MATERNAL EXPOSURE TO PHTHALATES AND MALE REPRODUCTIVE SYSTEM DEVELOPMENT IN THE OFFSPRING: RESULTS FROM A CANADIAN BIRTH COHORT STUDY

by

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Dedication Page

This work is dedicated to the most important people in my life, my children Bernardo, Matheus and Nicholas; my wife Daniela; my parents Luiz and Adelina. And to my calling of helping children afflicted by surgical and urological disease.

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Abstract

Objectives: The primary objective of this thesis was to determine if maternal exposure to phthalates during the first trimester of pregnancy was associated with penile length (PL) or width (PW) at birth in full term singleton boys.

Materials & Methods: In the MIREC (Maternal-Infant Research on Environmental Chemicals) prospective cohort study, 2001 pregnant women from 10 Canadian cities provided biospecimens for measurement of chemicals, including phthalates. Urinary concentrations of phthalate metabolites were measured in the first trimester of pregnancy. Total high and low molecular weight phthalates and Di-2-ethylhexyl phthalate(DEHP) concentrations were calculated based on the molar sum of their respective metabolites. At birth, multiple anthropometric measurements including PL and PW were conducted for 215 male offspring. Univariate and multivariate linear regressions were performed to study the association between penile measurements and maternal urinary concentrations of each phthalate metabolite and composite measures adjusting for potential confounders.

Results: 170 term male singletons had complete data for analysis. On univariate analysis, no association was found between the individual levels of 7 phthalates metabolites, DEHP, low or high molecular weight phthalates and PL. On univariate analysis, PW showed a statistically significant inverse relationship with the log-transformed, specific gravity adjusted concentration of MCPP. On multivariate analysis controlling for confounders, a positive association between MEHP and PL was the only one to approach statistical significance.

Conclusions: Based on our results, exposure to phthalates at levels representative of the Canadian population in the first trimester of pregnancy did not have a significant impact on PL/PW at birth in singletons born at term.

List Of Abbreviations Used

PL – penile length

PW – penile width

AGD – anogenital distance

ASD – anoscrotal distance

MPW – masculinizing programming window

TDS – testicular dysgenesis syndrome

MIREC – Maternal Infant Research on Environmental Chemicals

MIREC-ID – Maternal Infant Research on Environmental Chemicals – Infant Development

LOD – limit of detection

ICC – intraclass coefficient

EDC – endocrine disrupting chemicals

MnBP Mono-n-butyl phthalate

MBzP Mono benzyl phthalate

MCHP Mono cyclohexyl phthalate

MCPP Mono-3-carboxypropyl phthalate

MEHHP Mono-(2-ethyl-5-hydroxyhexyl) phthalate

MEHP Mono-2-ethylhexyl phthalate

MEOHP Mono-(2-ethyl-5-oxohexyl) phthalate

MEP Mono ethyl phthalate

MMP Mono-methyl phthalate

MiNP Mono-isononyl phthalate

MnOP Mono-n-octyl phthalate

DEHP - Di-2-ethylhexyl-phthalate

DnBP - Di-n-butyl phthalate

BBzP - Butyl-benzyl-phthalate

DnOP - Di-n-octyl-phthalate

DEP - Diethyl phthalate

LMW – low molecular weight phthalates

HMW – high molecular weight phthalates

EXP – phthalates implicated in the “phthalate syndrome’ in experimental studies

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Chapter 1 Introduction

1.1 The Problem

Numerous observational studies have suggested that disorders of the male reproductive system are on the rise worldwide in the last few decades.

An increase in the prevalence of hypospadias, a congenital anomaly of penile development, has been suggested by various population-based studies from across the globe (1-5). In a large study covering over 900,000 Danish boys in a 30-year span, Lund et al. identified a 2.4% (95% CI – 1.94-2.86) annual increase in hypospadias prevalence from 2.4/1000 in 1977 to 5.2/1000 live births in 2005(3). The same trend has been proposed for cryptorchidism, which refers to an abnormal position of the testicle(s) at birth, although with less emphasis than hypospadias (5-7). In a 2009 British infant cohort study, the prevalence of 5.9% among male live births was significantly higher than 2.7% and 4.1% reported in earlier decades in Western Europe populations (7).

Following a similar pattern, testicular germ cell cancer incidence rates are clearly rising worldwide (8-10). A modelling study in Western Europe estimated that 23,000 new cases will be diagnosed in that continent in 2025, representing a 24% increase compared to 2005, with 1/100 men receiving the diagnosis in higher risk countries (Croatia, Norway and Slovenia) (11).

Finally, a reduction in semen quality parameters and an increase in male infertility have been reported in recent years by multiple authors from around the world (12-14).

In summary, research studies concerning both congenital (e.g. hypospadias, cryptorchidism) and acquired (e.g. testicular cancer, infertility) diseases affecting males of all ages have led to the speculation that environmental factor(s) targeting the hormonal milieu responsible for male development and function may exert a causative effect (15-18).

Several chemicals ubiquitously present in the environment are known to have the potential to disrupt hormonal function. Such compounds are generically referred to as endocrine disrupting chemicals (EDCs) and include plasticizers (such as phthalates), pesticides, heavy metals and others.

Phthalates are a specific type of EDCs that have received significant attention from the scientific community and the media in the last decade due to their potential deleterious effects. Studies have suggested that parental exposure to phthalates impacts male reproductive development and leads to increased rates of hypospadias and cryptorchidism in the offspring (19-21). The major limitation of most published studies is the difficulty in providing an accurate estimate of the exposure to phthalates. Despite a robust body of experimental data in animals, which will be summarized in the "Background" section, few studies have reliably measured phthalate exposure in humans and assessed their association with reproductive outcomes.

Male reproductive development is a complex process mediated by timely genetic and biochemical events that start during embryogenesis and progress through pregnancy and after birth into adulthood. Modelling the association between exposure to phthalates and the aforementioned male reproductive disorders is challenging due to the low incidence of hypospadias and cryptorchidism and the temporal dissociation between exposure and diagnosis of testicular cancer and male infertility. Anthropometric measurements of the male external genitalia, such as penile length, width and anogenital distance are easy to obtain and are considered readouts of adequate exposure to male hormones (androgens) during development(22-24).

In this thesis, we set out to study the relationship between maternal phthalate exposure and objective penile measurements (length and width) in a Canadian birth cohort.

1.2 Objectives

Primary objective

The primary objective of this thesis is to determine if maternal phthalate metabolite concentrations during pregnancy are associated with penile length/width at birth in full term singleton boys.

Secondary objective

The secondary objective of this thesis is to explore if maternal phthalate metabolite concentrations during pregnancy could be associated with a higher occurrence of cryptorchidism (undescended testicle) and/or hypospadias in the male singleton offspring.

Chapter 2 Study Background And Literature Review

2.1 Development Of The Male Reproductive System And Markers Of Adequate Hormonal Action

Penile length (PL) and width (PW) are considered hallmarks of adequate exposure to androgens during reproductive development by the mechanisms summarized below.

The cornerstone of sex determination in humans is gonadal function (25). The gonads regulate all changes that will eventually lead to the definition of the phenotypic sex. The gonads are undifferentiated initially and develop into a testicle or ovary around the 6th week of embryogenesis.

The gene known as SRY (sex determining region mapped to the short arm of the "Y" chromosome) is regarded as the male-defining factor in humans. In its presence, the bipotential gonad will turn into a testicle.

Internal genitalia

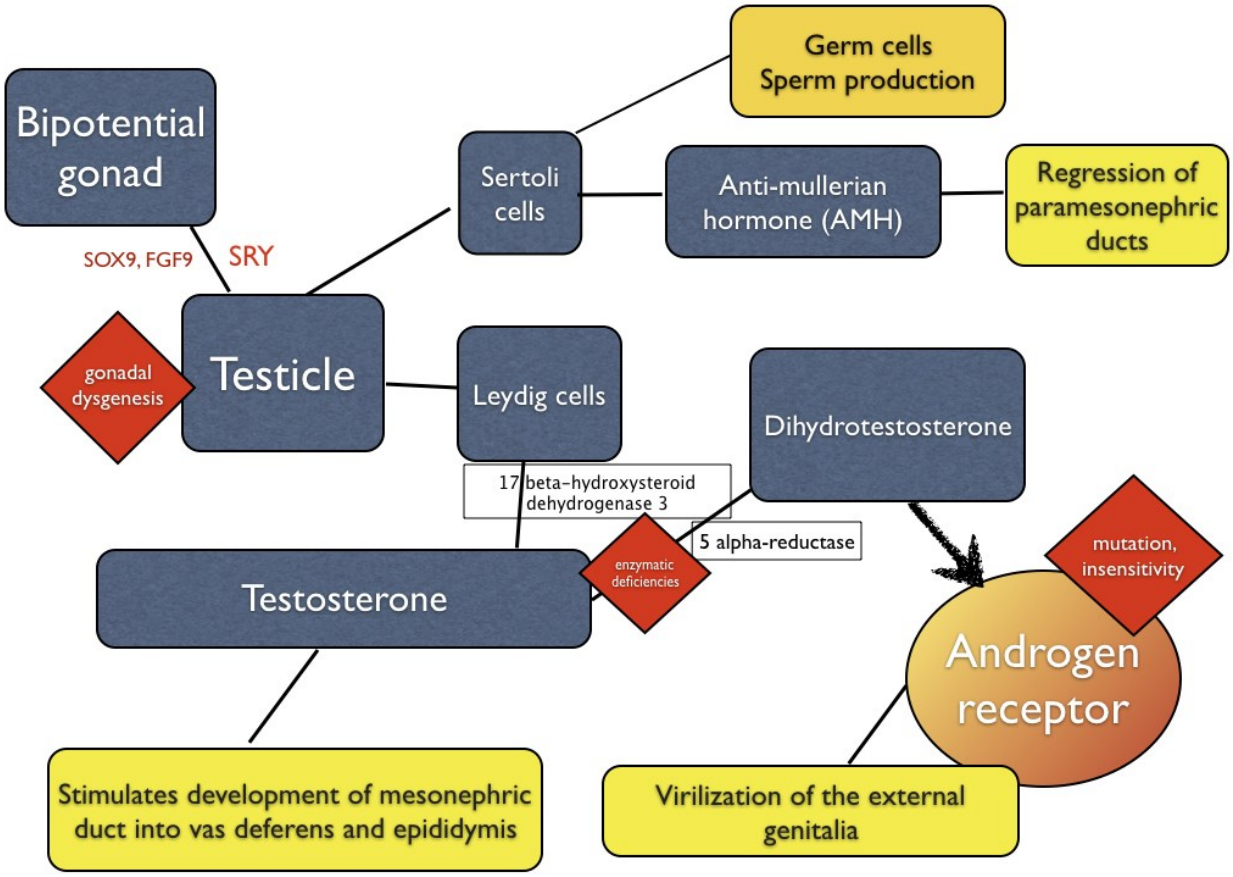
Once the gonad differentiates into a testicle, two hormones will drive differentiation of the internal genitalia, testosterone and anti-mullerian hormone (AMH). The former is an androgen produced by testicular Leydig cells from cholesterol and binds to the nuclear androgen receptor of the fetus, thus stimulating the development of male internal structures such as the vas deferens, epididymis, prostate and seminal vesicles; the latter hormone is

synthesized by testicular Sertoli cells and will inhibit the development of uterus, fallopian tubes, cervix and upper third of the vagina.

