MATERNAL FLUORIDE EXPOSURE AND OFFSPRING IQ: AN INVESTIGATION OF THE POTENTIAL MEDIATING ROLE OF THYROID DYSFUNCTION IN PREGNANCY

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I. Abstract.

Objective: Fluoride exposure has been associated with thyroid dysfunction; however, no studies to date have examined whether fluoride disrupts thyroid function in pregnant women. We evaluated the potential thyroid-disrupting effects of fluoride exposure in pregnancy and tested whether thyroid disruption in pregnancy would mediate the association between maternal fluoride exposure and child IQ in Canadian mother-child dyads.

Methods: Maternal thyroid dysfunction was estimated using both categorical measures of thyroid health status (i.e., euthyroid, subclinical, and primary hypothyroid) and continuous measures of thyroid hormone levels (i.e., TSH, FT4, and TT4).

Results: We observed a statistically significant association between water fluoride concentration and greater risk of primary hypothyroidism, and between primary hypothyroidism in pregnancy and lower IQ among male offspring. Further, higher urinary fluoride concentration was associated with higher TSH among women pregnant with female, but not male fetuses. Maternal thyroid hormone levels were not associated with offspring IQ.

Conclusion: Results suggest that maternal thyroid dysfunction in pregnancy may be one mechanism underlying the association between fluoride exposure in pregnancy and offspring IQ.

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IV. List of Abbreviations.

BMI: Body mass index

CH: Congenital Hypothyroidism

CHMS: Canadian Health Measures Survey

CNS: Central nervous system

CWF: Community Water Fluoridation

D2: Type 2 activating deiodinase enzyme

D3: Type 3 deactivating deiodinase enzyme

EPA: U.S. Environmental Protection Agency

FSIQ: Full-Scale Intelligence Quotient

HPT: Hypothalamic-Pituitary-Thyroid

INSPQ: Institut National de Santé Publique du Québec

IQ: Intelligence Quotient

IUCPQ: Institut Universitaire de Cardiologie et de Pneumologie de Québec

MAC: Maximum allowable concentration

MIREC: Maternal-Infant Research on Environmental Chemicals

MUF: Maternal urinary fluoride

NIS: Sodium-iodine symporter

NRC: National Research Council

NTP: National Toxicology Program

Tg: Thyroglobulin

TH: Thyroid hormone

TPO: Thyroid peroxidase

TR: Thyroid hormone receptor

TSH: Thyroid stimulating hormone

T3: Triiodothyronine (TT3: Total T3; FT3: Free T3)

T4: Thyroxine (TT4: Total T4; FT4: Free T4)

WHO: World Health Organization

WPPSI: Wechsler Preschool and Primary Scale of Intelligence

V. Manuscript.

1.0 Introduction.

Thyroid hormones in early pregnancy are critical for optimal fetal development. Because the fetal thyroid gland does not become fully functional until mid-gestation, the fetus is exclusively dependent on maternal supply of thyroid hormones during this period (Andersen et al., 2013; de Escobar et al., 2004; Krassas et al., 2010; Thompson et al., 2018). Insufficient supply of maternal thyroid hormones during pregnancy can contribute to preterm birth and lowered intelligence quotient (IQ) in the offspring (Ghassabian & Trasande, 2018). Fluoride exposure has been shown to disrupt normal thyroid function, including the production of thyroid hormones (Basha et al., 2011; Khandare et al., 2018; Kheradpisheh et al., 2018). However, to our knowledge, no studies to date have examined whether fluoride disrupts thyroid function in pregnant women. The proposed study will evaluate the potential thyroid-disrupting effects of fluoride exposure in pregnancy and test whether alterations to thyroid hormones in pregnancy may mediate the association between maternal fluoride exposure and child IQ. This research will contribute to the literature on neurotoxic mechanisms of fluoride exposure to help guide policy decisions on the safety of fluoride in pregnancy.

2.0 Literature review.

2.1 Susceptibility of the developing brain to neurotoxicants. Toxicant exposure to the developing brain is a concern because the brain is susceptible to injury and permanent damage during this critical period (Choi et al., 2012; Grandjean & Landrigan, 2014; Lanphear, 2015). This heightened vulnerability can be attributed to a number of factors. First, the developing blood-brain barrier is more permeable to toxicants than the mature brain (Rodier, 1995) and many neurotoxicants, including fluoride, are known to cross the placenta. Second, compared to

other organs, the brain develops over a longer period of time and growing cells are known to be more vulnerable to the effects of toxicants (Rice & Barone, 2000). Additionally, there are many different types of neurons that make up the brain, each having a distinct growth pattern and potentially different sensitivities to toxicants (Rodier, 1995). Further, due to differences in metabolism, mouthing behaviours, dietary intake, and respiratory rates, young children often experience greater exposure to toxicants overall (Lanphear, 2015).

Exposures to environmental toxicants, such as lead and mercury, at different developmental periods, including in utero, infancy, and early childhood, have been shown to disrupt neuronal migration and proliferation (Rice & Barone, 2000), neurotransmission, synaptogenesis, and synaptic trimming (Rodier, 1995), and epigenetic mechanisms such as DNA methylation (Perera & Herbstman, 2011). Importantly, there is growing evidence that these disturbances to the brain during development may contribute to increased risk of adverse neurodevelopmental outcomes, including learning disability, attention deficit hyperactivity disorder (ADHD), as well as deficits in memory, language, and motor skills (Jacobson & Jacobson, 1996; Mason et al., 2014; Sagiv et al., 2012). The economic and social toll of these neurodevelopmental impacts on individuals, families, and society is significant (Pelham et al., 2007). Thus, preventing prenatal and early-life exposure to environmental neurotoxicants is critical for ensuring optimal brain development and for protecting against learning and behavioural problems in children.

2.2 Sources of fluoride exposure in Canada. Fluoride is a naturally occurring mineral that is released from rocks into soil, water, and air (Aoun et al., 2018). In many parts of the world, fluoride is also added to drinking water and most dental hygiene products for the purpose of preventing tooth decay. The current optimal level of fluoride in drinking water is 0.7 mg/L in

many Western countries, including Canada and the United States (Heller et al., 1997; U.S. Department of Health and Human Services Federal Panel on Community Water Fluoridation, 2015). The recommended upper limit of fluoride in drinking water is 1.5 mg/L as set by the World Health Organization (WHO; 2004) and recognized as the maximum allowable concentration (MAC) in Canada; in some parts of the world where naturally occurring fluoride is found, this upper limit is surpassed.

Fluoridated water contributes the largest source of fluoride exposure in adolescents and adults, accounting for 40 to 70% of an individual's dietary fluoride intake and is even higher when considering fluoride from food and beverages made with fluoridated water (U.S. Environmental Protection Agency (EPA), 2010). Community water fluoridation (CWF), a process in which fluoride is added to community drinking water, was introduced in the mid-1940s in the United States and Canada as an intervention to reduce risk of dental caries (Wong et al., 2011; Marthaler, 2013). This practice continues to be supported by the WHO and is considered by the U.S. Centers for Disease Control and Prevention to be one of the greatest public health achievements of the 20th century (Pizzo et al., 2007). Studies published from the 1940s through to the 1970s found that CWF was successful in reducing dental caries by about two-thirds in children. Today, dental caries are reduced by about 25 to 30% (Centers for Disease Control and Prevention, 2015). There are several reasons for this reduced impact, including the introduction of topical sources of fluoride (e.g., toothpaste, mouthwash, etc.) starting in the late 1960s, better oral hygiene, and changes to diet (Pizzo et al., 2007). These factors have all contributed to the worldwide reduction in cavity rates over the past few decades.

Other sources of fluoride include food, such as some seafood and processed food, and beverages, such as green and black tea (Kempson, 2015). Tea plants, which absorb fluoride from

soil, can have very high levels of fluoride if grown in certain parts of the world where natural fluoride levels are high (Krishnankutty et al., 2021). Fluoride can also be found in juice, pop, and any other beverage that is made with fluoridated water (Rodríguez et al., 2018). As mentioned, fluoride is also found in most dental hygiene products. These topical sources of fluoride include fluoridated toothpastes, mouthwashes, and professionally applied or prescribed gels, foams, or varnishes (Ullah et al., 2019; Wong et al., 2011). Fluoride is often more highly concentrated in these products than it is in fluoridated water or food and drink products and is thus an important source of exposure in young children as they are more likely to inadvertently ingest these products, resulting in systemic fluoride absorption (Wong et al., 2011).

Importantly, the maximum acceptable daily fluoride intake level (i.e., dose) can be surpassed in some individuals who have high exposure for their size or body weight. The current EPA tolerable upper intake level for fluoride is 0.1 mg/day from birth to 6 months, 0.5 mg/day for 7 to 12 months, 0.7 to 1 mg/day for ages 1 to 8 years, and 3 mg/day for ages 14 and above (U.S. EPA, 2010). Despite different maximum intake levels by age, the optimal concentration of fluoride in drinking water remains consistent at 0.7 mg/L, regardless of weight and water consumption habits. The optimal concentration is set to a level that maximizes the protective role of fluoride while minimizing the risk of dental fluorosis (Heller et al., 1997).

Dental fluorosis is a dose-related mottling of enamel that can range from white opacity in mild cases to brown discoloration in more severe cases. This developmental defect results from over-exposure to fluoride during the first 8 years of tooth development (National Research Council (NRC), 2006), resulting in hypomineralization of the enamel prior to the eruption of permanent (adult) teeth (Wong et al., 2011). According to a national survey conducted between 1999-2004 by the Centers for Disease Control, 41% of American youth were reported to have

enamel fluorosis, ranging from very mild to more severe forms, an increase of over 400% from the rates found at the time community water fluoridation was first introduced. In 2012, the prevalence of dental fluorosis was 65% among American adolescents aged 12 to 15 years (age range displaying fluorosis most clearly; Wiener et al., 2018) and has been increasing since at least 1986 (Neurath et al., 2019). Reasons for the observed increase in dental fluorosis are not well understood. Potential explanations for the rise in fluorosis – at least among U.S. youth – include ingestion of fluoridated toothpaste, especially among young children, and increases in fluoride exposure from ready-to-drink (bottled) tea or iced-tea, mechanically deboned meats, and fluoride pesticide residues on foods (reviewed in Neurath et al., 2019). The implication for the rising prevalence of fluorosis is that it suggests overexposure to fluoride during early development; this is of concern given the growing body of evidence associating early-life exposure to fluoride with developmental neurotoxicity (National Toxicology Program (NTP), 2020).

2.3 Fluoride metabolism and beneficial properties.

After fluoride is absorbed in the stomach and small intestine, it is taken up by calcified tissues in the body, with nearly 99% of total body fluoride being stored in bone and teeth (Aoun et al., 2018; NRC, 2006). Because of fluoride's anticariogenic and antimicrobial properties, fluoride is commonly delivered topically through toothpaste or professional dental treatments to reduce risk of dental caries (Ullah et al., 2019). Systemic fluoride exposure, such as drinking fluoridated water, can also be said to deliver topical fluoride via saliva. The beneficial effects of fluoride predominantly occur at the tooth surface, after teeth have erupted (Limeback, 1999; Berg et al. 2011). Because fluoride is not essential for growth and development (Scientific Committee on Health and Environmental Risks, 2011), there is no recommended intake level of

fluoride during fetal development or in the first six months of life before teeth have erupted. Accordingly, the Canadian Pediatric Society recommends administering supplemental fluoride (i.e., fluoride tablets) only when primary teeth begin to erupt at approximately 6 months and only if the child is susceptible to high caries activity and is not exposed to other fluoride-based interventions, such as toothbrushing or fluoridated water (Godel et al., 2002). Likewise, use of fluoridated toothpaste for children from birth to 3 years of age is determined by the level of risk of tooth decay. If such a risk exists, the Canadian Dental Association recommends that children's teeth be brushed by an adult using a minimal amount (a portion the size of a grain of rice) of fluoridated toothpaste (Canadian Dental Association, 2022). However, if a child between birth and 3 years of age is not considered to be at risk, it is recommended that teeth be brushed by an adult using a toothbrush and water. After age 3, a small amount of fluoridated toothpaste (a portion the size of a green pea) is recommended.

In bone, fluoride is readily incorporated into the crystalline structure and accumulates over time. Further, at high levels, fluoride increases osteoblast activity and bone density. While this may suggest potential benefits of fluoride for bone growth, other studies suggest that fluoride may reduce bone strength. In the late 1990s, some researchers thought fluoridated water was protective against bone fracture whereas others did not find evidence of reducing risk of fracture (Meunier et al., 1998). A recent epidemiologic study by Helte and colleagues (2021) observed increased incidence of hip fractures among postmenopausal women with high consumption of drinking water at fluoride concentrations ranging from 0 to 1 mg/L (i.e., below the maximum level of 1.5 mg/L as recommended by the WHO). The increased risk of fractures may be attributed to reduced elasticity and bone strength (Fratzl et al., 1994).

2.4 Fluoride and neurocognitive outcomes.

2.4.1 Animal studies. Experimental studies show that fluoride induces neurological and behavioural changes. Specifically, fluoride accumulates and induces neurochemical changes and neurodegeneration in various regions of the hippocampus, amygdala, and cerebellum (Bhatnagar et al., 2002; Ghosh & Ghosh, 2020; Pereira et al., 2009). Accordingly, fluoride has been associated with deficits in learning and memory in mice and rats treated with varying doses of fluoride during gestation, shortly after weaning, and in adulthood (Chioca et al., 2008; Dong et al., 2015; Gao et al., 2009; Jiang et al., 2014; Liu et al., 2010; Liu et al., 2014; Wang et al., 2004). When reviewing the animal literature, fluoride concentrations often far exceed what humans would be exposed to; this is because rodents require almost five times the amount of exposure to reach the same blood-fluoride concentrations as humans (NRC, 2006). Jiang and colleagues (2014) found that Wistar rats exposed to 45.3 mg/L fluoride in drinking water during the prenatal period displayed significantly longer latency to complete the classical maze test when compared to controls. Male Sprague-Dawley rats, whose mothers were exposed to 0.13 mg/kg/day sodium fluoride during gestation, displayed disrupted behaviour, including hyperactivity, at 9 weeks of age (Mullenix et al., 1995). More broadly, a systematic review conducted by the NTP (2016) found a low-to-moderate level of evidence supportive of adverse effects on learning and memory in fluoride-exposed animals. Contrary to the human studies included in this review, the level of evidence was strongest in animals exposed as adults and weaker in animals exposed during development; however, concerns were raised about fluoride-induced indirect effects (e.g., degree to which fluoride-induced deficits in motor activity might compromise neurobehavioural assessments of learning and memory) and risk of bias (e.g., lack of control for potential litter effects, lack of randomization, lack of blinding, etc.). More recent experimental studies have been conducted to address these concerns (for a review, see NTP,

2020). While evidence from experimental studies supports an association between fluoride exposure and neurodevelopmental effects, the data are not sufficient to support this association in humans. This body of evidence has been helpful, however, in examining potential mechanisms underlying fluoride neurotoxicity.

2.4.2 Human neurodevelopmental studies from fluoride endemic areas. Most epidemiologic studies that have examined the effects of fluoride on cognition in children have been conducted in fluoride endemic regions of China, India, and Iran where there are naturally high levels of fluoride in drinking water (i.e., >1.5 mg/L). In these studies, exposure to high levels of fluoride in drinking water was associated with diminished Intelligence Quotient (IQ; Li et al., 1995; Lu et al., 2000; Xiang et al., 2003; Zhao et al., 1996). Additionally, a meta-analysis of 27 studies concluded that children living in areas with high fluoride exposure had significantly lower IQ scores when compared to those living in low-fluoride areas (Choi et al., 2012); standardized weighted mean difference in IQ score was -0.45, which corresponds to about 6.75 IQ points per 1 mg/L increase in water fluoride concentration. These results were supported by a more recent meta-analysis, concluding that exposure to high levels of fluoride in drinking water was significantly associated with reduced intelligence in children (Duan et al., 2018).

The presence of dental fluorosis has also been associated with neurocognitive deficits in children and adults, potentially resulting from overexposure to fluoride. More specifically, studies from China have found presence of moderate to severe dental fluorosis in children to be associated with higher water fluoride concentrations, as well as poorer attention and working memory, and lower IQ (Choi et al., 2015; Zhao et al., 1996). Accordingly, there is growing global concern regarding fluoride exposure and the potential risk of toxicity to the body and brain.

2.4.3 Human neurodevelopmental studies from fluoride non-endemic areas. Over the past decade, a growing number of studies have investigated neurodevelopmental effects of low fluoride exposure (i.e., <1.5 mg/L). One ecologic study conducted using data from the United States, where water fluoride levels were between 0.7 to 1.2 mg/L, found an association between water fluoridation and increased prevalence of ADHD (Malin & Till, 2015). Yet, another semi-ecologic study conducted in New Zealand (Broadbent et al., 2015) compared IQ scores of youth and adults living in areas with and without CWF, and reported that there was no effect of fluoride exposure on IQ; however, the difference in exposure between those living in the fluoridated area vs. the non-fluoridated area may have been too small to detect a difference in IQ, given that a large number of individuals living in the non-fluoridated area were taking fluoride supplements (i.e., 0.5 mg/day; Hirzy et al., 2017). Overall, ecologic studies have shown mixed results and have been criticized for their lack of individual exposure measures and failure to control for important confounders.

2.4.4 Human neurodevelopmental studies with measures of individual exposure. More recent studies have examined the effects of fluoride exposure in pregnancy on neuropsychological outcomes in children. In the past five years, there have been four prospective birth cohorts conducted in populations living in areas with optimal fluoridation: two from Mexico (Bashash et al., 2017; Cantoral et al., 2021), one from Canada (Green et al., 2019), and one from Spain (Ibarluzea et al., 2022). The first birth cohort study (Bashash et al., 2017) was conducted in Mexico City where fluoride is added to salt. Results showed that a 1 mg/L increase in maternal urinary fluoride was associated with a 5 to 6-point lower IQ score in boys and girls at ages 4 (*n*=211) and 6 to 12 years *(n*=287; Bashash et al., 2017). Another prospective study conducted in a different birth cohort from Mexico City (*n*=103; Cantoral et al., 2021) found that

a 0.5 mg/day higher dietary fluoride intake in pregnancy was associated with a 3.5-point lower cognitive score on the Bayley Scales of Infant Development in boys, but not girls. Among the two prospective studies conducted in areas with community water fluoridation, the results were inconsistent. The Canadian study of 512 children at ages 3 to 4 years found that exposure to higher levels of fluoride during gestation was associated with lower IQ scores (Green et al., 2019). Specifically, an increase of 1 mg/L in maternal urinary fluoride concentration was associated with a 4.5-point lower IQ score in boys, and a 1 mg/L increase in mothers' daily fluoride intake was associated with a 3.7 lower IQ score among both boys and girls (Green et al., 2019). In contrast, a study conducted in Spain (*n*=316; Ibarluzea et al., 2022) found that higher maternal urinary fluoride during pregnancy was associated with higher cognitive scores for boys; yet, the positive association between maternal urinary fluoride and child IQ was only statistically significant among mother-child dyads living in *non*-fluoridated communities and the association was attenuated (i.e., smaller) when adjusting for other neurotoxicants.

Overall, these findings are cause for concern because three of the four prospective birth cohort studies suggest that fluoride exposure in pregnant women living in areas with optimal fluoridation may have adverse consequences for offspring neurodevelopment. Accordingly, the safety of fluoridation remains a controversial topic, especially for the developing fetus for whom there is no direct benefit of fluoride (Limeback, 1999).

To date, little is known about the potential mechanisms that may explain fluoride's adverse effects on neurocognitive development; however, some potential hypotheses have been proposed. For one, fluoride may reduce brain-lipid or phospholipid content, and the enzymes that metabolize them, or inhibit cholinesterase activity and reduce acetylcholine (NRC, 2006). Another potential mechanism relates to fluoride's effects on immune function, which has been

explored in some experimental studies (Guo et al., 2017; NRC, 2006). Neurotoxicity of fluoride has also been associated with disruption of the thyroid gland, a mechanism that underlies various other endocrine disrupting chemicals (Grandjean, 2019; NRC, 2006). In fact, sodium fluoride was used as a medical treatment for hyperthyroidism up until the 1950s, at which time medications were developed to reduce thyroid gland overactivity (Galletti & Joyet, 1958). The current thesis will focus on examining thyroid toxicity as a potential mechanism of fluoride neurotoxicity.

