 33:317–323.

Suzuki Y, Yoshinaga J, Mizumoto Y, Serizawa S, Shiraishi H. 2011. Foetal exposure to phthalate esters and anogenital distance in male newborns. Int J Androl 35(3):236–244.

Swan SH, Main KM, Liu F, Stewart SL, Kruse RL, Calafat AM, et al. 2005. Decrease in anogenital distance among male infants with prenatal phthalate exposure. Environ Health Perspect 113:1056–1061.

Thankamony A, Ong KK, Dunger DB, Acerini CL, Hughes IA. 2009. Anogenital distance from birth to 2 years: a population study. Environ Health Perspect 117:1786–1790.

Torres-Sanchez L, Zepeda M, Cebrian ME, Balkind-Gerson J, Garcia-Hernandez RM, Balkind-Valdovinos U, et al. 2008. Dichlorodiphenyldichloroethylene exposure during the first trimester of pregnancy alters the anal position in male infants. Ann NY Acad Sci 1140:155–162.

Van Den Heuvel RL, Koppen G, Staessen JA, Hond ED, Verheyen G, Nawrot TS, et al. 2002. Immunologic biomarkers in relation to exposure markers of PCBs and dioxins in Flemish adolescents (Belgium). Environ Health Perspect 110:595–600.

Warner M, Eskenazi B, Patterson DG, Clark G, Turner WE, Bonsignore L, et al. 2005. Dioxin-Like TEQ of women from the Seveso, Italy area by ID-HRGC/HRMS and CALUX. J Expo Anal Environ Epidemiol 15:310–318.

WHO (World Health Organization). The WHO Child Growth Standards. 2006. Available: http://www.who.int/childgrowth/standards/en/ [accessed 17 October 2012].

Wolf CJ, Ostby JS, Gray LE Jr. 1999. Gestational exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) severely alters reproductive function of female hamster offspring. Toxicol Sci 51:259–264.

5.4 Paper IV

Prenatal exposure to persistent organochlorines and anogenital distance in the mother-child cohort in Crete, Greece (RHEA study)[4]

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Prenatal exposure to persistent organochlorines and anogenital distance in the mother-child cohort in Crete, Greece (RHEA study).

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List of abbreviations:
ACD, anoclitoral distance
AFD, anofourchetal distance
AGD, anogenital distance
ASD, anoscrotal distance
BPA, Bisphenol-A
BMI, body mass index
DDE, dichlorodiphenyl dichloroethene

DDT, dichlorodiphenyl trichloroethane

GAMs, Generalized additive models

GWG, gestational weight gain

HCB, hexachlorobenzene

IOM, Institute of Medicine

LOQ, limit of quantification

OCs, Organochlorine compounds

PCBs, polychlorinated biphenyls

POPs, persistent organic pollutants

PW, penis width

SD, standard deviation

95% CI, 95% Confidence Intervals

Abstract

Organochlorine compounds (OCs), are identified as endocrine disrupting chemicals (EDCs) which can alter the function of endocrine systems in humans and animals. Anogenital distance (AGD) has been inversely associated with prenatal exposure to environmental endocrine disruptors and several adult reproductive health outcomes. We evaluated the association between in utero exposure to organochlorine compounds and anogenital distance in newborns and young children from the Rhea study in Crete, Greece. Anogenital distance (AGD; anus to upper penis), anoscrotal distance (ASD; anus to scrotum), and penis width (PW) were measured in 77 newborn and 319 young boys; anoclitoral (ACD; anus to clitoris) and anofourchetal distance (AFD; anus to fourchette) were measured in 73 newborn and 304 young girls. Concentrations of several PCBs and other OCs (dichlorodiphenyl dichloroethene [DDE], and hexachlorobenzene [HCB]) were determined in 1st trimester maternal serum by triple quadrupole mass spectrometry. In multivariate models, negative but not statistically significant associations were observed for AGD and HCB, DDE and total PCBs in young boys. In young girls, ACD and AFD were positively associated with all OCs. Our results provided some evidence of an endocrine disruptive effect of OCs, expressed as phenotypic alterations of the reproductive system of young boys and girls.

INTRODUCTION

Organochlorine compounds (OCs), an important kind of persistent organic pollutants (POPs), are synthetic chemicals that were widely used as pesticides [e.g., dichlorodiphenyltrichloroethane (DDT), dichlorodiphenyl dichloroethene (DDE), hexachlorobenzene (HCB)] and in industrial processes [polychlorinated biphenyls (PCBs)], through most of the 20th century; until they were banned or their use tightly restricted in the seventies and eighties (1). They are ubiquitous and persist in the environment, accumulate in high concentrations in fatty tissues and are bio-magnified through the food-chain. Human exposure to POPs occurs primarily through diet and studies have demonstrated that maternal concentrations of POPs are transmitted to the developing fetus prenatally through the placenta and postnatally via breast milk (2). Although the use of these chemicals is presently banned or restricted; due to their persistence in the environment, the general population is still exposed to these substances at low doses (3) and adverse health outcomes related to background levels of exposure are still a concern for the general population (4).

Organochlorines at high levels are known to be toxic for wildlife and humans. Although there has been a growing concern about the effect of low-level exposure to organochlorine compounds on human fetal development, variation in results across studies remains unexplained (5,

6). Several of these compounds are identified as endocrine disrupting chemicals (EDCs) which can alter the normal function of endocrine systems in humans and wildlife (7). Chemical compounds with endocrine disrupting effects, that overall mimic estrogens and/or antagonize androgens, might interfere with several pathways of the reproductive system and could have different effects on males and females.

Anogenital distance (AGD), the distance from the anus to the genitalia, is a developmental endpoint in animal toxicology studies routinely used as a measure of fetal androgen action. AGD is sexually dimorphic; males have a longer AGD than females due to higher in utero androgen exposure and the use of AGD to sex newborns is common (8, 9). AGD usually tracks through life, varies by dose of anti-androgen, and can be predictive of other androgen-responsive outcomes such as hypospadias and cryptorchidism (10). AGD has been identified as an important clinical measure to address endocrine-sensitive endpoints in the U.S. Environmental Protection Agency guidelines for reproductive toxicity studies in humans (11) but has only been examined in recent years in human studies. Anogenital distance is sexual dimorphic also in humans and associated with body size (12-16). We previously reported inverse associations between prenatal exposure to dioxins and dioxin-like compounds and shorter AGD in human male infants (12). Moreover, AGD has been

inversely associated with prenatal exposure to other environmental endocrine disruptors, namely phthalates and bisphenol A (BPA) (17-20). Two studies have investigated the association between prenatal exposure to DDE and anogenital distance in children and reported inconsistent results (21, 22). Findings of recent studies have linked decreased AGD with reproductive parameters in adulthood such as poorer semen quality (23, 24), fertility (25), hypogonadal testosterone levels (26) and prostate cancer (27).

The purpose of the current analysis was to evaluate the association between in utero exposure to current low levels of different organochlorine compounds and anogenital distance in newborns and young children from the Rhea study in Crete, Greece.

MATERIALS AND METHODS

Study population

The Rhea study prospectively examines a population-based sample of pregnant women and their children at the prefecture of Heraklion, Crete, Greece. Methods are described in detail elsewhere (28). Briefly, female residents (Greek and immigrants) who became pregnant during a period of one year starting in February 2007 were contacted and asked to participate in the study. The first contact was made at the time of the first major ultrasound examination (~12th week of gestation) and several contacts followed (6th month of pregnancy, at birth, 6 months, 1st year and 4

years after birth). To be eligible for inclusion in the study, women had to have a good understanding of the Greek language and be older than 16 years of age. Face-to-face structured questionnaires along with self-administered questionnaires and medical records were used to obtain information on several psychosocial, dietary, and environmental exposures during pregnancy and early childhood. 1,765 eligible women were approached during the enrollment period, 1,610 (91%) agreed to participate and 1,388 (86%) were followed up until delivery. 1135 blood samples provided by the study participants were analyzed for exposure. Anthropometric measurements were conducted for 165 newborns (84 boys and 81 girls) and 732 young children (374 boys and 358 girls). The present analysis included 150 newborns (77 boys and 73 girls) and 623 young children (mean age, 17.2 months; 319 boys and 304 girls), all of whom were singletons with information on anthropometry and exposure. The study was approved by the ethical committee of the University Hospital in Heraklion, Crete, Greece, and all participants provided written, informed consent after complete description of the study.

Physical examination

The measurement protocol of anogenital distances has been previously described in details (12, 13). In brief, in boys we recorded AGD, the distance from the anterior base of the penis to the center of the anus; anoscrotal distance

(ASD), the distance from the posterior base of the scrotum to the center of the anus; and penis width (PW), the diameter of the penis in its base. In girls we recorded, anoclitoral distance (ACD), the distance between the clitoris and the center of the anus; and anofourchettal distance (AFD), the distance from the posterior convergence of the fourchette to the center of the anus. Each measurement was repeated three times and the average of the three measurements was recorded. Weight, length, and head circumference were measured twice, and average values were used for analysis. Gestational age was based on the interval between the last menstrual period and the date of delivery of the baby for the majority of the subjects. When the menstrual estimate of gestational age was inconsistent by ≥ 7 days with the ultrasound measurement taken in the first trimester of pregnancy, a quadratic regression formula describing the relationship between crown–rump length and gestational age was used instead.

Biological sample collection and exposure assessment

Maternal serum samples were collected at the first prenatal visit around the 3rd and 4th month of pregnancy, in 10 ml vacutainer tubes, were centrifuged, and were then stored in aliquots at -80 C until assayed. The POP analyses were performed in the National Institute for Health and Welfare, Chemical Exposure Unit, Kuopio, Finland with an Agilent 7000B gas chromatograph triple quadrupole mass spectrometer (GC-MS/MS). Pretreatment of serum samples

for GC-MS/MS analysis has been described elsewhere (29). Serum concentrations of six individual PCB congeners (IUPAC numbers: 118, 138, 153, 156, 170 and 180), HCB, DDT and DDE were determined. All the results were reported on whole weight and expressed in pg/ml serum, while samples bellow the limit of quantification (LOQ) were assigned the value 0.5×LOQ. LOQ was 6 pg/ml for PCB118 and PCB 156; 10 pg/ml for HCB, DDE, PCB138, PCB153, PCB170, and PCB180, and 50 pg/ml for DDT. Due to high percentages of samples below the LOQ, DDT was not used in the analyses. Regarding PCB 156, all analyses were repeated excluding this pollutant with no change in the results. We chose to use wet-weight levels for the POPs but adjusted for serum triglycerides and cholesterol as continuous variables in all multivariable models to minimize potential biases associated with automatic lipid adjustment (30). POPs were treated as continuous variables on a $\log_{10}$ scale. We defined total PCBs as the sum of the 6 congeners, non dioxin-like PCBs as the sum of PCB 153, 138, 170 and 180, and dioxin-like PCBs as the sum of PCB 118 and 156. The respective total PCB concentrations were calculated by summing the concentrations of individual PCB congeners. Moreover we constructed an overall exposure score based on the concentrations of HCB, DDE and total PCBs that took values of 0 for mothers with all chemicals below the median of each chemical, to a maximum of 3 for those with all chemicals above the median. We studied the

associations of interest separately for each pollutant, for the 3 groups of PCBs, the overall exposure score and in a multipollutant model including DDE, HCB and PCBs.

Statistical analysis

We examined descriptive and summary statistics for all variables, including maternal characteristics, serum levels OCs and AGD measures. Multivariate linear regression models were used to examine the association between POPs in maternal serum and anogenital distances in newborns and young children. Birth weight, gestational age and examiner were included in all models for newborns while weight and age at examination and examiner were included in all models for young children. In addition, maternal age (years), pre-pregnancy body mass index (BMI) (kg/m^2), maternal educational level [low level: ≤9 years of mandatory schooling, medium level: >9 years of schooling up to attending post-secondary school education (but not attending university or having a technical college degree) and high level: attending university or having a university/technical college degree]), sample batch (first, second) and serum triglycerides and cholesterol were a-priori considered as potential confounding factors and included in all multivariate models. Additionally, we assessed whether parity (primiparous, multiparous), maternal weight before pregnancy (kg), weight gain during pregnancy (kg), maternal origin (Greek, non Greek), residence (urban, rural), alcohol consumption during

pregnancy (yes, no), delivery type (vaginal delivery, Cesarean section), delivery hospital (private, public), marital status (married, not married), working during pregnancy (yes, no), smoking during pregnancy (never, ever) and breastfeeding [(never, ever) only for the models of young children] had further influence on the effect estimates. Potential confounding by these factors was examined by adding each at a time in the basic models already containing the a priori defined confounders. If inclusion of a variable altered the contaminant coefficient by 10% or more we retained the variable in the final set of covariates. The same set of covariates was used for all exposure-outcome combinations. In alternative analyses we used weight-standardized z-scores of AGD as the outcome. Generalized additive models (GAMs) were applied to explore the shape of the relationships between OCs in maternal serum and AGDs and test departures from linearity. These models indicated linear relationships for all AGDs in newborns and young children. Analyses were conducted using STATA software, version 13.0 (Statacorp, College Station, TX).

