Dietary exposure to persistent organic pollutants during pregnancy and child health

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ABSTRACT

Introduction

Diet is the main source of exposure to persistent organic pollutants (POPs), including dioxins and PCBs. During pregnancy the fetus is exposed to POPs which can lead to adverse health effects. The research hypothesis of this thesis is that maternal diet, as a source of prenatal exposure to POPs, may be linked to impaired fetal growth and endocrine disruptive effects.

Methods

This thesis included 604 mother-child pairs from the European NewGeneris project, 50,651 mother-child pairs from the Norwegian Mother and Child Cohort (MoBa) and 707 mother-child pairs from the Rhea and the Hmar studies. Dietary data were collected during pregnancy by food frequency questionnaires. Three approaches were used to derive dietary estimates of prenatal exposure, either related to levels of POPs in maternal and cord blood or in food. Birth outcome information was obtained by medical registries. Anogenital distance measurements were collected and used as a marker of endocrine disruptive effects. Main predictors of anogenital distance were assessed and a reliability study was conducted.

Results

In the NewGeneris project, a dioxin-diet characterized by high maternal intakes of meat and fish was positively related to dioxins and dioxin-like compounds in maternal blood. High adherence to the dioxin-diet was associated with a reduction of -115g in birth weight. In the MoBa study, an inverse dose-response association was found between dietary dioxins and PCBs intakes during pregnancy and birth size. The negative association remained even for intakes lower than the tolerable weekly intake. In the Rhea and Hmar studies, anogenital distances were related to growth, tracked

through early life and were highly reliable anthropometric measurements. A high-fat diet score during pregnancy was positively related to POPs in maternal blood and was associated with 15% reduction in anogenital distance of newborn boys.

Conclusions

Diet during pregnancy can influence maternal and fetal body burden of POPs. Prenatal exposure to POPs, through maternal diet, may lead to impaired fetal growth and endocrine disruptive effects, even in populations with low background exposures to POPs.

RESUM

Introducció

La dieta és la principal font d'exposició als contaminants orgànics persistents (COP), com les dioxines i els PCBs. Durant l'embaràs el fetus està exposat als COPs, que poden donar lloc a efectes adversos per a la salut. La hipòtesi principal d'aquesta tesi és que la dieta materna, com una font d'exposició prenatal als COPs, podria estar relacionada amb alteracions en el creixement fetal i efectes endocrins perjudicials.

Mètodes

En aquesta tesi es van incloure 604 parelles mare-fill del projecte europeu NewGeneris, 50.651 parelles mare-fill de la cohort noruega (MOBA) i 707 parells de mares i fills dels estudis RHEA (Grècia) i Hmar (Catalunya). Les dades dietètiques es van recollir durant l'embaràs mitjançant qüestionaris de freqüència d'aliments. Tres mètodes s'han aplicat per derivar estimacions de l'exposició dietètica prenatal, ja sigui en relació als nivells de COP en la sang materna i del cordó o en els aliments. Informació sobre el naixement va ser obtinguda pels registres mèdics. Mesures de les distàncies anogenitals es van recollir i s'han utilitzat com marcadors d'efectes endocrins pertorbadors. Els determinants principals de la distància anogenital van ser avaluats i es va fer un estudi de fiabilitat de les mesures.

Resultats

En el projecte NewGeneris, una dieta alta en dioxines es caracteritza per una alta ingesta materna de carn i peix, i estava positivament relacionada amb dioxines i compostos similars a les dioxines a la sang materna. Alta adherència a una dieta alta en dioxines es va associar amb una reducció de 115 g de pes al néixer. En l'estudi Moba, es va trobar una relació de dosi-resposta inversa entre la ingesta de dioxines i PCBs durant l'embaràs i el pes en néixer. L'associació es va mantenir fins i tot per una ingesta inferior al límit de ingesta tolerable. En els estudis Rhea i Hmar, les distàncies anogenitals estan relacionades amb el pes al néixer, les mesures al

naixement s'associaven amb aquestes dels primers anys de vida, i les mesures antropomètriques van ser altament fiables. Una dieta alta en greixos durant l'embaràs va ser positivament relacionada amb els COP en la sang materna i es va associar amb un 15% de reducció en la distància anogenital dels nounats.

Conclusions

La dieta durant l'embaràs pot influir en la càrrega corporal materna i fetal dels COP. L'exposició prenatal als COP, a través de la dieta materna, pot conduir a alteracions en el creixement fetal i als efectes pertorbadors endocrins, fins i tot en poblacions amb exposicions sota els límits d'ingesta estipulats.

PREFACE

What is the problem addressed in this thesis?

During pregnancy the developing fetus is extremely vulnerable. Prenatal exposure to environmental toxicants might lead to a suboptimal intra-uterine environment and permanently affect the fetus and health throughout life. Maternal diet might be related to the adverse health effects caused by toxicants that are introduced in the mother's body from food. This thesis aims to investigate the role of maternal diet, as a source of prenatal exposure to POPs, and child health.

How is the problem addressed?

This thesis consists of a compilation of scientific publications according to the normative of the Doctoral Program in Biomedicine of the Department of Experimental and Health Sciences at the Pompeu Fabra University. The included publications are:

i) Maternal diet, prenatal exposure to dioxins and birth outcomes in a European prospective mother-child study (NewGeneris).

ii) Maternal dietary intake of dioxins and polychlorinated biphenyls and birth size in the Norwegian Mother and Child Cohort Study (MoBa).

iii) Anogenital distances in newborns and children from Spain and Greece: predictors, tracking and reliability.

iv) Maternal diet, prenatal exposure to dioxins and other persistent organic pollutants and anogenital distance in children.

The publications are included in the results of this thesis which further consists from an abstract, a general introduction, rationale, objectives, methods, a global discussion and final conclusion.

What is the novelty of this thesis?

The novelty of this study bears upon the methodology used to estimate prenatal exposure to persistent organic pollutants through maternal diet. We defined three different dietary estimates using three different nutritional epidemiology approaches that have been scarcely used to study the relationship between prenatal exposure to environmental contaminants and child health. An additional novelty in our methodology is the use of anogenital distance as a marker of endocrine disruption during pregnancy. The novelty concerning the results is the identification of effects through the diet of the mother, even at current low levels of exposure to dioxins and other POPs.

What is the main contribution of this thesis?

We provide epidemiological evidence for the role of maternal diet in prenatal exposure to POPs using novel dietary estimates. Even in low exposed populations the effect of dietary contaminants on child health was found to be substantial, suggesting that further measures should be taken to lower population exposures.

CONTENTS

1 INTRODUCTION

1.1 Persistent organic pollutants

Persistent Organic Pollutants (POPs) are a group of toxic chemicals that are persistent and ubiquitous in the environment and the food chain [1]. Being semi-volatile compounds, POPs are able to travel for long distances through the atmosphere, while being very stable against degradation makes them persistent. Their high lipid solubility makes them lipophilic and enables them to bio-accumulate in the tissues. Exposure to POPs has been associated with carcinogenicity, as well as with a wide range of immunological, neurological, reproductive and birth defects in humans and other animal species. The first world-wide initiative on controlling and monitoring POPs was the "Stockholm Convention" in 2001, which aimed on global ban, reduction of emissions and eventual elimination from the environment. Among the targeted POPs were the polychlorinated dibenzo-p-dioxins (PCDDs), dibenzofurans (PCDFs), polychlorinated biphenyls (PCBs) and the organochlorine pesticides, hexachlorobenzene (HCB) and dichlorodiphenyl trichloroethane (DDT) [2] (Figure 1).

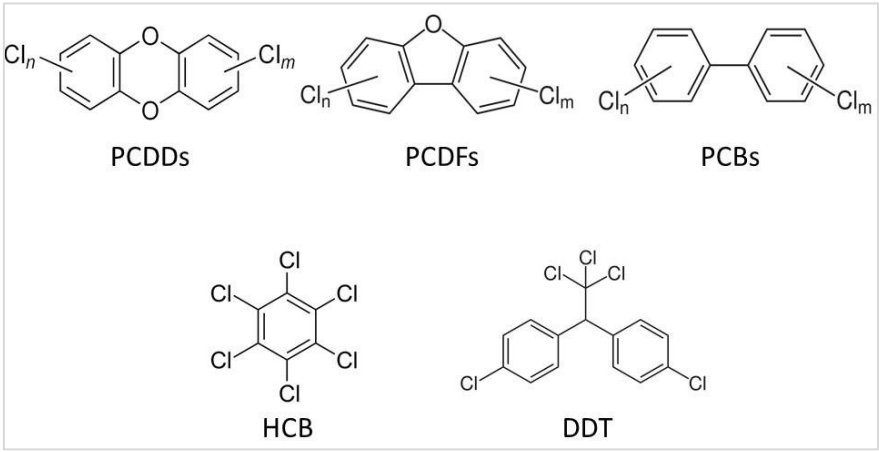

Figure 1. General chemical structures of PCDDs, PCDFs, PCBs, HCB and DDT.

PCDDs and PCDFs are termed as “dioxin” and are produced unintentionally as combustion by-products. Important sources of dioxin emissions are waste incineration, wood and coal burning as well as processing of metal and petroleum products. PCBs were manufactured for several industrial and commercial applications from 1930s until the 1980s when the production was banned, while PCB-containing equipment is still either in use or stocked [3,4]. PCBs are a group of 209 congeners which can be distinguished according to their toxicological properties. Twelve PCB congeners show toxicological properties similar to dioxins. In this thesis I will refer collectively to PCDDs, PCDFs and dioxin-like PCBs with the term “dioxins and dioxin-like compounds”. Dioxins and dioxin-like compounds have structural and chemical similarities and are able to bind to the aryl hydrocarbon receptor (AhR), a cytosolic receptor present in most tissues. The toxicity of dioxins and dioxin-like compounds is mainly mediated by their binding affinity to the AhR, a property that was used to create toxicity metric, the Toxic Equivalency Factor (TEFs). This factor has been assigned to each dioxin-like congener by comparison with the 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD), the most potent ligand of AhR and the most toxic dioxin-like compound (Van den Berg et al. 2006). The TEFs of 1998 and the updated TEFs of 2005 adapted by Van den Berg et al., are presented at Table 1 [5]. The sum of the products of the concentration of each compound multiplied by its TEF, represents the total Toxic Equivalent (TEQ) of a mixture of congeners. The use of TEQ has been established for risk assessment of dioxins and dioxin-like compounds in biological and environmental samples.

Table 1. Toxic equivalency factors (TEFs).

Compound	WHO 1998 TEF	WHO 2005 TEF
PCDDs		
2,3,7,8-TCDD	1	1
1,2,3,7,8-PeCDD	1	1
1,2,3,4,7,8-HxCDD	0.1	0.1
1,2,3,7,8,9-HxCDD	0.1	0.1
1,2,3,6,7,8-HxCDD	0.1	0.1
1,2,3,4,6,7,8-HpCDD	0.01	0.01
OCDD	0.0001	0.0003
PCDFs		
2,3,7,8-TCDF	0.1	0.1
1,2,3,7,8-PeCDF	0.05	0.03
2,3,4,7,8-PeCDF	0.5	0.3
1,2,3,4,7,8-HxCDF	0.1	0.1
1,2,3,7,8,9-HxCDF	0.1	0.1
1,2,3,6,7,8-HxCDF	0.1	0.1
2,3,4,6,7,8-HxCDF	0.1	0.1
1,2,3,4,6,7,8-HpCDF	0.01	0.01
1,2,3,4,7,8,9-HpCDF	0.01	0.01
OCDF	0.0001	0.0003
PCBs (IUPAC #)		
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'-PeCB (PCB 105)	0.0001	0.00003
2,3,4,4',5-PeCB (PCB 114)	0.0005	0.00003
2,3',4,4',5-PeCB (PCB 118)	0.0001	0.00003
2',3,4,4',5-PeCB (PCB 123)	0.0001	0.00003
3,3',4,4',5-PeCB (PCB 126)	0.1	0.1
2,3,3',4,4',5-HxCB (PCB 156)	0.0005	0.00003
2,3,3',4,4',5'-HxCB (PCB 157)	0.0005	0.00003
2,3',4,4',5,5'-HxCB (PCB 167)	0.00001	0.00003
3,3',4,4',5,5'-HxCB (PCB 169)	0.01	0.03
2,3,3',4,4',5,5'-HpCB (PCB 189)	0.0001	0.0001

HCB and DDT are organochlorine pesticides. HCB was manufactured and used mainly as a fungicide for food crops. Use of HCB in agriculture was banned in Europe at 1981 and its production declined as a result of world-wide regulations [6].

However, HCB is still used as an industrial chemical and is released as an incineration by-product in the environment. DDT is an insecticide and its use in agriculture is banned in most countries. However, it is also still used for vector-born disease control, like malaria, in African and Asian countries, mainly [7].

Effective regulations resulted in reduced contemporary POPs releases. However, POPs can be re-introduced in the environment from soil, sediment, biota and materials, through the environmental cycling of historical releases. Hence, exposure could be sustained due to numerous reservoir environmental sources [8,9]. Human and animal exposure occurs through contaminated air inhalation, soil ingestion, soil dermal contact and food consumption. The main source of exposure to POPs for non-occupationally exposed humans is through their diet [3,10,11].

1.2 Dietary exposure to dioxins, PCBs and organochlorine pesticides

Food consumption plays an important role to the human exposure, since lipophilic POPs can bioaccumulate and biomagnify though the food chain. Bioaccumulation refers to the increase of contaminant concentration within an organism, while biomagnification refers to the increase of contaminant concentration within a food chain (Figure 2). Hence, tissue concentrations of contaminants tend to increase as the level of the food chain is increasing reaching top predators, like humans. Diet contributes to 90% of total human exposure to dioxins and PCBs and is also the main source of exposure for HCB and DDT [10,12-14].

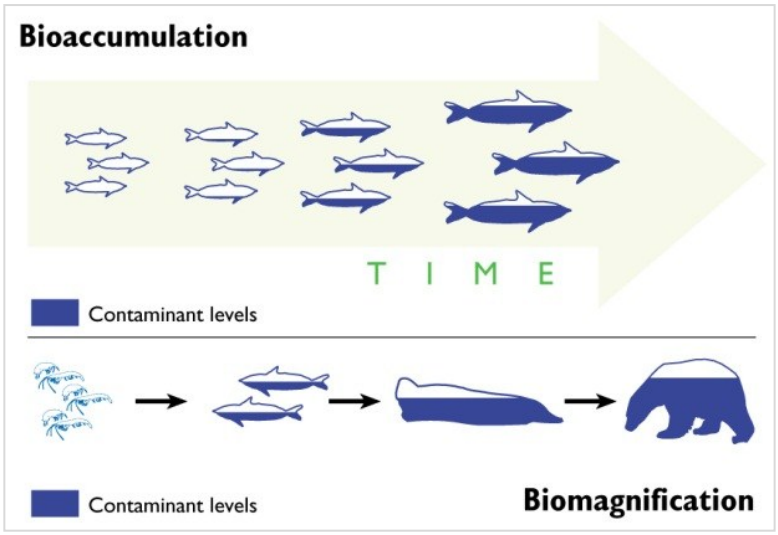

Figure 2. Bioaccumulation & Biomagnification of contaminants in food chain [15].

The European Scientific Committee on Food has established the Tolerable Weekly Intake (TWI) of dioxins and dioxin-like compounds at 14 pg TEQ/kg of body weight per week [16]. Recently, the average exposure of the adult European population was estimated between 0.57 to 1.67 pg TEQ/kg of body weight per day and 1 to 26% of the population exceeded the TWI [17]. Likewise, average dietary intakes of non-dioxin-like PCBs in Europe has been estimated between 10 to 45 ng/kg of body weight per day, while there is no established limit of intake for non-dioxin-like PCBs [18]. Additionally, the dietary HCB intake is in the range of 0.1 to 5 ng/kg of body weight per day, which is far below the suggested health-based limit of 170 ng/kg of body weight per day [10]. Regarding DDT, 5 to 30 ng/kg of body weight per day is the estimated dietary intake, which is much lower than the provisional tolerable daily intake of 10000 ng/kg of body weight [12].

Dioxins, PCBs and organochlorine pesticides have common dietary sources and the major sources are foods of animal and marine origin. The contaminants are introduced in meat and dairy products when the animals used for meat and milk production consume contaminated soil, feed and pasture.

Higher concentrations of lipophilic contaminants have been detected in meat and milk from sheep, pigs and cows raised outdoors and free-range chickens, compared to indoor-raised animals, due to soil ingestion [19,20]. The contaminants are stored in meat tissues and most of all in adipose tissue, which explains the higher concentrations of POPs in meat with higher fat content, like bovine and pork compared to poultry. Lipophilic contaminants tend to accumulate also in animal liver, which is linked to the processing of dietary fats, resulting in greater concentrations to this organ than in animal muscle meat [20].

Aquatic sediments are believed to be the biggest environmental sink of dioxins and PCBs, so seafood levels are higher than those of terrestrial animals [4,17]. Farmed fish has lower levels of lipophilic contaminants than wild fish, while contaminated fish-feed can lead to higher levels in fish [21,22]. Additionally, contaminant concentration is increasing as the fish size and the quantity of fat are increasing [23]. It has been reported that the transfer rate of dioxins from the fish feed to the edible part of the fish is approximately 5 times higher for fish than terrestrial animals [24]. Moreover, fish has low capacity for PCB metabolisation and tend to accumulate more POPs than terrestrial animals [25]. Hence, the bio-accumulation of POPs in seafood is more efficient than in terrestrial animals, supporting the leading role of seafood consumption to human dietary exposure. Similar intake, distribution and accumulation pathways have been described for HCB and DDT [10,12].

Human dietary exposure is a combination of food contamination and food consumption. The market basket and total diet studies are a traditional risk assessment and monitoring tool which combines the concentration of contaminants in foods with the food consumption. Findings from such studies depict the major contribution of seafood consumption to dietary exposure, followed by meat, dairy products and eggs [17]. However, different dietary habits and different food contamination levels can contribute to large variation.

As reported by European studies, total dietary intake of dioxins and dioxin-like compounds can vary substantially, as well as the contribution of each food group to total intake (Table 2).

Table 2. Relative contribution of main food groups to total dietary intake of dioxins and dioxin-like PCBs in European adults.

Contribution to total dietary intake (%)			Total dietary intake (in pg TEQ/kg body weight/day)	Country	
Seafood	Meat & products	Dairy products			
30 to 75	9 to 34	7 to 25	0.57 to 1.67	26 European countries	17
40	33	27	2.04		26
43	13	17	1.74	Belgium	27
18	22	51	0.72		28
72	5	14	1.30	Finland	29
39	10		0.47	France	30
44	7	27	2.28	Italy	31
16	23	27	1.20	Netherlands	32
12	17	38	0.80		33
11	37	29	3.22		34
58	6	8	1.12		35
59 to 78	5	7 to 12	2.86	Spain	36
49	6	12	0.60		37
33	15	20	1.30	Sweden	38
49	15	22	0.60		39
58	5	14	0.78	Norway	40

Additionally, dioxins, PCBs and organochlorine pesticides have been found in fruits, vegetables and cereals, and their participation to total dietary intake has been reported in several population [27,30,32,33,37,38,41,42]. However, organochlorine compounds, due to their chemical properties, are detected in higher concentrations in food of animal origin, establishing them as major sources of exposure.

Food consumption is associated with human serum and adipose tissue levels of contaminants, which strengthens the effect of food consumption, mainly seafood and meat, on human body burden of organochlorine compounds [11,43].

Likewise, estimates of the overall diet and calculated dietary intakes of organochlorine compounds have been linked to human body burden [40,44-46]. Notably, a decreasing trend on dietary exposure has been observed, explained by lower concentrations of lipophilic contaminants in food and by changes in dietary habits [47]. The declining trend in food concentrations in Europe is attributed to measures to reduce exposure to POPs, including regulations on emissions, phasing out of PCBs and establishing maximum tolerated levels in feed and food [28,30,37,39,48]. Similar regulations resulted in a decline of dietary intake in non-European populations [42,49]. Lower concentrations in human serum are following the worldwide decreasing trend of dietary intake, depicting that reduction of food concentrations can lead to lower human background exposure to dietary contaminants [50,51].

1.3 Maternal diet and prenatal exposure

Mother's body and diet is the only prenatal source of nutrient and non-nutrients and can influence fetal growth and offspring's long-term health. The essential role of early exposures including the maternal diet was shown by David Barker who first developed what is widely known as the "Barker hypothesis". Barker showed for the first time that a suboptimal intra-uterine environment, caused by maternal undernutrition, can increase the risk for the offspring to develop chronic diseases in adult life, such as coronary heart disease, type 2 diabetes and cancer [52-54]. The procedure that links the period of pregnancy with chronic diseases has been described as fetal programming and includes several structural, functional and epigenetic modifications, leading to morphologic, physiologic or metabolic changes to the fetus that can influence adult health.

On the other hand, optimal nutrient flux from the mother to the fetus is protective for pregnancy–related disease as preterm birth, preeclampsia and gestational diabetes, and for adverse birth outcomes, such as low birth weight [55-60] (Figure 3).

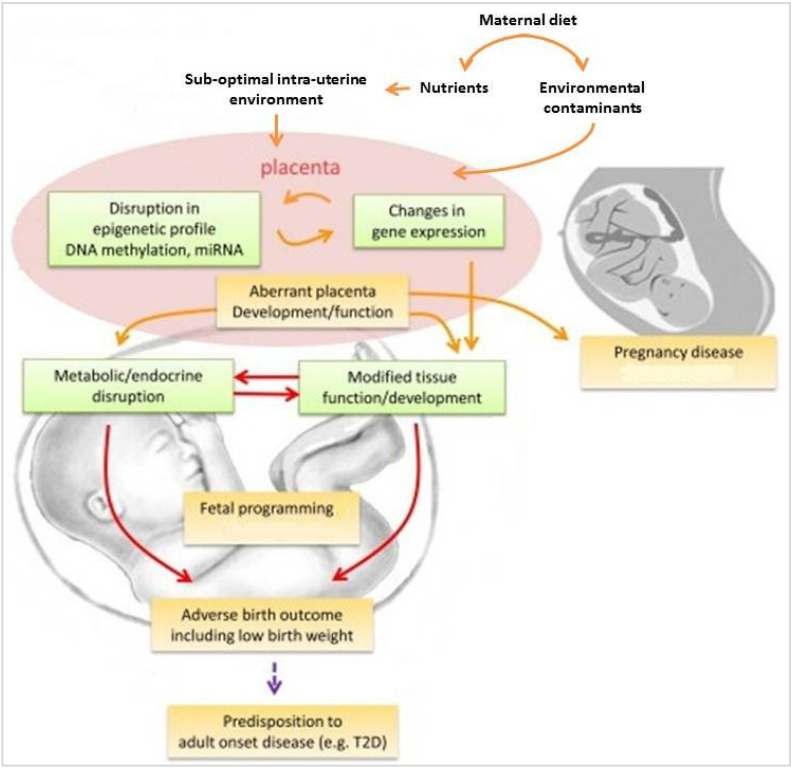

Figure 3. The developmental origins of chronic diseases (modified picture from Novacovic et al. [61])

However, maternal diet is also the main source of environmental contaminants, including POPs, for the developing fetus. Starting from dietary fat consumption, the ingested lipophilic compounds are distributed to maternal organs via blood and are stored in maternal body [62]. During pregnancy the accumulated contaminants can pass through the placenta and reach the circulation and the tissues of the fetus [63]. Measurements of contaminants in maternal blood, cord blood and placenta samples are used as estimates of prenatal exposure to organochlorine contaminants and have been linked to maternal dietary habits.

Fish and shellfish consumption during pregnancy has been identified as the major predictor of serum levels of dioxins, PCBs, HCB and DDE in maternal and cord blood [64-67]. Fatty fish is considered the main contributor of prenatal exposure to

organochlorine compounds, through maternal diet [68]. Moreover, studies in contaminated sites reported that fish consumption during pregnancy was the main intake pathway of PCBs and DDE for pregnant women[69,70]. Nevertheless, high consumption of meat and dairy products during pregnancy can also contribute to high prenatal exposure to organochlorine compounds, while there is one report linking fruits and cereals consumption during pregnancy to higher levels of DDT in maternal plasma [66,67,71,72]. Consequently, maternal diet can affect prenatal exposure to organochlorine compounds and can be related to potential health effects in the fetuses.

