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ΜΕΤΑΠΤΥΧΙΑΚΗ ΕΡΓΑΣΙΑ

«Exposure to compounds with dioxin-like activity in pregnant women, maternal socio-demografic and lifestyle factors and anogenital distance-mother-child cohort in Crete»

Βαφειάδη Μαρίνα
Βιολόγος

Επιβλέποντες:
1. **Ε. Κογεβίνας,** Καθηγητής, Τομέα Κοινωνικής Ιατρικής, Τμήμα Ιατρικής, Παν. Κρήτης
2. **Α. Κούτης,** Δρ. Κοινωνικής Ιατρικής, Αν. Δ/ντης ΕΣΥ, Τομέας Κοινωνικής Ιατρικής ΠΑ.Γ.Ν.Η

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Στους γονείς μου

Ευχαριστίες

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Της: Μαρίνας Βαφειάδη

Υπό τη επίβλεψη των: 1. Κογεβίνα Εμμανουήλ

2. Κούτη Αντωνίου

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Εισαγωγή: Η έκθεση σε περιβαλλοντικούς μολυντές, οι οποίοι έχουν οιστρογονική ή αντι-ανδρογονική δράση, επεμβαίνοντας στο μονοπάτι σηματοδότησης των ανδρογόνων, έχει συνδεθεί με αναπαραγωγικά προβλήματα. Οι διοξίνες και τα μόρια με παρόμοια δράση δρουν ως ορμονικοί διαταρράκτες. Παρόλο που η πρωκτογεννητική απόσταση χρησιμοποιείται ευρέως σε μελέτες σε ζώα για την μελέτη της ανδρογονικής δράσης, έχει χρησιμοποιηθεί ελάχιστα σε μελέτες σε ανθρώπους.

Στόχοι: Ο βασικός στόχος της παρούσας μελέτης ήταν ο σχεδιασμός και η εφαρμογή ενός πρωτοκόλλου ανθρωπομετρήσεων συμπεριλαμβανομένων των πρωκτογεννητικών αποστάσεων αλλά και η συσχέτιση κοινωνικό-δημογραφικών χαρακτηριστικών και τρόπου ζωής των εγκύων με την πρωκτογεννητική απόσταση των απογόνων τους.

Μέθοδοι: Αρχικά πραγματοποιήθηκε μια πιλοτική μελέτη σε 9 βρέφη. Στη συνέχεια πραγματοποιήθηκαν μετρήσεις σε 305 βρέφη (158 αρσενικά, 147 θηλυκά) των οποίων οι μητέρες συμμετείχαν στη μελέτη μητέρας-παιδιού ΡΕΑ.

Αποτελέσματα: Σχεδιάστηκε και εφαρμόστηκε ένα αναλυτικό πρωτόκολλο για τις ανθρωπομετρήσεις. Η απόσταση από το κέντρο του πρωκτού ως τη βάση του όσχεου στα αγόρια(διάμεσος, 31.54mm) βρέθηκε σχεδόν 2 φορές μεγαλύτερη από την απόσταση από το μέσο του πρωκτού ως το χαλινό στα κορίτσια(διάμεσος, 16.66mm). Η πρωκτογεννητική απόσταση του βρέφους σχετίζεται με την ηλικία της μητέρας και με κοινωνικο-δημογραφικούς παράγοντες όπως η κατοικία και ο τοκετός σε δημόσια ή ιδιωτικά μαιευτήρια.

Συμπεράσματα: Η ανάπτυξη ενός καλά σταθμισμένου πρωτοκόλλου στη μελέτη Ρέα κατέστησε δυνατή την μέτρηση των πρωκτογεννητικών παραμέτρων. Ο φυλετικός διμορφισμός στην πρωκτογεννητική απόσταση στους ανθρώπους υποδεικνύει ότι ο φαινότυπος αυτός ίσως είναι αποτέλεσμα ενδομητριακής έκθεσης σε ορμονικά ενεργές ενώσεις. Η πρωκτογεννητική απόσταση ίσως σχετίζεται με διαφορετικές οδούς έκθεσης σε χημικές ουσίες ανάλογα με την κοινωνικο-οικονομική θέση.

Λέξεις Κλειδιά: πρωκτογεννητική απόσταση, διοξίνες, ορμονικοί διαταρράκτες, εγκυμοσύνη, ενδομητριακή έκθεση, κοινωνικο-δημογραφικοί παράγοντες

Abstract

Title: Exposure to compounds with dioxin-like activity in pregnant women, maternal socio-demographic and lifestyle factors and anogenital distance - mother-child cohort in Crete.

By: Marina Vafeiadi

Supervisors: 1. Manolis Kogevinas
2. Antonis Koutis

Date: June 2009

Background: Exposures to environmental pollutants that have estrogenic or anti-androgenic action interfering to the androgen signaling pathway, have been linked to adverse reproductive outcomes. Dioxins are considered potent endocrine disruptor compounds. Although anogenital distance is commonly used as a measure of fetal androgen action in animal studies, it has been scarcely used in humans.

Objectives: The main objective of the study was the development of an assessment protocol for anthropometric measurements including anogenital distance and the association of maternal socio-demographic and lifestyle factors with anogenital distance.

Methods: A pilot study was initially conducted in 9 infants. Measurements were taken on an additional 305 infants (158 males, 147 females) whose mothers were participants of the 'Rhea' mother and child-cohort study.

Results: An analytical measurement protocol for anthropometric measurements was assessed. The anoscrotal distance (ASD) measure was about two-fold greater in males (median, 31.54mm) than the AFD in females (median, 16.66mm). Anogenital distance was associated with maternal age and with socio-economic factors such as residence and birth in private or public maternity clinics.

Conclusion: The development of a well standardized protocol has shown that it is possible to measure anogenital parameters in the Rhea study. The sexual dimorphism of anogenital distance in humans suggests that this phenotype may respond to *in utero* exposure to hormonally active agents. Anogenital distance may be related to different patterns of exposure to chemicals by socio-economic position.

Key words: anogenital distance, dioxins, endocrine disruptors, pregnancy, *in utero* exposure, socio-economic factors.

1. Background

In the past few years, the scientific community has studied with big interest the effects of environmental pollutants on human health. Such pollutants include dioxins, which consist of polychlorinated dibenzodioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs). Some of these, specially 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), are among the most toxic compounds that are known [1].

Other environmental pollutants such as polychlorinated biphenyls (PCBs) present similar properties to dioxins. The term "dioxins" refers to a group of dioxin-like chemical compounds that share similar chemical structures. Of these chemicals, 7 dioxins, 10 furans, and 12 dioxin-like PCBs (dl-PCBs) containing 4 to 8 chlorine atoms pose a major health risk. The toxicities of these compounds vary over more than five orders of magnitude and largely depend on the number and position of the chlorine atoms.

The overall toxicity of such a complex mixture is generally expressed as a 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD) Toxic Equivalent (TEQ) concentration, where 2,3,7,8-TCDD is the most toxic of these 29 dioxin and dioxin-like compounds. A Toxic Equivalency Factor (TEF) of 1 is given to 2,3,7,8-TCDD, and the TEFs for the other 28 congeners, which generally range from 0.00001 to 1, are assigned based on their toxicity relative to the toxicity of 2,3,7,8-TCDD. A TEQ is calculated by summing the multiplication of congener concentrations with congener-specific TEFs [2]. The body burden is often used for risk assessment purposes. It is defined as the concentration of 2,3,7,8-tetrachloro-dibenzodioxin (TCDD) and related compounds in the body, expressed as ng kg^{-1} body weight, and it is a useful dose metric to estimate potential effects on humans following exposure to dioxins, as the body burden is a function of the uptake, distribution, metabolism, and excretion of this complex mixture of related compounds. In humans the body burden is estimated either by taking into account the intake rate and the half-life of TCDD or on the basis of lipid adjusted toxicity equivalent (TEQ) concentration in serum or in adipose tissue [3].

PCDD/Fs are emitted via incomplete combustion processes from both anthropogenic and natural sources like waste incineration, chemical production, metal industry and to a small extent by forest fires and volcanic eruptions. In contrast, PCBs are released to the environment mainly via waste disposal. PCBs were produced

commercially since the 1920s, and were used in various applications, namely the manufacture of electronic appliances. Since dioxins and PCBs are lipophilic compounds, they accumulate in the food chain. Contamination of foods occurs through deposition of contaminated emissions on farmland, while fish accumulate dioxins through contamination of the aquatic environment [4]. PCDDs, PCDFs and dioxin-like PCBs have long half-times in the body and will therefore accumulate in the body during continuous exposure. Exposure through food is the main source of dioxin exposure for humans, estimated at over 95% of the total intake for non-occupationally exposed persons [5]. More specifically, foods with high content in fat, such as dairy products, meat and fish, are these with the higher concentration in dioxins.