RESULTS

Participants' characteristics

Overall, the population was fairly homogeneous and was predominantly Greek (Table 1). On average, mothers were 29 years of age at the time of delivery, had a pre-pregnancy body mass index (BMI) within the normal range (24.5 kg/m^2), were multiparous, nonsmokers, and living in urban

areas. Approximately half of the newborns (56 %) and (49 %) of the young children were vaginally delivered, with a mean (SD) birth weight of 3210 (398) g and 3169 (442) g respectively. Percentages of boys and girls were similar in newborns (51.3 % boys and 48.7 % girls) and young children (51.2 % boys and 48.8 % girls). Young children were measured at a mean age of 17.2 (6.6) months and most of them had breastfed (87.5 %).

POPs and anogenital parameters

Mean (SD) AGDs were longer in male newborns [AGD = 50.5 (4.6 mm), ASD = 27.0 (4.3) mm] than in female [ACD = 35.2 (2.9) mm, AFD = 14.5 (3.1) mm] (Table 2). Similarly, in young children mean AGDs were longer in males [AGD = 80.6 (7.9) mm, ASD = 39.7 (7.1) mm] than females [ACD = 49.9 (7.0) mm, AFD = 21.7 (3.9) mm]. The mean of PW was 11.2 (1.0) mm in newborns and 14.0 (1.6) mm in young boys. Mean maternal POPs concentrations were slightly lower in mothers of newborns than in mothers of young children (Table 2). The highest concentrations were found for DDE, followed by PCBs, HCB, and DDT.

Relationship between maternal serum POPs and anogenital parameters

GAMs examining the shape of the relationships between PCBs, HCB and birth weight showed no significant departures from linearity overall and separately in boys and girls (Figure 1).

In crude analyses, in newborn boys, AGD showed trends towards positive associations with maternal serum POPs levels, while ASD was negatively associated with total PCBs (Table 3). Because models were run with POPs on the $\log_{10}$ scale, a unit of increase would mean a 10-fold increase in the concentration of each contaminant. An increase in newborn AGD was observed for increasing levels of HCB (adjusted β= 1.7 mm; 95% CI: -4.1, 7.6), DDE (adjusted β= 3.9 mm; 95% CI: 1.3, 6.5) and total PCBs (adjusted β= 4.0 mm; 95% CI: -2.2, 10.3). In girls, crude models showed a trend towards negative associations between anogenital distances and POPs. In the fully adjusted models, ACD was negatively associated with all POPs except HCB, while AFD was negatively associated with the respective sums of PCBs and positively associated with DDE. Except for the association of AGD and DDE, none of the associations were statistically significant.

Negative but not statistically significant associations were observed for AGD and HCB (adjusted β= -0.8 mm; 95% CI: -5.2, 3.5), DDE (adjusted β= -1.7 mm; 95% CI: -4.3, 1.0) and total PCBs (adjusted β= -1.5 mm; 95% CI: -6.3, 3.3) in young boys. ASD was negatively associated with HCB (adjusted β= -2.4 mm; 95% CI: -6.5, 1.8) and positively associated with the rest of the POPs. Negative associations were also observed for PW. In young girls, crude models showed a trend towards positive associations between anogenital distances and all POPs. In the fully adjusted

models, an increase in ACD was observed for increasing levels of HCB (adjusted β= 0.6 mm; 95% CI: -2.5, 3.7), DDE (adjusted β= 1.1 mm; 95% CI: -1.1, 3.2) and total PCBs (adjusted β= 2.2 mm; 95% CI: -1.9, 6.3). We also noted a borderline significant reduction in AFD related to increasing levels of DDE (adjusted β= 1.0 mm; 95% CI: -0.2, 2.2) and total PCBs (adjusted β= 2.0 mm; 95% CI: -0.3, 4.3).

In analyses using weight-standardized z-scores of AGD as the outcome the results were unchanged in both the newborns and the young children (not shown).

DISCUSSION

We examined the association between maternal serum levels of different POPs and anogenital parameters of newborns and young children in a prospective mother-child cohort in Greece. Our results provided some evidence of an adverse effect of POPs on AGDs of young boys and girls, although the observed associations were small and not statistically significant. We found no evidence that in utero exposure to POPs is associated with newborn AGDs.

In our study, prenatal exposure to DDE, HCB and PCBs was negatively associated with AGD in young males. The antiandrogenic effects of DDE are consistent with animal models, were exposure to DDE in utero has been found to affect anogenital distance (31). In humans, the association between prenatal exposure to DDE and anogenital distance has been investigated by two cross-sectional studies in Mexico. Longnecker et al., in a study of 781 male infants

found no evidence that exposure in utero to relatively high levels of DDE, was related to reduced androgen action as reflected by anogenital distance or penile dimensions at birth (21). The other study of 71 infants also found no association with AGD but reported a significant reduction in one of the indices measured (Anal Position Index) which is a non-age-dependent measurement of AGD (22).

In utero exposure to endocrine disruptors can affect the reproductive system and AGD has been inversely associated with prenatal exposure to other environmental endocrine disruptors, namely phthalates. Two studies have found that in utero exposure to phthalates was associated with shortened AGD (17, 20). However, a third study reported no statistical association between phthalates and male newborns' AGI (AGD/weight) (19). A recent study reported that in utero exposure to BPA was associated with decreased AGD (18). To our knowledge this is the first study to estimate directly the effect of HCB and several PCBs on the development of the human genital system. We previously reported inverse associations between prenatal exposure to dioxins and dioxin-like compounds and shorter AGD in human male infants (12). Moreover, in the same population, prenatal exposure to persistent organic pollutants, through maternal diet was associated with reduction in anoscrotal distance (ASD) of newborn males. In this study in utero exposure to POPs was not associated with newborn AGDs but this could also be related to the

small sample size of newborns. Based on evidence from animal studies, genitalia distances are mainly defined in-utero and the effect of additional postnatal androgen action or production is considered minor.

Although AGD as a ”health” outcome has only been investigated in recent years, results show that it might be an important measure of the development of the human genital system. Short AGD may be associated with hypospadias and cryptorchidism, as a result of endocrine disruption (32-34). Findings of recent studies have linked AGD length and reproductive parameters in adulthood. Decreased AGD was associated with poorer semen quality (23, 24), fertility (25), hypogonadal testosterone levels (26), while prostate cancer patients found to have shorter distances than non-patients (27).

In our study, prenatal exposure to POPs was associated with an increase in female anogenital distances. In animal models high prenatal exposure to testosterone was related to an increase of female offspring genitalia distance, suggesting masculinization of female rodents (35). Recently, prenatal stress was associated with significantly longer AGD in female infants suggesting that prenatal stress may masculinize some aspects of female reproductive development in humans (36). Only one study has examined the relationships between AGD and female reproductive system characteristics in adult women and they reported that AGD was positively and strongly

associated with the presence of greater ovarian follicular number (37). Results on females are scarce and further follow-up of this cohort is needed to investigate possible effects of prenatal exposures on the reproductive health of females.

In conclusion, our findings provide some evidence that we found that prenatal exposure to current low levels of different organochlorine compounds might me associated with a reduction of male and an increase of female anogenital distance. Additional research is needed to examine whether these endocrine disruptive effects, expressed as phenotypic alterations of the reproductive system, are evident in other populations.

Table 1. Maternal and Child Characteristics in Newborns (n=150) and Young Children (n=623), "Rhea" Mother–Child Cohort Study in Crete, Greece

	Newborns			Young Children		
	No.	**mean (SD)**	**%**	**No.**	**mean (SD)**	**%**
Maternal characteristics						
Maternal age, years	150	29.7 (4.8)		621	30.1 (4.7)	
<20	2		1.3	9		1.5
≥20-30	68		45.3	249		40.1
≥30-40	77		51.4	349		56.2
≥40	3		2.0	14		2.3
Maternal weight before pregnancy, kg	149	65.8 (14.3)		622	65.2 (13.8)	
Maternal height, m	149	1.6 (0.1)		622	1.6 (0.1)	
Pre-pregnancy BMI, kg/m^2	149	24.5 (4.7)		621	24.5 (4.8)	
Underweight (<18,5)	6		4.0	23		3.7
Normal (≥18.5-25)	87		58.4	388		62.5
Overweight (≥25-30)	39		26.2	139		22.4
Obese (≥30)	17		11.4	71		11.4
Weight gain during pregnancy, kg	89	13.5 (5.2)		517	13.5 (5.2)	
Inadequate	16		18.0	120		23.3
Adequate	35		39.3	181		35.1
Excessive	38		42.7	215		41.7
Maternal origin						
Other	8		5.3	34		5.5
Greek	142		94.7	588		94.5
Maternal Education						
Low	34		22.7	103		16.6
Medium	74		49.3	314		50.5
High	42		28.0	205		32.9
Residence						
Urban	109		74.7	465		81.0
Rural	37		25.3	109		19.0
Parity						
Primiparous	63		42.6	234		38.5
Multiparous	85		57.4	374		61.5
Smoking during pregnancy						
Never	90		60.0	402		65.5
Ever	60		40.0	212		34.5
Smoking status during pregnancy						
Non smoker	90		60.0	402		65.5
Quit smoking	33		22.0	117		19.0
Smoker	27		18.0	95		15.5
Alcohol consumption						
No	42		80.8	292		68.2
Yes	10		19.2	136		31.8

	Newborns			Young Children		
	No.	**mean (SD)**	**%**	**No.**	**mean (SD)**	**%**
Type of delivery						
Vaginal	84		56.0	305		49.0
Caesarian	66		44.0	317		51.0
Delivery hospital						
Public	87		58.0	358		57.6
Private	63		42.0	264		42.4
Marital status						
Married	140		93.3	558		89.7
Other	10		6.7	64		10.3
Working during pregnancy						
No	80		53.3	279		44.5
Yes	70		46.7	343		55.1
Gestational hypertension						
No	136		95.1	542		95.9
Yes	7		4.9	23		4.1
Gestational diabetes						
No	131		92.9	519		90.7
Yes	10		7.1	53		9.3
Child characteristics						
Gender						
Boy	77		51.3	319		51.2
Girl	73		48.7	304		48.8
Birth weight,Kg	150	3.2 (0.4)		620	3.2 (0.4	
Birth length, cm	148	50.2 (2.1)		614	50.5 (2.3)	
Head circumference, cm	148	34.2 (1.1)		608	34.2 (1.5)	
Gestational age, weeks	127	38.6 (1.3)		548	38.2 (1.5)	
Age at examination, months				623	17.2 (6.6)	
Weight at examination,Kg	150	3.1 (0.4)		623	11.1 (2.1)	
Length at examination, cm	150	49.9 (1.9)		621	81.9 (8.1)	
Breastfeeding						
Never				77		12.5
Ever				537		87.5

Table2. Distribution of POPs Measured in 1st Trimester Maternal Serum (pg/ml), Anogenital Distances, and Penis Width in Newborns and Young Children, "Rhea" Mother–Child Cohort Study in Crete, Greece

