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ΜΕΤΑΠΤΥΧΙΑΚΗ ΕΡΓΑΣΙΑ

« Exposure to compounds with dioxin-like activity in pregnant women, maternal dietary patterns and anogenital distance – mother-child cohort in Crete»

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Ευχαριστίες

Θα ήθελα να ευχαριστήσω την συντονιστική επιτροπή και ιδιαίτερα το συντονιστή του μεταπτυχιακού προγράμματος κ. Αναστάσιο Φιλαλήθη, που μου έδωσαν την ευκαιρία να παρακολουθήσω το πρόγραμμα αυτό.

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Τέλος, ένα πολύ θερμό ευχαριστώ οφείλω στη Μαρίνα Βαφειάδη, βιολόγο, συμφοιτήτρια και στενή συνεργάτιδά μου. Χωρίς την πολύτιμη βοήθειά της οποίας δε θα μπορούσα να ολοκληρώσω την εργασία αυτή. Τις οφείλω πολλά περισσότερα αλλά κυρίως το ευχάριστο κλίμα καθόλη τη διάρκεια της συνεργασίας μας και τη στενή φιλική σχέση που δημιουργήθηκε.

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Της: **Παπαδοπούλου Ελένη-Ζουμπουλιά**

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ΕΙΣΑΓΩΓΗ: Οι διοξίνες και τα μόρια με παρόμοια δράση συσσωρεύονται κατά μήκος της τροφικής αλυσίδας, δρώντας ως ορμονικοί διαταράκτες. Η διατροφή της γυναίκας, στη διάρκεια της εγκυμοσύνης, αποτελεί την βασικότερη οδός ενδομήτριας έκθεσης σε αυτά τα συστατικά, επηρεάζοντας έτσι την έκβαση της γέννας και άλλες σχετικές παραμέτρους, όπως η πρωκτογεννητική απόσταση του βρέφους.

ΣΤΟΧΟΙ: Οι στόχοι αυτής της έρευνας είναι: ο σχεδιασμός και η εφαρμογή ενός πρωτόκολλου μετρήσεων των πρωκτογεννητικών αποστάσεων στα βρέφη, όπως και η εκτίμηση της συσχέτισης μεταξύ της πρόσληψης λίπους μέσω της διατροφής κατά τη διάρκεια της εγκυμοσύνης και της πρωκτογεννητικής απόστασης των αρσενικών και θηλυκών βρεφών. Επίσης σε ένα μικρότερο δείγμα, διερευνήθηκε η σχέση της έκθεσης της μητέρας σε διοξίνες και σε παρόμοιες διοξινών ουσίες με την πρόσληψη λίπους, μέσω της διατροφής.

ΜΕΘΟΔΟΙ: Αρχικά διενεργήθηκε μια πιλοτική μελέτη, που αποτελούνταν από 9 βρέφη. Συνολικά μετρήθηκαν 305 βρέφη (158 αγόρια και 147 κορίτσια), ηλικίας 0 έως 16 μηνών. Ενώ συλλέχθηκαν διατροφικές πληροφορίες από τις μητέρες, που συμμετείχαν στη μελέτη μητέρας-παιδιού. Οι πρωκτογεννητικές μετρήσεις συσχετίστηκαν με την πρόσληψη λίπους μέσω της διατροφής για 150 ζευγάρια μητέρας-παιδιού.

ΑΠΟΤΕΛΕΣΜΑΤΑ: Για το σύνολο του υπό μελέτη πληθυσμού, βρέθηκε μια αρνητική συσχέτιση μεταξύ της πρόσληψης λίπους από τη μητέρα και των πρωκτογεννητικών αποστάσεων του βρέφους. Τα αποτελέσματα είναι στατιστικά σημαντικά μόνο για την απόσταση μεταξύ του μέσου του πρωκτού και της ανώτερης βάσης του πέους, όπως και για την απόσταση μεταξύ του μέσου του πρωκτού και της βάσης του όσχεου, αλλά και το πλάτος του πέους, στα αγόρια. Σε πληθυσμό νεογνών, βρέθηκε ότι οι γυναίκες με υψηλή πρόσληψη λίπους κατά τη διάρκεια της εγκυμοσύνης, είχαν περισσότερες πιθανότητες να γεννήσουν παιδί με μικρή πρωκτογεννητική απόσταση, ανεξάρτητα το φύλο του, έπειτα από τον έλεγχο για παράγοντες σχετιζόμενους με τη μητέρα και την εγκυμοσύνη.

ΣΥΜΠΕΡΑΣΜΑ: Η πρόσληψη λίπους από τη μητέρα μέσω της διατροφής, κατά τη διάρκεια της εγκυμοσύνης, η οποία μπορεί να σχετίζεται με αυξημένη έκθεση σε ορμονικούς διαταράκτες, μάλλον σχετίζεται με μικρότερη πρωκτογεννητική απόσταση στα αρσενικά βρέφη. Επιπλέον ανάλυση με μεγαλύτερο δείγμα και τη χρήση βιολογικών δεικτών απαιτείται.

Λέξεις κλειδιά:

Ορμονικοί διαταράκτες, πρωκτογεννητική απόσταση, δράση παρόμοια διοξινών, πρόσληψη λίπους, εγκυμοσύνη, ενδομήτρια έκθεση, διατροφή, μελέτη μητέρας-παιδιού, Κρήτη, μελέτη Ρέα

Abstract

Title: **Exposure to compounds with dioxin-like activity in pregnant women, maternal dietary patterns and anogenital distance - mother-child cohort in Crete.**

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Date: June 2009

BACKGROUND: Dioxins and dioxin-like compounds bioaccumulate through the food chain and act as endocrine disruptors. Nutrition during pregnancy has been assessed as the main route of in-utero exposure to these compounds, affecting birth outcome and parameters, as anogenital distance of the infant.

OBJECTIVES: The aims of this study were to develop and implement a protocol for the measurement of anogenital distances in infants and to evaluate the association between maternal fat intake and anogenital distance in male and female infants. The association of mother's exposure to dioxin-like compounds with fat intake during pregnancy was also examined in a subsample.

METHODS: A pilot study was conducted and comprised of 9 infants. In the current study we measured anogenital distance for 305 infants (158 males and 147 females), 0 to 16 months old. Dietary information was obtained for their mothers, participating in the mother-child cohort study. Measurements were associated with maternal fat intake for 150 mother-child pairs.

RESULTS: In the total study population a negative correlation was found between maternal fat intake and anogenital distances, but results were statistically significant only for anogenital distance (AGD), anoscrotal distance (ASD) and penis width (PW) in males. Among newborns, women with high fat intake during pregnancy had more possibilities to give birth to an infant with a phenotype of short anogenital distance regardless of sex, after controlling for maternal and pregnancy related factors.

CONCLUSION: Maternal fat intake during pregnancy that could be related to a higher uptake of endocrine disruptors was associated with shorter anogenital distances in males. Further analyses with a larger sample and the use of biomarkers are needed.

Key words:

Endocrine disruptors, anogenital distance, dioxin-like activity, fat intake, pregnancy, in-utero exposure, nutrition, mother-child cohort, Crete, Rhea study

1. TITLE

Exposure to compounds with dioxin-like activity in pregnant women, maternal dietary patterns and anogenital distance - mother-child cohort in Crete.

2. BACKGROUND

2.1 Introduction

Polyhalogenated compounds (PHCs) are organic compounds with multiple substitutions of halogens, such as chlorine and bromine. They are of particular interest and importance because as poly-halogens generally are highly reactive and bioaccumulate in humans. Such types of compounds are polychlorinated (PCBs, PCDDs, Hexachlorophene, PCDFs, PCPPs, and PCDEs), polybrominated (PBDEs, PBBs), perflourinated and polyiodinated (www.foodsafety.gov, 2008)

More specifically, polychlorinated-dibenzo-p-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) are often designated as 'dioxins' because of their similarities in structure and biological properties. In addition, other organic compounds possess dioxin-like properties, particularly the coplanar polychlorinated biphenyls (PCBs). They naturally occur in nature and act as environmental pollutants.

Dioxins and dioxin-like compounds derive mainly from a variety of industrial and combustion sources. They are produced as by-products in the manufacturing of organochlorides, in the incineration of chlorine-containing substances, in the bleaching of paper and from natural sources also, like volcanoes and forest fires. Cigarette smoke contains small amounts of dioxins as well. According to the most recent US EPA data the major sources of dioxins are man-made emissions like: coal fired utilities, municipal waste incinerators, metal smelting, diesel trucks, burning treated wood, trash barrels' burning. Over the last 30 years, governmental agents and industry have worked together in order to implement new emission requirements which goals on reduction of man-made sources. These regulations have been very successful in reducing stuck emissions from incinerators more than 90% from past environmental levels (U.S Environmental Protection Agency-National Center for Environmental Assessment, 2005).

In addition, by the early 1900s PCBs were commercially produced as ingredients of coolants, lubricants, pesticides, flame retardants, paints and other daily used products. However, PCB production was banned in the 1970's due to the high toxicity of most congeners and mixtures.

Due to their chemical characteristics, dioxins and dioxin-like compounds are extremely persistent in the environment, hence characterized as "persistent organic pollutants" (Porta M., 2008). These compounds bioaccumulate in human and animal tissue due to their high lipid solubility resulting in the ability to pass through biological phospholipid membranes and to biomagnify in food chains (Smith AG., 2002).

These compounds are found worldwide, including areas where they have never been used or without industrial activity. This is due to their ability to travel long distances through indirect (particulate matter, food chain, water and soil sediments) or direct (atmosphere, water) routes. Therefore, despite the significant reduction of emissions in recent years, past releases of dioxins from man-made sources still contaminate the environment.

2.2 Sources of Exposure

Owning to the fact that a large part of today's exposure is from releases occurred decades ago, the public concern increases and focuses in planning minding on reducing human exposure to these compounds.

Occupational exposure is a determinant issue for workers in the chemical industry or in the application of chemicals. Very high levels have been observed in workers producing chlorophenoxy herbicides and chloropohenols with lower levels measured among professional sprayers of these herbicides (Kogevinas M, 1997). A recent study by Chia et al (Chia T., 2008) referring to the effect of occupation on oxidative damage for 41 workers at a zinc recovery plant in Taiwan reports that the lipid peroxidation levels are higher for those working at production departments than those who worked at managerial departments. They also observed that occupational exposure to hazards like PCDDs and PCDFs for heavy metal recovery workers contributed stronger on the rising of lipid peroxidation than smoking. Furthermore, other studies of exposure in several work sites specify that working at metal recycling sites could increase exposure on dioxins and are reporting measurements equal and lower than 9pgWHO-TEQ/m^3 (Sweetman A., 2004).

We can consider three routes of dioxin intake: ingestion, inhalation and dermal exposure. A few studies only, have examined the contribution of dermal absorption and the results suggest a 1% bioavailability across the skin (Weber LW., 1991). Inhalation of dioxin compounds could be a strong contributor mainly in worksite conditions. A large amount of studies suggest that all the dioxin that is attached on inhaled dust is taken into the body, resulting on 100% bioavailability (Nessel CS., 1990; Diliberto JJ., 1996).

2.3 Dietary intake

For people without occupational exposure, ingestion in terms of dietary intake is the primary source of dioxin exposure. The dioxins emitted from sources are transported aerially and deposited on crops, soils, and water. Because of their lipophilic nature, they tend to be stored in animal fats, including all animal products in terrestrial environments, and in fish and seafood in aquatic environments. Approximately 90% to 98% of human exposure is reported to occur through diet, with foods of animal origin being the major source (Liem AK., 2000; Parzefall, 2002).

