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The Influence of Environmental Contaminants on Time to Pregnancy

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Résumé

Cette thèse porte sur l'évaluation de l'impact de certains composés environnementaux sur la fécondité féminine, tel que mesuré par le délai de conception (« time to pregnancy » en anglais, ou TTP). Cette recherche a été réalisée dans le cadre de l'Étude mère-enfant sur les composés chimiques de l'environnement (MIREC), une cohorte de grossesse de 2001 femmes recrutées durant le premier trimestre dans dix villes canadiennes de 2008 à 2011. Les données des questionnaires et les échantillons biologiques ont servi à évaluer l'effet de deux groupes de composés : les persistants [composés perfluorés – perfluorooctanesulfonate (PFOS), perfluorooctanoate (PFOA) et perfluorohexane sulfonate (PFHxS)] et les non persistants (bisphénol A, triclosan et phtalates). Cette thèse comprend également une analyse du potentiel du ratio index-annulaire (2D:4D) comme mesure de sensibilité endocrinienne. À ce jour, des mesures anthropométriques ont été collectées sur environ 800 mères-enfants dans le cadre de l'Étude mère-enfant sur les composés chimiques de l'environnement : biomonitoring et neurodéveloppement à la petite enfance (MIREC CD Plus), un suivi de la cohorte MIREC portant sur la croissance et le développement des enfants jusqu'à 5 ans.

Sur l'ensemble, les résultats de cette thèse permettent d'étoffer les preuves concernant les effets adverses potentiels de plusieurs contaminants environnementaux sur la fécondité féminine, telle que mesurée par le TTP. Dans le premier article, nous avons montré une association entre les PFOA et les PFHxS et une baisse de fécondité, ce que d'autres recherches avaient déjà révélé. Dans le deuxième article, nous avons évalué l'effet du triclosan sur le TTP, ce qui n'avait jamais été examiné, pour montrer un délai plus élevé chez les femmes du quartile supérieur d'exposition. De plus, nos résultats sont en accord avec ceux de la seule

étude ayant évalué l'effet du Bisphénol A sur la fécondité féminine, qui n'avait pas détecté d'effet. Finalement, nos données semblent indiquer une association entre l'exposition des femmes aux phtalates et un TTP plus court, mais ces résultats ne sont pas statistiquement significatifs.

En ce qui a trait au potentiel du ratio index-annuaire (2D:4D) pour mesurer la sensibilité endocrinienne chez les femmes, nos données ne permettent pas d'établir une association entre ce ratio et le TTP. Pour ce qui est des enfants, nous n'avons pas trouvé d'effet adverse entre le tabagisme de la mère durant la grossesse et leur ratio 2D:4D. Par conséquent, nos données ne semblent pas justifier l'utilisation du ratio 2D:4D pour mesurer la sensibilité endocrinienne en lien avec le potentiel reproducteur (basé sur le TTP) ou l'exposition des enfants au tabac durant le premier trimestre de grossesse.

Mots-clés : fécondité, délai de conception, composés perfluorés, bisphénol A, triclosan, phtalates, perturbateurs endocriniens, ratio index-annuaire, 2D:4D

English Summary:

In this thesis, we aimed to evaluate the impact of selected environmental compounds on female fecundity as measured by time to pregnancy (TTP). This research was conducted in the framework of the Maternal-Infant Research on Environmental Chemicals (MIREC) study, a pregnancy cohort of 2001 women recruited during the first trimester of pregnancy in ten cities across Canada between 2008 and 2011. Questionnaire data and biological samples were analyzed to assess the effect of two groups of compounds: persistent [perfluorinated compounds - perfluorooctane sulfonate (PFOS), perfluorooctanoate (PFOA), and perfluorohexane sulfonate (PFHxS)-] and nonpersistent chemicals (Bisphenol A, Triclosan, and phthalates). In addition, this thesis aimed to examine the potential of the second to fourth finger digit ratio (2D:4D) as a sensitive-endocrine endpoint. To this end, anthropometric measurements were obtained in about 800 children and their mothers during the Early Childhood Biomonitoring and Neurodevelopment Study (MIREC-CD Plus), a MIREC follow-up conducted to measure growth and development up to age five.

Overall, the results of this thesis have contributed to the evidence regarding the potential adverse effect of several environmental contaminants (ECs) on female fecundity as measured by TTP. In the first article, we found that PFOA and PFHxS were associated with diminished fecundity, supporting previous evidence that suggested a similar effect. In the second article, we assessed for the first time the effect of Triclosan on TTP, presenting evidence of delayed fecundity at the highest quartile of exposure. In addition, our findings agreed with those of the only study that has assessed the effect of Bisphenol A on female

fecundity, and which showed no effect. Finally, we found some indication that female exposure to phthalates might be associated with a shorter TTP, although this finding did not reach statistical significance.

With regard to the potential of the digit length ratio (2D:4D) as an endocrine-sensitive endpoint in women, our data do not support a strong association between 2D:4D and TTP. In children, we did not find an adverse impact of maternal smoking during pregnancy on children's 2D:4D. Thus, our data do not support evidence to suggest that 2D:4D could be used as a potential reproductive endocrine-sensitive endpoint in women as measured by TTP, and in their offspring as measured by exposure to maternal smoking during the first trimester of pregnancy.

Key Words: fecundity, time to pregnancy, perfluorinated compounds, Bisphenol A, Triclosan, phthalates, endocrine disruptors, digit ratio, 2D:4D

Table of Contents

List of Tables

List of Figures

List of Acronyms

2D:4D	Second to fourth finger digit ratio
ART	Assisted reproductive technologies
BMI	Body mass index
BPA	Bisphenol A
CAH	Congenital adrenal hyperplasia
CDC	Centers for Disease Control and Prevention in the U.S.A.
CEPA	Canadian Environmental Protection Act
CHMS	Canadian Health Measures Survey
CI	Confidence interval
CIHR	Canadian Research Institutes for Health Research
DAG	Directed acyclic graph
DEHP	Di-(2-ethylhexyl) phthalate
ECs	Environmental contaminants
EDCs	Endocrine-disruptor chemicals
EEF	Estrogenicity equivalency factor
ENDO Study	Endometriosis—Natural History, Diagnosis and Outcomes Study
FORs	Fecundability odds ratios
HMW	High-molecular-weight
HPG	Hypothalamic-pituitary-gonadal
ICCs	Intraclass correlations
INSPQ	Institut national de santé publique du Québec
IVF	In vitro fertilisation
LMW	Low-molecular-weight
LOD	Limit of detection
MBzP	Mono-benzyl phthalate
MCHP	Mono-cyclo-hexyl phthalate
MCPP	Mono-(3-carboxypropyl) phthalate
MEHHP	Mono-(2-ethyl-5-hydroxy-hexyl) phthalate
MEHP	Mono-(2-ethylhexyl) phthalate
MEOHP	Mono-(2-ethyl-5-oxo-hexyl) phthalate
MEP	Mono-ethyl phthalate
MiNP	Mono-isononyl phthalate
MIREC	Maternal-Infant Research on Environmental Chemicals study
MIREC-CD Plus	Early Childhood Biomonitoring and Neurodevelopment Study
MMP	Mono-methyl phthalate
MnBP	Mono-n-butyl phthalate
MnOP	Mono-n-octyl phthalate
MoBa	Norwegian Mother and Child Cohort

NHANES	National Health and Nutrition Examination Survey in the U.S.A.
NSFG	National Survey of Family Growth
ODS	Ovarian dysgenesis syndrome
ORs	Odds ratios
p,p'-DDE	p,p'-Dichlorodiphenyldichloroethylene
PBDEs	Polybrominated diphenyl ethers
PCBs	Polychlorinated biphenyls
PCOS	Polycystic ovary syndrome
PFCs	Perfluorinated compounds
PFHxS	Perfluorohexane sulfonic acid
PFNA	Perfluorononanoic acid
PFOA	Perfluorooctanoate
PFOS	Perfluorooctane sulfonate
PFOSA	Perfluorooctane sulfonamide
PVC	Polyvinyl chloride
QTNPR	Quebec Training Network in Perinatal Research
SD	Standard deviation
STIRRHS	Strategic Training Initiative in Research in Reproductive Health Sciences
TCCD dioxin	Tetrachlorodibenzodioxin
TCS	Triclosan
TDS	Testicular dysgenesis hypothesis
TTP	Time to pregnancy
UPLC	Ultra Performance Liquid Chromatography
β-HCH	Beta-Hexachlorocyclohexane

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INTRODUCTION

There is increasing concern about the potential effects of environmental contaminants (ECs) on human reproduction, including fecundity and fertility. Fecundity impairments appear to be increasing in males, as suggested by the declining semen quality reported by several (1-5) but not all authors, as recently reviewed (6). In addition, the prevalence of urogenital malformations seems to be higher in men having fecundity impairments or cancers of the reproductive system compared to unaffected individuals (7-9). In women, as proposed by Buck Louis (10), the relatively high prevalence of reproductive endocrinology disorders such as fibroids, polycystic ovarian syndrome, and endometriosis (11-13) may be associated with impaired female fecundity, while increasing infertility rates may be indicative of diminished couple fecundity (14, 15). Such observations, together with increased awareness of the reproductive toxicity of several ECs in wildlife and experimental animals, have led to increased research attention on the effect of ECs on human reproduction. Nonetheless, little is known about the effect of most ECs on human fecundity and fertility.

Terminology for describing human reproduction differs across disciplines. Fecundity denotes the biological capacity to reproduce, irrespective of pregnancy intentions (16). Fecundity is difficult to measure due to the complex interaction of many biological factors. Therefore, fecundability, the probability of conceiving in any given menstrual cycle (or month), conditional on not achieving pregnancy in the previous cycle, has been used as a measurement tool for the study of fecundity. This measure depends on the health and environment of both the male and female partners, as well as the interaction between them (17, 18).

Fecundability is often measured by time to pregnancy (TTP). This metric was first developed in response to the need for a sensitive and convenient screening tool to identify environmental risk factors for conception (19). TTP provides an estimate of the per-cycle probability of conceiving a clinically detectable pregnancy. An increase in the time to pregnancy can indicate reproductive loss in any of several conditional processes underlying human conception, implantation, and the viability of the conceptus (20). TTP studies complement more mechanistic research on the biological processes necessary for fertility and studies of specific medical conditions (21).

Subfertility generally describes any form of delayed fecundity with a prolonged undesired delay in conception (22). Clinically, a couple is considered to be subfertile after more than six menstrual cycles of unprotected intercourse with unsuccessful conception, while infertility refers to more than 12 or 24 months (23, 22). However, the clinical dichotomy of subfertility and infertility is an arbitrary oversimplification that can result in misclassification (19, 20). Biologically, there is a wide range of reproductive capacity, even among couples who achieve pregnancy. This heterogeneity within populations is expressed in the gradual decrease in conception rates during successive months of trying (19, 20), which is not a true time effect, but evidence of sorting among a population who are heterogeneous in their capacity to conceive (20). Heterogeneity among couples raises the possibility that some of this variation may be explained by identifiable factors. If so, well-conducted epidemiological studies are needed to identify the potential adverse effect of ECs on human fecundity.

It is estimated that one in seven couples experiences some period of subfertility during their reproductive life (24). Approximately 50% of these couples can conceive spontaneously, with the remainder requiring some form of infertility treatment (25). Compared with women

who have no difficulties conceiving, delayed conception appears to be associated with increased obstetric and perinatal risks, independently of infertility treatment. Taken together, delayed time to pregnancy brings substantial physical, emotional, and socioeconomic burdens to individuals, families, and societies.

The life-cycle approach provides a useful framework for assessing environmental chemicals and human fecundity (26). In this paradigm, health depends on interactions across the lifespan, extending from the genome to the interplay of the individual with the environment. As an example, the testicular dysgenesis hypothesis (TDS) has been proposed as an endpoint of the early origins of male fecundity and later onset disease (27). The TDS suggests that poor semen quality, testis cancer, undescended testis and hypospadias are a common entity, which results from the disruption of embryonal programming and gonadal development during fetal life. In addition, some evidence suggests that the ubiquitous exposure to environmental contaminants is associated with the increased prevalence of TDS today (27). In counterpart, ovarian dysgenesis syndrome (ODS) has been proposed as a framework for conceptualizing an early origin for female fecundity, and subsequently, health across the woman's lifespan. ODS is defined as an alteration in ovarian structure or function that may manifest as a fecundity impairment, a gynecologic disorder, a pregnancy disease, or a later-onset adult disease (26). As well, the widespread presence of chemicals, especially those that interfere with any aspect of hormone action (known as endocrine-disrupting chemicals or EDCs) (28), has been linked to its etiology.

There is a paucity of solid endocrine-sensitive endpoints that allow the assessment of diverse life-course stressors on developmental and reproductive functions. Epidemiological studies have linked a number of outcome measurements (e.g., anogenital distance, breast size,

vaginal cytology, location of the testis, testicular size, and penis growth, among others) with exposure to EDCs (29). However, little is known about how predictive these endpoints are of later reproductive health or other chronic health conditions. A new endpoint that has been proposed as an indicator of sexual development is the 2nd-to-4th-finger digit ratio (2D:4D). The 2D:4D reflects sexual differentiation early in life and is likely an endpoint for the organizational effects of prenatal androgens in the human body (30). Associations between digit ratios and several health outcomes including male fertility, sexual orientation, physical performance, and autism have been reported. The direction and magnitude of these associations, however, have not been consistent. Moreover, as in most of the literature regarding the effect of ECs on reproductive outcomes, the 2D:4D has been mainly studied in males.

The purpose of the present thesis is to assess the effect of selected ECs on female fecundity as measured by TTP. In addition, this research program aims to evaluate the predictive role of 2D:4D as a sensitive-endocrine endpoint in women and their offspring. To this end, questionnaire and biomonitoring data from approximately 2000 pregnant women recruited in ten cities across Canada as part of the Maternal-Infant research on Environmental Chemicals (the MIREC Study) were analyzed (31) (Appendix 1). To assess the potential of 2D:4D as a sensitive-endocrine endpoint, anthropometric measurements were obtained in about 800 children and their mothers during a MIREC follow-up conducted to measure growth and development up to age five: the Early Childhood Biomonitoring and Neurodevelopment Study (MIREC-CD Plus).

Chapter 1 - REVIEW OF THE LITERATURE

1.1 Environmental contaminants (ECs) and reproductive function

The production and use of chemicals continue to grow worldwide. There are currently more than 80,000 synthetic chemicals, and most of these were introduced after 1950 (32). However, only a small fraction of these substances has undergone human toxicity testing for safety. Furthermore, approximately 2800 chemicals are produced in quantities of more than 1 million tons per year (32). These high-production-volume chemicals are dispersed ubiquitously in the environment: in the air, in soil, in water, in food, and in consumer products.

Numerous studies have been conducted to evaluate risks to human reproduction by exposure to various chemicals, principally in animal models. Nonetheless, only a limited number of ECs have been studied with respect to their effects on human reproduction. Although some chemicals are highly toxic, their exposures are often restricted to certain occupational sub-populations or residents close to specific plants or pollutant dumping sites. However, some pollutants are widespread and exist almost ubiquitously in the environment. Additionally, some of these can be enriched through the food chain and accumulate in specific tissues, raising the exposure levels in the human body to well beyond their concentrations in natural habitats (33). In this manner, ubiquitous chemicals as well as persistent pollutants are of particular public health concern and are major health research targets.

The recognition that several environmental contaminants have the capacity to interfere with hormone biosynthesis, metabolism, or action resulting in a deviation from normal homeostatic control or reproduction, has increased concern that exposure to these chemicals

may have serious adverse consequences on human health (34). These compounds are generally referred to as endocrine-disruptor chemicals (EDCs) and include, among other elements, several metals (35-39), polybrominated diphenyl ethers (PBDEs) (40), polychlorinated biphenyls (PCBs), bisphenol A (BPA) (41), and phthalates (42).

Detection of contaminant residues in human serum, ovarian follicular fluid, and seminal plasma (43-45), together with reports of a decline in semen quality (1-5), have raised concern that exposure to ECs is affecting fertility. Reports of impaired fecundity linked with occupational exposure to pesticides further support this hypothesis (46, 47). However, the number of studies that have assessed the effect of ECs at current levels of exposure is limited, as recently reviewed (10).

The organization of the endocrine control of reproduction does not end at birth, but remains sensitive to the interaction of steroids or ECs throughout the life course, as has been shown for the control of ovulation in rodents (34, 48, 49). Moreover, there are coexisting mechanisms not directly mediated in the hypothalamic-pituitary-gonadal (HPG) system that may alter fecundity. For instance, fertility impairments in women exposed to ECs might be mediated through menstrual cycle alterations (50), thyroid disruption (51), or other gynecologic endocrine disorders such as the polycystic ovary syndrome (PCOS) (49), and endometriosis (52).

1.2 Assessment of exposure to environmental contaminants

The quality of exposure measurement is often the most critical determinant of the validity of epidemiological studies assessing the health effects of ECs. While toxicological studies draw on relatively precise dosing information, epidemiological studies must rely on

other sources of exposure information such as questionnaires, environmental monitoring, or measurements of biological samples (biomonitoring) to measure exposure (53). Indirect techniques, primarily questionnaires, have been used historically to estimate the degree and frequency of exposure. However questionnaires rely on human knowledge and memory, and hence, are subject to error. Personal interviews may also elicit the underreporting of many phenomena (54). Today, the emphasis is shifting to biomonitoring, which is an important tool to assess human exposures and health risk. Biomonitoring provides information as to which chemicals are being absorbed into the body and in which quantities. Nonetheless, biomonitoring studies generally only have samples collected from individuals at a single point in time due to the high cost of collecting and analyzing biological specimens for ECs. The accuracy of this snapshot of exposure will depend on the chemical of interest, the life stage of the population, the availability and proper collection of the biological sample, the critical developmental stage of interest, the consistency of exposure over the time period, and the half-life of the chemical (53, 55). Biomonitoring studies provide physicians and public health officials with reference values so that they can determine whether people have been exposed to higher levels of ECs than are found in the general population. Biomonitoring data can also help scientists plan and conduct research on exposure and health effects.

Human biomonitoring surveys at the population level such as the National Health and Nutrition Examination Survey (NHANES) (56) in the U.S.A. and the recent Canadian Health Measures Survey (CHMS) (57, 58) have identified quantifiable levels of a number of ECs in most of the people tested. Biomonitoring for ECs with a focus on women of reproductive age is rare in most countries. In Canada, maternal or umbilical cord blood heavy metals have been studied in small select populations such as the Cree First Nation, (59) the Inuit, (60)

subsistence fishing groups, (61), and populations in Montreal (62) and southern Quebec (63-65). More recently, the Maternal-Infant Research on Environmental Chemicals (the MIREC study) (31), a pregnancy and birth cohort of 2000 pregnant women recruited during the first trimester of pregnancy from 2008 to 2011, started releasing much-needed national data on the exposure of the Canadian general population and of pregnant and lactating women to several priority ECs (66, 67).

1.3 Time-to-pregnancy (TTP) studies

Time to pregnancy is a measure of how long a couple takes to conceive. Studies using TTP obtain more detailed information, beyond the usual dichotomy of fertile and infertile couples. TTP measures can be collected retrospectively or prospectively. Both designs have their strengths and limitations, and their roles are complementary (21).

The gold standard for TTP is prospective data collection (68, 16). In studies using prospective design, couples who intend to conceive or are attempting to conceive can enroll, exposures are ascertained, and the participants are followed until they either achieve pregnancy, change their minds and resume contraception, or reach a certain maximum follow-up time without achieving pregnancy (20). Although it is difficult to define a representative population sample if the study is conducted outside an occupational setting (69), recent efforts have proven to be successful using population-based sampling frameworks (70-72). A limitation, however, is that due to research protocols that are very time-consuming and/or require invasive procedures, prospective studies may result in selection bias due to the participation of select highly motivated participants (21).

Another feasible design is the current duration approach (73, 74). This uses a cross-sectional survey design and focusses on couples who are currently having unprotected intercourse, asking participants about the duration of their ongoing attempt. In addition, a combination design is possible with follow-up of the couples, based on principles from the case-cohort design (75).

On the other hand, women (or their partners) can be asked to reconstruct their TTP retrospectively for a particular pregnancy. In this retrospective approach, both the exposures and the time to an index pregnancy are based on recall (20). Retrospective designs, although lacking detailed information about the duration of exposure, have good response rates and are simpler and less expensive to conduct than prospective studies (21). One strength of the retrospective design is that it allows researchers to obtain a sample that is representative of the target population. Additionally, validity studies suggest that retrospective TTP is a valid measure when collected within a short time frame (76, 77).

To ensure validity, retrospective TTP information should be derived from a well-defined source population (21). The three main sampling frames are: pregnancy-based studies, cross-sectional population-based or occupationally-based studies, and population-based birth cohort studies. In pregnancy-based studies, the recall bias is minimal, as women are asked about their current pregnancy. The disadvantage is that sterile couples are excluded, resulting in systematic underrepresentation of subfertile women (21). Thus, an exposure that causes complete sterility in a subpopulation and has no effect on the remainder of the population would be missed in the pregnancy-based retrospective TTP design (20). Nonetheless, these studies have been successful in identifying male and female exposures with adverse effects on

fertility, as most reproductive toxicants can be expected to cause fecundity impairments among the exposed couples who have not been rendered sterile (20).

Attention should also be paid to those pregnancies that are the consequence of inconsistent use and/or failure of a contraceptive method (21). These couples are generally excluded from the main pregnancy-based analysis as they do not have an eligible TTP value. A screening question is therefore recommended, asking whether the pregnancy resulted from contraceptive failure. However, if one exposure group (e.g., smokers) has a higher degree of risk-taking and/or a lower degree of conscious planning, the members of this group are more likely to have accidental pregnancies that are excluded from the main TTP analysis, so that their eligible pregnancies apply only to a subset who are less prone to "accidents", and very likely associated with lower fecundity, resulting in "planning bias" (78). In addition, some couples may change their attitude about the pregnancy when retrospective questioning is used, reporting it as planned even though it resulted from contraceptive failure. This has been called "wantedness bias" (78), as reporting the pregnancy as an accident could be considered as saying the child was unwanted. This would cause bias if differential propensity to reinterpret one's recall were associated with one or more covariates.

Another behavioural factor that could result in a bias is differential persistence in trying to conceive, for example, when couples do not pursue the attempt to have a child with similar persistence according to their age group (79).

1.4 Time to pregnancy and adverse health effects

Delayed TTP has been reported to be associated with adverse pregnancy and perinatal adverse outcomes independently of infertility treatment. The suspected relationship is

grounded in the assumption that impaired fecundity may be associated with adverse health implications for human conception, implantation, and fetal growth and development (80, 26). One possible explanation may be that exposures at critical windows of human development are manifested in varying adverse outcomes. Hence, the timing of an exposure may dictate the type of outcome observed. Some have suggested a shared aetiology for the diverse outcomes (81-83). An alternative explanation could be that conception delay is a marker of past exposure adversely impacting the gametes in one partner or the other (80, 26). Thus, impaired fecundity as measured by delayed conception may be an early marker of eventual disturbances in human development.

However, despite the high proportion of babies born to subfertile couples, only a few studies have evaluated the association between delayed fecundity and pregnancy or perinatal outcomes. In the Danish National Birth Cohort, the prevalence of congenital malformations increased with a longer TTP (84), as did the need for a caesarean section (85), preeclampsia (86), small-for-gestational age (87), and neonatal mortality (88). In one study, women who required more than 12 months to conceive experienced approximately a 70% increase in the baseline risk of delivering a preterm infant in comparison with women requiring less time (83). Similarly, women who delivered a preterm infant were reported to require a 15% longer time for conception compared with women delivering at term (82). However, no association was reported in a subsequent study (80). With respect to the risk of low birth weight, a longer TTP increased the risk in one study (81), but no relationship was observed in a second study (82).

Delayed TTP generates high emotional and socioeconomic burdens for individuals, couples, and society. The same is true for adverse pregnancy and perinatal outcomes. The

identification of exposures and the underlying biological mechanisms that can cause fertility impairments and adverse perinatal outcomes would have dual benefits for preventing both adverse outcomes.

1.5 Contaminants of emerging concern (CECs) and time to pregnancy

Contaminants of emerging concern are chemicals that had not been previously detected in the environment or have been detected at higher levels than expected (89). The uncertainty about the risk to human health associated with their presence, frequency of occurrence, and routes of exposure is a cause for concern.

Some of these chemicals have long half-lives, allowing bioaccumulation and persistence in the environment. On the opposite end of the spectrum are those chemicals with short elimination half-lives considered nonpersistent, although their high-volume production makes them a common source of human exposure. Recently, Buck Louis summarized the available literature regarding persistent environmental pollutants and couple fecundity (10). Basically, ten cohort studies constitute the principal source of information to date. Except in two studies, fecundability odds ratios (FORs) were always lower than 1, which is indicative of a longer TTP, although not all findings were statistically significant. Persistent chemicals associated with reduced couple fecundity as measured by a longer TTP included β-HCH, cadmium, lead, mercury, p,p'-DDE, TCCD dioxin, select polybrominated diethers (PBDEs), polychlorinated biphenyls (PCBs), and perfluorinated compounds (PFCs). For the purpose of this thesis, we reviewed the literature regarding our priori-defined exposures of interest, which were categorized within two groups: persistent (perfluorinated compounds - PFOA, PFOS, and PFHxS-) and nonpersistent chemicals (Bisphenol A, Triclosan, and phthalates).