In addition and equally important to diet, several other factors can influence maternal body burden of POPs and determine prenatal exposure, including maternal age, parity, socio-economic status, ethnicity, maternal weight, previous breastfeeding history and weight gain during pregnancy [65,70,73].

1.4 Prenatal exposure and restricted fetal growth

Pregnancy is a period of numerous metabolic changes and organogenesis, when the developing fetus is extremely vulnerable. Exposures to environmental toxic chemicals during early gestation, especially during short time windows, can disrupt the development of the fetus and lead to adverse health effects with a life-long health impact (Figure 4).

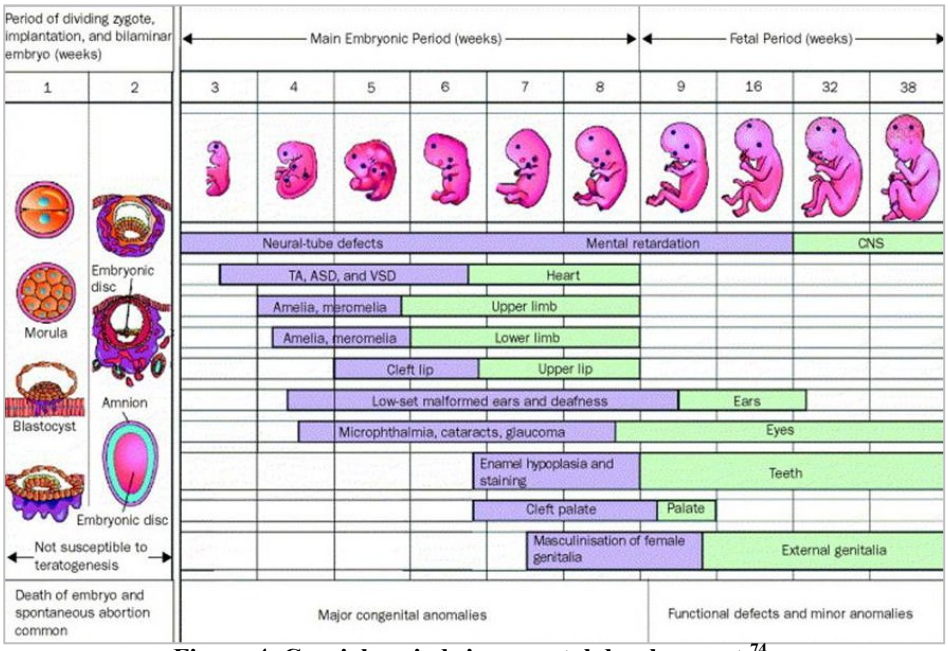

Figure 4. Crucial periods in prenatal development [74].

Several mother-child studies have investigated the hypothesis that prenatal exposure to organochlorine compounds, especially during early gestation, can negatively affect fetal growth and a wide range of health defects has been reported [75,76]. Birth weight is the most studied outcome and is used as a proxy for fetal growth, while low birth weight of less than 2,500 grams or birth weight below the 10th percentile for gestational age, depict intrauterine growth restriction. It is well established that intra-uterine growth retardation, measured as small birth size, is associated with increased risk of childhood asthma and obesity, as well as adult coronary heart disease and diabetes [77,78].

High accidental exposures of pregnant women have occurred at the Yusho and Yu-cheng accidents where cooking oil was contaminated with PCBs and the Seveso industrial accident where a large population was exposed to high levels of TCDD. In these cases, exposure during pregnancy led to stillborn and preterm delivery, modification of the sex ratio, impaired fetal growth and cognitive development, immunotoxicity and other defects of the thyroid and

the reproductive system [79-82]. Moreover, high consumption of contaminated fish during pregnancy has been linked to a reduction of 190 grams in birth weight and a 2-fold higher risk to deliver a low birth weight neonate [69,83].

However, following the worldwide declining trend, a decrease in background exposures has been observed for pregnant women, which has been attributed to lower dietary intakes [84,85]. In studies of non-accidentally exposed pregnant women with low levels of exposure, maternal and cord blood levels of POPs have been inconsistently linked with restriction in fetal growth and adverse health effects [86,87]. Regarding maternal diet, fish-eaters are often identified as high-risk population due to high background exposure levels, but relationship of seafood consumption with fetal growth is controversial and inconclusive [88-91].

The biological mechanisms under which prenatal exposure to environmental contaminants may lead to impaired fetal growth are very complex. Endocrine disruption is one of the pathways. Chemicals that act as endocrine disruptors can disrupt or mimic the normal endocrine functions. Endocrine disruptive chemicals can either bind to a hormone receptor, acting as agonists or antagonists of the hormone, or directly induce alterations of key enzymes and proteins and lead to non-normal hormone levels. Moreover, an indirect genomic pathway of toxicity has been also suggested, where contaminants can permanently modify the transcription of genes, by interfering at the epigenetic level of DNA functioning. A non-optimal intra-uterine environment, especially during crucial time windows for organ development, can lead to permanent structural and functional changes of the organs and increase the risk for impaired growth and development.

The most studied pathway for dioxins and dioxin-like compounds is the one mediated by the aryl hydrocarbon receptor (AhR). The AhR is a ligand-activated transcription factor, which mediates most of the toxic and carcinogenic effects of dioxins and dioxin-like

compounds. The activated AhR-ligand complex can alter the expression of a large network of genes and elicit hormone disruptive effects, by modulating the responsiveness of various hormone receptors and by interacting with various intracellular signaling pathways [92].

Organochlorine pesticides and PCBs are known immunotoxicants, neurotoxicants and endocrine disruptive chemicals and similar receptor-binding mechanisms of action have been suggested. [93-95]

1.5 Prenatal exposure and reduced anogenital distance

Convincing evidence exists from animal studies that prenatal exposure to chemicals with endocrine disruptive effect is associated with impaired reproductive health. Organochlorine compounds, as endocrine disruptors, can affect steroidogenesis and other androgens and estrogens involving pathways and can cause alterations of the reproductive system of male and female offspring. Prenatal exposure to organochlorine compounds has been linked to impaired testicular function, cryptorchidism and hypospadias in males and endometriosis, irregularities of the menstrual cycle, miscarriages in females. Impaired fertility and other morphological malformations of the reproductive system, including anogenital distance, have been also reported.

In animal studies, anogenital distance, measured from the anus to the genitalia, has been established as a marker of prenatal androgen exposure and hormonal disruption [96,97]. Anogenital distance is sexually dimorphic and males have longer distances than females, as a response to higher prenatal androgen exposure [98]. High prenatal exposure to compounds with anti-androgenic activity has been linked to shorter neonatal and adult anogenital distance as well as permanent impairments of the reproductive tracks in male pups [99-101]. On the other hand, high prenatal exposure to androgens has been linked to increased anogenital distance and permanent masculinization of the reproductive track in female pups[97].

In-utero exposures during a sensitive fetal masculinization window and not postnatally, caused the observed alterations in anogenital distance, which persist throughout adult life [102]. Such findings from animal studies suggest that anogenital distance can be used as a life-long guide to prenatal androgen exposure and a predictor of adult reproductive health [103].

In humans, few studies have examined anogenital distance. Descriptive studies have reported that anogenital distance is sexually dimorphic and in part determined by body dimensions [104,105]. A longitudinal study showed that neonatal anogenital distance increases from birth to the 1st year of life when it reaches a plateau [106]. Regarding the use of anogenital distance as a marker of fetal androgen disruption and predictor of adult reproductive health, findings in humans are suggestive. There is clear evidence that prenatal exposure to phthalates and bisphenol A, which act as anti-androgens, can reduce anogenital distance in boys [107-109]. One report exists on the reductive effect of prenatal phthalate exposure on anogenital distance of girls [110], while inconsistent results exist for organochlorine compounds in both genders [111-113].

In newborn and young boys shorter anogenital distance has been linked to hypospadias and cryptorchidism [114]. There are some reports linking maternal diet to higher risk for hypospadias and cryptorchidism in boys, mainly a vegetarian diet, but results are inconclusive [115,116]. There is no report on the effect of maternal diet on anogenital distance. In adult men shorter anogenital distance predicted poor semen quality and hypogonadal testosterone levels while prostate cancer patients found to have shorter distances than non-patients, suggesting that anogenital distance can be a novel metric of testicular function [114,117-123]. Such reports strengthen the use of anogenital distance as a predictor of adult reproductive health, especially in men.

1.6 Challenges in dietary exposure assessment

In mother-child studies and pregnancy cohorts, maternal diet is often used as an estimate of prenatal exposure to organochlorine compounds, while it might be a challenging methodology. The first issue lies on the appropriate dietary assessment method [124]. Several methods can be used to collect dietary information, while Food Frequency Questionnaires (FFQs) is the most frequently used method to study the links between human diet and disease [125].

The FFQs are designed to assess habitual diet by asking about the frequency with which foods are consumed over a reference period. In a semi-quantitative FFQ respondents are asked to additionally indicate their usual portion size and intakes of nutrients can be further estimated with the combination of information from food composition tables. With the same methodology and the use of information from food contamination databases, dietary intakes of contaminants can be estimated.

An additional key issue for using an FFQ to derive dietary exposure estimates is the list of food items included in the FFQ. The food list of the FFQ should include foods that are commonly consumed by the population under study, foods that are established and potential sources of contaminants and foods that contribute to population variation in the exposure. Moreover, the validity of the FFQ as a prenatal dietary and exposure assessment tool is very important. The relationship between FFQ estimates and maternal blood or urine biomarkers of dietary intake of nutrients and environmental contaminant is often used to assess validity [126-129].

The relationship between dietary intake and health is very complex and total dietary exposure can be more informative regarding the potential human health risks. Three methodologies have been suggested to study the overall diet. The challenge in dietary exposure estimation lies upon the decision for the most appropriate method. The first method is the researcher-defined food scores,

which are based on predefined food sources of nutrients or contaminants. A widely used example of this approach is the diet quality scores, including the Mediterranean diet scores [56,130,131]. The second approach is by defining dietary patterns driven by the underlying dietary data. Principal component and cluster analysis can be applied to derive dietary patterns. This approach is the most commonly used in mother-child studies [40,132-134]. The third approach includes dietary patterns, defined by reduced rank regression, that are driven by the underlying dietary data and biological pathways [135]. This approach has been scarcely used in mother-child studies [136-138].

After overcoming all the challenges, prenatal dietary exposure to environmental contaminants can be estimated by relating daily consumption of food items, food groups, dietary patterns or food scores with biomarkers of exposure. Additionally, prenatal dietary intake of contaminants can be calculated by using databases of contaminants in foods, while further comparison with biomarkers of exposure is more informative. Finally, the effect of all the different prenatal dietary exposure indices on child's health can be estimated (Figure 5).

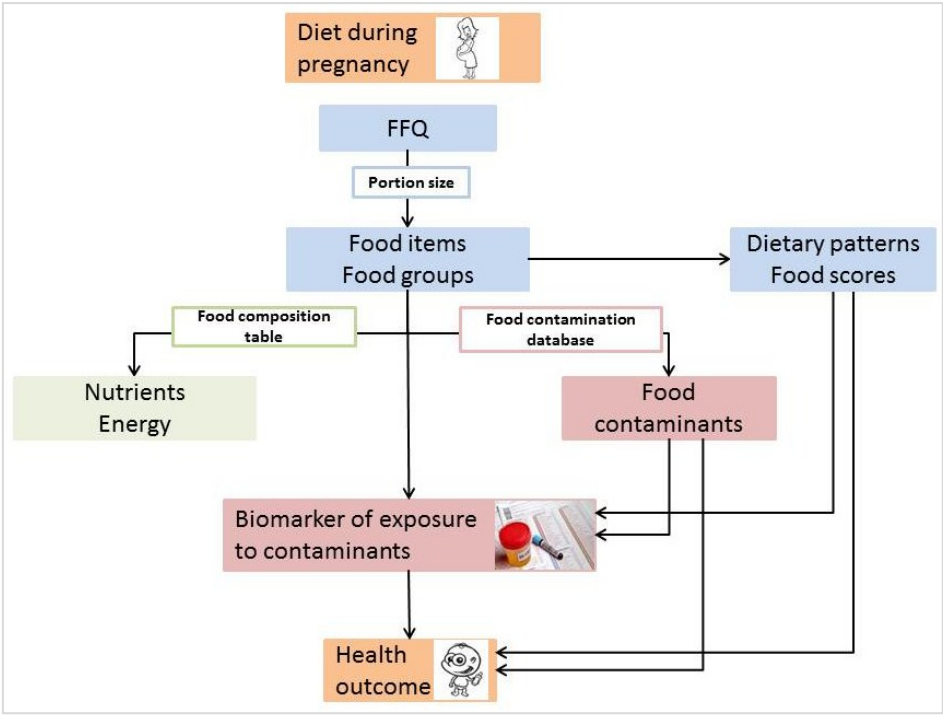

Figure 5. Scheme of dietary exposure assessment during pregnancy in mother-child studies.

2 RATIONALE

The developing organism is extremely sensitive to environmental toxic chemicals that can interfere with normal functions and can lead, through fetal programming, to increased susceptibility to adult disease [54,139,140]. Hence, fetal exposure to environmental contaminants, even in low levels, is of primary concern.

Human studies confirm that during pregnancy organochlorine compounds are transferred from the mother to the fetus [63,141]. Additionally, several reports have linked maternal diet to prenatal exposures, assessed as concentrations of organochlorine compounds in maternal and cord blood and the placenta. Epidemiological studies have established the negative association between high levels of exposure to dioxins, PCBs and organochlorine pesticides with child health[75]. However, the evidence on low prenatal exposure to organochlorine compounds is scarce and inconclusive. In low levels of background exposure, the extent to which maternal diet can affect fetal exposure, body burden and adverse health outcomes is not well appreciated.

In large mother-child studies there is a controversy on the relationship between maternal diet, as a source of contaminants, and fetal growth. Researchers have been focusing on single food groups, mainly seafood, but there is no study on the effect of overall maternal diet, as a source of prenatal exposure to contaminants, on fetal growth. Additionally, organochlorine contaminants can act as endocrine disruptors. In animal studies anogenital distance is established as an early marker of endocrine disruption and predictor of adverse reproductive health outcomes in adult life. The use of anogenital distance in human studies is increasing and methods for reliable measurement are still being developed. Inconsistent results exist for the effect of prenatal exposure to organochlorine compounds on anogenital distance of children, while the effect of the maternal diet has never been investigated [111-113]. There is a lack of studies that combine total maternal diet and biomarkers of

exposure with early biomarkers of hormone disruption and impaired fetal growth.

Why study diet?

Organochlorine compounds occur in the environment as mixtures and not as single congeners, sharing common food sources. It has been suggested that the study of contaminant mixtures can be more relevant to human exposure and health risks than single congeners, while the interpretation of mixture effects is complex [142,143]. Since diet is the main exposure pathway, assessing prenatal exposure to organochlorine compounds through maternal diet can provide an overall estimate of exposure and can further assist to the identification of main food sources of exposure. The importance of studying exposures through diet has also been stressed out by the observed interactions between environmental toxicants and nutrients, suggesting that overall diet can modulate the toxicity of compounds [144]. It is thought that the concentrations and congener patterns of POPs in foods are directly linked to congener patterns detected in serum[145,146]. Overall diet might provide a better image of the congener pattern of POPs which is important because not all the congeners can reach the fetus during pregnancy. Hence the consumption of single food groups might not be representative for prenatal exposure to organochlorine compounds and related health outcomes. Furthermore, for large epidemiological mother-child studies, the use of biomarkers might be restricted due to difficulties to collect maternal and fetal samples and due to the high cost of biomarker analysis. Hence, dietary assessment might be an alternative method to estimate prenatal exposure to organochlorine compounds that provides information within a wider, not compound specific context.

Nevertheless, dietary data collected by FFQ are subject to measurement error that can affect the analysis and the observed relationships. The combination of biomarkers of exposure and

maternal dietary information might reduce such uncertainties and provide a more specific estimate of exposure through diet [147].

From a public health perspective, exposure assessment through diet can be applied in large population-based epidemiological studies and can be essential for the identification of high-exposed populations. Furthermore, dietary habits are modifiable factors and food contributors of excess exposure can be a target of interventions aiming to reduce population exposure levels. It has been reported that by modifying maternal diet, a reduction of organochlorine compounds intake can be achieved on an individual level [148,149].

This study aims to contribute to a better scientific understanding of exposure to dioxins, PCBs and organochlorine pesticides, through maternal diet and the impact on fetal growth and anogenital distance, using data from European prospective birth cohorts.

3 OBJECTIVES

3.1 General objective

The overall aim of the thesis is to assess the effects of prenatal exposure to persistent organic pollutants, through maternal diet, on fetal growth and anogenital distance within five European population-based cohort studies in Greece, Spain, England, Denmark and Norway.

3.2 Specific objectives

- To investigate the association between prenatal exposure to dioxins and dioxin-like compounds, through maternal diet, and fetal growth in 5 European mother-child studies, within the NewGeneris project.

- To evaluate the association between dietary intake of dioxins and PCBs during pregnancy and fetal growth in the Norwegian Mother and Child Cohort Study (MoBa).

- To assess the main determinants and the reliability of anogenital distance of males and females measured in newborns and young children from the Rhea study in Crete, Greece and the Hmar study in Barcelona, Spain.

- To investigate the association between prenatal exposure to persistent organic pollutants, through maternal diet, and anogenital distance of males and females measured in newborns and young children from the Rhea study in Crete, Greece and the Hmar study in Barcelona, Spain.

4 METHODS

This section provides a brief summary integrating the methods used for the different papers included in this thesis. Further methodological details regarding each paper can be found in the results section.

4.1 Study population

This thesis is based on data from the NewGeneris project and on additional data from the Greek, Spanish and Norwegian mother-child studies (Figure 6 and Table 3). The NewGeneris project included five mother-child studies from Greece, Spain, England, Denmark and Norway and aimed to investigate the relationship between fetal exposure to dietary contaminants and the occurrence of early health effects, using biomarkers of exposure [150]. Detailed information on inclusion criteria can be found in paper I. Additionally, in the Rhea study in Greece and the Hmar study in Spain, anogenital distance and other anthropometric measurements were collected for children. Detailed information on the measurements protocol can be found in papers III, IV and in section 4.4 of this thesis. Finally, dietary intake of dioxins and PCBs was estimated for all participant of the MoBa study in Norway, by using a food contamination database. Detailed information on the study sample and methods can be found in paper II and at section 4.3 of this thesis.

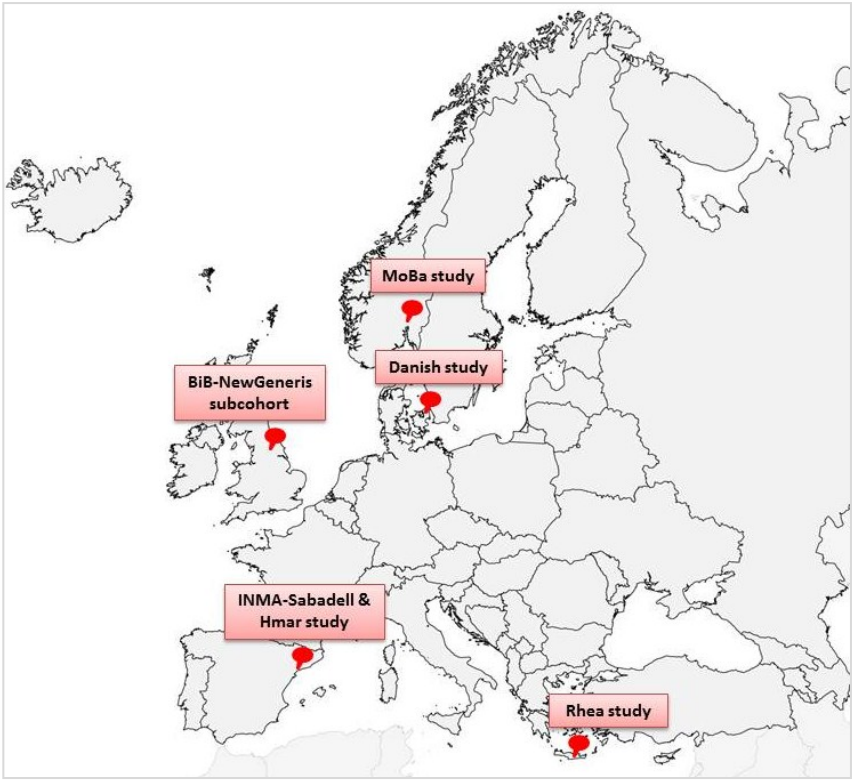

Figure 6. Location of five cohort studies included in this thesis.

Table 3. Description of population included in this study and available data information.

Study		Location	Period of recruitment	Inclusion criteria for this study	Maternal blood analysis	Cord blood analysis	Maternal dietary information	Birth size measurements	Anogenital distance measurements	Paper
Rhea Study		Heraklion, Crete, Greece	February 2007 to February 2008	Singleton pregnancies	Yes	Yes	Yes	Yes	Yes	I, III, IV
INMA-NewGeneris subcohort	INMA-Sabadell cohort	Sabadell, Spain	May 2007 to June 2007	Singleton pregnancies	Yes	Yes	Yes	Yes	No	I
	Hmar study	Barcelona, Spain	October 2008 to March 2010	Singleton pregnancies	Yes	Yes	Yes	Yes	Yes	I, III, IV
BiB-NewGeneris subcohort (Born in Bradford)		Bradford, England	January 2008 to December 2009	Planned C-sections of singleton pregnancies	Yes	Yes	Yes	Yes	No	I
Danish study		Copenhagen, Denmark	December 2006 to December 2007	Planned C-sections of singleton pregnancies	Yes	Yes	Yes	Yes	No	I
			September 2009 to December 2009	Singleton pregnancies	Yes	Yes	Yes	Yes	No	I
MoBa study (Norwegian Mother and Child Cohort Study)		Whole Norway	1999 to 2008	Full-term singleton pregnancies	No	No	Yes	Yes	No	II
	MoBa-NewGeneris sub-cohort	Oslo and Akershus, Norway	April 2007 to July 2008	Full-term singleton pregnancies	Yes	Yes	Yes	Yes	No	I

4.2 Maternal dietary assessment

Information on maternal diet during pregnancy was collected using validated food frequency questionnaires (FFQs). The main characteristics of dietary assessment in each cohort are presented in Table 4 and detailed information can be found in papers I, II and IV. In brief, all FFQs were semi-quantitative and included similar questions on food consumption, use of dietary supplements and dietary changes due to pregnancy. The reported frequency of consumption for each food item was multiplied with the portion size and converted into daily food intake (g/day). Further, nutrient calculation was performed using national or established international food composition tables.

Table 4. Main characteristics of the Food Frequency Questionnaires (FFQs) designed and implemented by each study.

	Rhea study	**INMA-NewGeneris sub-cohort**	**BiB-NewGeneris sub-cohort**	**Danish study**	**MoBa study**
FFQ type	Semi-quantitative	Semi-quantitative	Semi-quantitative	Semi-quantitative	Semi-quantitative
Number of food items	250	100	120	431	255
Time of administration	Mid pregnancy (14-18th week)	Around delivery or earlier	Around delivery	Around delivery or ealrier	Mid pregnancy (22nd week)
Administration	Interview administered by trained research stuff	Interview administered by trained research stuff	Self-administered	Self-administered & supported by research stuff	Self-administered
Serving sizes	Reference and individual	Reference	Reference	Standard	Standard Norwegian
Applied food composition tables	International & national sources	International & national sources	National	National	National

4.3 Prenatal exposure to environmental contaminants through maternal diet

Prenatal exposure to environmental contaminants through maternal diet was estimated by three different measures, a dioxin-dietary pattern, calculated dietary intake of dioxins and PCBs and a "high-fat diet" score.

Dioxin-dietary patterns

Using dietary information collected for mothers participating in the NewGeneris study, we identified food items, recognized as food sources of dioxins and dioxin-like compounds. Daily consumption of these food items was aggregated in 13 main food groups: red meat, white meat, processed meat, fatty fish, lean fish, mixed fish dishes, high-fat milk/yoghurt, high-fat cheese, low-fat dairy, eggs, butter, salty snacks and fast-food. Then, we used reduced rank regression (RRR) to derive dietary patterns, as estimates of prenatal exposure to dioxins and dioxin-like compounds.