According to the FAO/WHO 2002 report regarding safety evaluation of certain food additives and contaminants, mean concentrations of PCDDs, PCDFs and PCBs in 6 main food groups are:

Food category	PCDDs/PCDFs (WHO-TEQ pg/g whole food)	PCBs (WHO-TEQ pg/g whole food)
Dairy	0,07	0,08
Eggs	0,16	0,07
Fish	0,47	2,55
Meat	0,08	0,41
Vegetable products	0,04	0,04
Fats and oils	0,21	0,07

Likewise, fish and seafood are some of the major factors of exposure to dioxin-like compounds in adults, contributing in levels between 11-63% (Bilau M., 2007; Bocio A., 2005; EU SCOOP, 2000). Studies have shown that modest consumption of farm salmon contaminated with dioxin-like compounds raises human exposure levels above the lower end of the tolerable daily intake for adults in the US (Foran J.A., 2005). Furthermore, due to different dietary habits, some researchers report that in Europe the primary source of exposure in dioxins and PCBs are meat, eggs and dairy products (Schecter A., 1997). The contribution of milk and dairy products in total intake may range from 16 to 39%. Additionally, the contribution of meat and meat products varies between populations from 6 to 32% (EU SCOOP, 2000).

A common methodology of such studies is the analysis of food samples in order to estimate the exposure, but only a few studies have investigated the correlation between diet and exposure measured in biological samples like blood or human milk. A major benefit of analyzing biological samples is the estimation of the cumulative exposure and moreover the cumulative health risk of exposure, given the fact that dioxins and dioxin-like compounds not only bioaccumulate through the food chain but are also not cleared readily from the body. In addition, studies have report that consumption of contaminated feed or grazing of cattle on treated land is likely to increase PCDD/PCDF levels in meat products (Rideout K., 2004).

Donato F studied a population leaving near a chemical factory in Italy (Donato F., 2006). Their results endorse the hypothesis of strong association between high blood PCB's levels and consumption of food produced in polluted areas, which is strengthened by a dose-effect association. Furthermore, in other exposure studies a positive correlation with fish intake has often been observed, although correlations with other foods of animal origin have also been reported (Halldorsson TI., 2008; Huisman M., 1995; Nawrot TS., 2002).

As a result of the low dioxin concentration in foods it is common that correlations between foods are often ignored and dietary patterns rarely receive any attention. Conclusively, more detailed information is needed in order to elucidate the role of dietary intake in human exposure to dioxins.

2.4 Exposure of infants and young children

Analyzing biological samples provides the opportunity to appraise exposure of infants and young children, which has been a prominent concern for human health risk assessment of dioxins. Main routes of exposure for that population group is breastfeeding, where lipophilic compounds are transferred from mother to infant, consumption of dairy and other commonly consumed foods and the hand-to-mouth contact as a mediate to the environmental exposure (Kerger BD., 2007). Furthermore, recent studies support the possibility of infant exposure to dioxin via the placenta, due to the known accumulation of dioxins in adipose tissue.

Suzuki et al (Suzuki G., 2005) investigated the transferring mode of dioxins to the unborn child via the placenta and estimated the distribution of dioxins and dioxin-like compounds in maternal samples of blood, cord blood, adipose tissue, placenta and maternal milk. Results indicated that some congeners are being transferred from maternal blood to the cord blood via the placenta. The type of compounds being restricted or transferred by the placenta depends on factors such as molecular size, protein-bound, level of dissociation and level of affinity with the Ah receptor. The activation of the Ah receptor has been directly related to dioxin exposure (Ko HP., 1996).

After ingestion congeners are distributed to organs via blood circulation and accumulate in the liver and adipose tissue of the mother. Due to metabolism and their lipophilic nature, an amount of dioxins circulate through mother's body, transferred through membranes, stored in tissues or excrete. Thus, they act as a regular source of exposure for newborns and breast-fed children (Suzuki G., 2005; Donato F., 2006).

Milbrath et al (Milbrath MO., 2009) reported that 20% of the maternal load of some persistent pollutants can be transferred during 6 months of lactation. As reported above, accumulation in breast milk depends on molecular weight. More specifically, PCB's concentration appears to be 4-10 times higher in breast milk than in maternal blood and also higher than PCDD/PCDF concentration. Furthermore, breast milk contamination depicts regional variation derived from different maternal exposure scenarios (Solomon G.M., 2002).

Data from countries with ongoing breast milk monitoring programs indicate that over the past decade dioxin concentration has decreased by 50% (LaKind JS., 2001). Exclusive breastfeeding should be strongly supported and promoted. Negative effects of dioxins in child's health have been shown to be the result of transplacental rather than lactational transfer of dioxins (Hedley A.J., 2006).

2.5 Calux assessment

Recently, a relatively cheap and rapid screening tool has replaced the conventional instrumental chemical analysis of dioxins. The *c*hemically *a*ctivated *lu*ciferase gene e*x*pression (CALUX) in vitro cell bioassay is a bioanalytical tool that is increasingly being used by laboratories for the screening and relative quantification of dioxins and dioxin-like compounds in blood, sediments, food matrices, and milk. It uses genetically modified cells that respond to chemicals that activate the aryl hydrocarbon receptor (AhR). It has been reported that the main toxic activity of dioxins and dioxin-like compounds is by blocking the AhR receptor (Safe, 1995). The results are converted into a bioassay toxic equivalency (CALUX-TEQ) value by the direct comparison of the response for a given sample to a dose-response curve obtained with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD or dioxin) (Windal I., 2005). The Ahr receptor has an essential role given the fact that PCDDs/PCDFs are considered to exert their toxic effects through a common mechanism mediated via the receptor by altering the regulation of the expression of various genes (Arisawa K., 2005).

2.6 Levels of dioxin

During the last 100-200 years, human activity contributed extensively on the environmental increase of dioxin release. In July 1976, a large population living in a residential area in Seveso, Italy was exposed to high TCDD doses, caused by an industrial accident. TCDD is the most toxic congener and it became well known as a herbicide and defoliant contaminant of Agent Orange which was used by the US military during the Vietnam War, around 1960's. Recently, several scandals occurred concerning contaminated food with high doses of dioxin around Europe. These include the Naples mozzarella crisis at 2008, which dropped the sales of mozzarella across Italy by about 50%, the Irish pork crisis at the same year, regarding contaminated animal feed that effected pork, beef and milk markets across Europe. In 1999 the outburst of the dioxin food scandal in Belgium concerning mainly contaminated poultry feed created a major problem throughout Europe and resulted to a restructuring of Food and Safety policies. Taking into account the above, we can support the fact that although environmental levels of dioxins have been decreasing (Liem AK. 2000) 10% of the general population in Europe exceed the tolerable daily intake limit set by the WHO, as recent market-basket studies have reported (Baars A.J., 2004; Bilau M., 2007; Darnerud P.O., 2006).

The overall toxicity of a dioxin containing mixture is assumed to be the Toxic Equivalent (TEQ) of a stated amount of pure TCDD. Furthermore, the concept of Toxic Equivalency Factor (TEF) has been developed to facilitate the risk assessment and regulatory control (Linhardt, 2007).

WHO has assessed at1990 the Tolerable Daily Intake (TDI) for dioxins and dioxin-like PCB's as 10 pg TEQ/kg of body weight per day. The tolerable intake represents the level considered safe over a lifetime of consumption and is calculated with the use of safety margins. In 1998, after further research, TDI was re-evaluated as1-4 pg TEQ/kg/day, also setting as an ultimate goal to reduce human intake levels to less than 1pg TEQ/kg/day.

Finally in 2002, WHO/FAO set a limit of 70pg TEQ/kg/month (approximately 2pg/kg per day). Studies have reported that exposure for the general adult population varies between 0.33-3.57 pg TEQ/kg/day which is less than the upper limit. However, breast-fed babies have a mean intake between 26-170 pg TEQ/kg/day which is far more than the threshold (Arisawa K., 2005)**.**

Body burdens of dioxins should also be included to estimate and classify levels of exposure. Body burden is considered as a good measure for lifelong exposure because of the long half-life of such congeners (Tuomisto J., 2006). Estimating maternal body burden in human milk or blood lipids could reflect the exposure of infants. In contrast, it should be noted that in young children and especially breast-fed, half-life of dioxins (T ½= 0.27-0.46 years) are extremely lower than those in adults (T ½ =5.8-11.3 years) (Dai D., 2008). Thus, young children are highly exposed due to maternal body burden and breast-feeding.

2.7 Health Effects

A wide range of health effects due to dioxin exposure have been reported from several human and animal studies. Cancer risk as an exposure outcome has been widely studied and current results demonstrate a dose-response association pictured by a J-shaped curve (Tuomisto J., 2006). Furthermore, occupational cohort studies suggest a modest increase in total cancer risk and increase of soft-tissue sarcoma in higher levels of occupational exposure (Kogevinas, 2000; Steenland K., 1999). Thus, the hazard has been recognized and several precaution and protective initiatives have been planed. In addition, TCDD has been categorized in Group 1 of IARC as carcinogenic to humans, since 1997. PCDFs and PCDDs are unclassifiable as to its carcinogenicity to humans (Group 3). Generally, the toxic and biochemical responses induced by PCDDs/PCDFs include carcinogenicity, focused in liver, lung, oral mucose and skin, endocrine, reproductive, neurobehavioral and immune effects. Furthermore, in 1998 PCBs have been classified as probably carcinogenic to humans (Group 2A), while may also have non-carcinogenic health effects, particularly as endocrine disruptors (IARC, 1997).

As mentioned, human exposure begins in utero via placental transfer and may continue in early postnatal life via breast feeding (Wang SL., 2004).The effects of in utero and/or lactation exposure to dioxins and PCBs may persist for many years following the initial insult. Several human studies have shown that exposure to dioxins during pregnancy and breast-feeding may result in neurological, cognitive and motor development deficiencies in children (Boersma E.R., 2000; Vreugdenhil HJ., 2002), as well as neurodevelopmental disorders and brain injuries (Grandjean P., 2006).

Further studies on laboratory animals prenatally exposed to high levels of PCDDs/PCDFs and dioxin-like PCBs show reproductive and developmental effects (Ohsako S., 2001). It has been suggested by human studies that in utero exposure to environmental chemicals may have contributed to the reported decline in human sperm counts, the increased incidence of urogenital malformations and altered sex ratio over the last 40-50 years (Gray L.E., 2000).

Hypospadias, cryptorchidism and testicular cancer are abnormalities which the above endocrine disruptors may be responsible for (Toppari J., 2002) The main hypothesis is that exposure in environmental chemicals that have an estrogenic or anti-androgenic activity, by interfering with the androgen signaling pathway, might be responsible for the increased incidence of these alterations (Huang PC., 2008).

Morphological alterations are the most common adverse effects of dioxin exposure in the time point of development (Jin MH., 2008). Such an effect is the decreasing of the anogenital distance (AGD) of the infant (i.e. the distance from the anus to the base of the scrotum in males and from the anus to the base of the genitals in females). Measuring AGD in animals as an effect of exposure in hormonally active agents is a routine measurement and has been used as a bioassay of fetal androgen action (Nagao T., 2000; Gray L.E., 2001; McIntyre BS., 2002; Wang XQ., 2002; Bowman C.J., 2003). Such studies indicate that prenatal exposure in phthalates is positive associated with reduced neonatal anogenital distance with a dose-response relationship (Saillenfait AM., 2008). Thus, as AGD is a sensitive measure of prenatal anti-androgen exposure, it has been recently used in very few human studies. Unlike phthalate exposure, infant's AGD has not been studied as an effect of dioxin exposure in humans, while current results support the association between environmental phthalates exposure and adverse effects on the reproductive human system (Latini G., 2006; Swan SH., 2005).