	Newborns						Young children					
			Percentile						Percentile			
Anogenital distances	**No.**	**Mean (SD)**	**25th**	**50th**	**75th**		**No.**	**Mean (SD)**	**25th**	**50th**	**75th**	
Males												
AGD (mm)	77	50.5 (4.6)	47.2	50.1	53.0		317	80.6 (7.9)	75.6	80.7	86.5	
ASD (mm)	77	27.0 (4.3)	23.8	26.8	29.8		319	39.7 (7.1)	34.1	39.2	44.2	
PW (mm)	76	11.2 (1.0)	10.6	10.9	11.9		313	14.0 (1.6)	13.0	14.0	14.9	
Females												
ACD (mm)	73	35.2 (2.9)	33.8	35.8	37.1		302	49.9 (7.0)	45.8	49.1	53.7	
AFD (mm)	73	14.5 (3.1)	12.5	14.3	16.1		302	21.7 (3.9)	18.7	21.3	24.0	
Contaminants						**% >LOQ[a]**						**% >LOQ[a]**
HCB	150	95.5 (78.2)	57.4	77.4	99.0	100	623	114.2 (111.0)	62.8	84.9	122.2	100
DDT	150	63.4 (79.3)	25.0[b]	25.0[b]	64.4	37.3	623	68.7 (133.7)	25[b]	25[b]	67.0	37.4
DDE	150	2844 (2915)	1162.7	1960.9	3383.1	100	623	3199 (3704)	1246	2091	3641	100
PCB118	150	18.7 (13.2)	10.2	15.3	23.3	98.7	623	21.1 (12.8)	12.9	18.3	25.9	97.8
PCB153	150	127.9 (75.2)	79.7	108.3	161.4	100	623	150.8 (92.6)	89.2	129.2	188.3	100
PCB138	150	69.1 (41.6)	42.6	59.1	89.3	100	623	81.4 (49.5)	47.5	71.1	102.0	99.8
PCB156	150	7.4 (6.1)	3.0[b]	6.5	9.1	53.3	623	8.1 (6.8)	3[b]	6.64	10.93	55.5
PCB180	150	69.4 (46.9)	36.1	57.9	87.1	100	623	84.5 (64.6)	45.7	70.1	105.2	99.8
PCB170	150	36.0 (24.6)	17.8	30.8	47.4	95.3	623	42.6 (33.7)	22.2	35.6	52.8	97.1
Total PCBs	150	328.4 (198.3)	194.6	272.8	425.2		623	388.4 (248.7)	226.1	330.5	483.1	
DL PCBs	150	26.1 (18.3)	14.6	21.9	32.3		623	29.1 (18.1)	17.2	23.9	37.1	
Non DL PCBs	150	302.3 (183.4)	180.7	251.6	394.5		623	359.3 (234.5)	208.1	306.7	446.2	

[a] LOQ was 6 pg/ml for PCB118 and PCB 156; 10 pg/ml for HCB, DDE, PCB138, PCB153, PCB170 and PCB180; 50 pg/ml for DDT.
[b] Value is LOQ/2, DL PCBs=Dioxin-like PCBs , Non DL PCBs = non Dioxin-like PCBs

Table 3. Crude and Adjusted Associations Between POPs Measured in 1st Trimester Maternal Serum and Anogenital Distances (mm) and Penis Width (mm) in Newborns, "Rhea" Mother–Child Cohort Study in Crete, Greece

	Basic model[a]				
	Change in AGD	**Change in ASD**	**Change in PW**	**Change in ACD**	**Change in AFD**
	n=68	**n=68**	**n=67**	**n=58**	**n=58**
Contaminants	**β (95%CI)**	**β (95%CI)**	**β (95%CI)**	**β (95%CI)**	**β (95%CI)**
log_{10} HCB	0.7 (-4.4, 5.8)	0.01 (-5.1, 5.2)	0.1 (-1.2, 1.3)	2.1 (-1.1, 5.2)	-0.5 (-3.4, 2.5)
log_{10} DDE	3.1 (0.8, 5.5)	1.5 (-1.0, 4.0)	-0.1 (-0.7, 0.5)	-0.5 (-2.7, 1.7)	-0.2 (-2.3, 1.8)
log_{10} Σ all PCBs	0.9 (-3.5, 5.2)	-1.6 (-6.0, 2.8)	-0.2 (-1.3, 0.9)	-0.2 (-3.7, 3.4)	-2.0 (-5.2, 1.2)
log_{10} Σ dioxin-like PCBs	2.1 (-2.6, 6.8)	-1.2 (-6.0, 3.6)	-0.1 (-1.3, 1.1)	-1.0 (-4.7, 2.6)	-2.8 (-6.0, 0.5)
log_{10} Σ non dioxin-like PCBs	0.7 (-3.5, 5.0)	-1.6 (-5.9, 2.7)	-0.2 (-1.3, 0.9)	-0.1 (-3.6, 3.4)	-1.9 (-5.0, 1.3)
Exposure score[c]	0.5 (-0.5, 1.4)	0.5 (-0.4, 1.5)	0.1 (-0.2, 0.3)	-0.1 (-0.9, 0.6)	-0.4 (-1.1, 0.3)
	Fully adjusted model[b]				
	Change in AGD	**Change in ASD**	**Change in PW**	**Change in ACD**	**Change in AFD**
	β (95%CI)	**β (95%CI)**	**β (95%CI)**	**β (95%CI)**	**β (95%CI)**
log_{10} HCB	1.7 (-4.1, 7.6)	0.5 (-5.5, 6.5)	0.2 (-1.2, 1.6)	2.1 (-1.8, 6.1)	0.05 (-3.2, 3.3)
log_{10} DDE	3.9 (1.3, 6.5)	1.9 (-0.9, 4.7)	0.01 (-0.7, 0.7)	-1.4 (-4.5, 1.7)	1.1 (-1.4, 3.6)
log_{10} Σ all PCBs	4.0 (-2.2, 10.3)	-2.0 (-8.4, 4.4)	-0.2 (-1.8, 1.4)	-1.4 (-6.7, 4.0)	-0.8 (-5.1, 3.5)
log_{10} Σ dioxin-like PCBs	4.9 (-1.0, 10.9)	-1.6 (-7.8, 4.5)	0.003 (-1.5, 1.5)	-2.0 (-6.4, 2.5)	-1.8 (-5.4, 1.8)
log_{10} Σ non dioxin-like PCBs	3.8 (-2.4, 10.0)	-2.0 (-8.4, 4.3)	-0.2 (-1.7, 1.4)	-1.2 (-6.5, 4.1)	-0.7 (-5.0, 3.6)
Exposure score[c]	0.9 (-0.2, 2.1)	0.9 (-0.3, 2.1)	0.1 (-0.2, 0.4)	-0.4 (-1.5, 0.6)	-0.2 (-1.1, 0.7)

[a] Model includes sample batch, maternal serum triglycerides and cholesterol, birth weight, gestational age and examiner

[b] Model includes sample batch, maternal serum triglycerides and cholesterol, birth weight, gestational age, examiner, maternal age, pre-pregnancy BMI, maternal educational level, working status during pregnancy and delivery hospital.

[c] Exposure score: based on the concentrations of HCB, DDE and total PCBs.

Table 8. Crude and Adjusted Associations Between POPs Measured in 1st Trimester Maternal Serum and Anogenital Distances (mm) And Penis Width (mm) in Young Children, "Rhea" Mother–Child Cohort Study in Crete, Greece

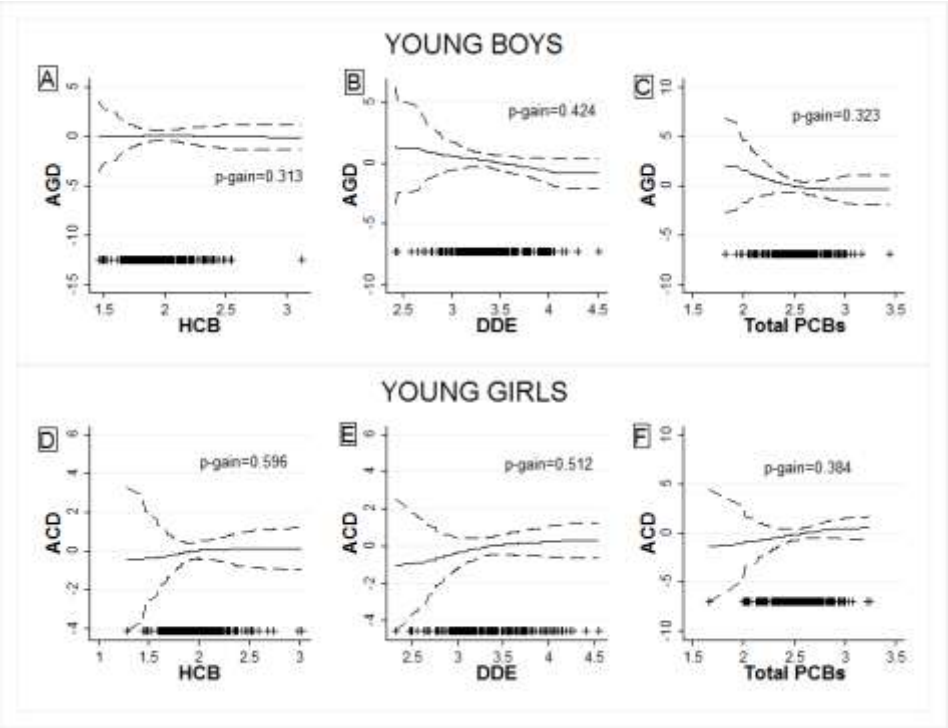
	Basic model[a]				
	Change in AGD	**Change in ASD**	**Change in PW**	**Change in ACD**	**Change in AFD**
	n=272	**n=274**	**n=268**	**n=269**	**n=269**
Contaminants	**β (95%CI)**	**β (95%CI)**	**β (95%CI)**	**β (95%CI)**	**β (95%CI)**
log_{10} HCB	0.7 (-3.0, 4.5)	-1.4 (-4.9, 2.1)	-0.2 (-0.9, 0.5)	1.3 (-1.5, 4.1)	1.4 (-0.2, 2.9)
log_{10} DDE	-0.6 (-2.9, 1.7)	0.4 (-1.8, 2.6)	0.1 (-0.4, 0.5)	1.5 (-0.4, 3.4)	1.2 (0.1, 2.3)
log_{10} Σ all PCBs	0.4 (-3.2, 4.0)	0.03 (-3.4, 3.5)	-0.4 (-1.1, 0.4)	1.6 (-1.5, 4.8)	1.5 (-0.2, 3.3)
log_{10} Σ dioxin-like PCBs	0.7 (-3.2, 4.5)	0.7 (-3.0, 4.5)	-0.5 (-1.3, 0.3)	0.8 (-2.3, 3.9)	1.5 (-0.2, 3.3)
log_{10} Σ non dioxin-like PCBs	0.4 (-3.1, 3.9)	-0.01 (-3.4, 3.4)	-0.3 (-1.0, 0.4)	1.6 (-1.5, 4.7)	1.5 (-0.3, 3.2)
Exposure score[c]	0.2 (-0.5, 0.9)	0.03 (-0.7, 0.7)	-0.1 (-0.2, 0.1)	0.5 (-0.2, 1.2)	0.3 (-0.1, 0.7)
	Fully adjusted model[b]				
	Change in AGD	**Change in ASD**	**Change in PW**	**Change in ACD**	**Change in AFD**
	β (95%CI)	**β (95%CI)**	**β (95%CI)**	**β (95%CI)**	**β (95%CI)**
log_{10} HCB	-0.8 (-5.2, 3.5)	-2.4 (-6.5, 1.8)	-0.1 (-0.9, 0.8)	0.6 (-2.5, 3.7)	0.7 (-1.0, 2.5)
log_{10} DDE	-1.7 (-4.3, 1.0)	0.4 (-2.2, 3.0)	0.2 (-0.3, 0.7)	1.1 (-1.1, 3.2)	1.0 (-0.2, 2.2)
log_{10} Σ all PCBs	-1.5 (-6.3, 3.3)	0.2 (-4.4, 4.8)	-0.3 (-1.3, 0.6)	2.2 (-1.9, 6.3)	2.0 (-0.3, 4.3)
log_{10} Σ dioxin-like PCBs	-1.0 (-5.6, 3.6)	0.7 (-3.7, 5.2)	-0.5 (-1.4, 0.5)	0.4 (-3.1, 3.9)	1.3 (-0.7, 3.2)
log_{10} Σ non dioxin-like PCBs	-1.5 (-6.3, 3.2)	0.2 (-4.4, 4.8)	-0.3 (-1.2, 0.7)	2.3 (-1.8, 6.3)	2.0 (-0.3, 4.2)
Exposure score[c]	-0.2 (-1.1, 0.8)	-0.02 (-0.9, 0.9)	-0.04 (-0.2, 0.2)	0.5 (-0.4, 1.3)	0.2 (-0.3, 0.7)

[a] Model includes sample batch, maternal serum triglycerides and cholesterol, weight and age at examination, and examiner

[b] Model includes sample batch, maternal serum triglycerides and cholesterol, weight and age at examination, examiner, maternal age, pre-pregnancy BMI, maternal educational level, working status during pregnancy and delivery hospital.

[c] Exposure score: based on the concentrations of HCB, DDE and total PCBs.

Figure 6. GAMS; adjusted associations (95% CIs) of AGD with HCB (A), DDE (B) and total PCBs (C), and ACD with HCB (D), DDE (E) and total PCBs (F). Adjusted for batch, maternal serum triglycerides and cholesterol, weight and age at examination, examiner, maternal age, pre-pregnancy BMI, maternal educational level, working status during pregnancy and delivery hospital. ++, observations.