External genitalia

Virilization of the external genitalia, characterized by fusion of labioscrotal folds forming the scrotum, urethral plate tubularization and development of the genital tubercle into a penis, are the result of the action of dihydrotestosterone (DHT) on the androgen receptor (AR) present on genital skin. DHT, a much more potent androgen, is converted from testosterone by 5-alpha reductase type 2 (SRD5A2). A schematic overview of the process can be seen in figure 1.

Figure 1 - Diagram of the biochemical events leading up to normal male development (reprinted from Romao et al.(26)**)**



Inadequate testicular development, enzymatic and/or receptor deficiencies (highlighted by the red checkpoints in the figure) may lead to undervirilization of the male genitalia. Hypospadias is a clinical example of such undervirilization. Undoubtedly, androgen (i.e. testosterone and DHT) stimulation is key for adequate male development(25,27-29). Nonetheless, morphological changes of the genitalia during development do not necessarily coincide with the periods of more intense hormonal stimulation and the concept of a ***masculinizing programming window*** must be explained.

Masculinizing programming window (MPW)

The existence of a tight prenatal MPW has been well documented in rats with plausible parallels drawn in humans (24,29). In summary, gestation in rats lasts on average 22 days and the onset of testicular function / testosterone production as well as male differentiation start in the last third of this period (e15.5). Even though most androgen-driven morphological events (development of the prostate, seminal vesicles, elongation of the phallus and testicular descent from the abdomen to the scrotum) are only seen close to term or even postnatally, pharmacological blockage of androgen action in the so-called early and middle windows of androgen stimulation (e15.5-e17.5 and e17.5-e19.5, respectively) impair male development significantly. Anti-androgen action in the late or morphological window (e19.5-e21.5) does not (24,28,29).

Similarly, through androgen (testosterone) supplementation to rats both in the fetal and postnatal periods, some authors have observed that adult penile length was critically dependent on adequate androgen exposure during MPW. Furthermore, they also concluded that inadequate androgen action during MPW was not recoverable by androgen administration later on in pregnancy or postnatally (30).

Based on morphological studies in humans, the external genitalia is identical between males and females at 8 weeks of gestation and completely discernible around 12 weeks(31). Interestingly, around 12-14 weeks of gestation the length of the penis and clitoris are comparable and penile length increases in a linear fashion from the 14^{th} week to term at an approximate rate of 0.7mm per week (32). Both penile length and width

have been shown to be amenable to reproducible prenatal sonographic measurements and show excellent linear correlation with gestational age(31). Testosterone levels start to rise around the 8^{th}-9^{th} week, peak around the 12^{th} and plateau through to the end of pregnancy(27,33). It has thus been hypothesized that the MPW in humans would be around 8-12 weeks of gestation with continuous testosterone stimulation thereafter (27,29). Penile length increases throughout pregnancy in a linear fashion and depicts a strong correlation with gestational age (31,34).

Penile measurements at birth, particularly penile length, have been widely accepted as key clinical parameters of adequate androgen stimulation during development(35). A linear relationship between newborn serum testosterone levels and penile length at birth in newborn males has been demonstrated(36). Moreover, both penile length and width can be measured reliably in the neonatal period(37,38). Testicular descent, particularly in term neonates, is also viewed as a clinical marker of proper androgenization that can be easily assessed in neonates and infants by trained practitioners. After its initial utilization in rodents, standards and nomograms have been published for the measurement of anogenital distance (AGD), another reliable measure of interest in this area that has gained momentum in the last few years (37,38) (39). Together, these anthropometric variables seem to constitute a readout of adequate androgen exposure during fetal life, which may correlate with clinical phenotypes observed later in life. Indeed, Eisenberg et al. reported that infertile men had significantly shorter PL, AGD and lower testicular volumes compared to fertile controls. On

multivariate analyses, only AGD showed statistically significant correlation with semen parameters (40).

The timing of penile measurements postnatally is a very important aspect of the assessment of androgen stimulation during MPW. After birth, infants go through a "mini-puberty" period, where a surge in testosterone is observed briefly peaking from 1 to 3 months of age and declining to minimal levels by 6 months. Levels then remain minimal until the wake of puberty (22). Penile length exhibits significant increase during mini-puberty with a velocity of 1mm/month during the first 3 months of life (41). Hence, to truly reflect prenatal androgen stimulation, penile measurements should ideally be performed early in the neonatal period.

2.2 The Testicular Dysgenesis Syndrome Hypothesis

Based on the diagram depicted in Figure 1, only a primary problem with testicular function could explain concomitant rising rates of hypospadias, cryptorchidism, testicular cancer and male infertility at the population level. In other words, since both Sertoli (semen production and quality) and Leydig (testosterone production and action) cell functions seem to be affected, the Testicular Dysgenesis Syndrome (TDS) hypothesis has been coined to provide an all-encompassing explanation to a possible climbing occurrence of different problems affecting the male reproductive system (18,27,42,43).

Hypospadias refers to an abnormal position of the external urethral opening; rather than being situated at the tip of the penis, it lies somewhere along the ventral shaft with variable degrees of severity. It can be associated with decreased penile length (23). Hypospadias may lead to significant functional issues, such as inability to void standing up and ejaculate inside the vagina during intercourse, not to mention self-image issues for those affected (44-46). It often requires corrective surgery that is technically demanding and associated with a significant rate of complications (47,48). Hypospadias (more severe cases in particular) is observed with increased frequency in infants with a history of prematurity or that are small for gestational age; in such instances, maternal-placental rather than genetic or infant-related causes seem to drive the abnormal virilization process (49-51) .

Cryptorchidism is the most common congenital anomaly in boys affecting approximately 3-5% of the male newborn population (6,7). It is defined as a testicle that is not situated in the scrotum at birth and it can affect one or both testicles. Cryptorchidism is associated with an increased risk of testicular cancer and impaired spermatogenesis, which can be associated with infertility, especially when bilateral (52-55). Spontaneous resolution (testicular descent) in the first few months of life is possible, with surgical treatment being reserved for those testicles that do not accomplish it.

Thankamony et al. has demonstrated that infant boys with hypospadias and/or cryptorchidism depict significantly lower age-adjusted PL and AGD standard deviation

scores compared to healthy controls followed through a longitudinal birth cohort in the UK (23).

In addition to male congenital reproductive abnormalities, several studies have suggested a recent trend towards decreased quality of semen parameters with consequent higher rates of male infertility(14). A similar trend for testicular cancer incidence rates (8) has also been proposed. Taken together, these observations point to androgen action being negatively affected by some exogenous factor during male reproductive development.

According to the TDS hypothesis, the increase in hypospadias, cryptorchidism and testicular cancer rates coupled with lower sperm counts and higher rates of male infertility are interconnected. Furthermore, it suggests that their concomitance implies the existence of a common environmental link contributing to reduced androgen exposure during key steps of development (such as the MPW).

It has thus been postulated that environmental chemicals with potential to have endocrine activity, commonly referred to as endocrine disruptors could represent such a link.

2.3 Environmental Chemicals

According to the World Health Organization (WHO), “an endocrine disruptor is an exogenous substance or mixture that alters function(s) of the endocrine system and

consequentially causes adverse health effects in an intact organism, or its progeny, or (sub)populations" (33,56). The U.S. Environmental Protection Agency (EPA) defines an endocrine disruptor as "an exogenous agent that interferes with synthesis, secretion, transport, metabolism, binding action, or elimination of natural blood-borne hormones that are present in the body and are responsible for homeostasis, reproduction, and developmental process"(57).

Several chemicals have been demonstrated to possess endocrine activity in vitro and in experimental models; moreover, some associations have been identified in humans as well. Contrary to the initial belief that nuclear hormone receptors (e.g. androgen, estrogen, progesterone, thyroid receptors) are the main targets of endocrine disrupting chemicals (EDCs), it is now common knowledge that the mechanisms disrupted by them are complex and variable; their common denominator is anti-androgenic and/or estrogenic activity. The list of molecules identified as EDCs is thus heterogeneous and includes: polychlorinated biphenyls (PCBs), polybrominated biphenyls (PBCs), bisphenol A, fungicides, legacy pesticides, phthalates and others(57).

2.4 Phthalates

Phthalates are substances related to diesters of 1,2- benzenedicarboxylic (phthalic) acid. They are detectable in indoor environments, dust and air. They have also been measured in food, milk and drinking water(58,59).

Low molecular weight (MW<250g/mol) phthalates, such as di-n-butyl phthalate (DnBP), butylbenzyl phthalate (BBzP), di-methyl phthalate (DMP) and diethyl phthalate (DEP) are used as solvents and plasticizers. They can be found in a wide array of personal care products, such as cosmetics and perfumes; furthermore, LMW phthalates are also utilized as coating for pharmaceutical products (20,60,61).

High molecular weight phthalates (MW>250g/mol), like di(2-ethylhexyl) phthalate (DEHP) and di-isononyl phthalate (DINP) are used as plasticizers in the production of polyvinyl chloride (PVC), which in turn is utilized widely in flooring, wall covering, food packaging and medical devices (intravenous tubing and blood bags). DEHP is also found in toys and bath books for children. (20,60).

Due to their ubiquitous presence, humans are exposed to phthalates by multiple routes. Direct dermal application of personal care products, inhalation of volatilized PVC from nail polish and hair spray, oral ingestion of DEHP-contaminated foods (the largest source of DEHP exposure) and parenteral exposure from IV tubing in hospitals constitute some examples of routine human exposure to phthalates (58). Virtually every person has detectable levels of phthalates in their bodies, including pregnant women.

2.5 Evidence of the impact of phthalates on reproductive development

2.5.1 Experimental Evidence

Phthalates have been one of the main targets of experimental studies on potential EDCs. Experimental data on the hormonal activity and potential deleterious effects of phthalates on male sexual development are abundant. Wolf et al. administered endocrine disruptors to pregnant rats during the period of sexual differentiation including DnBP and DEHP. The authors observed that the profile of reproductive malformations was chemical-specific, thereby corroborating the existence of different mechanisms of action for EDCs. Phthalate exposure was associated with reduced AGD, undescended testes and testicular atrophy in the offspring; furthermore, adult rats depicted impaired sperm production and infertility (62). High rates of reduced AGD, cryptorchidism, hypospadias, testicular abnormalities and infertility have been consistently observed in other studies where rats were exposed mainly to DnBP, DEHP, BBzP and DINP(17,63-66). Conversely, DEP and DMP have not demonstrated significant anti-androgen effects(63).

Experimental studies have also offered insight into the possible mechanisms whereby phthalates affect male development in rodents. Unlike chemicals such as flutamide and linuron, where AGD changes are mediated through antagonism to the androgen receptor, phthalates appear to impact Leydig cell function and thus testosterone synthesis directly(27,67,68).

While most studies focus on AGD, the aforementioned congenital malformations, semen parameters and infertility, penile length / width as proxies of inadequate androgen stimulation during development have received less attention in the experimental setting. Drake et al. observed a reduction in penile length and AGD as well as a higher occurrence of hypospadias and cryptorchidism in the offspring of pregnant rats treated with DnBP (69).

The constellation of findings including shortened AGD, hypospadias, cryptorchidism and abnormalities of the male internal genitalia (vas deferens, seminal vesicle, prostate) secondary to reduced fetal testosterone levels in the setting of experimental exposure to phthalates such as DnBP, DEHP and BBzP has been referred to as the "phthalate syndrome"(60,68). Interestingly and maybe not coincidentally, this syndrome shares many features with the TDS mentioned above. Fisher went as far as suggesting that DnBP administration to pregnant rats could be used as an experimental model of TDS(17).

Some have argued that in real life humans are exposed to much lower concentrations of these chemicals than the ones used in the laboratory raising the issue of potency, i.e. the magnitude of exposure would not be high enough to lead to reproductive abnormalities (56).

