ASSOCIATION BETWEEN FLUORIDE EXPOSURE AND IMMUNE SYSTEM BIOMARKERS IN PREGNANCY

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Abstract

Background: Fluoride may disrupt critical immune and inflammatory processes. Activation of the maternal immune response during pregnancy has been associated with increased risk of mood and neurodevelopmental disorders and altered fetal neurodevelopment. To date, no studies have investigated the relationship between gestational fluoride exposure and immunotoxicity.

Objective: The present study investigated whether fluoride exposure is associated with maternal immune system function in a Canadian pregnancy cohort.

Methods: Urinary fluoride concentrations were measured in each trimester, dilution-adjusted, and averaged across pregnancy. Drinking water fluoride concentrations (WFC) were collected from water treatment plants. Maternal immune system biomarkers included interleukin (IL)-6, IL-8, tumor necrosis factor-alpha (TNF-α), monocyte chemoattractant protein-1 (MCP-1), and C-reactive protein (CRP) from plasma samples collected during the third trimester of pregnancy.

Results: We observed a departure from linearity in the association between WFC and CRP, such that CRP levels increased with increasing WFC at levels below 0.63 mg/L. Urinary fluoride concentrations were not associated with maternal immune system biomarkers.

Conclusion: Water fluoride concentrations were associated with changes in some maternal immune system biomarkers, suggesting fluoride may be implicated in inflammatory processes during the third trimester. Further characterization of the timing of maternal immune activation is needed to advance our understanding of critical vulnerability periods during pregnancy.

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List of Abbreviations

BMI: Body mass index

CNS: Central nervous system

CRP: C-reactive protein

CWF: Community water fluoridation

IQ: Intelligence quotient

IL: Interleukin

MIA: Maternal immune activation

MIREC: Maternal-Infant Research on Environmental Chemicals

MCP-1: Monocyte chemoattractant protein-1

MUF: Maternal urinary fluoride

NaF: Sodium fluoride

NRC: National Research Council

NTP: National Toxicology Program

PNS: Peripheral nervous system

Th: Helper T cells

TNF-α: Tumor necrosis factor alpha

VIF: Variance Inflation Factor

WHO: World Health Organization

WFC: Water fluoride concentration

Introduction

One-third of Canadians have fluoride added to their municipal water supplies to prevent tooth decay (Public Health Agency of Canada, 2017). A growing body of evidence has suggested that ingestion of fluoride may be a developmental neurotoxicant, even at levels considered optimal for preventing dental caries (Choi et al., 2015; NTP, 2022). Studies have associated early-life fluoride exposure with lower intelligence quotient (IQ), internalizing symptoms, and increased risk of attention disorders among children (Adkins et al., 2022; Bashash et al., 2018; Green et al., 2019; Malin & Till, 2015; Riddell et al., 2019), although other studies have not found an association between fluoride exposure and lower IQ in children (Broadbent et al., 2015; Ibarluzea et al., 2022). Several mechanisms have been suggested to explain how fluoride exposure may contribute to adverse neurodevelopmental outcomes. These include biochemical effects in the brain and neurons, changes to neurotransmitters, thyroid hormone disruption, and oxidative stress on embryonic and fetal development (Hall et al., 2023; Strunecka & Strunecky, 2020; Wang et al., 2020; Wells et al., 2009). Disruption of the maternal immune system may also be implicated given its susceptibility to fluoride and putative relationship to neurodevelopmental disorders (Estes & McAllister, 2016; Knuesel et al., 2014). Pregnant women may be especially sensitive to immune disruption as a result of underlying inflammatory responses associated with gestation. In-vitro studies demonstrate both downregulation and overexpression of immune markers as a result of fluoride exposure (De La Fuente et al., 2016; Hosokawa et al., 2009). The proposed study evaluated the association between maternal fluoride exposure and markers of immune system function in a Canadian pregnancy cohort. This research will contribute to the literature on fluoride immunotoxicity to inform whether immune dysregulation in pregnancy may serve as a potential mechanism underlying the developmental neurotoxicity of fluoride.

Literature Review

Immune System Basics

The immune system, which is comprised of a network of proteins, cells, and organs, confers the host defense against pathogenic microbes via innate and adaptive responses (Chaplin, 2010). The innate, or non-specific, immune system responds rapidly to pathogens by generating a protective inflammatory response (Turvey & Broide, 2010). Conversely, the adaptive, or specific, immune system is slower to respond and requires prior exposure to produce an immune memory (Netea et al., 2019). While differing in their mechanisms of action, coordination between the innate and adaptive subsystems is critical in mounting an effective immune response. Misdirected defenses, such as failure of the immune system to differentiate "self" antigens from "non self" pathogens, are characteristic of autoimmune disorders, such as type 1 diabetes, rheumatoid arthritis, and multiple sclerosis (Lang et al., 2007). Interactions between cells of the immune system and soluble proteins regulate communication between innate and adaptive responses (Belardelli & Ferrantini, 2002).