REFERENCES

1. Stockholm Convention on Persistent Organic Pollutants. 2004. (http://www.pops.int.). (Accessed 30/08/2013).
2. Needham LL, Grandjean P, Heinzow B, et al. Partition of environmental chemicals between maternal and fetal blood and tissues. *Environ Sci Technol* 2011;45(3):1121-6.
3. Porta M, Puigdomenech E, Ballester F, et al. Monitoring concentrations of persistent organic pollutants in the general population: the international experience. *Environ Int* 2008;34(4):546-61.
4. Organization WH. Persistent Organic Pollutants: Impact on Child Health. 2010. (http://www.who.int/ceh/publications/persistent_organic_pollutant/en/index.html). (Accessed 5 November 2013).
5. Govarts E, Nieuwenhuijsen M, Schoeters G, et al. Birth weight and prenatal exposure to polychlorinated biphenyls (PCBs) and dichlorodiphenyldichloroethylene (DDE): a meta-analysis within 12 European Birth Cohorts. *Environ Health Perspect* 2012;120(2):162-70.
6. Al-Saleh I, Al-Doush I, Alsabbaheen A, et al. Levels of DDT and its metabolites in placenta, maternal and cord blood and their potential influence on neonatal anthropometric measures. *Sci Total Environ* 2012;416:62-74.

7. Diamanti-Kandarakis E, Bourguignon JP, Giudice LC, et al. Endocrine-disrupting chemicals: an Endocrine Society scientific statement. *Endocr Rev* 2009;30(4):293-342.
8. Greenham LW, Greenham V. Sexing mouse pups. *Lab Anim* 1977;11(3):181-4.
9. Marty MS, Chapin RE, Parks LG, et al. Development and maturation of the male reproductive system. *Birth Defects Res B Dev Reprod Toxicol* 2003;68(2):125-36.
10. Gray LE, Jr., Wolf C, Lambright C, et al. Administration of potentially antiandrogenic pesticides (procymidone, linuron, iprodione, chlozolinate, p,p'-DDE, and ketoconazole) and toxic substances (dibutyl- and diethylhexyl phthalate, PCB 169, and ethane dimethane sulphonate) during sexual differentiation produces diverse profiles of reproductive malformations in the male rat. *Toxicol Ind Health* 1999;15(1-2):94-118.
11. Arbuckle TE, Hauser R, Swan SH, et al. Meeting Report: Measuring Endocrine-Sensitive Endpoints within the First Years of Life. *Environ Health Perspect* 2008;116(7).
12. Vafeiadi M, Agramunt S, Papadopoulou E, et al. In utero exposure to dioxins and dioxin-like compounds and anogenital distance in newborns and infants. *Environ Health Perspect* 2013;121(1):125-30.
13. Papadopoulou E, Vafeiadi M, Agramunt S, et al. Anogenital distances in newborns and children from Spain

and Greece: predictors, tracking and reliability. *Paediatr Perinat Epidemiol* 2013;27(1):89-99.

14. Salazar-Martinez E, Romano-Riquer P, Yanez-Marquez E, et al. Anogenital distance in human male and female newborns: a descriptive, cross-sectional study. *Environ Health* 2004;3(1):8.
15. Sathyanarayana S, Beard L, Zhou C, et al. Measurement and correlates of ano-genital distance in healthy, newborn infants. *Int J Androl* 2010;33(2):317-23.
16. Thankamony A, Ong KK, Dunger DB, et al. Anogenital distance from birth to 2 years: a population study. *Environ Health Perspect* 2009;117(11):1786-90.
17. Swan SH, Main KM, Liu F, et al. Decrease in anogenital distance among male infants with prenatal phthalate exposure. *Environ Health Perspect* 2005;113(8):1056-61.
18. Miao M, Yuan W, He Y, et al. In utero exposure to bisphenol-A and anogenital distance of male offspring. *Birth Defects Res A Clin Mol Teratol* 2011;91(10):867-72.
19. Huang PC, Kuo PL, Chou YY, et al. Association between prenatal exposure to phthalates and the health of newborns. *Environ Int* 2009;35(1):14-20.
20. Suzuki Y, Yoshinaga J, Mizumoto Y, et al. Foetal exposure to phthalate esters and anogenital distance in male newborns. *Int J Androl* 2011.
21. Longnecker MP, Gladen BC, Cupul-Uicab LA, et al. In utero exposure to the antiandrogen 1,1-dichloro-2,2-bis(p-

chlorophenyl)ethylene (DDE) in relation to anogenital distance in male newborns from Chiapas, Mexico. *Am J Epidemiol* 2007;165(9):1015-22.

22. Torres-Sanchez L, Zepeda M, Cebrian ME, et al. Dichlorodiphenyldichloroethylene exposure during the first trimester of pregnancy alters the anal position in male infants. *Ann N Y Acad Sci* 2008;1140:155-62.
23. Eisenberg ML, Shy M, Walters RC, et al. The relationship between anogenital distance and azoospermia in adult men. *Int J Androl* 2012;35(5):726-30.
24. Mendiola J, Stahlhut RW, Jorgensen N, et al. Shorter anogenital distance predicts poorer semen quality in young men in Rochester, New York. *Environ Health Perspect* 2011;119(7):958-63.
25. Eisenberg ML, Hsieh MH, Walters RC, et al. The relationship between anogenital distance, fatherhood, and fertility in adult men. *PLoS One* 2011;6(5):e18973.
26. Eisenberg ML, Jensen TK, Walters RC, et al. The relationship between anogenital distance and reproductive hormone levels in adult men. *J Urol* 2012;187(2):594-8.
27. Castano-Vinyals G, Carrasco E, Lorente JA, et al. Anogenital distance and the risk of prostate cancer. *BJU Int* 2012;110(11 Pt B):E707-10.
28. Chatzi L, Plana E, Daraki V, et al. Metabolic syndrome in early pregnancy and risk of preterm birth. *Am J Epidemiol* 2009;170(7):829-36.

29. Koponen J, Rantakokko P, Airaksinen R, et al. Determination of selected perfluorinated alkyl acids and persistent organic pollutants from a small volume human serum sample relevant for epidemiological studies. *J Chromatogr A* 2013;1309:48-55.
30. Schisterman EF, Whitcomb BW, Louis GM, et al. Lipid adjustment in the analysis of environmental contaminants and human health risks. *Environ Health Perspect* 2005;113(7):853-7.
31. Gray LE, Ostby J, Furr J, et al. Effects of environmental antiandrogens on reproductive development in experimental animals. *Hum Reprod Update* 2001;7(3):248-64.
32. Hsieh MH, Eisenberg ML, Hittelman AB, et al. Caucasian male infants and boys with hypospadias exhibit reduced anogenital distance. *Hum Reprod* 2012;27(6):1577-80.
33. Hsieh MH, Breyer BN, Eisenberg ML, et al. Associations among hypospadias, cryptorchidism, anogenital distance, and endocrine disruption. *Curr Urol Rep* 2008;9(2):137-42.
34. Jain VG, Singal AK. Shorter anogenital distance correlates with undescended testis: a detailed genital anthropometric analysis in human newborns. *Hum Reprod* 2013;28(9):2343-9.
35. Hotchkiss AK, Lambright CS, Ostby JS, et al. Prenatal testosterone exposure permanently masculinizes

anogenital distance, nipple development, and reproductive tract morphology in female Sprague-Dawley rats. *Toxicol Sci* 2007;96(2):335-45.

36. Barrett ES, Parlett LE, Sathyanarayana S, et al. Prenatal exposure to stressful life events is associated with masculinized anogenital distance (AGD) in female infants. *Physiol Behav* 2013;114-115:14-20.

37. Mendiola J, Roca M, Minguez-Alarcon L, et al. Anogenital distance is related to ovarian follicular number in young Spanish women: a cross-sectional study. *Environ Health* 2012;11:90.

6 GENERAL DISCUSSION

This thesis presents a compilation of epidemiological studies to assess the impact of prenatal exposure to environmental chemicals on fetal growth indicators and the development of the human genital system in European populations using biomarkers of exposure to these contaminants. To our knowledge, this is the first study to estimate the effect of such chemicals on child development in the Greek population.

This section provides a global discussion and provides a broader and more integrated interpretation of the entire study project. It is an elaboration and expansion of the detailed discussions of each manuscript presented in the results section of this thesis.

6.1 Contribution and main strengths of this thesis

6.1.1 Exposure biomarkers

As described in detail in papers I and II, previous reports of associations between exposure to POPs and pregnancy outcomes are inconclusive. Several factors may account for these inconsistencies. First, discrepant findings may result from differences in study design, biological matrices sampled, time of sample collection, differences in classification and

assessment of exposure and outcome variables, and population differences such as confounding variables used or different susceptibility of studied populations. Second, regarding PCBs, different congeners are measured and the congeners may be classified differently. Moreover, differences in the toxicities of particular congener mixtures may contribute to inconsistent results and make comparisons between studies difficult.

Regarding exposure to dioxins and dioxin-like compounds, exposure assessment has been conducted in different ways in each study. Previous epidemiologic studies relied on surrogate indicators of exposure (eg, fish consumption (Tawara et al. 2009), residence near waste sites (Sonneborn et al. 2008) or in areas with high consumption of contaminated fish etc.) (Fein et al. 1984; Weisskopf et al. 2005), some have focused only on PCBs (Fein et al. 1984; Patandin et al. 1998; Hertz-Picciotto et al. 2005; Sagiv et al. 2007; Sonneborn et al. 2008; Rogan et al. 1986; Longnecker et al. 2005), others on PCDDs/PCDFs (Nishijo et al. 2008; Tawara et al. 2009), while others have measured the congeners in milk (Tajimi et al. 2005; Nishijo et al. 2008; Tawara et al. 2009; Vartiainen et al. 1998) and others in maternal (Hertz-Picciotto et al. 2005; Sonneborn et al. 2008; Konishi et al. 2009; Longnecker et al. 2005; Weisskopf et al. 2005; Halldorsson et al. 2009) and cord plasma(Fein et al. 1984; Sagiv et al. 2007).

Dioxins and dioxin-like compounds exist in environmental and biological samples as complex mixtures of various congeners. Therefore calculating total TEQ concentration has been established since it simplifies risk assessment and regulatory control and is the most relevant exposure measure in studies of health effects of dioxins and dioxin-like compounds (Van den Berg et al. 1998; Warner et al. 2005). High-resolution Gas Chromatography/Mass Spectrometry (GC/MS) has become the "gold standard" for identifying dioxins and dioxin-like compounds in a sensitive and specific fashion. These analytical methods measure the concentrations of specific compounds and TEQs are calculated using the TEF scheme of the WHO (Van den Berg et al. 1998; Van den Berg et al. 2006). However, these methods are time-consuming and expensive; they require large sample volumes (frequently tens of ml of blood) and extensive sample clean-up (Warner et al. 2005).

In addition to the analytical approach to dioxin exposure assessment, in vitro reporter gene bioassays allow the measurement of specific receptor activations. In this study, as explained in detail in Papers I and III, exposure to dioxins and dioxin-like compounds was estimated in plasma with the DR CALUX® bioassay. The Dioxin-Responsive Chemically Activated LUciferase eXpression (DR CALUX®) assay, measures the ability of a chemical mixture to activate AhR-dependent gene expression of the firefly luciferase gene in

genetically modified cell lines, requiring for this purpose only small amounts of blood plasma (Brouwer et al. 2004). Moreover because of the extensive clean-up procedure followed, only stable compounds (e.g. dioxins, furans and dioxin-like PCBs) and no other contaminations or chemicals are measured. Unlike chemical analysis methods, which measure the concentrations of specific compounds, the DR CALUX® provides an overall biological response/potency of mixture and takes into account possible interactions (synergistic, additive and/or antagonistic interaction) between congeners (Long et al. 2007). Moreover, the use of DR CALUX® may reduce exposure misclassification, as variation in uptake and affinity to the receptor are integrated in the toxic potency measurements.

Paper I, within the NewGeneris project, was the first large population-based study to use DR CALUX® to estimate exposure to dioxins and dioxin-like compounds in mother-child cohorts from five European countries. The use of such a biomarker to measure exposure, both in cord and in maternal plasma is a key strength of the present study, since large biomarker-based studies are rare because of their costs and complexity. The DR CALUX® bioassay has shown a high agreement with congener-specific methods and can be a useful tool for estimating exposures in epidemiological studies, since it also offers a considerable reduction in

sample volume compared to instrumental methods, which is often a limiting factor.