The RRR is a dimension reduction technique that determines linear functions of predictors (food groups), called dietary patterns, by maximizing the explained variation of the response [137]. The response variable was dioxin-like activity in maternal plasma, as estimated by the DR-CALUX bioassay, resulting to one dioxin-dietary pattern. A "dioxin-diet" score was assigned to each woman according to her consumption of the 13 food groups. High "dioxin-diet" score was linked to high adherence to the dioxin-dietary pattern. This score was calculated as the sum of the products of consumption of 13 food groups, with the corresponding factor loading. The factor loadings represent the correlation of each food group with the dietary pattern score and high absolute values of factor loadings depicted higher contribution of the food group to the dietary pattern. More information on the methodology and the association between dioxin-dietary pattern and birth size can be found in paper I.

Dietary intake of dioxins and PCBs

Maternal dietary intake of dioxins and PCBs during pregnancy was calculated by multiplying maternal consumption of all food items included in the FFQ with the concentration of contaminants in each food item. The sum of the products was the total maternal dietary intake. A previously published database was used for the concentrations of dioxins and PCBs in Norwegian food [40].

We estimated maternal intake of seventeen PCDD/Fs and twelve dioxin-like PCBs, expressed as toxic equivalents (TEQs) and the intake of six indicator non-dioxin-like PCBs (ΣPCB-28, 52, 101, 138, 153, 180). Maternal dietary intakes of dioxins and dioxin-like compounds during pregnancy (in pg TEQs/kg body weight/day) and dietary intakes of non-dioxin-like PCBs (in ng/kg of body weight/day) were used as estimates of prenatal exposure to dioxins and PCBs. More information on the methodology used and the association between dietary intake of dioxins and PCBs with birth size can be found in paper II.

"High-fat diet" score

In the Rhea and the Hmar studies, maternal consumption of food of animal origin, recognized as food sources of dioxins, PCBs and other organochlorine contaminants were aggregated into 6 main food groups: red meat, processed meat, seafood, fatty fish, eggs and high fat dairy products. Maternal weekly intake of the 6 food groups was used to create a researcher-defined food score, the "high-fat diet" score. Intake of each of the 6 food groups was categorized in tertiles and a value of 0, 1 and 2 was assigned to lower tertile, middle tertile and upper tertile respectively. The "high-fat diet" score was equal to the sum of values for each participant, according to her intake of the 6 food groups. The "high-fat diet" score was created to estimate prenatal exposure to organochlorine contaminants from maternal diet. Detailed information on the

methods and the association between "high-fat diet" score and anogenital distances can be found in paper IV.

4.4 Plasma dioxin-like activity (DR-CALUX® bioassay) and serum concentrations of organochlorine compounds

Dioxins and dioxin-like compounds are ligands of the aryl hydrocarbon receptor (AhR) [92]. Within the NewGeneris project, dioxin-like activity was estimated in maternal and cord blood collected around delivery, by the DR-CALUX bioassay (Dioxin-Responsive Chemically Activated LUciferase eXpression®). The analysis was conducted at Biodetection Systems B.V, Amsterdam in the Netherlands. In brief, the DR-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. Upon exposure of the cells to dioxins or dioxin-like chemicals light is emitted, that is proportional to the strength of the AhR binding. The luminance is calibrated with respect to TCDD TEQs and results are expressed as picogram (pg) CALUX®-TEQ per gram of lipid. Detailed information on sample processing can be found in papers I and IV.

Within the Rhea study, maternal blood samples collected at 1st trimester were analyzed gravimetrically using gas chromatograph triple quadrupole mass spectrometry (GC-MS/MS) at the National Institute for Health and Welfare, Chemical Exposure Unit, Kuopio, Finland [151].

Serum concentrations of four individual PCB congeners (IUPAC numbers: 138, 153, 170 and 180), hexachlorobenzene (HCB), and dichlorodiphenyl dichloroethene (p',p'DDE) were analyzed and reported on whole weight as ng/ml serum. Detailed information on sample processing and estimates of organochlorine compounds can be found in paper IV.

4.5 Birth outcomes and anogenital distance

Birth outcomes studied were gestational age and preterm birth (<37 weeks), birth weight, low birth weight (birth weight <2,500 g), small-for-gestational age (birth weight <10th percentile of weight for gestational age), birth length and head circumference. Medical records were used to collect information on weight, length and head circumference at birth. Detailed information on methodology can be found at papers I and II.

The measurements protocol of anogenital distances was based on published protocols and included measurements in both males and females [105,108,152]. As described in Figure 7, anogenital distance (AGD) was measured from the anterior base of the penis to the center of the anus and anoscrotal distance (ASD) from the posterior base of the scrotum to the center of the anus. Additionally, penis width (PW) was recorded in male participants. Likewise, in females, anoclitoral distance (ACD) was recorded as the distance between clitoris and the anus center and anofourchetal distance (AFD) as the distance measured from the posterior convergence of the fourchette to the center of the anus. Weight, length, head and abdominal circumference were also reported. The mean value of three repeated measurements was used for genitalia distances and of two repeated measurements for other anthropometric measures.

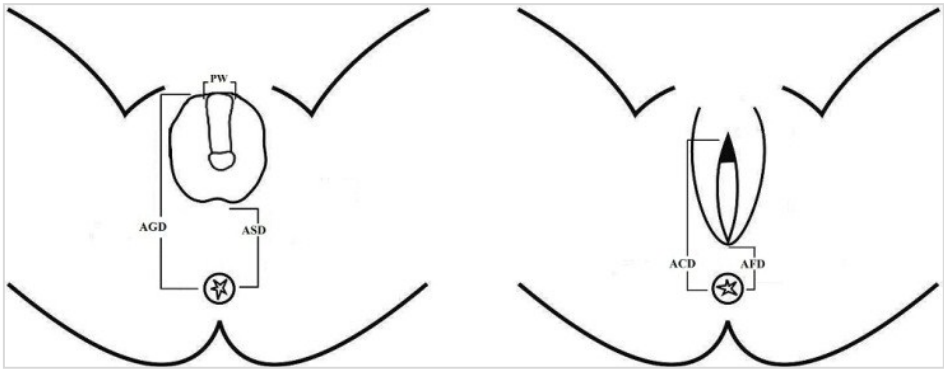

Figure 7. Schematic diagram of anogenital distance measurements in males and females.

Additionally, within the Rhea study, we conducted a reliability study for the genitalia measurements. Thirteen males and seventeen females (mean age: 23 months) participated and 1460 measurements were done in total, by two examiners. 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. Further details on the anogenital distance measurements protocol and the reliability study can be found in paper I.

5 RESULTS

<u>Paper I:</u> Maternal diet, prenatal exposure to dioxins and birth outcomes in a European prospective mother-child study (NewGeneris).

Main findings

- A dioxin-diet during pregnancy, characterized by combined high intakes of red and white meat, lean and fatty fish, was linked to higher levels of dioxins and dioxin-like compounds in maternal blood.
- Maternal diet during pregnancy was also contributing to cord levels of dioxins and dioxin-like compounds, mainly through meat consumption.
- High adherence to a dioxin-diet during pregnancy was associated with a reduction in birth weight.

<u>Paper II:</u> Maternal dietary intake of dioxins and polychlorinated biphenyls and birth size in the Norwegian Mother and Child Cohort Study (MoBa).

Main findings

- Dietary intake of dioxins and PCBs during pregnancy was negatively associated with birth size.
- Dietary intake of dioxins and dioxin-like PCBs above the tolerable weekly intake (TWI) was associated with reduction in birth size and a non-significant higher risk for small-for gestational age.
- After excluding women with dietary dioxins and dioxin-like PCBs intakes above the established TWI, negative associations with birth size persisted.
- The beneficial effects of seafood consumption on birth size were lower as the intake of dioxins and PCBs was increasing.

<u>Paper III:</u> Anogenital distances in newborns and children from Spain and Greece: predictors, tracking and reliability.

Main findings

- Anogenital distances in newborns and young children were sexually dimorphic and mainly predicted by body weight.
- Anogenital distances of males and females were increasing from birth to the 1st year of life where they reached a plateau.
- Anogenital distances found to track through early life.
- High reliability coefficients were found for all anogenital distances in males and females.

<u>Paper IV:</u> Maternal diet, prenatal exposure to dioxins and other persistent organic pollutants and anogenital distance in children.

Main findings

- A "high-fat-diet" during pregnancy, which consisted of high intakes of red meat, processed meat, fatty fish, seafood, eggs and high-fat dairy products, was positively linked to levels of dioxins and dioxin-like compounds in maternal and cord blood.
- The "high-fat-diet" was positively, though weakly, linked to serum concentrations of PCBs and HCB in maternal blood.
- The maternal "high-fat-diet" score was associated with a reduction in anoscrotal distance of newborn boys.
- Prenatal exposure to dioxins and dioxin-like compounds, through maternal diet, can induce endocrine disruptive effects and lead to alterations in the reproductive system.

5.1 Paper I

Maternal Diet, Prenatal Exposure to Dioxins and Birth Outcomes in a European Prospective Mother-Child Study (NewGeneris)

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Maternal Diet, Prenatal Exposure to Dioxins and Birth Outcomes in a European Prospective Mother-Child Study (NewGeneris).

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Running title: Maternal diet and dioxins
Key words: diet, dietary patterns, dioxins, DR CALUX, persistent organic pollutants, pregnancy

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Competing interests: The authors declare they have no competing financial interests.

List of abbreviations:
BMI, body mass index
DR-CALUX, Dioxin-Responsive Chemically Activated LUciferase eXpression
FFQ, Food Frequency Questionnaire
IQR, Interquartile Range
NA, not available
PAHs, polycyclic aromatic hydrocarbons
PCDD/Fs, polychlorinated dibenzo-p-dioxins and dibenzo-furans
PCBs, polychlorinated biphenyls RRR, Reduced Rank Regression
SD, standard deviation
TCDD, 2,3,7,8-Tetrachlorodibenzo-p-Dioxin
95% CI, 95% Confidence Intervals

Abstract

Background: Maternal diet may result in exposure of the foetus to environmental contaminants including dioxins and influence foetal growth.
Objective: We investigated the association between maternal diet and birth outcomes by defining a dioxin-rich diet.
Methods: We used food frequency questionnaires to assess the diet of pregnant women from five European countries and estimated plasma dioxin-like activity by the Dioxin-Responsive Chemically Activated LUciferase eXpression (DR-CALUX®) bioassay in 604 maternal and 198 cord blood samples collected at delivery. We applied reduced rank regression to identify a dioxin-rich dietary pattern based on dioxin-like activity (DR-CALUX®) levels in maternal plasma, and calculated a dioxin-diet score estimating adherence to this dietary pattern.
Results: In the five country population, dioxin-diet score was characterised by high maternal consumption of red and white meat, lean and fatty fish, low-fat dairy and low consumption of salty snacks and high-fat cheese. The upper tertile of the dioxin-diet score was associated with a change in birth weight of -115 g (95%CI: -224, -6) compared to the lower tertile after adjustment for confounders; a small non-significant reduction in gestational age was observed.
Conclusions: Maternal diet contributes to the exposure of the foetus to dioxins and dioxin-like compounds. A dioxin-rich diet during pregnancy may be related to reduced birth weight.

Introduction

Polychlorinated dibenzo-p-dioxins, dibenzo-furans (PCDD/Fs) and dioxin-like polychlorinated biphenyls (PCBs) are a group of environmental contaminants, unintentionally or industrially produced, with similar chemical and toxicological properties. Dioxins and dioxin-like compounds are lipophilic, stable against degradation and bio-accumulate, thus highly persistent and ubiquitous in the environment and the food chain (EFSA 2004;Kulkarni et al. 2008). Human exposure occurs mainly through food consumption (EFSA. 2012;Liem et al. 2000). Dioxins and dioxin-like compounds are introduced into meat and dairy food products when animals consume contaminated soil, feed and pasture (Rose et al. 2012). Additionally, aquatic sediments are considered the biggest environmental sink of dioxins and dioxin-like compounds. Persistent compounds accumulate more efficiently in the biota of the aquatic rather than the terrestrial environment, so seafood is the main source of dietary exposure (EFSA. 2012;Kulkarni et al. 2008).

The dominating role of seafood as a contributor to the dietary intake of dioxins and dioxin-like compounds has been confirmed by several studies (Fattore et al. 2006;Kiviranta et al. 2004;Sirot et al. 2012;Tornkvist et al. 2011), while others have reported a substantial contribution of meat and dairy products (De Mul et al. 2008;Fernandez et al. 2004;Schecter et al. 2001). Variations between and within populations are often explained by differences in food contamination or food consumption (Fernandez et al. 2004;Perello et al. 2012;Windal et al. 2010). Food consumption habits of populations can be described by dietary patterns. Dietary patterns can provide an overall exposure estimate, considering multiple dietary sources of exposure, but have been scarcely used (Arisawa et al. 2011;Kvalem et al. 2009).

Most importantly, dietary habits of pregnant women have been related to levels of dioxins and dioxin-like compounds in maternal, cord blood and human milk (Halldorsson et al. 2008;Huisman et al. 1995;McGraw and Waller 2009;Wang et al. 2009). During

pregnancy dioxins and dioxin-like compounds can be transferred from the mother to the fetus and may lead to a variety of toxic effects. The toxicity of dioxins and dioxin-like compounds is mainly mediated through their binding to the aryl hydrocarbon receptor (AhR) (Arisawa et al. 2005;Denison et al. 2011). The Dioxin-Responsive Chemically Activated LUciferase eXpression (DR-CALUX®) bioassay was developed to provide an estimation of the binding affinity of dioxins and dioxin-like PCBs to the AhR and is considered a valid and rapid screening tool for human exposure (Brouwer et al. 2004;Laier et al. 2003;Van Wouwe et al. 2004). There are few studies assessing blood levels of dioxins and dioxin-like compounds, by the DR-CALUX bioassay, and diet in pregnant women (Halldorsson et al. 2009;Koppen et al. 2009;Pedersen et al. 2012a). In the NewGeneris project we have investigated the association between dioxins and dioxin-like compounds in maternal and cord blood, assessed by the DR-CALUX bioassay, and birth outcomes (Vafeiadi et al. 2013).

The aim of our study was to identify a dietary pattern related to blood levels of dioxins and dioxin-like compounds of pregnant women from five European countries, and to estimate the association between the dietary pattern and birth outcomes.

Materials and methods

Study population

This study is part of the European NewGeneris project (Newborns and Genotoxic exposure risks) and includes five European mother-child studies (Merlo et al. 2009;Pedersen et al. 2012b): *The Rhea study* is a population-based mother-child study at the prefecture of Heraklion, Crete, Greece (Chatzi et al. 2009). Pregnant women were recruited within a year, from February 2007. *The INMA-NewGeneris sub-cohort* included women with singleton pregnancies, enrolled from May 2007 to March 2010, in Sabadell and Barcelona, Spain (Guxens et al. 2012).

The MoBa-NewGeneris sub-cohort included pregnant women with full-term singleton pregnancies from Oslo and Akershus in Norway, participants of the BraMat and BraMiljo studies, recruited from 2007 to 2008. These women were already enrolled in the Norwegian Mother and Child Cohort Study (MoBa) (Magnus et al. 2006). *The Danish study* included 2 enrollment periods, from December 2006 to December 2007 and from September to December 2009, in Copenhagen, Denmark. Women with singleton pregnancies and planned Caesarean sections were recruited in the 1st period, while singleton pregnancies were recruited during the 2nd period (Pedersen et al. 2009). *The BiB-NewGeneris sub-cohort* includes pregnant women participants of the Born in Bradford (BiB) study with elective Caesarean section, recruited from January 2008 to May 2009 in Bradford, UK (Hepworth et al. 2012;Wright et al. 2012).

Dietary information and plasma dioxin levels were available for 665 women with singleton pregnancies. After excluding 61 women due to implausible values of total energy intake (outside the range of 1000-4000 kcal/day), 604 women were included in this analysis (Davey et al. 2003). Questionnaires and medical records were used to collect information on several maternal lifestyle and socio-demographic characteristics, as well as birth outcomes. Gestational age for participants from Denmark, Greece, and Spain was estimated from the interval between last menstrual period and date of the delivery and was corrected by ultrasound measurement if there was a discordance of ≥ 7 days between estimates. Ultrasound-based estimation was provided for most participants from England and Norway (Pedersen et al. 2012b). Within our study population, 537 participants had available information on gestational age and birth weight, as well as on other socio-demographic and lifestyle characteristics.

Ethical approval was obtained from the research ethics committee in each country and written informed consent for participation of the women and their children was obtained from all participating women.

Dioxin-like activity in maternal and cord plasma (DR-CALUX® bioassay)

Peripheral blood samples from the mothers and umbilical cord blood were collected around delivery. Dioxin-like activity was determined through the DR-CALUX® bioassay at Biodetection Systems B.V., Amsterdam, The Netherlands. The protocol for sample processing has been presented elsewhere (Vafeiadi et al. 2013). In brief, the DR-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. Upon exposure of the cells to dioxins or dioxin-like chemicals light, that is proportional to the strength of the AhR binding, is emitted. The luminance is calibrated with respect to TCDD toxic equivalency quantities. Dioxin-like activity was estimated in 604 maternal and 198 cord blood samples and was expressed as pg CALUX®-TEQ per g of lipid. Analyses using cord blood were based on 198 samples for which cord blood lipids could be measured. Samples below the level of detection (LOD) were assigned a value of 0.5 x LOD.

Maternal diet and dietary patterns

Information on maternal diet during pregnancy was collected using validated cohort-specific food frequency questionnaires (FFQs) (Brantsaeter et al. 2008;Chatzi et al. 2011;Hepworth et al. 2012;Pedersen et al. 2012a;Vioque et al. 2007). All FFQs were semi-quantitative and included similar questions on food consumption, use of dietary supplements and dietary changes due to pregnancy. The reported frequency of consumption for each food item was multiplied with the portion size and converted into daily food intake (g/day). Further, intake of nutrients was calculated using food composition tables. More details for the FFQs are provided in Supplemental Table 1.

Food items which are established or potential sources of dietary dioxins and dioxin-like compounds were integrated in 13 food groups: red meat, white meat, processed meat, fatty-fish, lean fish, mixed fish dishes, high-fat milk/yoghurt, high-fat cheese, low-fat dairy, eggs, butter, salty snacks and fast-food (Supplemental Table 2). Consumptions of important food items were not asked in all studies and were additionally included in country-specific analysis. Intake of fish-oil dietary supplements is common for Norwegian pregnant women and was included in the analysis for the Norwegian study (Haugen et al. 2008) (Supplemental Table 3).

Reduced rank regression (RRR) was applied to identify dietary patterns. RRR is a dimension reduction technique that determines linear functions of predictors (food groups), called dietary patterns, by maximizing the explained variation of the response (Hoffmann et al. 2004). Methods mostly used to derive dietary patterns are data exploratory procedures, such as principal component or cluster analysis. RRR requires the definition of a response variable based on the scientific knowledge of the disease physiology and is more than a data exploratory method (Ambrosini et al. 2012).

In our study daily intakes of 13 food groups (g/day) were included as predictors and the response variable was dioxin-like activity in maternal plasma (pg CALUX-TEQ/g lipid), as estimated by the DR-CALUX bioassay. Hence, we extracted a dietary pattern, based on maternal dioxin-like activity, to study the association between maternal diet and birth outcomes, mediated by prenatal exposure to dioxins and dioxin-like compounds.

The extracted dietary pattern was termed “dioxin-dietary pattern”. The adherence of each woman to the “dioxin-dietary pattern” was described by the “dioxin-diet score”. This is a standardized score (with a mean of zero) calculated as the sum of the products of food intake multiplied by the corresponding factor loading. The factor loadings represent the correlation of each food group with the dietary pattern score and high absolute values of factor loadings depict higher contribution of the food group to the dietary pattern. The percentage of the variation of dioxin-like activity in maternal

blood (pg CALUX-TEQ/g lipid) explained by the dietary pattern, was further estimated.

Statistical analysis

Maternal dioxin-diet score was our exposure variable and was used as continuous or in tertiles (low, middle, upper tertile). We described maternal characteristics, gestational age and birth weight by tertiles of maternal dioxin-diet score.

Simple and multiple linear regression models were fitted to estimate the association between maternal dioxin-diet score (continuous or in tertiles) with either gestational age (days) or birth weight (g). The potential non-linear relationship was evaluated by including the exposure variable in tertiles and trend tests were performed to evaluate dose–response relations. Several potential confounders were assessed from a variety of maternal and pregnancy-related characteristics with established association with maternal dietary habits and birth outcomes. Characteristics, identified as predictors of maternal plasma dioxin-like activity (pg CALUX-TEQ/g lipid) by backward elimination in stepwise regression models, were included in the adjusted models. Child gender and gestational age were included as *a priori* confounders in the models of birth weight. The potential effect modification of gender was assessed by stratified analysis.

In the Norwegian study only full-term births were included, while all British women and Danish women from the 1st recruitment period had planned caesarean sections (Hepworth et al. 2012;Pedersen et al. 2009). Thus, we conducted sensitivity analysis for the association between dioxin-diet score and gestational age, after excluding 319 Norwegian, British and Danish women.

We repeated the RRR procedure to derive country-specific dietary patterns with more food groups. Finally, a dietary pattern was defined based on dioxin-like activity in cord blood (pg CALUX-TEQ/g lipid) and the association of this dietary pattern with gestational age and birth weight was estimated.

Analyses were performed using STATA 10.1 (Stata Corporation, College Station, Texas) and the PROC PLS procedure, in the SAS version 9.2 (SAS Institute, Inc.) was used for the RRR method (Hoffmann et al. 2004).

Results

The dioxin-dietary pattern was characterized by high intakes of red and white meat, fatty and lean fish, mixed fish dishes, low-fat dairy products (factor loadings≥0.20) and low intakes of salty snacks and high-fat cheese (factor loadings≤ - 0.20). This pattern explained 7.9% of the DR-CALUX variation in maternal plasma (pg CALUX-TEQ/g lipid) (Table 1). In country-specific analysis, the derived dioxin-dietary patterns varied substantially between countries and the explained DR-CALUX variation in maternal plasma, ranged from 9.8 to 27.6%. Additionally, the country-specific dioxin-diet scores were positively correlated with dioxin levels (pg CALUX TEQ/g lipid) in maternal plasma (range of correlation: 0.26-0.50) (Supplementary table 3).

The proportion of women participating in the Spanish or the Danish studies, non-Caucasian and non-smokers during pregnancy was higher in the upper tertile of the dioxin-diet score, compared to the middle and lower tertile (Table 2). Maternal age, daily energy and fat intake were increasing as the dioxin-diet score was increasing. Dioxin-like activity in maternal blood was also positively related to the dioxin-diet score (Spearman's rho= 0.29, P-value<0.001).

We found an inverse dose-response association between the dioxin-diet score and birth weight after adjustment for confounders (p for trend=0.041) (Table 3). The reduction in birth weight for infants born by mothers in the middle tertile of the dioxin-diet score was -35 g (95%CI:-125, 55) and for those in the upper tertile it was -115 g (95% CI:-224,-6) compared to the low tertile. Similar negative associations were obtained in a stratified analysis by gender, though statistically significant results were observed only in

boys (p for trend=0.049 and beta for high tertile of food score= -161 g, 95%CI=-323, 0) (data not shown).

Moreover, we observed that the dietary pattern of 198 mother-child pairs which was based on dioxin-like activity in cord blood (pg CALUX-TEQ/g lipid) explained 13.9% of the DR-CALUX variation in cord blood (Supplemental Table 4). We found an inverse though non-significant dose-response association between this dioxin-diet score, which was linked to cord dioxin levels, and birth weight (Supplemental Table 5). Additionally, a one-point increase of the maternal dioxin-diet score, linked to cord dioxin levels, was associated with 0.3 weeks (95% CI -0.5, 0.0) reduction in gestational age. The reduction associated with the upper tertile was 0.5 weeks (-1.0, 0.0) compared to the lower tertile of the dioxin-diet score.

Similar non-significant reductions in gestational age were obtained from a sensitivity analysis after restricting our sample to Greek, Spanish and Danish women, by 1-point increase of the dioxin-diet score, linked to either maternal or cord dioxins levels (β= -0.1 weeks, 95% CI -0.2,0.1 and -0.2 weeks, 95% CI -0.6,0.3, respectively).