The human health risk has not been eliminated, while it is elevated in case of occupational exposure and residence in polluted areas. Such high dioxin environmental releases are very common in developing countries where many hazardous productive activities have been transferred and little has been done to recognize and prevent the unfavorable effects of such activity.

Given the importance of early detection of effects on the capacity of humans to reproduce, intensive research should be carried out on both fundamental and epidemiological domains. Timing and duration of exposure during development, as well as dose, dictates the induced effects. In the field of risk assessment studies should try to investigate the dose response relationship in low dose range, which includes the exposure of general population. While this is already well studied in laboratory animals, several human health effects, mechanisms of action, cumulative risk, the connection of exposure and tissue dioxin levels are only a few of future study areas that should be conducted.

3. OBJECTIVES

This study aims on investigating the possible association between measurable alterations on infant's reproductive system and other birth outcomes and the estimated environmental exposure of the mother to dioxin and dioxin-like compounds, during pregnancy. More specifically the aims of the current study are:

- The development of an assessment protocol. It includes a pilot study and a field study in a population of infants, where several morphological alterations and anthropometric measurements are being estimated.
- The investigation of the relationship between maternal dietary habits during pregnancy, as the main source of dioxin exposure, and birth outcomes (anogenital distance).
- The correlation between maternal dietary patterns and maternal exposure measured by DR-Calux method in maternal blood samples, in a pilot study.

4. SUBJECTS AND METHODS

4.1 The mother-child cohort study in Crete (Rhea study)

This study is a part of the 'Rhea' mother and child-cohort study which includes approximately 1500 mothers and their children, who have consented to participate. All women have delivered in one of the maternal hospitals of Heraklion-Crete, either in public or private maternity clinic during a period of 1 year (October 2007-October 2008). Pregnant women were first contacted at the 12^{th} to 14^{th} week of pregnancy, and were re-contacted at the 28^{th} to 32th week and on the time of delivery. After birth, meetings are being scheduled in order to measure the newborn's development. In the Rhea cohort data have been collected from the mother (socio-demographic and lifestyle), the child (clinical examination) and biological samples (maternal and cord blood).

4.2 Study population

The current study is based on 305 pregnant women and their children (305 paired samples) selected from the 'Rhea' birth cohort, for whom anogenital distance has been measured in the children.

4.3 Socio-demographic and lifestyle data

All information about the maternal demographic data such as age, residence, marital status and education were obtained by validated questionnaires curried out at different time points during and after pregnancy. Likewise data concerning lifestyle at the same time period, like smoking, health status, lactation, weight, height and dietary habits were gathered. Interviews were performed via phone or in person by specialized personnel.

4.4 Dietary assessment

Dietary habits have been derived from 2 standardized food frequency questionnaires (FFQ) administered at the 20^{th} to 21^{st} (mid-pregnancy food frequency questionnaire) and 30^{th} week of pregnancy.

The mid-pregnancy food frequency questionnaire

The main instrument to collect information on maternal diet in the RHEA cohort was the food frequency questionnaire (FFQ) that was administered to the women in mid pregnancy (14^{th}-20^{th} week of gestation) asking the mother what she has eaten since she became pregnant.The FFQ was semi-quantitative and contained more than 150 items. The FFQ had the following components: food frequency, dietary supplements, information on organic food consumption, different types of vegetarianism, dietary changes due to pregnancy, conditions of pregnancy that could reflect diet (nausea, vomiting, etc). The questions were hierarchical so that the woman was first asked general questions and then more specific questions. The following food items or types were covered, in the following order: types of meals during the day (breakfast, morning snack, lunch, afternoon snack, dinner, evening snack); bread and spreads on bread; toppings on open sandwiches (cheese, meat, fish, etc.); eggs; cereals; dairy products; warm meals (beef pork, poultry, fish, seafood); soups; side dishes to warm meals (boiled potatoes, potatoes prepared in other ways, pasta, rice); sauces; vegetables raw, cooked and in mixed dishes; dressings used for salads; legumes; fats used in cooking, frying and in salad; fruits; nuts; desserts, cakes, sweets and snacks; fast-foods; and drinks (fresh juices, soft drinks, coffee and tea, alcoholic drinks). For each food item, participants were asked about both frequency of consumption and average portion size. The frequency of consumption was given per day, per week and/or per month, depending on the food item. Photographs were used to define portion sizes for each food item. Respondents had to choose one out of three pictures showing different amounts of foods or dishes. The latter are used to visualize small, medium and large portions. FFQs were filled up by face to face interviews between pregnant women and trained personnel. The interview lasted approximately 1 hour.

For the needs of the current study we also use food information as food groups and not as food items, for assessing the contribution of each food group to total exposure. Thus, for each food group there have been estimated the daily consumption in grams per day. It is also worth mentioned that all food items were converted in food groups, even those derived from complex-mixed dishes by using recipes from current literature. Groups included food items with high percentage of lipid, could also be

categorized as high fat groups, such as groups of red meat, poultry, fish, dairy and fats-oils. For further analysis, we have estimated total fat intake in grams per day, as a discrete nutrient.

4.5 DR-Calux Bioassay

Maternal and cord blood samples for the Calux analysis were collected by midwifes at the hospitals right after delivery. This analysis concerns approximately 20 of 305 participants. The samples were shipped overseas and analyzed by BioDetection Systems B.V (Amsterdam, Netherlands) supervised by Dr. Ir. Harrie Besselink. According to the protocol of analysis, for each participant there has been extracted a sample of 1-2gr of human plasma, which was cleaned-up and dissolved. All data were corrected for internal reference sample and procedure blank and total lipid content was determined gravitameticaly.

4.6 Anthropometric measurements

4.6.1 Pilot study

A pilot study was initially conducted, previous of all anthropometric measurements in study subjects. The objectives of the pilot study were to develop a detailed protocol for all anthropometric measurements and to train experts on these measurements. It was scheduled at May 2008 for the period of time of about a month at the two public maternity clinics of Heraklio in Crete. Five persons participated including 3 medical doctors, a dietitian-nutritionist (Papadopoulou Eleni) and a biologist. In each clinic the measurements took place in a particular examination room.

The sample included infants aged less than a year that were hospitalized for more than 1 day at the clinics. We excluded infants with serious health problems as well as the ones already participating to the Rhea cohort study. All the measurements where conducted with the consent of the mother and under the supervision of the clinic pediatrician.

Several meetings were scheduled between the examiners in order to discuss and decide upon the difficulties of the study. The initial draft protocol used was based on a leaflet with photos and measurement details from the only other published study by Professor SH Swan in Rochester, USA (Swan SH., 2005). Conference calls with researchers from Prof Swan's group were organised to discuss details of the protocol. A final protocol adapted to the situation in Crete was developed (see below).

4.6.2 Field study

The field study started at June 2008 and comprised of two parts. One part concerns measurements conducted at the three maternity clinics of Heraklion-Crete by 3 examiners, more specifically medical doctors. The total sample included 164 newborns, delivered from June until September 2008, whose mothers have consented to participate in the Rhea study. According to the procedure, each of the three clinics was assigned to one of the examiners, who had a list of all participants of the cohort. For each delivery, the examiner was informed and the measurement took place within 1-2 days since the birth date. The infant was measured in a specified examination room, with the assistance of a midwife engaged to the Rhea cohort.

The other part of the field study regards measurements at home and is being held by the other 2 examiners (Vafeiadi Marina and Papadopoulou Eleni) who work as a pair. At first, a convenient appointment for both, the examiners and the mother is being programmed, resulting to 3-5 appointments per day. Totally, 141 infants were measured from June 2008 till March 2009. The duration of each appointment is approximately 30 minutes and includes measurement of weight and height, abdominal and head circumference, body fat by skinfold thickness measurements and anogenital distances. One person examined the infant while the other one assisted.

4.7 Statistical analysis

4.7.1 Pilot study

Infants participating at the pilot study were very few, thus all anthropometric measurements are described by the median of each sample for boys and girls respectively.

4.7.2 Field study

Most of the data collected from mothers and their children didn't have normal distribution, thus descriptive statistics and univariate associations are conducted using non-parametric tests. For example, median (M) and intra-quartile range (IQR) of the sample describes daily intake of pregnant women and anthropometric measurements of infants.

In the current study, results of anogenital distance measurements are demonstrated with and without adjustment. In order to adjust our measurements for the size of each infant we have divided anogenital measurement by infant's weight in kilograms. The result equals to anogenital ratio.

Continuous variables of anogenital ratios and fat intake are modified as discrete variables using quartiles. First and third quartile (Q1 and Q3) were used as cut off points, splitting our sample in 3 parts. The first part includes 25% of the participants, the second part the middle 50% and the upper 25% are in the third part. Thus, we are able to conduct associations for levels of exposure for maternal fat intake, as well as for levels of the outcome, namely infant's anogenital distances.

Via liner regression we estimated the dependency of the studied outcome from the regression model. At first, regressions were conducted for crude correlations between each anogenital measure and maternal fat intake variables, separately. Then, daily fat consumption of the mother was adjusted for other pregnancy and infant related variables, used as covariates. Variables selected as covariates are significant when correlating with anogenital ratios ($p<0, 05$) and have few missing values for the specific sample. Via logistic regression we can estimate the relationship between anogenital ratios, as binary variables. Categories are selected as infants with anogenital ratios above and bellow the mean. Logistic regression is conducted only in the analysis for infants 0 to 7 days old.

Finally, association between maternal fat intake and DR-Calux measurements was estimated by bivariate correlations. The statistical package SPSS, version 15.0 was used.

5. RESULTS

5.1 Pilot analysis

The sample of the pilot study included 9 children, 5 boys and 4 girls. Each child was measured 2-5 times by a different examiner each time. The main difficulty of this study was the fact that not all examiners were able to measure every child at least 1 time (due to ethical reasons so as not to overburden the infants). This was therefore featured as a limitation of the training. It occurred mainly due to the small hospitalization period of the children, the time-restrictions dictated by the hospital personnel and the lower cooperation of the child as measurement time passed.

At Table 1, we show anthropometric measurements for the total sample of boys and girls respectively. We can observe that mean anogenital distance (AGD: distance from the middle of the anus to the top of the penis) is greater than the mean anoscrotal distance (ASD: distance from the middle of the anus to the bottom of the scrotum) of boys, and that the mean anoclitoris distance (ACD: distance from the middle of the anus to the top clitoris) is greater than the mean anofourchetal distance (AFD: distance from the middle of the anus to the bottom of the labia majora) of girls, respectively. Measurements of skinfold thickness and other measures of growth were also conducted (detailed below).

Table 1. Distribution of anthropometric measurements for boys and girls of the pilot study.

	BOYS (n=5)			GIRLS (n=4)	
Measurement	**Mean**	**SD**		**Mean**	**SD**
Age (months)	5,0	4,6	**Age (months)**	0,8	0,0
AGD(mm)	65,98	3,97	**ACD(mm)**	39,15	5,74
ASD(mm)	33,63	5,09	**AFD(mm)**	16,48	2,95
PW(mm)	12,22	5,39	-		
TRICEPS(mm)	8,96	1,06	**TRICEPS(mm)**	8,25	1,39
QUADRICEPS(mm)	15,26	1,88	**QUADRICEPS(mm)**	14,80	3,78
SUPRAILIAC(mm)	6,63	0,80	**SUPRAILIAC(mm)**	5,11	1,29
SUBSCAPULAR(mm)	7,00	0,97	**SUBSCAPULAR(mm)**	7,75	1,00
Length (cm)	62,83	10,54	**Length (cm)**	54,00	0,71
HC(cm)	41,81	2,57	**HC(cm)**	36,74	1,65
AC(cm)	38,21	1,55	**AC(cm)**	35,24	2,08

HC: head circumference, AC: abdominal circumference, AGD: anogenital distance, ASD: anoscrotal distance, PW: penis width, ACD: anoclitoral distance, AFD: anofourchetal distance.