Within the Rhea study, in papers II and IV we used state-of-the-art laboratory techniques for measuring serum levels of several POPs, including HCB, DDE and PCBs, in maternal serum samples collected at the first prenatal visit. The method that we used has been previously shown to be both sensitive and reproducible and requires only a small amount of serum (Koponen et al. 2013). Moreover we were able to assess the adverse effect of combined exposure to several contaminants. Since environmental contaminants coexist in exposure sources and may have additive or antagonistic effects, the importance of studying a broad array of contaminants that coexist in a specific population is apparent. Another notable strength of this study is that we were able to measure exposure at an early phase of pregnancy, reducing the influence of changing maternal physiology.

6.1.2 Novel outcomes

The majority of studies evaluating the endocrine-disrupting effects of environmental chemicals have focused on birth outcomes such as birth weight since it is an outcome easily collected from clinical records. Birth weight is indeed a very important outcome since it is reflective of developmental

progression from the time of conception to birth and may be intermediate between prenatal toxic exposures and various health outcomes in later life; hence the in utero effects of environmental agents on pregnancy outcomes are of interest. However, given the current interest in the reproductive and developmental effects of endocrine-disrupting chemicals, the identification of novel more sensitive health end-points, to assess early health effects that can predict later disease, is needed.

Anogenital distance, is a developmental endpoint in animal toxicology studies and is believed to be a marker of prenatal androgen exposure during a reproductive programming window (Welsh et al. 2008; van den Driesche et al. 2011; Macleod et al. 2010). Although it has been identified as an important clinical measure to address endocrine-sensitive endpoints in the U.S. Environmental Protection Agency guidelines for reproductive toxicity studies in humans it has only been examined in recent years in human studies.

In papers III and IV we examined anogenital distances in newborns and young children. All the examiners, including myself, received a common and extensive training before conducting the measurements. All AGD measurements were performed using a standardized analytical protocol. Anogenital distance has proven to be a highly reliable anthropometric measure, when conducted by trained

examiners (Papadopoulou et al. 2013; Romano-Riquer et al. 2007). Within the RHEA study, we were able to assess its reliability as an anthropometric measure. Two examiners, including myself, conducted a reliability study and we provided information on between- and within-examiners variation for boys and girls as well as repeatability coefficients (Papadopoulou et al. 2013). Moreover, measurements are well tolerated by all subjects and quick to perform.

The use of anogenital distance in epidemiological studies is relatively new, so methods for its reliable measurement are still being developed. Several alternative measurements have been used in examining AGD in humans and findings suggest that different genitalia measurements may reflect androgen exposures at different stages of life. In the present study we based our measurements on the protocol used in a previous study (Swan et al. 2005), which was modified to include additional measurements in both sexes (Salazar-Martinez et al. 2004; Callegari et al. 1987).

In males, AGD has been inversely associated with prenatal exposure to some environmental endocrine disruptors, hypospadias and cryptorchidism may be associated with reduced AGDs as a result of endocrine disruption and shorter AGD has been linked adverse to reproductive parameters in adulthood. Less is known about AGD in females. Within the RHEA study we were able to measure anogenital distances in

boys and girls. In paper III we found that prenatal exposure to dioxins and dioxin-like compounds might induce endocrine disruptive effects measured as a reduction in anogenital distance of boys in the context of overall low-level exposures in the general population. In paper IV, our findings provide some evidence that prenatal exposure to current low levels of different organochlorine compounds duch as HCB, DDE and PCBs might me associated with a reduction of male and an increase of female anogenital distance.

6.1.3 Confounders and effect modifiers

A key challenge in epidemiological studies that needs to be adequately addressed to ensure the validity, accuracy and reliability of the results, is confounding. The availability of relevant covariates in all the studies included in this thesis, including socioeconomic and life-style factors from both parents, as well as other harmful exposures, such as tobacco smoke, allowed the adjustment for potential confounders of association between prenatal exposure to environmental contaminants and fetal growth indicators. Moreover we were able to evaluate effect modification in the studied associations related to several factors such as child sex, smoking status and maternal pre-pregnancy BMI.

Scientific evidence on low-level prenatal exposure to environmental chemicals in relation to reproductive and

developmental health is inconclusive. Hence, the need to explore other factors that may interfere or explain these associations is apparent. A recent paper (Verner et al. 2013) reporting the use of pharmacokinetic models has questioned the validity of the association between PCBs and other POPs with lower birth weight and has suggested that the observed association may be due to uncontrolled confounding by maternal gestational weight gain (GWG). This hypothesis suggests that GWG is associated negatively with PCB levels in maternal and cord blood and positively with birth weight, hence, could substantially confound the association of PCBs and birth weight. The results of the pharmacokinetic models applied indicated that a previously observed association in a large meta-analysis (Govarts et al. 2012) of over 7,000 pregnancies of an estimated 150 g decrease in birth weight with each 1µg/L increase in PCB153, would practically disappear when controlling for GWG (Verner et al. 2013). In paper II we found that a 10-fold increase in total PCBs and HCB was associated with 174 g and 161 g decrease in birth weight respectively. Following Verner's et al. hypothesis we evaluated whether gestational weight gain (GWG) confounded the association between maternal serum levels of different POPs and fetal growth and length of gestation. When we adjusted for GWG the association with HCB was slightly reduced to a 154 g decrease and with PCBs to a 135 g decrease in birth weight. Therefore, evaluation of maternal gestational weight gain influenced only to a limited extent the

association of POPs and birth weight in our study, contrary to the prediction of the pharmacokinetic model proposed by Verner et al. (2013). Our results suggest that the association of POPs and birth weight is probably more complex than that hypothesized by Verner et al. (2013).

6.2 Limitations

One limitation of our study was that we did not measure anogenital distances in the same children at birth and in early childhood. In this population this was done only for 112 children and results showed that in both sexes, AGD at birth is correlated with AGD between ages 1 and 2 years (Papadopoulou et al. 2013) but more studies with larger sample size are needed to confirm these results. In the same line, anogenital distance was only measured in two of the participating studies (RHEA and HMAR) and not in all the children of the NewGeneris project. Although we did identify statistically significant results, the measurement of the same biomarkers and outcomes in a larger population, especially anogenital distance, would improve statistical power and provide more stable estimates.

Regarding the different exposures examined in the papers of this thesis, we were not able to measure the same contaminants in all the subjects. Furthermore, the measurement of a limited number of contaminants may not

provide a more global evaluation of exposure to endocrine disruptors. The issue of the evaluation of exposure to mixtures has been a classical problem in similar studies, without any easy solution. Measurement of a larger number of chemicals is one option, though the evaluation of the combination of their activity would still remain a problem. The use of DR CALUX® to some extent provides an alternative to individual estimation of chemicals with dioxin-like activity, while other assays evaluating global exposures have also been proposed such as the total effective xenoestrogen burden (TEXB) bioassay that is a marker of the combined effect of mixtures of xenoestrogens in the organism (Vilahur et al. 2013; Fernandez et al. 2008; Lopez-Espinosa et al. 2009; Fernandez et al. 2004).

Finally, unfortunately we lack a quantitative estimate of potential measurement error regarding gestational weight gain. For the calculation of this variable, we used measured weight at time of delivery, but self-reported pre-pregnancy weight.

6.3 Public health implications

The use of most chemicals examined in this thesis is presently banned or restricted and their concentrations in the environment and human samples have declined rapidly. However, due to their persistence in the environment, the

general population is still exposed to these substances at low doses and concerns about their effects in vulnerable populations remain. This thesis provides evidence about the adverse effects of prenatal exposure to environmental chemicals on fetal growth. We have shown that in utero low-level dioxin-like activity is associated with shorter gestational age and that increasing maternal serum levels of HCB and PCBs are associated with decreased birth weight. As mentioned in the introduction, reduced birth weight, has been associated with infant mortality as well as outcomes later in life such as asthma, lower IQ, and hypertension (Wilcox 2001). Thus, our findings might have implications for public health at earlier or later stages of life.

Our findings add to the existing literature that suggests that exposure to environmental endocrine disruptors is associated with shorter anogenital distance in male infants. In paper III prenatal exposure to dioxins and dioxin-like compounds was negatively associated with AGD in males in the context of overall low-level exposures in the general population. Moreover our findings provide some evidence that prenatal exposure to current low levels of different organochlorine compounds duch as HCB, DDE and PCBs might me associated with an increase of female anogenital distance. Since a reduction in male anogenital distance might be linked to impaired testicular function and men's reproductive health (Eisenberg et al. 2012a; Mendiola et al. 2011; Eisenberg et

al. 2011; Eisenberg et al. 2012b; Castano-Vinyals et al. 2012) and longer anogenital distance was associated with increased odds of multifollicular ovaries in women (Mendiola et al. 2012), our results could provide a link between prenatal exposures and reproductive function in adult life.

6.4 Future research

There are still several gaps in the current knowledge of the characterization of the exposure to environmental chemicals, their interrelation and their effects on child health. Although concentrations of established environmental toxicants have declined, concerns about their low-dose effects in vulnerable populations remain. Moreover, a huge numbers of “emerging” chemicals are only just starting to be characterized.

Epidemiological studies with good exposure assessment are few and have studied specific groups of exposures. Up to now, research has focused on single exposure-health associations and there are no available studies examining how different exposures co-exist and their joint impact on health outcomes. The concept of the “exposome” was first introduced by Cristopher Wild in 2005 in attempt to describe all the exposures of an individual in a lifetime and how those exposures relate to health (Wild 2005). In this line a new project, called HELIX, was funded under the FP7 European Comission Exposome Programme with the aim to exploit

novel tools and methods (biomarkers of exposure, 'omics'-based approaches, remote sensing and GIS-based spatial methods, personal exposure devices, statistical tools for combined exposures, and burden of disease methodologies), to characterize early-life exposure to a wide range of environmental hazards, and integrate and link these with data on major child health outcomes (growth and obesity, neurodevelopment, immune system), thus developing an "Early-Life Exposome" approach (http://www.projecthelix.eu/).

RHEA study is participating in this project and in this regard we will have the opportunity in the next years to collect the necessary data to unravel some of the research questions proposed. Future suggestions for epidemiological studies implement measurements of anogenital distance in boys and girls at several time-points as a sensitive marker of endocrine disruption.

Finally, during the last decade there has been increasing interest in whether environmental chemical exposures may contribute to the rising prevalence of obesity. However, there are few epidemiologic data on early-life chemical exposures that may be obesogenic. In the RHEA study, anthropometric measurements, including skinfold thickness measurements, have been collected at several follow-ups and a future investigation of their relationship with prenatal exposures to environmental contaminants has been planned.

7 CONCLUSIONS

- Results from the international NewGeneris project provide evidence supporting an association of low-level prenatal dioxin-like activity with shorter gestational age, particularly in boys, while weaker associations were found for birth weight.
- We found an association between increasing maternal serum levels of HCB and PCBs and decreased birth weight. Our findings suggest that prenatal exposure to PCBs and HCB impairs fetal growth and add to the growing literature that demonstrates an association between low-level environmental pollutant exposure and fetal growth. Adjustment for maternal gestational weight gain explained only to a small extent the association between POP levels and birth weight. Furthermore our results suggest that the association of POPs, maternal gestational weight gain and birth weight is probably more complex than that previously hypothesized.
- Prenatal exposure to dioxins and dioxin-like compounds was related to a reduction of anogenital distance in newborn boys. These results suggest that male infants may be susceptible to endocrine-disrupting effects of dioxins. Our findings are consistent with the experimental animal evidence used by the Food and Agriculture Organization/World Health Organization to set recommendations for human dioxin intake.

- Prenatal exposure to DDE, HCB and PCBs was negatively associated with anogenital distance in young males and possitively with female anogenital distances. Our findings provide some evidence of endocrine disruptive effects of prenatal exposure to current low levels of different organochlorine compounds, expressed as phenotypic alterations of the reproductive system.

8 REFERENCES

Grandjean P, Landrigan PJ. 2006. Developmental neurotoxicity of industrial chemicals. Lancet 368(9553): 2167-2178.

Thayer KA, Heindel JJ, Bucher JR, Gallo MA. 2012. Role of environmental chemicals in diabetes and obesity: a National Toxicology Program workshop review. Environ Health Perspect 120(6): 779-789.

Schug TT, Janesick A, Blumberg B, Heindel JJ. 2011. Endocrine disrupting chemicals and disease susceptibility. J Steroid Biochem Mol Biol 127(3-5): 204-215.