2.5 Thyroid hormone synthesis and transport. Synthesis of thyroid hormones (THs) occurs inside thyroid follicular cells via the iodination of thyroglobulin (Tg) tyrosine residues (Prezioso et al., 2018). THs are regulated by thyroid stimulating hormone (TSH), which is secreted by the pituitary gland. As such, when TSH is secreted, Tg is broken down by lysosomal enzymes into triiodothyronine (T3), the metabolically active hormone, and thyroxine (T4), a pro-hormone (Prezioso et al., 2018). Following production, T3 and T4 are released into the bloodstream primarily bound to thyroid-binding globulins (referred to as total circulating T3 and T4; TT3 and TT4), preventing them from entering target cells (Williams, 2008). A smaller proportion of T3 and T4 exist in unbound forms (referred to as free T3 and T4; FT3 and FT4), however, allowing them to travel into various target tissues throughout the body (Williams, 2008). At target cells, T3 and T4 are said to cross the membrane through specific membrane TH transporters with equal efficiency (i.e., they do not compete for uptake; Williams, 2008). T4 is activated or converted to T3 both intra- and extracellularly by the action of activating iodothyronine deiodinase enzymes (Préau et al.,2016), accounting for nearly 80% of all available T3 (Williams, 2008). Accordingly, T4 is generally produced in much higher amounts. Importantly, THs can exert non-genomic and genomic effects by binding to TH receptors (TRs)

on the membranes of various cell types throughout the body and on promotor regions of different target genes (Kumar et al., 2015; Prezioso et al., 2018). Given that T4 is most prevalent in circulation and that it is largely converted to T3, T4 tends to be the most important indicator of an individual's TH levels (Williams, 2008). FT4 is an especially representative measure of TH because it is the most readily available for cellular action (Williams, 2008).

Thyroid hormones play an important role in metabolic regulation, cardiac and skeletal muscle function, and central nervous system (CNS) activity (Senese et al., 2014). Notably, THs play an especially important role in regulating CNS activity during the early developmental period (Prezioso et al., 2018), which is described in more detail in a later section.

2.6 Fluoride exposure and thyroid dysfunction.

2.6.1 Animal studies. Exposure to fluoride has been shown to disrupt thyroid function in experimental studies, though findings are inconsistent. Decreases in FT4 and FT3 have been observed in Wistar rat offspring whose mothers were treated with very high levels of fluoride during gestation (i.e., > 100 ppm; Basha et al., 2011); this was in comparison to controls, whose mothers were treated with low levels of fluoride (i.e., < 1 ppm). Similarly, prolonged exposure to lower (i.e., ≤ 1 mg/kg; Bobek et al., 1976) and higher (i.e., 10 & 20 mg/kg; Dhurvey et al., 2017; 30 & 60 mg/L; Jiang et al., 2016) levels of fluoride have been associated with decreases in TT4 and TT3 in adult Wistar rats. In contrast, increases in TT4 and TT3 levels have been observed in rat offspring exposed to high levels of fluoride in drinking water (i.e., 45 mg/L; Ge et al., 2013). Notably, however, TT4 and TT3 levels were lower among those exposed to high fluoride in tandem with low dietary iodine (i.e., 0.086 mg iodine/kg of body weight; Ge et al., 2013). Other experimental studies have found no significant associations between fluoride exposure and TH levels (Clay & Suttie, 1987; McPherson et al., 2018); these studies were noted to vary in terms of

methodology, and quality and timing of the exposure matrix, and did not report on iodine intake levels.

Iodine is essential for the production of THs and may play a role in determining the magnitude of fluoride's effect on thyroid function (NRC, 2006). For instance, Guan et al. (1988) observed significant decreases in T3 and T4 among adult Wistar rats with sufficient iodine intake who were exposed to fluoride at a concentration of 30 mg/L. Interestingly, these same changes were observed among iodine-deficient rats who were exposed to fluoride at a concentration of only 10 mg/L (Guan et al., 1988). Another study conducted in adult Kunmim mice observed significantly lower levels of T3 among those with deficient (i.e., 0 μg/L) or excess (i.e., 2500 μg/L) iodine intake coupled with low (i.e., 0-0.6 mg/L) fluoride intake when compared to mice with moderate iodine intake (i.e., 20 μg/L); in comparison, iodine deficiency coupled with excessive fluoride intake (i.e., 30 mg/L) was associated with increased T3 (Zhao et al., 1998). Further, lower levels of T4 were observed among mice with deficient iodine intake across all levels of fluoride intake when compared to those with moderate or excess iodine intake (Zhao et al., 1998). Overall, these results suggest that the relationship between fluoride exposure and thyroid function may be modified by iodine intake.

2.6.2 Human studies. In children and adults, higher levels of fluoride in drinking water and elevated urinary fluoride concentration has been associated with higher TSH, lower T3 and T4 levels, and increased thyroid gland volume, all of which are typically associated with hypothyroidism (Du et al., 2021; Khandare et al., 2018; KheradPisheh et al., 2018; Wang et al., 2020). Higher levels of fluoride in drinking water have also been shown to be predictive of greater prevalence of hypothyroidism. Peckham and colleagues (2015) found that areas in England with higher water-fluoride concentrations (i.e., > 0.7 mg/L) were 1.6 times more likely

to have a high prevalence of hypothyroidism when compared to areas with lower water fluoride concentrations (i.e., ≤ 0.3 mg/L). Further, a recent systematic review found a positive correlation between level of fluoride exposure and prevalence of hypothyroidism (Chaitanya et al., 2018). Significant increases in TSH have further been observed in children living in fluorosis-endemic areas (i.e., water fluoride concentration range: 1.23 – 5.8 mg/L) when compared to those living in non-fluorosis-endemic areas (i.e., water fluoride concentration range: 0.58 – 1.08 mg/L; Kumar et al., 2018; Singh et al., 2014; Zhang et al., 2015). Increases in TT4, FT4, TT3, FT3, and TSH have also been observed in children and adults living in regions with endemic fluoride (i.e., water fluoride concentration range: 1.1 – 14.3 mg/L) when compared to individuals living in non-endemic areas (i.e., water fluoride concentration 0.14 – 0.81 mg/L; Susheela et al., 2005; Yasmin et al., 2013). Variability in findings of higher fluoride exposure being associated with lower or higher T4 and T3 levels may be attributed to differences in quality of experimental design and statistical methodology, timing and level of exposure, as well as age at exposure.

Less is known about the effects of exposure to optimal levels of fluoride (i.e., 0.7 mg/L) on THs. An epidemiological study using data from the Canadian Health Measure Survey (CHMS) did not report any significant associations between water fluoride or urinary fluoride concentration and abnormal THs or diagnosis of a thyroid disorder (Barberio et al., 2017). However, another study using the same CHMS sample found that iodine status was an important effect modifier of the relationship between fluoride exposure and thyroid hormones (Malin et al., 2018). Specifically, urinary fluoride concentration was significantly associated with higher TSH among non-pregnant adults with moderate-to-severe iodine deficiency (18% of overall sample); in contrast, this association was not significant among those who were iodine sufficient (Malin et al., 2018). This further emphasizes the importance of considering iodine status when examining

the relationship between fluoride and THs. Other factors that may moderate the relationship between fluoride and thyroid hormones include genetic polymorphisms, such as CREB1 (Xu et al., 2022).

2.7 Potential mechanisms of thyroid disruption. As mentioned, iodine is essential for thyroid function (Murcia et al., 2018; Thompson et al., 2018), and has been suggested to modify the effect of fluoride on thyroid function. This potential interaction may result from fluoride inhibiting the expression and activity of sodium-iodide symporters (NISs) that are necessary for mediating active iodide transport into the thyroid and other tissues, including the gastrointestinal mucosa (Greer et al., 2002; Waugh, 2019). Inhibition of NISs has been associated with impaired iodine absorption into the thyroid and iodine deficiency disorders (Greer et al., 2002; Waugh, 2019). Importantly, however, a recent experimental study (Buckalew et al., 2020) refuted this claim by showing that fluoride does not inhibit NIS activity in Fischer rat thyroid follicular cells (i.e., FRTL-5). Thus, it is unclear whether and how fluoride and iodine may interact to disrupt thyroid function. Further research is needed to understand the mechanism by which fluoride may result in thyroid disruption.

2.8 Sources of thyroid hormones in gestation. Thyroid hormones are critical for optimal growth and neurodevelopment, especially during gestation (Andersen et al., 2013; de Escobar et al., 2004; Zoeller et al., 2002). Because the fetal thyroid is not fully functionally mature until mid-gestation, the fetus is exclusively reliant on maternal THs during the first several weeks of gestation (de Escobar et al., 2004). At this point, minimum requirements for TH signaling are already in place in the developing fetus (Prezioso et al., 2018; Zoeller et al., 2002). Specifically, maternal THs can be detected in amniotic fluid as early as 8 weeks of gestation (Contempré et al., 1993); TRs are expressed in fetal brain tissues and TR occupancy by TH is in the range

known to induce physiological effects by 9 weeks of gestation (Ferreiro et al., 1988); lastly, the mRNAs that encode the two known classes of TRs (i.e., TR α and TR β) display complex temporal patterns of expression during early gestation and are also expressed in the human oocyte (Iskaros et al., 2000; Zhang et al. 1997). Thus, TH action and signaling begins very early in fetal development, prior to the maturation of the fetal thyroid (Zoeller et al. 2002).

The onset of fetal thyroid function begins at 10-12 weeks of gestation; however, the fetus does not produce sufficient levels of TH until 18-20 weeks of gestation, and thus continues to be reliant on maternal THs during this period (Bernal, 2005; Zoeller et al., 2002). In fact, even after the fetal thyroid is fully functionally mature, maternal THs continue to cross the placenta, with up to 50% of fetal blood-T4 at birth being of maternal origin (de Escobar et al., 2000; Vulsma et al., 1989). Therefore, maternal THs play a critical role throughout gestation and serve to protect the fetal brain until birth, even among offspring who have congenital hypothyroidism (CH; i.e., severe TH deficiency in neonates at birth) or other thyroid-related disorders (Bernal, 2005). While disruption to the fetal thyroid has been associated with adverse neurodevelopmental outcomes in infancy and early childhood (Cherella & Wassner, 2017; Rastogi & LaFranchi, 2010), studies have shown that long-term consequences of CH are often minor with early detection and intervention (Hanukoglu et al., 2001; Rovet, 2000). In contrast, maternal TH deficiency, or hypothyroidism, during gestation has been shown to have potentially devastating effects on fetal brain development and neurocognitive outcomes, that are chronic and often permanent (Haddow et al., 1999; Ghassabian & Trasande, 2018). Considering the critical role of maternal THs in early gestation for normal fetal brain development, this thesis will focus on measuring the potential for fluoride to disrupt maternal THs as a mechanism to account for adverse neurocognitive outcome in offspring.

2.9 Thyroid hormones and fetal brain development. Findings suggesting that adverse effects on offspring neurodevelopment are more severe in cases of maternal hypothyroidism compared to CH also illustrate that the timing of TH action is critical for normal offspring neurodevelopment (Williams, 2008). While THs are not involved in very early developmental events such as neural induction and establishment of polarity, they are essential for regulating later processes like neurogenesis, myelination, dendrite proliferation, and synapse formation (Zoeller & Rovet, 2004), and many TH responsive genes have been identified to be essential for supporting these processes (Bernal et al., 2003).

Three main stages of TH-dependent neurological development have been identified through experimental work with animal models, as outlined in Figure 1 (Williams, 2008, p. 785). The first stage, which occurs before the fetal thyroid can produce sufficient levels of TH (i.e., up to ~ 14 weeks of gestation, as indicated in Figure 1), influences neuronal proliferation and migration in the cerebral cortex, hippocampus, and cerebellum (Ausó et al., 2004; Cuevas et al., 2005; Williams, 2008). In the cerebral cortex, THs play a particularly important role in arranging the six-layer pattern, which is formed by the timely migration of cells originating in the ventricular neuroepithelium (Bernal, 2005). The second stage occurs throughout the remaining period of gestation, once the fetal thyroid is functional, and therefore relies on THs produced by both the mother and fetus (Williams, 2008). In this stage, THs mediate many processes including neurogenesis, neuronal migration, axonal outgrowth, dendritic branching, and synaptogenesis, in addition to the initiation of glial cell differentiation and migration, and the onset of myelination in various brain regions (Bernal et al., 2003; de Escobar et al., 2000; Williams, 2008). The last stage occurs in the neonatal or postnatal period when all THs supplied to the brain are derived from the offspring itself and are critical for continuing maturation and development of the brain

(Williams, 2008). During this stage, THs mediate the migration of granule cells in the dentate gyrus of the hippocampus and cerebellum, pyramidal cells in the cortex and Purkinje cells in the cerebellum become more sensitive to TH action, and TH-dependent gliogenesis and myelination continue (Bernal et al., 2003; de Escobar et al., 2000; Williams, 2008).

Figure 1

Relationship between thyroid hormone action and development of the brain.

Figure 1 has been removed due to copyright restrictions. Please see the figure as published in the original article: "Neurodevelopmental and neuropsychological actions of thyroid hormone*," by* G. R. Williams, 2008, *Journal of Neuroendocrinology, 20*(6), p. 785 (https://doi.org/10.1111/j.1365-2826.2008.01733.x).

Figure 1 (Williams, 2008, p. 785) also illustrates that type 3 deactivating deiodinase enzyme (D3) expression is reduced when the fetal thyroid gland starts to develop at the end of the first trimester (i.e., 10-12 weeks of gestation). D3 inactivates T3, or prevents T4 from being converted to T3, and is initially expressed in fetal tissues and the placenta to prevent access of maternal T3 to the developing fetus (Wasco et al., 2003). Accordingly, TRs remain unoccupied during this period (Apo-TRs), which is essential for maintaining cell proliferation and preventing cell differentiation (Chassande, 2003). This decrease in the expression of D3 correlates with an increase in the expression of type 2 activating deiodinase enzyme (D2; which converts T4 to T3) in T3-target tissues and the maturation of the fetal hypothalamic-pituitary-thyroid axis. At this point, there is a surge in TSH secretion that triggers the onset of fetal TH production. Together, these changes result in the conversion of unoccupied TRs into occupied TRs and the initiation of

cell differentiation in the fetus (Flamant et al., 2002; Williams, 2008). Thus, the TR seems to act as a deiodinase-dependent developmental switch that regulates maturation of T3-dependent tissues in the brain (Williams, 2008).

2.10 Maternal thyroid hormone deficiency and fetal neurodevelopment. It is clear that maternal THs are critical for normal neurodevelopment (Andersen et al., 2013; de Escobar et al., 2004; Krassas et al., 2010; Thompson et al., 2018), and that the developing fetus is dependent on maternal THs throughout gestation (Bernal, 2005; de Escobar et al., 2004). As outlined above, the actions of THs differ during gestation, in that they are responsible for facilitating certain actions or processes during specific stages (Bernal, 2005). Accordingly, maternal TH deficiency in gestation, even of short duration can result in devastating, irreversible neurological deficits; the consequence of which have been shown to depend on both the severity of deficiency and the specific timing of onset and duration of deficiency (de Escobar et al., 2004).

Through experimental manipulation of TH availability during gestation in various animal models, TH deficiency has been shown to have many widespread adverse effects on brain development including reduced progenitor cell expansion; deficits in neuronal migration; delays in neuronal proliferation; decreased expression of neuronal differentiation factors; impaired generation of primitive network activity patterns; reduced cortical thickness; cortical dysplasia; abnormal layering of the cerebral and cerebellar cortices; impairments in dendrite and axon development; decreased expression of proteins involved in synaptic plasticity; as well as delayed myelination and reduced axonal guidance and fasciculation (Prezioso et al., 2018; Thompson & Cline, 2016). In rats specifically, TH deficiency during the first stage of TH-dependent neurodevelopment has been shown to result in disrupted neuronal migration, leading to less defined cortical layers in the cerebral cortex and misplaced cells in the neocortex and

hippocampus; these disturbances were further associated with audiogenic seizures (Berbel et al., 2001). Maternal TH deficiency later in gestation has been shown to result in delayed and poor deposition of myelin in the fetal rat brain due to delays in oligodendrocyte differentiation and myelin gene expression, as well as impairments in axonal maturation, preventing many axons from reaching the size needed to facilitate myelination (Bernal, 2005; Notterpek & Rome, 1994).

Animal studies have associated a lack of maternal TH during gestation with irreversible deficits in offspring brain cytoarchitecture and altered behavioural development (Prezioso et al., 2018). In humans, adverse neuropsychological outcomes associated with maternal TH deficiency in gestation also depend on severity, timing, and duration of deficiency (Zoeller & Rovet, 2004). For instance, if there is TH deficiency early in gestation (i.e., during the first stage of TH-dependent neurological development), offspring may display problems in visual attention and processing, and gross motor skills. If maternal TH deficiency continues later in gestation, offspring are at additional risk of subnormal visual and visuospatial skills, slower processing and response speeds, and fine motor deficits (Zoeller & Rovet, 2004). More broadly, adverse consequences of maternal thyroid dysfunction (i.e., hypothyroidism) in gestation on offspring development have been largely documented and include, but are not limited to, miscarriage, preterm birth, altered brain structure, and increased risk of seizure disorders, autism spectrum disorder, attention deficit hyperactivity disorder, and other neurodevelopmental disorders (Andersen et al., 2013; Ghassabian & Trasande, 2018; Haddow et al., 1999; Henrichs et al., 2010; Julvez et al., 2013; Levie et al., 2018).

2.11 Maternal thyroid hormone deficiency and offspring IQ. Maternal hypothyroidism in gestation has also been associated with lower offspring IQ. More specifically, low maternal FT4 (e.g., < 10 pmol/L or 7.8 pg/mL) and high TSH (e.g., > 10 μIU/mL) in early gestation has

been associated with lower full-scale and verbal IQ scores in children aged 5 to 9 years (Andersen et al., 2018; Haddow et al., 1999). A meta-analysis of individual participant data from three prospective birth cohorts concluded that low maternal FT4 (i.e., < 2.5th percentile) was associated with a 3.9-point lower non-verbal IQ and a 2.1-point lower verbal IQ in children; however, no independent associations were found with TSH (Levie et al., 2018). This is likely a result of the physiology that maternal T4, but not TSH, passes the placental barrier; thus, maternal FT4 is more likely to represent the availability of TH for the fetal brain (Korevaar et al., 2018). Yet, this should not discredit the reliability of TSH as a valid indicator of maternal thyroid function (Korevaar et al., 2018).

Using data from one of the prospective birth cohort studies mentioned above (i.e., Generation R Study from Rotterdam, Netherlands), the association between maternal TH deficiency in gestation and offspring IQ was further evaluated by examining offspring brain morphology later in childhood (Korevaar et al., 2015). Specifically, Korevaar and colleagues (2015) investigated the association between maternal TSH and FT4 measured earlier in gestation (< 18 weeks) with child non-verbal IQ scores (median age at assessment= 6 years) and brain morphology assessed through magnetic resonance imaging scans (median age at assessment= 8 years). Low maternal FT4 concentration was associated with a statistically significant 1.5 to 3.8-point reduction in mean child non-verbal IQ, dependent on the percentile cut-off (< 3rd to < 11th percentile of FT4). No significant association was observed between maternal TSH and child non-verbal IQ (Korevaar et al., 2015). Further, lower maternal FT4 was significantly associated with lower total grey matter volume and cortex volume among offspring; however, there were no statistically significant associations between maternal FT4 and child total brain volume, white matter volume, hippocampal volume, or subcortical grey matter volume (Korevaar et al., 2015).

Unlike FT4, there were no significant associations between maternal TSH and offspring brain morphology (Korevaar et al., 2015). The authors proposed that reductions in grey matter volume associated with lower levels of maternal FT4 in gestation may indicate differences in efficiency of information processing (Korevaar et al., 2015). This aligns with previous findings indicating that grey matter volume is positively associated with processing speed and IQ (Luders et al., 2009).

Findings from Korevaar et al. (2015) showing that maternal FT4 deficiency is associated with reductions in offspring total grey matter and cortex volume are in accordance with animal studies highlighting the importance of maternal THs for normal fetal brain development and showing that maternal TH deficiency in early gestation can result in widespread disruptions to neuronal migration in the developing fetus (Berbel et al., 2001; Prezioso et al., 2018; Thompson & Cline, 2016). Specifically, maternal FT4 levels may not have been associated with child subcortical grey matter volume in this study, given that FT4 was measured primarily in the first trimester of gestation and that many subcortical structures develop later in gestation and throughout early childhood when the offspring's thyroid gland is fully functional (Bernal, 2005; Gilmore et al., 2012). Importantly, Korevaar et al (2015) were unable to determine whether the observed changes in grey matter volume mediated the association between maternal FT4 and offspring IQ due to a small sample size.

Other studies conducted in this area have found that children born to mothers with clinical hypothyroidism had smaller hippocampal volumes (particularly in the right posterior and left anterior regions) and abnormal corpus callosum measurements (i.e., smaller anterior corpus callosum and genu, and larger posterior corpus callosum and splenium) when compared to controls, which were further linked to deficits in cognitive functions that contribute to IQ (i.e.,

memory, executive function, and verbal comprehension; Samadi et al., 2015; Willoughby et al., 2014). It is important to note, however, that women in these studies had hypothyroidism that was left untreated throughout the entire gestational period (Koreevar et al., 2015; Samadi et al., 2015; Willoughby et al., 2014), which likely affected the development of later-evolving brain structures as well. In accordance with the animal literature, these three studies illustrate the time-dependent effects of maternal TH deficiency on offspring neurodevelopment in humans and highlight the need for future research in this area.