1.5.1 Perfluorinated compounds

Perfluorinated chemicals (PFCs) have received attention because of their high-volume production and ubiquitous environmental presence. PFCs are widely used in the manufacture of both domestic and industrial products, having applications as grease-or-water repellents, protective coatings for clothes, furniture and other products, and components of floor polish, adhesives, firefighting foam, and insulate for electrical wiring (56).

The two PFCs made in the largest quantities in the United States were perflurooctanoic acid (PFOA) and perfluorooctane sulfonic acid (PFOS) (90). The key producer of PFOS phased out worldwide production in 2002 and a replacement for PFOA was introduced recently, which resulted in the withdrawal of PFOA in manufacturing processes (91, 92). In spite of this, national biomonitoring surveys in the U.S.A. (56) and in Canada (57, 58) have shown that nearly all participants contained low levels of PFOA, PFOS, and perfluorohexane sulfonic acid (PFHxS) in their blood.

There is concern about the potential adverse developmental and reproductive effects of PFCs. PFOS, PFOA, and PFHxS have long serum elimination half-lives (i.e., 5.4, 3.8 and 8.5 years, respectively) (93). Toxicological studies evaluating the potential role of PFCs as endocrine-disrupting chemicals (EDCs) are limited. In vitro, some PFCs have the potential to affect estrogen-receptor and androgen-receptor transactivity (94). In rats, exposure to PFOS has shown estrous cyclicity disruption and neurotransmitter imbalance (95).

In humans, recent studies have explored the potential association between PFCs and fecundity, as measured by time to pregnancy. In a retrospective pregnancy-based TTP study Fei et al. (96) utilized banked biospecimens for the quantification of plasma PFOA and PFOS concentrations among 1,240 pregnant women participating in the Danish National Birth

Cohort. Significant inverse trends were observed for both PFOA and PFOS and TTP, reflecting an approximate 30% reduction in fecundity for women in the highest three quartiles in comparison to women in the lowest. In a case-control study including 910 pregnant women from the Norwegian Mother and Child Cohort (MoBa), Whitworth et al. (97) reported twofold higher odds of subfecundity (TTP >12 months) at increasing concentrations of PFOA and PFOS in parous but not nulliparous women. Moreover, a recent analysis of the same MoBa cohort testing different statistical approaches reported significant diminished fecundity at increasing PFOA concentrations (98). In prospective couple-based cohort designs, Vestergaard et al. (99) observed no consistent pattern between eight PFCs and TTP in the Danish Cohort, and Buck Louis et al. (71) reported just one (perfluorooctane sulfonamide, PFOSA) of the seven PFCs assessed in the LIFE Study (Michigan and Texas) associated with decreased fecundability.

1.5.2 Bisphenol A

Bisphenol A (BPA) is a high-production-volume chemical used in a variety of commonly used consumer products. Most notably, BPA is present in polycarbonate plastics, the epoxy resin liners of aluminum cans, and in thermal receipts (100). Exposure to BPA is common, with over 90% of the populations of the U.S.A. and Canada having detectable urinary concentrations (101, 58).

BPA has recognized endocrine-disrupting properties in animals (102), however, there is limited information regarding the effect of BPA exposure on human fecundity.

As recently reviewed (102), BPA may have ovarian and uterine toxicity. In the ovary, BPA affects the onset of meiosis in animal and in-vitro models, interferes with germ-cell nest breakdown in animal models, accelerates follicle transition in several animal species, alters

steroidogenesis in multiple animal models and in women, and reduces oocyte quality in animal models and in women undergoing IVF (102). In the uterus, BPA exposure is associated with impaired uterine endometrial proliferation, decreased uterine receptivity, and increased implantation failure in animal models (102).

While several studies conducted in infertile couples seeking assisted reproductive technologies (ART) suggest adverse reproductive effects, only one study has assessed the impact of BPA on couple fecundity in a population-based setting (72). In the context of ART, higher concentrations of BPA have been associated with hormonal imbalances in both women and men (103-105), diminished antral follicle counts (106), decreased number of oocytes retrieved after ovarian stimulation (107, 108), lower fertilization rate (108), and implantation failure (109). At the population level, the only epidemiological study to date assessing the effect of BPA on TTP reported no association between female or male BPA urinary concentrations and TTP among participants from the LIFE Study, a prospective cohort of couples attempting pregnancy in the U.S.A. (72).

1.5.3 Triclosan

Triclosan (TCS), a halogenated phenol, is a broad-spectrum antimicrobial used as an ingredient in disinfectants, soap, detergent, toothpaste, mouthwash, fabric, deodorant, shampoo, and plastic additives, in addition to innumerable other personal care, veterinary, industrial, and household products (110, 111). TCS is effective against many types of bacteria and certain types of fungi, preventing bacterial propagation and/or eventually resulting in cell death. The use of TCS is not highly regulated, as the antimicrobial has a low acute toxicity and is generally accepted as well tolerated and safe (110, 111).

TCS was detectable in about 75% of the urine samples collected as part of the NHANES 2003-2004 survey of the U.S. population (112) and in the 2009-2011 Canadian Health Measures Survey (58). TCS has a similar structure to known EDCs, including polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs), and BPA, and to thyroid hormones (110). These structural similitudes, together with some limited evidence in laboratory animals of effects on diverse hormones, suggest that TCS may influence endocrine function, and possibly the reproductive axis (110). Epidemiological studies on TCS are limited. Two studies reported no significant impact of prenatal exposure to TCS on birth size (113, 114). A recent analysis of urine samples from NHANES 2003-2008 showed a positive association between TCS and body mass index (BMI) (115). No studies assessing the effect of TCS on TTP have been conducted to date.

1.5.4 Phthalates

Phthalates are a group of synthetic EDCs used as plasticizers in industrial production. They have received special attention because of their high-volume production, ubiquitous environmental presence, and possible association with adverse reproductive health outcomes (56). Human exposure to phthalates may result in changes to tissues in the testes and reduced sperm counts; lower levels of testosterone; increased prenatal mortality; decreases in fetal growth, birth weight, and anogenital distance; and a number of biological malformations (116).

Phthalates are used to impart flexibility and resilience to polyvinyl chloride (PVC) plastics (56). Many consumer products contain phthalates, including vinyl flooring, adhesives, detergents, lubricating oils, solvents, automotive plastics, some medical pharmaceuticals, plastic bags, blood-storage bags, intravenous medical tubing, children's toys, and personal

care products such as soap, shampoo, deodorants, fragrances, hair spray, and nail polish (56). Di-(2-ethylhexyl) phthalate (DEHP) is the most abundant phthalate in the environment, and mono-(2-ethylhexyl) phthalate (MEHP) is its primary metabolite, created by the breakdown of DEHP in the body (116).

Because phthalates are not chemically bound to PVC plastics, they gradually separate from consumer products over time. As a result, humans can experience phthalate exposure during their entire life cycle, whether it be through digestion, inhalation, dermal contact, the indirect leaching of phthalates into other products, or general environmental contamination (117). Although phthalates tend to rapidly metabolize in the body, their widespread use and ubiquitous presence in dust, soil, and indoor and outdoor air results in continuous human exposure to these chemicals and possible health effects as a result. Additionally, as phthalates metabolize in the body, they are better able to interfere with biological function (118).

Experimental studies have indicated that the effect of phthalates on human health is very much related to the time of exposure. Exposures during critical windows of vulnerability may have adverse effects in the short and long term. Phthalate exposure during periods regulated by steroid hormone levels, such as ovarian and testicular development in the womb and during puberty, pregnancy, and menopause, can lead to reproductive effects later in life. Evidence is also building regarding the cumulative health effects of simultaneous exposure to multiple phthalates and other EDCs (119, 120). For example, studies have demonstrated associations between male reproductive malformations in rats and the presence of a combination of phthalates and EDCs in the body (119).

Most phthalate studies to date have focused on male exposures and reproductive effects. There continues to be limited research exploring the effects of phthalates on women's health, despite the fact that female reproductive function and development are equally, if not uniquely, susceptible to exposure to hormonally active chemicals (121). Evidence is now emerging that shows females are more highly exposed to phthalates than males. For example, the Fourth U.S. National Report on Human Exposure to Environmental Chemicals examined data from National Health and Nutrition Examination Surveys (NHANES, 1999-2000, 2001-02, and 2003-2004) on urinary levels of phthalates in a subsample of the U.S. population aged six and over (56). The analysis identified gender differences in the concentration of the phthalates under assessment, with higher urinary phthalate levels observed in women than in men. This pattern was also observed more recently in the Canadian Health Measures Survey (57), where biomonitoring of phthalate metabolites in the urine samples of 3,236 individuals ranging from six to forty-nine years of age reported significantly higher concentrations in females than in males (122).

There is a paucity of studies assessing the effect of phthalates on women's fecundity. In Generation R, a large pregnancy cohort study conducted in the Netherlands, occupational exposure to phthalates was assessed using a job-exposure matrix, and was reported as being associated with a longer TTP (123, 124). In Italy, concentrations of several phthalate metabolites were assessed in 56 infertile couples from an ART centre, and were found to be significantly higher than in the control group of fertile couples (125). Recently, in the LIFE Study, no phthalate metabolite in female urine was statistically associated with a longer TTP, although one metabolite [mono (3-carboxypropyl) phthalate] was associated with a shorter

TTP. In men, urinary concentrations of monomethyl, mono-n-butyl, and monobenzyl phthalates were associated with a longer TTP (72).

In summary, epidemiologic evidence for the role of ECs in general, and particularly for perfluorinated compounds, BPA, TCS, and phthalates in TTP is limited. Moreover, in most cases, relationships supported by inadequate evidence reflect the scarcity of evidence as opposed to the strong evidence for no effect (126). There are major knowledge gaps and a need for better research data on the effects of ECs on fecundity. As mentioned above, the effects of ECs on reproductive health have been less studied in women than in men. However, female reproductive function and development are susceptible to endocrine toxicants, and effects on female reproductive organs and pregnancy outcomes are a major health concern (127). Prospective cohorts of couples attempting pregnancy, along with the utilisation of stored biological samples from existing pregnancy cohort studies with larger sample sizes and valid assessments of exposure (i.e., biomarkers) will be of great value in better characterizing degrees of risk.

1.6 The digit length ratio as an endocrine-sensitive endpoint

The second to fourth digit length ratio (2D:4D) reflects sexual differentiation early in life and has been suggested as an endpoint for the organizational effects of prenatal androgens in the human body (30). 2D:4D is a sexually dimorphic trait, with males having relatively shorter 2nd digits (index fingers) with respect to their 4th digits (ring fingers) (128). As such, lower 2D:4Ds may be potential indicators of greater androgen exposure during fetal development. Associations between digit ratios and several health outcomes including male

fertility have been reported. The direction and magnitude of these associations, however, have not been consistent.

Several cross-sectional studies have reported low sperm counts and impaired hormonal status in men having higher 2D:4D ratios (129-131). However, studies with larger sample sizes have failed to replicate these associations (132-137). In addition, two retrospective studies reported associations between lower 2D:4D ratios and delayed age at menarche (138, 139), while a prospective cohort found lower ratios as predictors of early age at menarche (140). Concerning fertility, the evidence is sparse and usually assessed by means of indirect markers. Thus, lower female digit ratios have been associated with low offspring counts (141), congenital adrenal hyperplasia (CAH) (142, 143), and polycystic ovary syndrome (PCOs) (144), although this finding was challenged by Lujan et al. (145, 146). TTP is an epidemiological metric widely used for the assessment of human fecundity (19, 16). In males, one study reported decreased fecundity (i.e., delayed TTP) at higher 2D:4Ds (130). However, the predictive role of 2D:4D on TTP has never been assessed in females.

Smoking has a recognized detrimental impact on fertility, both male and female (147, 16). Furthermore, maternal smoking during pregnancy has been associated with adverse reproductive outcomes in offspring (148), including impaired fecundity (149). The causal mechanism by which smoking adversely affects fertility is still the subject of active research. In rats, prenatal exposure to nicotine has been linked to increased testosterone levels (150). In humans, maternal smoking during pregnancy has been positively associated with concentrations of maternal testosterone (151). Thus, if the adverse effect of maternal smoking on the offspring's subsequent fertility acts through the homeostasis of fetal androgens, and under the hypothesis that the 2D:4D ratio is an indicator of the fetal concentrations of

androgens, then 2D:4D might differ among children whose mothers smoked during pregnancy compared to those whose mothers did not smoke. Only one cross-sectional study has assessed the impact of maternal smoking during pregnancy on 2D:4D (152), reporting lower digit ratios in boys prenatally exposed to smoking, although this association was not observed in females.

To sum up, there is limited information about the predictive role of 2D:4D as a reproductive endocrine-sensitive endpoint and additional research in this area is required.

Chapter 2 - RATIONAL AND OBJECTIVES

The Centers for Disease Control and Prevention (CDC) has declared fertility impairment as an emerging public health priority in the United States (153, 154). Data from the U.S 2006-2010 National Survey of Family Growth (NSFG) showed that infertility affects about 6% of married women 15–44 years of age, and that an additional 12% of women ages 15-44 suffer from impaired fecundity (i.e., the ability to become pregnant or carry a pregnancy to a live birth). As for the men, 9% reported male-related infertility (155). Moreover 12% (7.3 million women) or their husbands or partners had used infertility services (including counseling and diagnosis) in their lifetime (156). In the recently-released CDC National Public Health Action Plan for the Prevention, Detection and Management of Infertility, data gaps in knowledge related to fertility impairments were identified in many areas. Regarding research needed for primary prevention, the investigation of environmental exposures was evoked as a priority (153, 154).

While there is strong animal evidence of the reproductive toxicity of many environmental chemicals, epidemiologic studies are limited. There is mounting concern that exposure to chemicals with endocrine-disrupting activity causes reproductive disorders in human populations such as delayed time to pregnancy, precocious puberty, estrogen-sensitive cancers, and declines in semen quality, among others.

There is a paucity of adequate epidemiologic studies on the reproductive health effects of environmental contaminants (157). Furthermore, additional studies are needed to strengthen the limited or inadequate evidence suggesting that low doses of some biologically active contaminants can alter reproductive function; that periconceptional exposure can have long-

term adverse effects, including reproductive health effects; and that multiple exposures can act synergistically (157).

Epidemiologic studies are warranted, given that biomonitoring studies show varying body burdens in the general population, and preliminary animal toxicity data provide evidence of potential endocrine disruption for numerous environmental contaminants. It is of primordial importance that the impact of selected ECs (i.e., perfluorinated compounds, phenols, and phthalates) on reproductive health be elucidated.

The potential for exposure to ECs and evidence of the sensitivity of the developing reproductive system from studies in animals and humans, as well as the many current knowledge gaps, justify continuing concern about this issue.

The present research program aims to determine the effect of selected environmental contaminants (i.e., perfluorinated compounds, phenols, and phthalates) in women's time to pregnancy, making use of questionnaire and biomonitoring data, to assess periconceptional hazard exposures in a population of pregnant women. In addition, the study aims to assess the potential of 2D:4D as a reproductive endocrine-sensitive endpoint in women and their offspring.

The specific objectives of this thesis include:

2.1 Study #1

To evaluate the association between selected PFCs (i.e., PFOA, PFOS, and PFHxS) and time to pregnancy in women participating the MIREC Study

2.2 Study #2

To assess the effect of BPA, TCS, and phthalates on women's fecundity, as measured by TTP in the context of the MIREC Study

2.3 Study #3

In examining the potential of 2D:4D as a reproductive endocrine-sensitive endpoint, the objectives were:

1. To evaluate the association between female 2D:4D and fecundity, as measured by time to pregnancy (TTP) in women from the MIREC Study;

2. To evaluate the effect of prenatal exposure to maternal smoking on the 2D:4D of children born to women from the MIREC cohort.

Chapter 3 - PERFLUORINATED CHEMICALS AND FECUNDITY: THE MIREC STUDY (ARTICLE 1)

Running Title: Perfluorinated Chemicals and Time to Pregnancy

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3.1 Abstract

Study question: What is the effect of maternal exposure to perfluorooctane sulfonate (PFOS), perfluorooctanoate (PFOA), and perfluorohexane sulfonate (PFHxS) on Time to Pregnancy (TTP)?

Summary answer: Evidence is presented that increases in maternal plasma concentrations of PFOA and PFHxS may be associated with reduced fecundability and infertility.

What is known already: Endocrine-disrupting chemicals (EDCs) can alter hormone functions essential to development and reproduction. Perfluorinated chemicals (PFCs) are a group of synthetic EDCs used in industrial production. There is increasing concern about the effect of PFCs on fecundity, as measured by Time to Pregnancy (TTP). Although recent studies suggest that increasing concentrations of PFCs may decrease fecundity, divergences in the methodological approaches used to evaluate this association have prevented firm conclusions.

Study design, size, duration: The Maternal-Infant Research on Environmental Chemicals (MIREC) Study is a pregnancy and birth cohort of 2,001 women recruited before 14 weeks of gestation in 10 cities across Canada between 2008 and 2011. The present study utilizes a pregnancy-based retrospective TTP approach.

Participants/materials, setting, methods: A questionnaire was administered and medical chart data and biospecimens were collected from participants. After excluding women who withdrew, those for whom data was incomplete, and those whose pregnancies followed birth-control failure, and accounting for the male infertility factor, 1743 participants remained. TTP was defined as the number of months of unprotected

intercourse needed to become pregnant in the current pregnancy, as self-reported in the first trimester of pregnancy. Plasma concentrations of PFOA, PFOS, and PFHxS measured in the first trimester were considered as a proxy of preconception exposure. Fecundability odds ratios (FORs) were estimated using Cox proportional hazard models for discrete time. FORs <1 denote a longer TTP and FORs >1 a shorter TTP. The odds of infertility (TTP > 12 months or infertility treatment in the index pregnancy) were estimated using logistic regression. Each chemical concentration (ng/ml) was log transformed and divided by its standard deviation. Potential confounders were maternal age, household income, and body mass index (BMI).

Main results and the role of chance: The probabilities of pregnancy at 1, 6 and 12 months were 0.42 (95% CI 0.40-0.45), 0.81 (95% CI 0.79-0.83) and 0.90 (95% CI 0.89-0.92), respectively. The mean maternal age was 32.8 (SD 5.0) years. Half of the women had at least one prior pregnancy with a live birth, and about 15% were obese or active smokers during the preconception period. Maternal and paternal age, pre-pregnancy BMI, and parity were associated with TTP. Maternal or paternal active smoking, gestational age at which the sample was collected, country of birth, household income, and education were not associated with TTP. The geometric means (ng/ml) of PFOA, PFOS, and PFHxS were 1.66 (95% CI 1.61-1.71), 4.59 (95% CI 4.46-4.72) and 1.01 (95% CI 0.97-1.05), respectively. Crude FORs per one standard deviation increase in log-transformed serum concentrations of PFOA, PFOS, and PFHxS were 0.91 (95% CI 0.86-0.96), 0.97 (95% CI 0.92-1.03), and 0.94 (95% CI 0.89-1.00), respectively. After adjustment for potential confounders, PFOA and PFHxS were associated with a 11% and 9% reduction in fecundability per one standard deviation increase (FOR= 0.89; 95% CI 0.83-0.94;

p<0.001 for PFOA, and FOR=0.91; 95% CI 0.86-0.97; p=0.002 for PFHxS), while no significant association was observed for PFOS (FOR=0.96; 95% CI 0.91-1.02; p=0.17). In addition, the odds of infertility increased by 31% per one standard deviation increase of PFOA (OR=1.31; 95% CI 1.11-1.53; p=0.001) and by 27% per one standard deviation increase of PFHxS (OR= 1.27; 95% CI 1.09-1.48; p=0.003), while no significant association was observed for PFOS (OR= 1.14; 95% CI 0.98-1.34; p=0.09).

Limitations, reasons for caution: Pregnancy-based TTP studies may exclude women with the highest concentrations of PFCs if there is causal association with infertility. We did not assess the concentrations of PFCs in males, nor their semen quality. As well, information on menstrual cycle characteristics, ovulation, and intercourse frequency was not available.

Wider implications of the findings: Our results add to the evidence that exposure to PFOA and PFHxS, even at lower levels than previously reported, may reduce fecundability. The mechanism involved in this endocrine-disrupting effect needs to be assessed.

Study funding/competing interest(s): The MIREC Study is supported by the Chemicals Management Plan of Health Canada, the Canadian Institutes for Health Research (CIHR, grant # MOP – 81285), and the Ontario Ministry of the Environment. M.P. Velez is supported by a CIHR Fellowship Award. W.D Fraser is supported by a CIHR Canada Research Chair.

3.2 Introduction:

Perfluorinated chemicals (PFCs) have recently received attention because of their high-volume production, ubiquitous environmental presence, and possible association with adverse health effects. PFCs were introduced in 1950 and have since been widely used in the manufacture of both domestic and industrial products having applications as grease-or-water repellents and protective coatings for clothes, furniture, and other products, and also as constituents of floor polish, adhesives, firefighting foam, and insulation of electrical wire (CDC 2009).

The two PFCs made in the largest quantities in the U.S. were perflurooctanoic acid (PFOA) and perfluorooctane sulfonic acid (PFOS) (ATSDR 2009). The key producer of PFOS phased out worldwide production in 2002 and a replacement for PFOA was introduced recently which resulted in PFOA no longer being used in manufacturing processes (DuPont 2013, 3M 2014). Direct uses in Canada have largely been phased out (Health Canada 2008); voluntarily, by most of the manufacturers of PFOA, and by federal regulation through the *Canadian Environmental Protection Act* (CEPA) for PFOS (Environment Canada 2012). Nonetheless, the production of PFOS has continued in China, and therefore, products containing their precursors may be still entering the Canadian market (Lim et al. 2011).

Although the production of several PFCs has declined during the last decade, recent national biomonitoring surveys in the United States (Centers for Disease Control and Prevention 2009) and Canada (Health Canada 2010, 2013) have shown that nearly all participants were found to have low levels of PFOA, PFOS, and perfluorohexane sulfonic acid (PFHxS) in their blood . These PFCs have also been detected in cord blood

(Arbuckle et al. 2013) and breast milk (Fromme et al. 2010). While the primary source of exposure in the general population is food (Tittlemier et al. 2007), water is an important source in contaminated areas, for example, in communities near production facilities (Hoffman et al. 2011). In addition, PFCs have been detected in dust (Bjorklund et al. 2009) and in indoor and outdoor air (Shoeib et al. 2004, Shoeib et al. 2011).

There is concern about the potential adverse developmental and reproductive effects of PFCs. PFOS, PFOA and PFHxS have long serum elimination half-lives (i.e., 5.4, 3.8 and 8.5 years, respectively) (Olsen et al. 2007). Toxicological studies evaluating the potential role of PFCs as endocrine-disrupting chemicals (EDCs) are limited. In vitro, some PFCs have the potential to affect estrogen-receptor and androgen-receptor transactivity (Kjeldsen and Bonefeld-Jorgensen 2013). In rats, exposure to PFOS has shown estrous cyclicity disruption and neurotransmitter imbalance (Austin et al. 2003).

In humans, several studies have explored the potential association between PFCs and fecundity, as measured by Time to Pregnancy (TTP). In a retrospective pregnancy-based TTP study within the Danish National Birth Cohort, Fei et al. (2009) observed a strong association between higher concentrations of PFOA and PFOS and longer TTP. In a case-control study within the Norwegian Mother and Child Cohort (MoBa), Withworth et al. (2012) reported higher odds of subfecundity (TTP >12 months) at increasing concentrations of PFOA and PFOS. A recent analysis of the same MoBa cohort testing different statistical approaches replicated the association between PFOA and longer TTP (Ding et al. 2014). Nonetheless, Whitworth et al. (2012), under the assumption of reverse causation suggested by Olsen et al. (2009) (i.e., parous women with longer Time to Pregnancy have higher PFCs levels because they have long interpregnancy intervals

allowing re-accumulation of PFCs), found no association among nulliparous women after stratification by parity. The same stratification was further reapplied by Fei et al. (2012), which resulted in stronger associations for PFOS and PFOA in nulliparous women and for PFOA in multiparous women. In prospective couple-based cohort designs, Vestergaard et al. (2012) observed no consistent pattern between eight PFCs and TTP in a Danish cohort, and Buck Louis et al. (2013) reported just one (perfluorooctane sulfonamide, PFOSA) of the seven PFCs assessed in the LIFE Study (Michigan and Texas) associated with decreased fecundability. Questions remain on why these studies have reported conflicting results on the association between PFCs and Time to Pregnancy.

The aim of the present study was to evaluate the association between selected PFCs (i.e., PFOA, PFOS, and PFHxS) and Time to Pregnancy in the MIREC Study, a Canadian pregnancy and birth cohort.

3.3 Methods:

Population and study design

The Maternal-Infant Research on Environmental Chemicals (MIREC) Study was established to examine potential adverse health effects of exposure to priority environmental chemicals on pregnancy and infant health in Canada. The cohort profile of the MIREC Study was recently published (Arbuckle et al. 2013). Briefly, pregnant women from the general population who were attending early prenatal clinics in 10 cities across Canada between 2008 and 2011 were invited to participate, reaching a participation rate of 39%. Some two thousand women were followed during each trimester of pregnancy, at delivery, and in the early post-natal period.

In this specific pregnancy-based retrospective TTP study, socio-demographic and lifestyle data, as well as biospecimens collected during the first trimester of pregnancy (6 to less than 14 weeks) from 1743 participants were analyzed (Appendices 2 and 3). Pregnancies following birth control failure were excluded (Figure 1). Sixteen patients who became pregnant with sperm donation, three with egg donation, and fifteen whose male partners required some infertility treatment were also excluded.