Landrigan PJ, Goldman LR. 2011. Children's vulnerability to toxic chemicals: a challenge and opportunity to strengthen health and environmental policy. Health Aff (Millwood) 30(5): 842-850.

Meeker JD. 2012. Exposure to environmental endocrine disruptors and child development. Arch Pediatr Adolesc Med 166(10): 952-958.

Ferguson KK, O'Neill MS, Meeker JD. 2013. Environmental contaminant exposures and preterm birth: a comprehensive review. J Toxicol Environ Health B Crit Rev 16(2): 69-113.

Norman RE, Carpenter DO, Scott J, Brune MN, Sly PD. 2013. Environmental exposures: an underrecognized contribution to noncommunicable diseases. Rev Environ Health 28(1): 59-65.

Wright RJ, Brunst KJ. 2013. Programming of respiratory health in childhood: influence of outdoor air pollution. Curr Opin Pediatr 25(2): 232-239.

Llop S, Lopez-Espinosa MJ, Rebagliato M, Ballester F. 2013. Gender differences in the neurotoxicity of metals in children. Toxicology 311(1-2): 3-12.

Philippat C, Mortamais M, Chevrier C, Petit C, Calafat AM, Ye X, et al. 2012. Exposure to phthalates and phenols during pregnancy and offspring size at birth. Environ Health Perspect 120(3): 464-470.

Holtcamp W. 2012. Obesogens: an environmental link to obesity. Environ Health Perspect 120(2): a62-68.

Cantonwine DE, Hauser R, Meeker JD. 2013. Bisphenol A and Human Reproductive Health. Expert Rev Obstet Gynecol 8(4).

Valvi D, Casas M, Mendez MA, Ballesteros-Gomez A, Luque N, Rubio S, et al. 2013. Prenatal bisphenol a urine concentrations and early rapid growth and overweight risk in the offspring. Epidemiology 24(6): 791-799.

Patel CJ, Bhattacharya J, Butte AJ. 2010. An Environment-Wide Association Study (EWAS) on type 2 diabetes mellitus. PLoS One 5(5): e10746.

Stockholm, Convention. 2009. Stockholm Convention on Persistent Organic Pollutants.

IARC. 1997. IARC Working Group on the Evaluation of Carcinogenic Risks to Humans: Polychlorinated Dibenzo-Para-Dioxins and Polychlorinated Dibenzofurans. Lyon,

France, 4-11 February 1997. IARC Monogr Eval Carcinog Risks Hum 69: 1-631.

Baan R, Grosse Y, Straif K, Secretan B, El Ghissassi F, Bouvard V, et al. 2009. A review of human carcinogens--Part F: chemical agents and related occupations. Lancet Oncol 10(12): 1143-1144.

IARC. 2012. A Review of Human Carcinogens: Chemical Agents and Related Occupations. IARC monographs on the evaluation of carcinogenic risks to humans 100F.

Milbrath MO, Wenger Y, Chang CW, Emond C, Garabrant D, Gillespie BW, et al. 2009. Apparent half-lives of dioxins, furans, and polychlorinated biphenyls as a function of age, body fat, smoking status, and breast-feeding. Environ Health Perspect 117(3): 417-425.

Schecter A, Birnbaum L, Ryan JJ, Constable JD. 2006. Dioxins: an overview. Environ Res 101(3): 419-428.

Safe SH. 1995. Modulation of gene expression and endocrine response pathways by 2,3,7,8-tetrachlorodibenzo-p-dioxin and related compounds. Pharmacol Ther 67(2): 247-281.

Hankinson O. 1995. The aryl hydrocarbon receptor complex. Annu Rev Pharmacol Toxicol 35: 307-340.

Safe S, Krishnan V. 1995. Chlorinated hydrocarbons: estrogens and antiestrogens. Toxicol Lett 82-83: 731-736.

Kitamura M, Kasai A. 2007. Cigarette smoke as a trigger for the dioxin receptor-mediated signaling pathway. Cancer Lett 252(2): 184-194.

Denison MS, Soshilov AA, He G, DeGroot DE, Zhao B. 2011. Exactly the same but different: promiscuity and diversity in the molecular mechanisms of action of the aryl hydrocarbon (dioxin) receptor. Toxicol Sci 124(1): 1-22.

Van den Berg M, Birnbaum L, Bosveld AT, Brunstrom B, Cook P, Feeley M, et al. 1998. Toxic equivalency factors (TEFs) for PCBs, PCDDs, PCDFs for humans and wildlife. Environ Health Perspect 106(12): 775-792.

Van den Berg M, Birnbaum LS, Denison M, De Vito M, Farland W, Feeley M, et al. 2006. The 2005 World Health Organization reevaluation of human and Mammalian toxic equivalency factors for dioxins and dioxin-like compounds. Toxicol Sci 93(2): 223-241.

Carpenter DO. 2006. Polychlorinated biphenyls (PCBs): routes of exposure and effects on human health. Rev Environ Health 21(1): 1-23.

Wang G, Lu Y, Han J, Luo W, Shi Y, Wang T, et al. 2010. Hexachlorobenzene sources, levels and human exposure in the environment of China. Environ Int 36(1): 122-130.

Barber JL, Sweetman AJ, van Wijk D, Jones KC. 2005. Hexachlorobenzene in the global environment: emissions, levels, distribution, trends and processes. Sci Total Environ 349(1-3): 1-44.

WHO. 2002. Global Assessment of the State-of-the-science of Endocrine Disruptors. International Programme on Chemical Safety.

Diamanti-Kandarakis E, Bourguignon JP, Giudice LC, Hauser R, Prins GS, Soto AM, et al. 2009. Endocrine-disrupting chemicals: an Endocrine Society scientific statement. Endocr Rev 30(4): 293-342.

Olea N, Fernandez M. 2007. Endocrine disruption. J Epidemiol Community Health 61(5): 372-373.

WHO. 2012. State of the science of endocrine disrupting chemicals - 2012

An assessment of the state of the science of endocrine disruptors prepared by a group of experts for the United Nations Environment Programme (UNEP) and WHO.

Dietrich DR, Aulock Sv, Marquardt H, Blaauboer B, Dekant W, Kehrer J, et al. 2013. Scientifically unfounded precaution drives European Commission's recommendations on EDC regulation, while defying common sense, well-established science and risk assessment principles. Chemico-Biological Interactions 205(1): A1-A5.

Bergman A, Andersson AM, Becher G, van den Berg M, Blumberg B, Bjerregaard P, et al. 2013. Science and policy on endocrine disrupters must not be mixed: a reply to a "common sense" intervention by toxicology journal editors. Environ Health 12: 69.

Gore AC, Balthazart J, Bikle D, Carpenter DO, Crews D, Czernichow P, et al. 2013. Policy decisions on endocrine disruptors should be based on science across disciplines: a response to Dietrich et al. Endocrinology 154(11): 3957-3960.

El-Shahawi MS, Hamza A, Bashammakh AS, Al-Saggaf WT. 2010. An overview on the accumulation, distribution, transformations, toxicity and analytical methods for the monitoring of persistent organic pollutants. Talanta 80(5): 1587-1597.

Porta M, Puigdomenech E, Ballester F, Selva J, Ribas-Fito N, Llop S, et al. 2008. Monitoring concentrations of persistent organic pollutants in the general population: the international experience. Environ Int 34(4): 546-561.

Patandin S, Dagnelie PC, Mulder PG, Op de Coul E, van der Veen JE, Weisglas-Kuperus N, et al. 1999. Dietary exposure to polychlorinated biphenyls and dioxins from infancy until adulthood: A comparison between breast-feeding, toddler, and long-term exposure. Environ Health Perspect 107(1): 45-51.

Gasull M, Bosch de Basea M, Puigdomenech E, Pumarega J, Porta M. 2011. Empirical analyses of the influence of diet on human concentrations of persistent organic pollutants: a systematic review of all studies conducted in Spain. Environ Int 37(7): 1226-1235.

Covaci A, Jorens P, Jacquemyn Y, Schepens P. 2002. Distribution of PCBs and organochlorine pesticides in umbilical cord and maternal serum. Sci Total Environ 298(1-3): 45-53.

Tsukimori K, Morokuma S, Hori T, Takahashi K, Hirata T, Otera Y, et al. 2013. Characterization of placental transfer of polychlorinated dibenzo-p-dioxins, dibenzofurans and

polychlorinated biphenyls in normal pregnancy. J Obstet Gynaecol Res 39(1): 83-90.

Mannetje A, Coakley J, Mueller JF, Harden F, Toms LM, Douwes J. 2012. Partitioning of persistent organic pollutants (POPs) between human serum and breast milk: a literature review. Chemosphere 89(8): 911-918.

Needham LL, Grandjean P, Heinzow B, Jorgensen PJ, Nielsen F, Patterson DG, Jr., et al. 2011. Partition of environmental chemicals between maternal and fetal blood and tissues. Environ Sci Technol 45(3): 1121-1126.

Llop S, Ballester F, Vizcaino E, Murcia M, Lopez-Espinosa MJ, Rebagliato M, et al. 2010. Concentrations and determinants of organochlorine levels among pregnant women in Eastern Spain. Sci Total Environ 408(23): 5758-5767.

Cerrillo I, Olea-Serrano MF, Ibarluzea J, Exposito J, Torne P, Laguna J, et al. 2006. Environmental and lifestyle factors for organochlorine exposure among women living in Southern Spain. Chemosphere 62(11): 1917-1924.

Arrebola JP, Martin-Olmedo P, Fernandez MF, Sanchez-Cantalejo E, Jimenez-Rios JA, Torne P, et al. 2009. Predictors of concentrations of hexachlorobenzene in human adipose tissue: a multivariate analysis by gender in Southern Spain. Environ Int 35(1): 27-32.

Li QQ, Loganath A, Chong YS, Tan J, Obbard JP. 2006. Persistent organic pollutants and adverse health effects in humans. J Toxicol Environ Health A 69(21): 1987-2005.

Colborn T, vom Saal FS, Soto AM. 1993. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. Environ Health Perspect 101(5): 378-384.

Tanabe S. 2002. Contamination and toxic effects of persistent endocrine disrupters in marine mammals and birds. Mar Pollut Bull 45(1-12): 69-77.

Mrema EJ, Rubino FM, Brambilla G, Moretto A, Tsatsakis AM, Colosio C. 2013. Persistent organochlorinated pesticides and mechanisms of their toxicity. Toxicology 307: 74-88.

Barker DJ, Osmond C. 1986. Infant mortality, childhood nutrition, and ischaemic heart disease in England and Wales. Lancet 1(8489): 1077-1081.

Barker DJ, Winter PD, Osmond C, Margetts B, Simmonds SJ. 1989. Weight in infancy and death from ischaemic heart disease. Lancet 2(8663): 577-580.

Barker DJ, Gluckman PD, Godfrey KM, Harding JE, Owens JA, Robinson JS. 1993. Fetal nutrition and cardiovascular disease in adult life. Lancet 341(8850): 938-941.

Gluckman PD, Hanson MA, Cooper C, Thornburg KL. 2008. Effect of in utero and early-life conditions on adult health and disease. N Engl J Med 359(1): 61-73.

Hanson M, Godfrey KM, Lillycrop KA, Burdge GC, Gluckman PD. 2011. Developmental plasticity and developmental origins of non-communicable disease: theoretical considerations and epigenetic mechanisms. Prog Biophys Mol Biol 106(1): 272-280.

Godfrey KM, Barker DJ. 2001. Fetal programming and adult health. Public Health Nutr 4(2B): 611-624.

Hochberg Z, Feil R, Constancia M, Fraga M, Junien C, Carel JC, et al. 2011. Child health, developmental plasticity, and epigenetic programming. Endocr Rev 32(2): 159-224.

Bruner-Tran KL, Osteen KG. 2011. Developmental exposure to TCDD reduces fertility and negatively affects pregnancy outcomes across multiple generations. Reprod Toxicol 31(3): 344-350.

Barr DB, Bishop A, Needham LL. 2007. Concentrations of xenobiotic chemicals in the maternal-fetal unit. Reprod Toxicol 23(3): 260-266.

Selevan SG, Kimmel CA, Mendola P. 2000. Identifying critical windows of exposure for children's health. Environ Health Perspect 108 Suppl 3: 451-455.

Wigle DT, Arbuckle TE, Turner MC, Berube A, Yang Q, Liu S, et al. 2008. Epidemiologic evidence of relationships between reproductive and child health outcomes and environmental chemical contaminants. J Toxicol Environ Health B Crit Rev 11(5-6): 373-517.