5.2 Anogenital protocol

The measurement protocol which was developed following the pilot study is described below. The followed order of the measurements was prescribed by the infant's convenience and its maximum cooperation for as long as it was required.

At first, we measure the anogenital distances, penis, quadriceps and suprailical skinfold thickness and abdominal circumference, for which the infant has to be naked and lay down. Secondly, in the embrace of the assistant, examiner measures triceps and subscapular skinfold thickness and head circumference. Weight is measured afterwards where the infant, if measured at home, must wear a clean diaper. Height estimation is the last one because the body extension that is required is an unpleasant position for the infant.

The equipment which is required is:

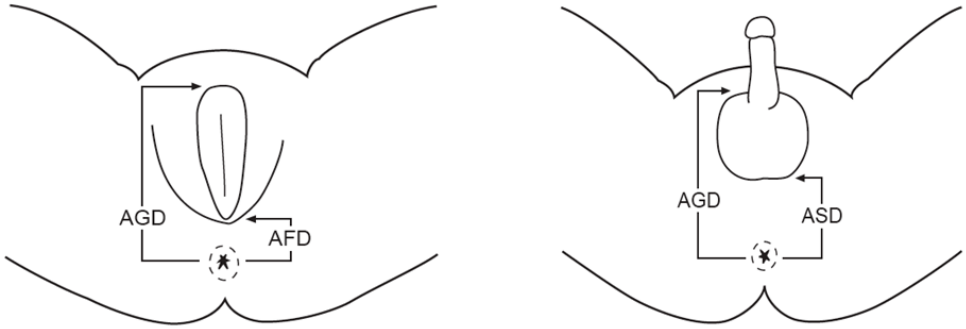
- Stable surface or examination table
- Electronic weight scale for infants
- Infant height measuring board (kiddimetre)
- Digital caliper => TESA-CAL IP67
- Skinfold thickness caliper => Lange caliper
- Non stretchable measuring tape
- Pen
- Recording form **(APPENDIX)**

After the appropriate place for the examination is found, [plenty of light, warm and providing the capability to place the lower part of neonate's trunk (buttocks) to the edge of examination table] a clean examination paper or a blanket (at home) was placed on the examination table. The callipers and the measuring tape were sterilized and the way they are used was demonstrated to the mother. A brief explanation of every measurement's purpose was done and the clothes and diaper were removed from the neonate. The assistant placed the neonate in supine position, on the examination table, with the head facing the mother and the genital area facing the examiner. The examination started with the anogenital distances, continued with the skinfold thickness and finished with the measurement of weight and height.

Measurement of anogenital distances

On all male infants anogenital distance (AGD), anoscrotal distance (ASD) and penis width (PW) have been estimated. AGD is the distance between the upper basis of penis and anus centre. ASD is the distance between the lowest point of the scrotum and the anus centre, while the PW is the diameter in the basis of penis. Respectively, on all female infants anoclitoral distance (ACD) and anofourchetal distance (AFD) have been measured. ACD is the distance between clitoris and anus center, whereas AFD is the distance between the fourchette and anus centre.

Figure 1. Anogenital distance measurements in males and females.



Before each measurement the infant should be calm so as not to alter measurements of the distance. The Digital caliper is switched on and set to zero. The examiner using his left hand holds the legs of the infant in abduction with its soles in touch and in contact with the abdominal wall. In case that the infant does not cooperate, its legs are held in abduction without its soles being in touch. The examiner, using his right hand, holds the digital caliper vertically to the body of the infant. The assistant (standing on the right side) distends the buttocks of the infant-in order to reveal the entrance of the anus- and, in case of male infant, to lift the scrotum (ASD measurement in boys), while in female infant, to distend the labia majora. The upper face of the calliper is placed on the upper point of each measured distance and faces of calliper are opened. The lower face of the caliper on the lowest point (always the centre of anus).

The examiner rechecks that the upper face has not moved from its initial position and announces the measurement to be recorded by the assistant. The calliper is closed, reset to zero and the measurement is repeated twice, while if there is a >1mm difference, a third measurement is performed.

Measurement of penis width (male infants)

While the infant is still laid supine with its legs in abduction the examiner gently applies pressure on the suprapubic area and the diameter in the basis of penis is measured. The measurement is repeated twice, while if there is a >1mm difference, a third measurement is performed.

Skinfold thickness measurement

Triceps, quadriceps, suprailiac and subscapular skinfold thickness measurements are always performed on the right side of the body. The examiner uses his left thumb and index finger to lift a double fold of skin and subcutaneous tissue. Gently but firmly grasps the fold of skin and subcutaneous adipose tissue approximately 1 cm above the site at which skinfold is to be measured. Only the skin and not the underlying muscle must be pinched. The examiner lifts the skinfold enough to separate it from underlying tissue.

The lever of the caliper, is gently depressed, so that the jaws separate and the jaws are applied 1cm below the pinch, at the same depth. The jaws should be vertical to the length of the skinfold. After 3 seconds, the caliper's value is recorded and the calliper is removed, keeping the left thumb and index finger in position. Measurement of skinfold thickness is always made in triplicate.

Measurement of subscapular skinfold thickness

The mother or the assistant takes the infant in her embrace (in upright position) with its back towards the examiner. The assistant holds infant's arm firmly extended (especially the upper arm), in contact with its trunk. The examiner identifies, by palpating with his left index, the inferior angle of the scapula. Then the examiner moves his left thumb downwards and diagonally, to identify the spinal column. Using these two fingers, grasps the skinfold formed by these two points and the jaws of caliper are applied just below the pinch. The measurement is recorded and repeated three times.

Measurement of triceps skinfold thickness

The infant is still on assistant's embrace with its back towards the examiner and its arm and elbow firmly extended. The distance between the acromial process (the most lateral bony protuberance of the back of the shoulder) and the olecranon (the bony structure that stands out when the elbow is flexed) is measured and the mid-point of the triceps muscle is marked. The examiner grasps the fold of the skin and subcutaneous adipose tissue approximately 1 cm above this point. The jaws of caliper are applied just below the pinch. The measurement is recorded and repeated three times.

Measurement of suprailliac skinfold thickness

The assistant places the infant on the examination table in the supine position. The examiner identifies the right iliac crest, by palpating with his left index and then drag his left thumb until the point traversed by the midclavicular line. Using these two fingers she grasps the skinfold formed by these two points. The measurement is recorded and repeated three times.

Measurement of quadriceps skinfold thickness

The infant should be lying supine and the examiner gently extends the right lower limb. The distance between the superior border of the patella and the level of the head of the femur is measured and the mid-point of the quadriceps muscle is marked. Then the examiner grasps the fold of the skin and subcutaneous adipose tissue approximately 1 cm above that point. The jaws of caliper are applied just below the pinch. The measurement is recorded and repeated three times.

Abdominal circumference

The infant is lying supine. Examiner places the measuring tape, in a horizontal plane, around the abdominal wall at the level of umbilical cord. The circumference is measured at the end of normal expiration and the measurement is recorded. The measurement is repeated twice, if there is a >1cm difference, a third one is performed.

Occipitofrontal circumference

The mother or the assistant takes the infant in their embrace (in upright position) with its back towards the examiner. The examiner places the measuring tape around the head of the infant and the circumference of the head, from the occiput of the skull to the most anterior portion of the frontal bone, is measured. The highest measurement value is recorded. The measurement is repeated twice, while if there is a >1cm difference, a third one is performed.

Measurement of infant's height

The height measuring tape is placed stretched on a stable examination surface. The infant is placed supine over the height measuring board. The top of the head of the infant is placed on the upper vertical surface of the height measuring board while the assistant holds it there steadily. The examiner brings the chin of the infant perpendicular to the trunk and both lower limbs are extended simultaneously. The measurement is recorded and repeated twice, while if there is a >1cm difference, a third one is performed.

Measurement of infant's weight

A cotton-paper or a small blanket (at home) is placed on the electronic weight scale and the scale is set to zero. The infant is placed on the scale naked or wearing only a clean dipper. In this case for data correction a clean dipper must be weighted. The measurement is recorded and repeated twice. In case of >10gr difference, a third measurement is performed.

Evaluation of reliability of measurements

When the examination is complete and all the measurements have been recorded, the examiner fills out a specific part of the recording form that aims to evaluate the reliability of measurements. This part includes three questions concerning the collaboration of the infant regarding to anogenital distances, the reliability of anogenital measurements according to the examiner's opinion and the collaboration of the infant regarding to skinfold thickness measurements. The answer is recorded in a four level scale where the first level reflects best collaboration and reliability while the last level reflects the worst. At the end of the form there is a fourth question where the assistant to the measurements is reported.

5.3 Study results

Main characteristics of pregnant women included in the sample of this study are shown in Table 3. They are approximately at the age of 30 years old, with a normal pre-pregnancy BMI. Regarding to pregnancy period, most of the women did not smoke during pregnancy (81,8%), blood glucose levels and blood pressure levels were normal for most of them (96,2% and 96,9%) and only very few had complications such as eclampsia. Furthermore, the 68% of them delivered at a public maternity clinic and for most of women this was not their first pregnancy (70,7%).

Table 2. Age, Body Mass Index (BMI) and several parameters regarding pregnancy for study participants.

	n	Mean	SD
Age(years)	296	30,13	4,74
Missing	9		
Pregnancy BMI	282	29,17	4,27
Missing	23		
Pre-pregnancy BMI	286	24,22	4,53
Missing	19		
	n	**%**	
Smoking[a]	285		
Yes	52	18,2	
No	233	81,8	
Missing	20		
Clinic	305		
Public	208	68,1	
Private	97	31,8	
Pregnant again	290		
Yes	205	70,7	
No	100	29,3	
Missing	15		
Breastfeeding	282		
Never	39	13,8	
Ever	243	86,2	
Missing	23		
Exclusive Breastfeeding	251		
Never	121	48,2	
Ever	130	51,8	
Missing	54		

[a] : Smoking variable is referring to whole pregnancy

Most women in this sample are Greek (94,4%), living mostly in urban areas (79,7%) and being married during pregnancy period (88,5%). Likewise, half of them are in a medium educational level (52,4%) and have been working during pregnancy (49%) (Table 4).

Table 3. Socio-economic characteristics of pregnant women in the study

	n	%
Residence	301	
Urban	240	79,7
Rural	61	20,3
Missing	4	
Nationality	304	
Greek	287	94,4
Other	17	5,6
Missing	1	
Marital Status	288	
Married	255	88,5
Engaged	27	9,4
Unmarried	5	1,7
Other	1	0,3
Missing	17	
Educational Status	286	
Low	57	19,9
Medium	150	52,4
High	79	27,6
Missing	19	
Working during pregnancy	290	
Yes	142	49
No	148	51
Missing	15	

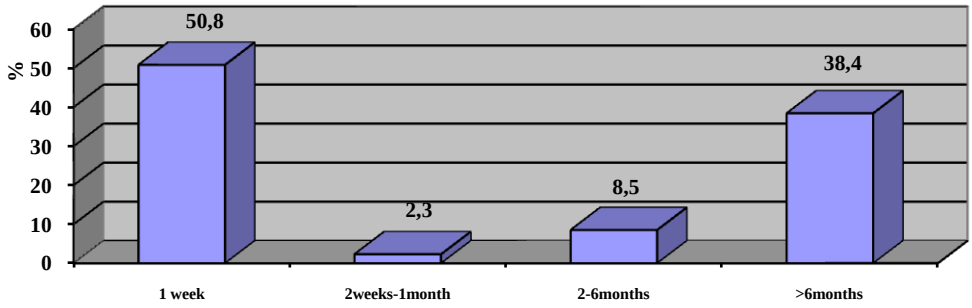
From Table 5, we can observe that the sample of 305 infants have an average gestational age of 38 completed weeks. Male and female are equally participating in the sample (52% and 48%).