Discussion

We investigated the association between maternal diet, dioxin levels in maternal and cord blood and birth outcomes in a large international study. Our main finding was that a dioxin-rich diet was associated with a reduction in birth weight while less consistent findings were observed for gestational age. These results are in agreement with a recent analysis within the NewGeneris project evaluating the association between low-level prenatal exposure to dioxins and dioxin-like compounds measured in cord blood and birth outcomes. They reported an association with shorter gestational age, particularly in boys, while weaker associations were found for birth weight (Vafeiadi et al. 2013).

We found that a diet high in red and white meat, lean and fatty fish, was linked to high levels of dioxins and dioxin-like

compounds in maternal plasma of European pregnant women. Fish and shellfish consumption have been identified as predictors of dioxin levels in maternal blood (Glynn et al. 2007;Halldorsson et al. 2008;Wang et al. 2009). Halldorsson et al. found that high fat intake, explained by high consumption of red meat and low consumption of fatty-fish was positively associated with plasma dioxin-like activity of 100 Danish pregnant women (Halldorsson et al. 2009). Additionally, a "meat", "seafood" and a "dairy" dietary pattern were independently and positively linked to blood levels of PCDD/Fs and dioxin-like PCBs of 1,656 Japanese adults (Arisawa et al. 2011). However, there are no reports for pregnant women and the effect of combined food consumption as dietary patterns, on dioxin and dioxin-like levels in maternal blood, is not known.

Findings from our own and other studies on the positive relationship between maternal fat intake and dioxin and dioxin-like levels in cord blood (correlation coefficient=0.15, p-value=0.031), support the fetal exposure pathway through prenatal dietary fat consumption (Koppen et al. 2009). Additionally, high maternal consumption of meat and fish was linked to high dioxin levels in maternal blood, while high consumption of meat but not of fish was linked to high dioxin levels in cord blood. In line with our results and despite the established contribution of fish intake during pregnancy to maternal and placenta dioxin levels, no link between fish intake and cord blood dioxin levels has been reported (Chevrier et al. 2013;Huang et al. 2007;Pedersen et al. 2012a). Accumulated maternal dioxins and dioxin-like compounds are transferred to the fetus through the placenta and this procedure is determined by several chemical properties of the lipophilic compounds (Suzuki et al. 2005). Similar congener concentrations and profiles have been reported for maternal blood and placenta samples, while differences have been found in cord blood, suggesting restricted transfer of dioxins and dioxin-like compounds from maternal to cord blood (Mose et al. 2012;Suzuki et al. 2005;Tsukimori et al. 2013). Thus, the contribution of fish to dioxin levels in maternal and not in cord blood might reflect and correspond to different congener profiles

between maternal and cord blood, since diet is the main intake pathway of dioxins and dioxin-like compounds. In the same study population, dioxin-like activity in maternal blood was weakly correlated with dioxin-like activity in cord blood, suggesting a difference between maternal and fetal body burden of dioxins and dioxin-like compounds (Vafeiadi et al. 2013). Hence this discrepancy might be reflected in the derived dioxin-dietary patterns. The low lipid content of cord blood might also introduce uncertainty in the exposure estimates. The different sample size of women included to derive each dietary pattern might also explain some discrepancies between dietary patterns estimated based on cord or maternal blood.

In our study, meat and fish intake during pregnancy was associated with reduced birth weight, mediated by high prenatal exposure to dioxins and dioxin-like compounds. High meat intake combined with low fish intake during pregnancy has been associated with lower birth weight and increased risk for being small-for-gestational age (Ricci et al. 2010;Timmermans et al. 2012) and vice versa, high fish and low meat consumption has been linked to protective effects on fetal growth (Chatzi et al. 2012). Findings on the relationship between consumption of seafood during pregnancy and fetal growth are discrepant, with reports of either negative effects (Halldorsson et al. 2007;Halldorsson et al. 2008;Mendez et al. 2010;Rylander et al. 2000;Weisskopf et al. 2005), positive or null effects (Brantsaeter et al. 2012;Drouillet-Pinard et al. 2010;Guldner et al. 2007;Heppe et al. 2011;Lucas et al. 2004;Thorsdottir et al. 2004). Hence, dietary patterns related to blood biomarkers of contaminants might be a promising methodology to study the link between overall maternal diet, prenatal exposure to environmental dietary contaminants and health effects.

Maternal diet explained a small percentage of the DR-CALUX variation (8-14%) that was smaller compared to other reports in pregnant women (25-70%) (Glynn et al. 2007; Halldorsson et al. 2008; Huisman et al. 1995). It has been pointed out that the

explained variation in an outcome variable (in our case dioxin levels) may not be a good metric of the association and the risk attributed to a predictor variable (in our case food groups) (Pearce 2011). This may happen if there is not enough variation in women's dietary habits. If, for example, intake of meat and seafood does not vary much among pregnant women then the explained plasma dioxin variation by diet would be small, even though diet would still be the main contributor to dioxin exposure. Moreover, given that there is a background long term exposure of the population to persistent environmental pollutants, the identification of risk factors, like diet, and high-risk groups is challenging. Finally a direct comparison between studies is difficult due to different methodologies in dietary assessment or blood analysis.

The large sample of pregnant women from 5 European countries, the use of a biomarker to assess maternal and fetal body burden and the use of RRR to derive dietary patterns, are among the strengths of our study. RRR derived dietary patterns have been linked to obesity, coronary heart disease, type 2 diabetes, breast cancer and other chronic diseases, by using blood estimates, anthropometric measurements or nutrient intake with an established association with the disease (Ambrosini et al. 2012;McNaughton et al. 2008;Meyer et al. 2011;Schulz et al. 2008). Since biomarker are reflecting the body burden of environmental toxicants that might lead to adverse health effects, dietary patterns based on exposure biomarkers might be more relevant for the effect of diet on disease. Nevertheless, uncontrolled measurement errors introduced by the use of FFQs and unmeasured long-term diet may have influenced our results. Moreover, individual differences in absorption, distribution and metabolism of persistent organic pollutants might add uncertainty to the estimation of dietary exposure.

In conclusion, we reported that maternal diet contributes to prenatal exposure to dioxins and dioxin-like compounds and is linked to reduced birth weight. More studies are needed to develop updated dietary guidelines for women of reproductive age, aiming to the reduction of dietary exposure to persistent organic pollutants.

Table 1. Factor loadings for 13 food groups included in dioxin-dietary pattern and the explained variation of dioxin-like activity in maternal blood (in pg CALUX-TEQ/g lipid) by the dietary pattern of pregnant women in the NewGeneris project.

	Dioxin-dietary pattern
Dioxin-dietary pattern loadings	
Red meat	0.27
White meat	0.27
Processed meat	-0.14
Fatty-fish	0.27
Lean fish	0.35
Mix fish dishes	0.24
High-fat milk/yoghurt	-0.15
High-fat cheese	-0.54
Low-fat dairy	0.30
Eggs	0.14
Butter	-0.10
Salty snacks	-0.35
Fast-food	0.16
Explained variation of dioxin-like activity in maternal blood (%)	7.9

Table 2. Maternal characteristics, birth weight and gestational age by tertiles of dioxin-diet score for pregnant women and their children in the NewGeneris project.

		Dioxin-diet score in tertiles			
	All	Low	Middle	Upper	
	N (%)	N (%)	N (%)	N (%)	P-value
All	604 (100)				
Greece	85 (14.1)	62 (30.8)	21 (10.4)	2 (1.0)	
Spain	114 (18.9)	18 (9.0)	31 (15.4)	65 (32.2)	
UK	74 (12.2)	36 (17.9)	24 (11.9)	14 (6.9)	<0.001
Denmark	169 (28.0)	5 (2.5)	55 (27.4)	109 (54.0)	
Norway	162 (26.8)	80 (39.8)	70 (34.8)	12 (5.9)	
Educational level					
Low	120 (19.9)	42 (23.1)	33 (18.1)	45 (25.3)	
Middle	202 (33.4)	77 (42.3)	67 (36.8)	58 (32.6)	0.132
High	220 (36.4)	63 (34.6)	82 (45.1)	75 (42.1)	
Missing	*62 (10.3)*				
Ethnicity					
Caucasian	523 (86.6)	188 (93.5)	181 (90.0)	154 (77.0)	<0.001
Non-Caucasian	79 (13.1)	13 (6.5)	20 (10.0)	46 (23.0)	
Missing	*2(0.3)*				
Parity					
Primiparous	216 (35.8)	66 (33.3)	79 (40.1)	71 (35.5)	0.361
Multiparous	379 (62.7)	132 (66.7)	118 (59.9)	129 (64.5)	
Missing	*9(1.5)*				
Smoking during pregnancy					
No	518 (85.8)	158 (79.4)	177 (88.9)	183 (92.0)	0.001
Yes	79 (13.1)	41 (20.6)	22 (11.1)	16 (8.0)	
Missing	*7 (1.1)*				
	N	Median (IQR)	Median (IQR)	Median (IQR)	P-value
Age (years) [a]	604	30.1 (5.5)	31.5 (4.2)	32.2 (4.5)	<0.001
Pre-pregnancy BMI (kg/m2)	556	23.0 (5.6)	23.1 (5.1)	22.6 (5.3)	0.620
Energy intake (kcal/day)	604	2,141 (822)	2,204 (940)	2,557 (1,078)	<0.001
Fat intake (g/day)	604	92.0 (44.3)	87.3 (51.5)	113.8 (73.5)	<0.001

Dioxin-like activity in maternal blood (pg CALUX-TEQ/g lipid)	604	35.5 (31.1)	38.5 (30.5)	45.5 (30.0)	<0.001
Dioxin-like activity in cord blood (pg CALUX-TEQ/g lipid)	198	33.0 (26.0)	37.9 (28.8)	35.6 (39.0)	0.706
Gestational age (weeks)	604	39 (2)	39 (2)	39 (2)	0.143
Birth weight (kg)	604	3,425 (614)	3,520 (620)	3,440 (650)	0.363

[a] Presented values are mean (SD).

Table 3. Association between dioxin-diet score with gestational age and birth weight for pregnant women and their children in the NewGeneris project.

		Gestational age (weeks) [a]			Birth weight (g) [b]		
	N	β	(95% CI)	P-value	β	(95% CI)	P-value
Continuous dioxin-diet score	520	0.0	(-0.1,0.1)	0.995	-22	(-60,16)	0.256
Dioxin-diet score in tertiles							
Low (< -0.4 points)	175	Ref.			Ref.		
Middle (-0.4 to 0.4 points)	173	-0.1	(-0.4,0.2)	0.605	-35	(-125,55)	0.442
Upper (>0.4 points)	172	-0.2	(-0.5,0.1)	0.251	-115	(-224,-6)	0.039
P for trend [c]			*0.254*			*0.041*	

[a] Model is adjusted for maternal educational level (low, middle, high), energy (kcals/day), maternal age (years), pre-pregnancy BMI (kg/m^2), parity (primiparous/multiparous) and country.

[b] Model is adjusted for same variables and additionally for gestational age (weeks) and gender.

[c] P for trend is estimated for the adjusted change across increasing tertiles of the dioxin-diet score.

Supplemental material

Supplemental Table 1. Main characteristics of the Food Frequency Questionnaires (FFQs) designed and implemented by each study.

	Rhea study	INMA-NewGeneris sub-cohort	BiB-NewGeneris sub-cohort	Danish study	MoBa-NewGeneris sub-cohort
Recruitment period	2007-2008	2007-2010	2008-2009	2006-2009	2007-2009
FFQ type	Semi-quantitative	Semi-quantitative	Semi-quantitative	Semi-quantitative	Semi-quantitative
Number of food items	250	100	120	431	255
Time of administration	Mid pregnancy (mean: 14.6 weeks, SD: 3.2)	Around delivery or earlier	Around delivery	Around delivery or earlier	Mid pregnancy (17^{th}-24^{th} week)
Dietary assessment period covered	Since the beginning of pregnancy (~4 months)	whole pregnancy period	Last 4 weeks of pregnancy	Last month of pregnancy	Since the beginning of pregnancy (~4 months)
Way of administration	Interview administered by trained research nurses	Interview administered by trained research nurses	Self-administered	Self-administered & partly supported by research staff	Self-administered
Serving sizes	Reference and individual servings sizes (photos were used for small, medium and large portions)	Reference servings sizes	Standard portion sizes	Standard portion sizes	Standard Norwegian portion sizes for women

Supplemental Table 2. Food items included in the food groups and available information for pregnant women in the NewGeneris project.

Food groups	Food items included in the food group	Available information
Red meat	Beef, veal, pork, lamb, mutton, minced meat, burger, meatballs	All studies (n=604)
White meat	Chicken, turkey, rabbit	All studies (n=604)
Processed meat	Cold turkey/chicken/ham cuts smoked or boiled, bacon, sausages (pork, chicken, beef), hot-dogs, mortadella, salami, chicken/turkey nuggets, ham/chicken croquettes, doner kebab, cracklings	All studies (n=604)
Fatty fish	Anchovy, sardines, mackerel, salmon, trout, herring, canned sardines/mackerel, smoke salmon/trout	All studies (n=604)
Lean fish	Cod, tuna, saithe, haddock, halibut, plaice, flounder, perch, pike, hake, sole, canned tuna, dried cod	All studies (n=604)
Mix fish dishes	Fish dishes with not defined fish type (fish croquette, fried fish, fish soup, fish cake/balls, fish paste with mayonnaise, sushi, fish curry)	All studies (n=604)
High-fat milk/yoghurt	Milk and yoghurt with total fat content higher than 2%	All studies (n=604)
High-fat cheese	Cheese with total fat content higher than 25% (feta, Roquefort, edam, gouda, white cheese, cured cheese, goat cheese, blue cheese, brie, smoked cheese)	All studies (n=604)
Low-fat dairy	Milk and yoghurt with total fat content lower than 2% and cheese with total fat content lower than 20%	All studies (n=604)
Eggs		All studies (n=604)
Butter	Added butter	All studies (n=604)
Salty snacks	Potato chips, crackers, pop-corn, salty snacks	All studies (n=604)
Fast-food	French-fries, pizza, wrap/tortilla/taco/pitta, burger/kebab (fast-food)	All studies (n=604)
Meat offals	Liver, liver paste, kidney, brain, tripe, pate (beef, pork, chicken, lamb, venison), foie gras	Greek, Spanish, Norwegian and Danish studies (n=530)
Mix meat dishes	Meat dishes with not defined meat type (pasta/lasagna/noodles with meat, meat pie)	Norwegian, Danish and British studies (n=405)
Game	Birds, hare	Greek and Norwegian studies (n=247)
Shellfish	Shrimps, lobster, crab, mussels, squid, cuttlefish, octopus, oysters, clams, cockles	Greek, Spanish, Norwegian and Danish studies (n=530)
Fish offals	Cod roe, cod roe paste, fish liver, fish liver paste	Norwegian and Danish studies (n=331)

Supplemental Table 3. Maternal dioxin-dietary pattern loadings for 14 to 19 food groups, explained variation of dioxin-like activity in maternal blood (in pg CALUX TEQ/ g lipid) by the dietary pattern and correlation of dioxin-diet score with dioxin-like activity in maternal blood, by country.

	Greece (n=85)	Spain (n=114)	Norway (n=162)	Denmark (n=169)	UK (n=74)
Dioxin-dietary pattern loadings					
Red meat	0.56	0.03	-0.04	-0.17	0.28
White meat	0.63	-0.08	0.41	0.20	0.22
Processed meat	-0.21	-0.28	-0.36	-0.36	0.09
Fatty-fish	0.31	-0.29	-0.29	0.37	-0.06
Lean fish	-0.18	-0.37	0.03	0.08	-0.11
Mix fish dishes	0.10	0.33	-0.14	-0.14	0.00
High-fat milk/yoghurt	-0.19	0.36	0.24	-0.26	0.33
High-fat cheese	-0.03	-0.15	-0.22	0.02	0.39
Low-fat dairy	-0.03	0.21	0.19	-0.04	0.38
Eggs	-0.09	0.07	0.01	0.09	-0.16
Butter	0.07	-0.09	0.22	-0.37	0.23
Salty snacks	0.02	0.19	-0.06	-0.42	-0.27
Fast-food	0.07	0.40	-0.14	-0.37	-0.47
Additional food groups					
Meat offals	-0.03	0.31	0.00	-0.06	NA
Mix meat dishes	NA	NA	-0.16	0.01	0.28
Game	NA	NA	0.30	-0.01	NA
Shellfish	0.23	0.28	-0.47	-0.32	NA
Fish offals	NA	NA	-0.22	NA	NA
Fish-oil dietary supplements	NA	NA	0.10	NA	NA
Explained variation of maternal dioxin-like activity (%)	27.6	14.0	10.4	16.3	9.8
Correlation of dioxin-dietary pattern score with maternal dioxin-like activity [Spearman's rho (p-value)]	0.50 (<0.001)	0.36 (<0.001)	0.26 (0.001)	0.35 (<0.001)	0.32 (0.006)

Supplemental Table 4. Dioxin-dietary pattern loadings of 13 food groups and explained variation of dioxin-like activity (in pg CALUX-TEQ/g lipid) in cord blood by the dietary pattern.

	Maternal dioxin-dietary pattern linked to cord blood (n=198)
Dioxin-dietary pattern loadings	
Red meat	0.20
White meat	0.36
Processed meat	0.00
Fatty-fish	-0.47
Lean fish	-0.10
Mix fish dishes	-0.06
High-fat milk/yoghurt	0.00
High-fat cheese	-0.29
Low-fat dairy	0.23
Eggs	0.01
Butter	0.08
Salty snacks	0.06
Fast-food	0.67
Explained variation of plasma dioxin-like activity (%)	13.9
Correlation between dioxin-diet score and dioxin-like activity in cord blood [Spearman's rho (p-value)]	0.35 (<0.001)

Supplemental Table 5. Association between maternal dioxin-dietary pattern scores, linked to cord blood dioxin-like activity (in pg CALUX TEQ/g lipid), with gestational age and birth weight.

		Gestational age (weeks) [a]			Birth weight (g) [b]		
	N	β	(95% CI)	P-value	β	(95% CI)	P-value
Maternal dioxin-dietary pattern linked to cord blood							
Continuous	158	-0.3	(-0.5,0.0)	0.026	-12	(-91,67)	0.772
Tertiles							
Low	58	Ref.			Ref.		
Middle	46	-0.1	(-0.5,0.4)	0.637	-10	(-156,137)	0.895
Upper	54	-0.5	(-1.0,0.0)	0.048	-73	(-237,90)	0.376
P for trend [c]			*0.094*			*0.392*	

[a] Model is adjusted for maternal educational level (low, middle, high), energy (kcals/day), maternal age (years), pre-pregnancy BMI (kg/m^2), parity (primiparous/multiparous) and country.

[b] Model is adjusted for same variables and additionally for gestational age (weeks) and gender

[c] P for trend is estimated for the adjusted change across increasing tertiles of the dioxin-diet score.

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5.2 Paper II

Maternal dietary intake of dioxins and polychlorinated biphenyls and birth size in the Norwegian Mother and Child Cohort Study (MoBa)

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Maternal dietary intake of dioxins and polychlorinated biphenyls and birth size in the Norwegian Mother and Child Cohort Study (MoBa).

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Abstract

Maternal diet provides essential nutrients to the developing fetus but is also a source of prenatal exposure to environmental contaminants. We investigated the association between dietary intake of dioxins and PCBs during pregnancy and birth size. The study included 50,651 women from the Norwegian Mother and Child Cohort Study (MoBa). Dietary information was collected by FFQs and intake estimates were calculated by combining food consumption and food concentration of dioxins, dioxin-like PCBs and non-dioxin-like PCBs. We used multivariable regression models to estimate the association between dietary intake of dioxins and PCBs and fetal growth. Further stratified analysis by quartiles of seafood intake during pregnancy was conducted. We found an inverse dose-response association between dietary intake of dioxins and PCBs and fetal growth after adjustment for confounders. Newborns of mothers in the upper quartile of dioxin and dioxin-like PCBs intake had 62 g lower birth weight (95%CI: -73, -50), 0.26 cm shorter birth length (95%CI: -0.31,-0.20) and 0.10 cm shorter head circumference (95%CI: -0.14,-0.06) than newborns of mothers in the lowest quartile of intake. Similar negative associations for intake of dioxins and dioxins-like PCBs were found after excluding women with intakes above the tolerable weekly intake (TWI). The reduction of fetal growth in association with dietary dioxins and PCBs persisted in analyses stratified by seafood intake. No association was found between dietary dioxins and PCB intake and the risk for small-for-gestational age neonate. In conclusion, dietary intakes of dioxins and PCBs during pregnancy were negatively associated with fetal growth, even at intakes below the TWI. Furthermore, the beneficial effects of maternal seafood consumption on fetal development might be compromised due to the adverse effects of contaminant exposure.

Keywords: diet, pregnancy, dioxins, PCBs, birth weight, MoBa

1. Introduction

During pregnancy the developing fetus is dependent on nutrient supply from the mother, and the maternal diet can influence the status of the intra-uterine environment (Cetin et al., 2013). A suboptimal intra-uterine environment, caused by malnutrition or nutrient restriction, can affect fetal growth and contribute to the risk of developing adult diseases (Barker, 1998). Additionally, maternal diet is linked to prenatal exposure to several environmental pollutants which enter the mother's body as food contaminants, such as dioxins and polychlorinated biphenyls (PCBs) (Arisawa et al., 2005).

Dioxins and PCBs are toxic, lipophilic and highly persistent environmental pollutants which bioaccumulate in the food chain. For non-occupationally exposed populations, diet is the main source of exposure to such contaminants. Seafood consumption is the major intake pathway followed by meat, dairy products, eggs and added fats, but main food contributors might differ with differing food patterns between populations (EFSA, 2012; Liem et al., 2000). The ingested compounds are distributed to the organs via the blood and stored in the adipose tissue (Henderson and Patterson, Jr., 1988). During pregnancy, accumulated dioxins and PCBs are transferred from the mother to the fetus through the placenta (Mose et al., 2012; Tsukimori et al., 2013).

Prenatal exposures to dioxins and PCBs can influence fetal body burden and have been related to impaired fetal growth, although the reported results are inconsistent (El Majidi et al., 2012; Lundqvist et al., 2006). In a recent meta-analysis of 12 European birth cohorts, researchers reported a 150 gram reduction in birth weight per 1-µg/L increase in PCB 153 in maternal blood (Govarts et al., 2012). Several mother-child studies have reported a negative association between prenatal exposure to concentrations of dioxins and PCBs in maternal blood and birth size (Halldorsson et al., 2008; Kezios et al., 2012; Patandin et al., 1998; Tan et al., 2009), while others have not found any association (Givens et al., 2007; Halldorsson et al., 2009; Longnecker et al., 2005; Wolff et al., 2007). Some studies

have found negative associations between prenatal exposure to dioxins and PCBs and fetal growth in boys and null associations in girls, suggesting a different effect between genders (Hertz-Picciotto et al., 2005; Konishi et al., 2009).

Seafood intake during pregnancy is related to concentrations of dioxins and PCBs in maternal blood (McGraw and Waller, 2009; Wang et al., 2009). However, studies examining the relationship between fish consumption and fetal growth have reported controversial results (Brantsæter et al., 2012; Grandjean et al., 2001; Guldner et al., 2007; Halldorsson et al., 2007; Thorsdottir et al., 2004), even for highly exposed populations eating very contaminated fish (Karmaus and Zhu, 2004; Rylander et al., 2000; Weisskopf et al., 2005). Hence, the effect of prenatal exposure to dioxins and PCBs through the maternal diet on fetal growth is still not clear.

The aim of our study was to investigate the association between dietary intake of dioxins and PCBs during pregnancy and fetal growth in the Norwegian Mother and Child Cohort Study (MoBa). Furthermore, we assessed gender differences and differences by seafood intake during pregnancy.

2. Material and Methods

2.1 Study population

This study is conducted within the Norwegian Mother and Child Cohort Study (MoBa), a prospective population-based pregnancy cohort study conducted by the Norwegian Institute of Public Health (Magnus et al., 2006). In brief, participants were recruited from all over Norway from 1999-2008 by a postal invitation at 17-18 weeks of pregnancy and 38.7% of invited women consented to participate. The cohort includes 91,000 mothers and 109,000 children. Data used in this study are based on version 5 of the quality-ensured data files, released for research in June 2010. The study was approved by The Regional Committee for Medical Research Ethics in South-Eastern Norway.