Wilcox AJ. 2001. On the importance--and the unimportance--of birthweight. Int J Epidemiol 30(6): 1233-1241.

Mocarelli P, Pocchiari F, Nelson N. 1988. Preliminary report: 2,3,7,8-tetrachlorodibenzo-p-dioxin exposure to humans--Seveso, Italy. MMWR Morb Mortal Wkly Rep 37(48): 733-736.

[Anonymous].

Mocarelli P, Brambilla P, Gerthoux PM, Patterson DG, Jr., Needham LL. 1996. Change in sex ratio with exposure to dioxin. Lancet 348(9024): 409.

Eskenazi B, Mocarelli P, Warner M, Chee WY, Gerthoux PM, Samuels S, et al. 2003. Maternal serum dioxin levels and birth outcomes in women of Seveso, Italy. Environ Health Perspect 111(7): 947-953.

Ngo AD, Taylor R, Roberts CL, Nguyen TV. 2006. Association between Agent Orange and birth defects: systematic review and meta-analysis. Int J Epidemiol 35(5): 1220-1230.

Masuda Y. 1985. Health status of Japanese and Taiwanese after exposure to contaminated rice oil. Environ Health Perspect 60: 321-325.

Tsukimori K, Uchi H, Mitoma C, Yasukawa F, Chiba T, Todaka T, et al. 2012. Maternal exposure to high levels of dioxins in relation to birth weight in women affected by Yusho disease. Environ Int 38(1): 79-86.

Yamashita F, Hayashi M. 1985. Fetal PCB syndrome: clinical features, intrauterine growth retardation and possible alteration in calcium metabolism. Environ Health Perspect 59: 41-45.

Rogan WJ, Gladen BC, Hung KL, Koong SL, Shih LY, Taylor JS, et al. 1988. Congenital poisoning by polychlorinated biphenyls and their contaminants in Taiwan. Science 241(4863): 334-336.

Guo YL, Lambert GH, Hsu CC, Hsu MM. 2004. Yucheng: health effects of prenatal exposure to polychlorinated biphenyls and dibenzofurans. Int Arch Occup Environ Health 77(3): 153-158.

Tsukimori K, Tokunaga S, Shibata S, Uchi H, Nakayama D, Ishimaru T, et al. 2008. Long-term effects of polychlorinated biphenyls and dioxins on pregnancy outcomes in women affected by the Yusho incident. Environ Health Perspect 116(5): 626-630.

Fein GG, Jacobson JL, Jacobson SW, Schwartz PM, Dowler JK. 1984. Prenatal exposure to polychlorinated biphenyls: effects on birth size and gestational age. J Pediatr 105(2): 315-320.

Patandin S, Koopman-Esseboom C, de Ridder MA, Weisglas-Kuperus N, Sauer PJ. 1998. Effects of environmental exposure to polychlorinated biphenyls and dioxins on birth size and growth in Dutch children. Pediatr Res 44(4): 538-545.

Hertz-Picciotto I, Charles MJ, James RA, Keller JA, Willman E, Teplin S. 2005. In utero polychlorinated biphenyl exposures in relation to fetal and early childhood growth. Epidemiology 16(5): 648-656.

Tajimi M, Uehara R, Watanabe M, Oki I, Ojima T, Nakamura Y. 2005. Relationship of PCDD/F and Co-PCB concentrations in breast milk with infant birthweights in Tokyo, Japan. Chemosphere 61(3): 383-388.

Sagiv SK, Tolbert PE, Altshul LM, Korrick SA. 2007. Organochlorine exposures during pregnancy and infant size at birth. Epidemiology 18(1): 120-129.

Nishijo M, Tawara K, Nakagawa H, Honda R, Kido T, Nishijo H, et al. 2008. 2,3,7,8-Tetrachlorodibenzo-p-dioxin in maternal breast milk and newborn head circumference. J Expo Sci Environ Epidemiol 18(3): 246-251.

Sonneborn D, Park HY, Petrik J, Kocan A, Palkovicova L, Trnovec T, et al. 2008. Prenatal polychlorinated biphenyl exposures in eastern Slovakia modify effects of social factors on birthweight. Paediatr Perinat Epidemiol 22(3): 202-213.

Konishi K, Sasaki S, Kato S, Ban S, Washino N, Kajiwara J, et al. 2009. Prenatal exposure to PCDDs/PCDFs and dioxin-like PCBs in relation to birth weight. Environ Res 109(7): 906-913.

Tawara K, Nishijo M, Honda R, Maruzeni S, Seto T, Kido T, et al. 2009. Effects of maternal dioxin exposure on newborn size at birth among Japanese mother-infant pairs. Environ Health Prev Med 14(2): 88-95.

Govarts E, Nieuwenhuijsen M, Schoeters G, Ballester F, Bloemen K, de Boer M, et al. 2012. Birth weight and prenatal exposure to polychlorinated biphenyls (PCBs) and dichlorodiphenyldichloroethylene (DDE): a meta-analysis within 12 European Birth Cohorts. Environ Health Perspect 120(2): 162-170.

Karmaus W, Zhu X. 2004. Maternal concentration of polychlorinated biphenyls and dichlorodiphenyl dichlorethylene and birth weight in Michigan fish eaters: a cohort study. Environ Health 3(1): 1.

Kezios KL, Liu X, Cirillio PM, Kalantzi OI, Wang Y, Petreas MX, et al. 2012. Prenatal polychlorinated biphenyl exposure is associated with decreased gestational length but not birth weight: archived samples from the Child Health and Development Studies pregnancy cohort. Environ Health 11: 49.

Lamb MR, Taylor S, Liu X, Wolff MS, Borrell L, Matte TD, et al. 2006. Prenatal exposure to polychlorinated biphenyls and postnatal growth: a structural analysis. Environ Health Perspect 114(5): 779-785.

Murphy LE, Gollenberg AL, Buck Louis GM, Kostyniak PJ, Sundaram R. 2010. Maternal serum preconception polychlorinated biphenyl concentrations and infant birth weight. Environ Health Perspect 118(2): 297-302.

Ribas-Fito N, Sala M, Cardo E, Mazon C, De Muga ME, Verdu A, et al. 2002. Association of hexachlorobenzene and other organochlorine compounds with anthropometric measures at birth. Pediatr Res 52(2): 163-167.

Rylander L, Stromberg U, Hagmar L. 2000. Lowered birth weight among infants born to women with a high intake of fish contaminated with persistent organochlorine compounds. Chemosphere 40(9-11): 1255-1262.

Tan J, Loganath A, Chong YS, Obbard JP. 2009. Exposure to persistent organic pollutants in utero and related maternal characteristics on birth outcomes: a multivariate data analysis approach. Chemosphere 74(3): 428-433.

Wu K, Xu X, Liu J, Guo Y, Huo X. 2011. In utero exposure to polychlorinated biphenyls and reduced neonatal physiological development from Guiyu, China. Ecotoxicol Environ Saf 74(8): 2141-2147.

Rogan WJ, Gladen BC, McKinney JD, Carreras N, Hardy P, Thullen J, et al. 1986. Neonatal effects of transplacental exposure to PCBs and DDE. J Pediatr 109(2): 335-341.

Longnecker MP, Klebanoff MA, Brock JW, Guo X. 2005. Maternal levels of polychlorinated biphenyls in relation to preterm and small-for-gestational-age birth. Epidemiology 16(5): 641-647.

Weisskopf MG, Anderson HA, Hanrahan LP, Kanarek MS, Falk CM, Steenport DM, et al. 2005. Maternal exposure to Great Lakes sport-caught fish and dichlorodiphenyl dichloroethylene, but not polychlorinated biphenyls, is associated with reduced birth weight. Environ Res 97(2): 149-162.

Halldorsson TI, Thorsdottir I, Meltzer HM, Strom M, Olsen SF. 2009. Dioxin-like activity in plasma among Danish pregnant women: dietary predictors, birth weight and infant development. Environ Res 109(1): 22-28.

Berkowitz GS, Lapinski RH, Wolff MS. 1996. The role of DDE and polychlorinated biphenyl levels in preterm birth. Arch Environ Contam Toxicol 30(1): 139-141.

Givens ML, Small CM, Terrell ML, Cameron LL, Michels Blanck H, Tolbert PE, et al. 2007. Maternal exposure to polybrominated and polychlorinated biphenyls: infant birth weight and gestational age. Chemosphere 69(8): 1295-1304.

Gladen BC, Shkiryak-Nyzhnyk ZA, Chyslovska N, Zadorozhnaja TD, Little RE. 2003. Persistent organochlorine compounds and birth weight. Ann Epidemiol 13(3): 151-157.

Khanjani N, Sim MR. 2006. Maternal contamination with dichlorodiphenyltrichloroethane and reproductive outcomes in an Australian population. Environ Res 101(3): 373-379.

Vartiainen T, Jaakkola JJ, Saarikoski S, Tuomisto J. 1998. Birth weight and sex of children and the correlation to the body burden of PCDDs/PCDFs and PCBs of the mother. Environ Health Perspect 106(2): 61-66.

Wolff MS, Engel S, Berkowitz G, Teitelbaum S, Siskind J, Barr DB, et al. 2007. Prenatal pesticide and PCB exposures and birth outcomes. Pediatr Res 61(2): 243-250.

Longnecker MP, Klebanoff MA, Zhou H, Brock JW. 2001. Association between maternal serum concentration of the DDT metabolite DDE and preterm and small-for-gestational-age babies at birth. Lancet 358(9276): 110-114.

Wassermann M, Ron M, Bercovici B, Wassermann D, Cucos S, Pines A. 1982. Premature delivery and organochlorine

compounds: polychlorinated biphenyls and some organochlorine insecticides. Environ Res 28(1): 106-112.

Al-Saleh I, Al-Doush I, Alsabbaheen A, Mohamed Gel D, Rabbah A. 2012. Levels of DDT and its metabolites in placenta, maternal and cord blood and their potential influence on neonatal anthropometric measures. Sci Total Environ 416: 62-74.

Kezios KL, Liu X, Cirillo PM, Cohn BA, Kalantzi OI, Wang Y, et al. 2013. Dichlorodiphenyltrichloroethane (DDT), DDT metabolites and pregnancy outcomes. Reprod Toxicol 35: 156-164.

Brucker-Davis F, Wagner-Mahler K, Bornebusch L, Delattre I, Ferrari P, Gal J, et al. 2010. Exposure to selected endocrine disruptors and neonatal outcome of 86 healthy boys from Nice area (France). Chemosphere 81(2): 169-176.

Eggesbo M, Stigum H, Longnecker MP, Polder A, Aldrin M, Basso O, et al. 2009. Levels of hexachlorobenzene (HCB) in breast milk in relation to birth weight in a Norwegian cohort. Environ Res 109(5): 559-566.

Fenster L, Eskenazi B, Anderson M, Bradman A, Harley K, Hernandez H, et al. 2006. Association of in utero organochlorine pesticide exposure and fetal growth and length of gestation in an agricultural population. Environ Health Perspect 114(4): 597-602.

Lopez-Espinosa MJ, Murcia M, Iniguez C, Vizcaino E, Llop S, Vioque J, et al. 2011. Prenatal exposure to organochlorine compounds and birth size. Pediatrics 128(1): e127-134.

Bjerregaard P, Hansen JC. 2000. Organochlorines and heavy metals in pregnant women from the Disko Bay area in Greenland. Sci Total Environ 245(1-3): 195-202.

Farhang L, Weintraub JM, Petreas M, Eskenazi B, Bhatia R. 2005. Association of DDT and DDE with birth weight and length of gestation in the Child Health and Development Studies, 1959-1967. Am J Epidemiol 162(8): 717-725.

Garced S, Torres-Sanchez L, Cebrian ME, Claudio L, Lopez-Carrillo L. 2012. Prenatal dichlorodiphenyldichloroethylene (DDE) exposure and child growth during the first year of life. Environ Res 113: 58-62.

Jusko TA, Koepsell TD, Baker RJ, Greenfield TA, Willman EJ, Charles MJ, et al. 2006. Maternal DDT exposures in relation to fetal and 5-year growth. Epidemiology 17(6): 692-700.

Pathak R, Ahmed RS, Tripathi AK, Guleria K, Sharma CS, Makhijani SD, et al. 2009. Maternal and cord blood levels of organochlorine pesticides: association with preterm labor. Clin Biochem 42(7-8): 746-749.

Warner M, Eskenazi B, Patterson DG, Clark G, Turner WE, Bonsignore L, et al. 2005. Dioxin-Like TEQ of women from the Seveso, Italy area by ID-HRGC/HRMS and CALUX. J Expo Anal Environ Epidemiol 15(4): 310-318.