Produced predominantly by helper T (Th) cells and macrophages, cytokines play a key role in mediating cell signaling and communication between immune and non-immune cells (Arango Duque & Descoteaux, 2014). Cytokines are ubiquitous in biological processes including, but not limited to, central nervous system (CNS) development, pathophysiology of mood disorders, and progression of neurodegenerative disorders (Allan & Rothwell, 2001; Deverman & Patterson, 2009; Ferro et al., 2021; Miller et al., 2009; Rosenblat et al., 2014; Smith et al., 2012). Broadly, cytokines include adipokines, chemokines, interferons, interleukins, mesenchymal growth factors, and tumor necrosis factors (Dinarello, 2007). These signaling proteins coordinate the body's defense against infections, while also facilitating communication between cells of non-

immune tissues, including the nervous system (Deverman & Patterson, 2009). Neuropoietic cytokines play crucial roles in cell survival, proliferation and differentiation, axonal growth, and synaptogenesis (Bauer et al., 2007). In the CNS and peripheral nervous system (PNS), neuropoietic cytokines also modulate responses following injury by promoting survival, upregulating expression of pro-inflammatory cytokines, and recruiting immune cells to the site of injury (Bauer et al., 2007).

The expression of cytokines and their receptors demonstrates regional specificity across developmental stages, suggesting that they may be implicated in important developmental functions (Deverman & Patterson, 2009). Indeed, region and age-specific patterns of cytokine expression emerge following activation of the maternal immune system during pregnancy (Garay et al., 2013). At birth, several cytokines are elevated in the frontal and cingulate cortices, followed by decreases during periods of synaptogenesis and plasticity, with subsequent increases in some regions in the adult mouse brain (Garay et al., 2013). These findings suggest long-lasting and age-specific changes in brain cytokines of the offspring following activation of the maternal immune system. An imbalance between pro- and anti-inflammatory cytokines, with a shift towards excess levels of pro-inflammatory cytokines, has been associated with the emergence of changes in brain and behaviour (Boksa, 2010; Depino, 2013; Ganguli & Chavali, 2021; Marques et al., 2013). In a study examining the association between circulating pro-inflammatory cytokines and mood disorders, adults with major depression had lower IL-10 levels and significantly higher IL-6/IL-10 ratios, suggesting that the apparent absence of immunoregulation may be contributing to a pro-inflammatory environment (Dhabhar et al., 2009). Similarly, a shift towards excess anti-inflammatory cytokines has also been associated with psychopathology, including major depressive disorder and schizophrenia (Meyer et al., 2007a; Meyer et al., 2009).

In the absence of a prenatal inflammatory event, increased prenatal serum IL-10 levels were associated with behavioural abnormalities in adulthood in a mouse model (Meyer et al., 2007a).

Cytokines may be classified based on functional status, cellular source, or role (pro-inflammatory, anti-inflammatory, or both; Liu et al., 2021). Release of pro-inflammatory cytokines from macrophages, such as interleukin (IL)-8, and tumor necrosis factor-alpha (TNF-α), provides signals to surrounding tissues that an injury or infection has occurred (Liu et al., 2021). Sudden, excessive levels of pro-inflammatory cytokines induced by infection and autoimmune conditions may result in a cytokine storm or cytokine release syndrome (Fajgenbaum & June, 2020). Pro-inflammatory cytokine response is modulated by anti-inflammatory cytokines, including IL-10, by repressing the expression of inflammatory cytokines and limiting sustained reactions (Zhang & An, 2007). Some cytokines may exhibit both pro- and anti-inflammatory properties depending on the amount and timing of cytokine release, nature of the activating signal, sequence of cytokine action, and other factors (Cavaillon, 2001). For example, IL-6 has both pro- and anti-inflammatory properties depending on the activation of different molecular mechanisms and signaling cascades (Scheller et al., 2011). Communication between cytokines and, more broadly, cells of the immune system is thereby critical for maintaining homeostasis.

Immune System Function During Pregnancy

Pregnancy presents a unique challenge for the immune system: when faced with a semi-allogeneic fetus, how does the maternal immune system respond to the expression of foreign paternal proteins? Historically, pregnancy has been associated with immune system suppression and increased susceptibility to infectious diseases; however, advances in reproductive immunology have led to the characterization of pregnancy as an immune condition that is

modulated, but not suppressed (Mor & Cardenas, 2010). One proposed mechanism underpinning tolerogenic states suggests that a combination of dynamic signals from the maternal-fetal interface may be implicated (Mor & Cardenas, 2010). The presence of innate and adaptive immune cells, activated by placental signals, are critical for fetal development and immune protection during early pregnancy (Mor et al., 2017). Indeed, distinct immunological profiles have been identified corresponding to each trimester.