Table 4.Gestational Age and Age of infants in the sample.

	n	Median	IQR
Gestational Age (weeks)	302	38,00	1,00
Missing	3		
	n	**%**	
Sex	305		
Male	158	52	
Female	147	48	

In addition, a substantial characteristic for the sample of infants is age distribution. From Graph 1, we can observe that approximately 50% of the sample is 0 to 7 days older and the rest is spread mainly around 5 to 15 months. Thus, distributions of anogenital measurements can not be normal and infant's age could be a major covariate for our further analysis.

Graph 2. Infants' age.



The characteristics of infants separately for males (n=158) and females (n=147) are shown in Table 6. Information is shown concerning birth outcomes, such as preterm births and small for gestational age.

Table 5.Birth outcomes and Breastfeeding for male and female infants.

	MALES			FEMALES		
	n	**Median**	**IQR**	**n**	**Median**	**IQR**
Gestational Age (weeks)	**155**	38,0	1,00	**147**	38,00	1,00
Infant Age(days)	**158**	12,0	278,3	**147**	3,0	253,0
	n	**%**		**n**	**%**	
Preterm	**155**			**147**		
Yes	14	9,0		12	8,2	
No	141	91,0		135	91,8	
Low Birth Weight	**158**			**147**		
Yes	9	5,7		9	6,1	
No	149	94,3		138	93,9	
SGA	**155**			**147**		
Yes	5	3,2		7	4,8	
No	150	96,8		140	95,2	
Breastfeeding	**149**			**133**		
Never	20	13,4		19	14,3	
Ever	129	86,6		114	85,7	
Missing	9			14		
Exclusive Breastfeeding	**136**			**115**		
Never	63	46,3		58	50,4	
Ever	73	53,7		57	49,6	
Missing	22			32		

Measurement of anogenital distances and other anthropometric variables

The male sample of infants has a median AGD distance equal to 58,95mm, median ASD to 31,54mm and penis width 12,21mm. Median ACD distance is 37,82 mm, which is greater than mean AFD distance (16,66 mm) (Table 7).

Table 6.Anthropometric measurements and anogenital ratios for male (n=158) and female (n=147) participants.

	MALES			FEMALES	
	Median	**IQR**		**Median**	**IQR**
Weight(gr)	4035,0	5,95	**Weight(gr)**	3630,0	5,10
Length(cm)	54,0	21,81	**Length(cm)**	52,0	20,75
HC(cm)	36,3	10,75	**HC(cm)**	36,0	10,00
AC(cm)	34,4	12,00	**AC(cm)**	34,0	11,4
Anogenital distances(mm)			**Anogenital distances(mm)**		
AGD	58,95	27,67	ACD	37,82	9,21
ASD	31,54	11,21	AFD	16,66	7,32
Penis Width	12,21	2,64	-	-	
Skinfold thickness(mm)			**Skinfold thickness(mm)**		
Tricept	7,00	5,50	Tricept	6,00	5,33
Quadricept	9,42	13,5	Quadricept	7,33	13,17
Suprailical	4,00	2,87	Suprailical	3,83	3,17
Subscapular	5,00	2,88	Subscapular	5,00	3,00
Anogenital ratios(mm/kg)			**Anogenital ratios(mm/kg)**		
AGD ratio	13,28	7,21	ACD ratio	9,79	6,53
ASD ratio	6,41	4,50	AFD ratio	3,46	2,22
Penis Width ratio	2,89	2,09	-	-	

HC: head circumference, AC: abdominal circumference, AGD: anogenital distance, ASD: anoscrotal distance, PW: penis width, ACD: anoclitoris distance, AFD anofourchetal distance.

Most male infants were cooperative (82,9%) and anogenital distance's measurements, as well as skinfold thicknesses' measurements were evaluated as reliable (84,8% and 87,7%) by the examiners. Female infants were also very cooperative, as the examiners reported (93,1%) (Table 8).

Table 7.Reliability of measurements for male (n=158) and female (n=147) participants.

	MALES		FEMALES	
	n	**%**	**n**	**%**
Cooperation Anogenital measurements	**158**		**147**	
Very Good	97	61,4	103	70,1
Good	34	21,5	34	23,1
Medium	21	13,3	8	5,4
Bad	6	3,8	2	1,4
Reliability Anogenital measurements	**158**		**147**	
High Reliability	84	53,2	93	63,3
Reliable	50	31,6	49	33,3
Medium Reliability	23	14,6	4	2,7
Not Reliable	1	0,6	1	0,7
Reliability Fat measurement	**158**		**147**	
High Reliability	104	65,8	106	72,1
Reliable	46	29,1	39	26,5
Medium Reliability	7	4,4	2	1,4
Not Reliable	1	0,6	0	0

Nutrition in mothers

Mothers during pregnancy (Table 9) were consuming high quantities of fruits (27,7%), dairy products and eggs (24,9% of total intake), as well as items from the food group of cereals and starchy food (13,2%). Non-alcoholic beverages, including soft drinks, stimulants and packed fruit juices, also contribute in daily energy intake (9,5%). In addition, fat intake is the 7% of total intake per day.

Table 8.Descriptives of maternal diet by food groups in grams per day (n=152).

Food Groups	Median (gr/day)	IQR	%
Starchy food	199	114	13,23
Meat & Products	65	97	4,32
Fish & Seafood	23	26	1,53
Dairy products & Eggs	375	292	24,93
Vegetables	196	161	13,03
Fruits	417	386	27,73
Nuts & Pulses	10	9	0,67
Fats & Lipids	48	33	3,19
Sweets & Salty snacks	26	46	1,73
Non-alcoholic beverages	143	240	9,51
Alcoholic beverages	0	4	0,00
Miscellaneous	2	2	0,13
Total fat	100	54	6,65

Conducting univariate associations by nonparametric tests, provides the information that food groups with high fat consistency and high daily intake such as meat, red meat, poultry, offals and processed meat as subgroups are associated with anogenital ratios (Table 10).

Table 9.Maternal food consumption and univariate associations with anogenital ratios.

	MALES(n=86)			FEMALES (n=64)	
Food consumption(gr/day)	**AGD RATIO**	**ASD RATIO**	**PW RATIO**	**ACD RATIO**	**AFD RATIO**
Starchy food	-0,252*	-0,225*	-0,247*	-0,185	-0,275*
Meat	-0,627*	-0,615*	-0,634*	0,644*	-0,574*
Red meat	-0,631*	-0,614*	-0,639	-0,595*	-0,531*
Poultry	-0,647*	-0,588*	-0,658	-0,630	-0,493*
Offals	-0,319*	-0,239*	-0,254	-0,217	-0,214
Processed meat	-0,152	-0,157	-0,139	-0,288*	-0,260*
Fish	0,095	0,080	0,127	-0,003	0,004
Fatty fish	0,009	0,012	0,064	-0,124	0,102
Dairy products & Eggs	-0,010	-0,042	-0,024	-0,035	-0,010
Milk & products	0,003	-0,029	-0,019	-0,051	-0,027
Vegetables	-0,173	-0,287*	-0,268*	-0,173	-0,168
Fruits	-0,096	0,109	0,121	-0,096	-0,105
Nuts & Pulses	-0,008	-0,003	0,037	0,138	0,147
Fats & Lipids	-0,117	-0,116	-0,077	-0,171	-0,191
Sweets & Salty snacks	-0,128	-0,161	-0,168	-0,252*	-0,333*
Non-alcoholic beverages	-0,239	-0,151	-0,221*	-0,239	-0,029
Alcoholic beverages	0,108	-0,139	-0,141	0,108	-0,730
Miscellaneous	-0,126	-0,150	-0,173	-0,126	-0,108
Total fat	-0,262*	-0,259*	-0,217*	-0,162	-0,226

*p<0,05.
AGD: anogenital distance, ASD: anoscrotal distance, PW: penis width, ACD: anoclitoris distance, AFD anofourchetal distance.

The 35% of total intake derives from food groups with high fat conciseness such as meat, dairy, fish, eggs, nuts, pulses, fats and lipids. In addition, 26,6% of total daily intake reflects consumption of meat and dairy, groups that are either correlated with anogenital ratios or are highly consumed by pregnant women. No significant association with anogenital ratios was found (Table 11).

Table 10.Combined food groups and univariate associations with anogenital ratios.

		MALES(n=86)			FEMALES (n=64)	
Food groups (gr/day)	**Median(%)**	**AGD RATIO**	**ASD RATIO**	**PW RATIO**	**ACD RATIO**	**AFD RATIO**
Fatty group[a]	527(35)	-0,163	-0,173	-0,175	-0,186	-0,165
Red meat-Dairy	385(26)	-0,108	-0,130	-0,125	-0,167	-0,129

[a]: Fatty group include: meat, dairy, fish, eggs, nuts, pulses, fats and lipids.
AGD: anogenital distance, ASD: anoscrotal distance, PW: penis width, ACD: anoclitoris distance, AFD: anofourchetal distance.

Generally, most boys and mothers have medium levels of anogenital ratios and fat intake, respectively. Regarding to children with low level of AGD ratios, mothers have medium to higher levels of fat intake, for all intake variables. The same pattern occurs for all adjusted measurements of anogenital distances. (Table 12, 13, 14).

Table 11.Association between the level of fat consumption and the level of Anogenital ratio in males.

MALES (N=86 mother-child pairs)			
	AGD ratio		
Fatty group	**Low n (%within AGD)**	**Medium n (%within AGD)**	**High n (%within AGD)**
Low	7(23,3)	11(25,6)	3(23,1)
Medium	12(40,0)	19(44,2)	9(69,2)
High	11(36,7)	13(30,2)	1(7,7)
Red meat-Dairy			
Low	9(30,0)	9(20,9)	3(23,1)
Medium	11(36,7)	24(55,8)	8(61,5)
High	10(33,3)	10(23,3)	2(15,4)
Total fat			
Low	7(23,3)	11(25,6)	5(38,5)
Medium	14(35,0)	18(45,0)	8(20,0)
High	9(30,0)	14(32,6)	0(0,0)

AGD: anogenital distance.

Data of Table 13, shows that mothers with high fat intake gave birth to boys with lower ASD ratio, either if fat intake is estimated as consumption of food groups or as a discrete nutrient.

Table 12.Association between the level of fat consumption and the level of Anoscrotal ratio in males.

MALES (N=86 mother-child pairs)			
	ASD ratio		
Fatty group	**Low n (%within ASD)**	**Medium n (%within ASD)**	**High n (%within ASD)**
Low	7(21,9)	5(20,0)	9(30,0)
Medium	13(40,6)	10(40,0)	18(60,0)
High	12(37,5)	10(40,0)	3(10,0)
Red meat-Dairy			
Low	9(28,1)	6(24,0)	6(20,0)
Medium	11(34,4)	13(52,0)	20(66,7)
High	12(37,5)	6(24,0)	4(13,3)
Total fat			
Low	7(21,9)	5(20,0)	11(36,7)
Medium	17(53,1)	9(36,0)	15(50,0)
High	8(25,0)	11(44,0)	4(13,3)

ASD: anoscrotal distance.