Brouwer A, Sonneveld E, Botschuijver S, Besselink H, Burg van der B. 2004. A low volume method for the analysis of dioxins and dioxin-like compounds in serum and whole blood using BDS' DR CALUX® bioassay. Organohalog Compd 66: 687–690.

Wilcox AJ, Bonde JP. 2013. On environmental threats to male infertility. Asian J Androl 15(2): 199-200.

Fernandez MF, Olmos B, Granada A, Lopez-Espinosa MJ, Molina-Molina JM, Fernandez JM, et al. 2007. Human exposure to endocrine-disrupting chemicals and prenatal risk factors for cryptorchidism and hypospadias: a nested case-control study. Environ Health Perspect 115 Suppl 1:8-14.

Crain DA, Janssen SJ, Edwards TM, Heindel J, Ho SM, Hunt P, et al. 2008. Female reproductive disorders: the roles of endocrine-disrupting compounds and developmental timing. Fertil Steril 90(4): 911-940.

Greenham LW, Greenham V. 1977. Sexing mouse pups. Lab Anim 11(3): 181-184.

Marty MS, Chapin RE, Parks LG, Thorsrud BA. 2003. Development and maturation of the male reproductive system.Birth Def Res B Dev Reprod Toxicol.68(2):125-36.

Gray LE, Jr., Wolf C, Lambright C, Mann P, Price M, Cooper RL, et al. 1999. Administration of potentially antiandrogenic pesticides (procymidone, linuron, iprodione, chlozolinate, p,p'-DDE, and ketoconazole) and toxic substances (dibutyl- and diethylhexyl phthalate, PCB 169, and ethane dimethane sulphonate) during sexual differentiation produces diverse profiles of reproductive malformations in the male rat. Toxicol Ind Health 15(1-2): 94-118.

Welsh M, Saunders PT, Fisken M, Scott HM, Hutchison GR, Smith LB, et al. 2008. Identification in rats of a programming

window for reproductive tract masculinization, disruption of which leads to hypospadias and cryptorchidism. J Clin Invest 118(4): 1479-1490.

Foster PM. 2006. Disruption of reproductive development in male rat offspring following in utero exposure to phthalate esters. Int J Androl 29(1): 140-147; discussion 181-145.

Jiang JT, Sun WL, Jing YF, Liu SB, Ma Z, Hong Y, et al. 2011. Prenatal exposure to di-n-butyl phthalate induces anorectal malformations in male rat offspring. Toxicology 290(2-3): 322-326.

Christiansen S, Scholze M, Dalgaard M, Vinggaard AM, Axelstad M, Kortenkamp A, et al. 2009. Synergistic disruption of external male sex organ development by a mixture of four antiandrogens. Environ Health Perspect 117(12): 1839-1846.

van den Driesche S, Scott HM, MacLeod DJ, Fisken M, Walker M, Sharpe RM. 2011. Relative importance of prenatal and postnatal androgen action in determining growth of the penis and anogenital distance in the rat before, during and after puberty. Int J Androl 34(6 Pt 2): e578-586.

Hotchkiss AK, Parks-Saldutti LG, Ostby JS, Lambright C, Furr J, Vandenbergh JG, et al. 2004. A mixture of the "antiandrogens" linuron and butyl benzyl phthalate alters sexual differentiation of the male rat in a cumulative fashion. Biol Reprod 71(6): 1852-1861.

Macleod DJ, Sharpe RM, Welsh M, Fisken M, Scott HM, Hutchison GR, et al. 2010. Androgen action in the masculinization programming window and development of male reproductive organs. Int J Androl 33(2): 279-287.

Scott HM, Hutchison GR, Jobling MS, McKinnell C, Drake AJ, Sharpe RM. 2008. Relationship between androgen action in the "male programming window," fetal sertoli cell number, and adult testis size in the rat.Endocrinology149(10):5280-7.

Hotchkiss AK, Lambright CS, Ostby JS, Parks-Saldutti L, Vandenbergh JG, Gray LE, Jr. 2007. Prenatal testosterone exposure permanently masculinizes anogenital distance, nipple development, and reproductive tract morphology in female Sprague-Dawley rats. Toxicol Sci 96(2): 335-345.

Wolf CJ, Hotchkiss A, Ostby JS, LeBlanc GA, Gray LE, Jr. 2002. Effects of prenatal testosterone propionate on the sexual development of male and female rats: a dose-response study. Toxicol Sci 65(1): 71-86.

Dean A, Smith LB, Macpherson S, Sharpe RM. 2012. The effect of dihydrotestosterone exposure during or prior to the masculinization programming window on reproductive development in male and female rats. Int J Androl 35(3): 330-339.

Wu XY, Li ZL, Wu CY, Liu YM, Lin H, Wang SH, et al. 2010. Endocrine traits of polycystic ovary syndrome in prenatally androgenized female Sprague-Dawley rats. Endocr J 57(3): 201-209.

Arbuckle TE, Hauser R, Swan SH, Mao CS, Longnecker MP, Main KM, et al. 2008. Meeting Report: Measuring Endocrine-Sensitive Endpoints within the First Years of Life. Environ Health Perspect 116(7).

Sathyanarayana S, Beard L, Zhou C, Grady R. 2010. Measurement and correlates of ano-genital distance in healthy, newborn infants. Int J Androl 33(2): 317-323.

Salazar-Martinez E, Romano-Riquer P, Yanez-Marquez E, Longnecker MP, Hernandez-Avila M. 2004. Anogenital distance in human male and female newborns: a descriptive, cross-sectional study. Environ Health 3(1): 8.

Thankamony A, Ong KK, Dunger DB, Acerini CL, Hughes IA. 2009. Anogenital distance from birth to 2 years: a population study. Environ Health Perspect 117(11): 1786-1790.

Papadopoulou E, Vafeiadi M, Agramunt S, Basagana X, Mathianaki K, Karakosta P, et al. 2013. Anogenital distances in newborns and children from Spain and Greece: predictors, tracking and reliability. Paediatr Perinat Epidemiol 27(1): 89-99.

Romano-Riquer SP, Hernandez-Avila M, Gladen BC, Cupul-Uicab LA, Longnecker MP. 2007. Reliability and determinants of anogenital distance and penis dimensions in male newborns from Chiapas, Mexico. Paediatr Perinat Epidemiol 21(3): 219-228.

Miao M, Yuan W, He Y, Zhou Z, Wang J, Gao E, et al. 2011. In utero exposure to bisphenol-A and anogenital distance of

male offspring. Birth Defects Res A Clin Mol Teratol 91(10): 867-872.

Swan SH, Main KM, Liu F, Stewart SL, Kruse RL, Calafat AM, et al. 2005. Decrease in anogenital distance among male infants with prenatal phthalate exposure. Environ Health Perspect 113(8): 1056-1061.

Torres-Sanchez L, Zepeda M, Cebrian ME, Belkind-Gerson J, Garcia-Hernandez RM, Belkind-Valdovinos U, et al. 2008. Dichlorodiphenyldichloroethylene exposure during the first trimester of pregnancy alters the anal position in male infants. Ann N Y Acad Sci 1140: 155-162.

Hsieh MH, Eisenberg ML, Hittelman AB, Wilson JM, Tasian GE, Baskin LS. 2012. Caucasian male infants and boys with hypospadias exhibit reduced anogenital distance. Hum Reprod 27(6): 1577-1580.

Hsieh MH, Breyer BN, Eisenberg ML, Baskin LS. 2008. Associations among hypospadias, cryptorchidism, anogenital distance, and endocrine disruption. Curr Urol Rep 9(2): 137-142.

Jain VG, Singal AK. 2013. Shorter anogenital distance correlates with undescended testis: a detailed genital anthropometric analysis in human newborns. Hum Reprod 28(9): 2343-2349.

Barrett ES, Parlett LE, Sathyanarayana S, Liu F, Redmon JB, Wang C, et al. 2013. Prenatal exposure to stressful life events is associated with masculinized anogenital distance (AGD) in female infants. Physiol Behav 114-115: 14-20.

Eisenberg ML, Shy M, Walters RC, Lipshultz LI. 2012a. The relationship between anogenital distance and azoospermia in adult men. Int J Androl 35(5): 726-730.

Mendiola J, Stahlhut RW, Jorgensen N, Liu F, Swan SH. 2011. Shorter anogenital distance predicts poorer semen quality in young men in Rochester, New York. Environ Health Perspect 119(7): 958-963.

Eisenberg ML, Hsieh MH, Walters RC, Krasnow R, Lipshultz LI. 2011. The relationship between anogenital distance, fatherhood, and fertility in adult men. PLoS One 6(5): e18973.

Eisenberg ML, Jensen TK, Walters RC, Skakkebaek NE, Lipshultz LI. 2012b. The relationship between anogenital distance and reproductive hormone levels in adult men. J Urol 187(2): 594-598.

Castano-Vinyals G, Carrasco E, Lorente JA, Sabate Y, Cirac-Claveras J, Pollan M, et al. 2012. Anogenital distance and the risk of prostate cancer. BJU Int 110(11 Pt B): E707-710.

Mendiola J, Roca M, Minguez-Alarcon L, Mira-Escolano MP, Lopez-Espin JJ, Barrett ES, et al. 2012. Anogenital distance is related to ovarian follicular number in young Spanish women: a cross-sectional study. Environ Health 11: 90.

Verner MA, McDougall R, Glynn A, Andersen ME, Clewell HJ, 3rd, Longnecker MP. 2013. Is the Relationship between Prenatal Exposure to PCB-153 and Decreased Birth Weight Attributable to Pharmacokinetics? Environ Health Perspect.

Merlo DF, Wild CP, Kogevinas M, Kyrtopoulos S, Kleinjans J. 2009. NewGeneris: a European study on maternal diet during pregnancy and child health. Cancer Epidemiol Biomarkers Prev 18(1): 5-10.

Pedersen M, von Stedingk H, Botsivali M, Agramunt S, Alexander J, Brunborg G, et al. 2012. Birth weight, head circumference, and prenatal exposure to acrylamide from maternal diet: the European prospective mother-child study (NewGeneris). Environ Health Perspect 120(12):1739-1745.

Pedersen M, Halldorsson TI, Mathiesen L, Mose T, Brouwer A, Hedegaard M, et al. 2010. Dioxin-like exposures and effects on estrogenic and androgenic exposures and micronuclei frequency in mother-newborn pairs. Environ Int 36(4): 344-351.

Murk AJ, Leonards PE, van Hattum B, Luit R, van der Weiden ME, Mit M. 1997. The CALUX assay adapted and validated for measuring TCDD equivalents in blood plasma. Environ Toxicol Chem 16: 1583–1589.

Koponen J, Rantakokko P, Airaksinen R, Kiviranta H. 2013. Determination of selected perfluorinated alkyl acids and persistent organic pollutants from a small volume human serum sample relevant for epidemiological studies. J Chromatogr A 1309: 48-55.

Callegari C, Everett S, Ross M, Brasel JA. 1987. Anogenital ratio: measure of fetal virilization in premature and full-term newborn infants. J Pediatr 111(2): 240-243.

Long M, Deutch B, Bonefeld-Jorgensen EC. 2007. AhR transcriptional activity in serum of Inuits across Greenlandic districts. Environ Health 6: 32.

Vilahur N, Molina-Molina JM, Bustamante M, Murcia M, Arrebola JP, Ballester F, et al. 2013. Male specific association between xenoestrogen levels in placenta and birthweight. Environ Int 51: 174-181.

Fernandez MF, Aguilar-Garduno C, Molina-Molina JM, Arrebola JP, Olea N. 2008. The total effective xenoestrogen burden, a biomarker of exposure to xenoestrogen mixtures, is predicted by the (anti)estrogenicity of its components. Reprod Toxicol 26(1): 8-12.

Lopez-Espinosa MJ, Silva E, Granada A, Molina-Molina JM, Fernandez MF, Aguilar-Garduno C, et al. 2009. Assessment of the total effective xenoestrogen burden in extracts of human placentas. Biomarkers 14(5): 271-277.

Fernandez MF, Rivas A, Olea-Serrano F, Cerrillo I, Molina-Molina JM, Araque P, et al. 2004. Assessment of total effective xenoestrogen burden in adipose tissue and identification of chemicals responsible for the combined estrogenic effect. Anal Bioanal Chem 379(1): 163-170.

Wild CP. 2005. Complementing the genome with an "exposome": the outstanding challenge of environmental exposure measurement in molecular epidemiology. Cancer Epidemiol Biomarkers Prev 14(8): 1847-1850.