3.0 Rationale.

To date, no studies have assessed the association between fluoride exposure and thyroid function during pregnancy in humans. If evidence is found supporting an association between fluoride and maternal thyroid function, this may provide a potential mechanism explaining prior findings showing developmental neurotoxicity of gestational exposure to fluoride (Bashash et al., 2017; Green et al., 2019; Cantoral et al., 2021). Moreover, considering the ubiquity of fluoride exposure and the lack of scientific consensus regarding optimal or safe levels of exposure for vulnerable populations, including pregnant women and the developing fetus, in addition to the long-term health consequences associated with TH deficiency in pregnancy, this is an area of increasing concern that warrants additional research. Accordingly, the current thesis will aim to assess maternal thyroid function as a potential mediator of the association between maternal fluoride exposure and child IQ in pregnant women living in Canada. Considering our prior work found a significant association between maternal urinary fluoride (MUF) and child IQ, we will determine whether changes in maternal TH levels mediate this previously established association (i.e., whether MUF is associated with maternal TH levels and whether maternal TH levels are associated with child IQ). Furthermore, given that sex differences have been identified in

children's sensitivity to neurotoxicant exposures, including differences in neurodevelopmental and thyroid related outcomes (Ballesteros et al., 2017; Kern et al., 2017), and that women's THs have also been shown to differ depending on the sex of the fetus that they are carrying (Sitoris et al., 2022; Wang et al., 2019), the role of fetal sex as an effect moderator will also be assessed in the associations between fluoride exposure, maternal TH levels, and child IQ.

4.0 Aims and Hypotheses.

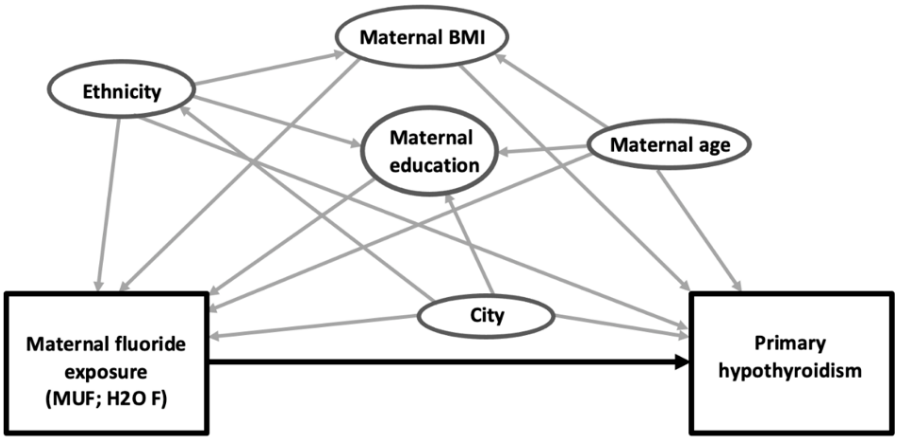
Aim 1. Fluoride exposure, maternal hypothyroid status, and child IQ.

Aim 1a. To determine if exposure to fluoride predicts thyroid health status (i.e., euthyroid versus hypothyroid) in Canadian pregnant women living in areas with or without community water fluoridation. Women will be classified as euthyroid, subclinical hypothyroid, or primary hypothyroid based on their FT4 and TSH levels measured in trimester one. Women classified as primary hypothyroid will be grouped with those who reported a clinical diagnosis of primary hypothyroidism at time of study enrolment. Risk of subclinical and primary hypothyroidism as a function of higher water fluoride and MUF concentration will be assessed while controlling for relevant covariates (Figure 2). Fetal sex-specific effects will not be explored among these associations considering many women with pre-existing diagnoses of hypothyroidism were likely diagnosed prior to pregnancy.

Hypothesis 1a. Higher water fluoride and MUF concentration will predict greater risk of subclinical and primary hypothyroidism among this group of women (Chaitanya et al., 2018; Peckham et al., 2015).

Figure 2

Directed acyclic graph depicting the model of interest for Aim 1a.



Note. This DAG depicts primary hypothyroidism as the outcome variable; the same DAG was used to conceptualize the model with subclinical hypothyroidism as the outcome variable.

Aim 1b. To assess whether subclinical or primary hypothyroidism in pregnancy is associated with offspring IQ while controlling for relevant covariates (Figure 3). Child sex-specific effects will also be explored.

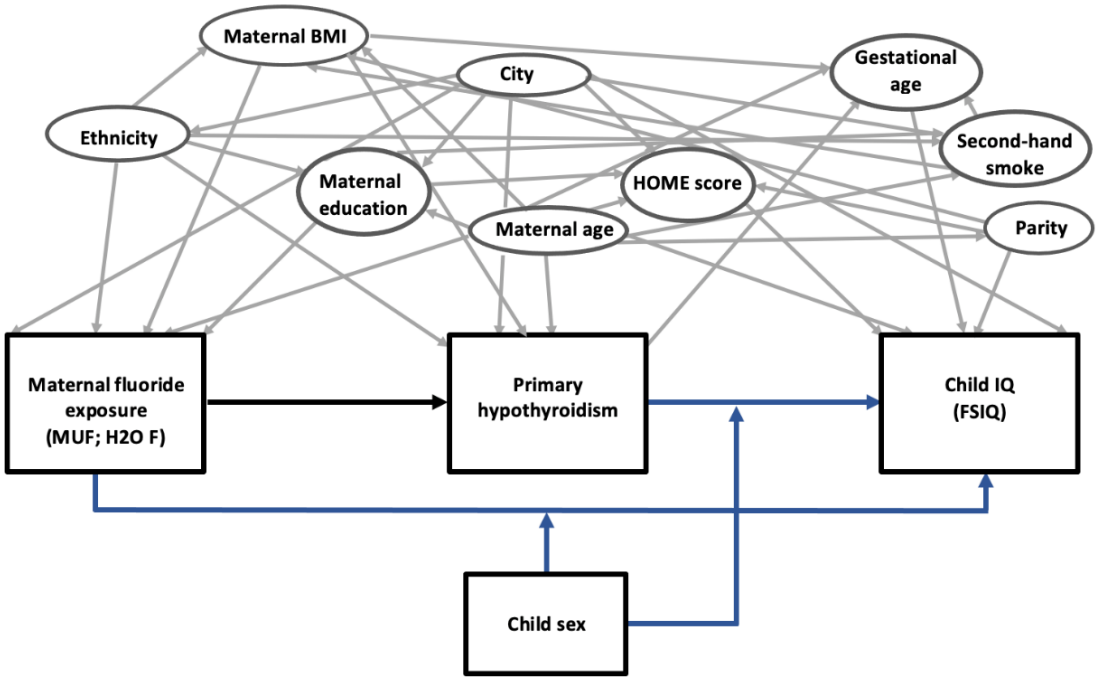
Hypothesis 1b. Primary hypothyroidism in pregnancy will be associated with lower offspring IQ (Levie et al., 2018). No specific hypothesis was made regarding subclinical hypothyroidism given mixed findings in the literature. Similarly, no prediction was made in the context of sex-specific effects given the exploratory nature of this sub-aim.

Aim 1c. To assess whether subclinical or primary hypothyroidism in pregnancy mediates the association between maternal fluoride exposure and offspring IQ while controlling for relevant confounders (Figure 3). Child sex-specific effects will also be explored.

Hypothesis 1c. Primary hypothyroidism, but not subclinical hypothyroidism, in pregnancy will mediate the previously reported association of higher maternal fluoride exposure and lower child IQ in the same study sample (Green et al., 2019). No hypothesis was made regarding sex-specific effects given the exploratory nature of this sub-aim.

Figure 3

Directed acyclic graph depicting the model of interest for Aim 1b and Aim 1c.



Note. The black line represents the association explored in Aim 1a whereas the blue lines represent the associations explored in Aims 1b and 1c. This DAG depicts primary hypothyroidism as the mediating variable; the same DAG was used to conceptualize the model with subclinical hypothyroidism as the mediating variable.

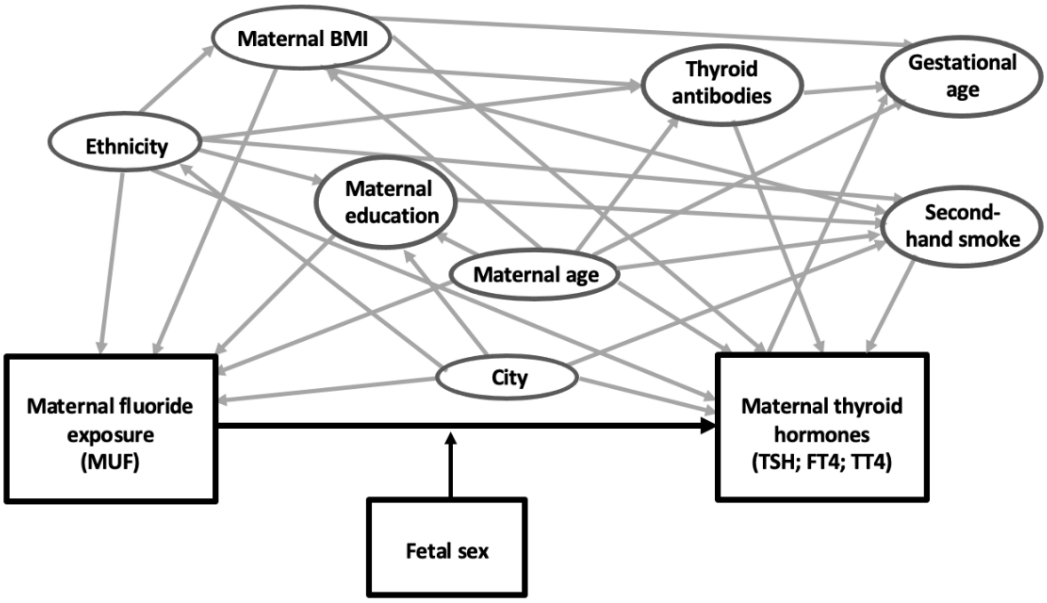
Aim 2. Maternal urinary fluoride concentration, thyroid hormones, and child IQ.

Aim 2a. To evaluate the potential thyroid-disrupting effects of fluoride exposure in pregnancy. Associations between MUF concentrations and TH levels (i.e., FT4, TT4, and TSH) will be assessed while controlling for relevant confounders (Figure 4). Effect modification by fetal sex will be assessed in these associations.

Hypothesis 2a. Higher concentrations of MUF in pregnancy will be associated with lower FT4 and higher TSH levels measured in maternal plasma from trimester one (Khandare et al., 2018; Kheradpisheh et al., 2018; Wang et al., 2020). No hypothesis was made regarding effect modification by fetal sex given the exploratory nature of this sub-aim.

Figure 4

Directed acyclic graph depicting the model of interest for Aim 2a.



Aim 2b. To evaluate whether maternal TH levels (i.e., FT4, TT4, and TSH) in pregnancy are associated with offspring IQ while controlling for relevant confounders (Figure 5). Effect modification by child sex will be assessed in these associations.

Hypothesis 2b. Lower maternal FT4 and higher maternal TSH levels will be associated with lower child IQ scores (Andersen et al., 2018; Haddow et al., 1999; Levie et al., 2018). No hypothesis was made regarding effect modification by child sex given the exploratory nature of this sub-aim.

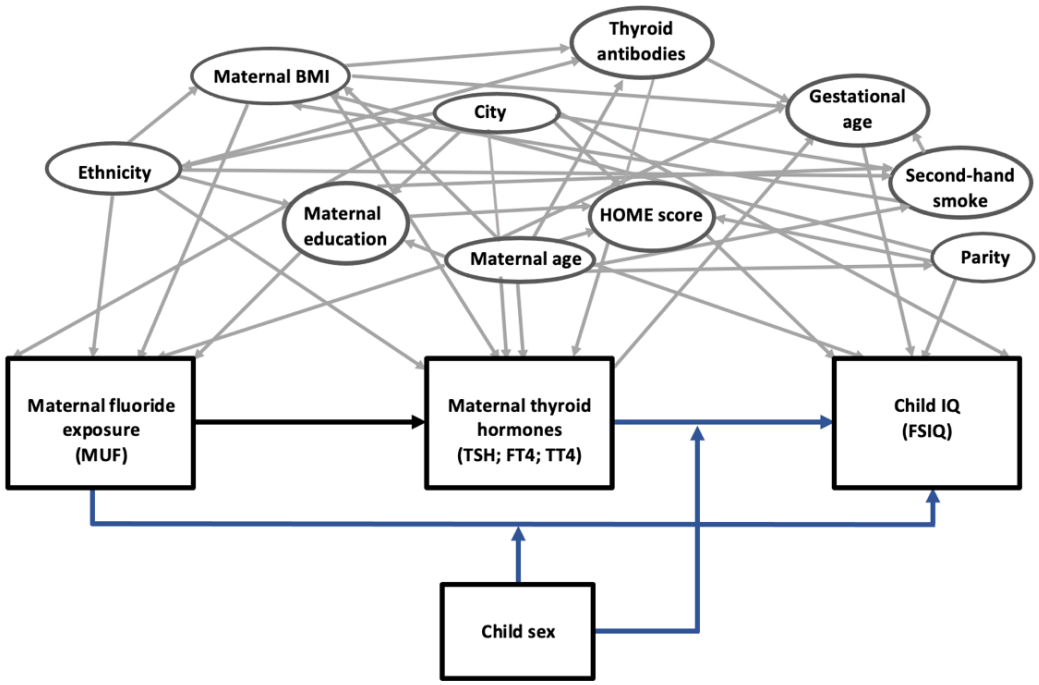
Aim 2c. To assess whether indicators of thyroid dysfunction in pregnancy mediate the association between maternal fluoride exposure and offspring IQ while controlling for relevant confounders (Figure 5). Effect modification by child sex will also be explored.

Hypothesis 2c. Thyroid hormone abnormalities will mediate fluoride-IQ scores in offspring. Specifically, lower FT4 and higher TSH levels will mediate the association of higher

maternal fluoride exposure with lower child IQ previously reported in the study sample (Green et al., 2019). No hypothesis was made regarding effect modification by child sex given the exploratory nature of this sub-aim.

Figure 5

Directed acyclic graph depicting the model of interest for Aim 2b and 2c.



Note. The black line represents the association explored in Aim 2a whereas the blue lines represent the associations explored in Aims 2b and 2c.

5.0 Methods.

5.1 Participants. Two thousand and one mother-child dyads were recruited through the Maternal-Infant Research on Environmental Chemicals (MIREC) Study between 2008 and 2011 (Figure 6). MIREC is a longitudinal pregnancy and birth cohort study that was initiated to examine the effects of prenatal exposure to environmental chemicals on the health of both pregnant women and their children. Women were recruited from prenatal clinics in hospitals with established clinical obstetrical research infrastructure in ten cities across Canada, seven of

which add fluoride to drinking water (Toronto, Hamilton, Ottawa, Sudbury, Halifax, Edmonton, Winnipeg; *n* = 1259) and three of which do not (Vancouver, Montreal, Kingston; *n* = 742). Women were eligible to participate if they were 18 years of age or older, were able to communicate in English or French, and were within their first 14 weeks of pregnancy, and were excluded if there were known fetal abnormalities, medical complications, or reported drug use. These women were slightly older on average at time of pregnancy (mean=32.2, *SD*=5.10), when compared to the Canadian average, well educated (62.3% had a university degree or higher), and predominately White (90%; Arbuckle et al., 2013).

A subset of women who met the inclusion criteria from the original cohort of women (*n*= 1207) were contacted to participate in MIREC's neurodevelopmental follow-up study called MIREC-Child Development (CD) Plus. Eligible mother-child dyads included mothers of singleton children born > 28 weeks' gestation who were between the ages of 3-4 years of age at time of study, with no congenital abnormalities, major neurological disorders, or history of convulsions. Due to limited resources, IQ testing was only offered in six of the study sites. Of the 808 women who consented to participate in either the Biomonitoring or Neurodevelopmental visit (67% of those contacted), 610 (76%) agreed to child IQ testing (i.e., the neurodevelopmental visit; Figure 6). These mother-child dyads lived in six of the most populous MIREC study sites, three of which are fluoridated (Toronto, Hamilton, and Halifax) and three of which are not (Vancouver, Kingston, and Montreal; Figure 6).

Figure 6

MIREC cohort participation in neurodevelopmental follow-up study.

Site Number	City	Participating Institutions	MIREC	MIREC-CD Plus	
				Bio-monitoring	Neuro-development

01	Vancouver	BC Children's and Women's Health Centre	162	83	55
02	Edmonton	University of Alberta	20		
03	Winnipeg	St. Boniface General Hospital/Health Sciences Centre	90		
04	Toronto	Mount Sinai Hospital/Sunnybrook Health Sciences Centre	325	108	72
05	Hamilton	McMaster University	275	112	85
06	Sudbury	Sudbury Regional Hospital	130		
07	Kingston	Kingston General Hospital	255	122	126
08	Ottawa	The Ottawa Hospital	119		
09	Montreal	CHU Sainte-Justine	300	175	145
10	Montreal	Jewish General Hospital	25	11	9
11	Halifax	IWK Health Centre	300	192	118
		Total	2001	803	610

Note. Figure was adapted from table titled "MIREC Platform Sites Participation" at: https://www.mirec-canada.ca/en/about/some-facts-and-figures/.
Eight-hundred and three of 808 respondents consented to the Biomonitoring visit and 610 of the 808 consented to Neurodevelopmental visit.
MIREC: Maternal-Infant Research on Environmental Chemicals study; MIREC-CD Plus: MIREC-Child Development Plus follow-up study.

5.2 MIREC study procedures. Women who consented to participate in the MIREC study were contacted during each trimester, at delivery, and in the early postnatal period (< 10 weeks) for data and biospecimen (e.g., blood and urine) collection. During the first and third trimesters, participants completed questionnaires administered by trained research staff to collect data on demographics, lifestyle factors (e.g., alcohol use and smoking), medical histories, use of health products and medications, and potential sources of exposures. Medical chart information was extracted at time of ultrasound in the first trimester, and at trimester two and post-delivery. Women's blood pressure, height, and weight were measured at each trimester.

Maternal urine and blood were collected during each trimester between 6-12 weeks, 16-21 weeks, and 32-34 weeks, respectively. Urine spot samples were collected in Nalgene® containers, and collection containers for both urine and blood samples were pretested for phthalates and BPA, and field blanks were incorporated for each chemical analyte. Women's urine and blood samples from each trimester were aliquoted into smaller cryovials and stored at -20 or -80^{o} as required. All biospecimen collection was done in clinic. For more information on data and biospecimen collection in the MIREC cohort, see Arbuckle et al., 2013.

5.3 Ethical considerations. The current thesis received approval from the research ethics boards at Health Canada and York University. The original MIREC study was approved by research ethics boards at Health Canada and all participating sites. MIREC implements high ethical standards in all follow-up studies to ensure that the privacy and confidentiality of participants are protected. To do this, participants are identified by a unique code so that their names are not stored in the biobank. Further, every woman provided informed consent to the collection and storage of their personal information and biospecimens. Every specimen is labelled with a barcode and securely stored in a freezer room. Only select staff have access to this room, which requires fingerprint recognition and is monitored by video surveillance 24/7. Despite participants being required to provide consent at every stage of the MIREC study, there is always a risk when handling personal data and sharing findings from analyses of participants' biological information. To further protect participants against this risk, results are never shared with identification codes or barcodes still intact.

5.4 Measures.

5.4.1 Exposure Measures. Maternal fluoride exposure was assessed using two different measures: drinking water fluoride concentration and maternal urinary fluoride

concentration.

Water fluoride. Water fluoride concentration was determined for each woman who reported drinking tap water during pregnancy. Water fluoride was derived by linking participants' postal codes to areas serviced by municipal water treatment plants. Water treatment plants measured fluoride levels daily if fluoride was added to public drinking water, and weekly or monthly if fluoride was not added to public water (Till et al., 2018). We estimated average water fluoride concentration (i.e., geometric means; mg/L) for each woman by averaging water fluoride concentrations across each woman's pregnancy; thus, each woman has a water fluoride concentration that is matched in time to the levels of fluoride found in tap water for the duration of her pregnancy.

Maternal Urinary fluoride (MUF). MUF concentration was analyzed in spot urine samples that were collected in each trimester of pregnancy, using a modification of the hexamethyldisiloxane (HMDS; Sigma Chemical Co., USA) microdiffusion method with ion-selective electrode at the Indiana University School of Dentistry (Martínez-Mier et al., 2011). In neutral solutions, fluoride concentrations were measured down to 0.02 mg/L. MUF values were excluded if they fell above the highest concentration standard of the instrument (>5 mg/L). Each MUF concentration was standardized for urine specific gravity to account for variability due to urinary dilution. An average, specific gravity adjusted MUF concentration was derived for each woman by taking the average across all three trimesters. Considering the relatively short half-life of fluoride of about 6 hours (Whitford, 1994), and the influence of dietary factors (e.g., high vegetable intake can increase fluoride excretion) and potential intake of high-fluoride foods or beverages (e.g., tea) immediately before sample collection, women were excluded if they did not provide all three urine samples.