The toxicity of PCDDs, PCDFs and dioxin-like PCBs (referred to as dioxins from this point forward) is mostly traced to their blocking of the aryl hydrocarbon receptor (AhR) present in all mammalian species, including humans [6] in part by modulating estrogen and androgen signaling [7]. AH receptor, upon exposure to TCDD, translocates into the nucleus, where it heterodimerizes with AH receptor nuclear translocator (ARNT). This complex then binds to its specific DNA recognition sites to activate the transcription of dioxin responsive genes [8]. The molecular properties of the Ah receptor are similar to those described for the steroid and thyroid hormone receptor superfamily, which are zinc finger-DNA binding proteins that act as ligand-induced transcription factors (LTFs) for transactivation of target genes. The Ah receptor gene has been cloned and sequenced and results show that the Ah receptor is a helix-loop-helix (HLH) DNA binding protein [9]. The AhR induces expression of direct target genes such as the drug metabolizing enzymes CYP1A1 and CYP1A2. AhR exhibits other regulatory functions by modulating the function of other transcription factors, including Rb/E2F, NF-κB, and the estrogen (ERa and ERb) and androgen (AR) receptors. Additionally complexes of the AhR with ERs or AR appear to regulate transcription as functional units by multiple mechanisms [10].

The toxic effects of dioxins have been extensively studied in several animal studies. TCDD affects bone growth, modeling and mechanical strength in vivo. Differentiation of osteoblasts and osteoclasts from bone marrow stem cells seems to be a very sensitive target for TCDD. Disrupting effects in osteoblastic cells, in addition to disturbed osteoclastogenesis, may thus play a role in adverse effects on bone quality in TCDD exposed animals [11]. *In-utero* and lactational TCDD

exposures alter cardiac gene expression and cardiac and renal morphology in adulthood, which may increase the susceptibility to cardiovascular dysfunction in mice [12]. In mammals dioxin exposure also leads to hepatotoxicity of varying severity and several studies have shown that dioxins extensively alter hepatic mRNA levels [13].

A wide variety of effects in human health have been associated with dioxin exposure. Exposure to high levels of dioxins in humans causes a severe form of persistent acne, known as chloracne. Diabetes, thyroid disorders, damage to the immune, gastrointestinal, respiratory and urinary systems, cardiovascular diseases and effects on the reproductive hormones and function are also some of the known effects caused by dioxins. In 1997, the International Agency for Research on Cancer (IARC) -- part of the World Health Organization -- published their research into dioxins and furans and announced on February 14, 1997, that the most potent dioxin, 2,3,7,8-TCDD, is a now considered a Group 1 carcinogen, meaning a "known human carcinogen." Also, in January 2001, the U.S. National Toxicology Program upgraded 2,3,7,8-TCDD from "Reasonably Anticipated to be a Human Carcinogen" to "Known to be a Human Carcinogen." Finally, a 2003 re-analysis of the cancer risk from dioxin reaffirmed that there is no known "safe dose" or "threshold" below which dioxin will not cause cancer.

Maternal exposure to dioxins and dioxin-like compounds has been related to a number of adverse health outcomes in infants [6]. The fetuses and neonates are believed to be more vulnerable to the effects of environmental pollutants as their organs and detoxification enzymatic systems are relatively immature [14]. Infant's exposure starts *in utero*, through the placenta, and continuous postnatally through breast feeding. Exposure to persistent organic pollutants (POPs) *in-utero* has been linked to various adverse effects on developing fetuses including intrauterine growth retardation (IUGR), neurocognitive deficits and hormonal dysfunctions [15]. It has been suggested by several human studies that *in-utero* exposure to environmental pollutants might have contributed to the reduction of sperm numbers, to the increase of incidence in malformations of the reproductive system and to the sex ratio alteration during the last 40-50 years. The underlying hypothesis is that exposure to environmental pollutants that have estrogenic or anti-androgenic action, interfering to the androgen signaling pathway, might be responsible for the elevated incidence of the changes mentioned above.

Anogenital distance is the distance from the anus to the base of the scrotum in males and from the anus to the base of the genitals (the fourchette) in females. In animal experiments, anogenital distance is used as a measure of fetal androgen action. Anogenital distance usually tracks through life, varies by dose of antiandrogen, and can be predictive of other androgen-responsive outcome[16].In rodents, perineal growth is dihydrotestosterone-dependent, males have a greater AGD than females, and use of AGD to sex newborns is often [17]. The anogenital distance is scarcely used in human studies [16, 18-22]. Results show that prenatal phthalate exposure at environmental levels may adversely affect male reproductive development in humans and that *in-utero* exposure to phthalates in general may have anti-androgenic effects on the fetus. Therefore, the necessity for further study on environmental pollutants is apparent in order to clarify their possible adverse health effects on the human reproductive system**.**

To date, analytical methods to quantify exposure to dioxins and dioxin-like compounds in biologic samples have included very sensitive and specific techniques such as high-resolution gas chromatography/high-resolution mass spectrometry (HRGC/HRMS). However, these methods are time-consuming and expensive; require large sample volumes and extensive sample clean-up. Exposure to dioxins and dioxin-like compounds can be estimated with the Calux bioassay, which is less expensive and quicker. The CALUX methodology (reporter gene assay) is a mechanistically based technique that detects all compounds that can activate the aryl hydrocarbon receptor (AhR) and AhR-dependent gene expression (i.e., AhR agonists). Among them dibenzodioxins, dibenzofurans and polychoro biphenyls are target compounds. These compounds are lipophilic and cross the cell membrane, presumably by diffusion; they bind to the AhR and activate the receptor. The dioxin-AhR complex then travels to the nucleus of the cell and binds to specific sequences in the DNA called dioxin responsive elements (DRE). The binding of the dioxin-AhR complex to the DRE causes the expression of the associated genes to be altered. It is this alteration in gene expression that causes the toxic effects observed.

Genetically engineered cell lines have been created which use this regulatory system to inducibly express other genes not ordinarily under control of the AhR system, such as the luciferase gene from the firefly. In the CALUX assay, mouse or rat hepatoma cell lines are used which contain a stably transfected vector (the pGudLuc1.1 vector) containing the firefly luciferase gene under transcriptional

control of several DREs. The resulting recombinant cell lines respond to dioxin exposure with the induction of luciferase, which in the presence of its substrate produces a luminescent signal proportional to the cells' response. The measured luminescence is converted into a TEQ value by direct comparison of the response for a given sample to a dose response curve obtained with 2,3,7,8-TCDD. The results are presented as CALUX-TEQs in pg/g lipid.

The Mother-child cohort in Crete (Rhea study) is prospectively examining a population sample of approximately 1500 pregnant women and their children, at the prefecture of Heraklion. Pregnant women (Greek and immigrants) residents at the prefecture of Heraklion that become pregnant during one year starting in February 2007 have been contacted and participate in the study. The main health problems that are evaluated in the study are reproductive outcomes (intrauterine growth, early births and SGA), allergies and asthma, infant and child neurodevelopment and behaviour, child development and obesity, metabolic syndrome, infections, thyroid function and thyroid diseases, and postnatal depression of the mothers. The Rhea study provides a strong study design to study the risk factors of several child and mother's health outcomes, including in-utero exposure to dioxins and dioxin-like compounds and ano-genital distance during the first year of life.

The main aim of this project was the development of an assesment protocol for the anthropometric measurements, including for anogenital distance, in children of the RHEA cohort. Further aims were to examine:

- The association of the anogenital distance with basic maternal socio-demographic and lifestyle factors.
- The association between infant's *in-utero* exposure to dioxins and dioxin-like compounds and the anogenital distance at one year.

2. Materials and Methods

2.1. The birth cohort

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 in private maternity clinics during a period of 1 year(October 2007-Oktober 2008). Pregnant women were first contacted at the 12th to 14th week of pregnancy, and then were re-contacted at the 28th to 32th week and on the time of delivery. After birth, meetings were scheduled in order to measure the newborn's development. In the Rhea cohort data have been collected from the mother (socio-demographic, lifestyle, medical history, environmental and nutrition), the child (clinical examination) and biological samples (maternal and cord blood, maternal and child urine).

2.2. Sample selection

The current study is based on 305 pregnant women selected from the 'Rhea' birth cohort and the infants that they delivered (paired sampled) for whom anogenital distance has been measured in the children.

2.3 Data collection

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

2.4. Blood sample collection

The maternal and the cord blood samples for the biochemical analysis were collected by midwifes at the hospitals right after delivery. The samples were shipped overseas and analyzed by BioDetection Systems B.V (Amsterdam, Netherlands) supervised by Dr. Ir. Harrie Besselink.

2.5. The Calux bioassay

There are three kinds of calux bioassays: The DR-Calux which is responsive to arylhydrocarbon receptor and the ER and AR calux responsive to estrogen receptor α and androgen receptor respectively.

DR CALUX®

Approximately 1-2 grams of human plasma was extracted by means of shake-solvent extraction (hexane:diethylether, 97:3). Extracted fat was used for clean-up on an acid silica column (20% and 33% H2SO4), topped with sodium sulphate. Cleaned extracts were dissolved in DMSO (8 μl); the DR CALUXR activity is determined following 24 hrs of exposure. Data were corrected for internal reference sample and procedure blank. Total lipid content was determined gravitameticaly. (BDS protocols: p-bds-042 ; p-bds-005 ; p-bds-042 ; p-bds004 ; p-bds-007)

ERα/AR CALUX®

0.5 ml of human plasma was extracted by means of shake-solvent extraction (MTBE (methyltertiairbutylether)). In case extracts are unclear, extracts were cleaned-up. Extracts were dissolved in DMSO (40 μl); the ER and AR CALUXR activity was determined following 24 hrs of exposure at various dilutions of the redisolved extracts. (BDS protocols: p-bds-006; p-bds-025; p-bds-045; p-bds-039; p-bds-055; p-bds-056)

2.6. Anthropometric measurements

2.6.1. Pilot study

A pilot study was initially conducted, before doing the 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 Heraklion in Crete. Five persons participated including 3 medical doctors, a dietitian-nutritionist (Eleni Zoumpoulia Papadopoulou) and a biologist(Marina Vafeiadi) In each clinic the measurements took place in a particular examination room.