The MIREC study was approved by the Research Ethics Board of Health Canada, the research ethics committee of the coordinating centre of Ste-Justine's Hospital in Montreal, and the academic and hospital ethics committees of the 10 study sites across Canada. All the participants signed informed consent forms.

Study variables

Time to Pregnancy

Time to Pregnancy was collected as a discrete variable (i.e. number of months of unprotected intercourse before conception) by this question: "How long did it take you to get pregnant with this pregnancy?" (number of months)

Infertility was defined as having a TTP of more than 12 months or requiring infertility treatment for this pregnancy.

PFOA, PFOS, and PFHxS exposure

Maternal blood samples collected during the first trimester of pregnancy were considered a surrogate of the preconception exposure to PFOS, PFOA and PFHxS. Maternal blood was collected in 10-ml sterile vacutainer tubes. Within 2 hours of the blood draw, the samples were centrifuged and the plasma aliquoted into smaller cryovials to be stored at -80 °C until analysis.

Chemical analyses were carried out by the toxicology laboratory of the Institut national de santé publique du Québec (INSPQ), https://www.inspq.qc.ca/ctq/, which is accredited by the Standards Council of Canada under ISO 17025 and CAN-P-43. The analytes were extracted at alkaline pH with methyl tertbutyl ether, and ion-pairing done with tetrabutylammonium hydrogensulfate, evaporated to dryness and dissolved in the mobile phase. Samples were analyzed with a Waters Acquity UPLC-MS-MS (Milford MA) operated in the MRM mode with an electrospray ion source in negative mode.

Statistical Analysis

Descriptive statistics, including the percentage detected, medians, and geometric means were computed for all chemicals, and arithmetic means were found for important

demographic variables. Concentrations below the limit of detection (LOD) were set to the LOD divided by two. Each chemical concentration (ng/ml) was log-transformed and divided by its standard deviation to facilitate the biologic interpretation of the effect measure (Buck Louis et al. 2013).

Fecundity odds ratios (FOR) were estimated using the Cox model, modified for discrete time data (Allison PD 2010). FORs estimate the odds of becoming pregnant each cycle, given exposure to the specific PFC, conditional on not being pregnant in the previous cycle. FORs less than 1 denote reduction in fecundity or longer TTP, and FORs greater than 1 denote a shorter TTP. TTP was censored at the 13th month. The proportional hazard assumptions were verified for the discrete-time models (Allison PD 2010). In addition, we used logistic regression to estimate the odds ratios (ORs) for infertility (TTP > 12 months or infertility treatment). Statistical significance was assessed using an alpha level of 0.05.

Potential confounders included gestational age at blood draw, maternal age, country of birth, education, household income, maternal and paternal smoking, and pre-pregnancy body mass index (BMI). Maternal and paternal age where highly correlated (r= 0.73), which precluded inclusion of paternal age into the model. Variables with a p-value <0.20 in the univariate analysis were potentially eligible for the multivariate model. In a second step, based on our literature review and the statistical assessment of covariates, we depicted a directed acyclic graph (DAG) to represent the covariates included in our causal model (Figure 2).

There is controversy on how to consider parity in the assessment of the toxicological effect of PFCs on TTP. Biologically, parity is influenced by a woman's fecundability.

TTP is used as an epidemiological metric for the assessment of women's fecundability. Thus, we consider that conditioning (i.e., adjusting, stratifying, or restricting) on parity is redundant and would cause over-adjustment, as parity is the result, among other factors, of proven fecundability. This argument proposes that parity is a type of covariant known as a collider in the modern theory of diagrams for causal inference using Directed Acyclic Graphs (DAGs) (Hernan and Robins 2013). A collider is a covariate that is affected, directly or indirectly, by two other covariates. In our DAG, parity lies in between the determinants of fecundability included in our analysis (i.e., age, BMI, income), and TTP (Figure 2). Conditioning on a collider is susceptible to collider-stratification bias (Greenland 2003, Schisterman et al. 2009). It would open the blocked backdoor path between exposure and outcome, inducing conditional bias for at least one stratum of parity (Howards et al. 2012, Hernan and Robins 2013).

Statistical analysis was performed using STATA 10.0 (Stata Corporation, College Station, TX) and SAS 9.3 (SAS Institute Inc., Cary, NC), specifically for the discrete-time Cox proportional models.

3.4 Results

The probabilities of pregnancy for the cohort at months 1, 6 and 12 were 0.42 (95% CI 0.40-0.45), 0.81 (95% CI 0.79-0.83), and 0.90 (95% CI 0.89-0.92), respectively. Socio-demographic characteristics of the population and their association with TTP are presented in Table 1. The mean maternal age was 32.8 (SD 5.0) years. About two thirds of the women had a university degree, most were born in Canada, more than one third reported a household income higher than $100.000 CAD, half had at least one prior pregnancy with a live birth, and about 15% were obese or active smokers during the preconception period. Maternal and paternal age, pre-pregnancy BMI, and parity were associated with TTP. Maternal or paternal active smoking, gestational age at which the sample was collected, country of birth, household income, and education were not associated with TTP.

Table 2 presents the distribution of PFC concentrations in maternal plasma. PFOA, PFOS and PFHxS were detected in at least 95% of the samples. The geometric means (ng/ml) of PFOA, PFOS, and PFHxS were 1.66 (95% CI 1.61-1.71), 4.59 (95% CI 4.46-4.72), and 1.01 (95% CI 0.97-1.05), respectively.

Crude FORs per one standard deviation increase in log-transformed serum concentrations were significantly lower for PFOA 0.91 (95% CI 0.86-0.96) and PFHxS 0.94 (95% CI 0.89-1.00), while there was no statistically significant association with PFOS 0.97 (95% CI 0.92-1.03) (Table 3). Fecundability decreased by 4% per one year increase in maternal age (FOR=0.96; 95% CI 0.95-0.97). Obesity (BMI >30) was associated with a 25% reduction in fecundability (FOR=0.75; CI 95% 0.63-0.90), and annual household income

> $100.000 CAD with a 21% decrease (FOR=0.79; CI 95% 0.67-0.93). Education and smoking status were not associated with fecundability reduction.

In models adjusted for maternal age and BMI, PFOA and PFHxS were associated with an 11% (FOR=0.89; 95% CI 0.83-0.94) and 9% (FOR=0.91; 95% CI 0.86-0.97) reduction in fecundability per one standard deviation increase in log-transformed serum concentrations, respectively; no significant association was observed for PFOS (FOR=0.96; 95% CI 0.91-1.02) (Table 4). When added to the model with the other variables, household income was no longer significant.

The adjusted odds of infertility (TTP > 12 months or infertility treatment to become pregnant) increased by 31% per one standard deviation increase of PFOA (OR=1.31; 95% CI 1.11-1.53) and by 27% per one standard deviation increase of PFHxS (OR=1.27; 95% CI 1.09-1.48). No significant association was observed between concentrations of PFOS and the odds of infertility (OR=1.14; 95% CI 0.98-1.34) (Table 5).

3.5 Discussion

The MIREC Study is the largest cohort of pregnant women to date measuring the concentrations of PFOA, PFOS and PFHxS in plasma samples collected during the first trimester of pregnancy. The participation rate of 39% in the MIREC Study is consistent with participation rates of several large prospective cohort studies. There is evidence from similar pregnancy cohorts that this level of participation does not increase the risk of bias (Nohr et al. 2006). The time-frame of the study, from 2008 to 2011, reflects current levels of exposure to these chemicals, especially after the move to reduce and phase out several PFCs over the last decade. We observed that increased concentrations of PFOA and PFHxS were associated with decreased fecundability as measured by a longer TTP, and increased odds of infertility, even at lower levels of exposure than previously documented. The median concentrations in our participants (1.7 ng/ml for PFOA, and 4.7 ng/ml for PFOS) were lower than those reported for women in the Danish National Birth Cohort conducted between 1996 and 2002 (5.3 ng/ml for PFOA and 33.7 ng/ml for PFOS) (Fei et al. 2009), the Norwegian Mother and Child Cohort Study conducted between 2003 and 2004 (2.2 ng/ml for PFOA and 13.0 ng/ml for PFOS) (Whitworth et al. 2012), and a Danish study conducted among trade union workers between 1992 and 1995 (5.6 ng/ml for PFOA and 36.3 ng/ml for PFOS) (Vestergaard et al. 2012). Also, our geometric mean concentrations (1.66 ng/ml; 95% CI 1.61-1.71 for PFOA and 4.59 ng/ml; 95% CI 4.46-4.72 for PFOS) were lower than those reported in the LIFE Study conducted in Texas and Michigan between 2005 and 2007 (3.11 ng/ml; 95% CI 2.91-3.33 for PFOA and 11.76 ng/ml; 95% CI 11.01-12.57 for PFOS) (Buck Louis et al. 2013).

Two previous studies have evaluated the effect of PFOA and PFOS on TTP using a retrospective-pregnancy TTP design. Our results are in agreement with those on PFOA reported by Fei et al. (2009) in a subset of 1400 women randomly selected from the Danish National Birth Cohort; however we did not find an association between TTP and PFOS, which might be explained by the differences in exposure. Indeed, while our median PFOA level was 1/3 of those reported by Fei, our median PFOS was 1/14 their level. The covariates included in our models were similar, except for the consideration of parity as a confounder by Fei et al. (2009).

A second study, a case-control analysis within the Norwegian Mother and Child Cohort, reported an increased odds of subfecundity (TTP > 12 months) with elevated PFOA and PFOS levels in a model (not adjusted for parity) (Whitworth et al. 2012). A recent analysis of the MoBa cohort, using the discrete-time Cox proportional hazard model, also reported diminished fecundability at increasing concentrations of PFOA (FOR 0.83; 95% CI 0.75-0.91) (Ding et al. 2014). In the study by Whitworth et al. (2012), however, the authors considered that reverse causation could account for their reported association in parous women (Olsen et al. 2009). Thus, women with longer TTP would have higher PFCs levels due only to a longer time since their previous pregnancy, allowing re-accumulation of the PFCs that had decreased during pregnancy and postpartum through placenta transfer and breastfeeding. Based on this assumption, they reported their final conclusion according to a second model, stratifying by parity. In this model, the odds ratios for subfecundity were elevated only among parous women, which, according to the authors, supports the reverse causation hypothesis (Whitworth et al. 2012). However, these results were not replicated by Fei et al. (2012) after stratification by parity, where

stronger associations were reported for PFOS and PFOA in nulliparous women, and for PFOA in the case of multiparous women, thus refuting reverse causation.

Based on our DAG (Figure 2), we considered that neither adjustment nor stratification for parity should be conducted when studying the reproductive adverse effects of PFCs, as this will introduce over-adjustment through collider-stratification bias (Greenland 2003, Schisterman et al. 2009). To describe this type of bias, Greendland (2003) uses the example of the effect of exposure to an unopposed estrogen therapy (E) and endometrial cancer (D), stratifying (conditioning) on uterine bleeding (C). It was common practice to assess the effect of estrogen therapy on endometrial cancer only among women presenting with uterine bleeding, as a marker of estrogen use and cancer. However, it was demonstrated that the measure of effect among these women would be much smaller than the true causal association because of the strong effects of both the therapy and the cancer on bleeding risk, and therefore, the bias induced by stratification on bleeding was toward the null (Greenland 2003). The same bias would be introduced if the association of PFCs (E) and TTP (D) were conditioned on parity (C).

In addition, we included women in our study who had had fertility treatment for the index pregnancy, i.e., 7.3% of the study population, as was also done by Fei et al. (2009). In our descriptive phase of analysis, we found that the geometric mean levels of PFOA and PFHxS were significantly higher in participants receiving fertility treatment for the index pregnancy than those of untreated participants (data not shown). We also noted that half of these patients reported a TTP < 12 months, even if these treatments (i.e., ovulation induction, intrauterine insemination, and *in-vitro* fertilization) are rarely prescribed before 12 months of attempting pregnancy. Our hypothesis is that some of the women

reported their TTP as the number of months since their first cycle of fertility treatment until the successful cycle, some considered the number of months of trying before starting fertility treatment, and others counted both. Moreover, if they had used fertility treatment for a previous pregnancy and therefore were able to access it for this pregnancy without having to wait during 12 months of trying, the reported TTP would probably be < 12 months. This misclassification may have introduced a selection bias in the Whitworth et al. (2012) study. For their cases, the authors randomly selected women who reported a TTP > 12 months but not according to fertility treatment, leading to the exclusion of, according to our study, approximately 50% of the potential cases that received fertility treatment but reported a TTP < 12 months. As proof of this misclassification, the authors reported that after the selection of cases and controls, they found that 39% of the cases (n=163) and 1% of the controls (n=7) had received fertility treatment for the current pregnancy. They decided, however, to exclude these 7 controls instead of reclassifying them as cases.

Two additional studies have addressed the association between PFCs and TTP using a prospective couple-based cohort design. In a survey conducted among trade union workers in Denmark from 1992 to 1995, no consistent pattern was observed between TTP and eight PFCs, including PFOA, PFOS, PFHxS, and PFOSA, measured in the serum of 222 women attempting pregnancy for the first time followed for up to six cycles of trying (Vestergaard et al. 2012). More recently, Buck Louis et al. (2013) reported the results from the LIFE Study, including biomonitoring data from 501 couples followed for up 12 months of attempting during 2005 and 2007. Decreased fecundability was observed at higher concentrations of PFOSA in females in the adjusted model; however, no

association was found for the other 6 PFCs assessed, including PFOA and PFOS in their unadjusted analysis. PFOSA was not measured in the MIREC Study. Detection of this chemical is currently very low in the United States (Centers for Disease Control and Prevention 2009) due to its phasing-out since 2002 (3M 2014). In fact, PFOSA was not detected in 90% of the samples from the LIFE Study (Buck Louis et al. 2013).

Some methodological aspects of our study need to be considered. Firstly, in pregnancy-based TTP, infertile couples who do not opt for fertility treatment or have no access to it and those whose fertility treatment is unsuccessful are excluded from the study, resulting in the systematic underrepresentation of infertile women and the selection of a healthier population (Joffe et al. 2005). Nonetheless, pregnancy-based TTP studies have been successful in identifying environmental exposures that may adversely affect fertility, as it is expected that at current levels of exposure, the toxic effect of these contaminants do not lead to complete infertility (Weinberg and Wilcox 2008). Secondly, TTP was assessed at a mean gestational age of 12 weeks (SD 1.52) in MIREC, similar to the gestational age at which TTP was assessed by Fei et al. (2009), but earlier than in Whitworth et al. (2012), which was at approximately 17 weeks of gestation. Studies that have assessed the validity of this recall have reported reasonable validity if collected at the short-term (Zielhuis et al. 1992, Cooney et al. 2009). Thirdly, we used the first trimester concentration of PFCs as a surrogate of the preconception exposure. Since the half-lives of these chemicals are considerably long (Olsen et al. 2007) and they are persistent in the environment, we considered that these concentrations reflected those at the time of the pregnancy attempts. In addition, one recent study reported robust correlations between two subsequent measures of PFCs in blood samples of 53 men

collected 6 years apart, in 2001 and 2007 (Spearman's ρ =0.75 for PFOA and 0.81 for PFOS, and PFHxS) (Nost et al. 2014).

One limitation of our study is that we do not have information on male partner exposure to PFCs. The LIFE Study is the only study that has measured concentrations of PFCs in both partners (Buck Louis et al. 2013), reporting high correlations between partner concentrations. Since TTP is an epidemiological metric of couple fecundity, we are unable to determine if the effects are female, male, or both. Furthermore, we did not assess specific endpoints of fecundability such as menstrual cycle characteristics, markers of ovulation, or semen quality (Buck Louis et al. 2014).

With regard to the biological mechanism of action, there are limited toxicological studies assessing the effect of PFCs on reproductive outcomes. In vitro, some PFCs have the potential to affect estrogen-receptor and androgen-receptor transactivity (Kjeldsen and Bonefeld-Jorgensen 2013). In rats, exposure to PFOS has shown estrous cyclicity disruption and neurotransmitter imbalance (Austin et al. 2003). In a rainbow trout model (Oncorhynchus mykiss), several perfluoroalkyl acids were potent inducers of the estrogen-responsive biomarker protein vitellogenin (Vtg) at very high dietary exposures (Benninghoff et al. 2011). Although this small number of studies suggests a possible endocrine-disrupting effect, additional toxicological studies need to be assessed to support an effect and its mechanistic pathway. Recently, PFOA and perfluorononanoic acid (PFNA) were reported to be associated with endometriosis in a cohort of 495 women undergoing laparoscopy/laparotomy in the ENDO Study (Endometriosis—Natural History, Diagnosis and Outcomes Study) (Buck Louis et al. 2012). Endometriosis is a condition associated with impaired fecundability. Therefore, endometriosis or its

determinants could be on the etiologic or causal pathways of the association between PFCs and TTP.

In conclusion, our results add to the evidence that exposure to PFOA and PFHxS, even at lower levels than previously reported, may reduce fecundability, as measured by a longer TTP and increased odds of infertility (TTP > 12 months). Future research should focus on the mechanisms involved in this potential endocrine-disrupting effect. Methodological differences in the causal models of previous studies have impaired possible conclusions about the potential adverse effect of PFCs and TTP.

Author's roles

M.P.V., T.E.A, and W.D.F. were all involved in the conception and design of the study. M.P.V. carried out analysis and interpretation of data, in addition to drafting the manuscript. T.E.A and W.D.F. were the co-principal investigators of the MIREC Study and contributed to data interpretation and review of the manuscript.

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Conflict of interest

None to declare

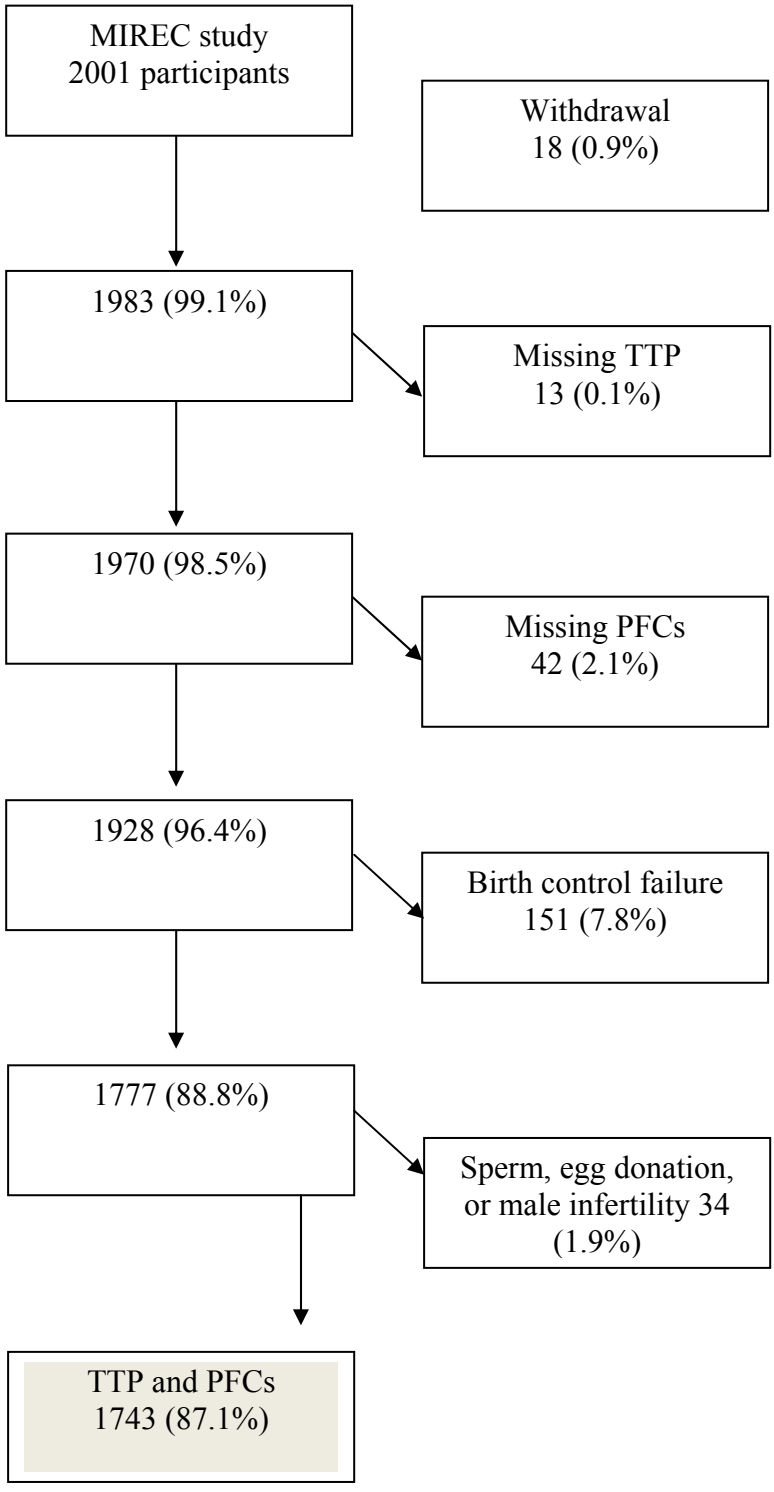

Figure 1. Participant's selection flow-chart

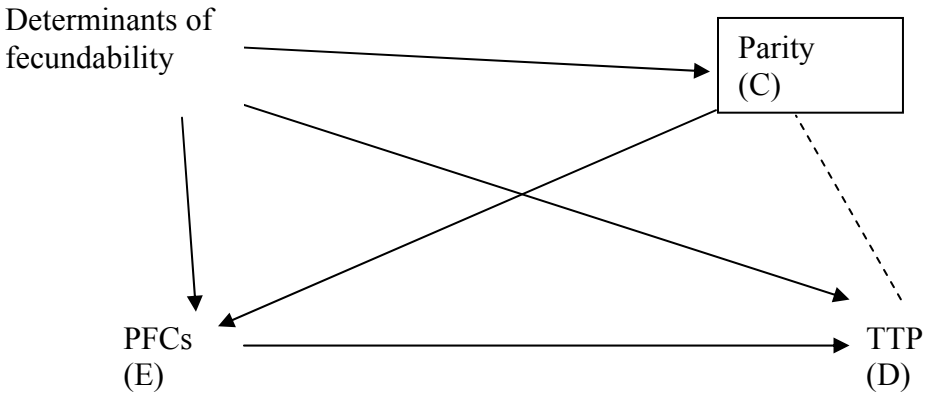

Figure 2. Directed acyclic graph representing covariates in the relationship between PFC (Exposure) and TTP (Disease) and the role of parity as a Collider (C)

Table 1. Characteristics of the study population and association with Time to Pregnancy: the MIREC Study

Continuous variables	N	Mean (SD)*	Median	Minimum	Maximum	P value**
Maternal age (yrs.)	1743	32.83 (4.96)	32.74	18.34	46.35	<0.001
Paternal age (yrs.)	1510	34.78 (5.68)	34.51	18.66	58.74	<0.001
Gestational age (wks.)	1741	12 (1.52)	12.43	6	14	0.19

Categorical variables	N	%	Time to Pregnancy (months)		
			Mean (SD)*	Median (p25-p75)	
Education					0.46
Some college or less	236	13.5	5.8 (12.8)	1 (1-5)	
College diploma	399	22.9	5.2 (8.9)	2 (1-5)	
Undergraduate	644	37.0	5.7 (12.2)	2 (1-5)	
Graduate (MSc PhD)	464	26.6	4.3 (6.4)	2 (1-4)	
Country of birth					0.93
Canada	1412	81.0	5.3 (10.8)	2 (1-5)	
United States	27	1.5	4.7 (6.4)	2 (1-6)	
Mexico	8	0.5	5.1 (4.8)	4.5 (1-9)	
China	17	1.0	3.8 (4.8)	1 (1-5)	
Other	279	16.0	5.1 (8.4)	2 (1-6)	
Household Income					0.06
< $60,000	361	20.7	4.5 (10.1)	2 (1-4)	
$60,001 – 100,000	609	35.0	5.2 (9.9)	2 (1-5)	
> $100,000	691	39.6	5.7 (10.7)	2 (1-6)	
No response	82	4.7	5.2 (10.9)	2 (1-4)	
Parity conditional on gravidity					<0.001
No prior pregnancy	501	28.8	6.5 (13.7)	2 (1-6)	
Prior pregnancy without live birth(s)	270	15.5	6.8 (12.1)	2 (1-6)	
Prior pregnancy with live birth (s)	971	55.7	4.2 (7.2)	2 (1-4)	
Maternal smoking					0.85
Never	1077	62.9	5.1 (8.9)	2 (1-5)	
Former	392	22.5	5.3 (11.8)	2 (1-5)	
Current †	272	15.6	5.7 (13.1)	2 (1-4)	
Pre-pregnancy BMI††					0.003
<24.9	1034	63.6	4.9 (9.6)	2 (1-5)	
25-29.9	355	21.9	5.2 (11.4)	2 (1-4)	
>30	236	14.5	6.7 (11.3)	3 (1-7)	
Current paternal smoking					0.83
No	1217	83.0	5.2 (9.9)	2 (1-5)	
Yes	249	17.0	5.6 (12.0)	2 (1-5)	

* Arithmetic mean and standard deviations (SD)

** P values for the association with Time to Pregnancy: Likelihood ratio for continuous variables, Log Rank test for categorical variables. †Includes women that quit during pregnancy or one year before. †† Body Mass Index

Table 2. Perfluoroalkyl acid levels (ng/ml) in maternal plasma. (n=1743)

	LOD*	n (%) <LOD	Median	Minimum	Maximum	GM** (95% CI)
PFOA	0.1	2 (0.12)	1.7	<LOD	16	1.66 (1.61-1.71)
PFOS	0.3	2 (0.12)	4.7	<LOD	36	4.59 (4.46-4.72)
PFHxS	0.2	69 (4.28)	1	<LOD	25	1.01 (0.97-1.05)

*Limit of Detection (LOD)

** Geometric Mean

Table 3. Crude Fecundity Odds ratios for TTP

	N	Crude FORs	95% Confidence interval	P value**
PFOA ng/ml*	1743	0.91	0.86-0.96	0.001
PFOS ng/ml*	1743	0.97	0.92-1.03	0.33
PFHxS ng/ml*	1743	0.94	0.89-1.00†	0.04
Age	1743	0.96	0.95-0.97	<0.001
Gestational Age	1743	0.98	0.94-1.01	0.18
Pre-pregnancy BMI				0.04
<24.9	1034	1		
25-29.9	355	1.03	0.89-1.20	
>30	236	0.75	0.63-0.90	
Education				0.68
Graduate (MSc PhD)	464	1		
Undergraduate	644	0.95	0.82-1.10	
College diploma	399	0.91	0.77-1.08	
Some college or less	236	0.97	0.82-1.21	
Household income				
<60000	361	1		0.04
60000-100000	609	0.86	0.73-1.01	
>100000	691	0.79	0.67-0.93	
No response	82	0.89	0.65-1.20	
Maternal smoking				0.63
Never	1077	1		
Former	392	1.05	0.91-1.21	
Current††	272	1.08	0.91-1.27	
Current paternal smoking				0.68
No	1217	1		
Yes	249	1.05	0.91-1.21	

*Chemical plasma concentrations were log-transformed and divided by their standard deviations.