5.4.2 Maternal thyroid hormones. Thyroid hormones, including TSH, FT4 and TT4, Tg, and thyroid antibodies (e.g., anti-Tg, and anti-thyroid peroxidase (TPO)) were analyzed in maternal plasma samples collected during the first trimester (mean= 11.6 weeks gestation). FT4 and TT4 were measured in 1885 samples using gold standard equilibrium dialysis isotope dilution mass spectrometry (ED-ID-MS) and isotope dilution high performance liquid chromatography mass spectrometry (ID-HPLC-MS), respectively, by the accredited Toxicology Laboratory at the Institut National de Santé Publique du Québec (INSPQ). TSH, Tg, anti-Tg, and anti-TPO concentrations were quantified in 1873 samples using commercial immunoassays by an accredited biochemistry laboratory at the Institut Universitaire de Cardiologie et de Pneumologie de Québec (IUCPQ). TSH values below the limit of detection (LoD; *n*= 7) were replaced with the LoD (.0025 μIU/mL) divided by the square root of two (Hornung & Reed, 1990).

Women in MIREC were classified as euthyroid, subclinical hypothyroid, or primary hypothyroid based on their TH levels (Table 1). TSH levels were assessed in comparison to the reference range for trimester one of pregnancy as recommended by the American Thyroid Association (i.e., 0.1 – 2.5 μIU/mL; Stagnaro-Green & Pearce, 2012). FT4 was considered normal if it fell between the 10th and 90th percentiles of FT4 levels for all women in MIREC (i.e., 11 – 17 pg/mL). More specifically, women were deemed euthyroid if their TSH and FT4 levels fell within the normal range (i.e., 0.1 – 2.5 μIU/mL and 11 – 17 pg/mL, respectively); subclinical hypothyroid if their TSH levels were elevated but FT4 levels were normal (i.e., 2.5 – 10 μIU/mL and 11 – 17 pg/mL, respectively); and primary hypothyroid if their TSH levels were high and FT4 levels were low (i.e., > 2.5 μIU/mL and < 11 pg/mL, respectively; Stagnaro-Green et al.,

2011). Women classified as primary hypothyroid were further combined with those who reported a diagnosis of primary hypothyroidism at time of study enrollment (i.e., 2008-2011; *n*= 79).

Table 1

Maternal thyroid status categories.

Thyroid status	*n*	TSH (μIU/mL)	FT4 (pg/mL)
Euthyroid	1293	0.1 – 2.5	11 – 17
Subclinical Hypothyroid	100	2.5 – 10	11 – 17
Primary Hypothyroid	27[a]	> 2.5	< 11

[a] Does not include the 79 women who reported a previous diagnosis of clinical hypothyroidism.

5.4.3 Children's IQ. Intellectual abilities were assessed using the Wechsler Preschool and Primary Scale of Intelligence (WPPSI-III; Canadian norms; mean= 100, *SD*= 15). Testing was conducted in the child's home (*n*=598) or clinic (*n*=12) in their primary language (English or French) and was conducted by trained research assistants. Training of research assistants was facilitated by a PhD level research coordinator and was done locally for each participating site. Integrity of test administration was ensured by periodic observation of testers (i.e., six months following training, research assistants were observed while conducting assessments in participants' homes) and double scoring of all protocols by the research coordinator. Among the 610 children who consented to neurodevelopmental testing, 601 (99%) completed testing. We used Full-Scale IQ (FSIQ), a measure of global intellectual and cognitive functioning, as the primary outcome.

5.5 Covariates. All potential confounding factors were identified *a priori* based on both biologically plausible and reported associations between fluoride exposure, thyroid function, and child IQ (Brent, 1997; Buzalaf et al., 2015; Buzalaf & Whitford, 2011; Collares et al., 2017; Makharia et al., 2016; Ronfani et al., 2015; Till et al., 2018). Maternal covariates that are known

to be or likely to be associated with THs include maternal age, ethnicity, level of education, pre-pregnancy body mass index (BMI), gestational age, city of residence, and thyroid antibody levels. Covariates relevant to analyses with child IQ included sex and gestational age, quality of home environment (i.e., HOME score), city of residence, parity, maternal age and level of education. We also evaluated a broad number of other covariates (e.g., fetal sex, second-hand smoke exposure, household income, parity, and medication use). Relationships between potential covariates, fluoride exposure variables, maternal THs, and child IQ are outlined in the directed acyclic graphs above (Figures 2 to 5). Covariates were retained in the statistical models if their associated *p* values fell at or were less than .2 or their inclusion altered the regression coefficient of the main predictor by more than 10% (i.e., augmented backward elimination procedure; Dunkler et al., 2014).

Daily maternal iodine intake (μg/day) was further added as a covariate in the models used to address Aim 2a as part of a sensitivity analysis. Maternal iodine intake was estimated from women's creatinine-corrected urinary iodine concentration averaged across trimesters one and two as described in Krzeczkowski et al. (2023). Urinary iodine concentration was analyzed in spot urine samples collected in the first and second trimesters of pregnancy, using inductively coupled plasma mass spectrometry (ICP-MS) by the accredited Toxicology Laboratory at INSPQ. Values below the LoD (*n*=184) were replaced with the LoD (0.3 μmol/L) divided by the square root of two (Hornung & Reed, 1990).

Women who reported taking medication to treat a thyroid disorder at the time of study enrolment were excluded from analyses involving THs. This is because nearly all women who reported a diagnosis of hypothyroidism (i.e., high TSH and low FT4) also reported taking medication (e.g., Levothyroxine) meant to replace the missing T4, and thus, were more likely to

present with normal TH levels.

6.0 Statistical Analysis.

Descriptive statistics were run on all demographics, for both mothers and children, fluoride exposure variables, maternal THs, and child FSIQ. Mother-child dyads with water fluoride concentration and child FSIQ data were compared to those with water fluoride concentration data alone. Independent samples t-tests and Chi-squared tests were used to compare these two groups on continuous and categorical variables, respectively. Distribution plots were examined to look for outliers and to assess the distribution of each variable. Spearman correlations were used to examine preliminary associations between fluoride exposure variables and TH measures.

6.1 Aim 1a – maternal fluoride exposure and thyroid health status. Women classified as euthyroid, subclinical, and primary hypothyroid were compared on sociodemographic characteristics, fluoride exposure, child IQ, and other variables. Four logistic regression models were used to quantify risk of subclinical and primary hypothyroidism, separately, by both water fluoride and MUF concentrations. Primary hypothyroid women were excluded from the models examining associations between fluoride exposure and risk of subclinical hypothyroidism, and vice versa. All models were adjusted for maternal age, level of education, pre-pregnancy BMI, ethnicity, and city of residence when MUF was used as the predictor. The same covariates were applied in the models where water fluoride concentration was the predictor, apart from city due to collinearity between water fluoride concentration and city. For all models, odds ratios (ORs) and associated confidence intervals (CIs) were reported for every 0.5 mg/L increase in MUF (which corresponds approximately to the difference between the 20^{th} and 80^{th} percentile) or water fluoride concentration (which corresponds to the approximate difference in water fluoride

concentration in fluoridated versus non-fluoridated communities).

6.2 Aim 1b – maternal hypothyroid status and child FSIQ. Following examination of the associations between fluoride exposure (i.e., water fluoride and MUF concentration) and hypothyroidism in Aim 1a, the association between subclinical and primary hypothyroidism and child FSIQ was evaluated using two separate multiple linear regression models (with FSIQ as the outcome variable) controlling for maternal age, ethnicity, level of education, second-hand smoke exposure, parity, child sex, HOME score, and city of residence. Once again, women classified as primary hypothyroid were excluded from the model examining the association between subclinical hypothyroidism and child FSIQ, and vice versa. Sex-specific effects were explored in both models through the inclusion of interaction terms. Given the interaction terms consisted of two binary variables (i.e., thyroid status and child sex), stratified linear models were run for male and female children separately for ease of interpretation.

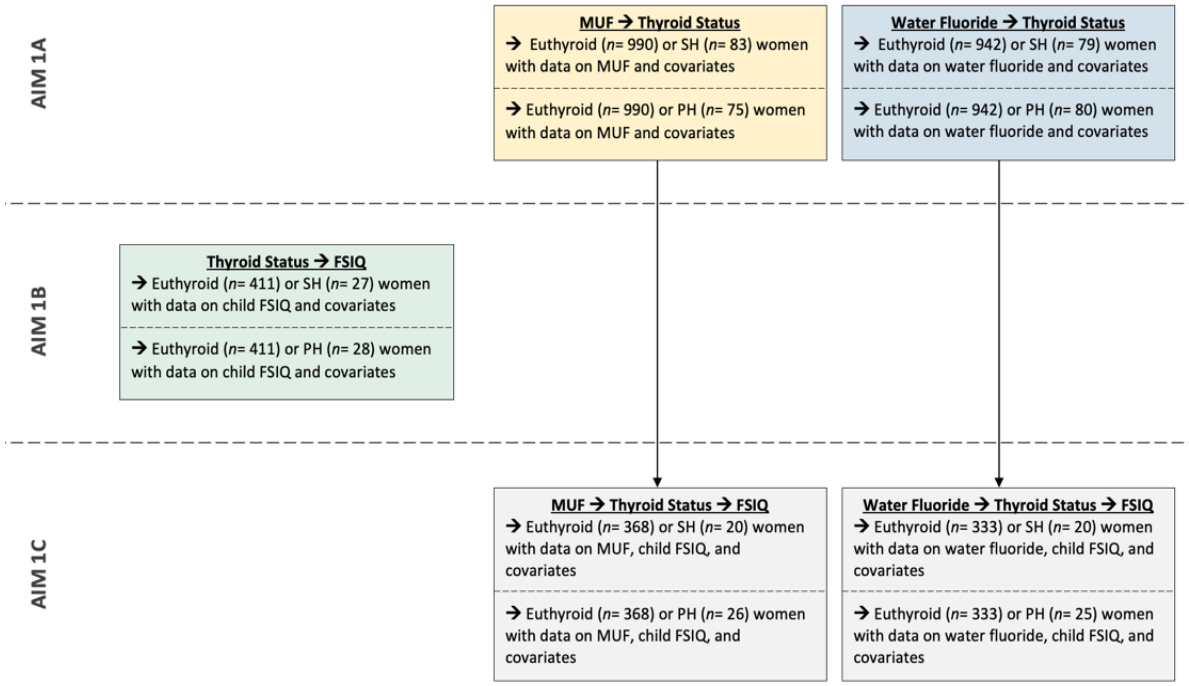
6.3 Aim 1c – maternal hypothyroid status as a mediator of fluoride-FSIQ association in children. The potential mediating effect of maternal thyroid status in the association between maternal fluoride exposure and child FSIQ was evaluated (Figure 3). Specifically, mediation analysis in the counterfactual framework was used to estimate the proportion of the effect of maternal fluoride exposure on child FSIQ that is mediated by hypothyroidism, through comparison of the natural direct and indirect effects (Valeri & VanderWeele, 2013; VanderWeele, 2014). The natural direct effect represents the unmediated effect (i.e., effect of fluoride exposure on child IQ that is not mediated through other mechanisms) whereas the natural indirect effect represents the average increase or decrease in the FSIQ score that is mediated by having hypothyroidism. Mediation analysis was performed using the *paramed* package in STATA, controlling for maternal age, level of education, ethnicity, second-hand

smoke exposure, HOME score, and child sex. The mediated effect (or indirect effect) was estimated and tested only when there was a significant association between maternal fluoride exposure and hypothyroid status (Aim 1a) and between maternal hypothyroid status and child FSIQ (Aim 1b). Once again, child sex-specific effects were explored through stratification for ease of interpretation considering the binary mediating variable (i.e., euthyroid vs. hypothyroid).

Figure 7 shows the study sample flow chart for all primary variables and subgroups of interest for Aim 1.

Figure 7

Study samples included in Aim 1 analyses.



Note. Full sample consisted of *n*=1293 women classified as Euthyroid; *n*= 100 women classified as Subclinical Hypothyroid (SH); and *n*=106 women classified as Primary Hypothyroid (PH). Only a subset of women from the original sample in Aim 1a participated in IQ testing with their child for Aims 1b and 1c. MUF = maternal urinary fluoride; FSIQ = full scale IQ.

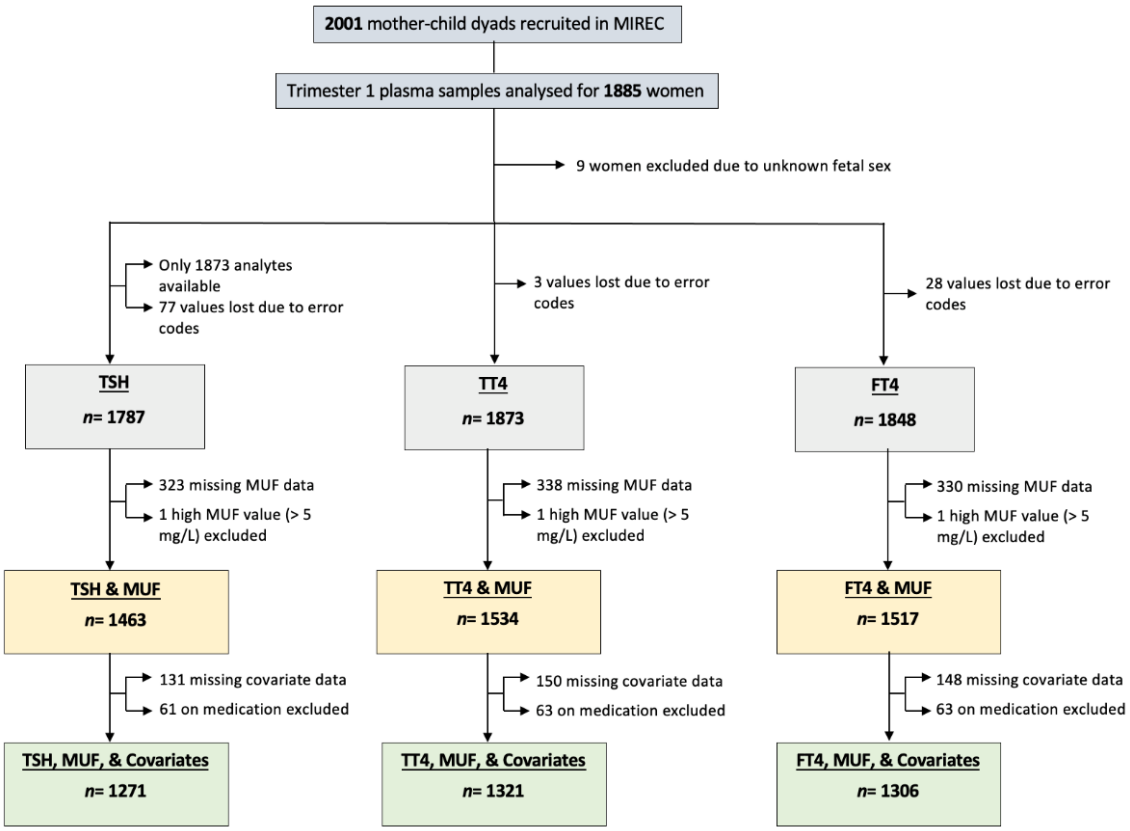
6.4 Aim 2a – maternal urinary fluoride concentration and thyroid hormones. Three

multiple linear regression models were used to test whether MUF predicted any changes in maternal TH levels (i.e., TT4, FT4, and TSH; Figure 4). Given the non-normal distribution of TSH, log transformed TSH was the primary outcome variable of interest. Change in log TSH predicted by MUF concentration was interpreted as a percent increase or decrease [i.e., (e^ (B coefficient log TSH) – 1) * 100] for every 1 mg/L increase in MUF, which corresponds to approximately to the difference between the 10^{th} and 95^{th} percentile. All models were adjusted for maternal age, level of education, pre-pregnancy BMI, ethnicity, parity, thyroid antibody levels, gestational age, fetal sex, second-hand smoke exposure, and city of residence. Effect modification by fetal sex was assessed in the above models through the inclusion of interaction terms; the predicted slopes for women carrying males and those carrying females were estimated by running each model twice, once with females set as the reference group, and again with males as the reference. As a sensitivity analysis, the models described above were rerun with maternal iodine intake added as a covariate considering both deficient and excess iodine intake can induce hypothyroidism in pregnant women (Leung & Braverman, 2014; Leung et al., 2011; Shi et al., 2015).

Figure 8 shows the study sample flow chart for all primary variables and subgroups of interest for Aim 2a.

Figure 8

Study sample flow chart for all primary variables and subgroups of interest for Aim 2a.



6.5 Aim 2b – maternal thyroid hormones and child FSIQ. Following examination of the associations between MUF concentration and maternal TH levels (i.e., TT4, FT4, and TSH) in Aim 2a, the associations between maternal TH levels and child FSIQ were evaluated using three separate multiple linear regression models controlling for maternal age, level of education, pre-pregnancy BMI, ethnicity, parity, gestational age, child sex, second-hand smoke exposure, HOME score, and city of residence. Effect modification by child sex was evaluated in the associations between maternal THs and child FSIQ through the inclusion of interaction terms. The predicted slopes for male and female children were estimated by running each model twice, once with females set as the reference group, and again with males as the reference

6.6 Aim 2c – maternal thyroid hormones as a mediator of fluoride-FSIQ association in children. The potential mediating effects of maternal TH levels in the association between

MUF and child FSIQ were considered (Figure 5). Specifically, mediation analysis in the counterfactual framework (as described above) was used to estimate and test the mediated effect (or indirect effect) only when there was a significant association between MUF and a maternal TH (Aim 2a) and between that maternal TH and child FSIQ (Aim 2b). If mediation analysis was performed, sex-specific effects were investigated using stratification.

Model diagnostics were examined for all regression models used to address Aims 1 and 2. Quartile and quadratic regression models were used to assess non-linearity among all associations investigated as part of Aim 2. All analyses were conducted using STATA version 17.0 (STATA corporation). The *p* value level of significance for probing main effects was .05 and all tests were two-tailed. Interactions were probed if the *p* value for the interaction term was less than .20. We used this more lenient *p* value given the reduction in sample size and therefore reduced power when estimating subgroup differences. See Rothman (2014) for why focusing on whether the coefficient of a product term is statistically significant (i.e., *p* value <.05) may miss important biological interactions. We did not adjust our threshold for significance (e.g., Bonferroni adjustment, familywise error rate, etc.). In Aim 1, we fitted separate models using one or two independent outcomes (i.e., subclinical or primary hypothyroidism in Aim 1a and FSIQ in Aim 1b). We predicted each of the outcomes in Aim 1a using two correlated predictors: MUF and water fluoride concentration. While use of these complementary predictors is advantageous because it allowed us to compare results of a model focused solely on fluoride in drinking water versus fluoride from all sources as measured in urine, it could also contribute to risk of a Type I error. Probability of making a Type I error may have increased in Aim 2 where we fit separate regression models for three related outcomes (TSH, TT4 and FT4) using MUF as the predictor. Importantly, however, adoption of a stringent *p* value could increase risk of a Type

II error and may possibly result in missing important findings. As such, we considered confidence intervals and the overall pattern of findings when interpreting results.

7.0 Results.

DISCLAIMER: *The results included in this thesis are preliminary and subject to revision. The results reported below were prepared to meet the requirements set by the Faculty of Graduate Studies at York University. Both the results and interpretation of the findings have not received final approval by co-investigators on this research project. Data reported in this thesis are not intended for distribution.*

7.1. Demographics. Demographic characteristics of the cohorts with blood plasma samples measured in the first trimester, MUF concentration data, water fluoride concentration data, and both water fluoride concentration and child IQ data are summarized in Table 2. The 485 mother-child dyads with data on water fluoride concentration and child IQ did not differ significantly from the 1522 with data only on water fluoride concentration on most characteristics except ethnicity, city of residence, second-hand smoke exposure, and MUF and water fluoride concentration. Compared to women without child IQ data, those with data on both water fluoride concentration and child IQ were more likely to be White, live in unfluoridated cities, and report no second-smoke exposure in the first trimester, and had lower MUF and water fluoride concentrations on average (Table 2).