The pilot sample included infants aged less than a year that were hospitalized for more than 1 day at the clinics. The sample was selected in a way that no infants with serious health conditions participated and they were all selected from non-participants at the Rhea cohort study. All measurements were 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 some measurement details from the only other published study by Prof SH Swan in Rochester, USA [18]**.** Conference calls with researchers from Prof Swan's group were organized to discuss details of the protocol. A final protocol adapted to the situation in Crete was developed (see below).

2.6.2. Field study

The field study started in June 2008 and comprised of two parts. One part concerns measurements at the three maternity clinics of Heraklion-Crete by 3 examiners and more specifically the 3 medical doctors. The total sample included 164 newborns, delivered from June until September 2008, whose mothers had consented to participate in Rhea cohort study. According to the procedure, each of three clinics was assigned to one of the examiners, who had a list of all participants to 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 homes and is being conducted by the other 2 examiners (Eleni Zoumpoulia Papadopoulou, Marina Vafeiadi) who work together as a pair. At first, at a date and time convenient for the mother and the examiners, an appointment was programmed, resulting to 3-5 appointments per day. A total of 141 infants were measured from June 2008 until March 2009. The duration of each appointment was approximately 30 minutes and included 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.

2.7 Statistical analysis

For the statistical analysis of the data SPSS version 15.0 (Statistical Package for the Social Sciences) was used. Descriptive and summary statistics for all study variables were examined. For nominal variables percentages were calculated while median and interquartile range (IQR) was calculated for continuous variables. Anogenital distances were divided by infant weight in kilos in order to construct a weight-normalized index of these distances (anogenital ratios). Anogenital ratios were categorized as discrete variables with three groups: low anogenital ratio, medium anogenital ratio and high anogenital ratio. The first group (low anogenital ratio) includes values from the lowest anogenital ratio to the 25^{th} percentile, the second group (medium anogenital ratio) includes the values from the 25^{th} percentile to the 75^{th} percentile and the third group (high anogenital ratio) includes values from the 75^{th} percentile to the highest anogenital ratio. In order to explore the relationship between several maternal socio-demographic and lifestyle variables with the infant's anogenital ratios, crosstab and chi square analysis was conducted. Spearman's rank correlation coefficient was used in order to assess the association between maternal age and anogenital ratios. In order to explore possible differences regarding to anogenital ratios between different residency and maternity clinic, Wilcoxon-Mann-Whitney test was used. One-way analysis of variance was used in order to explore possible differences regarding to anogenital ratios between different methods of contraception. General linear models were used to explore the relationships between maternal socio-demographic and lifestyle variables with the infant's anogenital ratios. Finally bivariate correlation analyses (Spearman's rank correlation coefficient) was used to assess the relation between maternal CALUX-TEQs and anogenital ratios.

3. Results

3.1. 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 the pilot 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). Therefore, this should be considered a limitation of the training mainly due to small hospitalization period of the children, some time-restrictions that were dictated by hospital personnel and the lower cooperation of the child as measurement time passed.

Results of anthropometric measurements for the total sample (9 infants) are presented in table 1. Mean anogenital (AGD-the distance from the middle of the anus to the top of the penis) and mean anoscrotal distance (ASD the distance from the middle of the anus to the bottom of the scrotum) of male infants are smaller than the mean anoclitoris (ACD the distance from the middle of the anus to the clitoris) and mean anofourchettal distance (AFD the distance from the middle of the anus to the bottom of the labia majora) of female infants, respectively. Apart from anogenital distances, other measurements such as skinfold thickness and growth measurements were conducted.

Table 1: Distribution of measured characteristics in 9 infants

	Males (n=5)				Females (n=4)			
Measurement	**Min**	**Median**	**Max**	**SD[a]**	**Min**	**Median**	**Max**	**SD[a]**
Age	0.8	5	11	4.58	0.8	0.8	0.8	0.0
AFD(mm)	-	-	-	-	12.61	16.48	21.57	2.95
ASD(mm)	26.93	33.63	42.44	5.08	-	-	-	-
PW(mm)	14.04	14.25	14.48	0.15	-	-	-	-
AGD(mm)	61.09	65.98	76.71	3.96	32.73	39.14	50.00	5.74
Triceps(mm)	6.5	8.96	10.33	1.06	7.00	8.25	11.17	1.39
Quadriceps(mm)	11.17	15.26	17.67	1.88	9.50	14.80	21.00	3.78
Suprailiac(mm)	4.50	5.95	7.00	0.80	3.17	5.11	7.00	1.29
Subscapular(mm)	5.17	6.63	8.83	0.97	6.33	7.75	9.33	1.00
Length(cm)	56.75	62.83	75.00	10.53	53.5	54.00	54.50	0.71
HC(cm)	39.5	41.81	46.5	2.57	34.00	36.74	38.00	1.65
AC(cm)	36.5	38.21	41.00	1.55	33.00	35.24	38.00	1.65

a: standard deviation

3.2. Protocol development

The measurement protocol which was developed following the pilot study is described below.

The order of measurements demonstrated is not according to the field procedure, which is designated by infant's convenience and the maximum cooperation. At first, we measure the anogenital distances, penis, quadriceps, suprailical skinfold thickness and abdominal circumference for which the infant has to be naked and lay down. After these measurements, the assistant embraces the infant and the examiner measures triceps, 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:

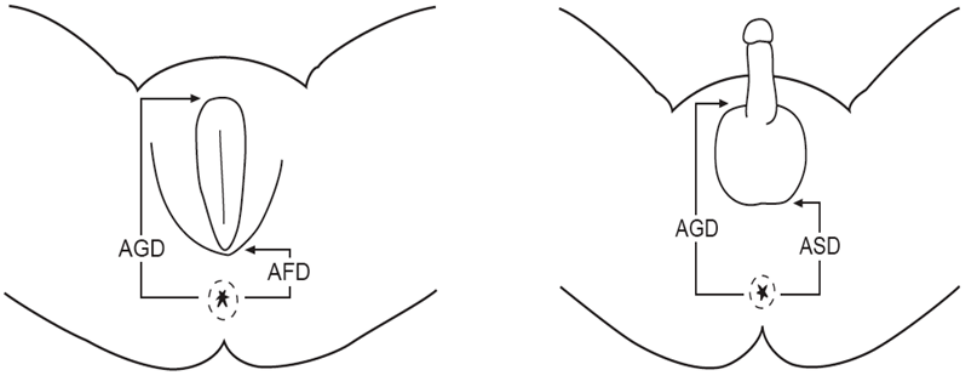
- Stable surface or examination table
- Electronic weight scale for infants
- Infant height measuring board (kiddimetre)
- Digital caliper => TESA-CAL IP67
- Skinfold thickness caliper => Lange caliper
- Non stretchable measuring tape
- Pen
- Recording form

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 the 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 anofourchettal 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: Schematic Diagram of Measurements Done, by Sex



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 calliper is placed 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, by palpating with his left index, the right iliac crest and then drags his left thumb until the point traversed by the midclavicular line. Using these two fingers, he 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 this 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. The 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, while 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.

3.3 Study results

3.3.1 Maternal characteristics

Several variables related to maternal socio-demographic status and life-style were examined. As presented in the table below the majority of mothers were living in urban areas (80%), were married (89%) and were Greek (94%). Most of the mothers were medium-educated (52%) and gave birth in public maternity clinics (68%), while almost half of them (49%) worked during pregnancy. Concerning to maternal life-style the majority of mothers did not smoke during pregnancy (82%), did not dye their hair during pregnancy (89%), and did not use pesticides at home (68%). More than half (65%) used cosmetics during their pregnancy.

Table 2: Maternal descriptive socio-demographic and life-style characteristics

	n	%
Residence		
Urban	**240**	**79.7**
Rural	**61**	**20.3**
missing	**4**	
Nationality		
Greek	**287**	**94.4**
Albanian	**10**	**3.3**
Bulgarian	**2**	**0.7**
Romanian	**3**	**1.0**
Other	**2**	**0.7**
missing	**1**	
Marital Status		
Married	**255**	**88.5**
Engaged	**27**	**9.4**
unmarried	**5**	**1.7**
other	**1**	**0.3**
missing	**17**	
Educational Status		
Low	**57**	**19.9**
Medium	**150**	**52.4**
high	**79**	**27.6**
missing	19	
Working During Pregnancy		
Yes	**142**	**49**
no	**148**	**51**
missing	**15**	
Smoking[a]		
Yes	**52**	**18.2**
no	**233**	**81.8**
missing	**20**	
Maternity Clinic		
Public	**208**	**68.2**
private	**97**	**31.8**
missing	0	
Hair-Dying During Pregnancy		
Yes	**32**	**11.2**
No	**253**	**88.8**
missing	20	
Cosmetics Use During pregnancy		

Yes	**187**	**65.4**
No	**99**	**34.6**
missing	**19**	
Pesticides Use at Home		
Yes	**90**	**31.8**
No	**193**	**68.2**
missing	**22**	

a: smoking during the whole pregnancy

The mean maternal age was 30 years and the mean maternal BMI during pregnancy was 29.17. Most of the mothers have been pregnant before (71%) and they breastfed their children (86%), while half (52%) of those mother which breastfed their children, breastfed exclusively. Finally 72.3% of the mothers did not use any contraception method around the time of conception and only 7.2% used contraception pill.