The first trimester necessitates an inflammatory environment to support blastocyst implantation and placentation (Zenclussen & Hämmerling, 2015). Cytokine profiling from longitudinal serum samples reveals that most inflammatory cytokines peak during the first trimester, then decrease in the second trimester (Jarmund et al., 2021). Ongoing pro-inflammatory signals beyond the first trimester may be indicative of infection, which has been associated with miscarriage and inflammation-induced preterm birth (Romero et al., 2007). One exception appears to be C-reactive protein (CRP) – a widely-used measure of acute inflammation in the body. Some studies report a marked increase in CRP in the second trimester (Ferguson et al., 2014; Jarmund et al., 2021) or increases throughout gestation (Yu et al., 2019), while other studies describe no consistent changes (Belo et al., 2005). During the second trimester, decidual macrophages and regulatory T cells (Tregs) producing anti-inflammatory cytokines help prevent maternal immune responses against paternal antigens and promote fetal development (Mor et al., 2017). Increased levels of Tregs activity-regulated cytokines implicated in immunoregulation and anti-inflammation have also been observed in pregnant women during this period (Tagoma et al., 2019). In the third trimester, mobilization of pro-inflammatory factors has been associated with the initiation of labour, and continued progress of labour and delivery (Mor et al., 2017). However, overexpression of pro-inflammatory cytokines, due to inflammation or infection,

towards the end of the third trimester may lead to preterm labour and lower birth weight (Dizon-Townson, 2001; Ferguson et al., 2014; Greig et al., 1997; Romero et al., 2006).

The impact of maternal cytokine expression dysregulation on the fetus has health implications beyond the prenatal period. Although the ability to adjust regulatory systems rapidly in response to environmental stressors confers a clear adaptive advantage, it can also exacerbate adverse, long-term effects on physiological functions should there be discordance between the pre- and postnatal environments (Hsiao & Patterson, 2012). Exposure to cytokines *in utero*, as a result of maternal immune activation, may program the fetal immune system such that subsequent postnatal exposure to the immune stimulus results in exacerbated responses (Morelli et al., 2015).

Implications of Maternal Immune Activation

The maternal immune activation (MIA) hypothesis posits that systemic increases in inflammatory mediators during pregnancy pose a risk for neurochemical and behavioural changes across the lifespan (Estes & McAllister, 2016; Knuesel et al., 2014). Throughout pregnancy, microglia – the resident macrophages of the central nervous system – are critical immune regulators of neurodevelopment, including neurogenesis, synaptogenesis, synaptic pruning, and axonal growth (Reemst et al., 2016). Manipulation of critical genes supporting microglial morphology reveals that perturbations in the functions of microglia are associated with changes in functional brain connectivity and behaviour (Zhan et al., 2014). Disruptions of microglial functions may subsequently result in loss of homeostasis; indeed, immune activation during pregnancy has been associated with accelerated microglia development and altered microglial phenotypes (Hanamsagar & Bilbo, 2017; Loayza et al., 2023; Matcovitch-Natan et al., 2016). Accordingly, microglia dysfunction has been suggested as one mechanism underlying

aberrant neurodevelopment (Biblo et al., 2018; Matcovitch-Natan et al., 2016; Prins et al., 2018). Microglia participate in these processes, in part, by responding to and releasing cytokines (Deverman & Patterson, 2009).

In humans, activation of the maternal immune system response due to infection, chronic inflammatory conditions, psychosocial stress, and nutrient conditions has been linked to increased risk of mood and neurodevelopmental disorders, as well as altered brain development (Estes & McAllister, 2016; Han et al., 2021; Knuesel et al., 2014; Marques et al., 2013). Maternal exposure to environmental chemicals, such as perfluorinated compounds, has also been associated with reduced antibody response to immunization and immunosuppression in children (Brieger et al., 2011; Grandjean et al., 2012; Granum et al., 2013). Pronovost and Hsiao (2019) suggest that interactions between the gut microbiome, immune system, and nervous system may be associated with increased risk of neurodevelopmental disorders, such as autism spectrum disorder and schizophrenia. These findings are corroborated by epigenetic studies, which indicate that environmental insults during the prenatal period can alter the epigenetic signatures of both the brain and immune system (Han et al., 2021). Additionally, several maternal cytokines have been implicated as risk factors for the development of central nervous system disorders in children across the lifespan (Estes & McAllister, 2016). In a United States-based cohort, maternal IL-8 levels during the second trimester were significantly elevated for mothers of children with schizophrenia spectrum disorders ($n = 59$), compared to controls (Brown et al., 2004). Similarly, maternal TNF-α was also significantly elevated among offspring diagnosed with schizophrenia and psychotic spectrum disorders (Allswede et al., 2020; Buka et al., 2001). Results from a longitudinal study demonstrated that maternal IL-6 concentrations during pregnancy were predictive of, and negatively correlated with, working memory performance in

early childhood (Rudolph et al., 2018). Increased inflammatory cytokines, such as monocyte chemoattractant protein (MCP)-1, IL-6 and TNF- α, measured in maternal amniotic fluid and sera have also been associated with increased risk of neurodevelopmental disorders in children (Abdallah et al., 2013; Goines et al., 2011; Jones et al., 2017). However, others have reported that mothers of autistic children have lower CRP levels in mid-pregnancy (15-19 weeks of gestation; Zerbo et al., 2016). Inconsistencies among these findings underlie the need to better understand determinants of MIA, as well as the biological mechanisms underlying the association between immune system disruption during pregnancy and altered neurodevelopmental trajectories in offspring. Exposure to neurotoxicants in pregnancy has been implicated in the maternal immune response; here, we propose that maternal exposure to fluoride during pregnancy may induce MIA, which in turn, may mediate changes in offspring neurodevelopment.