** Wald PHREG Procedure

† After rounding

†† Includes those who quit during pregnancy or less than one year prior to the study visit

Table 4. Adjusted Fecundity Odds Ratios for TTP (n=1625)*

	Adjusted FORs	95% Confidence interval	P value**
PFOA (ng/ml)	0.89	0.83-0.94	**<0.001**
Age	0.96	0.94-0.97	<0.001
Pre-pregnancy BMI			0.001
<24.9	1		
25-29.9	1.03	0.89-1.20	
>30	0.71	0.60-0.86	
PFOS (ng/ml)	0.96	0.91-1.02	**0.17**
Age	0.96	0.95-0.97	<0.001
Pre-pregnancy BMI			<0.001
<24.9	1		
25-29.9	1.03	0.89-1.20	
>30	0.72	0.60-0.86	
PFHxS (ng/ml)	0.91	0.86-0.97	**0.002**
Age	0.96	0.94-0.97	<0.001
Pre-pregnancy BMI			<0.001
<24.9	1		
25-29.9	1.05	0.90-1.22	
>30	0.73	0.61-0.87	

Chemical plasma concentrations were log-transformed and divided by their standard deviations.

* 1625 women (118 missing values for pre-pregnancy weight)

** Wald Chi-square test PHREG Procedure

Table 5. Adjusted Odd Ratios for Infertility (n = 1625)*

	Odds Ratios	95% Confidence interval	P value
PFOA (ng/ml)	1.31	1.11-1.53	0.001
PFOS (ng/ml)	1.14	0.98-1.34	0.09
PFHxS (ng/ml)	1.27	1.09-1.48	0.003

Chemical plasma concentrations were log-transformed and divided by their standard deviations.

* 1625 women (118 missing values for pre-pregnancy weight)

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Chapter 4 - FEMALE EXPOSURE TO PHENOLS AND PHTHALATES AND TIME TO PREGNANCY: THE MIREC STUDY (ARTICLE 2)

Running title: Phenols, Phthalates, and Fecundity

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Status: Under internal review at Health Canada. To be submitted to fertility & Sterility

Capsule: Elevated triclosan exposure may be associated with diminished fecundity. BPA and phthalates did not show any negative impact; on the contrary, phthalates might be associated with a shorter TTP.

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4.1 Abstract

Objective: to assess the potential effect of bisphenol A (BPA), triclosan (TCS), and phthalates on women's fecundity, as measured by time to pregnancy (TTP).

Design: Pregnancy-based retrospective Time to Pregnancy study

Setting: Ten cities across Canada

Patients: the MIREC Study (Maternal-Infant Research on Environmental Chemicals) recruited 2001 women during the first trimester of pregnancy between 2008 and 2011. 1742 women were included in our BPA analysis, 1699 in the TCS analysis, and 1597 in the phthalates analysis.

Intervention(s): None

Main Outcome Measure(s): Fecundability odds ratios (FORs) were estimated using the Cox model, modified for discrete time data.

Results: BPA concentrations were not significantly associated with diminished fecundity either in crude or adjusted models. Women in the highest quartile of TCS (>72 ng/ml) showed evidence of decreased fecundity (FOR 0.84; 95% CI 0.72-0.97) compared to the three lower quartiles used as the reference group. Exposure to phthalates was suggestive of a shorter TTP, as indicated by FORs greater than one, although the 95% CI always included one.

Conclusion: elevated TCS exposure may be associated with diminished fecundity. We found some indication that exposure to phthalates might be associated with a shorter TTP. A major limitation of the study was that only one measurement of exposure was available for each woman post conception. Further research is necessary to test these findings.

Keywords: Bisphenol A, triclosan, phthalates, fecundity, reproduction

4.2 Introduction

Endocrine-disrupting chemicals (EDCs) are chemicals that have the potential to interfere with hormone functions. Their ubiquitous presence in the environment, coupled with the detection of several EDCs in large biomonitoring surveys (1, 2), has raised concerns about their possible adverse health effects. Since the endocrine system is essential for sexual development and reproductive functions, new research has focussed on the effect of EDCs on human fecundity, defined as the biological capacity for reproduction (3). EDCs include natural and synthetic hormones, pesticides, compounds used in the plastics industry and in consumer products, and by-products of other industrial processes. Some of these chemicals have long half-lives, allowing bioaccumulation and persistence in the environment. On the opposite end of the spectrum are those chemicals with short elimination half-lives considered nonpersistent, although their high-volume production makes them a common source of human exposure. Bisphenol A (BPA), triclosan (TCS), and phthalates belong in this latter group.

Exposure to BPA is common, with over 90% of the populations of the U.S.A. and Canada having detectable urinary concentrations (1, 2). Although BPA has recognized endocrine-disrupting properties in animals (4), there is limited information regarding the effect of BPA exposure on human fecundity. While several studies conducted in infertile couples seeking Assisted Reproductive Technologies (ART) suggest reproductive effects, only one study has assessed the impact of BPA on couple fecundity in a population-based setting (5). In the context of ART, higher concentrations of BPA have been associated with hormonal imbalances in both women and men (6-8), diminished antral follicle counts (9), decreased number of oocytes retrieved after ovarian stimulation (10, 11),

lower fertilization rate (10), and implantation failure (12). In vitro, exposure of discarded human oocytes to BPA was associated with abnormal oocyte maturation (13). At the population level, Time to Pregnancy (TTP) has been widely used as an epidemiological metric for the study of fecundity (14). The only epidemiological study to date assessing the effect of BPA on TTP reported no association between female or male BPA urinary concentrations and TTP among participants from the LIFE Study, a prospective cohort of couples attempting pregnancy in the U.S.A. (5).

Triclosan (TCS) is a broad-spectrum phenolic biocide with activity against bacteria and fungi. It is widely used in personal care products ranging from soaps, cosmetics, and toothpaste to treated textiles and food contact materials (15). TCS was detectable in about 75% of the urine samples collected as part of the NHANES 2003-2004 survey of the U.S. population (16) and the 2009-2011 Canadian Health Measures Survey (2). It has a similar structure to known EDCs, including polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs), and BPA, and to thyroid hormones (17). These structural similitudes, together with some limited evidence in laboratory animals of effects on diverse hormones, suggest that TCS may influence endocrine function, and possibly the reproductive axis (17). Epidemiological studies on TCS are limited. Two studies reported no significant impact of prenatal exposure to TCS on birth size (18, 19). A recent analysis of urine samples from NHANES 2003-2008 showed a positive association between TCS and body mass index (BMI) (20). No studies assessing the effect of TCS on TTP have been conducted to date.

There is evidence suggesting that several phthalates may be endocrine disruptors (21) and affect development and reproduction (22, 23). Phthalates are extensively used in a variety

of industrial, consumer, and personal care products. Due to federal regulations, urinary concentrations of some phthalates have decreased during the last decade; however, a large number of phthalate metabolites are still detectable in more than 95% of the populations of the U.S.A. and Canada (2, 24). Reported use of personal care products has been positively associated with urinary concentration of multiple phthalate metabolites in women of reproductive age (25). Nonetheless, there is a paucity of studies assessing the effect of phthalates on women's fecundity. In Generation R, a large pregnancy cohort study conducted in the Netherlands, occupational exposure to phthalates was assessed using a job-exposure matrix, and was reported as being suggestive of a longer TTP (26, 27). In Italy, concentrations of several phthalate metabolites were assessed in 56 infertile couples from an ART centre, and were found to be significantly higher than in the control group of fertile couples (28). Recently, in the LIFE Study, no phthalate metabolite in female urine was statistically associated with a longer TTP, although one metabolite (mono [3-carboxypropyl] phthalate) was associated with a shorter TTP. In men, urinary concentrations of monomethyl, mono-n-butyl, and monobenzyl phthalates were associated with a longer TTP (5). Of note: while most of the literature assessing the adverse health effects of phthalates has focussed on the effect of individual metabolites, some studies suggest that simultaneous exposure to multiple phthalates might have a cumulative impact (29).

There is limited research exploring the effects of nonpersistent EDCs on TTP. Most studies to date have looked at ART outcomes and male exposure, despite the fact that the female reproductive function is also susceptible to hormonally-active chemicals (30). Aiming to fill this gap, data from the MIREC Study, a Canadian pregnancy and birth

cohort was analyzed to assess the effect of BPA, TCS, and phthalates on women's fecundity, as measured by TTP.

4.3 Methods

Population and study design

The Maternal-Infant Research on Environmental Chemicals (MIREC) Study is a pregnancy cohort of 2,001 women recruited in 10 cities across Canada between 2008 and 2011 (31). Women were approached during the first trimester of pregnancy at participating hospitals and clinics and followed for a total of five visits up to 10 weeks postpartum. A detailed questionnaire was administered during the first study visit (< 14 weeks gestation). Eighteen participants withdrew from the study and all their data and samples were destroyed. We excluded women with incomplete data for the specific compound/group studied (46 for BPA, 96 for TCS, and 211 for phthalates), TTP (14 for BPA and TCS, and 15 for phthalates), and specific gravity (n=3); women requiring egg donation (n=4) or reporting male factor infertility (n=26); and women whose index pregnancy was the result of birth control failure (148 for BPA, 141 for TCS, and 154 for phthalates). Thus, 1742 women were included in our BPA analysis, 1699 in the TCS analysis, and 1597 in the phthalates analysis.

The study was approved by the ethics committees of Health Canada, Sainte-Justine University Hospital Centre, and the hospitals affiliated with the study across Canada. Written informed consent was obtained from all the participants.

Data collection and urine sampling

Besides information on demographics, medical and obstetrical history, and lifestyle characteristics, women were asked about their Time to Pregnancy (i.e. number of months of unprotected intercourse before conception) by this question: “How long did it take you to get pregnant with this pregnancy?” (number of months).

As part of the biomonitoring component of MIREC, a spot urine sample was collected in polypropylene cups during the first trimester visit. This sample was aliquoted into 30-mL Nalgene® tubes, frozen at −20°C within 2 hours of collection, transported on dry ice to the MIREC coordinating centre in Montreal to be stored at −30°C, and subsequently shipped in batches to the Centre de Toxicologie du Québec of the Institut national de Santé Publique du Québec (INSPQ) for analysis. This laboratory is accredited by the Standards Council of Canada under ISO 17025.

Analytical Methods

As part of the initial MIREC protocol, urine samples were analyzed for bisphenol A (BPA) and 11 phthalate metabolites: mono-n-butyl phthalate (MnBP); mono-ethyl phthalate (MEP); mono-benzyl phthalate (MBzP); mono-methyl phthalate (MMP); mono-cyclo-hexyl phthalate (MCHP); mono-isononyl phthalate (MiNP); mono-n-octyl phthalate (MnOP); mono-(3-carboxypropyl) phthalate (MCPP); mono-(2-ethylhexyl) phthalate (MEHP); mono-(2-ethyl-5-oxo-hexyl) phthalate (MEOHP); and mono-(2-ethyl-5-hydroxy-hexyl) phthalate (MEHHP) (32). Additional research funds were obtained for the triclosan (TCS) analysis as part of methods development for the U.S. National Children's Study.

Urinary total BPA (free plus conjugated) concentrations were quantified using an established protocol. Samples were analyzed by GC–MS/MS coupled with a tandem mass spectrometer. Phthalate metabolites were analyzed by LC–MS/MS with an Ultra Performance Liquid Chromatography (UPLC) coupled with a tandem mass spectrometer. Further details are described in Arbuckle et al. (32).

For the TCS analysis, sensitive LC-MS/MS methods were developed for the analysis of free and conjugated forms of TCS in urine at the Centre de Toxicologie du Québec of the Institut national de Santé Publique du Québec (32). To account for urine dilution, specific gravity was measured in thawed urine samples by a refractometer (UG-1, Atago # 3461, Atago U.S.A. Inc., Bellevue, WA).

Field blanks were included to assess the potential contamination from the material used for collection and storage of urine samples as well as from the environment of collection sites. Results did not show any evidence of contamination (32, 33).

Statistical Analysis

Descriptive statistics, including the percentage detected, medians. and geometric means, were computed for all chemicals. Concentrations below the limit of detection (LOD) were set to the LOD divided by two. Total TCS was calculated by summing the free and conjugated forms. We considered the effect of total BPA, total TCS, and each individual phthalate metabolite independently. In the case of phthalate metabolites, we also categorized them into low and high molecular weight, and calculated the sum of their molecular weights in each category as a measure of total LMW and HMW phthalates. In addition, we calculated the estrogenicity equivalency factor (EEF) as proposed by Braun et al (34).

We also considered different alternatives for modelling exposure. First, biomarker concentrations were log-transformed and divided by their standard deviations (35). Second, concentrations were categorized *a priori* into quartiles using the lowest quartile as the reference group. In addition, since results from the MIREC cohort have shown that BPA and phthalate metabolite concentrations in maternal urine tended to be lower than

those reported in other pregnancy cohorts worldwide (32), we grouped the three lower quartiles as the reference group and compared it to the highest quartile. We also applied this categorization for TCS.

Fecundability odds ratios (FORs) were estimated using the Cox model modified for discrete time data (36). FORs estimate the odds of becoming pregnant each cycle, given exposure to the specific compound and conditional on not being pregnant in the previous cycle. FORs less than 1 denote reduction in fecundity or a longer TTP, and FORs greater than 1 denote a shorter TTP. TTP was censored at the 13th month. Linearity and proportional hazard assumptions were verified (36).

Potential confounders were maternal age, smoking, education, and household income, identified as predictors of exposure to BPA, TCS, and phthalates in the MIREC cohort (32, 33). In addition to these covariates that also impact on fecundity, body mass index (BMI) was included in the adjusted models (3). We did not include parity in our model, as it can lead to an overadjustment bias (37).

To account for urine dilution, specific gravity was included as a covariate in the regression model (38). We also evaluated possible interactions between specific gravity and time of urine collection as was evidenced in previous analyses conducted by our group (32). However, we did not include interactions in our final models, since none were observed. Statistical analysis was performed using STATA 10.0 (Stata Corporation, College Station, TX), and SAS 9.3 (SAS Institute Inc., Cary, NC) specifically for the discrete-time Cox proportional models.

4.4 Results

The characteristics of the study population are presented in Table 1. The distributions of demographic and lifestyle characteristics were similar for the three compounds/group studied (i.e., BPA, TCS, and phthalate metabolites). The mean maternal age was 32.8 years (SD 5.0), while the mean paternal age was 34.7 (SD 5.6). The median gestational age at interview was 12 weeks in a range between 6 to 14 weeks. Most participants included in the analysis (81%) were born in Canada, approximately two thirds had a university degree, and more than one third reported a yearly household income higher than $100.000 CAD. More than half of the women had at least one prior pregnancy with live birth, and about 15% were obese or active smokers during the preconception period. Maternal and paternal age, parity, and pre-pregnancy BMI were associated with TTP.

Detectable urinary concentrations of total BPA were found in 87% of the samples, while total TCS was detectable in more than 99% (Table 2). As for phthalates, five metabolites were detected in more than 98% of the samples (MnBP, MEP, MBzP, MEHP, MEOHP, MEHHP), while MCPP was detected in 82%. However, four metabolites (MMP, MCHP, MiNP, and MnOP) were detected in less than 14% of the samples, and for this reason, they were excluded from further analyses.

As Table 3 reflects, BPA concentrations were not significantly associated with diminished fecundity either in crude or adjusted models. This lack of association was independent of the manner in which BPA concentrations were considered (i.e., continuous, quartiles of BPA, or comparing the highest quartile with the three lower quartiles).

As for TCS, one standard deviation increase in the log transformed concentrations of TCS were associated with shorter TTP; however, the 95% CI included one (FOR 0.94; 95% CI 0.88-1.01). The same pattern was observed for the highest TCS quartile of exposure compared to the lowest quartile (FOR 0.89; 95% CI 0.74-1.07). It is noteworthy that when we considered the three lower quartiles as the reference group, women in the highest quartile of TCS (>72 ng/ml) showed evidence of decreased fecundity (FOR 0.84; 95% CI 0.72-0.97) (Table 3, Figure 1).

All phthalate metabolites had a similar pattern of association with TTP, independently of the variable transformation or the variables included in the statistical models. In general, exposure to phthalates was suggestive of a shorter TTP, as indicated by FORs greater than one, although the 95% CI always included one (Table 4). Total LMW and HMW metabolites were positively associated with TTP, although not statistically significant. Moreover, their FORs were of similar magnitude to those of the individual metabolites. The FORs according to the EEF were also of similar magnitude (data not shown).

4.5 Discussion

The MIREC Study is the largest cohort that has assessed the effect of ubiquitous plasticizers such as BPA and phthalates on women's fecundity as measured by TTP, and the first that has examined the potential effect of TCS. We found that urinary concentrations of TCS at the highest quartile of exposure were associated with a 16% reduction in fecundity. In addition, while BPA was not associated with TTP, it is noteworthy that in the case of phthalates, the FORs were almost universally greater than 1, suggesting a shorter TTP, although the 95% CI included 1.

Compared to the few studies available worldwide that have assessed concentrations of TCS in pregnant women, MIREC reported the highest urinary concentration of TCS (6784 μg/L) but had considerably lower median concentrations than other international studies (33). Higher socio-economic class and older age were determinants of TCS exposure in the MIREC cohort (33). The MIREC population tended to be more highly educated than the average woman giving birth in Canada (31). Higher education may be associated with postponed childbirth, hence increasing age at the time of pregnancy attempt. Despite accounting for all these factors in our statistical models, decreased fecundity at the highest quartile of TCS exposure was maintained. Since this is the first study conducted at the population level assessing the impact of TCS on TTP, interpreting our findings in the context of the available literature is difficult. As recently reviewed by Dann and Hontela (17), TCS may have endocrine-disrupting effects. In vitro, several studies have demonstrated the potential for TCS to act as an anti-estrogen and/or anti-androgen (39-41). Animal studies with male rats (42, 43) have shown that TCS decreases serum levels of testosterone and the activity of several important steroidogenic

enzymes. In addition, TCS has been shown to be a powerful inhibitor of estrogen sulfonation in sheep placental tissue (44), which could impair the maintenance of pregnancy. Finally, the homeostasis of thyroid hormones, critical for reproductive success (45, 46), might also be a target of TCS. The structural similarity of TCS to thyroid hormones has prompted experimental studies in this area (17). In vitro, TCS was capable of inhibiting sulfation of thyroid hormones (47). In animals, TCS exposure was associated with decreased levels of Thyroxine (T_4) in female (48) and male rats (42). Thus, we believe that the estrogenic and androgenic activity of TCS in rodents and its potential impact on thyroid hormones coupled with our finding of decreased fecundity warrant additional epidemiologic studies at the population level to elucidate the potential impact of TCS on human reproduction.

As for BPA, the only study that has assessed the effect of BPA on couple's fecundity reported results similar to ours. Neither female nor male BPA concentrations were associated with TTP (5). Our sample size is almost three times higher than the size of the LIFE Study. This would have helped to find an association if the absence of association in the LIFE Study was due to a lack of statistical power. Low BPA concentrations might also explain the absence of association in both studies. The geometric mean in our study (0.80 μg/L), and in the LIFE Study (0.63 μg/L), were lower than those reported in NHANES 2003-2004 for females ≥ 6 years of age (2.41 μg/L) (24) and in the Canadian Health Measures Surveys (CHMS) of 2007–2009 (1.26 μg/L) (49) and 2009–2011 (1.2 μg/L) for women 20–39 years of age (2). Since animal studies suggest that BPA has endocrine-disruption capacity, additional epidemiological studies in populations having

higher exposures to BPA should be conducted before making firm conclusions about the absence of its effect on fecundity.

With regard to phthalates, the interpretation of our results is even more challenging. Most of the FORs exceeded 1, suggesting a shorter TTP, although not statistically significant. In the LIFE Study, 9 out of the 14 metabolites assessed in women had FORs exceeding 1 in the adjusted models; however, with the exception of MCPP, the CI also included 1 (5). In the LIFE Study, men's urinary concentrations of MMP, MnBP, and MBzP were associated with a longer TTP (FOR 0.80; 95% CI 0.70-0.93; FOR 0.82, 95% CI 0.70-0.97; and FOR 0.77, 95% CI 0.65- 0.92, respectively). In general, median phthalate metabolite concentrations in maternal urine in MIREC were comparable to those reported for women 20–39 years of age in Cycle 2 of the CHMS (2009–2011) (2). Compared to female levels in the LIFE Study, MIREC reported similar or lower median concentrations. Some phthalates are recognized for their estrogenic activity, although they are weak in this regard when compared to 17B estradiol (50). One hypothesis is that this estrogenic activity may counteract the adverse impact of medical conditions associated with an androgenic environment (e.g., polycystic ovary syndrome), thus decreasing TTP. Furthermore, in-vitro studies have reported direct anti-androgenic activity of some phthalates (51, 52). This anti-androgenic effect has been the focus of recent epidemiological studies. In a recent analysis of the NHANES 2011-2012 cycle, most urinary female phthalate metabolites were associated with decreased concentrations of testosterone, especially in women 40-60 years old (53). In pregnant women, the concentrations of DEHP metabolites from samples collected between 1999 and 2002

were associated with decreased testosterone among all women, and between MBP and testosterone among women carrying female fetuses (54).

Our study has important limitations that need to be considered. Since this is a pregnancy-based TTP study, women that were infertile and/or did not have access to infertility treatment were excluded by design from our study. Thus, if BPA or phthalates have a negative impact on TTP, women with the highest exposures would have been excluded from our study. In addition, we measured the concentrations of chemicals only in women, and the process of reproduction involves not only the female and the male partner individually, but also many factors that are couple-mediated. Furthermore, we are assuming that the concentrations measured during the first trimester of pregnancy represent the concentrations that were present during the preconception period. A recent study evaluated the variability of urinary phthalate metabolites and BPA concentrations before and during pregnancy in a cohort of women receiving infertility treatment (55). The absolute differences in urinary concentrations for these chemicals were relatively small, which, according to the authors, might suggest that the women did not change their preconception behaviours to reduce exposure to these chemicals during pregnancy. Nonetheless, using the intraclass correlation coefficients (ICCs), the reliability of a spot urine sample to predict exposure over a few months is limited for repeated measures of BPA (56, 57) and several phthalate metabolites (55, 58-60). With respect to TCS, the reliability seems to be better (57, 61, 62).

In summary, our data suggest that elevated TCS exposure (> 72 ng/ml) may be associated with diminished fecundity, as evidenced by a longer TTP. Regarding phthalates and BPA, there was no evidence of any negative impact on TTP and even some suggestion

that exposure to phthalates might be associated with a shorter TTP. Further studies are necessary to test our findings and elucidate the potential impact of nonpersistent environmental contaminants on human fecundity.

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Table 1. Association of study population characteristics with TTP by chemical measured: the MIREC Study

	BPA (n=1742)		TCS (n=1699)		Phthalates (n=1597)	
Characteristics	N (%)[a]	p value[b]	N (%)[a]	p value[b]	N (%)[a]	p value[b]
Maternal age, y (Mean, SD)	32.84 (4.95)	<0.001	32.84 (4.91)	<0.001	32.85 (4.96)	<0.001
Paternal age, y (Mean, SD) [±]	34.76 (5.68)	<0.001	34.74 (5.58)	<0.001	34.74 (5.69)	<0.001
Gestational age, w (Median, min-max) [±]	12 (6-14)	0.04	12 (6-14)	0.03	12 (6-14)	0.05
Education		0.38		0.31		0.40
Some college or less	237 (13.6)		229 (13.5)		211 (13.2)	
College diploma	398 (22.8)		389 (22.9)		365 (22.9)	
Undergraduate	644 (37.0)		631 (37.1)		591 (37.0)	
Graduate (MSc PhD)	463 (26.6)		450 (26.5)		430 (26.9)	
Country of birth		0.92		0.93		0.81
Canada	1.408 (80.8)		1376 (81.0)		1294 (81.0)	
United States	27 (1.6)		27 (1.6)		24 (1.5)	
Mexico	8 (0.5)		8 (0.4)		6 (0.4)	
China	16 (0.9)		15 (0.9)		16 (1.0)	
Other	283 (16.2)		273 (16.1)		257 (16.1)	
Household Income		0.18		0.16		0.21
< $60,000	363 (20.8)		353 (20.8)		327 (20.5)	
$60,001 – 100,000	614 (35.3)		602 (35.4)		566 (35.4)	
> $100,000	685 (39.3)		671 (39.5)		633 (39.6)	
No response	80 (4.6)		73 (4.3)		71 (4.5)	
Parity conditional on gravidity		0.0001		0.002		0.001
No prior pregnancy	499 (28.7)		485 (28.5)		459 (28.6)	
Prior pregnancy without live birth(s)	270 (15.5)		266 (15.7)		253 (16.0)	
Prior pregnancy with live birth (s)	972 (55.8)		947 (55.8)		884 (55.4)	

[a] Values are N(%), unless otherwise stated. [b] p values for the association with Time to Pregnancy: Likelihood ratio for continuous variables, Log Rank test for categorical variables.