Additionally, boys with high PW ratio are fewer than those included in medium or low levels, while their mothers mainly have low fat intake.

Table 13.Association between the level of fat consumption and the level of Penis Width ratio in males.

MALES (N=86 mother-child pairs)			
	PW ratio		
Fatty group	Low n (%within PW)	Medium n (%within PW)	High n (%within PW)
Low	7(24,1)	10(23,3)	4(28,6)
Medium	11(37,9)	23(53,5)	7(50,0)
High	11(37,9)	10(23,3)	3(21,4)
Red meat-Dairy			
Low	8(27,6)	9(20,9)	4(28,6)
Medium	11(37,9)	27(62,8)	6(42,9)
High	10(34,5)	7(16,3)	4(28,6)
Total fat			
Low	5(17,2)	15(34,9)	3(21,4)
Medium	15(51,7)	17(39,5)	9(64,3)
High	9(31,0)	11(25,6)	2(14,3)

PW: penis width.

As reported for boys, mothers with high fat intake are paired with girls with low anogenital ratios. The difference within ACD ratio is clearer concerning maternal fat intake, measured as a specific nutrient (Table 15).

Table 14.Association between the level of fat consumption and the level of Anoclitoris ratio in females.

FEMALES (N=64 mother-child pairs)			
	ACD ratio		
Fatty group	Low n (%within ACD)	Medium n (%within ACD)	High n (%within ACD)
Low	4(15,4)	10(37,0)	3(27,3)
Medium	15(57,7)	13(48,1)	6(54,5)
High	7(26,9)	4(14,8)	2(18,2)
Red meat-Dairy			
Low	4(15,4)	9(33,3)	4(36,4)
Medium	15(57,7)	12(44,4)	4(36,4)
High	7(26,9)	6(22,2)	3(27,3)
Total fat			
Low	5(19,2)	8(29,6)	2(18,2)
Medium	12(46,2)	16(59,3)	6(54,5)
High	9(34,6)	3(11,1)	3(27,3)

ACD: anoclitoris distance.

We can observe that most mothers included in the higher level of high fat variable are paired with female children with low level of AFD ratio. For total fat intake the association is statistically significant (Table 16).

Table 15.Association between the level of fat consumption and the level of Anofourchetal ratio in females.

FEMALES (N=64 mother-child pairs)			
	AFD ratio		
Fatty group	Low n (%withinAFD)	Medium n (%within AFD)	High n (%within AFD)
Low	5(21,7)	9(26,5)	3(42,9)
Medium	11(47,8)	20(58,8)	3(42,9)
High	7(30,4)	5(14,7)	1(14,3)
Red meat-Dairy			
Low	5(21,7)	9(26,5)	3(42,9)
Medium	10(43,5)	18(52,9)	3(42,9)
High	8(34,8)	7(20,6)	1(14,3)
Total fat*			
Low	2(8,7)	12(35,3)	1(14,3)
Medium	12(52,2)	16(47,1)	6(85,7)
High	9(39,1)	6(17,6)	0(0,0)

*p<0,05. AFD: anofourchetal distance.

Table 17, demonstrates the distribution of anogenital ratios (mm/kg) categorized by levels of maternal intake. We can observe that there is a statistically significant difference between AGD measurements and fatty food group consumption, as well as between ASD and ACD measurements. Furthermore, in post hoc analysis we found that the differences of the mean ACD measurements are significant when comparing low fat intake with high fat intake. Likewise, in boys the difference is significant between medium and high fatty group consumption, for AGD and ASD ratios. In addition, when comparing with levels of consumption of red meat and dairy, significance remains only for AGD ratio.

Table 16.Distribution of anogenital measurements in males (n=86 pairs) and females (n=64 pairs) categorized by levels of maternal fat intake.

	MALES			FEMALES	
Fatty group	AGD RATIO Mean(±SD)	ASD RATIO Mean(±SD)	PW RATIO Mean(±SD)	ACD RATIO Mean(±SD)	AFD RATIO Mean(±SD)
Low	12,25(4,37)	6,16(2,76)	2,47(1,12)	9,43(3,39)	3,68(1,37)
Medium	11,86(3,91)	6,01(2,47)	2,32(1,03)	7,63(3,31)	3,31(1,23)
High	9,62(2,94)	4,59(1,79)	1,83(0,87)	6,48(3,22)	2,84(1,13)
Total	11,30(3,89)*	5,64(2,44)*	2,22(1,03)	7,87(3,43)*	3,31(1,26)
Red meat-Dairy	**AGD RATIO Mean(±SD)**	**ASD RATIO Mean(±SD)**	**PW RATIO Mean(±SD)**	**ACD RATIO Mean(±SD)**	**AFD RATIO Mean(±SD)**
Low	11,32(4,34)	5,54(2,68)	2,20(1,08)	9,47(3,43)	3,67(1,36)
Medium	12,14(3,74)	6,17(2,40)	2,38(1,00)	7,31(3,19)	3,25(1,29)
High	9,65(3,33)	4,67(2,06)	1,89(1,01)	7,28(3,53)	3,05(1,08)
Total	11,30(3,89)*	5,64(2,44)	2,22(1,03)	7,88(3,43)	3,31(1,26)
Total fat	**AGD RATIO Mean(±SD)**	**ASD RATIO Mean(±SD)**	**PW RATIO Mean(±SD)**	**ACD RATIO Mean(±SD)**	**AFD RATIO Mean(±SD)**
Low	12,53(3,96)	6,45(2,61)	2,47(1,00)	9,10(3,56)	3,55(1,04)
Medium	11,43(4,13)	5,64(2,55)	2,26(1,09)	7,83(3,26)	3,46(1,40)
High	9,87(2,97)	4,83(1,84)	1,88(0,89)	6,73(3,47)	2,74(1,01)
Total	11,30(3,89)	5,64(2,44)	2,22(1,03)	7,88(3,43)	3,31(1,26)

* p< 0,05.AGD: anogenital distance, ASD: anoscrotal distance, PW: penis width, ACD: anoclitoris distance, AFD: anofourchetal distance.

Linear regression models are describing the dependency of anogenital ratios from maternal exposure variables. Thus, estimating R square, could explain the proportion of anogenital measurements' total variance owning to the regression model. Furthermore, infant's age, as explained before, is a major covariate in estimating measurements' variance. In order to eliminate the influence of age, we are conducting regressions for boys and girls aged 0 to 7 days separately, where anogenital ratios fit better at normal distribution curve.

From Table 18 we can observe that when correlating maternal fat intake with AGD ratio, without adjustment, gives us negative beta coefficients. Thus, if fat consumption increases, AGD ratio decreases. The relationship is statistically significant only in the case of fat intake, measured as a nutrient. Furthermore, when adjusting diet variables for maternal health and pregnancy related characteristics (age, former pregnancy, gestational age, pregnancy and pre-pregnancy BMI) fat intake coefficients remain negative. Studying a model with maternal and infants' characteristics (infant age, skinfold thickness measurements) results in negative, though, very low diet coefficients.

Table 17.Linear regression for Anogenital ratio.

	CRUDE(n=86)		**ADJUSTED 1a(n=76)**		**ADJUSTED 2b(n=76)**	
	β*100	**95%CI**	**β*100**	**95%CI**	**β*100**	**95%CI**
Fatty group (gr/day)	-25.5	-0.007,0.0	-22.7	-0.007,0.0	-0.2	-0.002,0.002
Red meat-Dairy	-17.9	-0.007,0.001	-15.9		0.5	-0.002,0.002
(gr/day)				-0.008,0.001		
Total fat (gr/day)	-27.3	-0.031,-0.004	-26.1	-0.034,-0.003	-0.4	-0.007,0.007

a Adjusted for maternal age, former pregnancy, gestational age, pregnancy and pre-pregnancy BMI.

b Adjusted for maternal age, former pregnancy, infant age, skinfold thickness measurements, gestational age, pregnancy and pre-pregnancy BMI.

When adjusting for pregnancy related variables, diet coefficients are lower, while adding infant related variables causes further decreasing. Total fat intake estimated as a single nutrient, has higher βeta coefficients that remain significant for the first adjustment (Table 19).

Table 18.Linear regression for Anoscrotal ratio.

	CRUDE(n=86)		**ADJUSTED 1a(n=76)**		**ADJUSTED 2b(n=76)**	
	β*100	**95%CI**	**β*100**	**95%CI**	**β*100**	**95%CI**
Fatty group (gr/day)	-25.1	-0.005,0.0	-20.4	-0.004,0.0	-0.3	-0.001,0.001
Red meat-Dairy	-16.7	-0.004,0.001	-12.9		1.5	-0.001,0.002
(gr/day)				-0.004,0.001		
Total fat (gr/day)	-28.7	-0.020,-0.003	-25.6	-0.021, -0.002	-2.8	-0.007,0.004

a Adjusted for maternal age, former pregnancy, gestational age, pregnancy and pre-pregnancy BMI.

b Adjusted for maternal age, former pregnancy, infant age, skinfold thickness measurements, gestational age, pregnancy and pre-pregnancy BMI.

Same pattern is supported by data from Penis Width (Table 20).

Table 19.Linear regression for Penis Width ratio.

	CRUDE(n=86)		ADJUSTED 1a(n=76)		ADJUSTED 2b(n=76)	
	β*100	95%CI	β*100	95%CI	β*100	95%CI
Fatty group (gr/day)	-22.4	-0.002,0.0	-21.8	-0.002,0.0	2.0	0.0,0.0
Red meat-Dairy (gr/day)	-15.1	-0.002,0.0	-14.4	-0.002,0.0	1.3	0.0,0.0
Total fat (gr/day)	-24.3	-0.008,-0.0	-25.0	-0.009, 0.0	1.0	-0.001,0.002

a Adjusted for maternal age, former pregnancy, gestational age, pregnancy and pre-pregnancy BMI.
b Adjusted for maternal age, former pregnancy, infant age, skinfold thickness measurements, gestational age, pregnancy and pre-pregnancy BMI.

For girls participating, association is not that strong as for boys. For ACD ratio regression models, crude or adjusted for maternal characteristics, result in negative coefficients of diet, suggested that when fat intake increases, girls are born with a phenotype of lower ACD distance. No statistically significance was detected (Table 21).

Table 20.Linear regression for Anoclitoris ratio.

	CRUDE(n=64)		ADJUSTED 1a(n=60)		ADJUSTED2b(n=60)	
	β*100	95%CI	β*100	95%CI	β*100	95%CI
Fatty group (gr/day)	-25.6	-0.008,0.0	-21.2	-0.008,0.001	10.8	0.0,0.003
Red meat-Dairy (gr/day)	-20.7	-0.007,0.001	-15.1	-0.007,0.002	9.9	0.0,0.003
Total fat (gr/day)	-19.6	-0.036,0.004	-18.8	-0.037.0.007	8.9	0.0,0.015

a Adjusted for maternal age, former pregnancy, gestational age, pregnancy and pre-pregnancy BMI.
b Adjusted for maternal age, former pregnancy, infant age, skinfold thickness measurements, gestational age, pregnancy and pre-pregnancy BMI

As describe above, diet coefficients of AFD ratio regression models, are negative supporting our hypothesis, only crude and if adjusting for pregnancy related variables. If adding infants' age and skinfold thickness measurements, coefficients are under 0 and not significant (Table 22).

Table 21.Linear regression for Anofourchetal ratio.