Fluoride-Initiated Immune System Activation

Animal models: Exposure to fluoride has been associated with dysregulated immune system function in the animal literature; however, findings are inconsistent. Mice treated with sodium fluoride (NaF; ≥ 12 mg/kg) had significant decreases in percentages of T and B lymphocytes associated with innate and adaptive immune functions (Guo et al., 2017). This study also observed decreases in pro- and anti-inflammatory cytokine serum concentrations (TNF-α, IL-6, IL-10) compared to controls (Guo et al., 2017). Murine macrophage cell lines exposed to high concentrations of fluoride (≥ 50 mg/L) also had reduced macrophage populations, but increased levels of pro-inflammatory cytokines above 50 mg/L, and decreased IL-10 expression above 5 mg/L (De La Fuente et al., 2016). Similarly, rats treated with NaF had increased IL-6 protein expression levels and TNF-α immunoreactivity, as well as increased apoptotic neurons in the

cortex and hippocampus (Yan et al., 2016). An *in vitro* animal study performed on rabbit aorta also revealed that chronic exposure to fluoride increases RNA and protein expression levels of IL-6 and IL-8, suggesting that inflammatory responses may be implicated in fluoride toxicity (Ma et al., 2012). Male zebrafish exposed to fluoride expressed increased pro- (IL-1β and IL-6) and anti-inflammatory (IL-10, IL-13, TGF-β) cytokines 45 days, but not 90 days, after exposure (Wang et al., 2022). Several factors may be implicated in the differences observed at the two timepoints including exhaustion of the immune system, excessive anti-inflammatory responses to compensate for up-regulation, and neuroendocrine disturbances (Wang et al., 2022). These changes in inflammatory markers were significantly enhanced compared to female zebrafish, suggesting that males may be more sensitive to fluoride toxicity (Wang et al., 2022).

Human studies: In geographic regions where fluoride is endemic, ecological studies have revealed that adults had elevated levels of proteins involved in inflammatory processes, as well as decreased inhibition of cell proliferation and fewer T cells associated with immune system suppression (Hernández-Castro et al., 2011; Liu et al., 2014a). In children ages 6 to 12 exposed to fluoride in drinking water (5.3 mg/L), CD25 gene expression levels were negatively correlated with urinary fluoride concentrations (Estrada-Capetillo et al., 2014). The CD25 gene encodes a subunit of IL2-receptor, which binds IL-2 and regulates T cell function (Hinks et al., 2009). Among youth and adults chronically exposed to drinking water high in fluoride (4.02 – 5.44 mg/L) and arsenic (9.8 – 149.95 μg/L), there was a significant expression of genes associated with apoptosis and inflammation, and down-regulation of TNF-α, compared to non-exposed individuals (Salgado-Bustamante et al., 2010). In human leukocytes, fluoride has also been shown to inhibit locomotion and phagocytosis, and decrease cell adhesiveness (Gabler & Leong, 1979; Gómez-Ubric et al., 1992; Wilkinson, 1983). Incubation of monocytes with NaF resulted

in increased synthesis of reactive oxygen species and decreased production of ATP, consistent with oxidative stress (Gutowska et al., 2010). Similarly, fluoride has been associated with decreased activity of lipoxygenases implicated in the synthesis of inflammatory mediators (Gutowska et al., 2012). More recently, Den Besten et al. (2022) examined blood cell markers of inflammation in youth aged 6 to 19 living in the United States where water fluoride levels (0.56 ± 0.44 ppm) are typically below 1 mg/L. Plasma fluoride was positively associated with white blood cell count, segmented neutrophils, and monocytes, all of which are considered markers of inflammation. Taken together, these findings suggest that fluoride may disrupt critical immune and inflammatory processes underlying normal immune system functioning by altering the expression and function of various cellular components.