[±] Paternal age was missing in 234, 224, and 210 participants for BPA, TCS, and Phthalates, respectively. Gestational age was missing in two participants.

[±±] Includes women who quit smoking during pregnancy or one year before.

Table 1. Association of study population characteristics with TTP by chemical measured: the MIREC Study (cont'd)

	BPA (n=1742)		TCS (n=1699)		Phthalates (n=1597)	
Characteristics	N (%)[a]	p value[b]	N (%)[a]	p value[b]	N (%)[a]	p value[b]
Maternal smoking		0.77		0.78		0.54
Never	1.078 (61.9)		1049 (61.8)		993 (62.2)	
Former	398 (22.9)		390 (23.0)		360 (22.6)	
Current [±±]	264 (15.2)		258 (15.2)		242 (15.2)	
Pre-pregnancy Body Mass Index		0.01		0.01		0.01
<24.9	1.031 (63.5)		1003 (63.3)		960 (64.4)	
25-29.9	354 (21.8)		346 (21.9)		326 (21.8)	
>30	238 (14.7)		234 (14.8)		205 (13.8)	
Paternal smoking		1.00		1.00		0.98
No	1.219 (83.0)		1191 (83.0)		1117 (82.7)	
Yes	249 (17.0)		244 (17.0)		233 (17.3)	

[a] Values are N(%), unless otherwise stated. [b] p values for the association with Time to Pregnancy: Likelihood ratio for continuous variables, Log Rank test for categorical variables.

[±] Paternal age was missing in 234, 224, and 210 participants for BPA, TCS, and Phthalates, respectively. Gestational age was missing in two participants.

[±±] Includes women who quit smoking during pregnancy or one year before.

Table 2. Bisphenol A, triclosan, and phthalate metabolites (ng/ml) in maternal urine

Analyte (Abbreviation)	LOD[a]	n (%) <LOD	Median	Minimum	Maximum	GM[b] (95% CI)
Bisphenol A (BPA), n=1742	0.2	226 (13)	0.8	< LOD	130	0.78 (0.73-0.82)
Triclosan (TCS), n=1699	0.12	20 (0.1)	8.3	< LOD	6784	11.93 (10.67-13.34)
Phthalate metabolites (n=1597)						
Low molecular weight						
Mono-n-butyl phthalate (MnBP)	0.20	4 (0.25)	12	< LOD	3100	11.44 (10.78-12.15)
Mono-ethyl phthalate (MEP)	0.50	2 (0.13)	28	< LOD	13000	32.09 (29.67-34.70)
Mono-benzyl phthalate (MBzP)	0.20	10 (0.63)	5	< LOD	420	5.10 (4.79-5.44)
Mono-methyl phthalate (MMP)	5.0	1375 (86.1)	2.5	< LOD	1000	3.03 (2.95-3.11)
Intermediate molecular weight						
Mono-cyclo-hexyl phthalate (MCHP)	0.20	1482 (92.8)	0.1	< LOD	77	0.12 (0.11-0.12)
High molecular weight						
Mono-isononyl phthalate (MiNP)	0.40	1574 (98.6)	0.2	< LOD	6.2	0.21 (0.20-0.21)
Mono-n-octyl phthalate (MnOP)	0.70	1568 (98.2)	0.35	< LOD	7.9	0.36 (0.36-0.36)
Mono-(3-carboxypropyl) phthalate (MCPP)	0.20	290 (18.2)	0.93	< LOD	370	0.87 (0.81-0.93)
Mono-(2-ethylhexyl) phthalate (MEHP)	0.20	24 (1.5)	2.2	< LOD	340	2.27 (2.14-2.40)
Mono-(2-ethyl-5-oxo-hexyl) phthalate (MEOHP)	0.20	5 (0.31)	6.5	< LOD	980	6.42 (6.06-6.81)
Mono-(2-ethyl-5-hydroxy-hexyl) phthalate (MEHHP)	0.40	14 (0.88)	9.4	< LOD	1200	9.21 (8.65-8.79)

[a]Limit of Detection (LOD), [b] Geometric Mean

Table 3. Fecundability Odds Ratios (95% CIs) for Bisphenol A and Triclosan

	Unadjusted	Adjusted [a]	Adjusted [b]
Bisphenol A (BPA)			
BPA* (ng/ml)	0.99 (0.93-1.05)	0.99 (0.92 1.06)	1.0 (0.92-1.07)
BPA Quartiles (ng/ml)			
0.1-0.3	1	1	1
0.4-0.8	0.99 (0.84-1.17)	0.98 (0.83-1.16)	0.98 (0.82-1.18)
0.81-1.7	1.02 (0.87-1.20)	1.00 (0.83-1.21)	0.96 (0.79-1.17)
≥1.8	0.93 (0.79-1.10)	0.91 (0.74-1.12)	0.95 (0.77-1.17)
BPA dichotomized** (ng/ml)			
<1.8	1	1	1
≥1.8	0.93 (0.81-1.07)	0.92 (0.79 1.07)	0.97 (0.83-1.14)
Triclosan (TCS)			
TCS* (ng/ml)	0.96 (0.91-1.02)	0.96 (0.90-1.02)	0.94 (0.88-1.01)
TCS Quartiles (ng/ml)			
0.01-2.14	1	1	1
2.14-8.28	1.16 (0.98-1.37)	1.16 (0.98-1.39)	1.10 (0.91-1.31)
8.33-71.6	1.13 (0.95-1.33)	1.14 (0.95-1.36)	1.10 (0.92-1.32)
≥71.7	0.94 (0.79-1.11)	0.95 (0.79-1.13)	0.89 (0.74-1.07)
TCS dichotomized** (ng/ml)			
<71.7	1	1	1
≥71.7	0.86 (0.75-0.99)	0.86 (0.75-0.99)	0.84 (0.72-0.97)

[a] Adjusted for specific gravity. [b] Adjusted for specific gravity, maternal age, maternal smoking, education, income, BMI
* log transformed and rescaled by their standard deviation. ** Dichotomized as < 75th percentile versus ≥ 75th percentile

Table 4. Fecundability Odds Ratios (95% CIs) for Phthalate metabolites

Phthalate metabolites (Abbreviation)	Unadjusted	Adjusted [a]	Adjusted [b]
Continuous * (ng/ml)			
Mono-n-butyl phthalate (MnBP)	1.04 (0.97-1.10)	1.04 (0.95-1.14)	1.02 (0.93-1.12)
Mono-ethyl phthalate (MEP)	1.02 (0.96-1.08)	1.01 (0.94-1.08)	1.00 (0.93-1.08)
Mono-benzyl phthalate (MBzP)	1.05 (0.99-1.12)	1.06 (0.98-1.14)	1.02 (0.94-1.11)
Mono-(3-carboxypropyl) phthalate (MCPP)	1.04 (0.98-1.11)	1.05 (0.97-1.13)	1.08 (0.99-1.18)
Mono-(2-ethylhexyl) phthalate (MEHP)	1.04 (0.98-1.10)	1.04 (0.97-1.13)	1.04 (0.96-1.13)
Mono-(2-ethyl-5-oxo-hexyl) phthalate (MEOHP)	1.04 (0.98-1.10)	1.04 (0.96-1.13)	1.07 (0.98-1.17)
Mono-(2-ethyl-5-hydroxy-hexyl) phthalate (MEHHP)	1.03 (0.97-1.09)	1.02 (0.94-1.11)	1.06 (0.97-1.16)
Quartiles (ng/ml)			
Mono-n-butyl phthalate (MnBP)			
0.1-5.1	1	1	1
5.2-12	1.02 (0.86-1.21)	1.02 (0.85-1.22)	1.01 (0.84-1.22)
13-25	1.06 (0.89-1.26)	1.06 (0.86-1.31)	1.08 (0.87-1.35)
≥26	1.08 (0.91-1.28)	1.08 (0.84-1.38)	1.03 (0.80-1.33)
Mono-ethyl phthalate (MEP)			
0.25-11	1	1	1
12-28	0.93 (0.79-1.11)	0.92 (0.76-1.10)	0.89 (0.74-1.08)
29-89	0.95 (0.80-1.13)	0.93 (0.77-1.12)	0.88 (0.72-1.07)
≥90	1.09 (0.92-1.30)	1.06 (0.87-1.29)	1.01 (0.82-1.24)
Mono-benzyl phthalate (MBzP)			
0.1-2.2	1	1	1
2.3-5.0	0.98 (0.83-1.17)	0.99 (0.83-1.19)	0.90 (0.75-1.09)
5.1-12	1.10 (0.93-1.31)	1.11 (0.91-1.36)	1.03 (0.84-1.26)
≥13	1.10 (0.93-1.31)	1.12 (0.90-1.39)	1.00 (0.80-1.26)

[a] Adjusted for specific gravity. [b] Adjusted for specific gravity, maternal age, maternal smoking, education, income, BMI
* log transformed and rescaled by their standard deviation. ** Dichotomized as < 75th percentile versus ≥ 75th percentile

Table 4. Fecundability Odds Ratios (95% CIs) for Phthalate metabolites (cont'd)

	Unadjusted	Adjusted [a]	Adjusted [b]
Quartiles (ng/ml)			
Mono-(3-carboxypropyl) phthalate (MCPP)			
0.1-0.3	1	1	1
0.31-0.92	1.13 (0.95-1.35)	1.13 (0.94-1.35)	1.09 (0.91-1.31)
0.93-2.1	1.12 (0.94-1.33)	1.11 (0.91-1.35)	1.08 (0.88-1.33)
≥2.2	1.09 (0.92-1.29)	1.07 (0.86-1.34)	1.10 (0.87-1.38)
Mono-(2-ethylhexyl) phthalate (MEHP)			
0.1-1.0	1	1	1
1.1-2.2	1.08 (0.91-1.28)	1.08 (0.91-1.30)	1.07 (0.89-1.29)
2.3-4.4	1.08 (0.90-1.28)	1.09 (0.88-1.34)	1.06 (0.86-1.32)
≥4.5	1.13 (0.95-1.35)	1.15 (0.92-1.43)	1.13 (0.90-1.43)
Mono-(2-ethyl-5-oxo-hexyl) phthalate (MEOHP)			
0.1-2.9	1	1	1
3.0-6.5	1.09 (0.92-1.29)	1.08 (0.90-1.30)	1.08 (0.89-1.30)
6.6-13	1.04 (0.88-1.25)	1.04 (0.84-1.29)	1.10 (0.87-1.37)
≥14	1.11 (0.93-1.31)	1.10 (0.86-1.40)	1.18 (0.92-1.53)
Mono-(2-ethyl-5-hydroxy-hexyl) phthalate (MEHHP)			
0.2-4.1	1	1	1
4.2-9.4	1.07 (0.90-1.27)	1.06 (0.88-1.27)	1.08 (0.89-1.31)
9.5-20	1.04 (0.88-1.24)	1.02 (0.82-1.26)	1.09 (0.87-1.36)
≥21	1.07 (0.90-1.28)	1.04 (0.82-1.33)	1.14 (0.89-1.47)

[a] Adjusted for specific gravity. [b] Adjusted for specific gravity, age, smoking, education, income, BMI

[*] log transformed and rescaled by their standard deviation. [**] Dichotomized as < 75th percentile versus ≥ 75th percentile

Table 4. Fecundability Odds Ratios (95% CIs) for Phthalate metabolites (cont'd)

	Unadjusted	Adjusted [a]	Adjusted [b]
Quartiles dichotomized (ng/ml) [**]			
Mono-n-butyl phthalate (MnBP)			
<26	1	1	1
≥26	1.05 (0.91-1.21)	1.03 (0.87-1.23)	0.98 (0.82-1.17)
Mono-ethyl phthalate (MEP)			
<90	1	1	1
≥90	1.14 (0.99-1.31)	1.13 (0.97-1.31)	1.11 (0.95-1.30)
Mono-benzyl phthalate (MBzP)			
<13	1	1	1
≥13	1.07 (0.93-1.24)	1.06 (0.91-1.24)	1.02 (0.87-1.21)
Mono-(3-carboxypropyl) phthalate (MCPP)			
<2.2	1	1	1
≥2.2	1.01 (0.87-1.16)	0.98 (0.83-1.15)	1.03 (0.87-1.21)
Mono-(2-ethylhexyl) phthalate (MEHP)			
<4.5	1	1	1
≥4.5	1.08 (0.93-1.24)	1.07 (0.91-1.25)	1.07 (0.91-1.26)
Mono-(2-ethyl-5-oxo-hexyl) phthalate (MEOHP)			
<14	1	1	1
≥14	1.06 (0.92-1.22)	1.04 (0.88-1.23)	1.09 (0.92-1.30)
Mono-(2-ethyl-5-hydroxy-hexyl) phthalate (MEHHP)			
<21	1	1	1
≥21	1.04 (0.90-1.20)	1.01 (0.86-1.20)	1.10 (0.89-1.26)

[a] Adjusted for specific gravity. [b] Adjusted for specific gravity, age, smoking, education, income, BMI

[*] log transformed and rescaled by their standard deviation. [**] Dichotomized as < 75th percentile versus ≥ 75th percentile

Table 5. Fecundability Odd Ratios (95% CIs) for total Phthalate metabolites (*u*mol/L)

	Unadjusted	Adjusted [a]	Adjusted [b]
Continuous variable* (*u*mol/L)			
Low molecular weight	1.03 (0.97-1.09)	1.02 (0.95-1.10)	1.02 (0.94-1.10)
High molecular weight	1.03 (0.97-1.10)	1.03 (0.95-1.13)	1.07 (0.98-1.17)
Quartiles (*u*mol/L)			
Low molecular weight			
0.005-0.12	1	1	1
0.12-0.28	0.92 (0.77-1.09)	0.90 (0.75-1.08)	0.90 (0.74-1.09)
0.28-0.69	0.92 (0.76-1.10)	0.90 (0.73-1.09)	0.86 (0.70-1.06)
≥0.69	1.06 (0.89-1.27)	1.02 (0.82-1.26)	0.98 (0.78-1.23)
High molecular weight			
0.002-0.03	1	1	1
0.03-0.07	1.03 (0.87-1.23)	1.03 (0.86-1.24)	1.02 (0.85-1.24)
0.07-0.14	1.06 (0.89-1.26)	1.05 (0.85-1.31)	1.12 (0.90-1.41)
≥0.14	1.08 (0.91-1.29)	1.08 (0.85-1.37)	1.17 (0.91-1.51)
Quartiles dichotomized (*u*mol/L)			
Low molecular weight			
<0.69			
≥0.69	1.13 (0.98-1.30)	1.12 (0.96-1.31)	1.10 (0.94-1.29)
High molecular weight			
<0.14			
≥0.14	1.03 (0.88-1.22)	1.04 (0.88-1.22)	1.08 (0.91-1.29)

[a] Adjusted for specific gravity. [b] Adjusted for specific gravity, age, smoking, education, income, BMI

* log transformed and rescaled by their standard deviation. ** Dichotomized as < 75th percentile versus ≥ 75th percentile

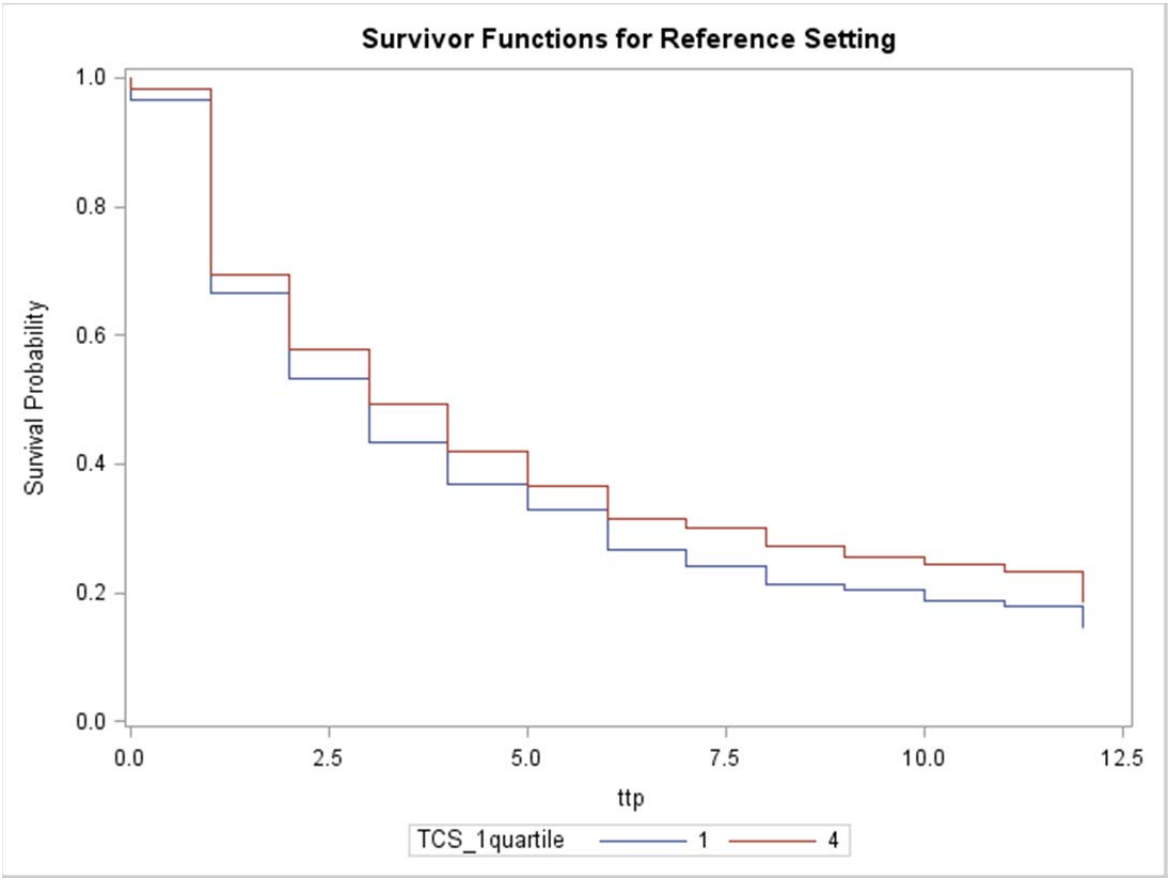

Figure 1. Survivor Function for triclosan concentrations adjusted for specific gravity, age, smoking, education, income, BMI

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Chapter 5 - THE POTENTIAL OF THE DIGIT LENGTH RATIO (2D:4D) AS AN ENDOCRINE-SENSITIVE ENDPOINT IN WOMEN AND THEIR OFFSPRING: THE MIREC STUDY (ARTICLE 3)

The potential of the digit length ratio (2D:4D) as an endocrine-sensitive endpoint in women and their offspring: the MIREC Study

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5.1 Abstract

Background: Solid endocrine-sensitive endpoints are needed to test the potential reproductive effects of endocrine-modulating chemicals. The 2th- to 4th-finger ratio (2D:4D) has been proposed as potential indicator of greater androgen exposure during fetal development.

Objectives: To evaluate the association between female 2D:4D and time to pregnancy (TTP) in women from the MIREC Study, a Canadian pregnancy and birth cohort, and to assess the effect of prenatal exposure to maternal smoking on the 2D:4D of children born to women from the MIREC cohort.

Methods: Digital pictures of the ventral surface of both hands were obtained in a subsample of some 800 mothers from the MIREC Study and their children (2-5 years). The 2D:4D was calculated as the ratio of the second and fourth fingers of each hand. The final sample included 638 mothers and 672 children (338 girls and 334 boys). Statistical analyses included discrete- time Cox proportional hazard models and multiple regression models, allowing adjustment for potential confounder factors.

Results: In women, there was no evidence of diminished/increased fecundability according to the 2D:4D, neither on the right, nor on the left hand (FOR 1.01; 95% CI 0.91-1.12 and 0.95; 95% CI 0.86-1.05, respectively). As for the children, boys had lower mean 2D:4Ds compared to girls, statistically significant for the right hand. However, the mean 2D:4D did not differ among children whose mothers had smoked during pregnancy compared to those whose mothers did not smoke, irrespective of gender.

Conclusions: our data do not support evidence to suggest that 2D:4D could be used as a potential reproductive endocrine-sensitive endpoint in women and in their offspring. Additional studies are needed.

5.2 Introduction

There is a paucity of solid endocrine-sensitive endpoints that allow the assessment of diverse life-course stressors on developmental and reproductive functions (Arbuckle et al. 2008). A novel endpoint in humans that has been proposed as an indicator of sexual development is the 2nd to 4th finger ratio (2D:4D). The 2D:4D ratio reflects sexual differentiation early in life and is likely an endpoint for the organizational effects of prenatal androgens in the human body (Manning 2002). The 2D:4D is a sexually dimorphic trait, with males having relatively shorter 2nd digit lengths than 4th digits (Bailey and Hurd 2005). As such, lower 2D:4Ds could be potential indicators of greater androgen exposure during fetal development. Associations between digit ratios and several health outcomes including male fertility, sexual orientation, physical performance, hand preference, and autism have been reported. The direction and magnitude of these associations, however, have not been consistent.

Several cross-sectional studies have reported low sperm counts and impaired hormonal status in men having higher digit ratios (i.e., implying low androgen exposure during fetal life) (Auger and Eustache 2011; Lu et al. 2012; Manning et al. 1998). However, several studies with larger sample sizes have failed to replicate the associations between 2D:4D and hormone levels in adults (Beaton et al. 2011; Honekopp et al. 2007; Neave et al. 2003), or to establish a number of male reproductive endpoints including semen quality (Bang et al. 2005; Firman et al. 2003; Seo et al. 2010). Menarche has also been assessed in association with 2D:4D. Two retrospective studies reported associations between lower digit ratios and delayed age at menarche (Manning and Fink 2011; Matchock 2008), while a prospective cohort found lower digit ratios as predictors of early age at

menarche (Oberg and Villamor 2012). Concerning fertility, the evidence is sparse and usually assessed by means of indirect markers. Thus, lower female digit ratios have been associated with low offspring counts (Manning and Fink 2008), congenital adrenal hyperplasia (CAH) (Brown et al. 2002; Oswiecimska et al. 2012), and polycystic ovary syndrome (PCOs) (Cattrall et al. 2005), although this finding was challenged by Lujan et al. (2010a; 2010b). Time to pregnancy (TTP) is an epidemiological metric widely used for the assessment of human fecundity (Baird et al. 1986; Buck Louis 2011a). In males, one study reported decreased fecundity (i.e., delayed TTP) at a higher 2D:4D (Auger and Eustache 2011). However, the predictive role of 2D:4D on TTP has never been assessed in females.

Smoking has a recognized detrimental impact on fertility, both male and female (Baird and Wilcox 1985; Buck Louis 2011a). Furthermore, maternal smoking during pregnancy has been associated with adverse reproductive outcomes in offspring (Ernst et al. 2012), including impaired fecundity (Weinberg et al. 1989). The causal mechanism by which smoking adversely affects fertility is still the subject of active research. In rats, prenatal exposure to nicotine has been linked to increased testosterone levels (Smith et al. 2003). In humans, maternal smoking during pregnancy has been positively associated with concentrations of maternal testosterone (Kandel and Udry 1999). Thus, if the adverse effect of maternal smoking on the offspring's subsequent fertility acts through the homeostasis of fetal androgens, and under the hypothesis that 2D:4D is an indicator of the fetal concentrations of androgens, then 2D:4D might differ among children whose mothers smoked during pregnancy compared to those whose mothers did not smoke. Only one cross-sectional study has assessed the impact of maternal smoking during

pregnancy on 2D:4D (Rizwan et al. 2007), reporting lower digit ratios in boys prenatally exposed to smoking, although this association was not observed in females.

In examining the potential of 2D:4D as a reproductive endocrine-sensitive endpoint, the purpose of this study was twofold: firstly, to evaluate the association between female 2D:4D and fecundity as measured by time to pregnancy (TTP) in women from the MIREC Study, a Canadian pregnancy and birth cohort; and secondly, to evaluate the effect of prenatal exposure to maternal smoking on the 2D:4D of children born to women from the MIREC cohort.

5.3 Methods

Population and study design

The MIREC Study (Maternal-Infant Research on Environmental Chemicals) is a pregnancy cohort conducted in 10 cities across Canada between 2008 and 2011 (Arbuckle et al. 2013). Approximately 2000 women attending prenatal clinics were recruited during the first trimester of pregnancy and followed up to 10 weeks postpartum. Subsequent follow-up studies have been conducted for the longitudinal assessment of child health and development.

The present analysis is part of the Early Childhood Biomonitoring and Neurodevelopment Study (MIREC-CD Plus), a follow-up study conducted to measure growth and development up to age 5 years in a subsample of some 800 MIREC children. Baseline demographic and lifestyle characteristics of the women were obtained from data collected at the first trimester visit. Gestational age at delivery, birth weight, and gender were obtained from the maternal delivery chart.