Conversely, others have demonstrated experimentally that mixtures of endocrine disruptors impair androgen activity and lead to reproductive malformations in a cumulative and additive manner, even when acting through different mechanisms(70,71) (57). That observation is relevant to humans, as combined exposure to small concentrations of a wide range of chemicals could suddenly become clinically significant. There is indeed experimental evidence that individual phthalates combined as a mixture cause testosterone depletion in a cumulative, dose-additive manner (70).

2.5.2 Evidence In Humans / Epidemiological Evidence

Since the late 90s, several reports exploring the association of exposure to environmental chemicals and development of congenital male reproductive disorders and infertility have been published. It is important to highlight that although many studies suggest a positive association (i.e. exposure is associated with higher rates of male reproductive abnormalities), there are significant methodological limitations that call for caution when interpreting the results. Firstly, due to the ubiquitous nature of these substances, adequately measuring the exposure is challenging and most studies rely on indirect methods based mostly on occupational data (e.g., job exposure matrix). Secondly, the outcome studied is usually the occurrence of hypospadias and / or cryptorchidism. Since these are rare, non-life-threatening, and in some cases, not readily identifiable anomalies, issues with ascertainment bias cannot be completely ruled out.

Few reports have focused on the relationship between exposure to endocrine disruptors and non-pathologic anthropometric proxies of androgen activity, such as penile length and anogenital distance. The existing evidence in humans is summarized below.

Indirect measurement of exposure

Van Tongeren developed a job exposure matrix for 7 classes of environmental chemicals; according to the authors, based on the parents' occupation it would be possible to make inference on the level of exposure to these substances at the time of conception and throughout pregnancy. Examples of exposure based on the matrix include: farm workers would be more likely to be exposed to pesticides; plastic workers, electricians, hairdressers to phthalates; dental practitioners, welders and goldsmiths to heavy metals(72).

Following the aforementioned matrix, Suarez-Varela et al. studied hypospadias and cryptorchidism rates in a Danish national birth cohort study with regard to parental occupation. Although a weak association was suggested for maternal exposure to phthalates and paternal exposure to heavy metals, none of the associations were statistically significant (73). A French case-control study using the same matrix also suggested a possible role for phthalates in the development of hypospadias (OR=2.53 for cosmetics), however the number of participants (both cases and controls) exposed was low and the confidence interval wide (0.98-7.32) (74).

In a case-control study of risk factors for cryptorchidism including over 6000 boys in Southern France, parents answered a questionnaire about environmental exposures. The final study population was comprised of 90 cases and 180 controls. Although the authors report a statistically significant risk associated with phthalate exposure, the small numbers (only 5 participants, 4 cases and 1 control, reported exposure to phthalates) and issues around recall bias challenge the validity of the conclusions (75). In a similar fashion, Kalfa et al. evaluated exposure to environmental chemicals in 408 boys with isolated hypospadias and 302 controls using multiple instruments, i.e. postal code / surrounding hazards, validated questionnaires and job exposure matrices. They concluded that fetal exposure to EDCs around the MPW was associated with an increased risk of hypospadias (40% vs. 17%, OR=3.13, 95% CI 2.11-4.65)(74).

Direct measurement of exposure

Individual biomarker concentrations are currently the most accurate method for estimating phthalate exposure. The pharmacokinetics of phthalates is such that they are quickly transformed into metabolites that are excreted in the urine. With such a short half-life, urinary measurement of the levels of phthalates metabolites rather than the parent compounds is still considered the preferred matrix to assess exposure to these chemicals(60,76).

Swan et al. were the first to evaluate the association between prenatal urinary levels of 9 phthalate metabolites in pregnant women and anthropometric measurements in

the offspring (n=134). These authors found that increased phthalate concentrations, mostly measured in the 2nd or 3rd trimester of pregnancy, were associated with reduced AGD, but not PL and PW; however, results need to be interpreted with caution because these measurements were not performed until after the "mini-puberty" period (mean age 15.9 months)(77).

Bustamante-Montes collected urine from 73 Mexican women in the 3rd trimester of pregnancy and obtained levels for 4 phthalate metabolites as well as total phthalate exposure levels. Anthropometric data from the male offspring were also collected between 24 and 48 hours of life. A statistically significant inverse relationship was detected for mono-2-ethylexhyl phthalate (MEHP) levels and stretched penile length. Higher total phthalatee8 levels were significantly associated with reduced PL, PW and AGD (21).

Bloom et al. recruited 500 men who were attempting to conceive and studied the association between urinary phthalate metabolite concentrations and sperm count / quality. They demonstrated an inverse relationship between phthalate levels and sperm count / quality(78).

In recent years, a few larger birth cohort studies described below have been designed to investigate the role played by exposure to environmental chemicals in the development of multiple clinical issues in the offspring. Some analyses from phthalate exposure have been made available from these studies. Because all of them were focused

on genital anthropometric measurements after birth, the literature is inevitably focused on exposure measured during pregnancy.

In the MIREC (Maternal-Infant Research on Environmental Chemicals) research platform, 2000 pregnant women from 10 Canadian cities were recruited during pregnancy and gave blood and urine samples to be tested for over 150 chemicals, including 11 phthalate metabolites determined in the first trimester of pregnancy(79). Multiple perinatal outcomes were ascertained including anthropometric measurements in the offspring. At least 5 phthalate metabolites were detected in over 95% of women included in the cohort(59). Agarwal found no association between phthalate levels and AGD in 199 male infants on multivariate analyses (80).

Following a similar design, the TIDES (The Infant Development and the Environment Study) enrolled 969 pregnant women from 4 American cities. First trimester urinary concentrations of 11 phthalate metabolites was measured and their association with AGD and PW, measured from 366 male neonates was determined. An inverse statistically significant association was found between levels of 3 di-ethylhexyl phthalate (DEHP) metabolites (~~both when modelled independently or~~both when modelled independently and summed) in the first trimester of pregnancy and AGD, but not PW. Outcomes measurements were performed before 3 days of age in 82% of the cohort. Penile length was not examined in this study (61).

The Swedish Environmental Longitudinal Mother and child, Asthma and allergy (SELMA) study enrolled more than 2,000 mother-child pairs. The associations between 10 phthalate metabolites measured in a first trimester urine sample and AGD at 21 months of age were tested. The authors reported a single statistically significant inverse association between diisononyl phthalate (DiNP) and AGD. Penile measurements were not examined in this study (81).

Finally, the Odense child cohort study in Denmark recruited over 2,000 pregnant women and measured 3rd trimester urinary levels for 12 phthalate metabolites. Associations between urinary concentrations, AGD and PW were determined at 3 months in 267 mother-child pairs. No statistically significant associations were found (82).

2.6 Summary And Study Rationale

The association between exposure to environmental chemicals, particularly phthalates, and negative male reproductive outcomes seems biologically plausible. The observation of similar findings in independent experimental and clinical scenarios (i.e. phthalate syndrome and TDS) provides compelling evidence that well-designed studies are required to specifically study this association. Given the rare occurrence of hypospadias and cryptorchidism as well as challenges in measuring the exposure to EDCs, it is arduous to draw meaningful conclusions from the published literature. Contrary to the usual extensive time lag between scientific knowledge generation and translation, calls for policy changes around regulation of endocrine disrupting substances have already been made with a focus on male reproductive issues. A European expert

panel recently estimated the yearly cost associated with male reproductive disorders caused by EDC to be around €15 billion (83) and suggested public health measures should be employed to reduce it. Domestically, Health Canada has instituted formal measures to limit exposure to phthalates particularly in toys used by young children (< 4 years of age) and taken in the mouth (84).

The use of biomarkers to assess exposure to EDCs in pregnant women and measurement of proxies of adequate endocrine function in healthy infants offers a promising avenue for research in this topic. Unfortunately, the limited available literature using this methodology has been very much focused on AGD, a promising, reproducible anthropometric measurement initially identified in rodents. To our knowledge, only one birth cohort study has focused on a variable accepted as an excellent proxy of adequate in utero exposure to androgens for decades, penile length; nonetheless, the number of participants and 3rd trimester phthalate metabolites measured were limited. Hence this thesis adds to the existing literature on the association between exposure to phthalates during pregnancy and endocrine disruption by focusing on penile length and width using an established maternal-infant research platform.

CHAPTER 3 METHODS

3.1 Study Population

This project was conducted with data obtained from the MIREC (Maternal-Infant Research on Environmental Chemicals) and MIREC-ID (ID=infant development) studies. Details about the structure and objectives of the MIREC Research Platform have been published elsewhere (79). In summary, 2001 healthy pregnant women attending a regular prenatal consult during the first trimester of pregnancy (6 to <14 weeks) across 10 Canadian cities (Vancouver, Edmonton, Winnipeg, Sudbury, Ottawa, Kingston, Hamilton, Toronto, Montreal and Halifax) were recruited to participate in this study from 2008 to 2011. Eligibility criteria included:

- Ability to communicate in English or French
- Age > 18 years
- Gestational age < 14 weeks
- Willingness to provide cord blood upon delivery and
- To deliver at a local hospital

Women with major medical conditions or carrying fetuses with major congenital or chromosomal abnormalities were excluded (79). Hence, the target population of the study was a healthy obstetric population.

A subset of the cohort (based on availability of funds) was enrolled in a follow-up study named MIREC-ID, where infant development was evaluated. In-clinic assessments were performed on approximately 400 infants at birth and 6 months of age, with the goal of measuring growth, behavior, sensory function and potential indicators of reproductive effects(79). Inclusion criteria for MIREC-ID were:

- Singleton infants
- No major congenital birth defects
- No neurological disorders.

Due to the known association between prematurity / intrauterine growth restriction and undervirilization as well as the described association of penile length with gestational age described in the Background, our analysis included full term infants only.

3.2 Study Design

This study followed a prospective observational (cohort) design. One of the main objectives of MIREC was to obtain contemporary biomarkers of in utero exposure to known environmental chemicals. Hence, participants had multiple biospecimens collected at different stages of pregnancy and after delivery, such as blood, urine, cord blood, breast milk, hair and meconium. In total, 126 aliquots of biomaterials were stored in the MIREC Biobank for each participant and analyzed for over 150 chemicals, including phthalates.

Data and biospecimen collection took place at 6 time points, namely 1st / 2nd / 3rd trimesters, delivery, post-partum day 1 or 2 and 2-10 weeks post-delivery. Trained

research staff administered questionnaires to participants about their demographic information, medical history and lifestyle. Medical chart data of relevant pre and perinatal information were abstracted at the aforementioned time points.

As part of MIREC-ID, demographic and perinatal information on the infants were collected. Furthermore, a detailed, standardized evaluation including physical exam was performed by trained practitioners on the offspring at the time of birth and 6 months of age.

3.3 Exposure – Phthalates

Exposure to phthalates was measured in a urine sample collected during the first trimester visit, between 6 and 13 weeks of gestation as part of the MIREC study. Maternal urine (minimal volume 80ml) was collected in 125 ml Nalgener® containers (Thermo-Fischer Scientific Inc., Rochester MN, USA) which were pretested to ensure a phthalate-free state. Samples were aliquoted into 30 ml Nalgener® containers, frozen at -20°C within 2 hours of collection and shipped on dry ice to the MIREC coordinating center at Saint-Justine's Hospital in Montreal, where they were stored at -30°C. Subsequently, samples were shipped in batches to the Centre de Toxicologie du Québec, Institut National de Santé Publique du Québec (INSPQ) for analysis. Samples were analyzed for a wide array of chemicals, including 11 phthalate metabolites. The laboratory is accredited under ISO 17025, which is the international standard for quality in all areas of testing and calibration. Due to their ubiquitous presence, the possibility of contamination was considered in every step from collection to analysis.