Sources of Fluoride Exposure in North America

Topical forms of fluoride include toothpaste, rinse, gels, as well as gels and varnishes applied by dental professionals for the purpose of caries prevention. These topical fluoridated products have high levels of fluoride that are not intended to be ingested, but children may be particularly susceptible to accidental ingestion. Indeed, children ages 6 years and younger accounted for more than 80% of reports of suspected over-ingestion of topical sources of fluoride (Shulman & Wells, 1997). The main source of systemic (*i.e.,* ingested) fluoride is fluoridated drinking water (Jean et al., 2018; Riddell et al., 2021), accounting for 40 to 70% of total fluoride intake (U.S. Department of Health and Human Services Federal Panel on Community Water Fluoridation, 2015). Other dietary sources of fluoride include seafood, deboned meat, processed foods, grapes (due to pesticide residue), and fluoride supplements (Aoun et al., 2018). Beverages made with fluoridated water, such as juices and carbonated drinks, and especially black tea (which

hyperaccumulates fluoride from soil), may also constitute a source of fluoride exposure (Rodríguez et al., 2018).

Introduced in 1945 and 1946 by the United States and Canada, respectively, community water fluoridation (CWF) has been implemented with the aim of preventing tooth decay (Marthaler, 2013). Named as one of the greatest public health achievements of the 20th century, the practice has been endorsed by government and national health organizations globally, including the World Health Organization (WHO) and the Centers for Disease Control and Prevention. The concentration of fluoride that is currently recommended for drinking water is 0.7 mg/L; this fluoride concentration is considered to be optimal for preventing caries while minimizing the potential for enamel fluorosis, considered to be an adverse health impact at moderate-to-severe levels (Heller et al., 1997; U.S. Department of Health and Human Services Federal Panel on Community Water Fluoridation, 2015). In comparison, the recommended upper limit of fluoride in drinking water set by the WHO is 1.5 mg/L, which is also considered the maximum allowable concentration in Canada.

Fluoride Absorption, Distribution, and Its Mechanism of Action

Once ingested, fluoride is absorbed in the stomach and small intestine, before it is taken up into calcified tissue (*e.g.*, bones, teeth) or excreted in urine (NRC, 2006; Whitford, 1994). In children, up to 80% of fluoride may be retained due to increased uptake in developing bones and teeth (Palmer & Gibert, 2012). Of the fluoride that is retained, approximately 99% is found in hard tissue, such as bones and teeth (Whitford, 1994). Fluoride appears to support bone formation; however, fluoride supplements, in conjunction with daily calcium supplements, has been shown to increase skeletal fragility (Riggs et al., 1990). Indeed, a systemic review revealed that treatment for osteoporosis with fluoride did not reduce vertebral fractures; rather, fluoride

has been associated with increased nonvertebral and hip fractures, among postmenopausal women (Haguenauer et al., 1996; Helte et al., 2021).

Fluoride mainly prevents dental caries through topical administration, in which coating of the tooth surface with fluoride significantly decreases enamel solubility (Shellis & Duckworth, 1994). After teeth have erupted, fluoride reduces enamel demineralization and accelerates remineralization of caries lesions due to its anticariogenic and antimicrobial properties (Auon et al., 2018; Ten Cate & Featherstone, 1991). However, if too much fluoride is ingested, fluoride can have detrimental effects on health. During critical periods of odontogenesis, exposure to high levels of fluoride may result in dental fluorosis which is characterized by hypomineralized enamel. The development of dental fluorosis results from overexposure to fluoride in children up to 8 years of age as permanent teeth are developing (NRC, 2006). Dental fluorosis can range from white opacity in mild cases to brown discolouration and pitting in its most severe form; protection of dentin and pulp of the teeth by the enamel is compromised in moderate-to-severe fluorosis (NRC, 2006). A survey of Canadian children, aged 6 to 12 years, found that 16% have very mild to mild dental fluorosis as assessed by evaluation of incisors (Health Canada, 2010); however, this may be an underestimate because the study only evaluated incisors whereas molars tend to have the highest degree of fluorosis. Worldwide, the prevalence and severity of fluorosis has been steadily increasing. A 2012 survey conducted in the United States found that 65% of children ages 12 to 15 had very mild to severe dental fluorosis based on an assessment of all teeth compared with only 30% in 2002 (Neurath et al., 2019). Several factors have been proposed as contributing to increased rates and severity of fluorosis, including more sources of fluoride in foods (*e.g.*, processed foods, deboned meat, fruits and vegetables with fluoride residue from pesticide use), beverages (*e.g.,* iced tea), and increased ingestion of fluoride

toothpaste in young children. This increasing prevalence of dental fluorosis in the United States has raised questions about the potential health effects of early life overexposure to systemic fluoride.

Vulnerability of the Developing Brain

The developing brain is particularly sensitive to environmental insults *in utero* (Grandjean & Landrigan, 2006). During this period of rapid growth when the blood-brain barrier is more permeable, CNS development is undergoing critical processes, such as proliferation, migration, and differentiation (Rice & Barone, 2000). As such, toxicants can more freely pass through the blood-brain barrier and interfere with these emerging biological processes (Rodier, 1995). Recent experimental studies have found that chronic fluoride exposure impairs neurogenesis and synaptic structure via disruptions in signaling pathways (Jiang et al., 2019; Niu et al., 2018). Unlike other tissues in the body with capability for cell regeneration, neurons have limited capacity to regenerate following damage or loss. Additionally, fetuses and young children have immature metabolic pathways and enzymes that are critical for metabolizing and excreting chemicals. As a result, exposure to environmental toxicants occurring during brain development is considered a more vulnerable period for long-lasting consequences than exposure in adulthood (Andersen et al., 2000).