One component of MIREC-CD Plus was the measurement of the index (second, 2D) and ring (fourth, 4D) digit lengths in mothers and their children to calculate the 2D:4D. Digital pictures of the ventral surface of both hands were obtained using a 16-GB iPOD TOUCH (Apple Inc., California), placed in a device specially designed to standardize this procedure (Figure 1) (Appendix 4). Measurements of the second and fourth digit lengths were conducted by a single experienced research assistant with the aid of Adobe Photoshop 8 software (Adobe Systems Incorporated, San Jose, CA). As in previous studies that had used digital hand-scans (Allaway et al. 2009; Lujan et al. 2010b), when needed, the brightness/contrast of each image was subtly modified to improve the

visualization of the tips and proximal creases of the fingers. The lengths of the second and fourth fingers of each hand were further measured using mouse-controlled calipers. Calipers were positioned midline along the finger's basal crease and expanded to the edge of the finger tip. 2D:4D was calculated as the ratio of the second and fourth fingers of each hand. In order to assess intra-observer reliability, each measurement was performed twice after a period of two weeks, with the observer unaware of the initial measurements. We then examined the reliability of the measurements by calculating intraclass correlations (ICCs) between the two measures of each finger, separately for each hand (Table 1).

As of August 15, 2014, a total of 716 visits had been conducted (89.5% of the initial MIREC-CD Plus target). Sixteen mothers were not present at the time of the child's visit. The hand pictures of three participants (one mother, and three children) were excluded because of visible malformations of their fingers (i.e., syndactyly). Eight mothers and 6 children refused to have the pictures taken. Six pictures were excluded due to poor quality (4 mothers, 2 children). Sixteen pairs of twins were excluded from the child analysis, although their mothers were included. Forty-six mothers were further excluded because the index pregnancy was the consequence of a birth control failure. Four women and three children had only one hand picture available and for this reason were excluded. This resulted in a sample of 638 mothers and 672 children for the respective analyses.

Operational definition of variables

For our first objective, the primary exposure of interest was the 2D:4D of the women expressed either as continuous (log-transformed and divided by their standard deviation) or categorized by tertiles. The primary endpoint was time to pregnancy (TTP) (i.e.

number of months of trying to conceive). TTP was assessed in the MIREC cohort by asking women during the first trimester of pregnancy the following question: "How long did it take you to get pregnant with this pregnancy?" (number of months). Potential confounders were maternal age and smoking, household income, maternal education, and BMI before pregnancy. We did not include parity in our model to limit overadjustment bias (Buck Louis et al. 2012).

For our second objective, the primary exposure was self-reported maternal smoking during pregnancy, assessed at the first visit of the MIREC Study (6-14 weeks of gestational age). The primary outcome was the 2D:4D of the children. Potential confounders were child age, gestational age, and birth weight. We also considered the effect of the maternal 2D:4D. The analysis was stratified by gender. Although collected in MIREC-CD Plus questionnaire, the children's hand preference (i.e., handedness) was not available at the moment of the presence analysis for the whole sample.

Statistical Analysis

The reliability of the digit length measurement was evaluated by calculating the intraclass correlation coefficient (ICC) using ANOVA analysis. The ICC is calculated by dividing the between-subject variability by the sum of the between- and within-subject variability. ICC values range from 0 to 1, with the maximum of 1 indicating complete reliability (Kirkwood and Sterne 2003).

The descriptive phase of analysis includes assessment of the distributions of the baseline characteristics of the mothers and their children. Fecundability odds ratios (FORs) were estimated using the Cox model, modified for discrete time data (Allison PD 2010). FORs estimate the odds of becoming pregnant each cycle, given exposure to the specific

compound, conditional on not being pregnant in the previous cycle. FORs less than 1 denote reduction in fecundity or longer TTP, and FORs greater than 1 denote a shorter TTP. TTP was censored at the 13th month. Linearity and proportional hazard assumptions were verified (Allison PD 2010). To assess the impact of maternal smoking during pregnancy on the children's 2D:4D, mean differences were calculated by unpaired *t*-test. To account for potential confounders, multiple regression analyses were conducted. Statistical analysis was performed using STATA 10.0 (Stata Corporation, College Station, TX) and SAS 9.3 (SAS Institute Inc., Cary, NC), specifically for the discrete-time Cox proportional models.

5.4 Results

For this analysis, the mean maternal age at recruitment was 33.50 years (SD 4.67), with a median gestational age of 12.43 weeks (minimum of 6.86 weeks and a 90th percentile of 13.43 weeks). The mean maternal 2D:4D was similar in both hands (right 0.984, ± 0.035 and left 0.979, ± 0.036). Most of the women were born in Canada, two thirds had a university degree, more than half had a prior pregnancy with a live birth, and 15% were obese previous to the index pregnancy (Table 2). Seventy-two women (11.3%) reported being smokers or having quit after knowing they were pregnant. As shown in Table 2, education was the only covariant significantly associated with right hand 2D:4D, with women who had less than a college diploma having a smaller ratio. In line with the literature, age and obesity were associated with a longer TTP. Differences in TTP were also present by country of birth; however, the small number of participants from countries other than Canada precludes conclusions about this estimate.

The probabilities of pregnancy for these women at 1, 6, and 12 months were 0.40 (95% CI 0.36-0.44), 0.80 (95% CI 0.76-0.83), and 0.90 (95% CI 0.88-0.92), respectively. As presented in Table 3, there was no evidence of diminished/increased fecundability per one standard deviation increase in log-transformed 2D:4D, neither on the right, nor on the left hand (FOR 1.01; 95% CI 0.91-1.12 and 0.95; 95% CI 0.86-1.05, respectively). In our analysis by tertiles, the highest FOR was associated with the smallest ratio in both hands, although the 95% CI included the null effect (FOR 1.16; 95% CI 0.90-1.49 in the right hand and 1.16; 95% CI 0.91-1.49 in the left hand).

With regard to the children, their mean age at follow-up was 3.38 years (SD 0.77). The mean gestational age at birth was 39 weeks (SD 1.77), with a mean birth weight of 3460

grams (SD 507). The gender distribution was similar, as represented by 338 girls (50.3%) and 334 (49.7%) boys. As shown in Table 4, boys had lower means 2D:4Ds compared to girls. As per exposure to maternal smoking during the first trimester of pregnancy, the mean 2D:4D did not differ among children whose mothers had smoked during pregnancy compared to those whose mothers did not smoke, irrespective of gender (Table 4). The same was observed in the multiple regressions analyses (Table 5), where the only factors associated with the child's 2D:4D were their age and maternal 2D:4D.

5.5 Discussion

To our knowledge, the MIREC Study is the first pregnancy and birth cohort that has assessed 2D:4D in association with TTP in women, and the effect of maternal smoking on children's 2D:4D. Our objective was to examine the potential of the 2D:4D as a reproductive endocrine-sensitive endpoint. In mothers, we found some evidence of association between lower 2D:4D and shorter TTP, although this was not statistically significant. In children, exposure to maternal smoking during the first trimester of pregnancy, a potential stressor of fecundity in a woman's progeny, failed to show any effect on the 2D:4D, independent of gender. Instead, we found that age and maternal 2D:4D were strong determinants of the children's 2D:4D.

Most of the studies assessing the predictive role of 2D:4D on human reproduction have been conducted in men. In relation to fertility/fecundity in women, low 2D:4Ds have been related to low offspring count (Manning and Fink 2008) and pathological conditions associated with impaired fecundity such as congenital adrenal hyperplasia (CAH) (Brown et al. 2002; Oswiecimska et al. 2012), and with polycystic ovary syndrome in one study (PCOs) (Cattrall et al. 2005), which was contradicted in Lujan et al. (2010a; 2010b). With regard to time to pregnancy, one study reported decreased fecundity in males (i.e., delayed TTP) at higher 2D:4D, representing a feminized phenotype (Auger and Eustache 2011). However, we are unaware of any study focusing on female 2D:4D and time to pregnancy (TTP).

Several critical and sensitive windows essential for human reproduction and development are present as early as in the intrauterine period (Buck Louis 2011b). If, as suggested by Manning et al. (1998), the 2D:4D is defined *in utero* by the homeostasis of reproductive

hormones, we would have been able to demonstrate some association between 2D:4D and TTP. Moreover, one would have expected lower FORs (delayed TTP) in women with the lowest 2D:4D, but our results went in the opposite direction (i.e., the highest FORs were associated with the lowest 2D:4Ds, although this was not statistically significant). This also disagrees with the hypothesis that increased 2D:4D in females may be associated with increased fertility/fecundity, as Sutcliffe et al. (2010) proposed after finding higher 2D:4D in girls born following intracytoplasmic sperm injection (ICSI) compared to naturally conceived controls.

There are some potential explanations for our failure to support this plausible association. First, 2D:4D may not be a precise measure of intrauterine exposure to testosterone in women. Although a prenatal reproductive hormonal imbalance might affect female fecundity, these hormonal changes might be too subtle to produce a detectable association between female 2D:4D and TTP. Second, some methodological aspects of our study need to be considered. The MIREC Study is a cohort of pregnant women, and therefore, women who are infertile and have no access to or success in infertility treatments were excluded by design from our study. If the 2D:4D of this population is different in women able to conceive, our study would not have been able to capture this association. We did, however, conduct sensitivity analysis in women who had undergone infertility treatment for the index pregnancy, and the results remained negative for any association between 2D:4D and TTP. Another aspect that needs to be considered is the retrospective nature of the assessment of TTP in our study. However, studies have reported reasonable validity of recall data if collected in the short-term (Cooney et al. 2009; Zielhuis et al. 1992).

In line with the evidence, we were able to demonstrate that boys have lower mean 2D:4D than girls, and that this effect is stronger in the right hand (Manning et al. 1998). In spite of this, we were unable to demonstrate any effect on 2D:4D of a potential reproductive-aggressor *in utero* such as maternal smoking. The only study that has assessed this association to date is a cross-sectional study conducted in children in primary school (Rizwan et al. 2007), which reported a significant association between maternal smoking during pregnancy and low mean right 2D:4D in male offspring only. Certain aspects of our respective studies may account for the different results. Firstly, the children in Rizwan et al. were older (mean age 8.3 years, SD 1.7) than the ones in our study. Although some authors suggest that 2D:4D is stable during life (Manning et al. 1998), in our multiple regressions analyses, the child's age was significantly associated with 2D:4D, which is in line with studies in children suggesting that the digit ratio increases with age (McIntyre et al. 2005). Secondly, although the prevalence of maternal smoking was comparable in both studies (12.9% in MIREC, versus 13% in Rizwan et al. (2007), the doses of exposure (i.e., number of cigarettes per day) may have been different. Our sample size was not sufficient to test this hypothesis. In fact, our sample size had a limited power (i.e., 64%) to detect mean differences in 2D:4D of 0.2 (SD 0.05), with 95% confidence ($\alpha = 0.05$) (Manning et al. 1998).Thirdly, Rizwan et al. (2007) recognized that they could not rule out that high fetal testosterone, smoking, and low 2D:4D are associated through a relationship of risk-taking behaviour, and that there are studies suggesting the heritability of the 2D:4D (Manning et al. 2000). Smoking, a trait of risk-taking behaviour, is heritable (Vink and Boomsma 2011), raising the possibility that more than a proxy of *in utero* androgen levels, 2D:4D could be a heritable trait. In fact, in our

multiple regression models, the respective maternal index of each hand was significantly associated with the children's 2D:4D.

The method used to measure digit lengths could also account for the differences among studies. Most of the studies have measured finger lengths by a physical method, while we used digital imaging of the hands. While Rizwan et al. (2007) reported a Pearson correlation coefficient of 0.52 using physical measurements, our correlation coefficient was 0.88. A high reliability of our measures is also supported by the ICCs, which were higher than 0.95 for most of the finger lengths and more than 0.80 for the indexes. Thus, the different methods used for the measurements of the digit length could explain why our mean 2D:4Ds (Table 4) were lower than those reported in Rizwan et al. (2007), not only in girls (right 0.978 ± 0.039 and left 0.999 ± 0.034), but also in boys (right 0.967 ± 0.035 and left 0.981 ±0.031). In fact, there is evidence suggesting that computer-assisted measurements of the 2D:4D are more accurate than physical measurements, photocopies, or printed scanned images measured with Vernier calipers (Allaway et al. 2009). This may explain why our mean maternal 2D:4Ds (right 0.984 ± 0.035 and left 0.979 ± 0.036) are similar to those reported in Lujan et al. (2010b), who also used digital hand scans to assess 2D:4D in 96 women with PCOs (right 2D:4D 0.981 ± 0.028 and left 2D:4D 0.982 ± 0.030) versus 48 female controls (right 2D:4D 0.972 ± 0.028 and left 2D:4D 0.974 ± 0.037).

Although we did not find associations between maternal smoking during pregnancy and the children's 2D:4D, numerous environmental contaminants were measured in the MIREC Study in maternal blood, urine, meconium, breast milk, and more recently, in the urine of offspring (Arbuckle et al. 2013). This will allow us to further test the possible

impact of potential endocrine disruptors during the prenatal period on children's 2D:4D. In addition, the MIREC biobank offers the future possibility of measuring the hormonal profile of mothers and children, a limitation of the present analysis.

In summary, our data do not support any strong association between 2D:4D and female fecundity as measured by TTP. In children, we were able to demonstrate a sexual dimorphic trait with boys having lower ratios than girls. As well, we found that age and maternal 2D:4D were strong determinants of the children's 2D:4D. However, we did not find an adverse impact of maternal smoking during pregnancy on children's 2D:4D. Thus, whether the 2D:4D is a reliable reproductive endocrine-sensitive endpoint has yet to be determined.

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Table 1. Intraclass correlation coefficient subsample (n=80)

	Mothers			Children		
	ICC	95% CI		ICC	95% CI	
Right hand						
2: index finger	0.98	0.97	0.99	0.98	0.97	0.99
4: ring finger	0.99	0.98	0.99	0.99	0.98	0.99
2:4 digit ratio	0.92	0.89	0.96	0.86	0.81	0.92
Left hand						
2: index finger	0.97	0.95	0.98	0.98	0.97	0.99
4: ring finger	0.99	0.99	0.99	0.99	0.98	0.99
2:4 digit ratio	0.88	0.83	0.93	0.87	0.82	0.93

Table 2. Digit ratio and estimated time to pregnancy according to baseline characteristics in 638 women: the MIREC Study

		Digit ratio				TTP	
		Right hand		Left Hand			
Characteristic	n (%)[a]	Mean	p[b]	Mean	p[b]	Median[c] (95% CI)	p[d]
Age, years			0.59		0.23		<0.001
<30	142 (22.3)	0.986		0.975		2 (1-4)	
30-34	254 (39.8)	0.985		0.978		2 (1-5)	
35-39	195 (30.5)	0.981		0.979		2 (1-6)	
≥40	47 (7.4)	0.985		0.988		4.5 (1-18)	
Education			0.04		0.90		0.48
Some college or less	55 (8.6)	0.978		0.977		1 (1-6)	
College diploma	136 (21.3)	0.991		0.978		2 (1-6)	
Undergraduate	270 (42.3)	0.984		0.980		2 (1-5)	
Graduate (MSc PhD)	177 (27.8)	0.981		0.980		2 (1-6)	
Country of birth			0.46		0.63		0.04
Canada	524 (82.0)	0.985		0.978		2 (1-5)	
United States	11 (2.0)	0.974		0.991		4 (1-12)	
Mexico	3 (0.5)	0.960		0.962		6 (1-12)	
China	4 (0.5)	0.970		0.970		1 (1-1)	
Other	96 (15.0)	0.984		0.981		3 (1-8)	
Household Income			0.13		0.10		0.23
< $60,000	115 (18.0)	0.978		0.972		2 (1-5)	
$60,001 – 100,000	242 (38.0)	0.985		0.978		2 (1-5)	
> $100,000	260 (40.7)	0.986		0.982		2 (1-6)	
No response	21 (3.3)	0.990		0.985		1 (1-2)	
Parity conditional on gravidity			0.84		0.53		0.10
No prior pregnancy	193 (30.2)	0.984		0.977		3 (1-6)	
Prior pregnancy without live birth(s)	91 (14.3)	0.983		0.976		2 (1-6)	
Prior pregnancy with live birth(s)	354 (55.5)	0.985		0.980		2 (1-5)	

[a] Total may be less than 638 because of missing values, [b] ANOVA F Test, [c] Median TTP in months, [d] Log-rank test

[±] Includes women who quit during the first trimester of pregnancy

Table 2. Digit ratio and estimated time to pregnancy according to baseline characteristics in 638 women: the MIREC Study (cont'd)

		Digit ratio				TTP	
		Right hand		Left Hand			
Characteristic	n (%)[a]	Mean	p[b]	Mean	p[b]	Median[c] (95% CI)	p[d]
Maternal smoking			0.70		0.80		0.33
Never	417 (65.4)	0.984		0.978		2 (1-6)	
Former	149 (23.3)	0.984		0.980		1 (1-4)	
Current [±]	72 (11.3)	0.988		0.977		2 (1-5)	
Pre-pregnancy BMI			0.19		0.23		0.07
<24.9	341 (63.7)	0.985		0.980		2 (1-5)	
25-29.9	98 (20.2)	0.979		0.973		2 (1-6)	
>30	80 (16.1)	0.986		0.980		3 (1-7)	

[a] Total may be less than 638 because of missing values, [b] ANOVA F Test, [c] Median TTP in months, [d] Log-rank test

[±] Includes women who quit during the first trimester of pregnancy

Table 3. Fecundability Odds Ratios (FORs) according to digit ratio in 638 women from the MIREC Study

	Right hand		Left Hand	
	Crude FOR (95% CI)	Adjusted (n=590)[a] FOR (95% CI)	Crude FOR (95% CI)	Adjusted (n=590)[a] FOR (95% CI)
Digit ratio, continuous[b]	1.01 (0.92- 1.11)	1.01 (0.91-1.12)	0.95 (0.87-1.05)	0.95 (0.86-1.05)
Age		0.95 (0.93-0.97)		0.95 (0.93-0.97)
Pre-pregnancy Body Mass Index				
<24.9		Reference		Reference
25-29.9		0.85 (0.66-1.10)		0.84 (0.65-1.09)
>30		0.74 (0.56-0.97)		0.74 (0.56-0.98)
Digit ratio tertile (right/left medians)				
1 (0.950/0.944)	1.15 (0.91-1.46)	1.16 (0.90-1.49)	1.17 (0.93-1.49)	1.16 (0.91-1.49)
2 (0.982/0.979)	1.04 (0.82-1.31)	0.97 (0.75-1.24)	1.09 (0.86-1.38)	1.02 (0.80-1.31)
3 (1.021/1.011)	Reference	Reference	Reference	Reference
Age		0.95 (0.93-0.97)		0.95 (0.93-0.97)
Pre-pregnancy Body Mass Index				
<24.9		Reference		Reference
25-29.9		0.84 (0.65-1.09)		0.84 (0.65-1.08)
>30		0.73 (0.55-0.96)		0.74 (0.56-0.98)

[a] Log-transformed and divided by its standard deviation

[b] Due to 48 missing values of Pre-pregnancy Body Mass Index

Table 4. Mean 2D:4D in children according to gender and maternal smoking during the first trimester of pregnancy

Digit ratio	Gender			Maternal smoking during 1st trimester of pregnancy					
	Female	Male	p value[a]	Female		p value[a]	Male		p value[a]
	(n=338)	(n=334)		No (n=299)	Yes (n=39)		No (n=296)	Yes (n=38)	
Right hand			0.001			0.74			0.45
Mean	0.947	0.937		0.947	0.945		0.937	0.932	
S.D	0.062	0.040		0.039	0.036		0.039	0.043	
Left Hand			0.05			0.38			0.94
Mean	0.943	0.937		0.944	0.938		0.937	0.937	
S.D	0.070	0.040		0.038	0.044		0.040	0.041	

[a] t-test

Table 5. Adjusted mean changes (β) for the 2D:4D in children per maternal smoking status during the first trimester of pregnancy: The MIREC Study

	Female				Male			
	n	β	IC 95%	p value	n	β	IC 95%	p value
Right hand	333				328			
Maternal smoking		-0.0052	-0.0178, -0.073	0.42		-0.0039	-0.0175, 0.0097	0.57
Maternal right 2D:4D		0.2986	0.1826, 0.4147	<0.001		0.2518	0.1326, 0.3710	<0.001
Age, years		0.0057	0.0002, 0.0112	0.04		0.0055	0.0001, 0.0109	0.04
Birth weight, grams[a]		-0.0019	-0.0069, 0.0031	0.46		0.0004	-0.0052, 0.0059	0.89
Gestational age, weeks		0.0019	-0.0011, 0.0049	0.22		0.0020	-0.0013, 0.0054	0.23
Left hand	331				327			
Maternal smoking		-0.0060	-0.0190, 0.0069	0.36		0.0002	-0.0140, 0.0136	0.98
Maternal left 2D:4D		0.1915	0.0749, 0.3080	0.001		0.0982	-0.0180, 0.2143	0.10
Age, years		0.0068	0.0012, 0.0125	0.02		0.0051	-0.0003, 0.0106	0.06
Birth weight, grams[a]		-0.0019	-0.0070, 0.0033	0.48		-0.0000005	-0.0056, 0.0056	1.00
Gestational age, weeks		0.0014	-0.0017, 0.0050	0.39		0.0007	-0.0026, 0.0041	0.66

[a]Log-transformed and divided by its standard deviation

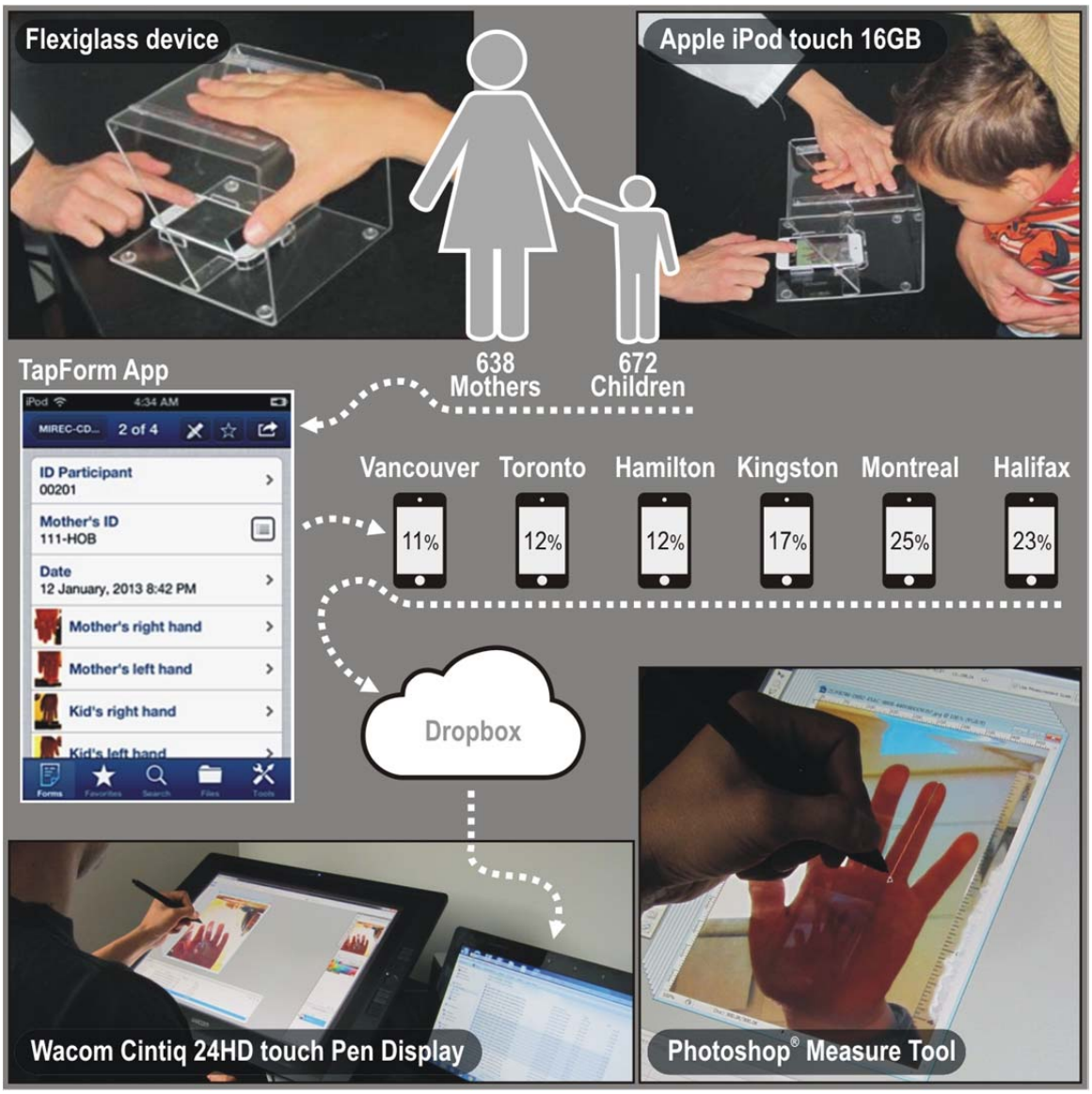

Figure1. Finger digital length image capture and measurement procedure.

5.6 References

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Chapter 6 - GENERAL DISCUSSION

This chapter summarizes the thesis, reviewing the key results, and discusses the more general methodological limitations of the research, in complement to the discussion sections of the three research articles presented above. The chapter ends with an outline of the implications of this research for public health and future research.