The phthalate monoester compounds were extracted by solid phase extraction with anion exchange media using the Janus robotic station (PerkinElmer; Waltham, Massachusetts, USA) (INSPQ Method E-453) after enzymatic deconjugation. The extracts were analyzed by LC–MS/MS with an Ultra Performance Liquid Chromatography (UPLC) Acquity (Waters; Milford, Massachusetts, USA) coupled with a tandem mass spectrometer Quattro Premier XE (Waters; Milford, Massachusetts, USA).

Measurements were expressed in μg/l. No laboratory reference values exist for phthalates, i.e. there is no known acceptable level of exposure in the general population. A measure of the additive effect of different phthalate metabolites ("total exposure") was calculated based on the molar sums of their individual concentrations (more details are provided in the "Analysis" section).

The following 11 phthalate metabolites were studied in the MIREC platform:

MnBP Mono-n-butyl phthalate

MBzP Mono benzyl phthalate

MCHP Mono cyclohexyl phthalate

MCPP Mono-3-carboxypropyl phthalate

MEHHP Mono-(2-ethyl-5-hydroxyhexyl) phthalate

MEHP Mono-2-ethylhexyl phthalate

MEOHP Mono-(2-ethyl-5-oxohexyl) phthalate

MEP Mono ethyl phthalate

MMP Mono-methyl phthalate

MiNP Mono-isononyl phthalate

MnOP Mono-n-octyl phthalate

Small concentrations of chemicals below the level of detection by the laboratory are indistinguishable from zero and are thus reported as < LOD (LOD=limit of detection). The LOD for the phthalate monoester metabolites varied from 0.2 to 5.0 µg/L.

Based on 2 previous studies using the MIREC platform and specifically looking at phthalate concentrations, it was known that for 4/11 of the aforementioned metabolites, over 50% of observations were below the level of detection (59,80). Those were therefore excluded from this study. Table 1 summarizes the 7 phthalate metabolites (representing their respective parent substances) that were considered in this thesis:

Table 1 - Phthalate metabolites and respective parent metabolites with over 50% of measurements > LOD analyzed in the MIREC study

Metabolite	**Metabolite Abbreviation**	**Parent phthalate**	**Parent phthalate Abbreviation**	**High (HMW) vs. Low (LMW) molecular weight**
Mono-n-butyl phthalate	MnBP	Di-n-butyl phthalate	DnBP	LMW

Metabolite	Metabolite Abbreviation	Parent phthalate	Parent phthalates Abbreviation	High (HMW) vs. Low (LMW) molecular weight
Mono-benzyl-phthalate	MBzP	Butyl-benzyl-phthalate	BBzP	LMW
Mono-3-carboxypropyl-phthalate	MCPP	Di-n-octyl-phthalate	DnOP	HMW
Mono-(2-ethyl-5-hydroxy-hexyl) phthalate	MEHHP	Di-2-ethylhexyl-phthalate	DEHP	HMW
Mono-(2-ethylhexyl) phthalate	MEHP	Di-2-ethylhexyl-phthalate	DEHP	HMW
Mono-(2-ethyl-5-oxo-hexyl-phthalate)	MEOHP	Di-2-ethylhexyl-phthalate	DEHP	HMW
Mono-ethyl phthalate	MEP	Diethyl phthalate	DEP	LMW

3.4 Outcomes

3.4.1 Primary Objective - Penile Length And Width

As part of the MIREC-ID study, several physical variables related to sexual characteristics were obtained from infants in the first 24-48 hours of life, including penile length and width.

Stretched penile length (PL) was measured from the base of the phallus to the tip of the glans in mm with calipers. Three separate measurements were obtained. If measures were not done or less than 3 measures were performed, a justification was required.

Penile width (PW) was measured as the diameter at the base of the flaccid penis in mm with calipers. Similarly to penile length, three separate measurements were obtained and a justification required if those were not performed. Measurements of PW using this technique have been shown to be both reliable and reproducible (38).

3.4.2 Secondary Objective – Hypospadias And Cryptorchidism

Within MIREC-ID, a physical exam including assessment of the external genitalia was performed. As part of this assessment, the exact position of the testicles and the presence of hypospadias were specifically collected.

Any testis that was not reported to be in a scrotal position was defined as undescended for the purpose of the analysis.

3.5 Covariates And Potential Confounders

The covariates that may have acted as confounders and were collected can be broadly categorized as: maternal demographic, clinical and socio-economic variables, infant anthropometric measurements (as PL and PW obviously vary as a function of the infant's size) and urine collection variables. Maternal and urine collection variables were collected from the MIREC dataset, while infant-related variables were collected from the MIREC-ID dataset. The following covariates were collected:

3.5.1 Maternal Variables

Maternal age, race, country of birth, active smoking status, level of education, centre where enrollment took place, annual household income, pre-pregnancy body mass index (BMI). The presence of pre-eclampsia (known to be associated with placental insufficiency, which in turn can affect in utero growth) was also collected.

The covariates were categorized as follows:

- Maternal age: <25, 25-29, 30-34, >=35
- Country of birth: Canada and Other
- Parity
- Race: White and Other
- Active smoking during pregnancy: Current, former, never, quit during pregnancy
- Level of education: High school or less, College diploma, University degree
- Household income: ≤ 50,000, 50,001-100,000, > 100,000 Canadian dollars

- Pre-pregnancy BMI: <25, 25-29.99, >=30
- Pre-eclampsia: Yes or No

3.5.2 Infant Variables

The infant variables included gestational age at birth, birth weight, and length. Weight-for-length (which is independent of age) and weight-for-gestational-age z scores were calculated based on WHO and Canadian standards, respectively (85) (86,87) (88). Since placental insufficiency is a known cause of IUGR, placental weight was collected as well.

3.5.3 Urine Collection Parameters

Urine specific gravity and the following parameters were collected:

- Season of urine collection: Fall, Winter, Spring, Summer
- Time of day of the collection: 6:00 – 9:00, 9:01-12:00, 12:01 – 15:00, 15:01 – 18:00, 18:01 – 24:00.
- Time since last void: ≤75 minutes, 76-120, 121-170, > 170

3.6 Statistical Analysis

Descriptive statistics were calculated for the maternal and infant covariates, phthalate concentrations, genital end points and urinary parameters (n, range, mean, standard deviation, median, interquartile ranges, percentiles, frequencies as appropriate).

The total number of urine samples with detectable levels of each phthalate metabolite was also recorded.

3.6.1 Exposure

The distribution of phthalate metabolite concentrations was assessed by inspecting histograms of the data. As projected based on previous MIREC studies analyzing phthalates (59,80,89), concentrations did not follow a normal distribution; hence a logarithmic (log) transformation of the phthalate concentrations was used in the exploratory analysis. Finally, chemical levels below the LOD were substituted by LOD/2. The LODs for each metabolite were provided by the INSPQ laboratory.

Total DEHP exposure was measured through molar sums (sum of concentrations after dividing individual levels by their respective molecular weight) of its three metabolites included in the study (MEHHP, MEHP, MEOHP). Furthermore, the additive effect of multiple exposures to different phthalates was accounted for by calculating the molar sums of low and high molecular weight phthalates separately for each participant. LMW and HMW phthalates were considered separately due to their unique exposure pathways as described in the Background. We also calculated cumulative exposure to phthalates implicated in the phthalate syndrome experimentally (molar sum of DEHP, DnBP and BBzP metabolites, referred to as EXP from here onwards) in each individual participant.

Since the state of hydration may affect phthalate metabolite concentration results, concentrations standardized by specific gravity (SG) were calculated using the formula:

$$P_c = P_i\,[(SG_m - 1)/(SG_i - 1)]$$

where Pc is the SG-adjusted concentration, Pi the observed concentration, SGm the median SG for the whole cohort and SGi the specific gravity of the individual urine sample (90).

Finally, phthalate metabolite concentrations were also categorized as low (below 25th percentile), intermediate (between 25th and 75th percentile) and high (above 75th percentile).

3.6.2 Outcome

The distribution of PL and PW measurements was also visually assessed with plots and histograms. Intra-class coefficients were computed to evaluate intra-rater reliability between repeated measurements of PL and PW for each subject. Levene's test was used to compare homogeneity of variances across centers for PL and PW. Analysis of variance (ANOVA) was then performed to compare means of individual PL and PW measurements across centers. A composite measure of PL and PW was calculated as the mean of the 3 measurements for each participant and compared between centres; these means of PL and PW measurements were utilized in the final analysis. Finally, a composite outcome measure was obtained based on the average PL/ average PW ratio, to assess the impact of phthalate exposure on both anthropometric measurements simultaneously.

Based on the distribution of PL and PW, the correlation between them was tested using Pearson correlation coefficient. Infants were also categorized based on the accepted definition of micropenis (penile length shorter than the sample mean minus 2.5 standard deviations(91)); however, only 2 infants would have met this definition. Hence, we considered those below the 10^{th} percentile for PL as having "micropenis" for a secondary analysis.

Given that anogenital and anoscrotal distances (AGD and ASD) were studied in a previous graduate level project using the same platform (80), we did not plan a primary analysis of these variables. Nonetheless, for quality control, we also assessed intra-rater reliability and between centre variability for these variables in the same way described above for PL and PW (intra-class coefficients, Levene's test to verify the homogeneity of variances and ANOVA to compare means between centres); correlation between the 4 anthropometric measurements in full term infants was also tested by calculating Pearson correlation coefficients.

3.6.3 Regression Analysis

Exploration of the data started with univariate (simple) linear regression to examine the relationship between PL, PW and PL/PW ratio with each of the exposure variables (crude and specific gravity-adjusted log transformed concentrations for each phthalate metabolite and molar sums for DEHP, LMW, HMW and EXP); the same approach was used to evaluate the relationship between the outcome measures and

covariates independently (to assess for potential confounding). Any covariates with a $p \leq 0.15$ were considered potential confounders and included in the multivariate analysis. The relationship between the outcome measures and each of the log-transformed phthalate concentrations was also visualized with plots and regression diagnostics were applied to ensure the assumptions of linear regression were not violated.

Association between outcomes and categories of phthalate metabolite concentrations (low, intermediate and high) were also explored using linear regression.

Multiple linear regressions were utilized to explore the association between PL, PW, PL/PW ratio and the exposures of interest controlling for the covariates selected in the univariate step. The following covariates were considered *a priori* for inclusion on multivariate models for both PL and PW: centre of enrollment, urinary specific gravity, gestational age and variables representing infant's size. For the other covariates, the best model was chosen based on the effect of each covariate on the phthalate concentration coefficient. Covariates that produced a change in the coefficient > 10% stayed in the final model. This analysis procedure was undertaken separately for each individual metabolite log-transformed concentration as well as the molar sums outlined above (DEHP, LMW phthalates, HMW phthalates, phthalate syndrome phthalates - EXP).

Regression assumptions were examined using residual plots; outliers with an absolute standardized residual above 3 were removed. Outliers with an absolute

standardized residual between 2 and 3 and high leverage based on Cook's *d* were also removed.

Penile length was also studied as a categorical variable (micropenis – PL below 10^{th} percentile) using logistic regression and following the same principles described above.

Calculation of the variation (delta) in penile length and width with relation to an increase in metabolite concentrations from the 10^{th} to the 90^{th} percentile was also planned, as described by Swan et al. (61) for the metabolite concentrations found to be statistically significant on multivariate analysis.