Developmental Neurotoxicity of Fluoride

Animal studies: Prenatal fluoride exposure has been shown to disrupt brain development and behaviour in experimental studies. Rats exposed to fluoride prenatally (0.13 mg/kg NaF) showed a different pattern of behaviour in a novel environment compared to those exposed as weanlings and adults (Mullenix et al., 1995). This change was also sex-specific as males were more sensitive to prenatal fluoride exposure, compared to females (Mullenix et al., 1995). Postnatal

exposure after weaning and in adulthood has also been associated with significant impairments in learning and memory (Chioca et al., 2008; Jiang et al., 2014; Liu et al., 2014b). These findings correspond to differences observed in neurochemistry and neurodegeneration. Rats exposed to high levels of fluoride (100 ppm) had pronounced, degenerative changes in the hippocampus, amygdala, motor cortex, and cerebellum (Shivarajashankara et al., 2002).

A systematic review of the animal literature conducted by the National Toxicology Program (NTP, 2016) examined the association between fluoride exposure and neurobehavioral outcomes at different exposure periods. Their analysis revealed that concentrations higher than 0.7 ppm had weak evidence that fluoride resulted in adverse effects of learning and memory among animals exposed to fluoride during development. The low level of evidence was attributed to potential confounding in these developmental studies. In contrast, in adults, the authors found a moderate level of evidence suggesting adverse effects of fluoride on learning and memory (NRC, 2016).

Human studies: The National Toxicology Program (NTP) conducted a comprehensive systematic review and series of meta-analyses evaluating the potential developmental neurotoxicity of fluoride (Taylor et al., in press). Among 59 studies reviewed, 52 (88%) reported a link between early-life exposure to fluoride and diminished intellectual abilities in children. On average, IQ scores were 7-points lower among children living in areas with high versus low fluoride exposure levels. When considering only the highest quality studies (n = 10), the association remained significant, but was attenuated to a 3-point lower IQ score among higher exposed groups. Importantly, negative associations between fluoride exposure and IQ scores remained consistent across age group, study location, child sex, exposure metric (urinary fluoride versus water fluoride), and group-level versus individual-level (*i.e.*, biomarker).

Recent prospective cohort studies conducted in areas with community-wide fluoridation have identified significant associations between fluoride exposure during pregnancy and adverse effects on offspring cognitive development (Bashash et al., 2017; Cantoral et al., 2021; Green et al., 2019), suggesting that the fetus may be particularly susceptible to fluoride neurotoxicity. In Mexico, where salt is fluoridated, higher prenatal exposure to fluoride was associated with lower scores on tests of cognitive function among children at the age of 4 and between the ages of 6 and 12 (Bashash et al., 2017). Our team has identified that prenatal fluoride exposure was significantly associated with lower IQ scores among children ages 3 to 4 in Canada (Green et al., 2019). A 1 mg/L increase in maternal urinary fluoride concentration was associated with a 4.5-point lower IQ score in boys; a 1-mg/L increase in daily fluoride intake was associated with a 3.7-point lower IQ score in both boys and girls (Green et al., 2019). Similarly, in Mexico City, a 0.5 mg/day increase in dietary fluoride intake from foods and beverages during pregnancy was associated with lower cognitive scores among boys, but not girls, at the age of 2 (Cantoral et al., 2021). In contrast, two other cohort studies did not find inverse associations between maternal fluoride exposure and child neurodevelopmental outcomes (Grandjean et al., 2023; Ibarluzea et al., 2022). The Odense Child Cohort (OCC) study in Denmark examined the association between maternal urinary fluoride concentrations in late pregnancy (0.52 mg/L) and child IQ at age 7 among 837 mother-child dyads and findings were null (Grandjean et al., 2023). The Infancia y Medio Ambiente (INMA) – Environment and Childhood cohort study in Spain investigated the relationship between first- (0.46 mg/L) and third-trimester (0.51 mg/L) maternal urinary fluoride concentrations and cognitive abilities of children at ages 1 and 4 among 248-316 mother-child dyads and findings revealed a positive association (Ibarluzea et al., 2022). Discrepancies among studies may be attributed to differences in cohort characteristics or outcome measures. For

example, the OCC study was conducted in a non-fluoridated area, where exposure contrasts may have been limited due to the study cohort drinking non-fluoridated water (Grandjean et al., 2023), whereas the INMA study measured children's cognitive abilities, rather than IQ (Ibarluzea et al., 2022). Finally, a recent review of animal and epidemiological studies suggests that males may be more susceptible to fluoride toxicity during critical periods of development, such as pregnancy, compared to females (Green et al., 2020). Taken together, these results suggest that exposure to fluoride during gestation may be associated with adverse effects on child neurodevelopment; however, the potential mechanism underlying this association remains unclear and warrants further research.