Spearman correlations indicated a moderate-to-large positive association between water fluoride and MUF concentrations (r= .49, p<.05), a moderate positive correlation between maternal FT4 and TT4 levels (r= 0.33, p<.05), a weak positive correlation between daily iodine intake and TSH (r= .06, p<.05), and weak negative correlations between daily iodine intake and FT4 (r= -.06, p<.05), TT4 and TSH (r= -.10, p<.05), and FT4 and TSH (r= -.18, p<.05)

(Supplemental Figure 1). There were no significant correlations between MUF concentration and any of the thyroid hormones (TSH, FT4, and TT4) or daily iodine intake (Supplemental Table 1); however, the association between MUF concentration and average, specific gravity-corrected urinary iodine concentration was significant but very small (r=.07, p<.05). Water fluoride concentration was not associated with daily iodine intake or any of the thyroid hormones, except for TT4 (r= .07, p<.05).

Table 2

Demographic characteristics of subsamples used in analyses for Aims 1 and 2.

	MIREC cohort participants with:			
	Plasma samples from trimester 1	Maternal urinary fluoride (MUF)	Water fluoride concentration	Water fluoride + IQ scores
n	1876[d]	1566	1522	485
Maternal age (years; mean; SD)	32.2 (5.1)	32.3 (4.9)	32.5 (5.1)	32.7 (4.7)
Ethnicity (%)				
White	86.0	86.0	83.5	87.8*
Other	14.0	14.0	16.5	12.2*
Marital status (%)				
Married or common law	95.1	95.8	94.8	95.5
Single	4.9	4.2	5.2	4.5
Level of education (%)				
College diploma or less	37.6	35.7	34.8	32.4
University degree	62.4	64.3	65.2	67.6
Household income (%)				
<100,000	59.8	59.2	58.6	59.1
≥100,000	40.2	40.8	41.4	40.9
City (%)				
Fluoridated[a]	61.5	60.8	61.9	46.0*
Unfluoridated[b]	38.5	39.2	38.1	54.0*
MUF concentration (mg/L; mean; SD)	0.59 (0.39)	0.59 (0.39)	0.61 (0.40)	0.55 (0.39)*
Water fluoride concentration	0.42 (0.25)	0.42 (0.26)	0.42 (0.25)	0.34 (0.24)*

(mg/L; mean; SD)				
Second-hand smoke in trimester 1				
Yes	6.1	5.6	5.7	3.3*
No	93.9	94.4	94.3	96.7*
Pre-pregnancy BMI (kg/m^2; mean; SD)	24.9 (5.5)	24.8 (5.4)	24.8 (5.3)	25.0 (6.0)
Parity (%)				
0	44.4	45.4	46.0	45.4
1	40.5	39.8	40.0	42.3
2+	15.1	14.8	14.0	12.3
Gestational age (weeks; mean; SD)[c]	11.6 (1.5)	11.6 (1.5)	11.7 (1.4)	11.7 (1.4)
Maternal thyroid hormones				
TSH (μIU/mL; mean; SD)	1.4 (1.2)	1.4 (1.2)	1.4 (1.2)	1.4 (1.1)
Log TSH (mean; SD)	0.01 (0.98)	0.04 (0.96)	0.02 (0.96)	0.03 (0.90)
FT4 (pg/mL; mean; SD)	13.7 (4.4)	13.7 (4.6)	13.6 (2.8)	13.5 (2.9)
TT4 (ng/mL; mean; SD)	106.7 (21.6)	106.6 (21.5)	106.9 (21.6)	105.3 (20.7)
Tg (ng/mL; mean; SD)	17.5 (16.8)	17.5 (16.7)	17.3 (16.1)	16.9 (14.6)
Maternal thyroid antibodies				
Anti-Tg (IU/mL; mean; SD)	11.8 (56.2)	12.1 (57.9)	11.6 (54.2)	11.5 (58.9)
Anti-TPO (IU/mL; mean; SD)	27.5 (106.5)	26.1 (100.7)	28.0 (109.4)	25.2 (100.2)
Child sex (%)				
Male	53.3	52.1	53.4	50.3
Female	46.7	47.9	46.6	49.7
Child IQ (mean; SD)				
Full Scale IQ	106.9 (13.6)	107.1 (13.3)	107.1 (13.7)	107.1 (13.7)
Verbal IQ	109.4 (13.3)	109.7 (12.9)	109.9 (13.1)	109.9 (13.1)
Performance IQ	103.0 (14.9)	103.1 (14.6)	102.8 (14.9)	102.8 (14.9)
HOME score (mean; SD)	47.3 (4.3)	47.3 (4.3)	47.3 (4.4)	47.3 (4.4)

[a] Edmonton, Winnipeg, Toronto, Hamilton, Sudbury, Ottawa, Halifax.
[b] Vancouver, Kingston, Montreal.
[c] Gestational age at time of maternal blood collection in trimester 1.
[d] Excluding those (n=9) with unknown fetal sex.
* Denotes significant differences (p value < .05) between participants with data on water fluoride concentration and children's IQ scores and those with data on water fluoride concentration.
Abbreviations: SD= Standard Deviation; IQ = intelligence quotient; HOME= home observation measurement of the environment; MUF = maternal urinary fluoride, adjusted for specific-gravity; TSH= thyroid stimulating hormone; FT4= free thyroxine; TT4= total thyroxine; Tg= thyroglobulin; TPO = thyroid peroxidase.

7.2 Aim 1.

7.2.1 Thyroid health status categories. Based on women's FT4 and TSH-blood plasma levels from the first trimester, 1293 women were classified as euthyroid, 100 met criteria for subclinical hypothyroidism, and 27 met criteria for primary hypothyroidism (Table 1). These 27 women were combined with an additional 79 women who reported a diagnosis of primary hypothyroidism at the time of study enrollment, to form the final primary hypothyroid group consisting of 106 women. Mean (*SD*) TSH and FT4 levels for women in the euthyroid, subclinical hypothyroid, and primary hypothyroid groups were 1.16 (0.55) μIU/m and 13.51 (1.69) pg/mL, 3.09 (0.69) μIU/m and 13.09 (1.48) pg/mL, and 3.14 (2.79) μIU/m and 14.02 (3.70) pg/mL, respectively. Primary hypothyroid women were slightly older, had higher BMI and elevated TH and antibody levels, were more likely to be White, live in fluoridated cities, take thyroid related medication, and have female children, and had children with lower FSIQ and performance IQ scores compared to the euthyroid group (Table 3). Women classified as subclinical hypothyroid were more likely to be pregnant with their first child at the time of study enrollment and to report taking prenatal vitamins in pregnancy, and had higher TSH, FT4, and thyroid antibody levels compared to euthyroid women (Table 3).

Table 3

Demographic characteristics of women classified as euthyroid, subclinical hypothyroid, and primary hypothyroid.

	Euthyroid	Subclinical Hypothyroid	Primary Hypothyroid
n	1293	100	106
Maternal age (years; mean; SD)	32.1 (5.0)	32.1 (4.8)	33.2 (5.1)**
Ethnicity (%)			
White	86.1	86.0	92.5*
Other	13.9	14.0	7.5*
Marital status (%)			
Married or common law	95.3	94.0	97.2

Single	4.7	6.0	2.8
Level of education (%)			
College diploma or less	36.8	37.0	35.9
University degree	63.2	63.0	64.1
Household income (%)			
<100,000	60.7	53.1	52.5
≥100,000	39.3	46.9	47.5
City (%)			
Fluoridated[a]	60.1	61.0	71.6**
Unfluoridated[b]	39.9	39.0	28.4**
Second-hand smoke in trimester 1			
Yes	6.1	6.0	6.7
No	93.9	94.0	93.3
Pre-pregnancy BMI (kg/m^2; mean; SD)	24.7 (5.3)	24.3 (5.9)	26.4 (6.5)**
Parity (%)			
0	43.7	52.0**	39.6
1	40.8	42.0	44.3
2+	15.5	6.0**	16.1
Gestational age (weeks; mean; SD)[c]	11.6 (1.6)	11.7 (1.4)	11.7 (1.4)
Maternal thyroid hormones			
Log TSH (mean; SD)	0.01 (0.57)	1.1 (0.19)**	0.68 (1.22)**
FT4 (pg/mL; mean; SD)	13.5 (1.7)	13.1 (1.5)**	14.0 (3.7)*
TT4 (ng/mL; mean; SD)	105.8 (19.9)	107.1 (18.9)	109.6 (26.6)*
Tg (ng/mL; mean; SD)	17.6 (16.5)	16.6 (17.2)	14.8 (19.5)*
Maternal thyroid antibodies			
Anti-Tg (IU/mL; mean; SD)	7.5 (39.3)	33.0 (107.8)**	52.7 (124.6)**
Anti-TPO (IU/mL; mean; SD)	15.0 (73.3)	51.9 (123.4)**	185.2 (253.3)**
Anti-TPO + (≥5.61 IU/mL; %)	10.5	33.0**	58.5**
Iodine intake (µg/day; mean; SD)	441.3 (357.3)	458.0 (273.8)	469.3 (209.7)
Reported taking a prenatal vitamin (%)	87.3	95.0**	87.7
Reported taking thyroid medication (%)	0	0	73.6**
Child sex (%)			
Male	53.5	50.0	37.6**
Female	46.5	50.0	62.4**
Child IQ (mean; SD)			
Full Scale IQ	107.6 (13.8)	108.5 (13.0)	101.5 (11.9)**
Verbal IQ	109.8 (13.6)	110.1 (11.5)	106.4 (13.8)
Performance IQ	103.9 (14.9)	104.7 (14.4)	96.3 (13.3)**

HOME score (mean; SD)	47.3 (4.2)	47.7 (4.9)	46.6 (4.4)

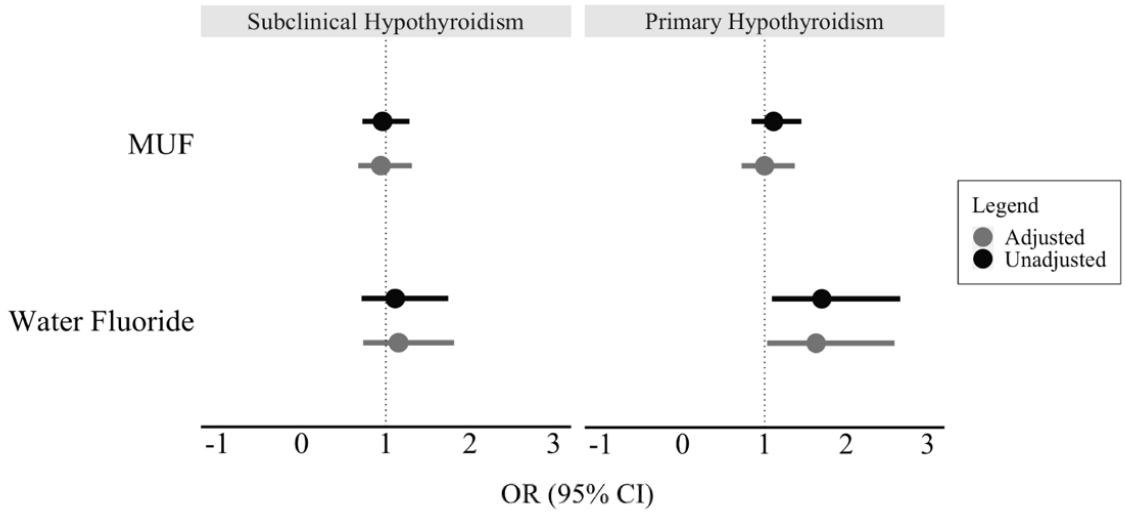
[a] Edmonton, Winnipeg, Toronto, Hamilton, Sudbury, Ottawa, Halifax.
[b] Vancouver, Kingston, Montreal.
[c] Gestational age at time of maternal blood collection in trimester 1.
* Denotes significant differences (* *p* value < .1; ** *p* value < .05) between euthyroid and subclinical hypothyroid women or between euthyroid and primary hypothyroid women.
Abbreviations: see note for Table 2.

7.2.2 Maternal fluoride exposure and thyroid health status (Aim 1a). Women in the euthyroid (*n*= 990), subclinical hypothyroid (*n*= 83), and primary hypothyroid (*n*= 75) groups had an average MUF concentration of 0.58 mg/L (*SD*= 0.39; range: 0.05 – 3.33), 0.57 mg/L (*SD*= 0.40; range: 0.11 – 2.43), and 0.62 mg/L (*SD*= 0.35; range: 0.11 – 1.47), respectively. Logistic regression analysis revealed that there was no statistically significant association between MUF and risk of subclinical hypothyroidism (OR= 0.94; 95% CI: 0.67, 1.31; *p*= .71) or primary hypothyroidism (OR= 1.00; 95% CI: 0.72, 1.37; *p*= .98; Figure 9).

Women in the euthyroid (*n*= 942), subclinical hypothyroid (*n*= 79), and primary hypothyroid (*n*= 80) groups had an average water fluoride concentration of 0.41 mg/L (*SD*= 0.26; range: 0.04 – 0.87), 0.42 mg/L (*SD*= 0.25; range: 0.04 – 0.85), and 0.48 mg/L (*SD*= 0.25; range: 0.04 – 0.87), respectively. There was no statistically significant association between water fluoride and risk of subclinical hypothyroidism (OR= 1.15; 95% CI: 0.73, 1.81; *p*= .55). However, there was a statistically significant association between water fluoride concentration and risk of primary hypothyroidism, whereby a 0.5 mg/L increase in water fluoride concentration was associated with a 1.63 times greater odds (95% CI: 1.03, 2.59; *p*= .04) of having a diagnosis or meeting criteria for primary hypothyroidism (Figure 9).

Figure 9

Covariate-adjusted and unadjusted effect estimates of associations between MUF and water fluoride, and subclinical and primary hypothyroidism relative to euthyroid women.



Note. Covariate-unadjusted effect estimates (i.e., odds ratios) shown in black; covariate-adjusted odds ratios shown in grey. MUF: maternal urinary fluoride.

7.2.3 Maternal hypothyroidism and child FSIQ (Aim 1b). Median (IQR) FSIQ score was 108 (19) for the overall sample of 439 children [females: 110 (17); males: 105 (19)]. Linear regression analysis revealed that FSIQ scores were 4.45 (95% CI: -9.17, 0.26; *p*= .06) points lower, on average, among children of primary hypothyroid women compared to children of euthyroid women. The interaction between maternal thyroid status (i.e., euthyroid vs. primary hypothyroid) and child sex in predicting child FSIQ met our threshold for model-selection purposes (*p* interaction term= .13). Stratification by child sex revealed that males (*n*=13) born to women with primary hypothyroidism had significantly lower FSIQ scores compared with males born to euthyroid women (*n*=201). Specifically, FSIQ scores were 8.78 (95% CI: -16.78, -0.79; *p*= .03) points lower, on average, among male children born to primary hypothyroid women compared to males of euthyroid women (Figure 10). In contrast, FSIQ scores did not differ significantly among females born to primary hypothyroid women (*n*=15) versus euthyroid women (*n*= 210; B = -1.11; 95% CI: -6.75, 4.54; *p*= .70).

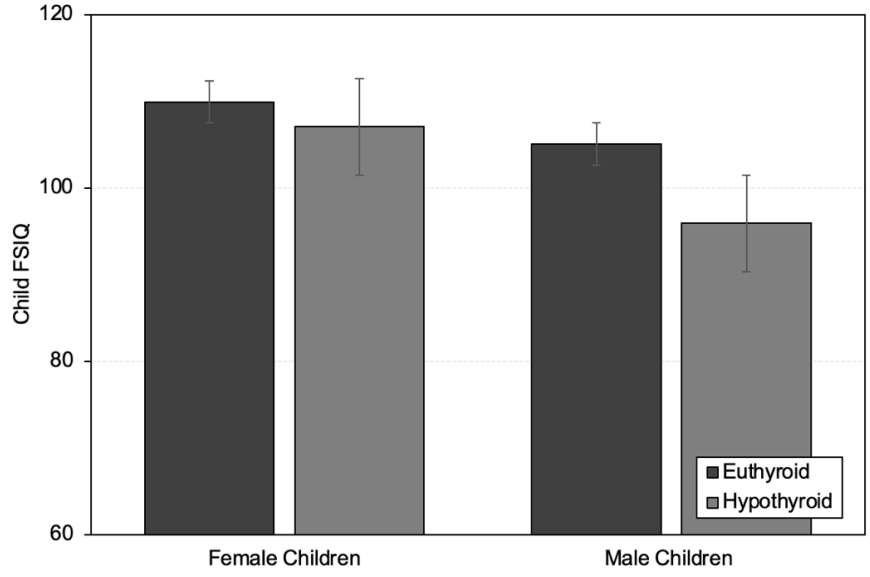
Results from linear regression revealed no significant association between maternal

subclinical hypothyroidism and child FSIQ (B= 0.05; 95% CI: -4.78, 4.89; *p*= .98). Further, the interaction between maternal thyroid status (i.e., euthyroid vs. subclinical hypothyroid) and child sex in predicting child FSIQ was not significant (*p* interaction term= .59). As such, this model was not stratified by child sex.

Regression diagnostics confirmed no issues with collinearity (variance inflation factor < 4 for all covariates), assumption violations (measured via plots of residuals versus fitted values and Cook-Weisberg test for heteroskedasticity), or influential cases or outliers (measured by Cook's distance) in any of the above models.

Figure 10

Sex-specific effects in the association between maternal primary hypothyroidism and child FSIQ.



Note. Left: mean FSIQ scores of female children born to euthyroid and primary hypothyroid women (total *n*=225). Right: mean FSIQ scores of male children born to euthyroid and primary hypothyroid women (total *n*=214).

7.2.4 Maternal hypothyroidism as a mediator of maternal water fluoride exposure-child FSIQ association (Aim 1c). Mediation analysis was conducted on the subsample of mother-child dyads with water fluoride and FSIQ data. Using the counterfactual framework, the

direct effect of water fluoride concentration on child FSIQ was significant (i.e., natural direct effect; *n*= 358; Table 4), whereas the natural indirect effect was not significant, indicating that maternal primary hypothyroidism did not significantly mediate the relationship between maternal water fluoride concentration and child FSIQ (*p*= .40; Table 4). Similarly, there was no evidence of mediation when the sample was stratified for women who had male children (*n*= 177; natural indirect effect estimate= -0.003; *SE*= 0.44; 95% CI: -0.86, 0.85; *p*= 0.99) and those who had female children (*n*= 181; natural indirect effect estimate= -0.013; *SE*= 0.21; 95% CI: -0.43, 0.41; *p*= .95).

Maternal subclinical hypothyroidism was not explored as a mediator given non-significant associations between fluoride exposure and risk of subclinical hypothyroidism, and between subclinical hypothyroidism and child FSIQ.

Table 4

Results from mediation analysis in the counterfactual framework to address Aim 1c.

Child FSIQ[a]	B	*SE*	*t*	P >| *t* |	95% Confidence Interval	
Water fluoride	-8.360	2.942	-2.84	0.005	-14.147	-2.573
Primary hypothyroidism	-6.031	5.107	-1.18	0.238	-16.075	4.014
Water fluoride*Primary hypothyroidism	1.847	11.682	0.16	0.874	-21.129	24.823
Maternal age	0.002	0.144	0.02	0.987	-0.281	0.285
Level of education	4.941	1.477	3.35	0.001	2.037	7.846
Ethnicity	4.400	2.146	2.05	0.041	0.180	8.621
Child sex	-4.041	1.345	-3.00	0.003	-6.687	-1.395
HOME score	0.911	0.165	5.52	0.000	0.587	1.236
Second-hand smoke	11.230	4.115	2.73	0.007	3.136	19.324
Primary hypothyroidism[b]			*z*	P > | *z* |		
Water fluoride	0.903	0.861	1.05	0.294	-0.784	2.590
Maternal age	0.009	0.047	0.19	0.848	-0.083	0.101
Level of education	0.371	0.492	0.76	0.450	-0.592	1.335
Ethnicity	0.569	0.787	0.72	0.469	-0.972	2.111

Child sex	-0.167	0.418	-0.40	0.690	-0.987	0.653
HOME score	-0.039	0.048	-0.82	0.411	-0.133	0.054
Second-hand smoke	1.266	0.873	1.45	0.147	-0.445	2.976
Mediation parameters				P > | *z* |		
Controlled direct effect	-2.801	4.864		0.565	-12.334	6.733
Natural direct effect	-3.549	1.225		0.004	-5.951	-1.148
Natural indirect effect	-0.125	0.147		0.396	-0.412	0.163
Marginal total effect	-3.674	13.771		0.790	-30.664	23.316

Note. B= unstandardized regression coefficient; *SE*=Standard Error.
[a] Multiple linear regression model evaluating the association between water fluoride and child FSIQ while controlling for maternal primary hypothyroidism and relevant covariates, with inclusion of the interaction between water fluoride and primary hypothyroidism (*n*= 358; R^2= 0.223; Adjusted R^2= 0.203; *F* (9, 348) = 11.09; Prob > *F*= 0.000).
[b] Logistic regression model evaluating risk of primary hypothyroidism by maternal water fluoride concentration while controlling for covariates (*n*= 360; Pseudo R^2= 0.025; Likelihood Ratio *Chi-Square* (7) = 4.66; Prob > *Chi-Square*= 0.701).