Logistic regression was performed to test the association of hypospadias and cryptorchidism in the male offspring with the phthalate metabolite concentrations; because the numbers of such malformations were small (hypospadias n=1; cryptorchidism n=4), we chose to combine all congenital malformations of the male external genitalia coded in the MIREC-ID data collection form (hypospadias, cryptorchidism, abnormal foreskin, abnormal genitalia, chordee) on univariate and multivariate analyses, following the same principles described above for identifying the best models.

3.6.4 Sample Size Calculation

Based on a previous publication using the TIDES data, a change of phthalate metabolite concentration from the 10^{th} to the 90^{th} percentile decreased AGD by 4%(61). Hence, our sample size (170) had > 90% power to detect a 5% change in mean penile length or width attributable to phthalate exposure based on the descriptive statistics of our outcome measures.

CHAPTER 4 RESULTS

4.1 Maternal And Birth Cohort Characteristics

Of the 2001 pregnant women recruited by MIREC, 978 gave birth to male singletons. Of those, 215 were included on MIREC-ID and formed the baseline population for this study. Contaminant data was not available for all phthalate metabolites in 25 infants, who were therefore excluded. There was no difference in the outcome measures (PL / PW) between infants with missing exposure data and the remainder of the study population. Out of the remaining 190 male infants, 176 had complete data on PW and PL and 6 were excluded due to prematurity. Hence, our final birth cohort was comprised of 170 mother-male singleton pairs. Mean gestational age, birthweight and length were 39.7±1.24 weeks, 3.55±0.46 kg and 51.4±2.39 cm, respectively.

Maternal characteristics and urine collection variables are presented in Tables 2 and 3. In our sample, 45% of the mothers were between 30 and 34 years of age, 64% had a university degree, and 92% were white and of Canadian origin (85%). Furthermore, most MIREC participants never smoked (64.7%), had an income over $50,000 CAD / year (79%) and a normal pre-pregnancy body mass index (55.5%). This was a first delivery for 43.5% of the participants. Urine collection typically took place in the morning between 9am and 12pm (50%) with a time elapsed since last void more commonly falling between 76 and 120 minutes (31%).

4.2 Descriptive Statistics – Contaminant Data (Exposure)

Table 4 provides a detailed account of the individual contaminant data. Descriptive statistics for unadjusted and specific gravity-adjusted concentrations of each phthalate metabolite are presented, as well as the geometric mean with confidence intervals and the percentage of observations below the level of detection (LOD). Most metabolites studied had a very low rate of observations below the LOD, except for mono-3-carboxypropyl-phthalate (MCPP – 21%). Observations below the LOD were substituted for LOD/2 as explained in the Methods.

Table 5 summarizes the descriptive statistics for the composite (additive) phthalate variables (DEHP, low molecular weight, high molecular weight and phthalates implicated in the phthalate syndrome experimentally) calculated based on molar sums of the individual metabolite concentrations. As expected, phthalate metabolite concentrations did not follow a normal distribution and were thus log-transformed.

4.3 Descriptive Statistics – Male External Genitalia Anthropometric Measurements (Outcome)

Of the 170 neonates included in the analysis, 157 had all 3 measurements for PL and PW performed. 167 and 166 had at least 2 measurements of PL and PW, respectively.

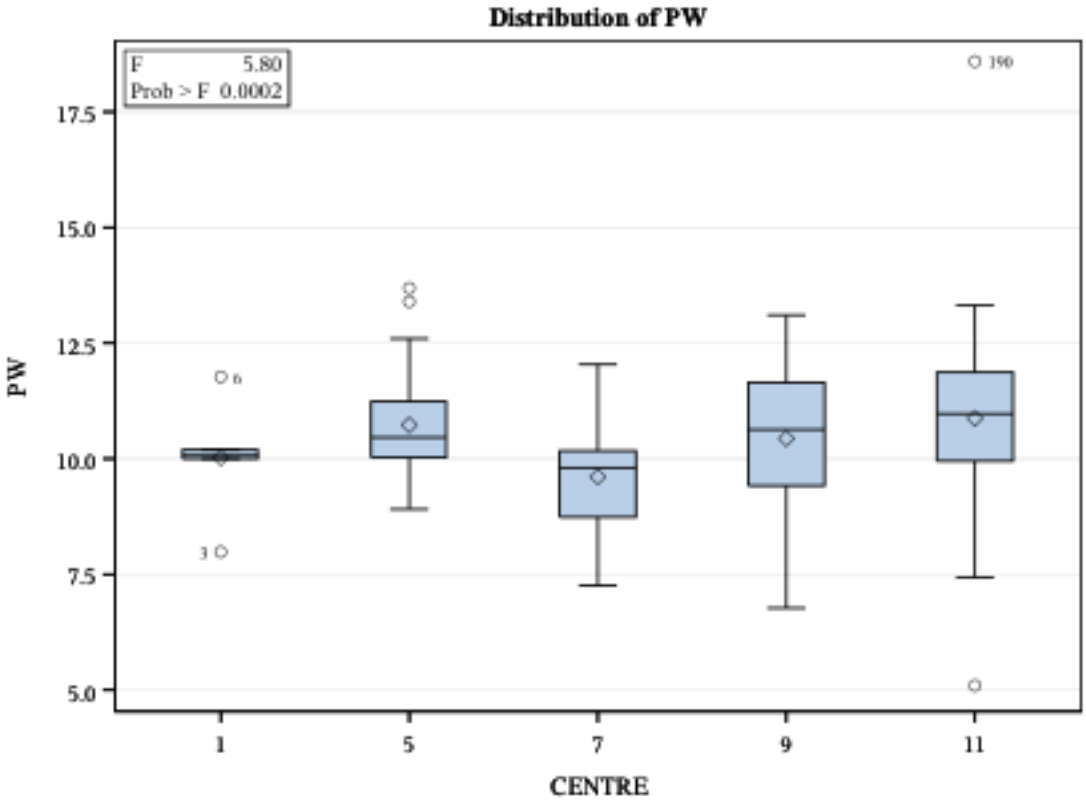
Both PL and PW measurements depicted strong intra-rater reliability. Intraclass coefficients (ICC) and 95% confidence intervals were 0.89 (0.86-0.91) and 0.72 (0.66-

0.77) for individual PL and PW measurements, respectively ($p<0.001$). Comparatively, AGD and ASD also exhibited excellent intra-rater reliability (AGD ICC=0.84 CI 0.80-0.87, ASD ICC= 0.85 CI 0.82-0.88).

Available PL and PW measurements for each subject were averaged to calculate a value used in the final analysis. Descriptive statistics for PL, PW and PL/PW ratio are summarized in table 6. Correlation between PL and PW was not statistically significant ($r=0.02$; $p=0.77$). The only genital anthropometric measurement that showed statistically significant correlation with the others was AGD, although the strength of the association was weak for all of them (PL: $r=-0.225$, PW: $r=0.186$ and ASD: $r=0.315$).

Overall, 5 participating centres enrolled infants in MIREC-ID; Levene's test for homogeneity of variances across centers was close to reaching statistical significance for both PL and PW ($p=0.05$ and $p=0.07$, respectively), but not for AGD and ASD ($p=0.87$ and 0.32, respectively); for that reason, comparison of means across centers for PL and PW was performed using both ANOVA and Welch's ANOVA. Mean PL was comparable across centres (ANOVA $p=0.16$; Welch's $p=0.13$), while PW was not (ANOVA $p=0.0014$; Welch's $p=0.0007$). The difference in PW means between centres was largely driven by 1 centre as illustrated in Figure 2. Difference between means across centres was performed using ANOVA and was statistically significant for AGD ($p<0.0001$), but not for ASD ($p=0.13$).

Figure 2 - Comparison of the means of 3 measurements of PW by centre; note that difference between centres is largely driven by centre #7

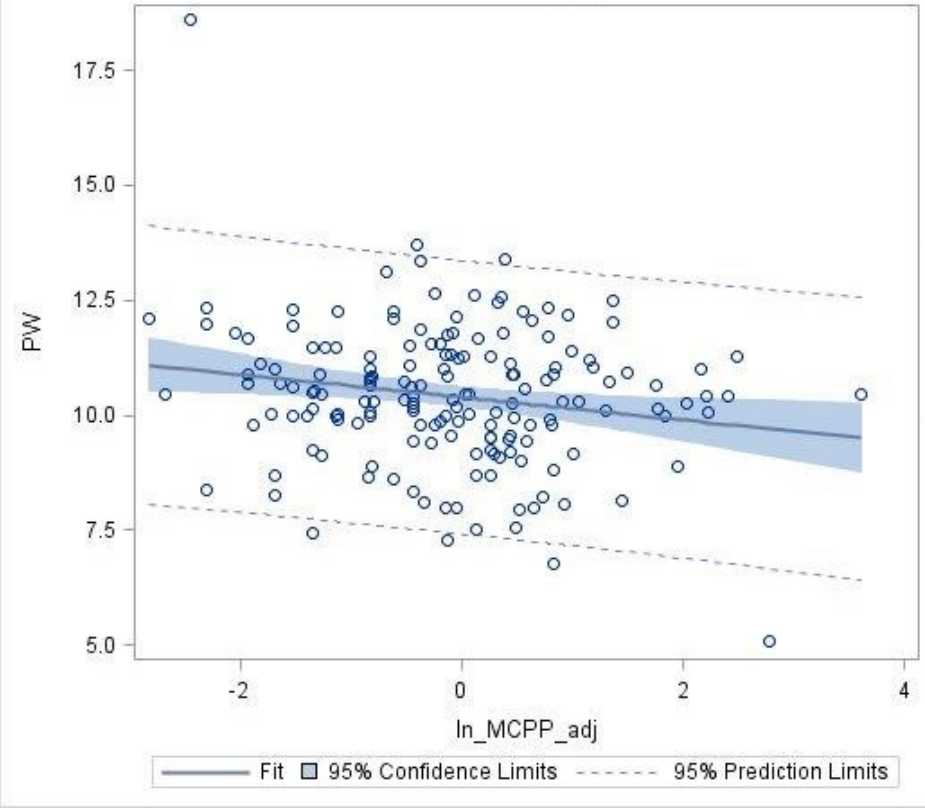


4.4 Univariate Analysis Of The Association Between Log-Transformed Phthalate Metabolite Concentrations And Genital Measurements

On univariate linear regression, no association was identified between PL and any of the crude and specific gravity-adjusted log-transformed phthalate concentrations (table 7). Conversely, PW showed a statistically significant inverse relationship with the log-transformed adjusted concentration of MCPP (estimate=-0.249, 95% confidence interval -0.048 / -0.500, p=0.0156). In practical terms, this means that a 10% increase in MCPP

adjusted concentration would lead to a 0.025mm reduction in penile width. A graphic representation of the association is seen in Figure 3.

Figure 3 - Linear regression of the association between PW and the log-transformed specific gravity-adjusted concentration of MCPP



No significant association was identified between log-transformed concentrations of the composite measures (DEHP, LMW, HMW, EXP – both crude and specific-gravity adjusted) and the outcomes of interest. When phthalate metabolites were analyzed as categorical variables (low, intermediate and high), no statistically significant association was identified with PL or PW.

Similarly, no association was found between crude and specific gravity-adjusted log-transformed phthalate concentrations and PL/PW ratio on linear regression. Finally, no association between belonging to the bottom percentile of penile length ("micropenis" or PL < 16.1167mm) and log-transformed phthalate concentrations was identified on logistic regression.

4.5 Selection Of Covariates

Penile length was not found to be associated with any of the covariates at a level below the selected threshold for inclusion in multivariate models ($p<0.15$). On the other hand, penile width depicted statistically significant associations with gestational age ($p=0.05$) and birth weight ($p=0.023$); furthermore, season of urine collection, weigh-for-gestational age z-score and active smoking during pregnancy also reached the $p<0.15$ cut-off ($p=0.08$, 0.13 and 0.14, respectively).