	CRUDE(n=64)		ADJUSTED 1a(n=60)		ADJUSTED 2b(n=60)	
	β*100	95%CI	β*100	95%CI	β*100	95%CI
Fatty group (gr/day)	-21.0	-0.003,0.0	-16.3	-0.002,0.001	10.3	0.0,0.002
Red meat-Dairy (gr/day)	-16.4	-0.002,0.001	-10.7	-0.002,0.001	10.2	0.0,0.002
Total fat (gr/day)	-23.9	-0.015,0.0	-21.9	-0.015,0.002	-1.2	-0.006,0.005

a Adjusted for maternal age, former pregnancy, gestational age, pregnancy and pre-pregnancy BMI.
b Adjusted for maternal age, former pregnancy, infant age, skinfold thickness measurements, gestational age, pregnancy and pre-pregnancy BMI.

Through logistic regression models we can estimate odds ratios for a child with a phenotype of short anogenital distance, when maternal fat intake is in high levels. Anogenital distance was recoded to a binary variable, using as cut off point the mean anogenital, anoscrotal, anoclitoris, anofourchetal and penis width ratio respectively (16.06mm, 8.52mm, 3.55mm, 11.70mm, 4.79mm). Likewise, maternal fat intake variables were transformed as binary to the median of the sample.

Table 23 provides the information that if the mother has high fat intake, the boy has more possibilities to have anogenital ratio bellow mean. Adjustment for pregnant related variables results in a lower odds ratio, while adding skinfold thickness measurements causes odds ratio increase.

Table 22.Logistic regression for Anogenital ratios of males aged 0-7 days (n=30).

		CRUDE		ADJUSTED 1[a]		ADJUSTED 2[b]	
Fatty group	**n**	**OR**	**95%CI**	**OR**	**95%CI**	**OR**	**95%CI**
Low*	17	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.
High	13	1.42	0.328,6.174	1.05	0.156,7.084	1.60	0.105,24.504
Red meat-Dairy							
Low*	18	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.
High	12	2.00	0.44,9.096	1.45	0.224,9.346	1.82	0.124,27.216
Total fat							
Low*	21	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.
High	9	1.82	0.357,9.272	1.51	0.161,14.061	21.37	0.196,2329.2

a Adjusted for maternal age, former pregnancy, gestational age, pregnancy and pre-pregnancy BMI.
b Adjusted for maternal age, former pregnancy (no former pregnancy is the reference group), skinfold thickness measurements, gestational age, pregnancy and pre-pregnancy BMI.
*: low group is the reference group.

As described above, odds ratios for a boy with short anoscrotal distance are greater than 1. Thus, if the mother consumes high quantities of fat the boy has 2 to 3.18 more possibilities to have short anoscrotal distance, than a boy whose mother belongs to the low fat intake group (Table 24).

Table 23.Logistic regression for Anoscrotal ratios of males aged 0-7 days (n=30).

		CRUDE		ADJUSTED 1[a]		ADJUSTED 2[b]	
Fatty group	**n**	**OR**	**95%CI**	**OR**	**95%CI**	**OR**	**95%CI**
Low*	17	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.
High	13	2.00	0.44,9.096	2.21	0.344,14.160	2.03	0.232,17.822
Red meat-Dairy							
Low*	18	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.
High	12	1.60	0.351,7.302	1.66	0.263,10.486	1.46	0.184,11.491
Total fat							
Low*	21	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.
High	9	3.18	0.531,19.051	4.92	0.418,57.824	6.13	0.191,196.8

a Adjusted for maternal age, former pregnancy, gestational age, pregnancy and pre-pregnancy BMI.
b Adjusted for maternal age, former pregnancy (no former pregnancy is the reference group), skinfold thickness measurements, gestational age, pregnancy and pre-pregnancy BMI.
*: low group is the reference group.

For the regression model of penis width ratio we can assume that high fat intake does not have an impact in the current measurement (Table 25).

Table 24.Logistic regression for Penis Width ratios of males aged 0-7 days (n=30).

		CRUDE		ADJUSTED 1[a]		ADJUSTED 2[b]	
Fatty group	**n**	**OR**	**95%CI**	**OR**	**95%CI**	**OR**	**95%CI**
Low*	17	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.
High	13	0.600	0.14,2.58	0.63	0.101,3.861	0.004	0.0,13.278
Red meat-Dairy							
Low*	18	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.
High	12	0.46	0.103,2.013	0.35	0.052,2.278	0.001	0.0,4.001
Total fat							
Low*	21	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.
High	9	0.60	0.124,2.894	0.97	0.126,7.495	0.918	0.023,37.003

a Adjusted for maternal age, former pregnancy, gestational age, pregnancy and pre-pregnancy BMI.
b Adjusted for maternal age, former pregnancy (no former pregnancy is the reference group), skinfold thickness measurements, gestational age, pregnancy and pre-pregnancy BMI.
*: low group is the reference group.

For girls aged 0 to 7 days, maternal fat intake in high levels results in a greater possibility for her girl to have low anoclitoris ratio, only if fat intake is estimated as a single nutrient. When adjusting for pregnant related variables the odds ratio also rises. For further adjustment the model could not be defined (Table 26).

Table 25.Logistic regression for Anoclitoris ratios of females aged 0-7 days (n=25).

		CRUDE		ADJUSTED 1[a]	
Fatty group	**n**	**OR**	**95%CI**	**OR**	**95%CI**
Low*	15	Ref.	Ref.	Ref.	Ref.
High	10	0.49	0.090,2.656	0.25	0.013,4.836
Red meat-Dairy					
Low*	15	Ref.	Ref.	Ref.	Ref.
High	10	0.49	0.090,2.656	0.25	0.013,4.836
Total fat					
Low*	13	Ref.	Ref.	Ref.	Ref.
High	12	2.25	0.439,11.522	3.23	0.330,31.679

a Adjusted for maternal age, former pregnancy, gestational age, pregnancy and pre-pregnancy BMI.
b Adjusted for maternal age, former pregnancy (no former pregnancy is the reference group), skinfold thickness measurements, gestational age, pregnancy and pre-pregnancy BMI.
*: low group is the reference group.

In addition, a high fat diet of the mother during pregnancy suggest more possibilities of having short anofourchetal ratio for the female infant, compared with an infant delivered by a mother with low fat intake during pregnancy. Odds ratio are increasing if adding pregnancy related variables, such as gestational age, maternal age and BMI, while results are based on small numbers (Table 27).

Table 26.Logistic regression for Anofourchetal ratios of females aged 0-7 days (n=25).

		CRUDE		ADJUSTED 1[a]	
Fatty group	**n**	**OR**	**95%CI**	**OR**	**95%CI**
Low*	15	Ref.	Ref.	Ref.	Ref.
High	10	2.00	0.304,13.173	3.08	0.168,56.213
Red meat-Dairy					
Low*	15	Ref.	Ref.	Ref.	Ref.
High	10	2.00	0.304,13.173	3.08	0.168,56.213
Total fat					
Low*	13	Ref.	Ref.	Ref.	Ref.
High	12	3.13	0.474,20.583	49.53	0.588,4175.5

a Adjusted for maternal age, former pregnancy, gestational age, pregnancy and pre-pregnancy BMI.
b Adjusted for maternal age, former pregnancy (no former pregnancy is the reference group), skinfold thickness measurements, gestational age, pregnancy and pre-pregnancy BMI.
*: low group is the reference group.

In order to explore the association between maternal dietary habits and DR-Calux results we conducted bivariate correlations. It appears that dioxin-like activity measured through DR-Calux measurements are decreased as maternal fat consumption rises from low to medium or higher levels, while no statistically significant difference was found (Table 28).

Table 27.Maternal DR-Calux measurements associated with fat intake (n=22).

	DR-Calux	P value
Fatty group	-0,010	0,964
Red meat-Dairy	-0,080	0,724
Total fat	-0,275	0,216

5. DISCUSSION

In the present study we attempted to investigate the hypothesis that environmental exposures are related to birth outcome, namely, the association between maternal fat intake and anogenital distance of the child, measured at birth or at early postnatal life.

The measurement protocol was based in the published study by Professor SH Swan (Swan SH., 2005) and was adapted to the local situation. Our final protocol includes 4 measurements in the genitalia area (2 for each sex), skinfold thickness measurements at 4 different points, 2 circumference measurements (head and abdominal), weight and length estimation. Furthemore, measurements were conducted for infants, as well as for childrens aged 1 to 16 months, for both sexes. The variety of cases in the field study contributed in the design of a complete and detailed protocol, were the main parameter is the infant's convenience.

As crude results indicate, for males anogenital distance (AGD) is 58.95mm and anoscrotal distance (ASD) is 31.54mm. Likewise, females' anoclitoris distance (ACD) is 37.82mm and anofourchetal distance (AFD) is 16.66mm. In addition, penis width (PW) median for boys aged 0 to 16 months was found equal to 12,21mm. In a recent study in Taiwan ASD and AFD of 65 newborns were measured (Huang PC., 2008). The results are comparable to ours for girls (AFD=16mm), and approximately for boys (ASD=23mm), taking into account the range from 10mm through 36mm.

In addition, Salazar-Martinez et al developed a method for reproducible evaluation of anogenital distance in human newborns (Salazar-Martinez E., 2004). They measure 87 infants and concluded that the distance from the anus to the base of the scrotum for boys was twice longer than the distance from the anus to the posterior fourchette for girls (22mm and 11mm respectively). The sexually dimorphism was also reported from our results, where median for ASD measurements of males is two-fold longer than median AFD of females (31,54mm and 16,66 respectively). According to our study, AGD and ACD distances have approximately twofold difference, suggesting sexually dimorphism as well.

In a previous study, Callegari et al (Callegari C., 1987) measured AFD and ACD distance in female newborns (n=115). Crude results are lower than ours, while AFD/ACD ratio is comparable to the ratio of the current study (0.37±0.07 and 0.44 respectively). On the contrary, a sample of 134 boys aged 15.9±8.6 months were studied by Swan et al, with AGD equals to 70.3±11mm and ASD equals to 37.4±7.5mm (Swan SH., 2005). Regarding their reported percentiles, our male sample belongs bellow 25^{th} percentile for the AGD median and between 25^{th} and 50^{th} percentile for the ASD median. Furthemore, in a cross-sectional study of 781 male new-delivered infants, our median AGD, ASD and PW rank above 90^{th} percentile (Longnecker MP., 2007). Moreover, in a study of 71 children (37 males and 34 females) measured at 3 through 18 months old, ASD was reported 42± 8mm and AFD 23± 6mm (Torres-Sanchez L, 2008). Thereupon, crude measurements of the present study could not be directly compared with studies of newborns, or children, due to the age variance of our sample.

In order to adjust measurements for infant's dimensions, we have used anogenital divided-by-weight ratios, as in previous studies (Swan SH., 2005; Huang PC., 2008). AGD ratio for boys studied by Swan et al, and for those studied by Huang et al is lower than the present one (7.1±1.9, 7.16, 13.2 respectively). An explanation of the observed difference could be that, due to the non-normal distribution of our anogenital measurements and ratios the median is greater than the mean of the sample. Thus, ratios described by the sample median are greater than those described by the sample mean, as in the case of Huang et al and Swan et al. On the contrary, Torres-Sanchez et al measured a sample of boys and girls, whose anogenital ratios are comparable to the current sample (6±13 for boys, 3.6±1.1 for girls) (Torres-Sanchez L, 2008).

Regarding to maternal dietary assessment, median of fat intake is 100 grams/day (6% of total intake). In addition, 35% (527gr/day) of total intake concerns meat, fish, dairy, eggs, nuts, pulses, fats and lipids, while red meat and dairy consumption equals 385 grams/day, namely, 26% of total intake. Dietary data of EDEN mother-child cohort in France are compared to the current study, where sample mean is 97.7gr of fat per day before pregnancy and 104.1gr/day during the last trimester of pregnancy (Drouillet P, 2009).