7.3 Aim 2.

7.3.1 MUF and maternal thyroid hormones (TSH, FT4, and TT4; Aim 2a). Women with data on TSH, TT4, or FT4 had a mean MUF concentration of 0.59 mg/L (S*D*= 0.39; range: 0.05 – 3.33).

MUF and TSH. Based on results from linear regression, there was an association between MUF and TSH, whereby a 1 mg/L increase in MUF was associated with a 0.125 logarithmic unit (i.e., 13.31%) increase in maternal TSH (Table 5); however, this association was not significant (*p*= .08). There was a statistically significant interaction between MUF and fetal sex in predicting maternal TSH (*p* interaction term= .04), indicating that higher MUF was associated with higher TSH among women carrying females. Probing of the predicted slopes revealed that an increase of 1 mg/L MUF was associated with a 0.30 (*SE*= 0.11; 95% CI: 0.08, 0.51; *p*= .01) logarithmic unit (i.e., 34.99%) increase in TSH among women carrying females (Figure 11). In contrast, MUF was not significantly associated with TSH among women carrying

males (B= 0.02; *SE*= 0.09; 95% CI: -0.15, 0.19; *p*= .83; Figure 11).

Table 5

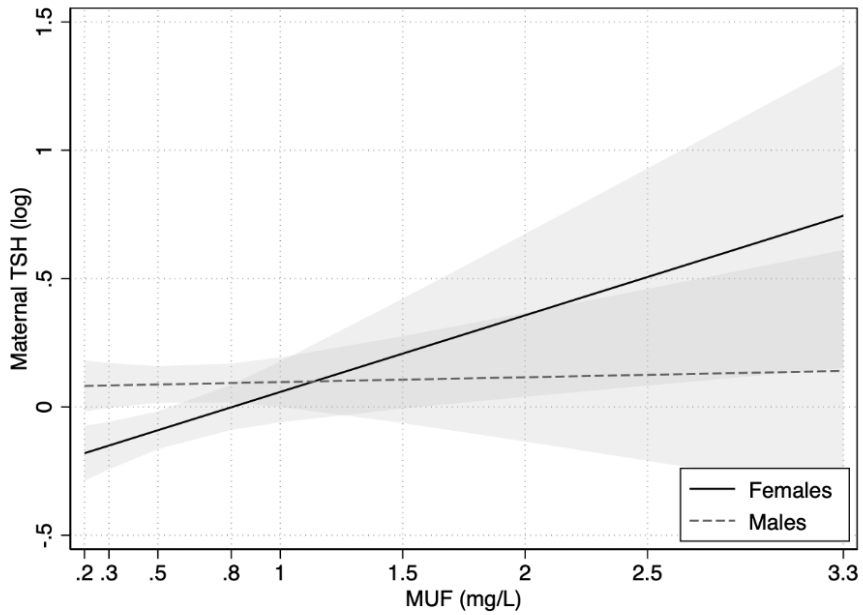
Output from linear regression model used to assess association between MUF and TSH.

TSH (log)[a]	B	*SE*	*t*	P >| *t* |	95% Confidence Interval	
MUF	0.125	0.072	1.73	0.083	-0.016	0.267
Pre-pregnancy BMI	0.006	0.005	1.14	0.254	-0.004	0.015
Ethnicity	0.142	0.077	1.86	0.064	-0.008	0.292
Level of education	0.043	0.058	0.75	0.453	-0.070	0.156
Maternal age	-0.011	0.006	-1.76	0.078	-0.023	0.001
Parity	-0.073	0.032	-2.30	0.021	-0.135	-0.011
Fetal sex	0.155	0.051	3.05	0.002	0.055	0.255
Gestational age	0.009	0.019	0.46	0.643	-0.028	0.046
City of residence[b]						
5[c]	Ref					
6	-0.224	0.249	-0.90	0.367	-0.712	0.264
7	-0.046	0.153	-0.30	0.765	-0.345	0.254
8	0.005	0.117	0.04	0.968	-0.225	0.234
9	-0.007	0.129	-0.05	0.957	-0.261	0.247
10	-0.104	0.162	-0.64	0.522	-0.422	0.214
11	0.089	0.122	0.73	0.463	-0.149	0.328
12	-0.058	0.146	-0.40	0.691	-0.344	0.228
13	0.001	0.115	0.01	0.993	-0.226	0.228
14	-0.047	0.117	-0.40	0.689	-0.276	0.183
Anti-Tg	0.001	0.001	2.25	0.025	0.000	0.002
Anti-TPO	0.001	0.000	3.38	0.001	0.001	0.002
Second-hand smoke						
No[d]	Ref					
Yes	0.016	0.114	0.14	0.889	-0.207	0.239
Do not remember	-0.404	0.904	-0.45	0.655	-2.177	1.369

Note. B= unstandardized regression coefficient; *SE*=Standard Error.
[a] Multiple linear regression model evaluating the association between MUF and maternal log transformed TSH while controlling for relevant covariates (*n*= 1271; R^2= 0.045; Adjusted R^2= 0.029; *F* (21, 1249) = 2.79; Prob > *F*= .000).
[b] Ten cities of residence were reported numerically; numbers were randomly assigned.
[c] City coded as 1 served as the reference category.
[d] "No" second-hand smoke exposure was the reference category.

Figure 11

Interaction between MUF concentration and fetal sex in predicting maternal TSH levels.



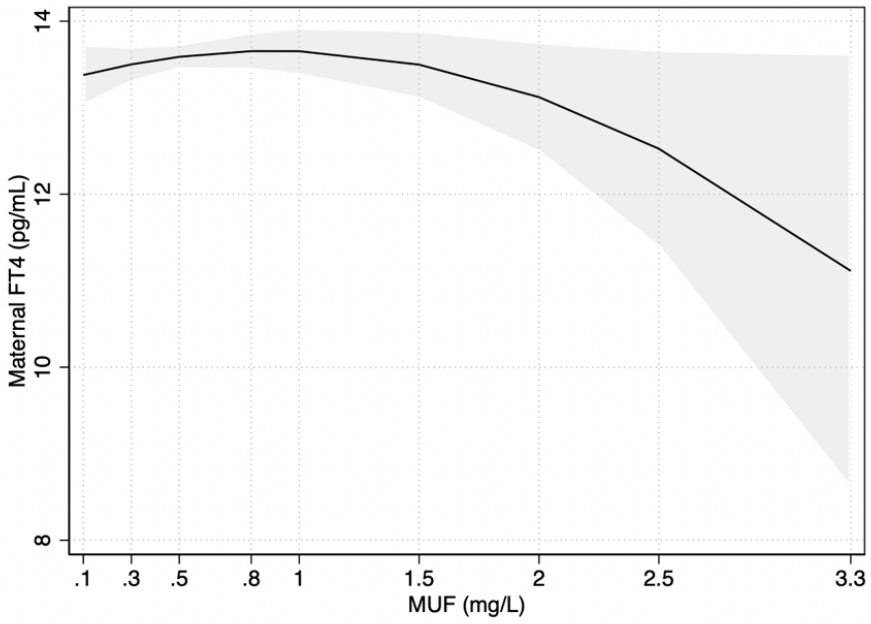
Note. Females: women carrying female fetuses; Males: women carrying male fetuses. Depicted associations were adjusted for covariates.

Results were similar when evaluating the associations between MUF and non-log transformed maternal TSH (Supplemental Table 2), and MUF and non-log transformed TSH when TSH values were trimmed at the upper end of the cohort-specific reference range (i.e., 4.94 μIU/mL; Supplemental Table 3). All three models of MUF predicting TSH violated the assumption of homogeneity of variance using the Cook-Weisberg test, indicating that the variance of the residuals is not equal across the levels of MUF. Further inspection revealed no outliers or influential cases for the model of MUF predicting log TSH (Cooks' d values < 0.1). Thus, violation of the assumption of homogeneity of variance is likely attributed to the large sample size (i.e., high power for the Cook-Weisberg test) and positively skewed distribution of maternal TSH, even after log transformation.

MUF and FT4. Regression analysis revealed no statistically significant linear association between MUF concentration and maternal FT4 (B= -0.13; *SE*= 0.20; 95% CI: -0.53, 0.27; *p*= .52). When the quadratic term was included in the model, the association between MUF and FT4 was such that at MUF concentrations above 0.30 mg/L, higher MUF was associated with lower FT4 (B= -0.44; *SE*= 0.24; 95% CI: -0.92, 0.04; Figure 12); however, the association was not statistically significant (*p* quadratic term= .07). There was no evidence of effect modification by fetal sex in the association between MUF and FT4 in the linear model (interaction term: B= 0.11; *SE*= 0.37; 95% CI: -0.62, 0.84; *p*= .77) or the quadratic model [(linear interaction term: B= 0.50; *SE*= 0.51; 95% CI: -0.51, 1.50; *p*= .33) (quadratic interaction term: B= -0.40; *SE*= 0.49; 95% CI: -1.35, 0.55; *p*= .41)]. The assumption of homogeneity of variance was violated for both the linear and quadratic models; however, when the models were rerun using log transformed FT4, there were no longer any violations. Using log FT4, results were consistent (Supplemental Table 4).

Figure 12

Quadratic association between MUF and maternal FT4.



MUF and TT4. Based on linear regression analysis, there was no statistically significant association between MUF concentration and maternal TT4 (B= -1.18; *SE*= 1.58; 95% CI: -4.29, 1.92; *p*= .46). Further, there was no evidence of effect modification by fetal sex in this association; the interaction between fetal sex and MUF in predicting maternal TT4 was not significant (interaction term: B= 0.30; *SE*= 2.91; 95% CI: -5.41, 6.02; *p*= .92).

Sensitivity analyses with maternal iodine intake. Women with data on MUF and TSH, TT4, or FT4 had a median (IQR) daily maternal iodine intake of 387.36 (293.97) µg/day, 387.90 (294.02) µg/day, and 386.14 (294.68) µg/day, respectively. Adding average daily maternal iodine intake as a covariate in the models of MUF predicting maternal THs (i.e., TSH, FT4, and TT4) did not produce significantly different results (Supplemental Tables 5-7). The general patterns observed in the associations between MUF and TSH, FT4, and TT4 remained consistent; however, there was a small decline in model fit when evaluating the association between MUF and TSH (i.e., R^2 declined from .045 to .044 and adjusted R^2 from .029 to .026; Supplemental Table 5). Importantly, however, the interaction between MUF and fetal sex in predicting maternal TSH remained significant (*p* interaction term= .02; Supplemental Table 5).

7.3.2. Maternal thyroid hormones (i.e., TSH, FT4, and TT4) and child FSIQ (Aim 2b). There was no statistically significant linear association between maternal TSH and child FSIQ (*n*= 480; B= 0.75; *SE*= 0.60; 95% CI: -0.42, 1.93; *p*= .21), between maternal FT4 and child FSIQ (*n*= 504; B= 0.02; *SE*= 0.20; 95% CI: -0.37, 0.40; *p*= .93), or between maternal TT4 and child FSIQ (*n*= 506; B= -0.00; *SE*= 0.03; 95% CI: -0.06, 0.05; *p*= .96). Further, there was no evidence of effect modification by child sex in any of these associations ([TSH interaction term: B= 0.67; *SE*= 1.23; 95% CI: -1.74, 3.08; *p*= .58]; [FT4 interaction term: B= -0.12; *SE*= 0.39; 95% CI: -

0.89, 0.65; *p*= .76]; [TT4 interaction term: B= -0.03; *SE*= 0.05; 95% CI: -0.13, 0.08; *p*= .62]). Mediation analysis in the counterfactual framework was not conducted given the lack of statistically significant associations between maternal THs (i.e., TSH, FT4, and TT4) and child FSIQ.

Regression diagnostics confirmed no issues with collinearity (variance inflation factor < 4 for all covariates), assumption violations (measured by plots of residuals vs. fitted values and Cook-Weisberg test for heteroskedasticity), or influential cases or outliers (measured by Cook's distance) in any of the above models other than those already described for the models of MUF predicting TSH and FT4.

8.0 Discussion.

This is the first cohort study to investigate the relationships between maternal fluoride exposure and thyroid function in pregnancy, and child IQ among mother-child dyads living in areas with and without water fluoridation.

8.1 Aim 1. Our findings indicate that maternal fluoride exposure was not associated with increased risk of subclinical hypothyroidism among this sample of pregnant women. In contrast, fluoride measured in women's drinking water, but not their urine (i.e., MUF), was significantly associated with increased risk of having a diagnosis or meeting criteria for primary hypothyroidism in the first trimester. Primary hypothyroidism in gestation was further associated with lower child FSIQ; however, this association was only significant among male children. Notably, we observed lower child IQ among hypothyroid women despite the majority of women (67.9%) being treated for their thyroid disorder.

Despite finding statistically significant associations between maternal water fluoride exposure and primary hypothyroidism in the full sample (*n*= 1022), and between primary

hypothyroidism and child FSIQ in the subsample with IQ (*n*= 439), results from formal mediation analysis were not statistically significant, even when the sample was restricted to only include women with male children. It is important to note, however, that we were unable to account for all relevant covariates in the mediation model due to collinearity between some of our main variables of interest (e.g., city of residence could not be controlled for due to collinearity with water fluoride concentration but is an important covariate in models predicting child IQ) and relevance to our main predictor (e.g., controlled for HOME score which is not relevant to water fluoride concentration). Moreover, considering only 25 of 106 (23.63%) children of mothers with primary hypothyroidism had IQ data (Figure 7), there may have been insufficient statistical power in the mediation model to detect a significant indirect effect. For these reasons, it is thus plausible that primary hypothyroidism in pregnancy could be a mediator of the previously found negative association between maternal water fluoride exposure and child FSIQ in the MIREC cohort, particularly among women with male children.

Findings from Aim 1 are consistent with those of previous studies. Specifically, we found that higher water fluoride concentration predicted greater risk of primary hypothyroidism in pregnancy, which aligns with reports from previous studies of a positive association between drinking water-fluoride level and prevalence of diagnosed hypothyroidism among non-pregnant adults (Chaitanya et al., 2018; Peckham et al., 2015). In contrast, we did not find evidence of an association between maternal fluoride exposure (i.e., water fluoride and MUF concentration) and risk of subclinical hypothyroidism in pregnant women in the present thesis. There are a few factors that may account for this finding. First, the subclinical hypothyroid status category was derived by classifying women based on their TH levels at trimester one, and thus does not equate to a formal diagnosis of subclinical hypothyroidism. On a similar note, it is well understood that

pregnancy-associated changes in TH levels are common and that it is not unusual for abnormalities in TH levels to be detected in routine blood work during this period (Brent, 1997; Glinoer, 1997). Lastly, the thyroid gland, and hypothalamic-pituitary-thyroid (HPT) axis more broadly, has been shown to be a dynamic and adaptive system that can continue to function normally even through periods of long-term stress, and therefore, may take longer to become disordered (Chatzitomaris et al., 2017; Glinoer, 1997). These physiological factors may explain the differences in results observed between women classified as subclinical versus primary hypothyroid in the MIREC cohort.

Results of previous studies examining the association between maternal hypothyroidism in gestation and child neurodevelopmental outcomes, namely IQ, further support our finding that women with primary hypothyroidism were more likely to have children with lower IQ scores (Andersen et al., 2018; Haddow et al., 1999; Levie et al., 2018). Regarding sex-specific associations, a previous study found that boys whose mothers were hypothyroid in pregnancy had an incidence rate for ADHD that was four times greater than for girls (Peltier et al., 2020). Sex-specific findings have also been identified in the association between maternal thyroid dysfunction in pregnancy and internalizing problems in the offspring (Andersen et al., 2018). Thus, sex differences in the association between maternal hypothyroidism in pregnancy and child IQ scores are plausible.

8.2 Aim 2. Associations between women's MUF concentrations and individual TH levels in pregnancy were variable. There was an association between MUF and TSH, where a 1 mg/L increase in MUF concentration was associated with a 13% increase in maternal TSH. Interestingly, however, this positive association was only found to be statistically significant among women carrying females, where a 1 mg/L increase in MUF was associated with a 35%

increase in maternal TSH. Further, MUF was not significantly associated with maternal FT4 or TT4. Despite finding a significant association between MUF and TSH, there was no evidence of a relationship between any maternal thyroid hormone levels and child FSIQ scores. Thus, results from Aim 2 do not support changes in maternal thyroid hormone levels as a mediator of maternal fluoride exposure-child FSIQ associations in this Canadian prospective pregnancy and birth cohort.

Findings from Aim 2 are consistent with some previous studies examining associations between fluoride exposure and individual TH levels in children and adults, where higher levels of fluoride in drinking water and elevated urinary fluoride concentration were associated with higher TSH and lower T4 levels (Khandare et al., 2018; Kheradpisheh et al., 2018; Wang et al., 2020). We did not find evidence of an association between these continuous measures of maternal TH levels in gestation and child IQ, which contradicts previous reports of an association between lower maternal FT4 in gestation and lower child IQ (Korevaar et al., 2015; Levie et al., 2018), but may be supported by others indicating that maternal TSH is unlikely to be associated with child IQ as it does not cross the placental barrier (Korevaar et al., 2018).

8.3 Comparing results from Aims 1 and 2. Having reviewed the main findings from Aims 1 and 2 and how they fit within existing literature, it is important to comment on the differences in results obtained for both aims. Specifically, results from Aim 1 suggest that higher water fluoride concentration increases risk of maternal thyroid dysfunction (i.e., primary hypothyroidism) in pregnancy, which in turn is associated with lower child FSIQ. However, evidence for maternal hypothyroidism as a main mechanism underlying fluoride-induced FSIQ decrements in males (Green et al., 2019) was not supported in our mediation analysis, most likely because our mediation analysis only included one quarter of the hypothyroid women.

Likewise, results from Aim 2 provide no evidence of mediation when maternal thyroid dysfunction was estimated using continuous measures of maternal THs (i.e., log TSH, FT4, and TT4). One reason for this may be a result of inherent differences between categorical classifications of thyroid health status and continuous measures of individual TH levels. Our primary hypothyroid category included not only those women with a pre-existing diagnosis (although self-reported), but those who met criteria for primary hypothyroidism based on both their TSH and FT4 levels at trimester one. This allowed us to capture a certain level of specificity regarding the underlying pathology of primary hypothyroidism, including the relationship between THs, that is not possible to quantify using individual TH levels (Korevaar et al., 2018). Moreover, by excluding the women who were being treated with medication for hypothyroidism, we may have reduced the sensitivity of our analysis to detect a relationship between fluoride exposure and THs in Aim 2.

8.3.1 Fluoride exposure measures. Differences in results by maternal fluoride exposure were observed in Aim 1, whereby fluoride in drinking water, but not MUF, was significantly associated with risk of primary hypothyroidism. These differences could be because water fluoride concentration may be more indicative of chronic fluoride exposure than urinary fluoride, which may be a more accurate measure of current exposure levels. As mentioned, thyroid disorders tend to develop over time due to well-developed compensatory mechanisms of the thyroid gland and HPT axis; thus, it is reasonable that our measure of chronic fluoride exposure would be more strongly associated with increased risk of primary hypothyroidism (Chatzitomaris et al., 2017; Glinoer, 1997). It is also important to note that women with previous diagnoses of hypothyroidism were diagnosed prior to pregnancy and their enrollment in the MIREC study; however, MUF was measured during pregnancy, and thus, likely following

diagnosis for most women. Therefore, these findings make sense temporally as well, in that we would not expect a variable measured in pregnancy to predict risk of a disorder diagnosed before pregnancy. Interestingly, other studies conducted by our group also found that water fluoride concentration was a predictor of different health outcomes (i.e., sleep quality and ADHD) where MUF concentration was not (Cunningham et al., 2021; Riddell et al., 2019).

Analyses in Aim 2 should be replicated using water fluoride concentration as the predictor to determine if there are any differences in associations between maternal fluoride exposure and TH levels when using the estimate of chronic fluoride exposure. Yet, this should not discount our use of MUF concentration as the main measure of maternal fluoride exposure in Aim 2. Urinary fluoride concentration is a high-quality biomarker of fluoride exposure (i.e., the gold standard) that allows for more precise estimates of individuals' fluoride intake from multiple sources. Furthermore, direct associations have been observed between biomarkers of other environmental chemicals and TH levels in pregnancy (Derakshan et al., 2021; Preston et al., 2020), providing additional support for the use of urinary fluoride concentration in our investigation.