Specifically, in terms of infant size covariates, PL was not found to be associated with birth weight, length, weigh-for-length z-score and weight-for-gestational-age z-score. PW showed a statistically significant association with birth weight but not with the other covariates.

Neither PL/PW ratio nor "micropenis" depicted statistically significant associations with covariates.

4.6 Multivariate Analysis Of The Association Between Log-Transformed Phthalate Metabolite Concentrations And Genital Measurements

Several multiple linear regression models were created to study the association of anthropometric measurements of the external genitalia and phthalate metabolite concentrations. Gestational age, centre of enrollment, urinary specific gravity and weight-for-gestational-age z-score were included in final models for PL, PW and PL/PW ratio; weight-for-gestational-age z-score was chosen over weight-for-length z-score due to missing data on length in 10 participants and after ensuring no change to phthalate metabolite coefficients >10% was observed when switching between z-scores.

For PW, maternal active smoking was included in the final models in addition to the aforementioned variables. No statistically significant associations were found between any of the phthalate metabolite concentrations and PL, PW or PL/PW ratio upon fitting the best multivariate models, as summarized in table 8. For PL, a positive association with MEHP (i.e., a 10% increase in MEHP concentration would lead to increased penile length by 0.08mm) approached statistical significance (p=0.05).

Final multivariate logistic regression models for the bottom 10^{th} percentile of PL ("micropenis") included gestational age, centre of enrollment, urinary specific gravity and weight-for-gestational-age z-score. No statistically significant associations were found between any of the phthalate metabolite concentrations and micropenis, as summarized on table 9.

4.7 Secondary Objective Results

Only 3 male singletons were born with cryptorchidism. A possible case of hypospadias was documented in 1 subject. Hence, exploratory analysis of the association between phthalate levels and these conditions was not performed as planned. We created a variable "congenital malformations", which included all infants with any documented non-specific abnormality of the external genitalia (total n=10, cryptorchidism n=2, hypospadias n=1; hydrocele n=4, "unusual" appearance of the genitalia n=5, chordee n=1, abnormal foreskin n=2; note that some infants had more than one abnormality). No association was found between log-transformed phthalate metabolite concentrations and having a congenital malformation of the external genitalia on univariate or multivariate analyses (controlled for the same variables specified above). Multivariate analyses results for congenital abnormalities of the external genitalia are summarized in table 10.

RESULTS - TABLES

Table 2 - Maternal characteristics

Variable	Categories	Number (n)	Percentage (%)
Age	< 25 years	6	3.5
	25-29 years	45	26.5
	30-34 years	77	45.3
	≥35 years	42	24.7
Education	High school or less	12	7.0
	College	49	29.0
	University	108	63.9
	Missing	1	0.1
Race	White	156	92.0
	Other	14	8.0
Country of birth	Canada	144	85.0
	Other	26	15.0
Smoking	Current	13	7.7
	Former	37	21.8
	Quit during pregnancy	10	5.8
	Never	110	64.7
Parity	0	74	43.5
	1	60	35.3
	2	26	15.3
	3 or more	10	5.9
Gestational hypertension	No	163	96.0
	Yes	7	4.0
BMI pre-pregnancy (kg/m^2)	<18	1	0.6
	18-24	91	55.5
	25-29	42	25.6
	≥30	30	18.3
	Missing	6	
Income (C$)	less or equal to 50,000 / year	32	18.8
	50,001 – 99,999 / year	80	47.1
	Higher or equal to 100,000 / year	54	31.8
	Missing	2	

Table 3 - Urine collection variables

Variable	Categories	Number (n)	Percentage (%)
Time of day	6-9 am	4	2.4
	9:01 am to 12 pm	86	50.6
	12:01 pm to 3pm	56	32.9
	3:01 to 6pm	24	14.1
Time since last void	≤ 75 minutes	42	25.6
	76-120 minutes	51	31.1
	121-170 minutes	24	14.6
	> 170 minutes	47	28.7
Season	Fall	53	31.2
	Spring	39	22.9
	Summer	35	20.6
	Winter	43	25.3

Table 4 - Descriptive statistics for individual phthalate metabolites (volumetric and specific gravity adjusted urinary concentrations in µg/L) – n=170

Parent phthalate	Metabolite	LOD / % < LOD	Geometric Mean (95% confidence intervals)	25th quartile	Median	75th quartile	95th percentile
DEHP	MEHHP	0.4 / 1.8	8.84 (7.27 – 10.75)	3.80	9.30	20.00	54.00
	MEHHP adjusted		10.15 (8.99 – 11.46)	6.50	9.65	15.89	37.38
	MEHP	0.2 / 1.2	2.24 (1.87 – 2.69)	1.00	2.20	4.40	18.00
	MEHP adjusted		2.58 (2.29 – 2.90)	1.52	2.37	3.71	10.31
	MEOHP	0.2 / 0.6	6.24 (5.19-7.50)	2.70	6.70	13.00	40.00
	MEOHP adjusted		7.17 (6.41 – 8.02)	4.77	6.50	9.75	25.16
DnBP	MnBP	0.2 / 0	11.58 (9.67 – 13.86)	5.00	13.50	26.00	67.00
	MnBP adjusted		13.30 (11.89 – 14.86)	9.49	12.40	18.32	37.63
BBzP	MBzP	0.2 / 0	5.70 (4.71 – 6.90)	2.90	5.80	13.00	40.00
	MBzP adjusted		6.55 (5.65 – 7.59)	3.21	6.27	12.50	41.32
DnOP	MCPP	0.2 / 21.2	0.76 (0.60 – 0.95)	0.20	0.89	2.30	9.30
	MCPP adjusted		0.87 (0.73 – 1.04)	0.43	0.88	1.71	7.00
DEP	MEP	0.5 / 0.6	31.81 (25.28 – 40.03)	14.00	30.00	69.00	550.00
	MEP adjusted		36.54 (29.76 – 44.86)	13.96	29.71	75.21	411.67

LOD – limit of detection

Table 5 - Descriptive statistics for composite phthalate metabolites (µg/L)

Phthalate	Geometric Mean (95% confidence intervals)	25th quantile	Median	75th quantile	95th percentile
DEHP	60.45 (50.14 - 72.87)	25.78	63.94	130.31	359.19
DEHP adjusted	69.42 (61.94 - 77.82)	46.59	64.74	97.48	255.37
LMW	290.57 (239.57 - 352.43)	146.40	280.12	577.08	2952.29
LMW adjusted	333.73 (284.78 - 391.10)	170.67	255.23	535.63	2208.55
HMW	65.86 (54.68 - 79.32)	27.80	70.81	142.53	374.08
HMW adjusted	75.64 (67.55 - 84.71)	48.79	71.74	108.78	259.33
EXP	147.04 (1.01 - 1.02)	72.19	178.48	314.10	836.10
EXP adjusted	172.40 (155.05 - 191.69)	118.87	161.30	251.90	543.85

Molar sum of DEHP metabolites – MEHP, MEOHP, MEHHP

Molar sum of LMW (low molecular weight) phthalate metabolites – MEP, MnBP, MBzP

Molar sum of HMW (high molecular weight) phthalate metabolites – MCPP, MEHHP, MEHP, MEOHP

Molar sum of EXP (phthalates with experimental evidence of anti-androgen effects) phthalate metabolites – DEHP, MnBP, MBzP

Table 6 - Descriptive statistics for the outcome measurements

	Number	Mean	Standard deviation	Median	Min	Max
Penile length (mm)	170	21.56	4.19	21.48	10.33	34.7
Penile width (mm)	168	10.42	1.52	10.42	5.1	18.6
Penile length / width ratio	168	2.12	0.53	2.05	0.99	4.27

Table 7 - Linear regression coefficients and confidence intervals for univariate analysis of the association between phthalate concentrations (µg/L) and penile length, width and length / width ratio

	PENILE LENGTH	
Phthalate metabolite (log-transformed concentrations adjusted for specific gravity)	**Estimate - beta coefficient (95% confidence interval)**	**p-value**
MBP (Mono-n-butyl phthalate)	0.475 (-0.389 / 1.340)	0.28
MBzP (Mono-benzyl-phthalate)	0.067 (-0.588 / 0.723)	0.84
MCPP (Mono-3-carboxypropyl-phthalate)	-0.230 (-0.780 / 0.321)	0.41
MEHHP (Mono-(2-ethyl-5-hydroxy-hexyl) phthalate)	0.547 (-0.245 / 1.339)	0.17
MEHP (Mono-(2-ethylhexyl) phthalate)	0.536 (-0.272 / 1.343)	0.19
MEOHP (Mono-(2-ethyl-5-oxo-hexyl-phthalate))	0.390 (-0.471 / 1.252)	0.37
MEP (Mono-ethyl phthalate)	-0.159 (-0.629 / 0.311)	0.51
∑ DEHP (Di-2-ethylhexyl-phthalate)	0.522 (-0.321 / 1.365)	0.22
∑ LMWP (low molecular weight)	-0.187 (-0.795 / 0.422)	0.55
∑ HMWP (high molecular weight)	0.464 (-0.387 / 1.315)	0.28
∑ EXP (experimental phthalate syndrome)	0.595 (-0.312 / 1.502)	0.20

	PENILE WIDTH	
Phthalate metabolite (log-transformed concentrations adjusted for specific gravity)	**Estimate -beta coefficient (95% confidence interval)**	**p-value**
MBP (Mono-n-butyl phthalate)	0.036 (-0.280 / 0.352)	0.82
MBzP (Mono-benzyl-phthalate)	0.017 (-0.221 / 0.256)	0.89
MCPP (Mono-3-carboxypropyl-phthalate)	***-0.249 (-0.449 / -0.048)***	***0.02***
MEHHP (Mono-(2-ethyl-5-hydroxy-hexyl) phthalate)	0.030 (-0.262 / 0.322)	0.84
MEHP (Mono-(2-ethylhexyl) phthalate)	0.034 (-0.261 / 0.330)	0.82
MEOHP (Mono-(2-ethyl-5-oxo-hexyl-phthalate))	0.057 (-0.260 / 0.373)	0.72
MEP (Mono-ethyl phthalate)	-0.041 (-0.213 / 0.130)	0.63
∑ DEHP (Di-2-ethylhexyl-phthalate)	0.034 (-0.276 / 0.344)	0.83
∑ LMWP (low molecular weight)	-0.062 (-0.283 / 0.160)	0.58
∑ HMWP (high molecular weight)	-0.023 (-0.335 / 0.290)	0.89
∑ EXP (experimental phthalate syndrome)	0.030 (-0.302 / 0.362)	0.86

	PENILE LENGTH / WIDTH RATIO	
Phthalate metabolite (log-transformed concentrations adjusted for specific gravity)	**Estimate - beta coefficient (95% confidence interval)**	**p-value**
MBP (Mono-n-butyl phthalate)	0.038 (-0.071 / 0.148)	0.49
MBzP (Mono-benzyl-phthalate)	0.004 (-0.079 / 0.087)	0.92
MCPP (Mono-3-carboxypropyl-phthalate)	0.033 (-0.037 / 0.104)	0.35
MEHHP (Mono-(2-ethyl-5-hydroxy-hexyl) phthalate)	0.033 (-0.068 / 0.134)	0.52
MEHP (Mono-(2-ethylhexyl) phthalate)	0.017 (-0.086 / 0.120)	0.74
MEOHP (Mono-(2-ethyl-5-oxo-hexyl-phthalate))	0.010 (-0.100 / 0.120)	0.86
MEP (Mono-ethyl phthalate)	-0.001 (-0.061 / 0.058)	0.96
∑ DEHP (Di-2-ethylhexyl-phthalate)	0.027 (-0.081 / 0.134)	0.63
∑ LMWP (low molecular weight)	0.004 (-0.073 / 0.081)	0.92
∑ HMWP (high molecular weight)	0.038 (-0.070 / 0.147)	0.49
∑ EXP (experimental phthalate syndrome)	0.042 (-0.074 / 0.157)	0.47