Table 3: Maternal descriptive health related characteristics

	n	**Mean**	**SD[c]**
Age(years)	**296**	**30.13**	**4.74**
BMI[a]	**282**	**29.17**	**4.27**
	n	**%**	
Pregnant Again			
Yes	**205**	**70.7**	
No	**85**	**29.3**	
Breastfeeding			
Yes	**243**	**86.2**	
No	**39**	**13.8**	
Exclusive breast feeding			
Never	**121**	**48.2**	
Ever	**130**	**51.8**	
Contraception			
None	**201**	**72.3**	
Pill	**20**	**7.2**	
Other	**57**	**20.5**	

a: BMI calculated with pregnancy weight
b: O.D.P. only during pregnancy
c: Standard deviation

3.3.2 Infant characteristics

As presented in table 4, the sample of 305 infants had an average age of approximately 4.4 months (132 days) and an average gestational age of 38 completed weeks. Half of the sample is 0 to 7 days old while the other half is 8 days to 16months old. Therefore the age distribution of the sample is not normal and this suggests that

age should be an important covariate for the further analysis. Sex is equally distributed in the sample since 52% of the infants are males and 48% are females.

Table 4: Gestational age, infant age and sex of the infants in the sample

	n	Mean	SD[c]
Age (days)	305	132.08	156.3
Gestational age (weeks)	302	38.32	1.33
	n	**%**	
Age			
0 to 7 days old	155	50.8	
Older than 7 days old	150	49.2	
Sex			
Male	158	52	
Female	147	48	

Information concerning birth outcomes, such as preterm births, small for gestational age and low birth weight are presented in table 5, for male and female infants separately. For both sexes the majority of infants are neither preterm nor small for gestational age.

Table 5: Birth outcomes organized by infant's sex

	Males		Females	
	n	**%**	**n**	**%**
SGA[a]				
Yes	5	3.2	7	4.8
No	150	96.8	140	95.2
Low birth weight[b]				
Yes	9	5.7	9	6.1
No	149	94.3	138	93.9
Preterm[c]				
Yes	14	9	12	8.2
No	141	91	135	91.8

a: small for gestational age, BW below 10 percentile
b: low birth weight, <2500gr
c: gestational age <37 weeks

Table 6: Infant's age, anthropometric measurements and skinfold thickness measurements organized by sex

	Males			Females		
	n	**mean**	**SD[e]**	**n**	**mean**	**SD[e]**
Gestational age (weeks)	155	38.25	1.48	147	38.40	1.24
Weight (gr)	157	6092.72	3233.222	146	5624.00	3145.033
Length (cm)	158	61.07	11.97	147	58.92	11.07
Head circumference (cm)	157	39.97	5.72	147	38.86	5.38
Abdominal circumference (cm)	158	37.41	8.41	147	36.60	6.31
STFI [a] (mm)	158	4.88	2.39	147	5.11	2.83
STQUAD [b](mm)	158	12.32	7.46	147	12.33	7.77
STTR [c](mm)	158	7.35	3.39	147	7.39	3.40
STSUB [d](mm)	158	5.39	1.79	147	5.60	2.03

a: suprailliac skinfold thickness
b: quadriceps skinfold thickness
c: triceps skinfold thickness
d: subscapular skinfold thickness
e: Standard deviation

Male infants have a median anogenital distance (AGD) equal to 58.94mm, median anoscrotal distance (ASD)equal to 31.54mm and penis width (PW) 12.20mm (table 7). Respectively, for female infants median anoclitoral distance (ACD) is 37.82mm, while median anofourchettal distance (AFD) is 16.66mm.

Table 7: Infant anogenital distances organized by sex

	Min	**Median(n)**	**Max**	**IQR[a]**
Males				
AGD	41.53	58.94(157)	96.56	27.68
ASD	17.96	31.54(158)	51.54	11.21
PW	9.33	12.20(156)	18.67	2.64
Females				
ACD	21.70	37.82(147)	63.17	9.21
AFD	8.42	16.66	33.33	7.32

a: interquartile range

Anogenital distances were divided by infant weight in order to construct a weight-normalized index of these distances (anogenital ratios). Because the distribution of anogenital ratios is skewed, median is presented in table 8. Male infants have a median anogenital ratio equal to 13.26, mean anoscrotal ratio to 6.41 and penis width ratio 2.88. Respectively, for female infants median anoclitoral ratio is 9.79, while mean anofourchettal ratio is 3.46.

Table 8: Infant anogenital ratios(anogenital distance/infant weight) organized by sex

	Min	Median(n)	Max	IQR[a]
Males				
AGD ratio	4.64	13.26(156)	21.59	7.21
ASD ratio	1.9	6.41(157)	12.48	4.50
PW ratio	1.03	2.88(155)	4.77	2.01
Females				
ACD ratio	2.07	9.79(146)	14.92	6.53
AFD ratio	0.84	3.46(146)	7.48	2.23

a: interquartile range

In order to explore the relationship between several maternal socio-demographic and lifestyle variables with the infant's anogenital distances (ratios), crosstab and chi square analysis was conducted. Anogenital ratios were categorized as discrete variables with three groups: low anogenital ratio, medium anogenital ratio and high anogenital ratio.

As seen in table 9, the statistics from the crosstab analysis concerning mother's residence, show that 89.5% of male infants with low AGD ratio were born by mothers who lived in an urban area while 10.5% of male infants with low AGD ratio were born by mothers who lived in a rural ratio. Since the p-value for Pearson's Chi-Square is <0.05 the relationship between residence of the mother and AGD ratio is statistically significant.

Likewise the crosstabs analysis for the ASD and PW ratio and residence showed that 86.8% of males with low ASD ratio were born by mothers living in an urban area while 13.2 % of males with low ASD ratio were born by mothers living in a rural area.

Similar results, concerning the PW ratio, were observed. 82.1% of males with low PW ratio were born by mothers who lived in an urban area whereas the 17.9% of males with low PW ratio were born by mothers that lived in a rural area. Neither of the two latter relationships was statistically significant.

Table 9: Residence * AGD, ASD and PW ratio Crosstabulation

		AGD ratio			
		low	**medium**	**high**	Total
Residence*	**urban**	34(89.5)	61(80.3)	25(64.1)	120(78.4)
	rural	4(10.5)	15(19.7)	14(35.9)	33(21.6)
	total	38(100)	76(100)	39(100)	153
		ASD ratio			
		low	**medium**	**high**	Total
Residence	**urban**	33(86.8)	61(79.2)	26(66.7)	120(77.9)
	rural	5(13.2)	16(20.8)	13(33.3)	34(22.1)
	total	38(100)	77(100)	39(100)	154(100)
		PW ratio			
		low	**medium**	**high**	Total
Residence	**urban**	32(82.1)	61(81.3)	25(65.8)	118(77.6)
	rural	7(17.9)	14(18.7)	13(34.2)	34(22.4)
	total	39(100)	75(100)	38(100)	152(100)

*: $p<0.05$

Similarly, the cross tab analysis for the female infants (table 10) showed that 75% of female infants with low AFD ratio and 86.1% of females with low ACD ratio were born by mothers that lived in an urban area. On the other hand 25% of females with low AFD ratio and 13.9% of females with low ACD ratio were born by mothers living in rural areas.80.8% of female infants with medium ACD ratio and 87.7% of female infants with low AFD ratio were born by mothers living in urban areas, whereas 19% of female infants with medium ACD ratio and 12.3% of female infants with medium AFD ratio were born by mothers living in rural areas. Regarding to high ACD and AFD ratio, 83.3% and 80% of female infants respectively were born by mothers that lived in urban areas, while 16.7% and 20% of female infants respectively, were born by mothers living in rural areas.

Table 10: Residence * ACD and AFD Crosstabulation

		ACD ratio			
		low	**medium**	**high**	Total
Residence	**urban**	31(86.1)	59(80.8)	30(83.3)	120(82.8)
	rural	5(13.9)	14(19.2)	6(16.7)	25(17.2)
	total	36(100)	73(100)	36(100)	145(100)
		AFD ratio			
		low	**medium**	**high**	Total
Residence	**urban**	27(75)	64(87.7)	28(80)	119(82.6)
	rural	9(25)	9(12.3)	7(20)	25(17.4)
	total	36(100)	73(100)	35(100)	144(100)

With regard to mother's educational status, the cross tab analysis showed that 26.3% of males with high AGD ratio and 28.2% of males with high ASD ratio were born by mothers with low educational status. 28.6% of male infants with low AGD ratio were born by mothers with high educational status. 52.6% of male infants with low PW ratio were born by mothers with medium educational status, while 26.3% of males with low PW ratio were born by mothers with high educational status.