According to our inclusion criteria, women with the first participation in MoBa, with singleton births recorded in the Medical Birth Registry of Norway (MBRN) and with complete information on socio-demographic characteristics, exposure to tobacco smoke, weight gain and maternal diet during pregnancy, as well as birth outcomes, where eligible for the present analysis (n=52,295). All information on maternal and pregnancy related characteristics was collected by questionnaires. After excluding 385 mother-child pairs with gestational age <28 weeks or >42 weeks, 796 mother-child pairs due to implausible maternal daily energy intake (<4500 kJ or >20000 kJ) and 463 mother-child pairs with implausible weight change during pregnancy (<-30 kilo or >50 kilo), a total of 50,651 women with their children were included in our study. The cut-offs for plausible maternal energy intakes were established previously (Meltzer et al., 2008).

2.2 Dietary intake of dioxins and PCBs

The MoBa food frequency questionnaire (FFQ) (downloadable at http://www.fhi.no/ dokumenter/011fbd699d.pdf) was used to calculate the dietary intake of dioxins and PCBs during pregnancy. This validated semi-quantitative FFQ was administered at 22nd week of pregnancy and was designed to assess dietary habits and supplement intakes during the first four to five months of pregnancy (Meltzer et al., 2008). The validation study was focused on energy, nutrients, and specific food groups including seafood (Brantsæter et al., 2008; Brantsæter et al., 2010). Pregnant women were asked to report their frequency of consumption of 255 food and beverage items, by selecting one of 8-10 possible frequencies, ranging from never to several times monthly, weekly or daily. Daily food consumption (in g/day), energy (kcal/day) and fat (g/day) intakes were estimated using FoodCalc (Lauritsen, 2005) and the Norwegian Food Composition table (Rimestad et al., 2001).

Maternal dietary intakes of PCDD/Fs and PCBs during pregnancy were calculated by multiplying consumption with concentration of contaminants for each food item. The sum of the

products was the total dietary intake. The concentrations of PCDD/Fs and PCBs in Norwegian food items have been published previously (Kvalem et al., 2009). Hence, we have estimated dietary intakes of seventeen 2,3,7,8-substituted PCDD/Fs, twelve dl-PCBs (four non-ortho substituted PCBs (no-PCBs) PCB-77, 81, 126, 169 and eight mono-ortho substituted PCBs PCB-105, 114, 118, 123, 156, 157, 167, 189) and six non-dl-PCBs (sum of PCB-28, 52, 101, 138, 153, 180). Maternal intake of PCDD/Fs and dl-PCBs was expressed as toxic equivalents (TEQs) using toxic equivalence factors established by the World Health Organization in 2005 (Van den Berg et al., 2006). The exposures under study were the total dietary intake of dioxins and dl-compounds (in pg TEQs/day) and the total dietary intake of 6 non-dl-PCBs (in ng/day). A more detailed description of dietary exposure to dioxins and PCBs and foods contributing to the exposure in the MoBa study is presented in an accompanying paper by Caspersen *et al* (Caspersen et al., 2013).

2.3 Birth outcomes

The methodology for birth outcome collection and definition has been described previously (Duarte-Salles et al., 2012). In brief, information on weight, length and head circumference measures at birth was obtained from the Medical Birth Registry of Norway (Irgens, 2000). Gestational age was estimated using the first trimester ultrasound in 98.2% of MoBa participants. In case of missing ultrasound measure, gestational age was calculated as the interval between the last menstrual period and the date of delivery.

For births during 34 to 42 weeks of gestation, small for gestational age (SGA) was defined as a newborn with birth weight below the 10th percentile of newborns in MoBa according to the week of gestational age at birth and parity. For births during 28 to 33 weeks, data from the Medical Birth Registry of Norway (MBRN) published in 2000 were used to determine the 10th percentile of birth weight according to gestational age and parity

(Skjaerven et al., 2000). Additionally, low birth weight (LBW) was defined as any full-term newborn with birth weight below 2,500 g.

2.4 Statistical analysis

We used weight-adjusted exposure variables, by dividing dietary intake of dioxins and dioxin-like compounds (in pg TEQs/kg bw/day) and non-dioxin-like PCBs (in ng/kg bw/day) with maternal pre-pregnancy weight, which was self-reported at week 17th of pregnancy. Additionally, maternal intake of dioxins and dioxin-like PCBs was dichotomized according to the Tolerable Weekly Intake (TWI) of 14 pg TEQ/kg bw/week (EU-SCF, 2001). Exposure and outcome variables were evaluated regarding normality. For non-normal exposure variables, medians (IQR) and non-parametric Mann–Whitney or Kruskal–Wallis tests were used for distributions and comparison between groups, while Spearman's correlation coefficient was used to assess correlations between continuous variables. Anthropometric measurements at birth were described as means (SD). Categorical data were expressed as frequency and percentage (%) and differences in frequencies were assessed by Pearson's chi-square test. Bonferroni correction was used for multiple comparisons.

Simple and multiple linear and logistic regression models were fitted to estimate the association between maternal dietary intake of either dioxins and dioxin-like PCBs or non-dioxin-like PCBs with fetal growth measures. Exposure variables were divided into quartiles to evaluate potential nonlinear relationships and trend tests were performed to evaluate dose–response relations. Several potential confounders were assessed from a variety of maternal, paternal and pregnancy-related characteristics with established association with maternal dietary habits and birth outcomes. The retained covariates that resulted in a change in estimate >10% for either birth weight, SGA or LBW were: maternal age, weight gain during pregnancy, daily energy intake, parity, smoking during pregnancy, pre-pregnancy BMI (normal or overweight/obese), gestational age and child's gender. Other covariates (type of

delivery, parental income and education, paternal BMI, marital status, percentage of energy from fat, alcohol consumption and exposure to passive smoking during pregnancy) were tested as potential confounders but did not meet our criteria of inclusion.

In this population, fish intake has been previously positively associated with fetal growth but is also the main source of dietary intake of dioxins and PCBs in the Norwegian population (Brantsæter et al., 2012; Kvalem et al., 2009). We conducted stratified analysis by fitting linear regression models to assess the association of dioxins and dioxin-like PCBs and non-dioxin-like PCBs intake and birth outcomes by quartiles of fish intake.

We repeated our regression analysis after excluding 1,129 (2.2%) women with dietary intakes of dioxins and dl-PCBs over the tolerable weekly intake to investigate the influence of extreme dioxin and dl-PCBs intake to our findings. Further sensitivity analysis was conducted for primiparous women only and by gender. All statistical analyses were performed with STATA (STATA version 11.1; Stata Corporation, College Station, Texas).

3. Results

Dietary intakes of dioxins and PCBs during pregnancy according to maternal characteristics and birth outcomes are presented in Table 1. The median intake of dioxins and dioxin-like PCBs was 0.55 pg TEQ/kg bw/day (IQR= 0.37) and of non-dioxin-like PCBs was 2.34 ng/kg bw/day (IQR=2.01). Dietary intakes of dioxins and dioxin-like PCBs were positively correlated with energy (rho=0.33, 95%CI= 0.32, 0.34), fat (rho=0.37, 95%CI=0.36, 0.38) and seafood intake (rho=0.52, 95%CI=0.51, 0.53). Similar positive correlations were found for dietary non-dioxin-like PCB intake with energy, fat and seafood intake during pregnancy. Higher maternal age, education and parental income were linked to higher dietary intakes of contaminants. Additionally, higher intakes of dioxins and PCBs were observed for normal weight compared to overweight and obese women. Within our study population, 1,129 (2.2%) women had dietary intake of dioxins and dioxin-like PCBs during

pregnancy above the TWI. Notably, women with a dietary intake of dioxins and dioxin-like PCBs above the TWI were more likely to have low education and income than women with intakes below the TWI (39 vs. 30% in the lowest educational level and 31 vs. 27% in the lowest income level). Additionally, dietary intake of dioxins and dioxin-like PCBs was highly and positively correlated with intake of non-dioxin-like PCBs (rho=0.94, 95% CI=0.94, 0.94).

Male and female newborns were equally distributed among the mother-child pairs included in our study (Table 1). All anthropometric measurements were negatively, though modestly, correlated with maternal dietary intake of dioxins and dioxin-like PCBs (rho= -0.05 to -0.04), as well as non-dioxin-like PCBs (rho=-0.06 to -0.05). The prevalence of preterm birth, LBW and SGA was 9%, 2.5% and 7.3% respectively. Women with intake of dioxins and dioxin-like PCBs above the TWI had approximately 1% higher prevalence of preterm birth, LBW and SGA compared to women with lower intakes, though the differences were not statistically significant (Table 2).

We found an inverse dose-response association between dietary intake of dioxins and dioxin-like PCBs and birth weight, length and head circumference (Table 3). Newborns of mothers in the highest quartile of dioxin intake had -61.6 g lower birth weight (95% CI -73.2, -50.0), -0.26 cm shorter birth length (95% CI -0.31,-0.20) and -0.10 cm shorter birth head circumference (95% CI -0.14,-0.06) compared to newborns of mothers in the lowest quartile of dioxin intake. For non-dioxin-like PCBs intake in the highest quartile the reduction was -40.5 g for birth weight (95% CI -51.6,-29.4), -0.21 cm for birth length (95% CI -0.26,-0.15) and -0.06 cm for birth head circumference (95% CI -0.09,-0.02). After stratifying by gender, we observed that the adjusted reduction in birth weight associated with intake of dioxins and dl-PCBs was -68.5 grams (95% CI=-84.8, -52.1) for boys and -54.2 for girls (95% CI=-70.2, -37.7) (Supplementary Table). The reduction in head circumference was more pronounce in females than in males.

Additionally, we observed a negative association between maternal dietary dioxin and dioxin-like PCB intake above the TWI and birth weight, length and head circumference, compared to women with lower dietary intakes during pregnancy (Table 4). The risk for SGA was not different for mothers with dioxins and dioxin-like PCB intakes above the TWI, compared with lower intakes (OR=1.18, 95% 0.95, 1.46). We found no dose-response association between maternal dietary intake of either dioxins and dioxin-like PCBs or non-dioxin-like PCBs with gestational age (data not shown).

The adjusted associations between dietary intake of dioxins and dioxin-like PCBs and birth weight stratified by seafood intake are presented in Figure 1. Each square represents the regression coefficient and line edges represent the 95% CI for the association between dioxins and dioxin-like PCBs intake in the 4th quartile and birth weight. The adjusted change in birth weight is compared with women in the 1st quartile of dioxins and dioxin-like PCBs intake. We observed that newborns of mothers with seafood intakes of less than 25 g/day and in the 4th quartile of dioxins and dioxin-like PCBs intakes had -87.8 g (95% CI=-55.7, -120.0) lower birth weight than newborns of mothers with dioxins and dioxin-like PCBs intakes in the 1st quartile and in the same category of seafood intake. The corresponding reduction in birth weight for newborns of mothers with seafood intake >60 g/day and in the 4th quartile of dioxins and dioxin-like PCBs intakes was -76.6 g (95% CI=-47.4, -105.8), compared to the 1st quartile of dioxins and dioxin-like PCBs intake. Likewise, a high dietary intake of non-dioxin-like was associated with -57 grams (95%CI=-24.4, -89.5) reduction in birth weight if the mother consumed <25g seafood/day and -49 grams (95%CI=-20.5,-77.4) reduction if the mother was consuming >60 g seafood/day.

4. Discussion

We investigated the association between dietary intake of dioxins and PCBs during pregnancy and birth outcomes in 50,651 mother-

child pairs from the Norwegian Mother and Child Cohort Study. We found that high dietary intake of dioxins and PCBs was associated with a significant reduction in birth weight, length and head circumference.

In our study, dietary intake of dioxins and dl-PCBs (0.56 pg TEQ/kg bw/day) was similar or lower than those reported previously for women (Kvalem et al., 2009; Llobet et al., 2008; Perello et al., 2012; Schecter et al., 2001; Sirot et al., 2012) Additionally, our calculation on dietary intake of non-dl PCBs (2.49 ng/kg bw/day) was between the ones reported for women in a Norwegian and a French study (Kvalem et al., 2009; Sirot et al., 2012). The current study and the accompanying study by Caspersen et al. are the first presentations of dietary intake of PCBs and dioxins in pregnant women (Caspersen et al., 2013). Overall, the dietary exposure to dioxins and PCBs during pregnancy was among the lowest reported for an adult population in Scandinavian (Kiviranta et al., 2004; Tornkvist et al., 2011) or other European countries (De Mul et al., 2008; Fattore et al., 2006; Fernandez et al., 2004; Windal et al., 2010).

We are the first to report a negative association between dietary intakes of dioxins and PCBs and fetal growth. Our findings are in agreement with studies which have shown a negative relationship between maternal or cord serum levels of dioxins and PCBs and birth weight (Govarts et al., 2012; Patandin et al., 1998; Sagiv et al., 2007; Tan et al., 2009). According to these studies, the highest level of exposure, as estimated by concentrations of the contaminants in blood samples, was associated with reductions in birth weight of more than 100g, which is more than our estimate. This discrepancy can be explained by the different methodologies used for prenatal exposure assessment, since blood levels of lipophilic contaminants reflect total body burden, while daily dietary intakes reflect background exposures and do not represent long-term exposure. Interestingly, the negative relationship between dietary intakes of dioxins and dioxin-like PCBs and fetal growth was observed even after excluding women with intakes above the tolerable weekly

intake level, implying that the observed negative relationships were not driven by extreme intakes.

Fetal growth restriction, as assed by SGA, was more prevalent among women with intakes of dioxins and dioxin-like PCBs above the TWI, but the difference was not significant. In a recent review, researchers concluded that there is not enough evidence to support a negative association between concentrations of PCBs in maternal blood, cord blood or breast milk and the risk for low birth weight (El Majidi et al., 2012). Similar null associations were found for high prenatal exposures to TCDD in a cohort of women from Seveso, Italy (Eskenazi et al., 2003).

The contribution of maternal diet and particularly seafood intake to the body burden of dioxins and PCBs is well established, while the extent to which dietary intake of dioxins and PCBs can predict smaller birth size is unknown. In large European mother-child studies of low-exposed populations, seafood has been differently linked to fetal growth, with negative associations reported in Danish, French, Spanish and Swedish populations (Guldner et al., 2007; Halldorsson et al., 2007; Mendez et al., 2010) and positive associations reported in Norwegian and in French women (Brantsæter et al., 2012; Drouillet-Pinard et al., 2010; Guldner et al., 2007), while no effect was observed for Dutch pregnant women (Heppe et al., 2011). Halldorsson et al. found that Danish women who consumed more than 60 g of seafood/day had 24% higher risk of giving birth to an SGA neonate, while Brantsæter et al. reported 44% lower risk of giving birth to a low birth weight neonate for the same level of seafood intake in Norwegian women (Brantsæter et al., 2012; Halldorsson et al., 2007). In our study, the negative association between dioxins and PCBs intake during pregnancy and birth weight remained also for women with a seafood intake more than 60g/day, but in lower seafood intake levels the observed reduction in birth weight was greater. Seafood consumption during pregnancy has been reported as an effective method to prevent preterm delivery and fetal growth retardation, as wells as later adult diseases, particularly due to the beneficial effects of omega-3 fatty

acids (Salvig and Lamont, 2011; Swanson etal., 2012). Our findings, taken together with the observed protective association for LBW in Norwegian women, suggest that the potential beneficial effects of seafood consumption on fetal growth could have been lowered due to the increasing intake of dioxins and PCBs. Evidence of the negative effect of prenatal exposure to environmental contaminants through maternal diet, on child's health is growing, while the debate between adequate nutrition and dietary toxicant exposure continues (Bushkin-Bedient and Carpenter, 2010; Genuis, 2008).

In our study, the reduction of fetal growth related to high prenatal exposure to dietary dioxins and PCBs, was greater in males than in females. Previous studies have also reported significant negative association between prenatal exposure to dioxins and PCBs and birth weight only in males, suggesting that they are more vulnerable than females (Hertz-Picciotto et al., 2005; Konishi et al., 2009; Sonneborn et al., 2008). Moreover, high dietary intake of dioxins and PCBs was related to reduced head circumference mostly in females, but the associations were weak. In epidemiological studies, neonatal head circumference is used as an estimate of fetal brain development, and prenatal exposure to dioxins and PCBs can induce neurotoxic effects and affect neurodevelopment. Additionally, reduction in head circumference at birth has previously been linked with high serum levels of PCBs in maternal and cord blood, as well as breast milk (Fein et al., 1984; Hertz-Picciotto et al., 2005; Nishijo et al., 2008; Tan et al., 2009). Our findings of head circumference related to dioxins and PCBs are interpreted in the context of a reduction in overall birth size and not as evidence of neurotoxicity. Further follow-up of these children is needed, which should focus on the study of low-level organochlorine exposure through maternal diet and neurodevelopment.

Dioxins and dioxin-like PCBs share common sources of exposure and are highly correlated with non-dioxin-like PCBs, hence is hard to attribute the estimated negative effects to one of the

compound groups. Studies indicate that dioxins and dioxin-like PCBs are more readily transferred from maternal blood to the placenta and cord blood than non-dioxin-like PCBs (Tsukimori et al., 2013). This procedure is mediated through the affinity of dioxins and dioxin-like PCBs for the aryl hydrocarbon (Ah) receptor which is also the mediator for the manifest of their toxic effects (Tsukimori et al., 2013; Van den Berg et al., 2006). AhR-ligand activation induces the expression of placental xenobiotic metabolizing enzymes and might lead to an unfavorable intra-uterine environment and finally to disruption of normal fetal growth (Bustamante et al., 2012; Suter et al., 2010). However, also hormone disruption, impaired immune function and other non-AhR mediated pathways have been suggested as potential mechanisms for the effect of prenatal dioxins and PCBs exposures on child's health, illustrating the complexity of the mechanism.

The present study has strengths and limitations. A main strength is the large sample size, including participants from both urban and rural regions representing all age groups and all socioeconomic groups. MoBa participants are not representative of all pregnant women in Norway. However, the relationships between variables were not necessarily distorted by exclusion of non-participants. For example, for eight exposure-disease relationships examined in the MoBa data, the associations were essentially the same as those found in an analysis of data for the entire pregnant population in Norway (Nilsen et al., 2009).

We have used an extensive FFQ which has been thoroughly validated and allowed us to estimate dietary intakes of dioxins and PCBs from the whole diet. However, all dietary assessment tools have uncertainties and imprecisions which might lead to underestimation of the true effect of the PCB and dioxin exposure on fetal growth (Grandjean and Budtz-Jorgensen, 2007; MacMahon et al., 1990). Additionally, we acknowledge that fetal exposure is a result of accumulated intakes of dioxins and PCBs and dietary intake estimates reflect short-term rather than long-term exposures and body burden. Several other lifestyle factors, along with diet, are

important predictors of blood levels of dioxins and PCBs, including age, parity, BMI and breastfeeding history, and can influence body burden (Knutsen et al., 2011). Nevertheless, in epidemiological studies, dietary intakes have often been used as a proxy of human exposure to dioxins and PCBs and as a tool for ranking individuals in the population (Bilau et al., 2008; Huisman et al., 1995; Kvalem et al., 2012).

5. Conclusions

In conclusion, within a large population-based mother and child study, we found that dietary intakes of dioxins and PCBs during pregnancy might adversely affect fetal growth. This is the first report relating maternal dietary dioxin and PCBs intakes with fetal growth. Our findings support the hypothesis that prenatal exposure to environmental contaminants, through maternal diet, can negatively affect fetal size, even at low dietary intakes. Maternal diet might affect fetal growth, since it is contributing to maternal and fetal body burden of contaminants and can influence the intra-uterine environment. Therefore, reduction of maternal body burden long before pregnancy can reduce prenatal exposure to environmental toxicants and consequently avoid impaired fetal growth.

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Table 1. Prenatal dietary intake of dioxins and dioxin-like PCBs (in pg TEQ/kg bw/day) and non-dioxin-like PCBs (in ng/kg bw/day) according to maternal and birth outcomes for 50,651 mother-child pairs.

			Dioxins and dioxin-like PCBs intake (pg TEQ/kg bw/day)			Non-dioxin-like PCBs intake (ng/kg bw/day)		
	N	(%)	Median	(IQR)	P-value [a]	Median	(IQR)	P-value [a]
Maternal age (years)								
<25	5,492	(10.8)	0.53	(0.38)		2.18	(2.00)	
25-29	17,411	(34.4)	0.54	(0.35)	0.0001	2.24	(1.93)	0.0001
30-34	19,329	(38.2)	0.56	(0.36)		2.37	(1.98)	
≥35	8,419	(16.6)	0.59	(0.38)		2.57	(2.17)	
Pre-pregnancy BMI status								
Normal (≤25 kg/m^2)	34,842	(68.8)	0.61	(0.37)	0.0001	2.57	(2.11)	0.0001
Overweight/Obese (>25 kg/m^2)	15,801	(31.2)	0.44	(0.29)		1.85	(1.59)	
Maternal education								
<12 years	15,243	(30.1)	0.52	(0.39)		2.20	(2.03)	
13-16 years	21,847	(43.1)	0.55	(0.35)	0.0001	2.31	(1.91)	0.0001
>17 years	12,539	(24.8)	0.59	(0.36)		2.55	(1.91)	
missing/other	*1,022*	(2.0)	*0.54*	(*0.36*)		*2.27*	(*1.96*)	
Parity								
Primiparous	26,320	(52.0)	0.55	(0.36)	0.0001	2.31	(2.01)	0.0001
Multiparous	24,331	(48.0)	0.56	(0.37)		2.37	(2.00)	
Type of delivery								
Normal	43,523	(85.9)	0.55	(0.36)	0.0005	2.34	(2.00)	0.0171
Caesarean	7,128	(14.1)	0.54	(0.38)		2.30	(2.07)	
Smoking during pregnancy								
No	46,420	(91.7)	0.55	(0.36)	0.0200	2.34	(2.00)	0.0907
Yes	4,231	(8.3)	0.54	(0.41)		2.27	(2.15)	
Alcohol during pregnancy								
No	44,726	(88.3)	0.55	(0.36)	0.0001	2.30	(1.97)	0.0001
Yes	5,925	(11.7)	0.61	(0.38)		2.64	(2.15)	

Parental income								
Low	13,501	(26.7)	0.54	(0.38)		2.26	(1.98)	
Medium	21,413	(42.3)	0.55	(0.36)	0.0001	2.31	(1.98)	0.0001
High	13,488	(26.6)	0.57	(0.35)		2.46	(2.02)	
missing	*2,249*	(4.4)	*0.57*	(*0.41*)		*2.36*	(*2.26*)	
Child's gender								
Male	25,906	(51.2)	0.55	(0.37)	0.870	2.34	2.03	0.848
Female	24,745	(48.9)	0.55	(0.36)		2.33	2.00	
Preterm birth (<37 weeks)								
No	46,071	(91.0)	0.55	(0.36)	0.043	2.34	2.00	0.048
Yes	4,580	(9.0)	0.55	(0.37)		2.30	2.08	
Low birth weight (<2,500 g)								
No	49,373	(97.5)	0.55	(0.37)	0.123	2.34	2.01	0.084
Yes	1,278	(2.5)	0.54	(0.39)		2.24	2.03	
Small-for-gestational age								
No	46,956	(92.7)	0.55	(0.37)	0.084	2.34	2.01	0.052
Yes	3,695	(7.3)	0.54	(0.37)		2.28	2.03	

[a] After Bonferroni correction statistically significant differences were for P-value<0.002.

Table 2. Birth outcomes and newborn characteristics according to prenatal dietary intake of dioxins and dioxin-like PCBs above the Tolerable Weekly Intake (TWI) for 50,651 mother-child pairs.