Table 8 - Measures of association between maternal urinary phthalate metabolite concentrations (μg/L) and penile length, width and length / width ratio on multivariate linear regression

MULTIVARIATE ANALYSIS	PENILE LENGTH [1]	
Phthalate metabolite (log-transformed concentrations)	Estimate - beta coefficient (95% confidence interval)	P-value
MBP (Mono-n-butyl phthalate)	0.390 (-0.403 / 1.182)	0.33
MBzP (Mono-benzyl-phthalate)	0.105 (-0.508 / 0.718)	0.74
MCPP (Mono-3-carboxypropyl-phthalate)	-0.300 (-0.840 / 0.239)	0.27
MEHHP (Mono-(2-ethyl-5-hydroxy-hexyl) phthalate)	0.698 (-0.081 / 1.477)	0.08
MEHP (Mono-(2-ethylhexyl) phthalate)	0.789 (0.0001 / 1.577)	0.05
MEOHP (Mono-(2-ethyl-5-oxo-hexyl-phthalate))	0.673 (-0.172 / 1.517)	0.12
MEP (Mono-ethyl phthalate)	0.036 (-0.420 / 0.492)	0.88
∑ DEHP (Di-2-ethylhexyl-phthalate)	0.744 (-0.085 / 1.573)	0.08
∑ LMWP (low molecular weight)	0.002 (-0.580 / 0.584)	0.99
∑ HMWP (high molecular weight)	0.680 (-0.153 / 1.513)	0.11
∑ EXP (experimental phthalate syndrome)	0.618 (-0.225 / 1.460)	0.15

MULTIVARIATE ANALYSIS	PENILE WIDTH [2]	
Phthalate metabolite (log-transformed concentrations)	Estimate - beta coefficient (95% confidence interval)	P-value
MBP (Mono-n-butyl phthalate)	0.011 (-0.245 / 0.266)	0.93
MBzP (Mono-benzyl-phthalate)	0.068 (-0.128 / 0.264)	0.50
MCPP (Mono-3-carboxypropyl-phthalate)	-0.140 (-0.321 / 0.041)	0.13
MEHHP (Mono-(2-ethyl-5-hydroxy-hexyl) phthalate)	-0.046 (-0.301 / 0.209)	0.72
MEHP (Mono-(2-ethylhexyl) phthalate)	-0.005 (-0.261 / 0.251)	0.97
MEOHP (Mono-(2-ethyl-5-oxo-hexyl-phthalate))	-0.064 (-0.338 / 0.209)	0.64
MEP (Mono-ethyl phthalate)	0.022 (-0.120 / 0.165)	0.76
∑ DEHP (Di-2-ethylhexyl-phthalate)	-0.053 (-0.323 / 0.217)	0.70
∑ LMWP (low molecular weight)	0.048 (-0.136 / 0.232)	0.61
∑ HMWP (high molecular weight)	-0.056 (-0.326 / 0.215)	0.68
∑ EXP (experimental phthalate syndrome)	0.005 (-0.269 / 0.278)	0.97

MULTIVARIATE ANALYSIS	PENILE LENGTH / WIDTH RATIO[1]	
Phthalate metabolite (log-transformed concentrations)	**Estimate -beta coefficient (95% confidence interval)**	**P-value**
MBP (Mono-n-butyl phthalate)	0.045 (-0.051 / 0.140)	0.36
MBzP (Mono-benzyl-phthalate)	0.013 (-0.061 / 0.087)	0.73
MCPP (Mono-3-carboxypropyl-phthalate)	0.0004 (-0.068 / 0.069)	0.99
MEHHP (Mono-(2-ethyl-5-hydroxy-hexyl) phthalate)	0.072 (-0.023 / 0.167)	0.14
MEHP (Mono-(2-ethylhexyl) phthalate)	0.058 (-0.039 / 0.154)	0.24
MEOHP (Mono-(2-ethyl-5-oxo-hexyl-phthalate))	0.068 (-0.036 / 0.171)	0.20
MEP (Mono-ethyl phthalate)	-0.001 (-0.057 / 0.055)	0.97
∑ DEHP (Di-2-ethylhexyl-phthalate)	0.074 (-0.027 / 0.175)	0.15
∑ LMWP (low molecular weight)	-0.006 (-0.078 / 0.066)	0.87
∑ HMWP (high molecular weight)	0.067 (-0.035 / 0.168)	0.20
∑ EXP (experimental phthalate syndrome)	0.065 (-0.037 / 0.167)	0.21

1 – controlled for gestational age, weight-for-gestational age z-score, urinary specific gravity, centre of enrollment

2 - controlled for gestational age, weight-for-gestational age z-score, urinary specific gravity, centre of enrollment and maternal smoking

Table 9 - Multivariate logistic regression results; odds ratios and confidence intervals of the association between phthalate metabolites and "micropenis"

	MICROPENIS (lower 10th percentile of PL)	
Phthalate metabolite (log-transformed concentrations)	**Odds ratio (95% confidence interval)**	**P-value**
MBP (Mono-n-butyl phthalate)	0.83 (0.39 – 1.76)	0.62
MBzP (Mono-benzyl-phthalate)	0.81 (0.45 – 1.44)	0.47
MCPP (Mono-3-carboxypropyl-phthalate)	0.95 (0.60 – 1.51)	0.84
MEHHP (Mono-(2-ethyl-5-hydroxy-hexyl) phthalate)	0.62 (0.29 – 1.32)	0.22
MEHP (Mono-(2-ethylhexyl) phthalate)	0.68 (0.32 – 1.44)	0.31
MEOHP (Mono-(2-ethyl-5-oxo-hexyl-phthalate))	0.63 (0.27 – 1.46)	0.28
MEP (Mono-ethyl phthalate)	1.05 (0.73 – 1.52)	0.78
∑ DEHP (Di-2-ethylhexyl-phthalate)	0.61 (0.27 – 1.39)	0.24
∑ LMWP (low molecular weight)	1.01 (0.63 – 1.63)	0.97
∑ HMWP (high molecular weight)	0.61 (0.27 – 1.38)	0.23
∑ EXP (experimental phthalate syndrome)	0.59 (0.26 – 1.33)	0.20

Controlled for gestational age, weight-for-gestational age z-score, urinary specific gravity, centre of enrollment

Table 10 - Multivariate logistic regression results; odds ratios and confidence intervals of the association between phthalate metabolites and congenital abnormalities of the external genitalia

	CONGENITAL ABNORMALITIES OF THE EXTERNAL MALE GENITALIA	
Phthalate metabolite (log-transformed concentrations)	**Odds ratio (95% confidence interval)**	**P-value**
MBP (Mono-n-butyl phthalate)	0.86 (0.36 – 2.03)	0.73
MBzP (Mono-benzyl-phthalate)	1.04 (0.52 – 2.07)	0.92
MCPP (Mono-3-carboxypropyl-phthalate)	1.44 (0.80 – 2.61)	0.23
MEHHP (Mono-(2-ethyl-5-hydroxy-hexyl) phthalate)	1.11 (0.49 – 2.54)	0.80
MEHP (Mono-(2-ethylhexyl) phthalate)	0.87 (0.35 – 2.16)	0.76
MEOHP (Mono-(2-ethyl-5-oxo-hexyl-phthalate))	1.00 (0.40 – 2.49)	0.99
MEP (Mono-ethyl phthalate)	1.01 (0.62 – 1.65)	0.97
∑ DEHP (Di-2-ethylhexyl-phthalate)	1.03 (0.42 – 2.53)	0.94
∑ LMWP (low molecular weight)	0.96 (0.52-1.78)	0.89
∑ HMWP (high molecular weight)	1.11 (0.46 – 2.71)	0.82
∑ EXP (experimental phthalate syndrome)	0.93 (0.37 – 2.32)	0.87

Controlled for gestational age, weight-for-gestational age z-score, urinary specific gravity, centre of enrollment

CHAPTER 5 DISCUSSION

5.1 Descriptive Data

As expected in studies recruiting healthy volunteers, the maternal cohort in our study was mostly from a higher than average socioeconomic stratum. More than 90% of participants had at least a college degree, with 64% holding a university degree. Over 30% of mothers in our study had an annual household income over $100,000 CAD. Two other studies with a comparable design (TIDES, USA and SELMA, Sweden) have identified similar trends(61,81).

In terms of the phthalate metabolite concentrations, 4 metabolites had more than 50% of samples below the LOD and were therefore not included in the analysis. Two other studies (TIDES and SELMA) modelled first trimester maternal urinary phthalate metabolite concentrations and anthropometric measurements of the external genitalia (AGD and PW) (61,81). Table 11 summarizes the geometric means with confidence intervals for each metabolite concentration in each of the 3 studies. In summary, concentrations reported in the Swedish study were much higher than the ones seen in North America; MEP depicted the highest geometric mean in all 3 studies. Overall, individual metabolite concentrations were higher in the present study compared to TIDES, except for MCPP. The DEHP concentration based on molar sums was also higher in TIDES, probably due to the inclusion of an extra DEHP metabolite not assessed in MIREC (MECPP).

Table 11 - Comparison between phthalate metabolite levels described in studies with similar design (all concentrations measured in µg/L or ng/ml)

	MIREC (Canada) N=170	TIDES (USA) N=753	SELMA (Sweden) N=196
Metabolite	**Geometric Mean (95% confidence intervals)**	**Geometric Mean (95% confidence intervals)**	**Geometric Mean (95% confidence intervals)**
MEHHP	8.84 (7.27 – 10.75)	6.04 (5.49 – 6.64)	14.42 (12.82 – 16.22)
MEHP	2.24 (1.87 – 2.69)	1.93 (1.76 – 2.11)	3.27 (2.87 – 3.73)
MEOHP	6.24 (5.19-7.50)	4.22 (3.84 – 4.63)	9.68 (8.59 – 10.91)
∑DEHP	60.45 (50.14 - 72.87)	71.7 (65.6 – 78.3)	142.61 (126.99 – 160.16)
MnBP	11.58 (9.67 – 13.86)	6.36 (5.77 – 7.00)	67.62 (60.02 – 76.17)
MBzP	5.70 (4.71 – 6.90)	3.31 (2.98 – 3.68)	15.99 (13.50 – 18.95)
MCPP	0.76 (0.60 – 0.95)	1.91 (1.72– 2.13)	n/a
MEP	31.81 (25.28 – 40.03)	28.4 (25.3 – 31.9)	63.64 (54.35 – 74.52)

With regards to outcome measurements, final PL measurements in our study were found to be lower than values reported by others while PW measurements were similar (table 12). Almost all studies targeting neonatal genital anthropometric measurements in the literature use the same technique used in MIREC to measure PL (stretched penile length measured from the base of the phallus to the tip of the glans); furthermore, PL demonstrated excellent intra-rater reliability on repeated measures and there was no statistically significant variation of PL between centres in our study, which suggests that the measurements were accurate. Interestingly, anogenital distance, a popular genital anthropometric measurement in the current literature, also depicts significant variability between studies worldwide; whether these represent true differences or are the product of measurement inconsistencies remains to be determined. In our study population, infant

covariates such as weight, length and gestational age were comparable to the ones described in TIDES (61,92).