Table 11: Mother's educational status * AGD, ASD and PW ratio Crosstabulation

		AGD ratio			
		low	**medium**	**high**	Total
Education	**low**	5(14.3)	14(18.7)	10(26.3)	29(19.6)
	medium	20(57.1)	41(54.7)	22(57.9)	83(56.1)
	high	10(28.6)	20(26.7)	6(15.8)	36(24.3)
	total	35(100)	75(100)	38(100)	148(100)
		ASD ratio			
		low	**medium**	**high**	Total
Education	**low**	6(16.2)	13(17,8)	11(28.2)	30(20.1)
	medium	20(54.1)	44(60.3)	19(48.7)	83(55.7)
	high	11(29.7)	16(21.9)	9(23.1)	36(24.2)
	total	37(100)	73(100)	39(100)	149(100)
		PW ratio			
		low	**medium**	**high**	Total
Education	**low**	8(21.1)	14(19.7)	8(21.1)	30(20.4)
	medium	20(52.6)	41(57.7)	21(55.3)	82(55.8)
	high	10(26.3)	16(22.5)	9(23.7)	35(23.8)
	total	38(100)	71(100)	38(100)	147(100)

Regarding female infants, results showed that 57.6% of female infants with low ACD ratio and 58.8% of female infants with low AFD (tables 12), were born by mothers with medium educational status. None of the relationships between mother's education and anogenital ratios was statistically significant

Table 12: Mother's educational status * ACD and AFD Crosstabulation

		ACD ratio			
		low	**medium**	**high**	Total
Education	**low**	3(9.1)	16(23.2)	7(21.2)	26(19.3)
	medium	19(57.6)	31(44.9)	16(48.5)	66(48.9)
	high	11(33.3)	22(31.9)	10(30.3)	43(31.9)
	total	33(100)	69(100)	33(100)	135(100)
		AFD ratio			
		low	**medium**	**high**	Total
Education	**low**	2(5.9)	15(22.7)	9(26.5)	26(19.4)
	medium	20(58.8)	28(42.4)	17(50)	65(48.5)
	high	12(35.3)	23(34.8)	8(23.5)	43(32.1)
	total	34(100)	66(100)	34(100)	134(100)

In order to explore the relationship between mother's nationality and anogenital ratios, cross tab and chi square analyses were conducted. Due to the sample's distribution (94.4% Greeks vs 5.6% others) no significant difference between nationality categories was observed.

Another variable, related to maternal socio-demographic status, examined is the maternity clinic that the infants were born in. The delivery of the infants took place in a public or a private maternity clinic. The cross tab and chi square analysis showed 84.6% of male infants with low AGD ratio, compared to 64.1% with medium or high AGD ratio, were born in a public maternity clinic. 84.6% of male infants with low ASD ratio, compared to 60.8% with medium and 71.8% with high, were born in a public maternity clinic. Since the p-value for Pearson's Chi-Square is <0.05 the relationship between the maternity clinic that that infant was born and the ASD ratio is statistically significant. Similarly, 82.5% of male infants with low PW ratio, compared to 52.6% with high PW ratio, were born in a public maternity clinic and this relationship is also statistically significant.

Table 13: Maternity clinic * AGD, ASD and PW Crosstabulation

		AGD ratio			
		low	**medium**	**high**	Total
Maternity clinic	**public**	33(84.6)	50(64.1)	25(64.1)	108(69.2)
	private	6(15.4)	28(35.9)	14(35.9)	48(30.8)
	total	39(100)	78(100)	39(100)	156(100)
		ASD ratio			
		low	**medium**	**high**	Total
Maternity clinic*	**public**	33(84.6)	48(60.8)	28(71.8)	109(69.4)
	private	6(15.4)	31(39.2)	11(28.2)	48(30.6)
	total	39(100)	79(100)	39(100)	157(100)
		PW ratio			
		low	**medium**	**high**	Total
Maternity clinic*	**public**	33(82.5)	55(71.4)	20(52.6)	108(69.7)
	private	7(17.5)	22(28.6)	18(47.4)	47(30.3)
	total	40(100)	77(100)	38(100)	155(100)

*: $p<0.05$

69.4% of female infants with low ACD ratio, compared to 63.5% with medium, and 72.2% of female infants with low AFD ratio, compared to 63% with medium, were born in a public maternity clinic (table 14). The relationship between the maternity clinic that the infant is born was not statistically significant with neither the ACD ratio nor the AFD ratio.

Table 14: Maternity clinic * ACD and AFD Crosstabulation

		ACD ratio			
		low	**medium**	**high**	Total
Maternity clinic	**public**	25(69.4)	47(63.5)	25(69.4)	97(66.4)
	private	11(30.6)	27(36.5)	11(30.6)	49(33.6)
	total	36(100)	74(100)	36(100)	146(100)
		AFD ratio			
		low	**medium**	**high**	Total
Maternity clinic	**public**	26(72.2)	46(63)	24(66.7)	96(66.2)
	private	10(27.8)	27(37)	12(33.3)	49(33.8)
	total	36(100)	73(100)	36(100)	145(100)

In point of maternal life-style characteristics, cross tab and chi square analysis was conducted as well. With regard to smoking during the whole pregnancy, 81.8% of the mothers did not smoke whereas 18.2% smoked during the whole pregnancy (table 2). Due to the fact that the majority of mothers did not smoke during the whole pregnancy, the relationship between smoking and anogenital ratios was not further explored.

The cross tab and chi square analysis concerning use of cosmetics during pregnancy showed that 70.3% of male infants with low AGD ratio, 68.4% of male infants with low ASD ratio and 66.7% of male infants with low PW ratio were born by mothers who used cosmetics during their pregnancy, while 29.7 % of male infants with AGD ratio, 31.6% with low ASD ratio and 33.3% with low PW ratio were born by mothers that did not use cosmetics during their pregnancy(table 15).

Table 15: Use of cosmetics during pregnancy * AGD, ASD and PW Crosstabulation

		AGD ratio			
		low	**medium**	**high**	Total
Cosmetics use	**No**	11(29.7)	28(37.8)	12(32.4)	51(34.5)
	Yes	26(70.3)	46(62.2)	25(67.6)	97(65.5)
	total	37(100)	74(100)	37(100)	148(100)
		ASD ratio			
		low	**medium**	**high**	Total
Cosmetics use	**No**	12(31.6)	25(34.2)	15(39.5)	52(34.9)
	Yes	26(68.4)	48(65.8)	23(60.5)	97(65.1)
	total	38(100)	73(100)	38(100)	149(100)
		PW ratio			
		low	**medium**	**high**	Total
Cosmetics use	**No**	13(33.3)	25(35.2)	14(37.8)	52(35.4)
	Yes	26(66.7)	46(64.8)	23(62.2)	95(64.6)
	total	39(100)	71(100)	37(100)	147(100)

Likewise, 70.6% of female infants with low ACD ratio and 77.1% of female infants with low AFD ratio were born by mothers that used cosmetics during their pregnancy, whereas 29.4% of females with low ACD and 22.9% with low AFD were born by mothers who used cosmetics during pregnancy (table 16). The relationship between usage of cosmetics during pregnancy was not statistically significant with any of the anogenital ratios.

Table 16: use of cosmetics during pregnancy * ACD and AFD Crosstabulation

		ACD ratio			
		low	**medium**	**high**	Total
Cosmetics use	**No**	10(29.4)	24(34.8)	12(37.5)	46(34.1)
	Yes	24(70.6)	45(65.2)	20(62.5)	89(65.9)
	total	34(100)	69(100)	32(100)	135(100)
		AFD ratio			
		low	**medium**	**high**	Total
Cosmetics use	**No**	8(22.9)	27(40.3)	11(34.4)	46(34.3)
	Yes	27(77.1)	40(59.7)	21(65.6)	88(65.7)
	total	35(100)	67(100)	32(100)	134(100)

Another variable which was examined with regards to maternal life-style characteristics was the use of pesticides at home during the pregnancy and cross tab and chi square analysis was also conducted.

As presented in table 17, 37.8% of male infants with low AGD ratio and 34.2% of male infants with low ASD ratio, were born by mothers who used pesticides at home during their pregnancy, while 62.2% of male infants with low AGD ratio and 65.8% with low ASD ratio, were born by mothers that did not use pesticides at home during pregnancy.

For the PW ratio, results were similar as 38.5% of male infants with low PW ratio were born by mothers who used pesticides at home during pregnancy and 61.5% by mothers that didn't use pesticides at home during their pregnancy. The relationship between usage of pesticides at home during pregnancy was not statistically significant with any of the anogenital ratios in male infants.

Table17: Use of pesticides at home during pregnancy * AGD, ASD and PW Crosstabulation

		AGD ratio			
		low	**medium**	**high**	Total
Pesticides use	**No**	23(62.2)	46(63)	26(70.3)	95(64.6)
	Yes	14(37.8)	27(37)	11(29.7)	52(35.4)
	total	37(100)	73(100)	37(100)	147(100)
		ASD ratio			
		low	**medium**	**high**	Total
Pesticides use	**No**	25(65.8)	45(62.5)	25(65.8)	95(64.2)
	Yes	13(34.2)	27(37.5)	13(34.2)	53(35.8)
	total	38(100)	72(100)	38(100)	148(100)
		PW ratio			
		low	**medium**	**high**	Total
Pesticides use	**No**	24(61.5)	43(61.4)	26(70.3)	93(63.7)
	Yes	15(38.5)	27(38.6)	11(29.7)	53(36.3)
	total	39(100)	70(100)	37(100)	146(100)

As expected, results for the female infants were not really different. 27.3% of female infants with low ACD ratio and 33.3% of female infants with low AFD ratio were born by mothers that did not use pesticides at home during pregnancy, whereas 72.3% of females with low ACD and 66.7% with low AFD were born by mothers who did not use pesticides at home during pregnancy (table 18). The relationship between usage of pesticides at home during pregnancy was not statistically significant with any of the anogenital ratios for the female infants also.