Rationale

Experimental and human epidemiological studies have identified that exposure to fluoride during critical periods of development may contribute to adverse health outcomes. Prenatal exposure to fluoride has been associated with altered neurodevelopmental trajectories (Bashash et al., 2018; Green et al., 2019; Lu et al., 2000; Xiang et al., 2003; Zhao et al., 1996). We propose that activation of the maternal immune system during pregnancy may be implicated in this process. Findings from both animal models and human studies reveal that exposure to fluoride may lead to changes in the expression of inflammatory immune markers and compromise immune system function (De la Fuente et al., 2016; Guo et al., 2017). The maternal immune system may also be activated during pregnancy due to infections, stress, or chronic inflammatory conditions, such as obesity or asthma (Estes & McAllister, 2016). This activation of the immune response, via immune mediators known as cytokines, has been linked to increased risk of neurodevelopmental disorders and altered brain development in children (Estes & McAllister, 2016; Han et al., 2021; Knuesel et al., 2014).

To date, no studies have investigated the association between fluoride exposure in pregnancy and immunotoxicity. Should an association exist between the two, this may provide insights into the mechanism underlying the relationship between developmental neurotoxicity and gestational fluoride exposure (Green et al., 2019; Taylor et al., 2024). Given the ubiquitous nature of fluoride exposure, further research is warranted to identify potential health risks associated with this exposure, especially as it concerns vulnerable populations, such as pregnant women and developing fetuses. The proposed study will evaluate the association between maternal fluoride exposure and markers of immune system function (*i.e.,* IL-6, IL-8, IL-10, TNF-α, MCP-1, CRP; Table 1) during pregnancy while controlling for potential covariates. Compared to females, male offspring are often more sensitive to prenatal exposure to fluoride (Green et al., 2020) or other neurotoxicants (Goodman et al., 2023). Likewise, in zebrafish, males show a greater inflammatory response in response to continuous fluoride exposure compared with females (Wang et al., 2022). Considering the evidence of sex differences in susceptibility to neurotoxicant exposures, we will also investigate effect modification by fetal sex. Based on the extant literature, we hypothesize that higher maternal urinary fluoride and water fluoride concentrations will be associated with elevated concentrations of immune system markers during pregnancy.

Table 1

Immune system biomarkers categorized by broad function

Function/Class	Biomarkers
Pro-inflammatory	Interleukin-6 (IL-6) Interleukin-8 (IL-8) Tumor necrosis factor alpha (TNF-α) Monocyte chemoattractant protein-1 (MCP-1)
Pro-inflammatory (acute)	C-reactive protein (CRP)
Anti-inflammatory	Interleukin-10 (IL-10)

Methods

Participants

Data for this study was obtained from an existing Canadian pregnancy and birth cohort: Maternal-Infant Research on Environmental Chemicals (MIREC) Study. Between 2008 and 2011, 2001 pregnant women (< 14 weeks gestation) were recruited from ten cities across Canada when attending prenatal clinics during the first trimester of pregnancy. Of these ten, seven (Toronto, Hamilton, Ottawa, Sudbury, Halifax, Edmonton, and Winnipeg) partake in community water fluoridation whereby fluoride is added to public water supplies to meet levels deemed optimal for preventing tooth decay in the community. Eligibility criteria for women's enrolment included: 18 years of age or older, ability to communicate in English or French, willing to provide a cord blood sample at time of delivery, planning to deliver at a local hospital, and no known fetal abnormalities or history of medical complications (Arbuckle et al., 2013). Of the 8716 participants approached at prenatal clinics, 5108 (58.6%) were eligible, 2001 agreed to participate (39%), and 1983 (39%) provided full consent to having data and biospecimens collected at each trimester, stored in the MIREC biobank, and used in future research (Arbuckle et al. 2013). Generally, MIREC participants tended to be older, married, more educated, less likely to be a current smoker, and born in Canada compared to Canadian birth statistics from 2009 (Arbuckle et al., 2013).

Ethical Considerations

The proposed study has received ethics approval from the York University Research Ethics Board and Health Canada. At the time of enrollment, participants provided informed consent for the collection and use of their data, as well as storage of their samples in the MIREC biobank. All participants were identified with a unique ID, so no data stored in the biobank includes

identifying information. Further, all biobank samples were labelled with a bar code and securely stored in freezers. In accordance with the MIREC Research Platform Knowledge Transfer Policy, no data files will be shared as part of publications and presentations. To further ensure that security is not compromised, no unique IDs or barcodes will be included in the dissemination of results.

Fluoride Exposure Measures

Maternal fluoride exposure was evaluated using fluoride measured from 1) urine samples collected during each trimester of pregnancy and 2) drinking water.