Table 12 -Comparison between neonatal / infant penile measurements between studies with a similar design

	MIREC (Canada) N=170	Bustamante-Montes (Mexico) N=73	TIDES (USA) N=753	Acerini et al. (UK) N=710	Boas et al. (Finland) N=1962
		Mean ± SD			
Penile length (mm)	21.56 ± 4.19	24.8 ± 4.40	n/a	31.70 ± 5	34.90 ± 4
Penile width (mm)	10.42 ± 1.52	10.2 ± 0.90	10.81 ± 1.32	n/a	n/a

5.2 Association Between Phthalate Metabolites And Penile Measurements

Our study of the association between maternal exposure to phthalates in the first trimester of pregnancy and anthropometric measurements of the genitalia in the male offspring yielded mostly non-significant results. We did not identify an association between any of the individual log-transformed phthalate metabolite urinary concentrations and PL or PW on multivariate analyses. Likewise, composite measures reflecting additive exposure to DEHP, low molecular weight, high molecular weight and phthalates found to have anti-androgenic effects on experimental animal models were not associated with PL or PW on univariate and multivariate analyses. There was a statistically significant inverse association between PW and the log-transformed adjusted concentration of MCPP on univariate analysis that did not persist after adjusting for

potential confounders. The association between PL and log-transformed concentration of MEHP approached statistical significance, albeit in a positive direction, which goes against our hypothesis.

We also failed to identify a statistically significant association between PL/PW ratio and "micropenis" (belonging to the bottom 10^{th} percentile for PL) and any of the metabolite concentrations on univariate and multivariate analyses.

To our knowledge, only one previous study has modelled the association between phthalate levels and penile length measured at birth. Bustamante-Montes et al. studied 73 Mexican mother-infant pairs and demonstrated an inverse relationship between DEHP phthalate metabolite levels and PL. However, of the 4 metabolites studied, only one (MEHP) had a substantial proportion of measurements above the LOD (67%, other 3 less than 15%); its multivariate linear regression coefficient modelling the association with PL, adjusted for birth length and creatinine, barely reached statistical significance ($p=0.05$) (21). Comparatively, our study had a larger sample size, encompassed a wider array of phthalate metabolites with higher proportions above the LOD; the association between PL and MEHP log-transformed concentrations was close to reaching statistical significance, although in the opposite direction (i.e. an increase in MEHP would lead to an increase in PL).

The association between maternal phthalate metabolite levels and penile width has been modelled by other authors. Akin to our findings, studies with comparable

designs to MIREC and MIREC-ID from the US (n=366) and Mexico (n=73) did not find any association between phthalate metabolite levels and PW (21,61) on multivariate analyses. A Danish (n=273) study focused on maternal phthalate levels in late pregnancy and PW also failed to identify any relationship between them (82). Our study demonstrated an inverse relationship between log-transformed adjusted MCPP concentrations and PW on univariate but not multivariate analysis. None of the aforementioned studies reported an association between PW and MCPP concentrations.

The TDS hypothesis is biologically plausible. The observation of an increase in the incidence of male reproductive tract abnormalities related to insufficient androgen stimulation (e.g. hypospadias, testicular cancer and male infertility), anti-androgen effects after phthalate exposure in the experimental setting coupled with the ubiquitous environmental human exposure to this chemical certainly raise rightful concerns about possible negative consequences to the human male offspring. Despite the inverse relationship between phthalate levels in pregnancy and AGD in the male offspring demonstrated by some authors (77,81,93,94), such findings have not been consistently reproduced by others (21,80,82). Moreover, the effect size demonstrated by studies suggesting a significant association has been modest (4 to 5% decrease in AGD for increase in metabolite concentration from the 10^{th} to the 90^{th} percentile (61) and 4% AGD reduction for more than an interquartile range increase in exposure (81)). The only statistically significant association found in our study (PW and MCPP levels on univariate analysis) confirm the same trend (10% increase in MCPP adjusted concentration would lead to a 0.025mm reduction in penile width). Hence, in light of the

existing evidence it seems premature to establish a causal relationship between phthalate exposure and undervirilization or TDS at this stage.

Given our negative results, the occurrence of a type II error should be entertained, i.e. not finding a statistically significant difference when one is present due to sample size / power issues. Nonetheless, our sample size is powered at 90% to detect a 5% change in PL using our cohort's mean and standard deviation. A larger sample size would allow for smaller changes to be detected, although the practical significance of such detection would be of debatable relevance.

With regards to our secondary objective, occurrence of congenital malformations of the male external genitalia, the number of cases of hypospadias (1) and cryptorchidism (3) was very low; exploratory analysis combining all reported abnormalities of the external genitalia (n=10) did not identify an association with any of the phthalate metabolite levels. A recent report from TIDES suggests that increased exposure to DEHP in the first trimester of pregnancy was associated with an increased risk of male congenital abnormalities (OR=2.54, CI 1.09-5.92); however, a closer look at the results reveals that, similarly to our cohort, there were only 3 cases of hypospadias, 5 undescended testes and 30 hydroceles (total sample size 371) (95). Hydroceles (accumulation of fluid in the scrotum) are common in newborn males and the vast majority undergo spontaneous resolution without any intervention; moreover, there is no evidence that hydroceles have a hormonal background contributing to their pathophysiology. Hence, at this stage the available data from birth cohort studies such as

MIREC do not allow firm conclusions to be drawn in terms of genital congenital malformations in the offspring.

Lastly, if PL, PW and AGD are truly surrogates of adequate androgen stimulation during the MPW, it would be reasonable to expect some degree of correlation between them. In our study, we did not find any correlation between PL and PW measurements. AGD was the only anthropometric measurement of the external genitalia that depicted statistically significant correlation with the other 3; nevertheless, the strength of the association was weak (all coefficients < 0.4).

5.3 Strengths And Limitations

The main strength of our study are the prospective collection of maternal, infant and urinary variables and the robustness of both exposure and outcome measures. Exposure was assessed through direct measurement of multiple phthalate metabolite concentrations in the urine during pregnancy, in the critical stages of sexual development. Moreover, our main outcome measure (PL) is a well-known surrogate of adequate androgen stimulation that has seldom been studied in the context of EDC; in addition, outcomes were measured repeatedly by trained personnel, allowing for assessment of their reliability.

Our study also offered some insight into the relationship between different anthropometric measurements of the male external genitalia (PL, PW, AGD, ASD) and

the (lack of) association between phthalate concentrations and having smaller genitals at birth.

Some limitations of this thesis should be highlighted. It is possible that our study and others have been affected by volunteer bias, a type of selection bias. In this case, highly educated women with access to information would limit their exposure to environmental chemicals and choose overall healthier behaviors, resulting in lower exposure to phthalates than what would be seen in the average expectant Canadian mother. Although a study describing the MIREC cohort profile did confirm that participants were not comparable to the average Canadian(79), phthalate metabolite levels in MIREC were similar to those reported in the Canadian Health Measures Survey for women aged 20 to 39 years old(59), suggesting that the impact of volunteer bias was minimal. It is possible that the ubiquitous nature of phthalate exposure may limit the ability to deliberately avoid contact with these substances. Nevertheless, the unique composition of the MIREC cohort may affect generalizability of the results to other populations.

Second, it is possible that our study was affected by measurement biases, both related to the exposure and outcomes. With regards to the phthalate concentrations, measurements followed a standardized process and were centralized in one laboratory. Hence, it is possible that true concentrations were under or overestimated; nonetheless, such bias would be unlikely to affect only women with high levels of exposure compared to those with lower ones, characterizing nondifferential misclassification.

It is also noteworthy that the mean penile length in our study (21.5mm) was lower than the stretched penile length observed in other birth cohort studies. Reports from the UK (7), different parts of Africa (36,96), Japan (97), and Finland (41) systematically describe average neonatal stretched penile length above 30mm. Studies on penile width are much less abundant, with results well within the ones observed in this cohort (21,38). Both outcome measures are subject to measurement bias. Nevertheless, the intra-rater reliability of repeated measures for PL and PW was high, which suggests that the measurements were consistent. Hence, even if PL and PW were underestimated for the entire cohort, smaller measurements related to the exposure would still potentially be identified.

Third, our study is based on one isolated measurement of urinary phthalate metabolite concentrations in the first trimester of pregnancy, which may have led to misclassification of the exposure. In a recent publication using the TIDES platform, the association of PW and AGD with DEHP metabolite levels in each trimester of the pregnancy was studied; the authors concluded that higher exposure to DEHP in the 1st trimester was inversely associated with AGD but not PW. Conversely, increased 2nd trimester exposure was associated with reduced PW but not AGD; finally 3rd trimester exposure did not show any significant correlations with either measurement(92). Although reasonable reproducibility over months and years has been reported for urinary biomarker concentrations of nonpersistent chemicals (76), studies of phthalates ICCs for

samples collected at different stages of pregnancy reveal overall low reproducibility(98,99), with most ICCs < 0.40.

Braun et al. compared urinary levels of phthalate metabolites before and during the pregnancy in American women attending a fertility clinic and suggested that MBP and MEP concentrations could be assessed by a single spot urine with relative accuracy (ICC=0.40 and 0.56, respectively), but the same would not be true for DEHP, BzBP metabolites and BPA (99). Fisher et al. performed multiple measurements of urinary levels of phthalate metabolites in Canadian women during pregnancy and the postpartum period; although they conclude that a single spot urine might correctly represent exposure to MEP and MBzP, the overall reproducibility of metabolite concentrations was low and multiple measurements collected at different times of the day would be desirable to paint a true picture of the exposure (98).

Fourth, our analysis was focused on one class of chemicals with the potential to disrupt endocrine function, phthalates; we did not factor in other known EDCs, such as polychlorinated byphenyls (PCBs), polybrominated byphenyls (PBBs), bisphenol A (BPA), dioxins, pesticides, fungicides and others. It is possible that male development is affected by the synergistic effect of phthalates with these other substances and our study did not model such effects.

Finally, although modelling the association between phthalate levels and anthropometric measurements of the external genitalia is appealing from a sample size

and feasibility perspective, the ideal study design would be to evaluate the impact of phthalate exposure on actual clinical endpoints. In other words, it would be desirable to compare exposures in individuals with congenital abnormalities of the external genitalia, affected by testicular cancer or infertility and controls. Nonetheless, the incidence of these conditions is low and very large sample sizes would be required for such case-control studies to be adequately powered, which might not be practical. Perhaps a case-control study nested in one of the large population-based studies that collect chemical data such as the Canadian Health Measures Survey might aid in answering this question in the future.

CHAPTER 6 CONCLUSIONS

Our study of the association between maternal urinary levels of phthalate metabolites in the 1st trimester of pregnancy and penile measurements (length and width) yielded no significant findings on multivariate analyses. This was the case for individual phthalate metabolite levels and the molar sums of DEHP, low or high molecular weight phthalates as well as phthalates implicated with anti-androgen effects experimentally. Penile width depicted a statistically significant inverse relationship with MCPP levels; however, this association was not significant on multivariate analyses and the effect size was small.

Our study did not find an association between phthalate metabolite levels and belonging in the bottom 10th percentile of penile length ("micropenis") or having congenital abnormalities of the male external genitalia on univariate and multivariate analyses. The number of cases of hypospadias or cryptorchidism was too small to allow any meaningful conclusions to be reached for these specific malformations.

Subtle changes in offspring penile length and width do not seem to be influenced by maternal urinary phthalate concentrations in this cohort of Canadian women.

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