	Dioxins and dioxin-like PCBs intake above TWI				
	No (N=49,522)		Yes (N=1,129)		
	Mean	(SD)	Mean	(SD)	P-value
Gestational age (weeks)	40	(2)	40	(1)	0.004
Birth weight (g)	3602	(539)	3526	(527)	<0.001
Birth length (cm)	50.4	(2.4)	50.0	(2.4)	<0.001
Birth head circumference (cm)	35.3	(1.6)	35.2	(1.6)	<0.001
	N	(%)	N	(%)	P-value
Child's gender					
Male	25,351	(51.2)	555	(49.2)	0.177
Female	24,171	(48.8)	574	(50.8)	
Preterm birth (<37 weeks)					
No	45,056	(91.0)	1,015	(89.9)	0.211
Yes	4,466	(9.0)	114	(10.1)	
Low birth weight (< 2,500 g)[a]					
No	46,960	(99.3)	1,067	(99.3)	0.903
Yes	337	(0.7)	8	(0.7)	
Small-for-gestational age					
No	45,921	(92.7)	1,035	(91.7)	0.178
Yes	3,601	(7.3)	94	(8.3)	

[a] Only full-term births.

Table 3. Associations of maternal dietary intake of dioxins and dl-PCBs and non-dl-PCBs with anthropometric measurements at birth.

	Birth weight (in g) (n=50,651)				Birth length (in cm) (n=49,684)				Birth head circumference (in cm) (n=49,684)			
	Crude model		Adjusted model		Crude model		Adjusted model		Crude model		Adjusted model	
	beta	(95% CI)	beta	(95% CI)	beta	(95% CI)	beta	(95% CI)	beta	(95% CI)	beta	(95% CI)
Dioxins and dioxin-like PCBs intake (pg TEQ/kg bw/day)												
Q1 (<0.39)	Ref.		Ref.		Ref.		Ref.		Ref.		Ref.	
Q2 (0.39-0.55)	-24.2	(-37.5,-11.0)	-28.3	(-38.9,-17.8)	-0.03	(-0.09,0.03)	-0.08	(-0.13,-0.02)	-0.05	(-0.09,-0.01)	-0.04	(-0.08,-0.01)
Q3 (0.56-0.77)	-49.2	(-62.4,-35.9)	-40.7	(-51.7,-29.8)	-0.17	(-0.23,-0.11)	-0.18	(-0.23,-0.13)	-0.11	(-0.15,-0.07)	-0.07	(-0.10,-0.03)
Q4 (>0.77)	-79.6	(-92.8,-66.3)	-61.6	(-73.2,-50.0)	-0.27	(-0.33,-0.21)	-0.26	(-0.31,-0.20)	-0.17	(-0.21,-0.13)	-0.10	(-0.14,-0.06)
P trend		*<0.001*		*<0.001*		*<0.001*		*<0.001*		*<0.001*		*<0.001*
Non-dioxin-like PCBs intake (ng/kg bw/day)												
Q1 (<1.59)	Ref.		Ref.		Ref.		Ref.		Ref.		Ref.	
Q2 (1.59-2.34)	-25.3	(-38.6,-12.1)	-23.2	(-33.7,-12.7)	-0.09	(-0.15,-0.03)	-0.11	(-0.16,-0.05)	-0.05	(-0.09,-0.01)	-0.03	(-0.06,0.01)
Q3 (2.35-3.60)	-33.8	(-47.0,-20.5)	-24.8	(-35.6,-14.0)	-0.13	(-0.19,-0.07)	-0.14	(-0.19,-0.09)	-0.08	(-0.12,-0.04)	-0.04	(-0.08,0.00)
Q4 (>3.60)	-65.3	(-78.6,-52.1)	-40.5	(-51.6,-29.4)	-0.25	(-0.31,-0.19)	-0.21	(-0.26,-0.15)	-0.13	(-0.17,-0.09)	-0.06	(-0.09,-0.02)
P trend		*<0.001*		*<0.001*		*<0.001*		*<0.001*		*<0.001*		*0.003*

Adjusted models include maternal age, energy intake, pre-pregnancy BMI, parity, weight gain and smoking during pregnancy, gestational age and child's gender

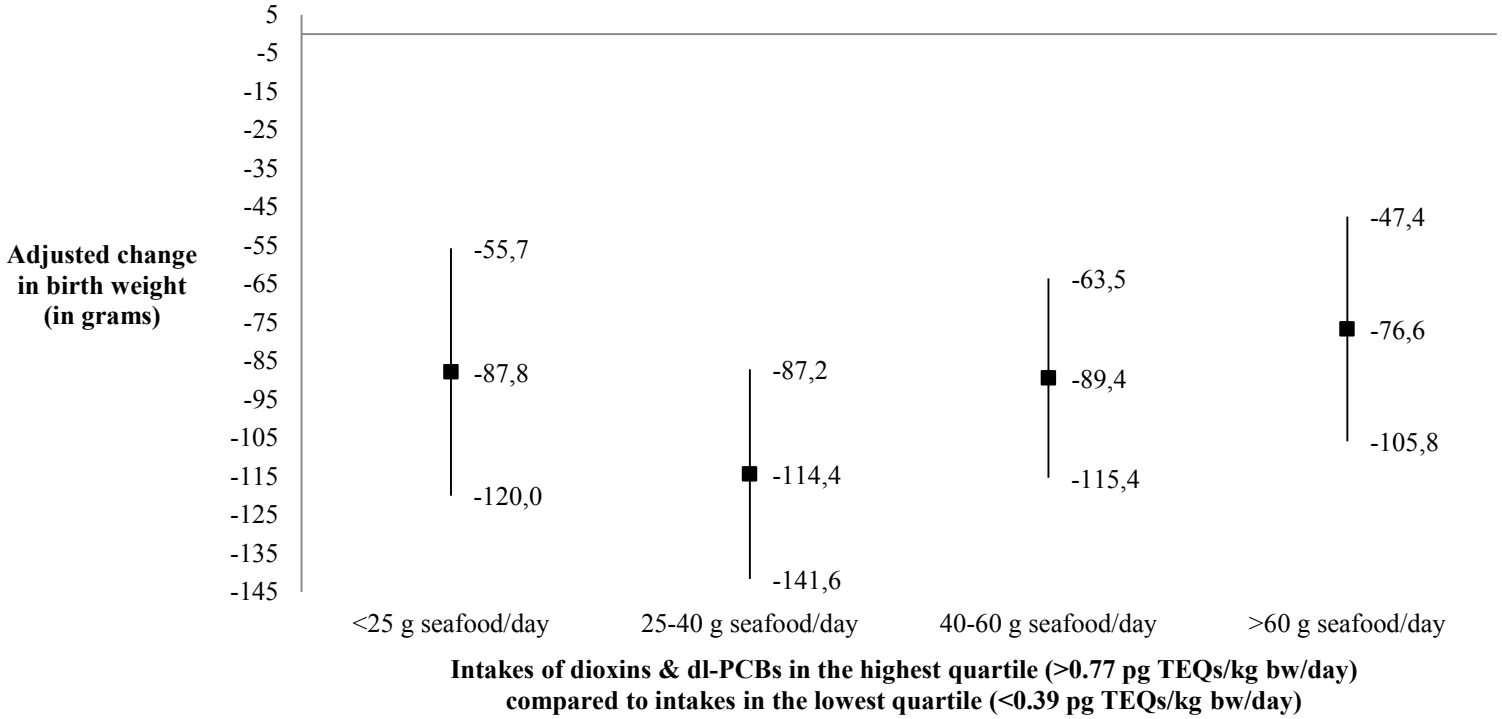
Table 4. Associations between maternal dietary intake of dioxins and dl-PCBs above the TWI and birth outcomes.

	Crude model		Adjusted model	
	beta	(95% CI)	beta	(95% CI)
Birth weight (grams)				
Dioxins and dioxin-like PCBs intake above TWI				
No	Ref.		Ref.	
Yes	-76.4	(-108.1,-44.6)	-42.4	(-67.1,-17.7)
Birth length (cm)				
Dioxins and dioxin-like PCBs intake above TWI				
No	Ref.		Ref.	
Yes	-0.36	(-0.51,-0.22)	-0.23	(-0.35,-0.11)
Birth head circumference (cm)				
Dioxins and dioxin-like PCBs intake above TWI				
No	Ref.		Ref.	
Yes	-0.17	(-0.26,-0.07)	-0.06	(-0.14,0.03)
	OR	(95% CI)	OR	(95% CI)
Risk for small-for-gestational age				
Dioxins and dioxin-like PCBs intake above TWI				
No	Ref.		Ref.	
Yes	1.2	(0.9,1.4)	1.2	(0.9,1.5)
Risk for low birth weight (<2,500g)				
Dioxins and dioxin-like PCBs intake above TWI [a]				
No	Ref.		Ref.	
Yes	1.1	(0.5,2.1)	0.9	(0.4,1.8)

Adjusted model includes maternal age, weight gain during pregnancy, energy intake, parity, smoking during pregnancy, pre-pregnancy BMI status, gestational age and child's gender.

[a] Only full-term births.

Figure 1. Adjusted association between high (4th quartile) maternal dietary intake of dioxins and dioxin-like PCBs and birth weight, compared to low intake (1st quartile) of dioxins and dioxin-like PCBs, by categories of seafood intake during pregnancy.



Supplemental material

Supplemental material, Table 1. Adjusted associations of maternal dietary intake of dioxins and dl-PCBs and non-dl-PCBs with anthropometric measurements at birth, by gender.

	Birth weight (in g)				Birth length (in cm)				Birth head circumference (in cm)			
	Boys (n=25,906)		Girls (n=24,745)		Boys (n=25,398)		Girls (n=24,286)		Boys (n=25,398)		Girls (n=24,286)	
	beta	(95% CI)	beta	(95% CI)	beta	(95% CI)	beta	(95% CI)	beta	(95% CI)	beta	(95% CI)
Dioxins and dioxin-like PCBs intake (pg TEQ/kg bw/day)												
Q1 (<0.39)	Ref.		Ref.		Ref.		Ref.		Ref.		Ref.	
Q2 (0.39-0.55)	-32.4	(-47.2,-17.6)	-24.1	(-39.0,-9.1)	-0.06	(-0.13,0.02)	-0.09	(-0.17,-0.02)	-0.03	(-0.08,0.03)	-0.06	(-0.11,-0.01)
Q3 (0.56-0.77)	-45.0	(-60.5,-29.5)	-36.0	(-51.5,-20.4)	-0.18	(-0.26,-0.10)	-0.18	(-0.25,-0.10)	-0.05	(-0.11,0.00)	-0.08	(-0.13,-0.03)
Q4 (>0.77)	-68.5	(-84.8,-52.1)	-54.2	(-70.7,-37.7)	-0.28	(-0.36,-0.20)	-0.23	(-0.31,-0.15)	-0.08	(-0.14,0.02)	-0.13	(-0.18,-0.07)
P trend		*<0.001*		*<0.001*		*<0.001*		*<0.001*		*0.003*		*<0.001*
Non-dioxin-like PCBs intake (ng/kg bw/day)												
Q1 (<1.59)	Ref.		Ref.		Ref.		Ref.		Ref.		Ref.	
Q2 (1.59-2.34)	-32.1	(-46.8,-17.3)	-14.3	(-29.2,0.6)	-0.12	(-0.19,-0.04)	-0.10	(-0.17,-0.03)	-0.02	(-0.07,0.03)	-0.03	(-0.08,0.02)
Q3 (2.35-3.60)	-28.6	(-43.9,-13.4)	-20.6	(-35.9,-5.3)	-0.16	(-0.23,-0.08)	-0.12	(-0.20,-0.05)	-0.03	(-0.08,0.03)	-0.06	(-0.11,-0.01)
Q4 (>3.60)	-52.5	(-68.2,-36.9)	-28.0	(-43.7,-12.3)	-0.24	(-0.32,-0.16)	-0.17	(-0.25,-0.09)	-0.05	(-0.10,0.01)	-0.07	(-0.12,-0.01)
P trend		*<0.001*		*<0.001*		*<0.001*		*<0.001*		*0.089*		*0.011*

Adjusted model includes maternal age, weight gain during pregnancy, energy intake, parity, smoking during pregnancy, pre-pregnancy BMI status, and gestational age.

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5.3 Paper III

Anogenital distances in newborns and children from Spain and Greece: predictors, tracking and reliability

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5.4 Paper IV

Maternal diet, prenatal exposure to dioxins and other persistent organic pollutants and anogenital distance in children.

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Maternal diet, prenatal exposure to dioxins and other persistent organic pollutants and anogenital distance in children.

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Abstract

We investigated the potential endocrine disruptive effect of prenatal exposure to persistent organic pollutants (POPs) through maternal diet, by measuring anogenital distance in newborns and young children. We included 231 mothers and their newborns measured at birth from the Rhea study in Crete, Greece and the Hmar study in Barcelona, Spain and 476 mothers and their children measured between 1 to 2 years from the Rhea study. We used food frequency questionnaires to assess maternal diet and estimated plasma dioxin-like activity by the Dioxin-Responsive Chemically Activated LUciferase eXpression (DR-CALUX®) in maternal and cord blood samples, and other POPs in maternal samples. We defined a "high-fat diet" score, as a prenatal exposure estimate, that incorporated intakes of red meat, processed meat, fatty fish, seafood, eggs and high-fat dairy products during pregnancy. Increasing maternal "high-fat diet" score was related to increasing dioxin-like activity in maternal and cord blood and serum concentrations of lipophilic persistent organic pollutants in maternal blood. An inverse dose-response association was found between "high-fat diet" score and anoscrotal distance in newborn males. The highest tertile of the maternal score was associated with -4.2mm (95%CI -6.6 to -1.8) reduction in anoscrotal distance of newborn males, compared to the lowest tertile. A weak positive association was found between the "high-fat diet" score and anofourchetal distance in newborn females. In young children we found no association between maternal "high-fat diet" score and anogenital distances. In conclusion, maternal high-fat diet may be linked to high prenatal exposure to persistent organic pollutants and endocrine disruptive effects, resulting to phenotypic alterations of the reproductive system.

Keywords: pregnancy, maternal diet, persistent organic pollutants, DR-CALUX, anogenital distance, Rhea study

1. Introduction

Maternal diet during pregnancy is crucial because it provides essential nutrients to the developing fetus (Chatzi et al., 2012;Knudsen et al., 2008). However, the fetus is also exposed through the mother to persistent organic pollutants (POPs) (Liem et al., 2000). Human exposure to POPs such as dioxins, biphenyls (PCBs) and organochlorine pesticides occurs mainly through diet. Studies combining food consumption and contamination levels have identified seafood, meat, eggs and dairy products as main sources of dietary exposure to POPs (Darnerud et al., 2006;De Mul et al., 2008;Perello et al., 2012;Tard et al., 2007).

Diet of pregnant women is associated with levels of lipophilic organochlorine contaminants in maternal and cord blood as well as in the placenta (Glynn et al., 2007;Halldorsson et al., 2008;Huang et al., 2007;Llop et al., 2010). During pregnancy, absorbed compounds pass through the placenta and reach the fetus (Lopez-Espinosa et al., 2007;Suzuki et al., 2005). After birth, exposure continues through breastfeeding and concentrations of POPs in maternal blood have been related to concentrations in breast milk (Solomon and Weiss, 2002). Therefore, maternal body burden of POPs is important because of potential health effects in the fetuses.

Organochlorine contaminants can disrupt normal endocrine function and *in-utero* exposures have been linked to several adverse health effects (Lundqvist et al., 2006;Wigle et al., 2008). In animals, prenatal exposure to organochlorine contaminants may induce anti-androgenic effects and alterations in the reproductive system of offspring, including a reduction in anogenital distance (Gray et al., 2001;Ohsako et al., 2002). Anogenital distance, measured from the anus to the genitalia, is used as marker of prenatal exposure to androgens. Additional postnatal androgen production does not affect anogenital distance in animals and may assist as a predictor of androgen responsive outcomes in adulthood (McIntyre et al., 2002;van den Driesche et al., 2011).

In humans, prenatal exposure to phthalates, which can act as hormone disrupters, has been linked to shorter anogenital distance

mainly in boys (Huang et al., 2009;Suzuki et al., 2012;Swan et al., 2005). Two studies have investigated the association between prenatal exposure to DDE and anogenital distance in children and reported inconsistent results (Longnecker et al., 2007;Torres-Sanchez et al., 2008). Our research group recently showed that high dioxin-like activity in maternal blood was associated with a reduction in anogenital distance of newborn boys (Vafeiadi et al., 2012). There is no study on the effect of maternal diet, as a source of exposure to POPs, on anogenital distance of children. In adult men, shorter anogenital distance predicted poorer semen quality and hypogonadal testosterone levels, while prostate cancer patients had shorter anogenital distances than healthy adult men (Castano-Vinyals et al., 2012;Eisenberg et al., 2012a;Mendiola et al., 2011). Anogenital distance in humans has been also suggested as a novel marker of adult testicular function (Eisenberg et al., 2012a;Eisenberg et al., 2012b;Eisenberg et al., 2012c).

We examined the association between prenatal exposure to organochlorine compounds, through maternal high-fat diet, and anogenital distance measured in males and females, in two mother-child cohorts in Greece and Spain.

2. Methods

2.1 Study population

Mothers and their children included in this analysis were from the mother-child cohort in Crete, Greece ("Rhea study") and the Hospital del Mar cohort in Barcelona, Spain ("Hmar study"). The Rhea study examines prospectively a population-based cohort of pregnant women and their children at the prefecture of Heraklion, Crete, Greece (Chatzi et al., 2009). Women were recruited within a year (from February 2007) at around 12 weeks of gestation. The inclusion criteria were: to be residents of the study area, to be more than 16 years old, to have the 1st visit at hospitals or private clinics at 10-13 week of gestation for the first major ultrasound examination and to have no communication handicap. The Hmar

study includes women with singleton pregnancies enrolled at delivery in a public hospital of Barcelona, Spain, from October 2008 to March 2010. Women less than 18 years old, with multiple pregnancies or with pregnancy complications (HIV/B hepatitis/C hepatitis infections, urgent C-sections, postpartum excessive hemorrhage) were excluded.

In the Rhea study, 795 (49.4% of the total "Rhea study" population) women with singleton pregnancies agreed to participate in the anogenital measurement protocol of their children and 647 children were measured. In the Hmar study, 187 (66.5% of the total "Hmar study" population) newborns were measured and 127 mother-newborn pairs were eligible for this analysis with complete maternal dietary information. In both studies, 34 women were excluded due to missing information on maternal socio-demographic characteristics and 33 women were excluded due to implausible maternal energy intake (outside the range of 4184-16736 kj/day) (Davey et al., 2003). Hence, 231 mothers with theirs newborns (n=128 from the Rhea study, n=103 from the Hmar study) and 476 mothers with their children measured between 1 to 2 years (all from Rhea study) were included in our analysis.

All procedures of the study were approved by the ethical committee of the University Hospital in Heraklion, Crete, Greece and by the Clinical Research Ethical Committee at Hospital del Mar (CEIC), Barcelona, Spain. Written informed consent was obtained from all women participating in the studies concerning themselves as well as their children.

2.2 Dietary assessment and maternal "high-fat diet" score

In the Rhea study, a validated food frequency questionnaire (FFQ) was used to assess dietary habits over pregnancy. It was administered by trained research nurses between14 and 18 weeks of gestation (Chatzi et al., 2011). Frequency of intake was obtained for 250 food items. The FFQ used by the Hmar study was adapted by the INMA (INfancia y Medio Ambiente) Project and it has been validated for use among adults living in Spain (Guxens et al., 2012).

Women completed the FFQ after delivery and were asked to report the frequency of intake for 100 food items during the whole period of their pregnancy.

A "high-fat diet" score was created to estimate prenatal exposure to organochlorine contaminants from maternal diet. Foods of animal origin with high fat content, recognized as dietary sources of organochlorine compounds are included in this score as 6 food groups: red meat, processed meat, seafood, fatty fish, eggs and high fat dairy products (EFSA, 2012;EFSA, 2006a;EFSA, 2006b). Each food group was formed as a summary of the weekly frequency of intake of specific food items. The group of "red meat" included: pork, beef, lamb, goat, pork and beef burgers and minced meat. The group of "processed meat" included: cured meat (ham, sausage, salami, mortadella, and smoked turkey), bacon and boiled turkey. Tuna (canned and fresh) and salmon were included in the "fatty fish group", as well as smaller species of fatty fish (i.e sardines, mackerel). The group of "seafood" included: shrimps, squid and shellfish. The "eggs" food group was a summary of intake of boiled eggs, fried eggs and omelets. Finally, "high-fat dairy products" included: whole milk, high-fat cheese, whole yogurt, ice cream and whipped cream. Liver and offal were not included in the "high-fat diet" score because many women reported no intake of such foods (Rhea study: liver 74.7% and offal 85.7% of no intake; Hmar study: liver 63.1% and offal 80.6% of no intake).

Each food group was categorized in tertiles and a value was assigned in each woman, according to her level of intake, as follows: 0 for the lower tertile, 1 for middle tertile and 2 for the upper tertile. The summary of those values was the "high-fat diet" score of each woman. Many women reported no intake of seafood (n=406, 56%), thus we used 2 values for seafood intake. A value of 0 was assigned for no intake and 1 for intake. Hence, the "high-fat" score ranged from 0 to 11 and represents the cumulative weekly intake of 6 food groups (in times/week).

2.3 Dioxin-like activity (DR-CALUX® bioassay)

Dioxin-like activity was estimated in maternal and cord blood samples collected at delivery using the Dioxin-Responsive Chemically Activated LUciferase eXpression (DR-CALUX®) bioassay, in a subsample of our study population from the Rhea and the Hmar study (n=166 maternal samples and 148 cord blood samples). The analysis was conducted at Biodetection Systems B.V., Amsterdam. The protocol performed for the DR-CALUX® bioassay in this study has been described in details elsewhere (Vafeiadi et al., 2012).

2.4 Concentrations of organochlorine compounds in maternal blood

Additional concentrations of organochlorine compounds were measured in maternal blood samples collected at 1st trimester from 475 mothers from the Rhea study in Greece. Samples were analyzed with an Agilent 7000B gas chromatograph triple quadrupole mass spectrometry (GC-MS/MS) at the National Institute for Health and Welfare, Chemical Exposure Unit, Kuopio, Finland. Pretreatment of serum samples for GC-MS/MS analysis has been described elsewhere (Bjermo et al., 2013). Serum concentrations of four individual PCB congeners (IUPAC numbers: 138, 153, 170 and 180), hexachlorobenzene (HCB), and dichlorodiphenyl dichloroethene (p',p'DDE) were analyzed and reported on whole weight as ng/ml serum. Concentration of DDT was also estimated but due to high percentage of samples below the limit of detection (60%), results are not reported. The sum of the four PCB congeners (ΣPCB) was used in our analysis.

Samples below the limit of detection (LOD) for both DR-CALUX and organochlorine compound analyses were assigned the value 0.5×LOD.

2.5 Breastfeeding

Breastfeeding during the first 18 months of life was examined to assess postnatal exposure to lipophilic pollutants. Mothers were

asked if they had ever breastfed their child in the 6th and 18th month post-partum follow-up. If the woman had ever breastfed further information on breastfeeding duration and intensity was asked (Vassilaki et al., 2012).

2.6 Anthropometric measurements and gestational age

The measurement protocol of anogenital distances has been previously described in details (Papadopoulou et al., 2013). In brief, anogenital distance (AGD) was measured from the base of the penis to the anus, anoscrotal distance (ASD) from the base of the scrotum to the anus and penis width was also measured in males. In females, anoclitoral distance (ACD) was measured from the clitoris to the anus and anofourchettal distance (AFD) from the posterior convergence of the fourchette to the anus. Weight and length were also reported. The same protocol was followed by both studies. Additionally, within the Rhea study a reliability study was conducted and reported high reliability coefficients for anogenital distance measurements: AGD=0.89, ASD=0.96 and penis width=0.75 in males and ACD=0.91 and AFD=0.91 in females (Papadopoulou et al., 2013).

Gestational age was based on the interval between the last menstrual period and the date of delivery (84.2% for the Rhea study and 96.8% for the Hmar study). The menstrual estimate of gestational age was compared to the 1st trimester ultrasound measurement. In case of inconsistencies greater than 7 days, gestational age was estimated by a quadratic regression formula (15.8% for the Rhea study and 3.2% for the Hmar study) (Westerway et al., 2000).