Table18: Use of pesticides at home during pregnancy * ACD and AFD Crosstabulation

		ACD ratio			
		low	**medium**	**high**	Total
Pesticides use	**No**	24(72.7)	48(70.6)	25(78.1)	97(72.9)
	Yes	9(27.3)	20(29.4)	7(21.9)	36(27.1)
	total	33(100)	68(100)	32(100)	133(100)
		AFD ratio			
		low	**medium**	**high**	Total
Pesticides use	**No**	22(66.7)	49(73.1)	25(78.1)	96(72.7)
	Yes	11(33.3)	18(26.9)	7(21.9)	36(27.3)
	total	33(100)	67(100)	32(100)	132(100)

The use of contraception pill before this pregnancy is another variable that was examined. 8.3% of male infants with low AGD ratio and 8.1% of male infants with low ASD ratio were born by mothers who used contraception pill before their pregnancy compared to 5.4% and 5.3% of infants with high AGD and ASD ratio respectively (table 19). The percentages of infants, born by mothers who used the pill with low and medium AGD and ASD ratio are slightly higher than those for high respectively. Similarly 8.1% of male infants with low PW ratio, compared to 5.3% of males with high PW ratio, were born by mothers that used contraception pill before this pregnancy.

Table 19: Use of contraception pill before this pregnancy * AGD, ASD and PW Crosstabulation

		AGD ratio			
		low	**medium**	**high**	Total
Contraception pill	**No**	23(63.9)	50(68.5)	29(78.4)	102(69.9)
	Yes	3(8.3)	3(4.1)	2(5.4)	8(5.5)
	Other method	10(27.8)	20(27.4)	6(16.2)	36(24.7)
	total	36(100)	73(100)	37(100)	146(100)
		ASD ratio			
		low	**medium**	**high**	Total
Contraception pill	**No**	25(67.6)	48(66.7)	30(78.9)	103(70.1)
	Yes	3(8.1)	3(4.2)	2(5.3)	8(5.4)
	Other method	9(24.3)	21(29.2)	6(15.8)	36(24.5)
	total	37(100)	72(100)	38(100)	147(100)
		PW ratio			
		low	**medium**	**high**	Total
Contraception pill	**No**	26(70.3)	46(64.8)	30(81.1)	102(70.3)
	Yes	3(8.1)	4(5.6)	1(2.7)	8(5.5)
	Other method	8(21.6)	21(29.6)	6(16.2)	35(24.1
	total	37(100)	71(100)	37(100)	145(100)

The results from cross tab and chi square analysis for female infants showed that 18.8% of female infants with low ACD ratio, compared to 6.7% with high ACD ratio, were born by mothers that used contraception pill before their pregnancy. Since the p-value for Pearson's Chi-Square is <0.05 the relationship between the use of contraception pill and the ACD ratio is statistically significant. 9.1% of female infants

with low AFD ratio, compared to 3.2% with high AFD ratio, were born by mothers who used contraception pill before this pregnancy but this relationship was not statistically significant (table 20).

Table 20: Use of contraception pill during pregnancy * ACD and AFD Crosstabulation

		ACD ratio			
		low	**medium**	**high**	Total
Contraception pill*	**No**	16(50)	55(82.1)	25(83.3)	96(74.4)
	Yes	6(18.8)	4(6)	2(6.7)	12(9.3)
	Other method	10(31.3)	8(11.9)	3(10)	21(16.3)
	total	32(100)	67(100)	30(100)	129(100)
		AFD ratio			
		low	**medium**	**high**	Total
Contraception pill	**No**	22(66.7)	48(75)	26(83.9)	96(75)
	Yes	3(9.1)	8(12.5)	1(3.2)	12(9.4)
	Other method	8(24.2)	8(12.5)	4(12.9)	20(15.6)
	total	33(100)	64(100)	31(100)	128(100)

*: $p<0.05$

The last variable of interest concerning maternal life-style characteristics was the use of hair dyes during pregnancy. 88.8% of the mothers did not use hair dyes during their pregnancy while 11.2% used hair dyes during the pregnancy. Due to the fact that the majority of mothers did not use hair dyes during the whole pregnancy, the relationship between hair dyes and AG ratio was not further explored.

In order to explore the association between maternal age and anogenital ratios, Spearman's rank correlation coefficient was used. As presented in table 21 for both sexes, anogenital ratio decrease as mother's age increases. For male infants this correlation is statistically significant at the 0.05 level for the AGD and PW ratio and at the 0.01 level for the ASD ratio.

Table21: Correlation between mother's age and anogenital ratios for male and female infants

	MALES			**FEMALES**	
	AGD ratio	**ASD ratio**	**PW ratio**	**ACD ratio**	**AFD ratio**
Mother's age	-0.195*	-0.223**	-0.164*	-0.015	-0.036

*: correlation is significant at the 0.05 level
**: correlation is significant at the 0.01 level

In order to explore possible differences regarding to anogenital ratios between different residency and maternity clinic, Wilcoxon-Mann-Whitney test was used used.

As presented in table 22, the results suggest that there is a statistically significant difference between male infants born by mothers that lived in an urban or a rural area regarding to AGD, ASD and PW ratio. Male infants who were born by mothers living in an urban area had lower anogenital ratios compared to male infants born by mothers that lived in rural areas. A statistically significant difference between male infants born in a public or private maternity clinic, regarding to AGD and PW ratio, was also indicated. Male infants born in public maternity clinics had lower AGD and PW ratios compared to male infants born in private maternity clinics.

One-way analysis of variance was used in order to explore possible differences regarding to anogenital ratios between different methods of contraception. A statistically significant difference between female infants born by mothers that did not use any contraception method and those that used, regarding to ACD ratio, was detected.

Table 22: Comparison of mean values regarding to anogenital ratios

		Males			Females	
		AGD* ratio(153)	**ASD* ratio(154)**	**PW* ratio(152)**	**ACD ratio(145)**	**AFD ratio(145)**
Residence	**urban**	71.29	73.08	72.08	71.83	73.04
	rural	97.76	93.09	91.85	78.64	72.80
		AGD* ratio(156)	**ASD ratio(157)**	**PW* ratio(155)**	**ACD ratio(146)**	**AFD ratio(146)**
Maternity clinic	**public**	72.81	75.48	70.03	73.26	71.81
	private	91.29	87	96.32	73.98	76.84
		AGD ratio(146)	**ASD ratio(147)**	**PW ratio(145)**	**ACD* ratio(129)**	**AFD ratio(129)**
Contraception pill	**no**	13.05	6.64	2.67	9.4	3.88
	yes	11.55	5.99	2.21	7.35	3.34
	other	11.65	5.87	2.40	7.24	3.34

*: significant at the 0.05 level

Regression analysis was used in order to take into account the effect of several covariates (maternal and infant characteristics). Two multiple linear regression models were set up in order to predict anogenital ratios with residence, contraception, maternity clinic and maternal age. In the first model maternal health and pregnancy

related variables were included, such as pre-pregnancy BMI, BMI during this pregnancy, gestational age and former pregnancy. In the second model infant related variables were added, such as infant age and skinfold thickness measurements as an index of infant's body fat.

The results of the multiple regression analysis, as presented in table 23, suggest that residence is significantly related to AGD ratio. Residence alone explains 6.4% of the variance on AGD ratio for male infants and when including pre-pregnancy BMI, maternal BMI during pregnancy, gestational age and former pregnancy this increases to 21.9% and statistical significance remains. When including infant age and skinfold thickness measurements, the model explains 85.7% of the variance on AGD ratio. Regarding to contraception pill, a negative relation to AGD ratio is observed. The first model explains 20.5% while the second model explains 85.4% of the variance on AGD ratio.

Table 23: Linear regression analysis of AGD ratio

	AGD ratio								
	Crude (n=151)			**Adjusted [a](n=131)**			**Adjusted 2[b](n=131)**		
	β	**95% CI**	**R^2**	**β**	**CI 95%**	**R^2**	**β**	**CI 95%**	**R^2**
Residence (urban/rural)	0.25	0.96,4.02	0.064*	0.26*	-3.46,-0.73	0.219*	0.08*	0.04,1.42	0.857*
Contraception (none/pill/other)	-0.16	-1.49,0.03	0.024	-0.23*	-1.8,-0.32	0.205*	-0.08*	-0.7,-0.03	0.854*
Maternity clinic (public/private)	0.12	0.34,3.07	0.038*	0.13	-0.41,2.58	0.167*	-0.03	-0.91,0.41	0.852*
Mother's age (yrs)	-0.17	-0.29,0.01	0.03*	-0.17*	-0.28,-0.006	0.182*	-0.01	-0.07,0.05	0.852*

*: significant at the 0.05 level
a: adjusted for maternal pre-pregnancy BMI, maternal BMI during pregnancy, gestational age and former pregnancy
b: adjusted for maternal pre-pregnancy BMI, maternal BMI during pregnancy, gestational age, former pregnancy, infant's age and infant's skinfold thickness measurements

Maternity clinic and maternal age is also significantly related to AGD ratio. Similar are the results for the ASD ratio (table 24). Contraception pill and maternal age are negatively related to ASD ratio while residence is positively related to ASD ratio.