As suggested by studies, maternal dietary factors may influence outcomes either of pregnancy (length of gestation, fetal growth, birth defects, pre-eclampsia) or of childhood (cognitive development, blood pressure, adiposity and atopic disease) (Rifas-Shiman SL, 2006). High fat intake during pregnancy has been related to adverse birth outcomes, such as the elevated risk for small-for-gestational age infant (Siega-Riz AM, 2008). Likewise, the determinant and clustering effect of fat intake during pregnancy have been studied in relation to the development of wheeze, eczema and atopy in childhood (Miyake Y, 2009); Chatzi L, 2008; Romieu I, 2007). Additionally, a randomized clinical trial in pregnant women, showed that modifying fat intake from the 20^{th} week of gestation until birth is effective on the reduction of preterm delivery (Khoury J, 2005).

The effect of maternal diet at the anogenital distance of the newborn has not been explored, however, linear regression analysis of the present study indicates a correlation between maternal fat intake and anogenital distance of the child, while significant only for AGD, ASD and PW in boys (β*100: -27.3, -28.7, -24.3, $p<0.005$ respectively). In crude results the association is negative, where most mothers belonging to the high fat intake level, have children with lower anogenital ratios, regardless of sex. In adjusted for pregnancy related characteristics models, the pattern is reported mainly for AGD and ASD ratios, of boys (β*100: -26.1, -25.6, -25, $p<0.005$ respectively). In the case of adding infant's variables correlation is still negative, though weak and not statistically significant. In addition, focusing on newborns (0-7 days) depicts that is more likely for an infant to be born with short AGD and ASD for a boy, or ACD and AFD for a girl, if his/her mother has high fat intake, compared with pregnant women with low fat intake.

Following the same trend, mothers with high consumption of fatty food groups are more likely to give birth to a boy with short anoscrotal distance, compared with mothers of low consumption (OR_{crude},adj_1=2) .

Likewise, it is 2 to 3 times more possible for a girl to be born with short anofourchetal distance, if her mother consumes such food items in a high level (OR_{crude}=2, adj_1= 3.08). Fatty group intake and anogenital distance are not strongly related, possibly due to the variety of food items included. Different composition in macro- and micronutrients could be the reason of overlapping any association as the exposure variable is not clear.

Intake of red meat and dairy products during pregnancy has been reported as potential source of environmental exposure and a mediator affecting the birth outcome (Halldorsson TI, 2008; Huang MC, 2007), while, maternal milk intake during pregnancy has been associated with a reduced risk of small-for-gestational-age infant (Olsen SF, et al., 2007). The contrast might be explained due to the fact that dietary pattern do not always reflect actual nutrient intake, namely dairy products consumption may not contribute much in the total fat intake (Northstone K., 2008). According to our results, red meat and dairy products consumption are not strongly associated with anogenital distance of the child, even if adjust for covariates. However, it is remarkable that if mother consumes red meat and dairy products in a high level, infant's possibilities to be born with short anofourchetal distance for girls are 2 to 3 times higher (OR_{crude}=2, adj_1= 3.08).

In the current study, we have conducted a pilot analysis of 22 participants, investigating the associattion of maternal diettary patterns and dioxin-like activity in blood samples, detected by DR-Calux assay. By bivariate associattion we have seen that fat intake is negatively associatted with dioxin and dioxin-like compounds (-0.275). It is worthmentioned that red meat and dairy consumption is higher associatted than fatty group consumption (-0.08 and -0.01 respectively), though nothing statistically significance. An explanation could be that dairy consumption has been identified as a major contributor to the daily intake of dioxins and planar PCBs, reflecting 45-53% of total exposure (Huisman M., 1995; Liem AK., 2000). The association presented is based in very few measurements and in not very informative. Future analyses of samples will help evaluate this association in Crete and other European cohorts participating in the same study.

The biological pathway which can explain the association indicated by our results have not been extensively explored yet, while maternal diet as source of in-utero exposure in environmental chemicals has been a major area of research. Such research started in the early 1990, when a generalized decrease in sperm quality was identified and was attributed to higher exposure in recent years to chemicals with endocrine functions.

Animal models of proposed endocrine disruptors have associated prenatal exposure with hypospadias, cryptorchidism, and reduced anogenital distance. Disruption is attributed in the mediation at the androgen signaling pathway (Jin MH., 2008) Human studies have correlated shorter anogenital distance, especially for boys, to in utero exposure to phthalates and DDT metabolites. (Hsieh MH, 2008; Longnecker MP., 2007; Torres-Sanchez L, 2008).

The physiological and functional meaning of changes in anogenital distance is unknown, this measure appears to be a permanent characteristic as a phenotype), thus, a very good marker of the in-utero exposure to endocrine disrupters. Our study is at an initial phase and will be completed with more anogenital measurements and chemical analyses. We hope that this study will contribute identify the adverse effects to the child at exposure to these chemicals and help present such exposures in the population.

6. REFERENCES

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7. APPENDIX

- Recording form for anthropometric measurements in male infants

- Recording form for anthropometric measurements in female infants

Κλινική εξέταση Αγοριών

Κωδικός βρέφους:

Ονοματεπώνυμο μητέρας:

Τηλ.

Κωδικός μητέρας:

Ημερομηνία εξέτασης:
Ημερομηνία γέννησης:

Εξεταστής: ……..
Ηλικία βρέφους: …….
Φυλή……..
Εθνικότητα: ……..

ΚΛΙΝΙΚΗ ΕΞΕΤΑΣΗ ΑΓΟΡΙΩΝ
ANOGENITAL PROTOCOL

Anogenital distance

Πρωκτός-κατώτερη οσχεική (ASD)

1η……..mm

2η……mm

(σε απόκλιση>1mm:)

3η…..……mm

Πρωκτός-άνω βάση πέους (AGD)

1η……mm

2η…….mm

(σε απόκλιση>1mm:)

3η…….mm

Πλάτος πέους:

1η ……..mm

2η……mm

(σε απόκλιση>1mm:)

3η……mm

Πάχος δερματικής πτυχής

Υποπλατιαία

1η……mm

2η……..mm

3η……..mm

Τρικέφαλου

1η ……..mm

2η……..mm

3η ……mm

Υπερλαγόνια

1η……mm

2η……mm

3η……mm

Τετρακέφαλου

1η……..mm

2η……mm

3η……mm

Κοιλιακή περίμετρος: 1η……..cm
$2^{η}$……cm
(σε απόκλιση>1 cm:)
$3^{η}$……cm

Περίμετρος κεφαλής: $1^{η}$……..cm
$2^{η}$……cm
(σε απόκλιση>2 cm:)
$3^{η}$……cm

Μήκος: $1^{η}$……cm
$2^{η}$……cm
(σε απόκλιση >1 cm:)
$3^{η}$…….cm

Βάρος: $1^{η}$……..gr
$2^{η}$…….gr
(σε απόκλιση> 10 gm:)
$3^{η}$…….gr

Διαγνώσεις σχετικά με το βρέφος: ρωτάμε την μητέρα και κοιτάμε και το γαλάζιο βιβλιαράκι υγείας του παιδιού.

Κωδικός βρέφους:……

Αποκλειστικός θηλασμός:

Ναι…… Όχι……

Δύσμορφα χαρακτηριστικά:

Ναι…… Όχι…….

Η συνεργασία του βρέφους στις Πρωκτογεννητ. Μετρήσεις ήταν:

Πολύ καλή…1
Καλή…….2
Μέτρια……..3
Κακή…….4

Η αξιοπιστία των Πρωκτογεννητ. Μετρήσεων είναι κατά τη γνώμη σας:

Υψηλή……1
Γενικά αξιόπιστη……2
Με ερωτηματικά…….3
Μη ικανοποιητική…..4

Η αξιοπιστία των μετρήσεων των δερματ. Πτυχών είναι κατά τη γνώμη σας:

Υψηλή……1
Γενικά αξιόπιστη……2
Με ερωτηματικά…….3
Μη ικανοποιητική…..4

Οι μετρήσεις έγιναν με τη βοήθεια:
Συνεργάτριας της μελέτης……1
Μητέρας ή άλλου μέλους της οικογένειας…2
Νοσοκομειακό προσωπικό……3
Χωρίς άλλο άτομο……..4

Κλινική εξέταση κοριτσιών

Κωδικός βρέφους:

Κωδικός μητέρας:

Τηλ.

Εξεταστής:

Ονοματεπώνυμο μητέρας

Ηλικία Βρέφους:

Ημερομηνία εξέτασης:

Φυλή:

Ημερομηνία γέννησης:

Εθνικότητα

Κλινική εξέταση κοριτσιών
Anogenital Protocol

Anogenital distance

Πρωκτός-χαλινός (AFD) 1η……..mm

$2^{η}$……..mm

(σε περίπτωση απόκλισης πάνω από 1mm)

$3^{η}$…….mm

Πρωκτός- κλειτορίδα (AGD) 1η ……mm

$2^{η}$……mm

(σε περίπτωση απόκλισης πάνω από 1mm)

$3^{η}$…….mm

Anogenital ratio (AFD/AGD)=

Πάχος δερματικής πτυχής

Υποπλατιαία

1η…….mm

$2^{η}$…….mm

$3^{η}$…….mm

Τρικέφαλου

1η ……..mm

$2^{η}$……..mm

$3^{η}$…….mm

Υπερλαγόνια

1η…….mm

$2^{η}$…….mm

$3^{η}$…….mm

Τετρακέφαλου

1η……..mm

$2^{η}$…….mm

$3^{η}$…….mm

Κοιλιακή περίμετρος: 1η……..cm $2^{η}$……cm (σε απόκλιση> 1cm:) $3^{η}$……cm **Περίμετρος κεφαλής:** $1^{η}$……..cm $2^{η}$……cm (σε απόκλιση>2cm): $3^{η}$……cm **Μήκος:** $1^{η}$……cm $2^{η}$……cm (σε απόκλιση>1cm:) $3^{η}$…….cm **Βάρος:** $1^{η}$……..gr $2^{η}$…….gr (σε απόκλιση πάνω από 10 gm :) $3^{η}$…….gr **Διαγνώσεις σχετικά με το βρέφος: ρωτάμε την μητέρα και κοιτάμε και το γαλάζιο βιβλιαράκι υγείας του παιδιού.**	Κωδικός βρέφους: **Αποκλειστικός θηλασμός:** Ναι…… Όχι…… **Δύσμορφα χαρακτηριστικά:** Ναι…… Όχι……. **Η συνεργασία του βρέφους στις Πρωκτογεννητ. Μετρήσεις ήταν:** Πολύ καλή…1 Καλή…….2 Μέτρια……..3 Κακή…….4 **Η αξιοπιστία των Πρωκτογεννητ. Μετρήσεων είναι κατά τη γνώμη σας:** Υψηλή……1 Γενικά αξιόπιστη……2 Με ερωτηματικά…….3 Μη ικανοποιητική…..4 **Η αξιοπιστία των μετρήσεων των δερματ. Πτυχών είναι κατά τη γνώμη σας:** Υψηλή……1 Γενικά αξιόπιστη……2 Με ερωτηματικά…….3 Μη ικανοποιητική…..4 **Οι μετρήσεις έγιναν με τη βοήθεια:** Συνεργάτριας της μελέτης……1 Μητέρας ή άλλου μέλους της οικογένειας…2 Νοσοκομειακό προσωπικό……3 Χωρίς άλλο άτομο……..4