2.7 Potential confounders

Potential confounders included characteristics that have an established or potential association with maternal diet during pregnancy and anogenital distances of newborns and children including maternal age (years), pre-pregnancy BMI (kg/m^2),

paternal BMI (kg/m^2) and age (years), weight gain during pregnancy (kg), maternal education (primary/secondary/high-university), maternal ethnicity (Caucasian/non-Caucasian), smoking during pregnancy (yes/no), type of delivery (normal/caesarean), parity (primiparous/multiparous), gestational hypertension and/or preeclampsia (yes/no), gestational diabetes (yes/no), hospital of delivery (private/public), residence (urban/rural), breastfeeding in previous pregnancies (yes/no), alcohol intake during pregnancy (yes/no), vegetables and fruits consumption during pregnancy (times/week).

2.8 Statistical analysis

We examined summary statistics of maternal characteristics, dietary intakes and serum levels of dioxin-like activity and organochlorine compounds. Crude and adjusted linear regression models were used to estimate the association between maternal "high-fat diet" score (as continuous variable or categorized into tertiles) and genitalia distances of newborns and children. Mothers in the lower tertile of "high-fat diet" score were the reference group. Age at the time of examination was included in both crude and adjusted models of young children. The selection of confounders included in the adjusted models was based on directed acyclic graph (DAG) (Supplementary Graph) (Greenland et al., 1999). Maternal ethnicity, age and smoking status during pregnancy have been related to anogenital distances, hence were included in the adjusted models (Fowler et al., 2011;Longnecker et al., 2007;Papadopoulou et al., 2013;Suzuki et al., 2012). Study (Rhea study/Hmar study) was also included in multivariate models. Body weight has been identified as a predictor of anogenital distances but it can also be influenced by prenatal exposure to organochlorine compounds (Papadopoulou et al., 2013). Hence, we formed two adjusted models, with and without body weight, to test if the associations are driven by body weight. Similar results were obtained and the fully adjusted models were presented. Lastly, breastfeeding duration was added in adjusted models of young children. Finally, we examined

the association of each food component of the "high-fat diet" score with the genitalia measurements. Multicolinearity was not observed in our analysis, as assessed by the variance inflation factor (VIF>10). All statistical analyses were performed using STATA version 10.0 (Stata Corporation, College Station, TX).

3. Results

3.1 Maternal "high-fat-diet" score

Maternal and child characteristics of our study population are presented in Table 1. The mean "high-fat diet" score of 231 women with newborns was 5.7 (SD 2.8) and of 476 mothers with young children was 5.6 (SD 2.1). Mothers in the upper tertile of the "high-fat diet" score were most likely to be of normal BMI status, non-Caucasian, smokers and alcohol drinkers during pregnancy, compared to mothers in the lower tertile (Supplementary Table 1). Higher food scores were observed in non-Caucasian women compared to Caucasian, even when women from the Greek study were excluded (Greek study: 100% Caucasian and Spanish study: 55% Caucasian vs. 45% non-Caucasian) (data not shown). Intakes of vegetables, energy and fat were higher with increasing "high-fat diet" score (Supplementary Table 1).

Dioxin-like activity in maternal and cord blood was higher in the children born by mothers in the upper tertile of the "high-fat diet" score during pregnancy, compared to the lower and middle tertiles and were positively correlated (Spearman's rho=0.47, P <0.001and Spearman's rho=0.42, P <0.001) (Table 2). Median concentration of ΣPCB and HCB were increasing for increasing "high-fat-diet" score tertiles, while differences were not significant. A weak positive correlation was found between "high-fat-diet" score and ΣPCB and HCB in maternal blood (Spearman's rho=0.09, P =0.048 and Spearman's rho=0.10, P <0.029). Regarding the food groups included in the "high-fat diet" score, maternal and cord dioxin-like activity were positively correlated with all food groups, except high-fat dairy products. The highest coefficients were found

for red meat (Spearman's rho range=0.44, P<0.001), seafood (Spearman's rho range=0.45, P<0.001) and fatty fish (Spearman's rho range=0.46, P<0.001). Likewise, a positive correlation was found between ΣPCB and fatty-fish (Spearman's rho=0.09, P=0.043) and between HCB and red meat (Spearman's rho=0.10, P=0.029) (data not shown).

3.2 Associations between "high-fat-diet" score and anogenital distances in males and females

The maternal "high-fat diet" score was associated with a reduction of all genitalia distances measured in male newborns in a dose-response relationship, in the crude model (P for trend<0.001) (Table 3). In the adjusted model a one-point increase in maternal "high-fat diet" score was associated with 0.5mm (95% confidence interval (CI) = -0.9 to -0.2) reduction in anoscrotal distance (ASD) of newborn males and the inverse dose-response association between tertiles of maternal "high-fat diet" score remained. The reduction in ASD of males whose mothers were in the middle tertile was -1.9mm (95%CI= -3.8 to 0.1) and for those with mothers in the upper tertile was -4.3 (95%CI= -6.6 to -1.8mm), compared to mothers in the lower tertile. A negative but smaller effect of maternal "high-fat diet" score was also observed for anogenital distance (AGD) and penis width in newborn males in adjusted models.

In newborn females, there was no dose-response association between maternal "high-fat diet" score and genitalia distances. We observed an increase in the measured distances for medium maternal "high-fat diet" scores compared to the lower tertile of the score.

Further, we examined the adjusted association between maternal weekly intake of each of the food groups included in the "high-fat diet" score and anogenital distances. High intake of red meat, fatty fish and eggs was associated with a reduction in ASD of newborn males (Figure 1).

In young males and females, measured at 1 to 2 years, we found no association between maternal "high-fat diet" score and anogenital distances (Supplementary Table 2). Adding breastfeeding duration in the adjusted models provided similar null associations.

4. Discussion

In this study we found that increasing maternal "high-fat diet" score was associated with a reduction in anoscrotal distance of newborn males. Additionally, maternal "high-fat diet" score was positively related to maternal and cord dioxin-like activity and serum PCBs and HCB concentrations in maternal blood.

Maternal "high-fat diet" score was positively related to dioxin-like activity in cord blood indicating that the score captures adequately fetal exposure to dioxins and dioxin-like compounds. As recently published by our research group in the same population, maternal dioxin-like activity was associated with a reduction in anogenital distance of newborn males, even in low-levels of exposure (Vafeiadi et al., 2012). The toxicity of dioxins and dioxin-like compounds is mostly traced to their blocking of the aryl hydrocarbon receptor (AhR) that modulates the function of the estrogen and androgen receptors (Denison et al., 2011). In animal models suppression of the androgen receptor during gestation results in changes in the reproductive system including lower testosterone production and reduced anogenital distance (Hotchkiss et al., 2004). Hence, during pregnancy, environmental contaminants may disrupt or mimic the normal activity of androgens, which stimulate the growth of the perineal region, and result to a phenotype of a shorter anogenital distance of the male offspring (Bowman et al., 2003). In our study maternal dioxin-like activity was positively correlated with the "high-fat diet" score, while other studies in pregnant women have reported controversial results (Halldorsson et al., 2009;Pedersen et al., 2012). The correlation was mainly driven by high consumptions of red meat, seafood and fatty-fish. The "high-fat diet" score was also positively related with

maternal serum concentrations of HCB and non-dioxin-like PCBs (sum of PCB-138, 153, 170 and 180), mainly due to red meat and fatty fish consumption during pregnancy, respectively. Our findings are in line with other reports on the major contribution of fish and seafood consumption during pregnancy to body burden of dioxins and PCB, in low-exposed pregnant women (Chevrier et al., 2013;Halldorsson et al., 2008;Huang et al., 2007;Ibarluzea et al., 2011;Llop et al., 2010). Only two studies have found an association between red meat and dairy products consumption during pregnancy with levels of dioxins in maternal blood (Halldorsson et al., 2009;Huang et al., 2007).

We found that maternal "high-fat diet" score in the upper tertile was associated with a 15% reduction in anoscrotal distance (ASD) of newborn males. High consumption of red meat, fatty fish and eggs were the foods negatively linked to ASD in newborn males. No previous report exists on the association between maternal diet and anogenital distance in children. Nevertheless, high maternal fish and shellfish intake has been linked to higher risk for hypospadias, mediated by high prenatal exposure to HCB, while researchers did not distinguished between fatty fish, lean fish or shellfish and their link to maternal HCB levels (Giordano et al., 2010;Giordano et al., 2008). Another study reported that a dietary pattern of high meat, poultry and offals intake during pregnancy was linked to increased risk for hypospadias (de Kort et al., 2011). Hsieh et al., found that anogenital distance of young boys with hypospadias is shorter than healthy boys, hence the results of the studies on maternal diet and risk for hypospadias might be relevant to our results (Hsieh et al., 2012).

In adult men, high intakes of red and processed meat, has been related to poor semen quality, while researchers has been focused on the positive effects of antioxidants intake on semen parameters (Gaskins et al., 2012;Mendiola et al., 2009;Mendiola et al., 2010;Minguez-Alarcon et al., 2012). Eisenberg et al, through a series of studies, suggested that neonatal anogenital distance can be a novel metric to predict adult testicular function (Eisenberg et al.,

2012a). Consequently, impaired adult reproductive health might be related prenatal exposure to organochlorine compounds, through maternal diet.

High fat intake has been linked to poor semen quality in adult men (Mendiola et al., 2010). In our study, women with high food scores had high intakes of fat which might mediate the observed reduction in ASD. However, red meat and fatty fish were the main contributors of maternal and cord blood levels of toxic contaminants and were also related with a reduction in ASD. This strengthens our hypothesis towards the link of prenatal exposure to organochlorine compounds and the observed reduction in ASD.

Prenatal exposures to endocrine disruptive chemicals can be linked to small size at birth. Body size is positively related to genitalia distances, thus a reduction in anogenital distance might be mediated by small size at birth (Papadopoulou et al., 2013). According to the DAG approach, the association between prenatal exposure to contaminants and anogenital distance should not be adjusted for birth weight and the total effect, and not direct or indirect effects, would be estimated (Greenland et al., 1999). A suggested solution is to include in the regression model, body weight along with all maternal lifestyle factors that are confounding the association of body weight with anogenital distance. Since the estimated effects were not modified after adjusting for weight, then we can argue that the observed negative associations are not mediated by a reduction in weight. However, maternal factors that can influence anogenital distances have not been thoroughly studied and the relationship between fetal growth and genital development still remains unclear. The limitations of our observational study are acknowledged and our findings are interpreted as associations and not as causations.

In our study, maternal “high-fat diet” score was associated with an increase in anofourchetal distance (AFD) of newborn females, while due to lack of monotonicity we consider our findings weak. In animal models high prenatal exposure to testosterone was related to increase of female offspring genitalia distance, suggesting

masculinization of female rodents (Hotchkiss et al., 2007). Recently an association was reported between prenatal stress and longer anogenital distance in female newborns, suggesting masculinization of female reproductive development (Barrett et al., 2013). The link between neonatal anogenital distance and women reproductive health is unknown, while there is one recent report suggesting that increased prenatal androgen exposure can be linked to longer anogenital distance in adult women (Mendiola et al., 2012). 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 children measured at 1 to 2 years of life we observed no effect of maternal high-fat diet during pregnancy on genitalia measurements. In line with our findings, no association was found in the same population between maternal dioxin-like activity, assessed by the DR-CALUX bioassay, and anogenital distances of young children (Vafeiadi et al., 2012). 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. However, in humans the extent to which the postnatal exposures can affect anogenital distances measured in early childhood is unknown, while postnatal growth might mask any prenatal or postnatal effect on genitalia. In our study, exposure misclassification is high during the postnatal period, given that breastfeeding was short for most children and that there was no information concerning child´s diet during the first 2 years of life, which might contribute to total postnatal exposure.

Higher prevalence of non-Caucasian ethnicity, smoking and alcohol drinking during pregnancy was observed for women with higher food scores. Hence the relationship between high-fat-diet score and anogenital distances could be driven by unidentified maternal factors of an unhealthy lifestyle. Nevertheless, after adjusting for maternal characteristics our findings were similar, though weaker. Furthermore, it has to be acknowledged that maternal body burden of organochlorine compounds during

gestation might not reflect a long-term exposure history, due to the physiological changes occurring in pregnancy that can influence the disposition of chemicals (James et al., 2002;Wang et al., 2009). In addition, exposures of women in early life are important body burden determinants and reduction of maternal body burden long before pregnancy can lead to lower prenatal exposures (Glynn et al., 2007;Huisman et al., 1995).

5. Conclusions

In conclusion, we found that high maternal "high-fat diet" score is associated with a reduction of ASD in newborn males. Our results suggest that prenatal exposure to persistent organic pollutants, through maternal diet, may have an endocrine disruptive effect, expressed as phenotypic alterations of the reproductive system.

Acknowledgements

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Table 1. Characteristics of 707 mother-child pairs by age group.

	Newborns (n=231)		Children (n=476)	
	N	(%)	N	(%)
Rhea study, Crete, Greece	128	(55.4)	476	(100.0)
Hmar study, Barcelona, Spain	103	(44.6)	0	(0.0)
Maternal age (years) [a]	29.9	(5.2)	30.1	(4.4)
Pre-pregnancy BMI (kg/m^2) [a]	23.1	(4.9)	23.3	(5.0)
Maternal education				
Primary	55	(23.8)	76	(16.0)
Secondary	105	(45.5)	238	(50.0)
Higher/University	71	(30.7)	162	(34.0)
Smoking during pregnancy				
No	155	(67.1)	379	(79.6)
Yes	76	(32.9)	97	(20.4)
Parity (no. of previous pregnancies)				
0	110	(47.6)	189	(39.7)
≥1	121	(52.4)	287	(60.3)
Maternal ethnicity				
Non-Caucasian	57	(24.7)	0	(0.0)
Caucasian	174	(75.3)	476	(100.0)
Alcohol consumption during pregnancy				
No	178	(77.1)	321	(67.4)
Yes	53	(22.9)	155	(32.6)
Breastfeeding				
Never			67	(14.1)
Ever			409	(85.9)
Maternal "high-fat diet" score tertiles				
Lower	61	0 to 3	138	0 to 4
Middle	83	4 to 6	169	5 to 6
Upper	87	7 to 11	169	7 to 11
Child characteristics				
		Mean (SD)		Mean (SD)
Age at examination		Birth		15.0 (10.0)
Gestational age (weeks) [a]		39 (2)		38 (1)
Weight (kg)		3.2 (0.4)		10.9 (2.1)
Length (cm)		50.0 (2.0)		81.0 (7.9)
Anogenital distances				
Males	119		248	
AGD (mm)		49.0 (5.0)		80.3 (7.8)
ASD (mm)		25.4 (4.8)		39.5 (7.3)
Penis width (mm)		10.7 (1.1)		13.8 (1.7)

Females	112			228		
ACD (mm)		35.2	(3.2)		49.4	(5.9)
AFD (mm)		14.2	(2.9)		21.7	(3.9)

[a] Values presented are median (IQR)

Table 2. Maternal and cord dioxin-like activity and concentration of other compounds in maternal blood, in all mothers and by "high-fat diet" score tertiles.

				Maternal "high-fat diet" score tertiles						
				Lower (0 to 3)		Middle (4 to 6)		Upper (7 to 11)		
	N	Median (IQR)		Median (IQR)		Median (IQR)		Median (IQR)		P-value
Dioxin-like activity in maternal blood (in pg CALUX TEQ/ml plasma) [a]	166	0.29	(0.34)	0.05	(0.20)	0.29	(0.28)	0.34	(0.20)	<0.001
Dioxin-like activity in cord blood (in pg CALUX TEQ/ml plasma) [b]	148	0.11	(0.13)	0.05	(0.00)	0.05	(0.07)	0.15	(0.16)	<0.001
ΣPCBs (in ng/ml serum) [c]	475	306.5	(247.1)	271.0	(261.3)	308.4	(252.4)	318.8	(229.9)	0.201
HCB (in ng/ml serum) [c]	475	83.5	(58.0)	80.6	(41.6)	83.8	(63.1)	90.3	(64.3)	0.162
DDE (in ng/ml serum) [c]	475	1979.7	(2279.7)	2013.0	(1933.6)	1893.5	(2553.3)	2010.7	(1337.9)	0.943

ΣPCBs: sum of PCBs calculated by summing the concentrations of the individual congeners 153,138,180,170

[a] n=166, n=30 in lower tertile ; n=47 in middle tertile ; n=89 in upper tertile.

[b] n=148, n=27 in lower tertile ; n=37 in middle tertile ; n=84 in upper tertile.

[c] n=475, n=152 in lower tertile ; n=179 in middle tertile ; n=144 in upper tertile. Only women from the Rhea study in Greece.

Table 3. Adjusted association between maternal "high-fat diet" score during pregnancy and anogenital distances [a] of male and female newborns, in the Rhea and Hmar studies.

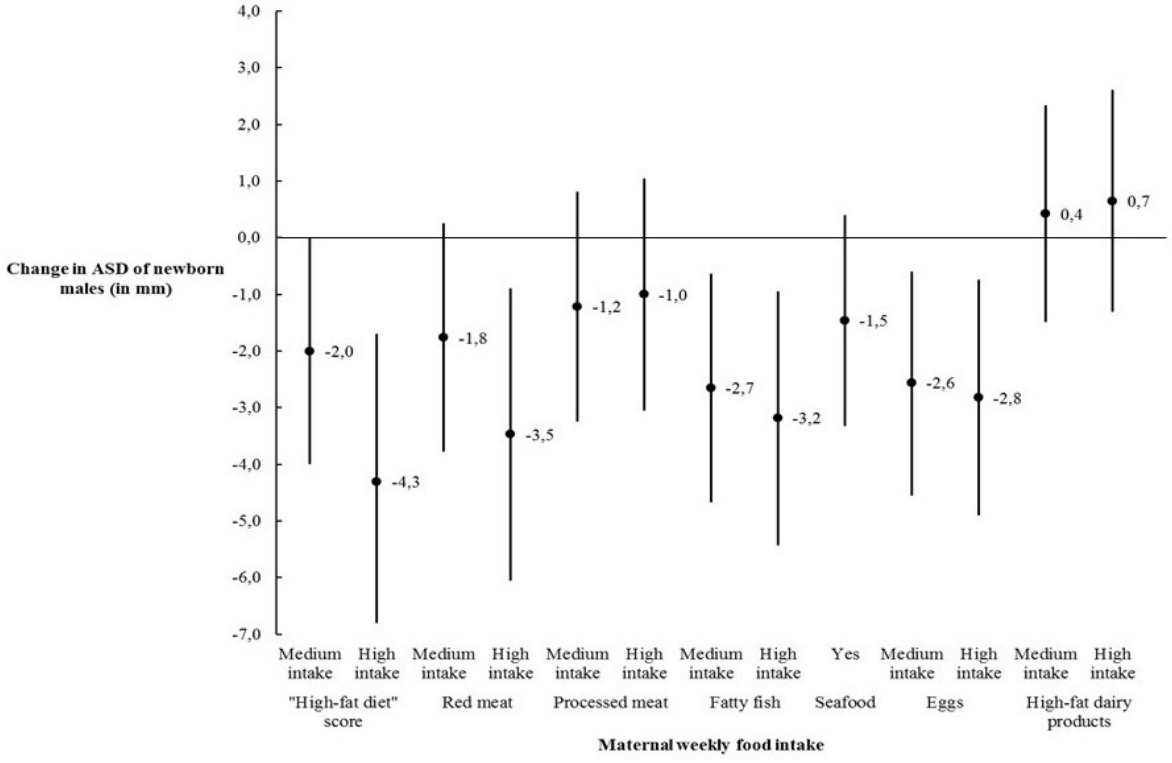
	Newborn males (n=119)						Newborn females (n=112)			
	N	AGD(mm)(95% CI)		ASD(mm)(95% CI)		PW(mm)(95% CI)	N	ACD(mm)(95% CI)		AFD(mm)(95% CI)	
Maternal "high-fat diet" score											
Crude model											
Continuous		-0.7	(-1.0 to -0.4)	-0.8	(-1.0 to -0.5)	-0.1 (-0.2 to -0.1)		-0.1	(-0.3 to 0.1)	0.0	(-0.2 to 0.1)
Tertiles											
Lower [b]	31	0.0		0.0		0.0	30	0.0		0.0	
Middle	44	-2.2	(-4.2 to -0.1)	-2.4	(-4.3 to -0.5)	-0.4 (-0.9 to 0.2)	39	1.1	(-0.4 to 2.6)	1.5	(0.2 to 2.9)
Upper	44	-4.3	(-6.3 to -2.2)	-5.4	(-7.3 to -3.5)	-1.0 (-1.4 to -0.5)	43	-0.3	(-1.8 to 1.2)	0.3	(-1.1 to 1.6)
Adjusted model [c]											
Continuous		-0.2	(-0.5 to 0.2)	-0.5	(-0.9 to -0.2)	0.0 (-0.1 to 0.1)		0.0	(-0.3 to 0.2)	0.2	(-0.1 to 0.4)
Tertiles											
Lower [b]	31	0.0		0.0		0.0	30	0.0		0.0	
Middle	44	-1.2	(-3.1 to 0.7)	-1.9	(-3.8 to 0.1)	-0.2 (-0.6 to 0.2)	39	1.4	(0.1 to 2.7)	2.0	(0.6 to 3.4)
Upper	44	-1.0	(-3.3 to 1.4)	-4.2	(-6.6 to -1.8)	-0.2 (-0.8 to 0.3)	43	0.2	(-1.5 to 1.9)	1.5	(-0.2 to 3.3)

[a] AGD: anogenital distance, ASD: anoscrotal distance, PW: penis width, ACD: anoclitoral distance, AFD: anofourchetal distance.

[b] Reference category.

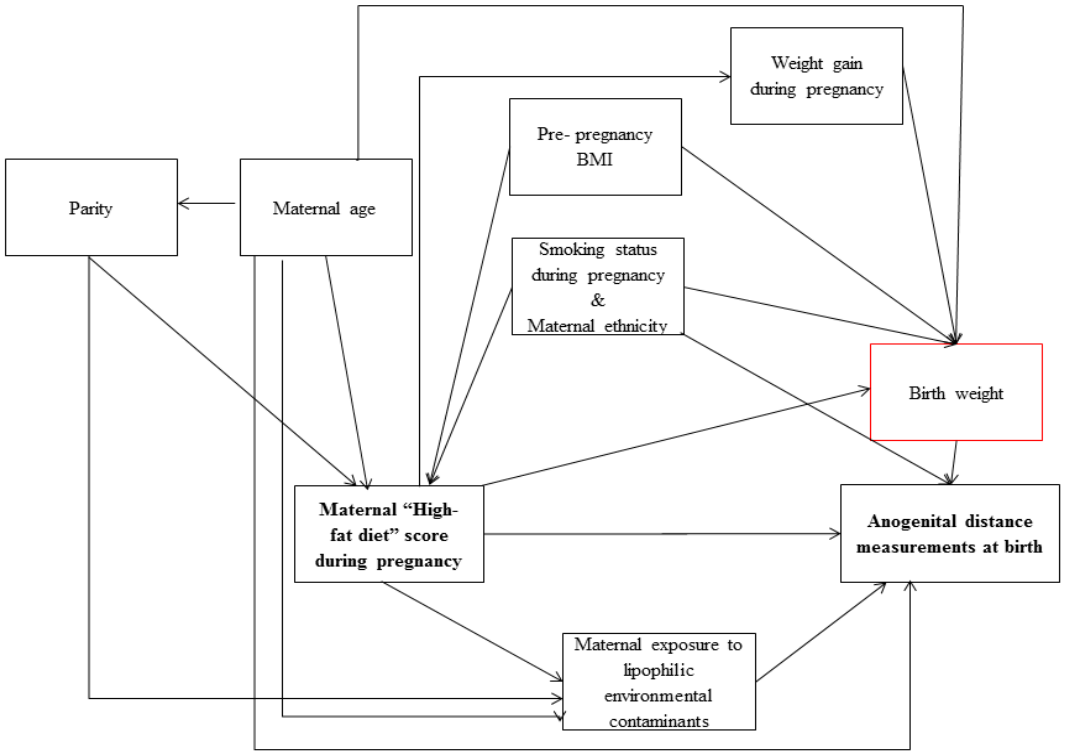
[c] Models are adjusted for study, maternal ethnicity, maternal age, smoking status during pregnancy and birth weight

Figure 1. Adjusted change in anoscrotal distance (ASD) of newborn males associated with medium and high maternal weekly intake of red meat, processed meat, seafood, fatty fish, eggs and high fat dairy products. Low weekly intake of each food group is the reference group.



Supplementary material

Supplementary Figure 1. Directed Acyclic Graph for the association between maternal "high-fat diet" and anogenital distances of newborns.