Table 24: Linear regression analysis for ASD ratio

	ASD ratio								
	Crude (n=154)			Adjusted 1[a](n=132)			Adjusted 2[b](n=132)		
	β	95% CI	R^2	β	CI 95%	R^2	β	CI 95%	R^2
Residence (urban/rural)	0.19	0.18,2.07	0.035*	0.18*	0.09,1.97	0.203*	-0.01	-0.56,0.48	0.784*
Contraception (none/pill/other)	-0.14	-0.87,0.08	0.02	-0.2*	-1.02,-0.11	0.216*	-0.05	-0.4,0.1	0.783*
Maternity clinic (public/private)	0.12	-0.2,1.5	0.014	0.05	-0.66,1.2	0.175*	-0.1*	-1.03,-0.05	0.792*
Mother's age (yrs)	-0.20	-0.19,-0,03	0.035*	-0.18*	-0.18,-0.01	0.204*	-0.03	-0.06,0.03	0.784*

*: significant at the 0.05 level
a: adjusted for maternal pre-pregnancy BMI, maternal BMI during pregnancy, gestational age and former pregnancy
b: adjusted for maternal pre-pregnancy BMI, maternal BMI during pregnancy, gestational age, former pregnancy, infant's age and infant's skinfold thickness measurements

The results of the multiple regression analysis, as presented in table 25, suggest that residence is related to PW ratio. Residence alone explains 5.4% of the variance on PW ratio for male infants and when including pre-pregnancy BMI, maternal BMI during pregnancy, gestational age and former pregnancy this increases to 19.5% and statistical significance remains. When including infant age and skinfold thickness measurements, the model explains 89.3% of the variance on PW ratio but the relation is not statistically significant. Maternity clinic and maternal age is also related to PW ratio.

Table 25: Linear regression analysis for PW ratio

	PW ratio								
	Crude (n=150)			Adjusted 1[a](n=130)			Adjusted 2[b](n=130)		
	β	95% CI	R^2	β	CI 95%	R^2	β	CI 95%	R^2
Residence (urban/rural)	0.23	0.2,1.02	0.054*	0.24*	0.2,1.03	0.195*	0.03	-0.08,0.24	0.893*
Contraception (none/pill/other)	-0.16	-0.36,0.06	0.013	-0.19*	-0.44,-0.02	0.167*	-0.02	-0.1,0.06	0.89*
Maternity clinic (public/private)	0.27	0.28,1	0.073*	0.21*	0.08,0.91	0.176*	0.07	-0.001,0.3	0.895*
Mother's age (yrs)	-0.16	-0.07,0.001	0.025	-0.17*	-0.08,-0.002	0.167*	0	-0.01,0.01	0.892*

*: significant at the 0.05 level
a: adjusted for maternal pre-pregnancy BMI, maternal BMI during pregnancy, gestational age and former pregnancy
b: adjusted for maternal pre-pregnancy BMI, maternal BMI during pregnancy, gestational age, former pregnancy, infant's age and infant's skinfold thickness measurement

For the female infants, results of the crude model suggest that ACD ratio is related to contraception pill. When including pre-pregnancy BMI, maternal BMI during pregnancy, gestational age and former pregnancy in the model the relation is still significant but when infant age and skinfold thickness measurements are included in the model the relation remains but is not statistically significant. The results from the second model also suggest that ACD is negatively related to maternity clinic.

Table 26: Linear regression analysis for ACD ratio

	ACD ratio								
	Crude (n=142)			**Adjusted 1[a](n=128)**			**Adjusted 2[b](n=133)**		
	β	**95% CI**	**R^2**	**β**	**CI 95%**	**R^2**	**β**	**CI 95%**	**R^2**
Residence (urban/rural)	0.08	-0.8,2.19	0.006	0.11	-0.48,2.17	0.047	0.03	-0.46,1.05	0.822*
Contraception (none/pill/other)	-0.26	-1.92,0.41	0.07*	-0.24*	-1.84,-0.26	0.09*	-0.05	-0.52,0.07	0.883*
Maternity clinic (public/private)	-0.02	-1.06,1.34	0	-0.01	-1.33,1.18	0.042	-0.13*	-1.46,-0.38	0.838*
Mother's age (yrs)	-0.02	-0.13,0.10	0	-0.06	-0.17,0.09	0.044	0.006	-0.05,0.06	0.822*

*: significant at the 0.05 level
a: adjusted for maternal pre-pregnancy BMI, maternal BMI during pregnancy, gestational age and former pregnancy
b: adjusted for maternal pre-pregnancy BMI, maternal BMI during pregnancy, gestational age, former pregnancy, infant's age and infant's skinfold thickness measurements

Table 27: Linear regression analysis for AFD ratio

	AFD ratio								
	Crude (n=145)			**Adjusted 1[a](n=128)**			**Adjusted 2[b](n=128)**		
	β	**95% CI**	**R^2**	**β**	**CI 95%**	**R^2**	**β**	**CI 95%**	**R^2**
Residence (urban/rural)	0	-0.62,0.62	0	0.04	-0.55,0.83	0.048	0.004	-0.44,0.48	0.617*
Contraception (none/pill/other)	-0.16	-0.62,0.03	0.025	-0.12	-0.56,0.11	0.053	0.04	-0.14,0.28	0.664*
Maternity clinic (public/private)	0.05	-0.36,0.64	0.002	0.02	-0.47,0.57	0.049	-0.09	-0.59,0.08	0.626*
Mother's age (yrs)	-0.54	-0.07,0,03	0.003	-0.08	-0.08,0.03	0.054	-0.02	-0.04,0.03	0.619*

a: adjusted for maternal pre-pregnancy BMI, maternal BMI during pregnancy, gestational age and former pregnancy
b: adjusted for maternal pre-pregnancy BMI, maternal BMI during pregnancy, gestational age, former pregnancy, infant's age and infant's skinfold thickness measurements

Bivariate correlation analyses (Spearman's rank correlation coefficient) was used to assess the relation between maternal CALUX-TEQs and anogenital ratios. Regarding to male infants, AGD and PW ratio decrease as DR Calux-teq increases, AGD, ASD and PW ratio decrease as AR Calux-teq increases and AGD and ASD ratio decrease as ERα Calux-teq increases. For female infants, ACD ratio decreases as DR Calux-teq and AR Calux-teq increases, while AFD ratio decreases as ERα Calux-teq increases. None of the relationships between maternal CALUX-TEQs and anogenital ratios was statistically significant.

Table 28: Correlation between DR Calux teq, ERα Calux teq and and infant anogenital ratios

		MALES			**FEMALES**
	AGD ratio	**ASD ratio**	**PW ratio**	**ACD ratio**	**AFD ratio**
DR Calux teq	-0.006	0.345	-0.091	-0.256	0.329
ERα Calux teq	-0.329	-0.26	0.027	0.207	-0.195
AR Calux teq	-0.7	-0.119	-0.519	-0.409	0.165

4. Discussion

The main aim of this project was the development of an assesment protocol for the anthropometric measurements, including anogenital distance, in children of the RHEA cohort. After the pilot study was conducted, an analytical measurement protocol for anthropometric measurements was developed. The protocol includes measurements of weight and height, abdominal and head circumference, body fat estimated by skinfold thickness measurements and anogenital distances. Anogenital distance was associated with maternal age and with socio-economic factors such residence and birth in private or public maternity clinic. Only few other studies have measured anogenital distance in newborns and there are several complexities regarding these measurements.

Callegari et al. in 1987 measured the distance from the center of the anus to the base of the clitoris (AC), the distance from the center of the anus to the posterior commissure of the fourchette, where the mucosa begins (AF) and distance from the fourchette to the base of the clitoris (FC) in premature and full-term female newborn infants. Salazar-Martinez et al. in 2004, developed a protocol for the measurement of AGD (the distance from the center of the anus to the posterior convergence of the fourchette (where the vestibule begins) in female infants and from the center of the anus to the junction of the smooth perineal skin with the rugated skin of the scrotum in male infants) in male and female newborns. Swan et al. in 2005 measured AGD (from the center of the anus to the anterior base of the penis) and the anoscrotal distance ASD (from the center of the anus to the posterior base of the scrotum) in male infants. Longnecker et al. in 2007 developed a protocol for the measurement of three anogenital distances (anterior base of penis to anus (AGD1), posterior base of penis to anus (AGD2), and posterior of scrotum to anus (ASD)) in human newborn males. They also measured penile width (PW) and stretched penile length (PL). Huang et al. in 2008 measured AGD, from the center of the anus to the posterior convergence of the fourchette in female newborns, and from the center of the anus to the junction of the perineal skin with the rugated skin of the scrotum in male newborns. Finally Torres-Sanchez et al. in 2008 measured the PA distance, measured between the center of the anus and the scrotal–PA junction for males or the vaginal fourchette for females.