6.1 Overview of the results

The research undertaken in this thesis represented an effort to: [1] enhance the knowledge regarding the potential effect of selected environmental contaminants on female fecundity as measured by TTP; and [2] in an effort to validate a novel endocrine-sensitive endpoint, to evaluate the potential of 2D:4D as a predictor of later reproductive health in women and their offspring.

To assess the effect of selected ECs on female fecundity, two groups of compounds were considered: persistent (perfluorinated compounds -PFOA, PFOS, and PFHxS-) and nonpersistent chemicals (Bisphenol A, Triclosan, and Phthalates). As explained above, this thesis was developed in the framework of the Maternal-Infant Research on Environmental Chemicals (MIREC) study, a pregnancy cohort of 2001 women recruited in ten cities across Canada between 2008 and 2011 (30). Upon thesis commencement, a follow-up study was planned to measure growth and development up to age five in a subsample of some 800 MIREC children: the Early Childhood Biomonitoring and Neurodevelopment Study (MIREC-CD Plus). A contribution of this doctoral work was to add to this project by developing the finger-length component of the study, which consisted of measuring the index (2D) and the

ring (4D) digit lengths in mothers and children to calculate the 2D:4D ratio. To do this, digital pictures of the ventral surface of both hands were obtained using a standardized procedure (Figure 1). The purpose of this component of the study was to evaluate the potential of 2D:4D as a reproductive endocrine-sensitive endpoint by first analyzing the association between female 2D:4D and maternal fecundity, and secondly, by assessing the effect of prenatal exposure to maternal smoking on the 2D:4D ratio of the children.

Overall, the results of this thesis have contributed to the evidence regarding the potential adverse effect of several ECs on female fecundity as measured by TTP. Firstly, we found that PFOA and PFHxS were associated with diminished fecundity (FORs 0.89; 95% CI 0.83-0.94 and 0.91; 95% CI 0.86-0.97, respectively), supporting previous evidence that suggested a similar effect. Secondly, we assessed for the first time the effect of Triclosan on TTP, presenting evidence of delayed fecundity at the highest quartile of exposure (>72 ng/ml; FOR 0.84; 95% CI 0.72-0.97). Thirdly, our findings agreed with those of the only study that has assessed the effect of BPA on female fecundity, and which showed no effect. Fourthly, we found some indication that female exposure to phthalates might be associated with a shorter TTP, although this finding did not reach statistical significance.

In line with the literature (10), the magnitude of FORs reported is smaller than those reported for biologic determinants of fecundity such as oligospermia or gynecologic disorders (i.e., FORs 0.34 and 0.46, respectively) (99), which were not available in our database. However, our estimates are comparable to those reported for other lifestyle factors involved in fecundity such as smoking, higher BMI, and parental ages (158, 159).

With regard to the potential of the digit length ratio (2D:4D) as an endocrine-sensitive endpoint in women and their offspring, our data do not support a strong association between 2D:4D and TTP. In children, we did not find an adverse impact of maternal smoking during pregnancy on children's 2D:4D. Thus, our data do not support evidence to suggest that 2D:4D could be used as a potential reproductive endocrine-sensitive endpoint in women as measured by TTP, and in their offspring as measured by exposure to maternal smoking during the first trimester of pregnancy.

6.2 Summary of Key Results

The results of this thesis support the evidence that exposure to potential endocrine-disrupting chemicals may have an impact on human reproduction, underscoring the importance of continued investigation of ubiquitous environmental chemicals and human reproduction and development. Such work will help inform the extent to which such exposures might adversely impact population health.

In regards to persistent ECs, our results add to the evidence that exposure to PFOA and PFHxS, even at lower levels than previously reported, may reduce fecundability. Future epidemiological studies at current levels of exposure, including male partner concentrations should be conducted to strength conclusions about causality. It also be important to follow couples trying to attempt pregnancy to obtain data relevant for understanding the toxicokinetics of chemicals and their impact on sensitive fecundity endpoints (10). Research should also be focused on the identification of strategies to reduce exposure to these chemicals in the general population. Controversy exists about how best to model exposures and TTP. We used DAGs to support the possibility of collider-stratification bias with the inclusion of

parity in models. Moreover, PFCs are not lipophilic and while placental and lactational transfer has been reported, the magnitude of change is minimal relative to lipophilic compounds. In addition, data have been reported for daily exposure to PFCs via diet, underscoring the continual exposure for women including pregnant women. Such exposure and toxicokinetic data offer support for the exclusion of parity in models. In this respect, our manuscript will help to guide the analytical phase of further studies.

In the case of non-persistent ECs, our data suggest that elevated TCS exposure (> 72 ng/ml) may be associated with diminished fecundity, as suggested by a longer TTP. In regards to phthalates and BPA, there was no evidence of any negative impact on TTP and even some suggestion that exposure to some phthalates might be associated with a shorter TTP. Since this is the first study to evaluate TCS in relation to TTP, and the second to evaluate the effect of BPA and phthalates we recommend further epidemiological and toxicological studies in order to test our findings and elucidate the potential impact of nonpersistent environmental contaminants on human fecundity.

As stated in the Scientific Statement of the Endocrine Society, in the absence of direct information regarding cause and effect, the precautionary principle is critical to enhancing reproductive and endocrine health, and should be used to inform decisions about exposure to, and risk from, potential EDCs (34).

6.3 Comments on Study Design and Limitations

The specific limitations of this research have already been described in detail in the previous chapters, and therefore, here, as in a recent overview conducted by Buck Louis (10), we will focus on the more important methodological limitations that need to be considered in

weighing the evidence of the effect of ECs on fecundity. These are: 1) reliance on pregnant women, which may exclude women with the highest exposures if related to the inability to conceive; 2) retrospectively reported TTP, which may be associated with bi-directional reporting errors; and 3) limited attention to male partners or couples' exposures.

Firstly, this thesis was developed within the context of the MIREC Study, a Canadian pregnancy and birth cohort. Thus, only women who became pregnant spontaneously or who opted/had access to ART and succeeded in their treatment were included in the study. Numerous studies have shown that women who seek medical help for fertility problems make up a highly selective group among women with fertility problems. Data from nationally representative surveys in the United States have shown that women with fertility impairments who use infertility services are more likely to be married, non-Hispanic white, older, more highly educated, and more affluent than non-users (156). The significant cost of medical services for infertility is probably the main reason for the disparities in the use of infertility services (160). In Canada, health care is the responsibility of the individual provinces. Quebec is the only province that universally covers the costs of ART, since August 2010. This new policy increased access to these types of treatments, as reflected by a 200% increase in the number of IVF cycles performed during the first year of the program compared to the year previous to the policy, as shown in our evaluation of this program (161).

As recently reviewed in Velez et al., (121) disadvantaged populations experience greater susceptibility to environmental hazards because of compounding health hazards (poor nutrition, poor housing, poor health care, and higher levels of environmental contaminants) (162, 163). Exposure to environmental toxicants varies according to race, income, gender, age,

and other demographic characteristics (164), in addition to susceptibilities related to genetics or physiologic differences related to sex. For example, data from NHANES revealed that non-Hispanic blacks and Mexican-Americans in the United States have higher levels of MEP, MiBP, and MEHP in their urine than non-Hispanic whites (165, 56). Marginalized groups disadvantaged by demographic characteristics are at greater risk of experiencing more toxic exposures and are more vulnerable to their toxic effects, due to a limited access to political power and to the living conditions and services needed to address contaminants in their communities (163). Although all social classes use consumer products containing chemicals, people with lower socio-economic status are more likely to be exposed to products manufactured outside North America that are subject to lower controls by regulatory authorities.. These include cosmetics, children's toys, and food containers, among other products (121).

Participants in MIREC, on average, tended to be older, better educated, born in Canada, married, and less likely to be current smokers than the general population of Canadian women giving birth in 2009 (31), characteristics that were also observed in our three manuscripts. The validity of a study is usually separated into two components: The validity of the inferences drawn as they pertain to the members of the source population (internal validity) and the validity of the inferences as they pertain to people outside that population (external validity or generalizability) (166). Most violations of internal validity can be classified into three general categories: confounding, selection bias, and information bias. Since information on socio-demographic and life-style characteristics were collected and considered in our analysis, we were able to control for potential confounding, a major factor affecting internal validity. In order to minimize selection bias we included all the potential

participant from the MIREC cohort, excluding women requiring egg donation or reporting male factor infertility, as well as women whose index pregnancy was the result of birth control failure. Sensitivity analysis including the latter group of women resulted in similar results. However, we recognize that since this is a retrospective-TTP study, women that were infertile and/or did not have access to infertility treatment were excluded by design from our study. Thus, the possibility of selection bias exists since women highly exposed to these chemicals were excluded if there is a causal association with infertility. This is acknowledged in the manuscripts. Regarding information bias, the retrospective assessment of TTP can introduce recall bias; however it is a reliable method when collected in the short term. Moreover, the effect of digit preference was addressed, suggesting that this was not a concern. Exposures were based on biomonitoring data conducted by an accredited laboratory, which decreases the possibility of measurement error. In terms of external validity, considering that the MIREC study population is largely Caucasian, more educated and of a higher income group than the Canadian population at large, generalization to other ethnic and socioeconomic populations should be done with caution. However, given the relatively large sample size in our analysis, there still were substantial numbers in the cell for the less economically advantaged women (n = 361 < $60,000) to still suggest some inferences for this population.

Secondly, because MIREC is a pregnancy cohort of women enrolled during the first trimester of pregnancy (6 to 14 weeks), TTP was assessed retrospectively. While reliability is reported to be good for retrospectively measured TTP, even with a long period of recall (167), only short recall periods (i.e., not longer than 20 months), are considered valid (76, 77). Therefore, this potential limitation of pregnancy-based retrospective TTP studies is unlikely to have biased our results.

Digit preference reporting can also affect retrospective TTP studies (168); however, it is estimated that stable estimates of the TTP distribution can be obtained with approximately 200 values per exposure group (21), a number that, due to our large sample size, was always attained in the different categories of exposure. Furthermore, to evaluate if digit preference had any effect on our results, we applied the method recently proposed in McLain, et al. (169). The histogram of our data suggested digit preference at 6, 12 and 18 months. We estimated a piecewise exponential model with three separate knot scenarios, each using seven knots; the locations for knot scenarios were $\{1,2,4,9,18,30,\infty\}$, $\{1,2,4,9,15,27,\infty\}$, and $\{1,2,4,10,17,29,\infty\}$. The estimates showed little bias (Table 1), suggesting that digit preference had little impact in our results.

Table 1. Digit preference Piecewise knot scenario

		Piecewise knot scenario		
	MIREC	A	B	C
		Mean (SD)	Mean (SD)	Mean (SD)
BPA	-0.01868	-0.0168	-0.0152	-0.0166
	(0.02872)	(0.0242)	(0.0246)	(0.0244)
TCS	-0.03531	-0.0357	-0.0343	-0.0352
	(0.02820)	(0.0238)	(0.0238)	(0.0236)
Phthalates				
MnBP	0.03108	0.0317	0.0329	0.0337
	(0.03027)	(0.0258)	(0.0253)	(0.0252)
MEP	0.02494	0.0169	0.0155	0.0173
	(0.02972)	(0.0258)	(0.0258)	(0.0247)
MBzP	0.04161	0.0409	0.0396	0.0404
	(0.02978)	(0.0249)	(0.0252)	(0.0256)
MCPP	0.03087	0.0369	0.0378	0.0378
	(0.02946)	(0.0249)	(0.0247)	(0.0244)
MEHP	0.03490	0.0389	0.0397	0.0390
	(0.02912)	(0.0245)	(0.0244)	(0.0244)
MEOHP	0.02987	0.0361	0.0358	0.0350
	(0.02908)	(0.0244)	(0.0238)	(0.0244)
MEHHP	0.02317	0.0285	0.0287	0.0290
	(0.02914)	(0.0250)	(0.0248)	(0.0246)

Thirdly, another limitation of current studies is the lack of attention given to male partners' or couples' exposures. In fact, although MIREC collected some demographic and lifestyle information regarding the babies' fathers, we did not collect biological samples in males. This could be a limitation, given recent results from the LIFE study reporting associations between concentrations of some phthalate metabolites in males and delayed TTP (71), as well as some associations between semen quality and exposure to select PFCs, although in directions that were not consistent (170). We did, however, take some precautions to limit the extent to which male fecundity determinants could affect our results. During our sample selection, we excluded participants whose partners had had some type of infertility treatment; also, during the statistical analysis, we took into account paternal age and smoking status. Paternal and maternal ages were highly correlated (r=0.73); therefore, to avoid collinearity, we did not include paternal age in the analyses. However, we conducted sensitivity analyses, including paternal age, and this did not modify the associations that we reported. Paternal active smoking during the first trimester of pregnancy was not associated with TTP, and therefore, was not included in the adjusted models.

In addition to the previously-mentioned limitations, there are some covariates for which we did not have information. One of the main difficulties in studying human fecundity is the large behavioural component. There is constant interplay between behaviour and biology. The frequency of sexual intercourse (19, 171, 172), as well as the timing of sexual intercourse in relation to ovulation, strongly influences the probability of conception (173). This information was not available in the MIREC study, which may have affected our results. In addition, we did not have information on menstrual cycle histories. TTP was assessed in months. However, since a woman can conceive only once in each menstrual cycle, it has been

argued that the proper way to measure TTP is in cycles (174). Nonetheless, the majority of studies investigating TTP have measured TTP in months. For women with regular menstrual cycles of 28 days, the effect of the choice of unit does not impact on the effect estimates. However, for women with long menstrual cycles, measuring TTP in months would overestimate the true TTP. On the contrary, if a chemical modifies the duration of the cycle, then looking only at conception per cycle would underestimate the effect of the exposure on TTP. Finally, only data on the first pregnancy can be considered unbiased, as relatively fertile couples may tend to have more children, and adverse reproductive experience may influence behaviour. This issue should be considered if later pregnancies are studied (21). Although detailed information about previous pregnancies of MIREC participants was obtained, no information regarding the TTP of previous pregnancies was available. Nevertheless, despite recognition of the importance of lifestyle factors in TTP (175), it is likely that at the population level, female biological factors, such as age and menstrual cycle length, are more important predictors of TTP than lifestyle factors (174). Thus, as previously mentioned, biologic determinants of fecundity such as oligospermia or gynecologic disorders (i.e., polycystic ovary syndrome, fibroids, and endometriosis among others), which have a higher impact on fecundity, were not assessed in MIREC. Furthermore, the hormonal profile of women and children, important in the study of reproductive function, was also absent.

There are additional methodological considerations related to laboratory analytical issues that need to be mentioned in this thesis. Additional potential limitations in the exposure assessment need to be considered. First, no exposure data was available for 2% of the eligible women for BPA, 5% for TCS, and 10% for phthalates. It is considered that complete case analysis is unlikely to introduce bias when the incomplete cases are less than about 5% (176). In most of

the cases, there was no laboratory result because the woman did not provide sufficient urine for all the chemical analyses that were done. More phthalate results were missing because they were analysed in the second aliquot of urine, while BPA was analysed in the first. TCS was analysed in the first aliquot, but we lost 2% of women who did not consent to further analyses of the biobanked specimens. We consider that the missing values for phthalates as consequence of being measured in the second aliquot of urine are independent of both observed and unobserved data, which is defined in the literature as "missing completely at random" (MCAR), in which case, complete case analysis is an acceptable approach (177). All chemical concentrations that were below the LOD were coded as the lowest concentration obtained for that congener divided by 2 (178), which has been shown to give similar results to division by the square root of 2 (56). However, recent evidence suggests that this simple substitution may lead to increased bias and the underestimation of the error variance, which results in a lower power for statistical hypothesis testing (179-181). However, simulation studies using alternative methods to account for exposures <LOD, have demonstrated that the LOD divided by two worked fairly well in simulations with ≤50% exposure data below the LOD (181). Methods have also been proposed for Cox regression models with covariates subject to a lower LOD, however they have not provided much improvement over the LOD divided by two (182). In our analyses, PFC, TCS and five phthalates metabolites (MnBP, MEP, MBzP, MEHP, MEOHP, MEHHP), were detected in at least 95% of the samples, which suggests that the probability of bias due to our substitution approach is very low for these particular chemicals. In the case of BPA and MCPP, detection rates were also high (87% and 82%, respectively), which is also reassuring. On the other hand, four phthalate metabolites were detectable in less than 14% of the samples (MMP, MCHP, MiNP, and MnOP). These

metabolites were excluded from further analyses, an approach used in large biomonitoring surveys when the proportion of results below the LOD is greater than 40% (56). However, an analysis of these metabolites as continuous variables showed that the adjusted FORs were approximately 1, although not statistically significant for MMP (FOR=1.03, 95% CI 0.96-1.10), MiNP (FOR=1.0, 95% CI 0.94- 1.06), and MnOP FOR=1.02, 95% CI 0.96- 1.09) using the continuous scale. In the case of MCHP, the FOR was lower than 1, but the 95% CI was large and non-significant (FOR=0.93, 95% CI 0.72-1.18). To account for urine dilution in the case of nonpersistent chemicals, and to be consistent with current practice, specific gravity was included as a covariate in the model (183, 66). Specific gravity has been proposed as a more appropriate method than creatinine for adjusting phthalate concentrations because their elimination by active tubular secretion might limit the accuracy of creatinine adjustment (184). Additionally, creatinine levels may be confounded by muscularity, physical activity, urine flow, time of day, diet, and disease states (184). In pregnant women, specific gravity appeared to be more effective than creatinine in adjusting for urine dilution late in pregnancy (185).

Another methodological consideration is the self-reported assessment of smoking. Although the MIREC study included the measurement of cotinine concentrations in plasma, these results were not available at the time of conducting the analyses for this thesis. However, the validity of self-reported smoking during pregnancy has been shown to be high compared to plasma levels of cotinine in early pregnancy (186).

It is also worth recalling that although for persistent environmental chemicals such as PFCs, blood samples collected during the first trimester of pregnancy are probably a reliable surrogate of periconceptional concentrations due to their long half-lives (93), the same cannot

be true for nonpersistent chemicals. Concentrations of BPA, TCS, and phthalates were measured in a single spot urine sample collected during the first trimester of pregnancy. As these chemicals have a short half-life (hours) and there are multiple sources and routes of exposure, intra-individual variability in results are expected. Previous studies have indicated that while the intra-class correlation coefficient (ICC) for BPA is low (< 0.25) across pregnancy (187-189), the ICC for TCS is better (> 0.47) (57, 61, 62). As for phthalates, the extent of the variability depends on the metabolite, with DEHP metabolites often displaying more variability than other metabolites (55, 58-60).

With regard to the assessment of 2D:4D as an endocrine-sensitive endpoint in women and their offspring, differences in the methods selected for measuring the digit ratios could in part explain divergences in previous studies that have assessed several outcomes, including male fertility endpoints such as semen quality, and TTP. In females, however, this is the first study that has evaluated TTP according to 2D:4D. Among the methods previously used to assess 2D:4D are direct physical measurements with calipers (129), measurements from photocopies (190), scanned images (128), radiographs (191, 192), inked handprints (193), scaled tubes (194), and digital pictures (195). Digital technology may provide a superior alternative to the more traditional techniques for the acquisition of images, and computer-assisted image enhancement may improve measurement reliability (196). We used Photoshop, a graphics editor software that has been shown to have very high precision compared to scales and calipers for the measurement of digit lengths (197). 2D:4D evaluations are technique-dependent and this has serious implications in a field that necessitates a highly precise tool to identify relatively small differences among study populations (197). Thus, we believe that our

method for capturing and measuring the images does not explain the absence of association that we found.

6.4 Power calculation

For the assessment of ECs and TTP, the final number of participants included in the adjusted models ranged from 1491 to 1625 (Table 2). These samples have a power higher than 95% (α= 0.05) to detect FORs = 0.90 per one standard deviation increase in log-transformed serum concentrations of the respective chemicals, and considering the potential effect of additional covariates (r^2 =0.14) (174). In the case of our third article, maternal 2D:4D and TTP, the power was 66% to detect FOR= 0.90, but higher than 95% to detect FOR = 0.85.

These power calculations were based on the formula developed by Hsieh and Lavori (198), which also corrects for a variance inflation factor (VIF) (Appendix 5).

Table 2. Power calculation

	Exposure	N	FORs		
			0.90	0.85	0.80
Article 1	Perfluorinated compound	1625	98%	100%	100%
Article 2					
	Bisphenol A	1623	98%	100%	100%
	Triclosan	1583	97%	100%	100%
	Phthalates	1491	97%	100%	100%
Article 3	2D:4D	590	66%	96%	100%

In relation to the children's 2D:4D, our study had a limited power (i.e. 64%) to detect mean differences in digit ratios of 0.2 (SD 0.05), with 95% confidence (α = 0.05) (129).

6.5 Public Health Relevance and Future Research Directions

The MIREC study offers a unique opportunity to examine the effect of several environmental contaminants on time to pregnancy in a large Canadian population of pregnant women. Overall, the results of this thesis are expected to support public health interventions to limit exposure in the general population to select ECs. Awareness of these potential risks may lead people to adopt corrective behaviours that should help them maintain their fecundity, hence reducing the burden of fertility impairment in Canada and worldwide.

Fecundity impairments and infertility have important public health implications. Given that the injudicious use of many infertility treatments results in twin and higher-order births, both mothers and infants are at higher risk for adverse health outcomes (161). While there is increasing recognition about the adverse effect of many factors, including environmental contaminants, on fecundity, there are still many opportunities to better understand and address population-level issues that contribute to infertility in men and women (154).

As suggested by Buck Louis, two opportunities of research may provide insight regarding the effect of ECs on fecundity (10). Firstly, existing pregnancy and/or birth cohorts conducted during the last decade, including the MIREC Study (31), have biobanks that may be suitable for continued investigation. However, since women who are not able to achieve pregnancy are excluded by design from these cohort studies, additional strategies are needed to target this population in different settings. Secondly, follow-up studies of children from existing birth cohorts can be conducted, with TTP studies being designed when they enter their reproductive years. This intergenerational approach will provide information on *in utero* exposures, as well as on exposures at the time of pregnancy attempts. In addition, as

recommended by Arbuckle et al. (29), research should continue to search for valid biomarkers of endocrine dysfunction, and preferably longitudinally, as we did in our 2D:4D study with women and their offspring, measuring additional endpoints such as anogenital distance, breast size, menarche, and hormonal profiles at different sensitive windows to facilitate comparability between study groups. Finally, further investigation is needed to better understand the mechanistic pathways by which select environmental chemicals are able to modulate the endocrine system and affect development and reproduction.

6.6 Student's contribution

In February 2007, I joined the MIREC coordinating team as a research assistant, a position that I held until June 2010. During that time I worked on the implementation of the MIREC study in the 10 study sites, including the preparation of the case report forms (questionnaires), manual of procedures, ethics submission, research nurses' training, quality control, and participation in the weekly conference call with the principal investigators and co-investigators of the study. I also worked on a manuscript assessing the effect of phthalates on women's reproductive health. This paper was selected by the National Network of Environments and Women's Health (NNEWH), a Canadian policy-oriented research centre, as a chapter for a book focused on Environmental Health Policy (199). In addition, I was co-investigator of a research proposal lead by Dr. Monnier to assess exposure to phthalates in children admitted to the pediatric intensive care unit at CHU Sainte-Justine that was funded by the Research Center. I started my PhD in September 2008, presented the written general exam in August 2009, and the oral defence of the protocol in August 2010. In agreement with Dr. Fraser, I started a clinical fellowship in Reproductive Endocrinology and Infertility at

Université de Montréal in July 2010 for two years . In addition to my clinical activities, I was the main research consultant for my fellow colleagues and residents. Several abstracts were presented in National and International meetings, four manuscripts (200-202, 161) and two book chapters were published (203, 204).

In 2012, I joined the MIREC research platform as a co-investigator of the anthropometry component of the MIREC-CD Plus Study (follow-up of about 800 MIREC children to age 5 years). During this time, I obtained a research grant from the CIHR-Quebec Training Network in Perinatal Research (QTNPR) to assess the utility of the Finger Digit Ratio (2D:4D) as a potential endocrine-sensitive endpoint in women and their offspring. I had an active role in the writing of various sections of the MIREC-CD Plus protocol and in its submission for ethics approval. For the 2D:4D component, I developed the protocol, questions to be included in the case report forms, and designed the device for obtaining standardized digital pictures of both hands in mothers and children. I also trained the nurses on how to use the device, and developed a detailed manual of procedures.

The decision about the exposures that were going to be assessed in relation to TTP was taken in conjunction with my supervisors Dr. Fraser and Dr. Arbuckle, and was based on the scarcity of data for these emerging contaminants and human fecundity. In regards to the 2D:4D, this ratio was measured in a previous MIREC follow-up study, [MIREC-ID (Infant Development)], but only in children, and using a tape metric. I was interested in measuring the 2D:4D in women in relation to their TTP, since it had never been done, to test the hypothesis that it reflects the endocrine environment during the perinatal period. I also wanted to measure it in children using a standardized method.

I carried out all the data management, statistical analysis and interpretation of results, in addition to drafting the manuscripts. I worked closely with my supervisors in the interpretation and review of the manuscripts. I submitted the first two manuscripts, first to internal approval at Health Canada, and later to the scientific journals. Both of them have were accepted for publication in December 2014. The 2D:4D manuscript will be submitted for internal review soon after including a few additional participants that were recruited up to December 2014 (N=35). The results on the complete sample remained similar to those reported in the thesis.