8.3.2 Iodine status. Iodine is an essential nutrient for TH synthesis and has been shown to play an important role in determining the magnitude of fluoride's effect on thyroid function (NRC, 2006). An epidemiological study using data from the CHMS found evidence of effect modification by iodine status, in that a statistically significant association between urinary fluoride concentration and higher TSH was observed among non-pregnant adults who were classified as having moderate-to-severe iodine deficiency (18% of overall sample) when compared to those who were iodine sufficient (Malin et al., 2018). Further, in a recent cross-sectional study, urinary iodine concentration was found to modify the positive association

between urinary fluoride concentration and thyroid volumes among school-aged children living in China, where children with higher urinary iodine were found to be less susceptible to fluoride's effects on the thyroid (Du et al., 2021). Effect modification by maternal iodine status in pregnancy was not examined in the present thesis as it was beyond the scope; yet, we did consider maternal iodine status as a potential confounder in associations between MUF and maternal TH levels by including maternal daily iodine intake as a covariate in sensitivity analyses. Although iodine intake was not a significant covariate (*p* value = .29), inclusion of this variable as a covariate in the association between MUF and TSH resulted in a slight reduction in model fit (Adjusted R^2 changed from .029 to .026). Notably, however, loss of the effect was explained by a decrease in sample size given that fewer women had data on iodine intake (*n* decreased from 1271 to 1199). Most notably, the sex specific effect found in this association (i.e., positive association for women carrying females) remained significant when daily iodine intake was added as a covariate. One reason that iodine intake did not appreciably change the results of our models is because the MIREC cohort was recently found to be a predominantly iodine sufficient sample (Krzeczkowski et al., 2023). Therefore, it is unlikely that maternal iodine status will modify the associations between MUF and maternal TH levels among women in this sample. The fact that women in the MIREC cohort are mostly iodine sufficient makes the current findings even more concerning given that inadequate iodine intake, a risk factor for thyroid dysfunction, including hypothyroidism, would be expected to result in a stronger association between water fluoride exposure and thyroid dysfunction. A recent study by our group involving 366 mothers and children found greater IQ loss from maternal fluoride exposure among boys whose mothers had low iodine during pregnancy (Goodman et al., 2022). Accordingly, further investigation is warranted to understand how fluoride may disrupt thyroid

function among pregnant women with adequate iodine intake. It may also be important to explore this interaction among iodine deficient women given that moderate-to-severe iodine deficiency was found in ~32% of non-pregnant Canadian women of childbearing age (Bertinato et al., 2021), and about one quarter of pregnant women living in Toronto (Katz et al., 2013).

8.3.3 Sex differences. Sex differences were observed in the results from both Aims 1 and 2. First, primary hypothyroidism in gestation was only significantly associated with lower FSIQ among male children. Few, if any, studies have explored effect modification by child sex when assessing the relationship between maternal thyroid function in pregnancy and offspring IQ. Importantly, however, sex differences have been observed in children's susceptibility to adverse neurodevelopmental outcomes, with males being disproportionally affected (Kozhemiako et al., 2020; Schore, 2017). In the context of neurotoxicants, the male brain has been shown to be more vulnerable to many toxic exposures (e.g., fluoride, lead, some organochlorine pesticides, air pollution, etc.) when compared with similarly exposed females (Green et al., 2020; Kern et al., 2017). Males increased vulnerability to neurodevelopmental disorders such as ADHD, and adverse outcomes like lowered IQ, may be explained by potentiating effects of co-exposure to neurotoxicants and testosterone, greater neuroinflammatory responses and increased vulnerability to oxidative stress, and the neuroprotective effects of estrogen and progesterone for females (Kern et al., 2017). Moreover, recent studies suggest that sex differences may exist in women's TH levels during pregnancy, where women pregnant with males were found to be more likely to have elevated TSH (Sitoris et al., 2022; Wang et al., 2019). Whether women pregnant with males are more likely to develop hypothyroidism in pregnancy, however, is unclear. The notion that the developing male brain may be more sensitive to disruption overall, together with findings suggesting sex-based

differences in maternal TH levels in pregnancy, may aid in providing an explanation for the sex differences observed in results from Aim 1.

In contrast, we found that higher MUF was significantly associated with higher maternal TSH among women pregnant with females, potentially suggesting that higher TSH in the context of fluoride exposure may be protective. Considering the sensitivity and resilience of the HPT axis, particularly in times of stress, it is plausible that maternal TSH, and in turn T4 and T3, would increase in response to exposure to endocrine disrupting chemicals during such a critical period as gestation. Importantly, it is likely that both the direction and magnitude of this compensatory response would be dependent on the level of exposure (Chatzitomaris et al., 2017). While sex differences have not been previously explored in the relationship between maternal fluoride exposure and thyroid function in pregnancy, a recent study observed sex differences in Chinese school-aged children's thyroid gland volumes in response to fluoride exposure (Du et al., 2021). Sex differences have also been found in pregnant women and children's TH levels (e.g., TSH) in response to other neurotoxicants such as perfluoroalkyl substances (Ballesteros et al., 2017). More specifically, these studies found significant positive associations between neurotoxicant exposures and thyroid function among women pregnant with males and male offspring themselves (Ballesteros et al., 2017; Du et al., 2021). In contrast, another study found evidence of a stronger positive association between maternal exposure to perfluorooctanoic acid in pregnancy and cord blood-TSH levels among female offspring (Liang et al., 2020). Given evidence of sex specific findings in associations between neurotoxicant exposures and thyroid function, it is plausible that there may be a protective effect among female fetuses, whereby mothers' TH production increases (hence the elevations in TSH) in response to greater fluoride exposure; the biological mechanism that may explain this, however, is unknown.

Considering the HPT axis is a complex system of feedback loops, elevated TSH may also be indicative of decreasing TH levels; thus, alternatively, it is possible that the developing female brain may be more resilient to fluctuations (drops) in maternal TH levels in utero (Kern et al., 2017). Overall, our sex specific findings may provide support for an optimal level of maternal TSH in pregnancy, where very high elevations in TSH may be indicative of disorder (i.e., hypothyroidism) and have potential adverse effects on fetal neurodevelopment, and slight elevations in TSH may be protective by ensuring adequate TH availability to the developing fetus.

8.4 Results in context. The present thesis provides evidence to suggest that primary hypothyroidism in pregnancy may play a role in the previously found negative association between maternal water fluoride exposure and child FSIQ among mother-child dyads in the MIREC cohort. Further, higher MUF concentration was associated (though not significantly) with both higher TSH and lower FT4 measured at trimester one among women in this study, suggesting that pregnant women may be especially vulnerable to thyroid disruption by fluoride exposure. Considering our findings, it is important to revisit how fluoride may impact thyroid function in pregnancy and the effects of TH deficiency on fetal neurodevelopment. The effects of fluoride on the thyroid gland may be especially pronounced during pregnancy when there is an increased demand on the maternal thyroid system to meet the requirements of the fetus (Brent, 1997) or in the presence of iodine insufficiency during pregnancy.

8.4.1 Fluoride and thyroid function. There are a few proposed mechanisms that may explain fluoride's potential adverse effects on thyroid function. For instance, it has been suggested that fluoride may interfere with thyroid function by inhibiting the deiodinase enzymes that are necessary for the production of THs (i.e., inhibiting activation of T4 to T3; Brent, 1997;

Moog et al., 2017; Susheela et al., 2005). This means that fluoride could decrease TH (namely T3) production and blood-T3 and T4 levels, subsequently increasing circulating TSH levels (Malin et al., 2018). Similarly, fluoride may also induce structural and functional changes to the follicular epithelial cells of the thyroid gland (e.g., decline in the colloidal content and damage to the endoplasmic reticulum) resulting in insufficient secretion of thyroglobulin, and thus disruption to thyroid hormone synthesis more broadly (Banji et al., 2013; Basha et al., 2011). Notably, we found evidence of a positive association between maternal fluoride exposure (i.e., MUF) and TSH levels, particularly among women carrying females. While the observed effects of MUF on TSH in this thesis may seem negligible, at the population level, a small increase in TSH during pregnancy could translate to a significant impact on children's neurodevelopment (Bellinger, 2012). In fact, there is some evidence to suggest that subclinical increases in maternal TSH in pregnancy may still be associated with adverse birth and neurodevelopmental outcomes among offspring (Korevaar et al., 2018).

It is possible that there may be other factors that place pregnant women at increased risk of fluoride-induced changes in thyroid function. Perhaps women who are at greater risk of developing hypothyroidism due to an underlying autoimmune condition (i.e., Hashimoto's disease; Brent, 1997) may be more vulnerable to fluoride induced changes in thyroid gland functioning, and more generally, less able to compensate for the increased stress put on the HPT axis. This hypothesis may be worth investigating considering ~ 15% of women in MIREC have TPO antibody levels (a marker of thyroid autoimmune disorders; Brent, 1997) above the upper end of the cohort-specific reference range (i.e., 5.61 IU/mL).

As mentioned, fluoride exposure has been associated with poorer sleep outcomes, including shorter sleep duration (Cunningham et al., 2021). Notably, sleep problems have been

shown to be both a predictor of thyroid dysfunction (Kim et al., 2019) and an outcome resulting from thyroid dysfunction (Green et al., 2021; Song et al., 2019), offering another potential area worth investigating.

Other studies have suggested that fluoride may interact with iodine to exert its negative effects on thyroid function. One proposed explanation for this relationship is that fluoride might inhibit the expression and activity of NISs that are necessary for mediating active iodide transport into the thyroid, resulting in lower iodine availability and the indirect suppression of TH production (Greer et al., 2002; Waugh, 2019). Importantly, however, a more recent experimental study (Buckalew et al., 2020) refuted this claim by showing that fluoride does not inhibit NIS activity in Fischer rat thyroid follicular cells. This, together with recent reports of the women in MIREC being largely iodine sufficient, may suggest that fluoride-iodine interactions are not at play in this case. Ultimately, further research in this area is needed to identify the mechanism by which fluoride exerts its negative effects on thyroid function.

8.4.2 Thyroid hormones and neurodevelopment. THs are critical for normal fetal neurodevelopment and have been shown to play a role in regulating nearly all stages of brain development, including neuron proliferation and migration, myelination, synapse formation, dendritic branching, etc. (Williams, 2008). THs of maternal origin are particularly critical in facilitating these processes, especially during early gestation, as the fetal thyroid does not become fully functional until mid-gestation (Andersen et al., 2013; de Escobar et al., 2004; Krassas et al., 2010; Thompson et al., 2018). In experimental studies, maternal TH deficiency in early gestation has been shown to have adverse effects on neurological development, including disrupted neuronal migration, leading to less defined cortical layers in the cerebral cortex and misplaced cells in the neocortex and hippocampus (Berbel et al., 2001). Deficits in offspring

brain cytoarchitecture and development resulting from maternal TH deficiency early in gestation have been observed in humans as well, with offspring being more likely to display problems in visual attention and processing, and gross motor skills (Zoeller & Rovet, 2004). More generally, adverse consequences of maternal TH deficiency (i.e., hypothyroidism) in gestation on offspring development have been largely documented and include increased risk of autism spectrum disorder, attention deficit hyperactivity disorder, and other neurodevelopmental conditions, as well as lowered IQ (Andersen et al., 2018; Haddow et al., 1999; Henrichs et al., 2010; Levie et al., 2018). Thus, it is clear that maternal TH deficiency or hypothyroidism in pregnancy can have adverse effects on offspring neurodevelopmental and neuropsychological outcomes. If fluoride does in fact have negative effects on maternal thyroid function in pregnancy, as some of our results suggest, this could have critical implications for those living in fluoridated communities. As such, this is an area of research that warrants urgent attention.

8.5 Strengths, limitations, and future directions. This thesis presents the first study to examine how exposure to optimal levels of fluoride may impact thyroid function in pregnancy, which is a strength in itself. Other strengths of this prospective study include the use of a large pregnancy and birth cohort with robust measures of maternal fluoride exposure and TH levels measured from women's urine and blood plasma, respectively. Further, we used the gold standard for TH measurement (ED-ID-MS and ID-HPLC-MS). The rich dataset associated with the MIREC study further allowed us to control for numerous potential confounding variables in our statistical analyses, including maternal iodine status, an essential nutrient for TH production. Considering we were able to address our aims in mother-child dyads living in cities with and without water fluoridation, and that majority of women were exposed to water fluoride levels below 0.7 mg/L (the optimal level), our results may be generalizable to other samples of mothers

and children living in areas with community water fluoridation.

Demographics of the MIREC cohort may be a limitation. Compared to the Canadian population, women in the MIREC cohort tend to be older, primarily White, with higher household incomes and education status, are more likely to be married/common law and less likely to smoke, and were more likely to report taking prenatal vitamins supplemented with iodine (Arbuckle et al., 2013). Some of these sociodemographic factors have been shown to be protective against exposures to chemicals in the environment and thyroid disorders (e.g., Caucasian ethnicity, non-smoking status, higher education), whereas others are established risks (e.g., older age; Huang et al., 2017; Diab et al., 2019). While this may limit our ability to generalize to the broader Canadian population (i.e., affecting external validity), it does not affect the internal validity of our work.

Regarding our measures of maternal thyroid function in pregnancy, there are some potential limitations worth considering. For one, there may be reporting bias related to self-reported diagnosis of primary hypothyroidism, in that there may have been some women who failed to report a thyroid disorder at study enrollment. Similarly, some participants may not have reported taking thyroid hormone medications. Considering these women were excluded from most analyses, inaccurate reporting of diagnoses or medication use could be an additional limitation. We were also missing important information regarding the timing of diagnosis and where the diagnoses were made. Further, the number of women with subclinical and primary hypothyroidism (and complete covariates etc.) in MIREC is relatively low, especially when looking at sex specific effects, which may result in reduced power to detect a significant effect and reduce precision of the model estimates. Furthermore, we did not control for thyroid-binding globulins (i.e., proteins that bind T4 to form TT4), which may increase measurement error in

TT4 levels given that levels have been shown to fluctuate over the course of pregnancy. We were also unable to control for biomarkers of other nutrients like selenium, which has been shown to play an important role in T4 to T3 activation by the iodothyronine deiodinases (Arthur et al., 1992). Future studies in this area may also want to obtain measures of maternal T3 as it may be more sensitive to fluoride action.

Another limitation is that we used spot urine samples without control for behaviours that could contribute to fluctuations in urinary fluoride concentration, such as consumption of fluoride-free bottled water or black tea prior to urine collection. However, effects of this limitation were mitigated by averaging urine fluoride across trimesters and adjusting for urinary dilution. MUF was also used as a proxy of prenatal fluoride exposure in the fetus, which may be a limitation given that maternal biomarkers do not always provide accurate estimates of fetal exposures because they do not account for variability in placental transport and metabolism (Andra et al., 2015). Finally, controlling for human chorionic gonadotropin levels in pregnancy may be more accurate than using estimates of gestational age reported in weeks.

Multiple comparisons and risk of a type 1 error may also be of concern considering we examined multiple thyroid hormone outcomes and diagnostic categories, and child IQ; however, given our main hypotheses were made a priori based on findings from previous experimental and epidemiological studies and that many of our findings were null, we did not adjust for multiple comparisons in our analyses. Specifically, we did not adjust our threshold for significance (e.g., Bonferroni adjustment, familywise error rate, etc.) because while we may have increased the probability of a Type I error by using multiple thyroid related outcomes (subclinical and primary hypothyroidism, and TSH, FT4, and TT4 levels) and two predictors (water fluoride and MUF concentration), adoption of a stringent *p* value could increase risk of a Type II error and

the probability of missing important findings.

A final limitation relates to the potential for confounding. In the current analysis, hypothyroidism was considered a potential mediator of the relationship between fluoride exposure and child IQ. However, it is conceivable that women with hypothyroidism may drink more water (or other fluoride-rich beverages, such as tea) because hypothyroidism is associated with increased thirst. In this case, hypothyroidism would be associated with higher fluoride exposure. The plausibility of reverse causality is unlikely, however, given that we used water fluoride concentration as the predictor, and not the volume of water consumed (i.e., fluoride intake or dose).

As a future direction, biomarkers of fluoride exposure (e.g., tooth dentin fluoride) and thyroid function (e.g., cord blood TH levels) should be acquired for children in MIREC to determine the relationship between fluoride and thyroid function in the fetus directly. Future studies may also want to obtain urine samples prior to conception when examining the link between this biomarker and risk of thyroid disorder in pregnancy. Considering findings that children's performance or non-verbal IQ may be more negatively affected by maternal TH deficiency in pregnancy than their verbal IQ scores (Levie et al., 2018), performance and verbal IQ should be explored as outcomes alongside FSIQ. Given that children's IQ may stabilize with age and that sex differences in IQ scores may fluctuate throughout childhood (Buczylowska et al., 2019), our aims should be explored using IQ scores obtained later in childhood.

8.6 Conclusions and implications. In this large Canadian pregnancy and birth cohort, higher exposure to fluoride from drinking water was strongly associated with higher risk of primary hypothyroidism in pregnant women. Higher levels of fluoride in maternal urine were associated with changes in TH levels that may be indicative of thyroid dysfunction and might be

specific to the sex of the fetus. Further, maternal primary hypothyroidism in pregnancy was associated with significantly lower FSIQ scores among male, but not female children in this cohort. Together, these findings provide some evidence to suggest that maternal thyroid dysfunction in pregnancy may be one mechanism that contributes to the association between maternal fluoride exposure, from drinking water, and lower offspring IQ, particularly among women with male children.

Pregnancy is a critical period that is dependent on the normal functioning of the maternal thyroid for optimal fetal growth and development. Maternal TH deficiency during pregnancy has been associated with increased risk of adverse neurodevelopmental outcomes in children, including lowered IQ. This area of research is important not only for understanding more about the effects of environmental chemicals on thyroid function, but the risks associated with maternal thyroid dysfunction in pregnancy and the proceeding effects on children's health. Considering the ubiquity of fluoride exposure, further research is needed to determine whether alterations in maternal THs may mediate neurodevelopmental outcomes in offspring in other populations, and to understand the mechanism through which fluoride may disrupt thyroid function in pregnancy.

Results from this thesis have notable implications. This research provides important contributions to the literature on the neurotoxic mechanisms of fluoride exposure that may help guide policy decisions on the safety of fluoride for vulnerable populations. More specifically, results of this study provide essential information to citizens, scientists, and policymakers about the safety of fluoride exposure for pregnant women and their children and form a significant contribution to the literature on the characterization of sensitive periods of exposure. This research may further inform risk assessment for Canadians and Americans living in fluoridated cities more generally.

VI. References.

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VII. Appendix.

Supplemental Table 1

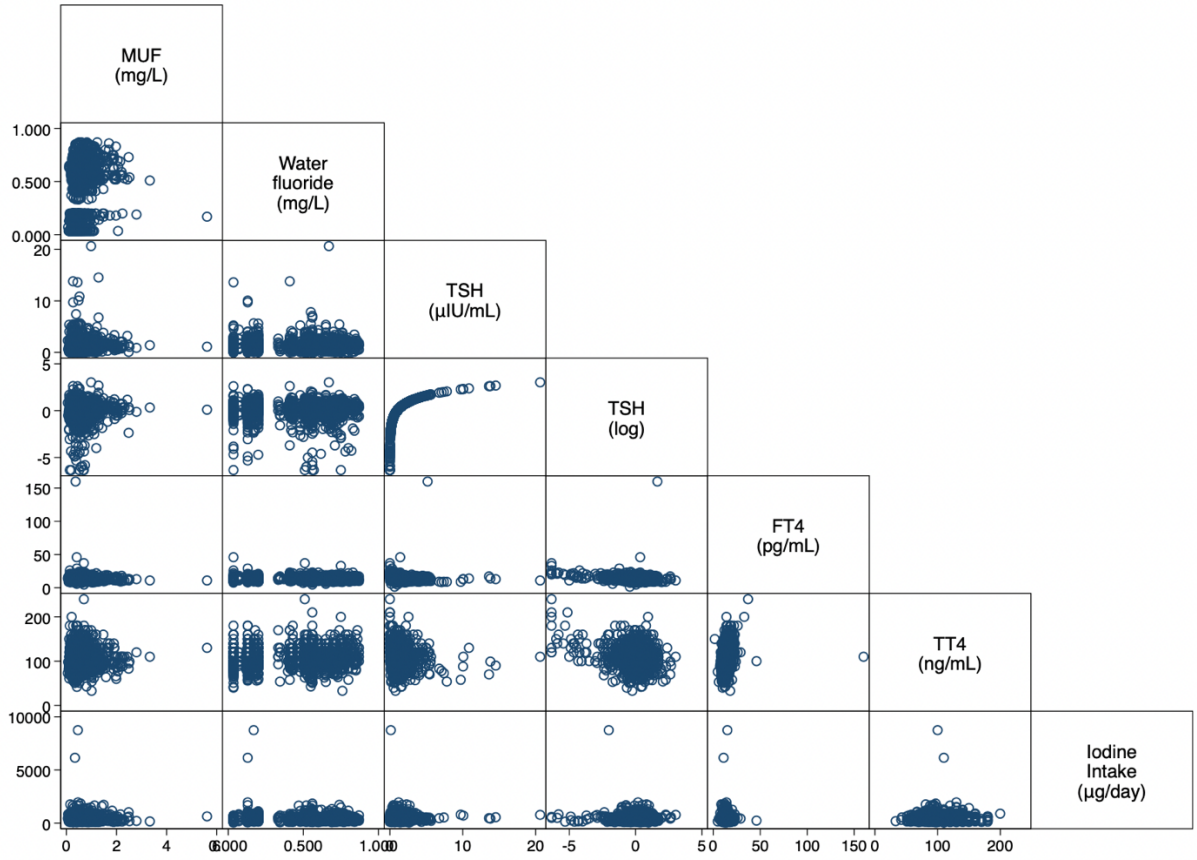
Spearman correlations between maternal fluoride exposure and thyroid hormone variables.