In this study, the following anogenital measurements were conducted: anogenital distance(AGD- the distance from the upper basis of penis to anus centre),

anoscrotal distance (ASD-the distance from the lowest point of the scrotum to the anus centre) and penis width (PW-the diameter in the basis of penis) in male infants, while anoclitoral distance (ACD- the distance from clitoris to anus center) and anofourchettal distance (AFD-the distance from the fourchette to anus centre) in female infants. In our study median ASD distance was 31.54 and median AFD distance was 16.66. This suggests that the two-fold difference between males and females (ASD and AFD respectively) represents sexual dimorphism, something that corresponds to the findings of Salazar-Martinez et al. In this study median AGD distance was 58.94 and median ACD distance was 37.82. This also suggests that the almost two-fold difference between males and females (AGD and ACD respectively) may represent sexual dimorphism.

The median AGD distance in male infants from this study was 58.94, while in the Swan et al. study was 70.3 and in the Longnecker et al. study it was 49.9. The median ASD distance in male infants from this study was 31.54, while in the Salazar-Martinez et al. study it was 22, in the Swan et al. study was 37.4, in the Longnecker et al. study it was 19.1, in the Huang et al. study it was 23 and in the Torres-Sanchez et al study it was 42. The median PW in this study was 12.20, whereas in the Longnecker et al. study it was 10.6. Regarding female infants, in this study median ACD distance was 37.82, while in the Callegari et al. study it was 29.6. In this study median AFD distance was 16.66, while in the Callegari et al. study it was 10.9, in the Salazar-Martinez et al. it was 11, in the Huang et al. study it was 16 and in the Torres-Sanchez et al. study it was 23. In the Callegari et al. study, the ratio of the lower segment (AF) to the whole (AC) (AF/AC) was calculated and it was 0.37, while in this study the same ratio (AFD/ACD) was 0.44.

Differences between the measurements of this and other studies may be due to the different samples that are examined. The Callegari et al. study was based on 115 premature and full-term female newborn infants, the Salazar-Martinez et al. study was based on 87 newborns (42 females, 45 males), the Swan et al. study was based on 134 males 2–36 months of age, the Longnecker et al. study was based on 781 newly delivered male infants, the Huang et al. study was based on sixty-five fetuses (32 female and 33 male) and the Torres-Sanchez et al. study was based on 71 infants (37 males and 34 females) 3-18 months of age. This study was based on 305 infants (158 male and 147 female). Half of the sample is 0 to 7 days old while the other half is 8 days to 16months old. 164 newborns were measured at the maternity clinic within 1-2

days since the birth date, while 141 infants were measured later at home. Therefore the comparison of our results to the results of the other studies is difficult.

Swan et al. defined the anogenital index [AGI =AGD/weight (mm/kg)] as a weight-normalized index of AGD, while Huang et al. defined the anogenital index in a slightly different way since they used birth weight to standardize AGD[AGI=anogenital index; AGI-W=AGD/birth weight(mm/kg)]. Torres-Sanchez et al. defined also defined a weight normalized index for PA [PA/W=PA/weight(mm/kg)]. In this study all anogenital distances were divided by infant weight in order to construct a weight-normalized index of these distances (anogenital ratios). In the Swan et al. study AGI was 7.1 while in this study AGD ratio was 13.26. In the Huang et al. study AGI-W was 7.16. in male infants and 5.37 in female infants, in the Torres-Sanchez et al. study PA/W was 6 in male infants and 3.6 in female infants, while in this study ASD ratio was 6.41 in male infants and AFD was 3.46 in female infants. Although anogenital ratios were calculated in order to construct a weight-normalized index of anogenital distances, our sample includes two different groups (half the sample were measured at birth and half later) and difference between anogenital ratios in this study and AGI in the Swan et al. and Huang et al. study may occur due to this characteristic of our sample. Our results seem to be closer to Torres-Sanchez et al. probably because our samples are not so different regarding infant age.

The linear relationships between a range of risk factors and poor birth outcomes have been extensively studied. Evidence exists for maternal and environmental factors such as socioeconomic status, nutrition and body mass index (BMI), maternal age, urinary tract and sexually transmitted infections, physical abuse, alcohol consumption, and smoking during pregnancy having an impact upon birth outcomes. Such factors have been linked with low birth weight, small for gestational age (SGA), pre-term delivery, low Apgar (Activity, Pulse, Grimace, Appearance and Respiration) score, rate of assisted deliveries, length of hospital stay, and infant mortality[23]. Air pollution and social characteristics have been shown to affect indicators of health since there is greater likelihood of reduced birth weight and preterm births among the more socially disadvantaged, and a greater risk of reduced birth weight associated with traffic exposures[24]. Advanced maternal age is associated with various obstetric complications including antepartum hemorrhage, pre-eclampsia, diabetes mellitus, and preterm birth[25]. Among adverse outcomes

found in some studies to occur more frequently in older women are prolonged labour, low birthweight, perinatal morbidity and mortality, and cesarean section[26].

Associations of anogenital ratios with maternal socio-demographic and life-style factors have not been explored yet. In our study maternal age was found to be negatively associated with anogenital ratios. For both sexes, anogenital ratios decrease as mother's age increases and or male infants this correlation was statistically significant. These results are supported by the results of regression analyses that show that maternal age is negatively related to anogenital ratios.

Results suggest that there is a statistically significant difference between male infants born by mothers that lived in an urban or a rural area regarding to AGD, ASD and PW ratio. Male infants who were born by mothers living in an urban area had lower anogenital ratios compared to male infants born by mothers that lived in rural areas. These results are supported by the results of the multiple regression analyses, which indicate that residence is significantly related to AGD, ASD and PW ratio. A statistically significant difference between male infants born in a public or private maternity clinic, regarding to AGD and PW ratio, was also indicated. Male infants born in public maternity clinics had lower AGD and PW ratios compared to male infants born in private maternity clinics. This fact is also supported by the results of the multiple regression analyses, which indicate that the maternity clinic that the infant is born in is significantly related to ASD and PW ratios. This may be related to different patterns of exposure to chemicals, by socio-economic position. A statistically significant difference between female infants born by mothers that did not use any contraception method and those that used, regarding to ACD ratio, was detected. Female infants born by mothers that used contraception method had lower ACD ratio compared to those born by mothers that did not use any contraception method. The results of the multiple regression analyses also indicate that contraception method is negatively related to ACD ratio in female infants.

Prenatal exposure to hormonally active agents, may affect infant's development. Exposures to environmental pollutants that have estrogenic or anti-androgenic action, interfering to the androgen signaling pathway, have been linked to adverse reproductive outcomes. Studies have shown that prenatal phthalate exposure at environmental levels may adversely affect male reproductive development in humans and that *in-utero* exposure to phthalates in general may have anti-androgenic effects on the fetus. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is considered to be one of the most potent endocrine disruptor compounds. Several animal studies have

indicated that exposure to TCDD induces multiple organ dysfunctions, especially in the male reproductive system, which is one of the most sensitive organ systems. In adult male rats, exposure to relatively high doses of TCDD affects testis function directly. TCDD decreases Leydig cell volume, induces severe histological distortion, and impairs steroidogenesis. Maternal exposure studies have also confirmed the adverse effects of TCDD on the development of reproductive organs, such as anogenital distance (AGD), testis, and prostate. Previous studies have shown that the effects of TCDD on those reproductive organs are associated with disruption of the androgen system[27]. Maternal exposure to dioxins and dioxin-like compounds has been related to a number of adverse health outcomes in human infants as well. Maternal exposure to dioxins and dioxin-like compounds can be estimated by the DR-Calux bioassay which is responsive to arylhydrocarbon receptor. The ER and AR calux bioassay, responsive to estrogen receptor α and androgen receptor respectively, can be used for the estimation of estrogen and androgen exposure respectively.

Attempting to assess the relation between maternal DR, AR and ERα CALUX-TEQs and anogenital ratios, bivariate correlation analysis was conducted. Since we had the Calux-teqs from only 22 maternal blood samples, this was actually a pilot analysis. Results indicate that AGD and PW ratio decrease as DR Calux-teq increases, AGD, ASD and PW ratio decrease as AR Calux-teq increases and AGD and ASD ratio decrease as ERα Calux-teq increases. For female infants, ACD ratio decreases as DR Calux-teq and AR Calux-teq increases, while AFD ratio decreases as ERα Calux-teq increases. Since the sample is so small, no significant relation could be detected.

The fetus and young infant appear to be susceptible to endocrine-disrupting effects of environmental chemicals. As exposure during critical developmental phases such as *in utero* and in the early postnatal period may have an adverse effect on reproductive health, research in this area should be expanded. Such research should aim on detecting valid biomarkers of endocrine dysfunction and at the same time include the appropriate measurements, to facilitate comparability between study groups. With respect to anogenital distances, major research is needed in order to identify the best approach to normalize these measurements by the size of the infant so that measurement of anogenital distances can become a well established method that provides reproducible results. Finally follow-up of the newborns until puberty will help evaluate whether early effects of endocrine disruption are also associated with effects in later life.

5. References

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Appendix A. recording form for anthropometric measurements in male 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

Appendix B. recording form for anthropometric measurements in female infants

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