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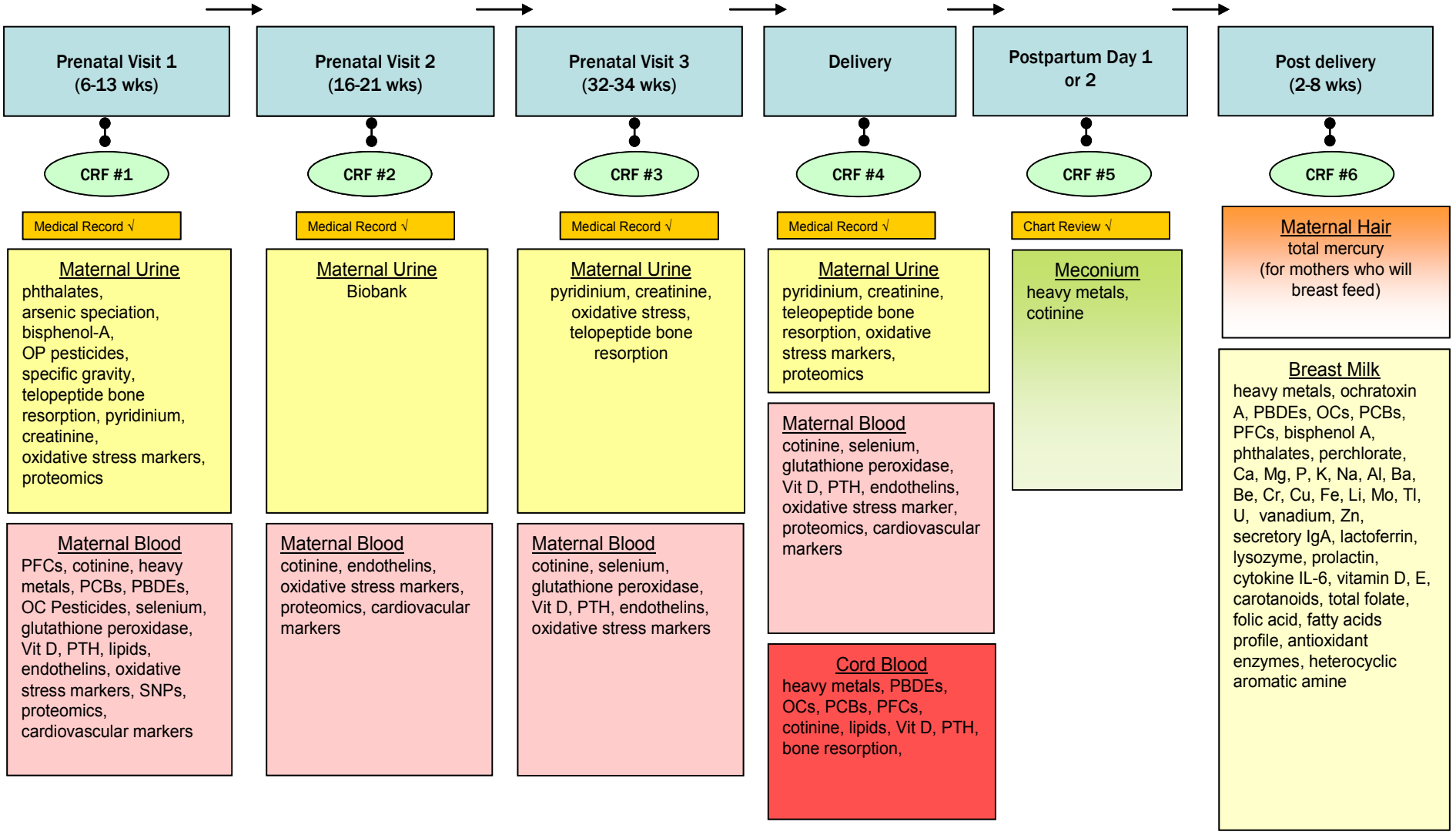
APPENDIX 1. COHORT PROFILE: THE MATERNAL-INFANT RESEARCH ON ENVIRONMENTAL CHEMICALS RESEARCH PLATFORM

APPENDIX 2. VARIABLES ASSESSED IN MIREC AT EACH VISIT

Measures	6-12 weeks	16-21 weeks	32-34 weeks	Delivery	1-2 days postnatal	2-10 weeks postnatal (breastmilk)
Demographics						
Maternal age, education, ethnicity, employment	✓		✓			
Paternal age, education, ethnicity, occupation	✓					
Marital status, income	✓					
Obstetric History						
Pregnancy history, time to preg	✓					
Use of ART and contraceptive	✓					
Pregnancy outcome	✓	✓	✓	✓		
Family Medical History	✓					
Current Medications	✓	✓	✓	✓		
Nutritional Supplements	✓	✓				
Environmental Exposures						
Hobbies, work, home renovations, cooking ustentils	✓		✓			
Lifestyle						
Active and passive smoking	✓		✓			✓
Alcohol consumption	✓		✓			✓
Residential History						
Addresses, home characteristics	✓		✓			
Activities	✓		✓			
Diet						
Meat	✓		✓			
Fish	✓		✓			✓
Food Frequency		✓				✓
Maternal anthropometric measures	✓	✓	✓	✓		✓
Blood pressure	✓	✓	✓	✓		
Protein urine dipstick test	✓	✓	✓			
Placenta weight				✓		
Co-factor Biomonitoring						
pyridinium, creatinine, selenium, glutathione peroxidase	✓		✓	✓		
oxidative stress, markers, endothelins	✓		✓	✓		
minerals, vitamins, fatty acids, enzymes						✓
Chemical Biomonitoring						
Metals	✓		✓	✓	✓	✓
Plasticizers	✓			✓		✓
Brominated flame retardants	✓			✓		✓
Surface coatings	✓			✓		✓
OP Pesticides	✓			✓		✓
POPs (PCBs, OCs)	✓			✓		✓
Cotinine	✓	✓	✓	✓	✓	✓
Genetic Polymorphisms		✓				
ALAD, VDR (fokI, bsmI, apaI, taqI), APOE (1-4), HFE (C282Y, H63D), metallothionein		✓				

APPENDIX 3: MIREC DATA COLLECTION



APPENDIX 4. MANUAL OF PROCEDURES FINGER DIGIT RATIO.

6.6.1 Measuring Fingers length using the iPod Touch

A novel endpoint that is showing promise as an indicator of sexual development is the 2nd to 4th finger digit ratio (2D:4D). The 2D:4D ratio shows sexual differentiation (lower in males) and is likely a biomarker for the organizational (permanent) effects of prenatal androgens on the human brain and body. Some evidence has suggested that low 2D:4D is related to high fetal testosterone. Several studies have suggested associations between 2D:4D and a multiplicity of sex-dependent traits. Furthermore, evidence is starting to emerge about the predictive role of 2D:4D on later reproductive health. For example, in regards to the age of menarche, an endpoint of the influence of the hormonal intrauterine environment, a recent prospective cohort study in pre-menarcheal girls (5-12 years) reported a lower median age at menarche for girls in the lowest digit ratio tertile of the right hand compared with those in the highest. Additionally, the 2D:4D has been linked to sperm counts and hormonal status in men with impaired fertility.

The TapForms application will be used for finger measurements. Seven fields will be entered for each participant; for information on entering data in the form, see Appendix 14:

- ID Participant refers to the Biospecimen ID - 99999).
- Mother's ID (from a pick list) refers to the study ID (99-999-ABC).
- The date is filled automatically.
- Mother's right hand image.
- Mother's left hand image.
- Child's right hand image.
- Child's left hand image.

The same application allows Transferred data, either by e-mail, web page or DropBox application, see Appendix 14. You must transfer the hand scans on a **regular basis** (e.g. every 2 weeks) in order to minimize any risk of potentially losing information, should unforeseen circumstances occur (ie. ipod is lost or stolen, etc). To follow each transfer and make sure that the files were really dropped, you must advise Gabriel Abad or Stéphanie Bastien of the upcoming transfer. Stéphanie will make sure that the files were transferred in the DropBox and if not, you will be informed. NEVER delete the transferred images. If you have any problem with the iPod, please contact Stéphanie.

The biometric measurements of the photos will be performed at the SCC.

Taking the hand pictures:

- Open the application and enter the Participant ID (Biospecimen ID: 99999), the Mother's ID (study ID: 99-999-ABC) and place the iPod Touch into the structure.
- You can start with the mother or with the child, depending on the situation; usually, it is easier to start with the mother.
- Place the structure on a table and position yourself face to face with the mother.
- Remove rings, if the mother is wearing any.

- Put the mother's right hand on the structure, with the palm facing down, within the ruled area, see **figure K** below. For the mother's hand, leave the thumb outside of the ruled area (note that for child's pictures, the thumb must be included, refer to **figure M**).
- Take the picture following the steps of the application, see Appendix 14.
- Repeat for the left hand.
- Ask the mother to place the child for each picture and to press on the hand to ensure the flateness of the child's hand, refer to **figure L**. For the child's hand, keep the thumb within the ruled area, see **figure M**.
- Remember that an image can be repeated if required.

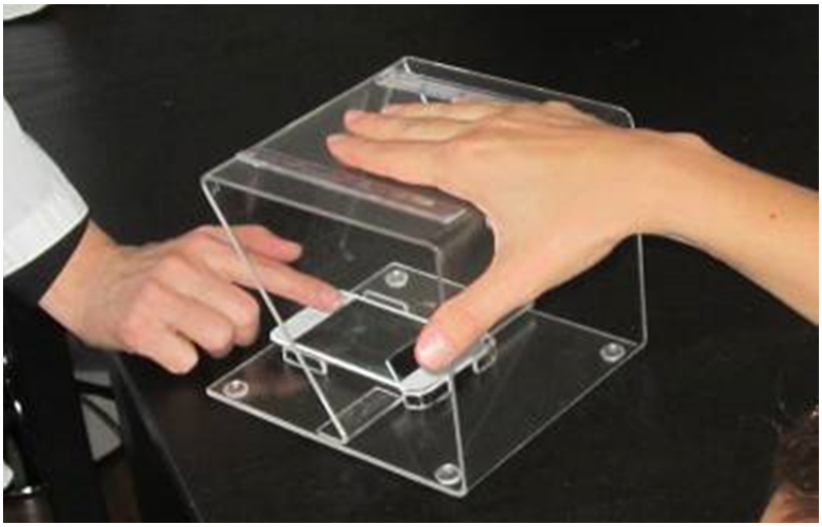


Figure K: *Positioning mother's hand*

Figure L: Positioning child's hand.

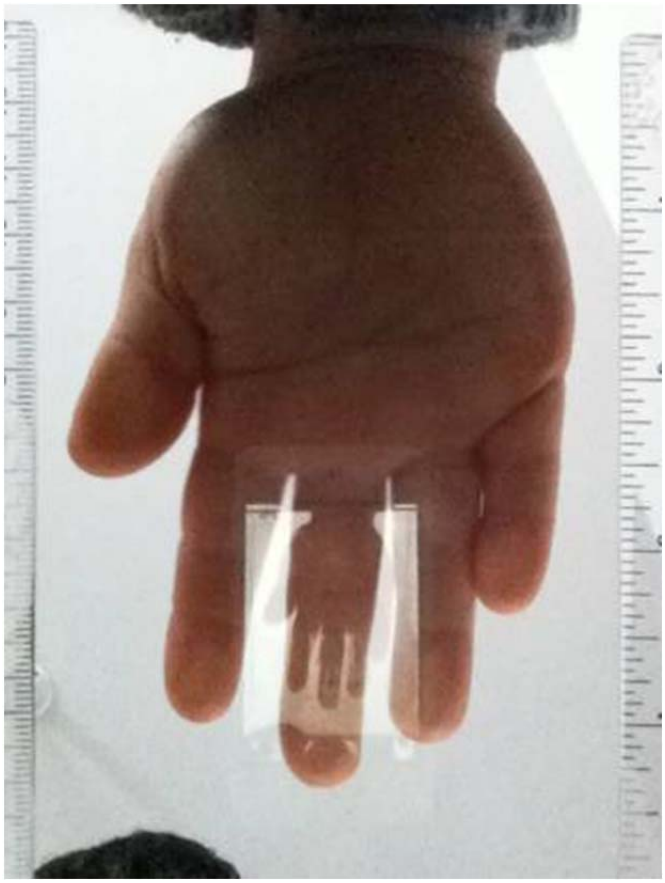
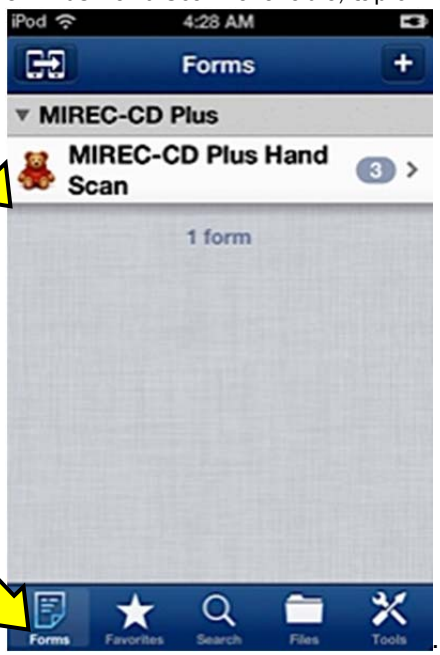


Figure M: Child's picture example.

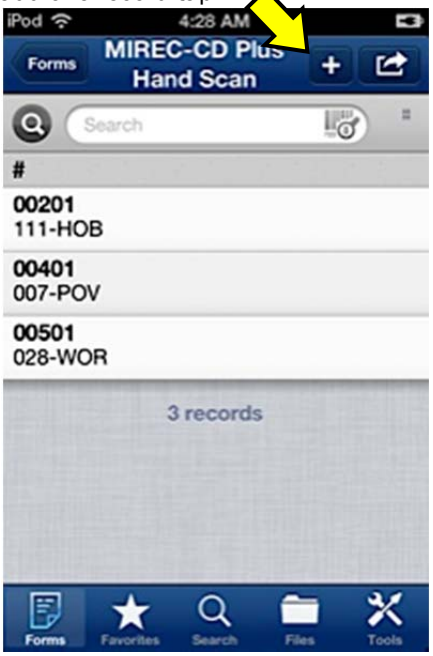
NOTE: there is no impact if the fingers are touching each other. Just make sure that the fingers are not "folded" and that they touch the acrylic surface."

Using the Hand Scan application:
1. Find “Tap Forms” icon:

2. On the Forms tab, you have the form “MIREC-CD Plus Hand Scan” available, tap on it.

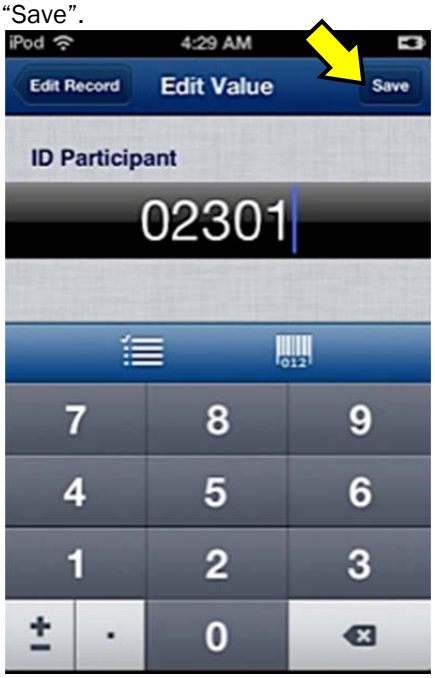


3. You see the list of records that you have. To add one record tap “+”.



4. You have seven fields for each record. The date is already filled. Tap “ID Participant”.

5. Enter the 5 digits “ID Participant” (this corresponds to Biospecimen Identifier) and tap “Save”.



6. Tap on the pick list icon of the next field to enter choose “Mother’s ID” (this is the study ID minus the site number; e.g 999-ABC). You could tap over the title of the field and enter the ID with keyboard, but the pick list is ready to help you.

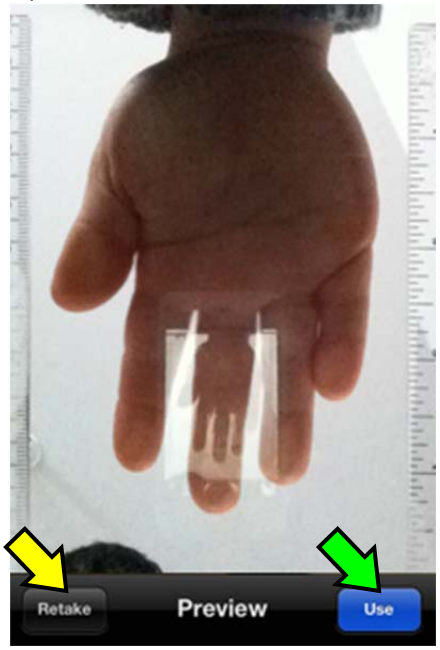
7. Choose the Mother’s ID and Tap “Done”

8. For the first picture tap “Mother’s right hand”

9. Choose “Camera” like image source.

10. This iPod has two cameras, be sure you are using the front camera, otherwise switch it. When the hand is well placed, take the picture.

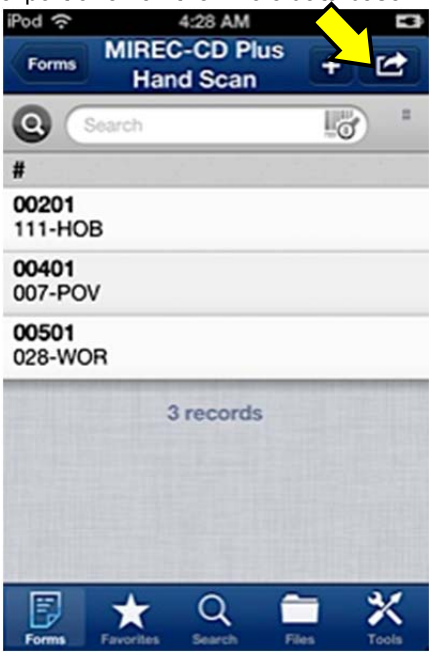
11. Confirm the picture by tapping “Use” or repeat with “Retake”.



12. Take the four pictures of the mother and the infant. With the form completed, you can block “Edit the fields”. The data is saved.

To export your data, be sure you have an Internet connection:

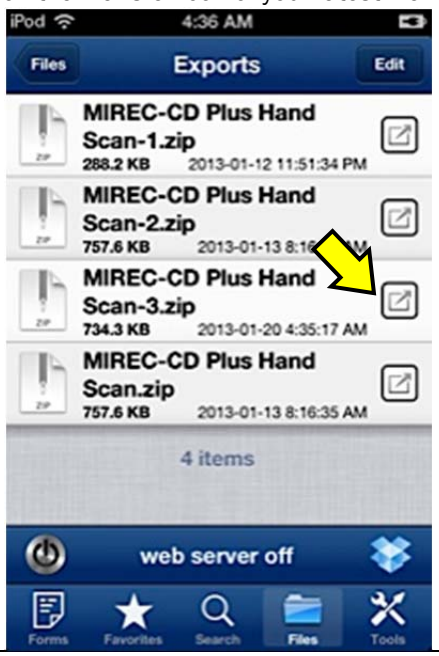
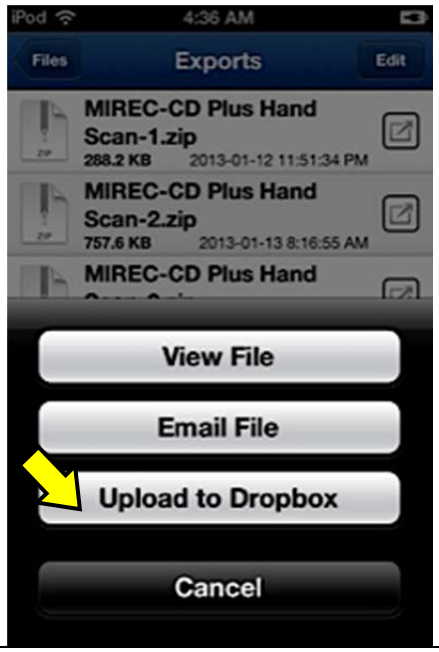
1. In the participants list, you will find the icon to export or email the whole data base.



Note: In the view of a particular patient, the icon for exporting a single record is present. But, we do not use it.

2. Tap on "Export Records" to generate a ZIP file of date base including the images. The "Delete All Records" option is too visible, avoid touching it, if you do by mistake, you can cancel the operation.

3. You will have a confirmation message, tap OK. The zip file is on the Exports folder of the Files tab ready to be sent, tap the File tab icon.

4. The application keeps all the export files. Tap on the Transfer icon of your latest file. 	5. Choose “Upload to Dropbox” to transfer your data. 

APPENDIX 5 . SAMPLE-SIZE METHOD FOR NONBINARY COVARIATES

In a univariate model, without making assumptions about the distributions of covariate X_1 and survival time T, the total number of deaths required is given by the following formula, derived in Appendix A:

$$D = (Z_{1-\alpha} + Z_{1-\beta})^2 [\sigma^2 (\log\Delta)^2]^{-1} \quad (2)$$

where σ^2 is the variance of X_1 and $\log\Delta = \theta^*$ is the log hazards ratio associated with a one-unit change in X_1. Formula (2) is similar to formula (1) except that the variance of X_1, $P(1 - P)$ in formula (1) is now replaced by a more general term, σ^2. The required sample size is then equal to the number of deaths divided by the overall proportion of death. In practice, investigators may have a good idea of the overall death rate. In clinical trials with specific numbers of years of patient recruitment and follow-up, the overall death rate can also be approximately calculated [2, 5].

In deriving formula (2), we assumed that either (1) X_1 is the only covariate and PH holds or (2) there are additional covariates, PH holds for the full model with all covariates, and X_1 is independent of other covariates. These assumptions are likely to be justified in an experiment where X_1 has been randomized and the other covariates are introduced specifically to improve the fit of the data to the PH assumption. In a later section, we relax the independence assumption.

VARIANCE INFLATION FACTOR

In a regression model, the variance of the estimate b_1 of the parameter θ_1 is inversely related to the variance of the corresponding covariate X_1. For example, if we increase the scale of X_1 by a factor of 10, the variance of X_1 will increase by 100 and the variance of b_1 will decrease by 100. If covariates explain some of the variance of X_1, the same effect results. Let R be the multiple correlation coefficient $\rho_{1.23\ldots k}$ relating X_1 with $X_2, \ldots, X_k$. Then R^2 is the proportion of variance explained by the regression of X_1 on $X_2, \ldots, X_k$. In a multiple regression model with covariates $X_1, X_2, \ldots, X_k$, the conditional variance of $X_1 \mid X_2, \ldots, X_k$ is smaller than the marginal variance of X_1 by a factor of $1 - R^2$. Therefore the variance of b_1 estimated from the multiple regression model will increase by a factor of $1/(1 - R^2)$. In multiple linear regression the variance inflation factor can also be shown directly from the ratio

$$\mathrm{Var}_k(b_1)/\mathrm{Var}_1(b_1^*) = 1/(1 - R^2)$$

where $\mathrm{Var}_k(b_1)$ and $\mathrm{Var}_1(b_1^*)$ are the variances of the parameter estimate b_1 and b_1^* obtained from multiple regression models with k and 1 covariates, respectively [8]. In the PH context, to preserve the power we propose that the required number of deaths be calculated as if there were only one predictor and then inflated by the same proportion that the variance of the estimate of the effect of the predictor has been inflated by the adjustment for the other covariates. That is, $D = D_1/(1 - R^2)$, where we define $1/(1 - R^2)$ as the VIF and D_1 is the required number of deaths calculated from formula (2). In Appendix B, we present simulations that allow θ_2 to vary from small to large and to be statistically significantly different from 0, and demonstrate that this only increases the variation of the approximation.

CURRICULUM VITAE

ACADEMIC BACKGROUND

09/2008 – 01/2015 PhD in Public Health/ Epidemiology, Université de Montréal

07/2010 – 06/2013 Fellowship Reproductive Endocrinology and Infertility, U. de Montréal

03/2005 – 11/2006 M.Sc. Epidemiology, Universidade Federal de Pelotas, Brazil

07/2002 – 01/2007 Obstetrics and Gynecology, Universidad Pontificia Bolivariana, Colombia

07/1992 – 05/1999 Medicine, Universidad Pontificia Bolivariana, Colombia

DISTINCTIONS/AWARDS

2014 CIHR-IHDCYH. Travel Award to attend the ASRM annual meeting and present my work on Perfluorinated Chemicals and Time to Pregnancy (oral presentation)

2014 CIHR-IHDCYH. Selected to participate at the CIHR-CFAS New Investigator Workshop. September 11, Quebec

2013 Quebec Training Network in Perinatal Research (QTNPR) Research grant: Finger Digit Ratio: A potential endocrine-sensitive endpoint in women and their offspring

2010 CIHR-IHDCYH Distinction: "Rising Star in Reproductive Health Research in the Postdoctoral Fellow"

2010 CIHR Fellowship for Health Professionals

2008 STIRRHS-CIHR Doctoral Research Award

2007 Society for Pediatric and Perinatal Epidemiologic Research (SPER). Heinz Berendes International Travel Award. Paper: "Birthweight and gestational age across generations: the 1982 Pelotas Birth Cohort Study"

2006 IHDCYH & NICHD Second Annual Summer Institute in Reproductive and Perinatal Epidemiology

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Monnier P, **Vélez MP,** Forges T. IVF and Perinatal Outcomes. In: Pregnancy disorders and perinatal outcomes, Julie Lafond & Cathy Vaillancourt (Eds), Bentham Science Publishers. 2012