Supplementary Table 1. Maternal characteristics by "high-fat diet" score tertiles, in the Rhea and Hmar studies [a].

	Maternal "high-fat-diet" score tertiles						
	Lower		Middle		Upper		
	N	(%)	N	(%)	N	(%)	P-value
Maternal age (years)							
<25	38	(22.8)	31	(14.2)	46	(19.4)	0.072
25-30	45	(27.0)	53	(24.2)	50	(21.1)	
30-35	66	(39.4)	92	(42.0)	93	(39.2)	
>35	18	(10.8)	43	(19.6)	48	(20.3)	
Pre-pregnancy BMI							
Normal (≤25 kg/m2)	98	(58.7)	150	(68.5)	176	(74.3)	0.004
Overweight/Obese (>25 kg/m2)	69	(41.3)	69	(31.5)	61	(25.7)	
Maternal ethnicity							
Non-Caucasian	0	(0.0)	11	(5.0)	46	(19.4)	<0.001
Caucasian	167	(100. 0)	208	(95.0)	191	(80.6)	
Maternal education							
Primary	35	(21.0)	35	(16.0)	45	(19.0)	0.092
Secondary	90	(53.9)	107	(48.9)	104	(43.9)	
Higher/University	42	(25.1)	77	(35.1)	88	(37.1)	
Smoking during pregnancy							
No	134	(80.2)	166	(75.8)	161	(67.9)	0.016
Yes	33	(19.8)	53	(24.2)	76	(32.1)	
Parity (no. of pregnancies)							
0	69	(41.3)	84	(38.4)	112	(47.3)	0.147
≥1	98	(58.7)	135	(61.6)	125	(52.7)	
Alcohol consumption during pregnancy							
No	132	(79.0)	157	(71.7)	142	(59.9)	<0.001
Yes	35	(21.0)	62	(28.3)	95	(40.1)	
Breastfeeding							
Never	24	(16.8)	29	(16.3)	14	(8.9)	0.080
Ever	119	(83.2)	149	(83.7)	143	(91.1	

Dietary intake during pregnancy

(times/week)

	Median	(IQR)	Median	(IQR)	Median	(IQR)	P-value
Red meat	0.5	(1.2)	1.2	(2.4)	3.0	(2.4)	<0.001
Processed meat	0.3	(1.2)	2.0	(2.5)	3.0	(3.6)	<0.001
Seafood	0.0	(0.0)	0.0	(0.5)	0.5	(1.0)	<0.001
Fatty fish	0.2	(0.5)	0.5	(0.8)	1.0	(1.5)	<0.001
Eggs	0.7	(1.2)	1.2	(2.0)	2.1	(2.0)	<0.001
High fat dairy products	7.8	(7.4)	12.0	(10.4)	17.0	(12.1)	<0.001
Vegetables	6.4	(4.3)	8.0	(4.6)	9.0	(8.0)	<0.001
Fruits	17.0	(16.5)	18.0	(15.1)	18.5	(15.9)	0.264
Energy (kcals/day) [b]	1,583	(899)	1,788	(763)	2,360	(1,042)	<0.001
Fat (g/day) [b]	79.8	(51.1)	92.0	(44.2)	114.9	(49.9)	<0.001

[a] n=623 (n=167 in lower tertile ; n=219 in middle tertile ; n=237 in upper tertile). Number is smaller than the total sample of mother-child pairs (n=707) because 84 children were measured at both time points, birth and 1st year.

[b] n=487 (n=107 in lower tertile ; n=160 in middle tertile ; n=220 in upper tertile).

Supplementary Table 2. Adjusted association between maternal "high-fat diet" score during pregnancy and anogenital distances [a] of male and female children, in the Rhea study.

	Males (n=248)						Females (n=228)			
	N	AGD(mm)(95% CI)		ASD(mm)(95% CI)		PW(mm)(95% CI)	N	ACD(mm)(95% CI)		AFD(mm)(95% CI)	
Maternal "high-fat diet" score											
Crude model											
Continuous		-0.1	(-0.6 to 0.4)	0.2	(-0.3 to 0.6)	-0.1 (-0.2 to 0.0)		-0.1	(-0.5 to 0.2)	0.2	(-0.1 to 0.4)
Tertiles											
Lower [b]	76	0.0		0.0		0.0	62	0.0		0.0	
Middle	84	1.0	(-1.4 to 3.4)	1.6	(-0.5 to 3.8)	0.0 (-0.5 to 0.5)	85	-1.1	(-2.9 to 0.7)	-0.3	(-1.5 to 0.9)
Upper	88	-0.5	(-2.9 to 1.9)	1.0	(-1.2 to 3.2)	-0.3 (-0.8 to 0.2)	81	-0.5	(-2.4 to 1.4)	0.6	(-0.7 to 1.8)
Adjusted model [c]											
Continuous		0.0	(-0.4 to 0.5)	0.2	(-0.2 to 0.6)	-0.1 (-0.2 to 0.0)		-0.1	(-0.4 to 0.3)	0.2	(0.0 to 0.4)
Tertiles											
Lower [b]	76	0.0		0.0		0.0	62	0.0		0.0	
Middle	84	0.5	(-1.6 to 2.7)	1.4	(-0.7 to 3.6)	0.0 (-0.5 to 0.5)	85	-1.0	(-2.8 to 0.8)	-0.1	(-1.3 to 1.0)
Upper	88	-0.1	(-2.3 to 2.1)	1.0	(-1.2 to 3.2)	-0.3 (-0.8 to 0.2)	81	-0.3	(-2.1 to 1.4)	0.7	(-0.5 to 1.9)

[a] AGD: anogenital distance, ASD: anoscrotal distance, PW: penis width, ACD: anoclitoral distance, AFD: anofourchetal distance.

[b] Reference category.

[c] Models are adjusted for maternal age, smoking status during pregnancy, child's birth weight, weight and age at the time of examination.

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6 GENERAL DISCUSSION

This thesis presents a compilation of epidemiological evidence for the effect of prenatal exposure to POPs through maternal diet, on fetal growth and anogenital distance, using estimates of overall maternal diet and biomarkers of prenatal exposure to these contaminants. This section is meant to be a global discussion and provides a broader and more integrated interpretation of the entire study project, avoiding repeating what has been already discussed in each of the manuscripts included in this doctoral thesis.

The main findings of the thesis are:

- Maternal diet is associated with levels of dioxins and other POPs in the mother and the fetus.
- Prenatal exposure to dietary dioxins and other POPs, estimated through overall maternal diet and exposure biomarkers, may reduce birth weight.
- Prenatal exposure to POPs, through maternal diet, might induce endocrine disruptive effects measured as a reduction in anogenital distance of boys.
- Anogenital distances of boys and girls are associated with body size, can track through life and are highly reliable anthropometric measurements.

6.1 The role of diet on prenatal exposure to POPs and related health effects

6.1.1 Overall maternal diet and prenatal exposure to POPs

We assessed prenatal exposure to dietary lipophilic contaminants by three different approaches: 1) by defining dietary patterns, which were based on maternal and cord blood biomarkers of exposure to dioxins and dioxin-like compounds, 2) by creating an *a priori* food score, which included established food sources of exposure and was

positively linked to maternal and cord blood levels of POPs and 3) by estimating daily dietary dioxins and PCBs intake from maternal diet. All three approaches aimed to study overall maternal diet, rather than consumption of single food groups, and were based on levels of the contaminants either in maternal and cord blood or in the food.

Our study is the first to report an association between contaminant-based dietary patterns of pregnant women, derived by reduced rank regression (RRR), and fetal growth. RRR requires the definition of response variables which are based on the scientific knowledge of the disease physiology. Since biomarkers are reflecting the body burden of environmental toxicants that can produce adverse health outcomes, dietary patterns based on biomarkers can be more relevant for the effect of diet on disease [153,154]. RRR has been used to identify dietary patterns in children which were further linked to obesity indices [136,155,156] and dietary patterns in adults which were linked to risk for mortality, CHD, type 2 diabetes, breast cancer and Alzheimer's disease [157-162]. There is only one report on biomarker-based dietary patterns in pregnant women and risk for spina bifida [138]. The RRR has never been used to study prenatal exposure to dietary environmental contaminants. We suggest that RRR is a more suitable method to study the effect of overall diet on human body burden and related health effects.

Moreover, *a priori* food scores are commonly used in epidemiological studies, as a combination of intake of foods related to a health outcome. There is one study using an *a priori* food score to assess prenatal exposure to dietary acrylamide and reported a positive association between the food score and the blood biomarker [130]. Our predefined food score was also positively related to levels of dioxins and dioxin-like compounds in maternal and cord blood. However, there is no other study using this method to assess dietary exposure to environmental contaminants. This methodology might be rather crude and might serve as a proxy marker for other factors that can be related to the observed health effect.

A way to assess the capacity of a food score to capture exposures to environmental contaminants is to investigate its correlation with the corresponding biomarker. Strong associations with biomarkers can consolidate the use of a food score as an estimate of dietary exposure to environmental contaminants.

Although dietary estimation of dioxins and PCBs is an established methodology for monitoring human exposure, our report is the first to use such an approach to study prenatal exposures to dietary dioxins and PCBs. Estimated dietary dioxins and PCBs intake have been previously linked to blood levels of contaminants and have been also used to predict blood levels [40,148,163]. Hence this methodology can be valid as a dietary exposure estimate.

6.1.2 Maternal diet and health outcomes: beneficial nutrients vs. environmental contaminants

As noted in the introduction, foods consumed during pregnancy are a source of both beneficial and harmful compounds for the developing fetus. There is extensive discussion between scientists on this controversy, especially regarding seafood (fish and shellfish) intake during pregnancy and the benefit-risk balancing.

Seafood consumption is an example of simultaneous exposure to toxic contaminants, such as dioxins, PCBs and mercury and beneficial nutrients, such as omega-3 fatty acids [164]. The overlapping nutrients and non-nutrients can result to confounding of the adverse effects of toxic contaminants by the competing nutrients' benefits. Vice versa, the beneficial effects of essential nutrients can be confounded by the competing adverse effects of the toxicants (Figure 8). This type of confounding where two variables are affecting the outcome in opposite directions has been described as negative confounding [165].

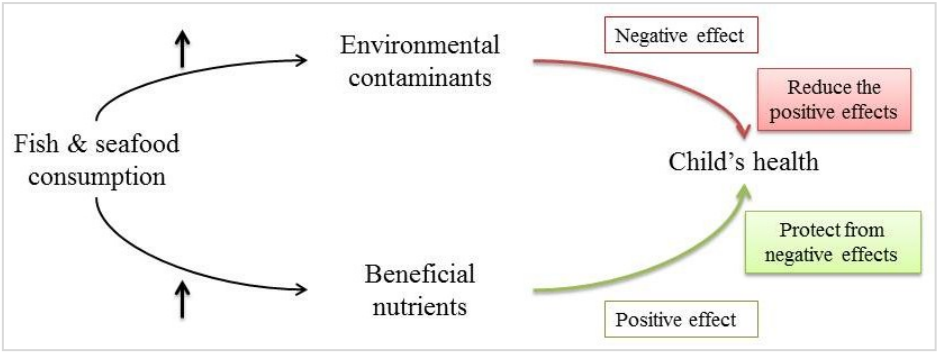

Figure 8. Competing effects of beneficial nutrients and environmental toxicants on child's health.

Under the effect of negative confounding, the potential negative association between environmental contaminants in seafood and child's health is under-estimated, because there is an additional negative effect that is masked by the beneficial effect of the omega-3 in seafood. A similar under-estimation of the beneficial effects of omega-3 occurs due to the competing effects with environmental contaminants in seafood [164].

A suggested solution is to use sensitive markers of intake of either beneficial nutrients or environmental toxicants. Measured concentrations of nutrients or environmental contaminants in the blood or urine can be used to derive "naked" un-confounded risk or benefit estimates. Another alternative would be to estimate the intake of environmental contaminants from accurate survey data [164]. In this thesis, we have estimated prenatal exposure to organochlorine compounds, by combining dietary information with biomarkers of exposure or with concentrations of dioxins and PCBs in food.

Sensitive health end-points that would respond to an increase in the prenatal exposure to contaminants and not in an increase of the prenatal exposure to beneficial nutrients can also assist to obtain unbiased risk estimates. In this thesis, anogenital distance measurements were sensitive to high prenatal exposures to dioxins and other POPs, through maternal diet.

Animal and human studies have used anogenital distance as a marker of fetal androgen disruption, which has been linked to genital defects in young and adult males [114]. Hence, anogenital distance might be a novel risk estimate of prenatal exposure to endocrine disruptive chemicals through maternal diet.

6.2 Contribution and main strengths of this thesis

We investigated the extent to which maternal overall diet can contribute to prenatal exposure to organochlorine compounds. We found that overall maternal diet can influence prenatal exposure to environmental contaminants and may lead to adverse health impacts. It is established that seafood intake is the main source of exposure to organochlorine compounds. However, overall diet approaches revealed that meat combined with fish consumption was contributing to maternal body burden of dioxins and other POPs and was associated with impaired fetal growth and reduction in anogenital distance. Hence, in populations of low-background exposure, where consumption of single food groups might not be related to health outcomes, combined food consumption might be more relevant to total body burden and adverse health effects.

Strengths

First, all observed associations derived from prospective mother-child cohort studies. The prospective design, allows the evaluation of longitudinal associations between prenatal exposure to dietary contaminants and early health outcomes. Within the NewGeneris project we studied pregnant women from 5 European countries. Both the Cretan Rhea study and the Norwegian MoBa study have large sample size relative to the population of each area and included participants from both urban and rural regions representing all age groups and all socioeconomic groups. Additionally, each cohort study had collected extensive information on several social, lifestyle and environmental factors and their effect as covariates was assessed within this thesis.

Second, all FFQs used in this thesis to assess maternal dietary habits, were extensive and validated for use in pregnant women, providing a comprehensive estimation of food consumption during pregnancy. The large study samples and the population-based designs provided great variability in maternal dietary habits. The regional design and application of each FFQ gave us the opportunity to study rare food items and country-specific dietary habits, such as the seagull eggs in Norway or the many different fish species throughout Europe. Moreover, within the MoBa study, we were able to calculate dietary intakes of dioxins and PCBs from the whole diet by using concentrations of contaminants in foods commonly consumed by the Norwegian population. Additionally, for the whole study we were able to calculate micronutrients intake, such as total fat. This allowed us to investigate the association between fat intake, as the main intake pathway of organochlorine compounds and biomarkers of exposure.

Third, the use of biomarkers of exposure and their positive relationship with our dietary estimates strengthen their validity as estimates of prenatal exposure. The derived dioxin-rich diets, either as dioxin-dietary patterns or as food scores were based on maternal and cord dioxin-like activity, measured by the DR-CALUX bioassay. This bioassay is sensitive to the binding of dioxins and dioxin-like compounds to the AhR. Hence, the observed negative associations between prenatal dietary exposures and health outcomes were mediated by prenatal exposure to dioxins and dioxin-like compounds and their binding affinity to the AhR. In addition, in some analyses we were also able to incorporate information on exposure to other POPs.

Finally, we have used birth outcomes information derived by medical records that are accurate regarding basic birth outcome data, such as birth weight and gestational age. Medical reports and registries are less prone to reporting errors. A further strength of our study was the measurement of anogenital distance, which has been suggested to be a sensitive marker of fetal endocrine disruption.

All the examiners, including myself, conducting the measurements in the Rhea study were trained under the same measurements protocol. In an absence of a "golden standard" for the measurement of anogenital distance, we were able to assess its reliability as an anthropometric measure. Two examiners, including myself, conducted a reliability study and under a novel statistical method we provided information on between- and within-examiners variation for boys and girls as well as repeatability coefficients.

6.3 Limitations

The main limitation of our study is that dietary data were collected by FFQs. All dietary assessment tools, including FFQs, have uncertainties and errors, such as misreporting and recall bias. FFQs are designed to assess the ranking of intakes within a population and they might not be relied on to produce reliable estimates of absolute intake. However, further formulation of the FFQ-derived dietary intakes into biomarker-based dietary estimates of exposure might reduce the uncertainty in our study. Additionally, it has been suggested that FFQs can be a valid tool for ranking populations according to their intake and dietary exposure to environmental contaminants [129]. However, the risk of over- or under estimation remains.

Within the NewGeneris project, each study designed and applied a different FFQ. Although the main covered areas were the same, the reported food items varied by study, leading to variation in the derived data and the dietary exposure estimates. However, this limitation is counterbalanced by the large variation in overall and country-specific maternal dietary intake, precisely due to the international characteristics of the study.

Additionally, in the MoBa study dietary intakes of dioxins and PCBs were calculated by using a database of contaminant concentrations in food. This might provide a biased exposure estimate since concentrations of nutrients and toxicants can vary

between food items. An alternative could be a duplicate diet study, where participants are asked to provide a portion of their daily consumed food in order to measure contaminant concentrations [166]. However, in a nation-wide study such as MoBa with more than 90,000 participants this was impossible.

Finally, we acknowledge that dietary estimates provide short-term exposure estimates, while maternal body burden is a result of cumulative environmental exposure to POPs. Hence we are able to capture only a part of the whole picture that links maternal lifestyle with environmental exposures and child health. Especially for persistent pollutants, as dioxins and PCBs, the mother's exposure long before she becomes pregnant can contribute to total body burden of contaminants and we were not able to capture such long-term exposures [73].

6.4 Endocrine disruptive effects of prenatal exposure to environmental contaminants through maternal diet: evidence for causality

Fetal development is influenced by the intrauterine environment, which is determined by several factors, including maternal lifestyle, nutrient supply and exposure to environmental chemicals. We provide evidence that a dioxin-rich diet might be linked to impaired fetal growth and anti-androgenic effects. The evidence provided by this study derived from observational studies, and our results were interpreted as associations and not as causal effects.

In epidemiological studies of prenatal exposures and adverse health effects there is a concern that the observed effect is related to the exposure of interest and not confounded by correlated factors. Hill's criteria can be applied to evaluate the level of causality of associations observed in epidemiology and include: consistency, strength of association, dose-response relationship, time order, specificity, consistency on replication, predictive performance, biological plausibility and coherence [167].

Observational epidemiologic studies can be cross-sectional, case-control or cohort studies and each study type contributes differently to epidemiological evidence for a specific causal relationship. In prospective cohort studies, participants are followed over time and exposures are assessed prior to the incidence of the health outcome. Evidence derived from prospective cohort studies have more weight on assessing causal relationships, than case-control and cross-sectional studies. Additionally, the validity of the exposure assessment method is a major challenge in studies of environmental toxicants and human health effects [168]. Adequate justification is also needed for the health outcome under study towards its use as an adverse health event.

Epidemiological studies, as the ones included in this thesis, can demonstrate statistically significant associations between contaminant exposure and health outcomes, but it should be noted that statistical significance does not imply a causal relationship. In the same way, absence of a statistically significant association does not prove the absence of a potential relationship.

In this thesis we have included large samples of European prospective mother-child studies, with extensive information on lifestyle and environmental factors. We have explored the effect of overall maternal diet using three different approaches, based on maternal and cord serum levels of contaminants or concentrations of contaminants in food. We have used birth outcomes collected from medical registries and anogenital distance. The consistency of our report on the negative association between prenatal exposure to dioxins and PCBs, through maternal diet, with fetal growth and anogenital distance can add further validity on our epidemiological evidence.

6.5 Public health implications

The public health implications of the findings presented in this thesis are substantial. This thesis provides evidence on the negative association of dioxins and POPs through maternal diet on fetal growth and anogenital distance. As mentioned in the introduction, reduced birth weight can be linked to increase susceptibility to diseases developed either in childhood or in adulthood. Likewise, a reduction in anogenital distance might be linked to impaired testicular function and men's reproductive health. Thus, our findings have implications that can affect earlier as well as later human health.

Women included in this thesis had low levels of exposure during pregnancy, as assessed by the blood biomarkers of dioxins and other POPs and still a dioxin-rich diet was negatively related to fetal growth and anogenital distance. Among all the factors that can influence maternal body burden of dioxins and other POPs, diet is the main modifiable factor. Hence, regulations on food consumption focused on women of reproductive age might be an effective intervention to reduce adverse health effects related to environmental toxicants. A reduction of dietary intake of dioxins and PCBs might lead to an overall reduction in body burden of environmental contaminants because they might share common dietary source of exposure, such as mercury and organochlorine pesticides.

Within the NewGeneris project we found that country-specific dietary habits can be related to maternal body burden of dioxins and dioxin-like compounds. Hence dietary advice for women of reproductive age should also incorporate knowledge on local exposure patterns through diet.

6.6 Future perspectives

This thesis presents epidemiological studies for the relationship of overall maternal diet, as a source of prenatal exposure to POPs, and fetal growth. The essential contribution of large epidemiological longitudinal studies on the relationship between fetal life and chronic diseases has been acknowledged. Future high quality epidemiological studies should be supported, such as mother-child prospective cohorts with large samples, biomarkers of exposure and extensive lifestyle, socio-demographic and environmental information. Dietary assessment is often incorporated, but overall maternal diet is not always well reported or studied and this could be an opportunity for future steps. Additionally, multicenter and multi-country studies are an excellent opportunity to investigate more thoroughly exposure-health outcome relationships in large populations and evaluate replication of findings in different settings.

Background exposure to environmental toxicants is constant and it will be impossible to virtually eliminate human exposure. More research is needed to define the role of diet in reducing prenatal exposure to toxicants that disrupt prenatal development and function. First, exposure biomarkers can play an important role to investigate prenatal exposure-outcome associations. However, in large epidemiological studies is not always a cost-effective assessment tool. In the studies presented in this thesis, questionnaire data were combined with biomarkers to get a biomarker-based dietary estimate of contaminant exposure. This might be an efficient method to assess prenatal exposure to dietary contaminants while more studies are needed to confirm our results. Second, innovative tools for dietary assessment are being developed and validated which are based on computers or smart phones [169]. Such techniques might be more cost-effective and less burdensome for dietary assessment in large populations, while the quality of data provided is still under discussion.

Future suggestions for epidemiological studies include the use of anogenital distance as a sensitive marker of endocrine disruption. Until now, prenatal exposures to anti-androgens have been linked to reduced anogenital distance in boys, while the effects in girls are unclear. Anogenital distance is a highly reliable anthropometric measure. We suggest the incorporation of anogenital distance in the measurements protocol of newborns in epidemiological studies. Additionally, other sensitive end-points are needed to assess early health effects that can predict later disease. An example of a sensitive marker might be the skinfold thickness measurements, which are used to assess body composition and to estimate body fat, but there are scarce reports for newborns and young children. Given the increasing evidence on the obesogenic effect of prenatal exposure to organochlorine compounds in humans, skinfold thickness measurements might assist to elucidate this relationship. In the Rhea study, skinfold thickness measurements were collected for newborns and young children and a future investigation of their relationship with prenatal exposures to environmental contaminants has been planned.

Finally, information on postnatal growth of children in the Rhea and MoBa study has been collected during follow-ups. Hence, it would be of interest to investigate whether the effects of prenatal exposures to dioxins and PCBs, through maternal diet, persist through early childhood and in which direction can affect postnatal growth.

7 CONCLUSIONS

- Diet during pregnancy can contribute to maternal and fetal body burden of dioxins and other POPs.
  - Combined dietary data with levels of POPs in blood samples or in food provided a valid dietary exposure estimate.
  - Reduced rank regression (RRR) provided biomarker-based dietary patterns which can be used as estimates of prenatal exposures to dietary contaminants.
  - High consumption of meat and fish during pregnancy was positively related to biomarker assessed levels of POPs in maternal and cord blood.
  - Dietary exposure estimates might reflect short-term exposures and might not be able to capture cumulative exposures to dioxins and other POPs.
- Prenatal exposure to dioxins and other POPs, through overall maternal diet, was negatively associated with birth size.
  - Maternal diet can be a source of prenatal exposure to POPs and be related to impaired fetal growth even in low exposed populations.
- Prenatal exposure to dioxins and dioxin-like compounds, through overall maternal diet, was related to a reduction of anogenital distance in newborn boys.
  - Maternal diet, as a source of dioxins and dioxin-like compounds, might induce anti-androgenic effects.
- Anogenital distances were related to body size, found to track through life and were highly reliable anthropometric measurements in both boys and girls.

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