	MUF	Water Fluoride	TSH	FT4	TT4	Iodine Intake
MUF	1.00					
Water Fluoride	0.49*	1.00				
TSH	0.01	-0.003	1.00			
FT4	-0.03	-0.05	-0.18*	1.00		
TT4	0.03	0.07*	-0.10*	0.33*	1.00	
Iodine Intake	0.01	0.02	0.06*	-0.06*	-0.05	1.00

Note. * p value < .05

Supplemental Figure 1

Correlation matrix for maternal fluoride exposure and thyroid hormone variables.



Supplemental Table 2

MUF predicting TSH (untransformed).

TSH (μIU/mL)[a]	B	*SE*	*t*	P >| *t* |	95% Confidence Interval	
MUF	0.126	0.071	1.78	0.076	-0.013	0.266
Pre-pregnancy BMI	-0.002	0.005	-0.36	0.716	-0.011	0.008
Ethnicity	0.020	0.075	0.26	0.795	-0.128	0.167
Level of education	0.010	0.057	0.18	0.860	-0.101	0.121
Maternal age	-0.005	0.006	-0.87	0.382	-0.017	0.007
Parity	-0.095	0.031	-3.05	0.002	-0.156	-0.034
Fetal sex	0.077	0.050	1.54	0.123	0.021	0.175
Gestational age	-0.002	0.019	-0.11	0.913	-0.038	0.034
City of residence[b]						
5[c]	Ref					
6	-0.120	0.245	-0.49	0.624	-0.599	0.360
7	-0.007	0.150	-0.04	0.965	-0.301	0.288
8	0.040	0.115	0.35	0.729	-0.186	0.265
9	-0.057	0.127	-0.45	0.654	-0.307	0.193
10	0.028	0.159	0.18	0.859	-0.284	0.341
11	0.119	0.119	1.00	0.319	-0.115	0.354
12	-0.038	0.143	-0.27	0.789	-0.320	0.243
13	0.067	0.114	0.59	0.553	-0.155	0.290
14	-0.007	0.115	-0.06	0.952	-0.232	0.218
Anti-Tg	0.001	0.001	1.59	0.113	-0.000	0.002
Anti-TPO	0.002	0.000	7.51	0.000	0.002	0.003
Second-hand smoke						
No[d]	Ref					
Yes	0.031	0.112	0.27	0.784	-0.188	0.250
Do not remember	-0.739	0.888	-0.83	0.406	-2.481	1.003
TSH (μIU/mL)[e]						
MUF	0.353	0.108	3.82	0.001	0.142	0.564
Fetal sex*MUF	-0.366	0.131	-2.80	0.005	-0.624	-0.109

Note. B= unstandardized regression coefficient; *SE*=Standard Error.

[a] Multiple linear regression model evaluating the association between MUF and maternal TSH (μIU/mL) while controlling for relevant covariates (n= 1271; R^2= 0.076; Adjusted R^2= 0.060; F (21, 1249) = 4.88; Prob > F= .000).

[b,c,d] See Table 5 note.

[e] Multiple linear regression model evaluating effect modification by fetal sex in the association

between MUF and maternal TSH (n= 1271; R^2= 0.082; Adjusted R^2= 0.065; F (22, 1248) = 5.03; Prob > F= .000); female fetuses were the reference category for the interaction term.

Supplemental Table 3

MUF predicting TSH with maternal TSH trimmed at 4.94 µIU/mL.

TSH (< 4.94 µIU/mL)[a]	B	*SE*	*t*	P >| *t* |	95% Confidence Interval	
MUF	0.079	0.059	1.32	0.189	-0.039	0.196
Pre-pregnancy BMI	0.000	0.004	0.08	0.938	-0.008	0.008
Ethnicity	-0.004	0.063	-0.06	0.955	-0.128	0.120
Level of education	0.029	0.048	0.62	0.539	-0.064	0.123
Maternal age	-0.006	0.005	-1.20	0.229	-0.016	0.003
Parity	-0.084	0.026	-3.21	0.001	-0.135	-0.033
Fetal sex	0.079	0.042	1.88	0.060	-0.003	0.162
Gestational age	0.013	0.016	0.85	0.394	-0.017	0.044
City of residence[b]						
5[c]	Ref					
6	-0.074	0.205	-0.36	0.718	-0.477	0.329
7	-0.049	0.126	0.39	0.697	-0.199	0.297
8	0.081	0.098	0.84	0.401	-0.108	0.271
9	-0.016	0.107	-0.15	0.881	-0.226	0.194
10	-0.074	0.134	-0.55	0.583	-0.337	0.190
11	0.108	0.101	1.07	0.286	-0.090	0.305
12	0.013	0.121	0.11	0.914	-0.224	0.250
13	0.071	0.096	0.75	0.456	-0.116	0.259
14	0.020	0.097	0.21	0.835	-0.170	0.210
Anti-Tg	0.001	0.000	2.88	0.004	0.000	0.002
Anti-TPO	0.001	0.000	5.00	0.000	0.001	0.002
Second-hand smoke						
No[d]	Ref					
Yes	0.067	0.094	0.72	0.472	-0.117	0.251
Do not remember	-0.636	0.746	-0.85	0.394	-2.099	0.827
TSH (< 4.94 µIU/mL)[e]						
MUF	0.186	0.091	2.04	0.042	0.007	0.365
Fetal sex*MUF	-0.173	0.111	-1.56	0.119	-0.390	0.045

Note. B= unstandardized regression coefficient; *SE*=Standard Error.

[a] Multiple linear regression model evaluating the association between MUF and maternal TSH trimmed at 4.94 µIU/mL while controlling for relevant covariates (n= 1264; R^2= 0.060; Adjusted

R^2= 0.044; *F* (21, 1242) = 3.77; Prob > *F*= .000).
[b,c,d] See Table 5 note.
[e] Multiple linear regression model evaluating effect modification by fetal sex in the association between MUF and maternal TSH trimmed at 4.94 μIU/mL (*n*= 1264; R^2= 0.062; Adjusted R^2= 0.045; *F* (21, 1241) = 3.71; Prob > *F*= .000); female fetuses were the reference category for the interaction term.

Supplemental Table 4

MUF predicting log transformed FT4 (linear and quadratic).

FT4 (log; LM)[a]	B	*SE*	*t*	P >| *t* |	95% Confidence Interval	
MUF	-0.009	0.014	-0.67	0.502	-0.037	0.018
Pre-pregnancy BMI	-0.004	0.001	-4.26	0.000	-0.006	-0.002
Ethnicity	-0.031	0.015	-2.08	0.38	-0.060	-0.002
Level of education	0.018	0.011	1.63	0.103	-0.004	0.040
Maternal age	-0.000	0.001	-0.26	0.795	-0.003	0.002
Parity	-0.004	0.006	-0.64	0.523	-0.016	0.008
Fetal sex	-0.009	0.010	-0.90	0.366	-0.028	0.010
Gestational age	-0.027	0.004	-7.44	0.000	-0.035	-0.020
City of residence[b]						
5[c]	Ref					
6	-0.038	0.049	-0.77	0.443	-0.133	0.058
7	-0.038	0.030	-1.28	0.199	-0.096	0.020
8	-0.045	0.023	-1.95	0.051	-0.089	0.000
9	-0.052	0.025	-2.09	0.037	-0.102	-0.003
10	-0.046	0.032	-1.45	0.148	-0.108	0.016
11	-0.059	0.024	-2.50	0.012	-0.106	-0.013
12	-0.006	0.028	0.21	0.835	-0.049	0.061
13	-0.033	0.022	-1.48	0.138	-0.077	0.011
14	-0.075	0.023	-3.31	0.001	-0.119	-0.031
Anti-Tg (IU/mL)	-0.000	0.000	-1.11	0.269	-0.000	0.000
Anti-TPO (IU/mL)	-0.000	0.000	-0.75	0.456	-0.000	0.000
Second-hand smoke						
No[d]	Ref					
Yes	-0.037	0.022	-1.69	0.092	-0.081	0.006
Do not remember	0.360	0.178	2.02	0.044	0.010	0.709
FT4 (log; QM)[e]						
MUF (centered)	0.017	0.021	0.84	0.403	-0.023	0.058
MUF (centered)^2	-0.030	0.017	-1.75	0.080	-0.063	0.004

Pre-pregnancy BMI	-0.004	0.001	-4.34	0.000	-0.006	-0.002
Ethnicity	-0.030	0.015	-2.03	0.042	-0.059	-0.001
Level of education	0.018	0.011	1.58	0.115	-0.004	0.040
Maternal age	-0.000	0.001	-0.33	0.744	-0.003	0.002
Parity	-0.003	0.006	-0.52	0.600	-0.015	0.009
Fetal sex	-0.009	0.010	-0.87	0.385	-0.028	0.011
Gestational age	-0.028	0.004	-7.49	0.000	-0.035	-0.020
City of residence[b]						
5[c]	Ref					
6	-0.045	0.049	-0.92	0.358	-0.141	0.051
7	-0.047	0.030	-1.56	0.119	-0.106	0.012
8	-0.051	0.023	-2.22	0.026	-0.097	-0.006
9	-0.060	0.025	-2.37	0.018	-0.110	-0.011
10	-0.054	0.032	-1.68	0.093	-0.117	0.009
11	-0.062	0.024	-2.63	0.009	-0.109	-0.016
12	0.000	0.028	0.01	0.990	-0.055	0.056
13	-0.032	0.022	-1.41	0.159	-0.075	0.012
14	-0.081	0.023	-3.53	0.000	0.125	-0.036
Anti-Tg (IU/mL)	-0.000	0.000	-1.05	0.294	-0.000	0.000
Anti-TPO (IU/mL)	-0.000	0.000	-0.77	0.443	-0.000	0.000
Second-hand smoke						
No[d]	Ref					
Yes	-0.036	0.022	-1.65	0.100	-0.080	0.007
Do not remember	0.351	0.178	1.97	0.049	0.001	0.700

Note. B= unstandardized regression coefficient; *SE*=Standard Error.
[a] Multiple linear regression model evaluating the association between MUF and maternal FT4 (log) while controlling for relevant covariates (n= 1306; R^2= 0.093; Adjusted R^2= 0.079; F (21, 1284) = 6.30; Prob > F= .000).
[b,c,d] See Table 5 note.
[e] Linear regression model with quadratic term included to evaluate the association between MUF centered around the mean MUF value for the sample (linear term) and MUF, centered and squared (quadratic term), and maternal FT4 (log) while controlling for relevant covariates (n= 1306; R^2= 0.096; Adjusted R^2= 0.080; F (22, 1283) = 6.16; Prob > F= .000).

Supplemental Table 5

MUF predicting TSH while controlling for daily maternal iodine intake.

Log TSH[a]	B	*SE*	*t*	P >| *t* |	95% Confidence Interval	
MUF	0.058	0.068	0.85	0.395	-0.076	0.192

Daily iodine intake	0.000	0.000	-1.06	0.290	0.000	0.000
Pre-pregnancy BMI	0.006	0.005	1.20	0.231	-0.004	0.015
Ethnicity	0.070	0.073	0.95	0.340	-0.074	0.214
Level of education	0.038	0.055	0.70	0.487	-0.069	0.146
Maternal age	-0.007	0.006	-1.18	0.236	-0.019	0.005
Parity	-0.076	0.030	-2.51	0.012	-0.135	-0.016
Fetal sex	0.128	0.049	2.62	0.009	0.032	0.223
Gestational age	0.008	0.018	0.45	0.653	-0.027	0.043
City of residence[b]						
5[c]	Ref					
6	-0.269	0.240	-1.12	0.263	-0.740	0.202
7	-0.083	0.147	-0.56	0.574	-0.370	0.205
8	0.023	0.114	0.21	0.837	-0.200	0.247
9	-0.019	0.127	-0.15	0.883	-0.268	0.231
10	-0.107	0.156	-0.69	0.493	-0.414	0.199
11	0.096	0.119	0..81	0.421	-0.138	0.330
12	-0.077	0.142	-0.54	0.587	-0.356	0.202
13	-0.050	0.112	-0.45	0.653	-0.271	0.170
14	-0.045	0.115	-0.39	0.699	-0.270	0.181
Anti-Tg	0.001	0.000	2.18	0.030	0.000	0.002
Anti-TPO	0.001	0.000	3.56	0.000	0.001	0.002
Second-hand smoke						
No[d]	Ref					
Yes	0.052	0.110	0.47	0.636	-0.164	0.269
Do not remember	-0.415	0.839	-0.50	0.620	-2.061	1.230
TSH (log)[e]						
MUF	0.236	0.103	2.28	0.023	0.033	0.439
Fetal sex*MUF	-0.288	0.126	-2.29	0.022	-0.535	-0.041

Note. B= unstandardized regression coefficient; *SE*=Standard Error.
[a] Multiple linear regression model evaluating the association between MUF and maternal log TSH while controlling for average daily maternal iodine intake and relevant covariates (n= 1199; R^2= 0.044; Adjusted R^2= 0.026; F (22, 1176) = 2.46; Prob > F= .000).
[b,c,d] See Table 5 note.
[e] Multiple linear regression model evaluating effect modification by fetal sex in the association between MUF and maternal log transformed TSH with maternal iodine intake added as a covariate (n= 1199; R^2= 0.048; Adjusted R^2= 0.030; F (23, 1175) = 2.59; Prob > F= .000); female fetuses were the reference category for the interaction term.

Supplemental Table 6

MUF predicting FT4 (linear and quadratic) while controlling for daily maternal iodine intake.

FT4 (pg/mL; LM)[a]	B	*SE*	*t*	P >| *t* |	95% Confidence Interval	
MUF	-0.086	0.203	-0.42	0.672	-0.484	0.312
Daily iodine intake	0.000	0.000	-0.69	0.491	-0.001	0.000
Pre-pregnancy BMI	-0.053	0.014	-3.81	0.000	-0.081	-0.026
Ethnicity	-0.389	0.217	-1.79	0.073	-0.814	0.036
Level of education	0.307	0.162	1.90	0.058	-0.010	0.625
Maternal age	-0.006	0.017	-0.32	0.747	-0.040	0.029
Parity	-0.065	0.090	-0.72	0.471	-0.241	0.111
Fetal sex	-0.137	0.144	-0.95	0.341	-0.421	0.146
Gestational age	-0.348	0.054	-6.49	0.000	-0.454	-0.243
City of residence[b]						
5[c]	Ref					
6	-0.611	0.719	-0.85	0.396	-2.022	0.800
7	-0.467	0.433	-1.08	0.280	-1.316	0.381
8	-0.636	0.338	-1.88	0.060	-1.298	0.026
9	-0.746	0.374	-1.99	0.046	-1.480	-0.012
10	-0.740	0.465	-1.59	0.111	-1.651	0.171
11	-0.834	0.353	-2.36	0.018	-1.526	-0.142
12	-0.065	0.418	-0.16	0.876	-0.885	0.755
13	-0.488	0.331	-1.47	0.141	-1.137	0.161
14	-1.014	0.339	-2.99	0.003	-1.680	-0.349
Anti-Tg (IU/mL)	-0.001	0.001	-0.83	0.407	-0.004	0.002
Anti-TPO (IU/mL)	-0.001	0.001	-0.81	0.421	-0.003	0.001
Second-hand smoke						
No[d]	Ref					
Yes	-0.487	0.327	-1.49	0.137	-1.129	0.155
Do not remember	5.181	2.520	2.06	0.040	0.236	10.125
FT4 (pg/mL; QM)[e]						
MUF (centered)	0.297	0.303	0.98	0.328	-0.299	0.892
MUF (centered)^2	-0.414	0.244	-1.70	0.090	-0.893	0.065
Daily iodine intake	0.000	0.000	-0.73	0.468	-0.001	0.000
Pre-pregnancy BMI	-0.055	0.014	-3.89	0.000	-0.082	-0.027
Ethnicity	-0.380	0.217	-1.75	0.080	-0.805	0.045
Level of education	0.298	0.162	1.84	0.065	-0.019	0.616
Maternal age	-0.007	0.017	-0.40	0.688	-0.041	0.027
Parity	-0.054	0.090	-0.60	0.549	-0.231	0.123

Fetal sex	-0.133	0.144	-0.92	0.356	-0.416	0.150
Gestational age	-0.352	0.054	-6.55	0.000	-0.457	-0.246
City of residence[b]						
5[c]	Ref					
6	-0.738	0.722	-1.02	0.307	-2.156	0.679
7	-0.597	0.439	-1.36	0.174	-1.458	0.264
8	-0.740	0.343	-2.16	0.031	-1.412	-0.067
9	-0.863	0.380	-2.27	0.023	-1.609	-0.117
10	-0.859	0.469	-1.83	0.067	-1.780	0.062
11	-0.883	0.354	-2.50	0.013	-1.577	-0.189
12	-0.149	0.421	-0.35	0.723	-0.974	0.676
13	-0.470	0.331	-1.42	0.155	-1.119	0.179
14	-1.101	0.343	-3.21	0.001	-1.773	-0.429
Anti-Tg (IU/mL)	-0.001	0.001	-0.78	0.438	-0.004	0.002
Anti-TPO (IU/mL)	-0.001	0.001	-0.84	0.402	-0.003	0.001
Second-hand smoke						
No[d]	Ref					
Yes	-0.482	0.327	-1.48	0.140	-1.124	0.159
Do not remember	5.048	2.520	2.00	0.045	0.104	9.991

Note. B= unstandardized regression coefficient; *SE*=Standard Error.
[a] Multiple linear regression model evaluating the association between MUF and maternal FT4 while controlling for average daily maternal iodine intake and relevant covariates (n= 1234; R^2= 0.080; Adjusted R^2= 0.063; F (22, 1211) = 4.78; Prob > F= .000).
[b,c,d] See Table 5 note.
[e] Linear regression model with quadratic term included to evaluate the association between MUF centered around the mean MUF value for the sample (linear term) and MUF, centered and squared (quadratic term), and maternal FT4 while controlling for average daily maternal iodine intake and relevant covariates (n= 1234; R^2= 0.082; Adjusted R^2= 0.065; F (23, 1210) = 4.70; Prob > F= .000).

Supplemental Table 7

MUF predicting TT4 while controlling for daily maternal iodine intake.

TT4 (ng/mL)[a]	B	*SE*	*t*	P >| *t* |	95% Confidence Interval	
MUF	-0.386	1.621	-0.24	0.812	-3.566	2.794
Daily iodine intake	-0.002	0.002	-1.01	0.311	-0.005	0.002
Pre-pregnancy BMI	0.305	0.111	2.75	0.006	0.087	0.522
Ethnicity	-6.251	1.733	-3.61	0.000	-9.650	-2.852
Level of education	-0.457	1.292	-0.35	0.723	-2.991	2.077

Maternal age	-0.374	0.139	-2.68	0.007	-0.648	-0.101
Parity	-0.719	0.717	-1.00	0.316	-2.126	0.688
Fetal sex	-2.599	1.152	-2.26	0.024	-4.859	-0.339
Gestational age	1.666	0.430	3.88	0.000	0.823	2.509
City of residence[b]						
5[c]	Ref					
6	-0.370	5.766	-0.06	0.949	-11.683	10.943
7	0.369	3.448	0.11	0.915	-6.395	7.132
8	5.688	2.692	2.11	0.035	0.406	10.970
9	-1.022	2.987	-0.34	0.732	-6.883	4.839
10	-0.134	3.704	-0.04	0.971	-7.402	7.134
11	0.456	2.807	0.16	0.871	-5.052	5.963
12	3.444	3.320	1.04	0.300	-3.069	9.957
13	0.272	2.645	0.10	0.918	-4.917	5.461
14	2.122	2.708	0.78	0.433	-3.191	7.436
Anti-Tg	0.007	0.012	0.58	0.559	-0.017	0.031
Anti-TPO	-0.011	0.008	-1.45	0.146	-0.026	0.004
Second-hand smoke						
No[d]	Ref					
Yes	-1.749	2.626	-0.67	0.505	-6.901	3.402
Do not remember	-70.558	20.232	-3.49	0.001	-110.251	-30.866

Note. B= unstandardized regression coefficient; *SE*=Standard Error.
[a] Multiple linear regression model evaluating the association between MUF and maternal TT4 while controlling for average daily maternal iodine intake and relevant covariates (n= 1249; R^2= 0.070; Adjusted R^2= 0.053; F (22, 1226) = 4.20; Prob > F= .000).
[b,c,d] See Table 5 note.