Maternal Urinary Fluoride

Maternal urinary fluoride (MUF) concentrations were analyzed in spot urine samples collected in each trimester of pregnancy using a modification of the hexamethyldisiloxane microdiffusion method with ion-selective electrodes (Martinez-Mier et al., 2011). Using this technique, fluoride concentrations could be detected in urine down to 0.02 mg/L. MUF concentrations were adjusted for specific gravity (SG) to account for variations in urine dilution using the following equation: $MUF_{SG} = MUF_i \times (SG_M - 1)/(SG_i - 1)$, where MUF_{SG} is the SG-adjusted fluoride concentration (mg/L), MUF_i is the observed fluoride concentration, SG_M is the median SG for the cohort, and SG_i is the SG of the urine sample (MacPherson et al., 2018). MUF values exceeding 5 mg/L were excluded from analyses as they surpassed the highest concentration standard of the instrument. Given the relatively short half-life of fluoride (~6 hours; Buzalaf & Whitford, 2011) and changes in MUF levels across trimesters due to physiological changes (Castiblanco-Rubio & Martinez-Mier, 2022), women were excluded from data analysis if they did not have urine samples available for all three trimesters. In our analyses,

MUF concentrations were measured during each trimester, dilution-standardized, and averaged across pregnancy. See Till et al. (2018) for additional details on urinary fluoride analysis.

Drinking Water Fluoride

Water fluoride concentrations (WFC) were available for women who reported drinking municipal tap water during pregnancy. As a result, only participants who reported drinking tap water from public sources during pregnancy were included in the WFC analyses. Water fluoride levels were derived from municipal water treatment plants associated with the participants' postal codes. Water treatment plants that added fluoride to drinking water measured fluoride levels on a regular basis (*i.e.,* daily or multiple times per day) whereas a plant that did not add fluoride to drinking water would measure fluoride levels quarterly. Concentrations were averaged across pregnancy, resulting in a geometric mean WFC (mg/L) for each participant corresponding to the fluoride levels found in the participant's tap water during pregnancy. For cities that reported fluoride concentrations that were equivalent to the limit of detection (LoD), we used an imputed value of the LoD divided by the square root of 2 (Hornung & Reed, 1990).

Maternal Immune System Markers

Immune system biomarkers were measured in third trimester (mean [SD] gestational age = 33.1 [1.49] weeks) plasma samples (Table 2). Maternal plasma samples were analyzed by affinity-based multiplex protein array assays using Bio-Plex Pro Human panels (Bio-Rad, Canada) and Milliplex Map kits (Millipore, Canada; Kumarathasan et al., 2018). Laboratory analyses were conducted by the Environmental Health Science and Research Bureau at Health Canada. Immune system biomarker concentrations below the detection limit of the assay were replaced by the LOD divided by $\sqrt{2}$ (Hornung & Reed, 1990). All six of the immune biomarkers were detected in over 99 percent of the sample (Table 2).

Table 2

Descriptive statistics for immune system biomarkers in third trimester maternal plasma samples

Immune system biomarkers	*n*	LoD	% < LoD	Min	25th percentile	Median	75th percentile	Max	GM (95% CI)
IL-6 (pg/mL)	1586	0.2	0.69	0.14	0.95	1.68	2.94	184.57	1.75 (1.67, 1.82)
IL-8 (pg/mL)	1586	0.05	0	0.18	1.46	1.98	2.75	40.39	2.07 (2.01, 2.14)
TNF-α (pg/mL)	1586	0.07	0	0.20	3.23	4.33	5.87	132.78	4.38 (4.28, 4.49)
MCP-1 (pg/mL)	1579	1.1	0	6.49	28.21	37.86	51.23	229.71	38.22 (37.32, 39.14)
CRP (μg/mL)	1583	1.0×10^{-6}	0	0.11	8.40	17.47	36.48	1602.60	17.34 (16.41, 18.31)
IL-10 (pg/mL)	1592	0.48	0.25	0.34	14.00	20.58	31.41	720.79	21.04 (20.21, 21.91)

Inflammatory Index

Based on immune system biomarker function, a composite index of inflammation was created using the same approach reported in a prior study conducted in the same cohort (Kumarathasan et al., 2018). Specifically, the inflammatory index was derived by summing the standardized z-scores of IL-6, IL-8, TNF-α, and MCP-1 using the means and standard deviations to calculate each z-score (Palaniyandi et al., 2023). Although CRP does have pro-inflammatory properties, it was excluded from the inflammatory index as it is often considered a measure of acute inflammation. Principal component analysis (PCA) was also used to confirm that these selected inflammatory biomarkers loaded most strongly on an inflammatory index. Results of the PCA showed that first principal component (consisting of IL-6, IL-8, TNF-α, and MCP-1) explained 45% of the total variance (Supplemental Table 1) with IL-6, IL-8, and TNF-α loading most on to the component with factor loadings of 0.46 or higher (Supplemental Table 2). MCP-1 had a weaker loading on the first principal component. To be consistent with prior work, we used

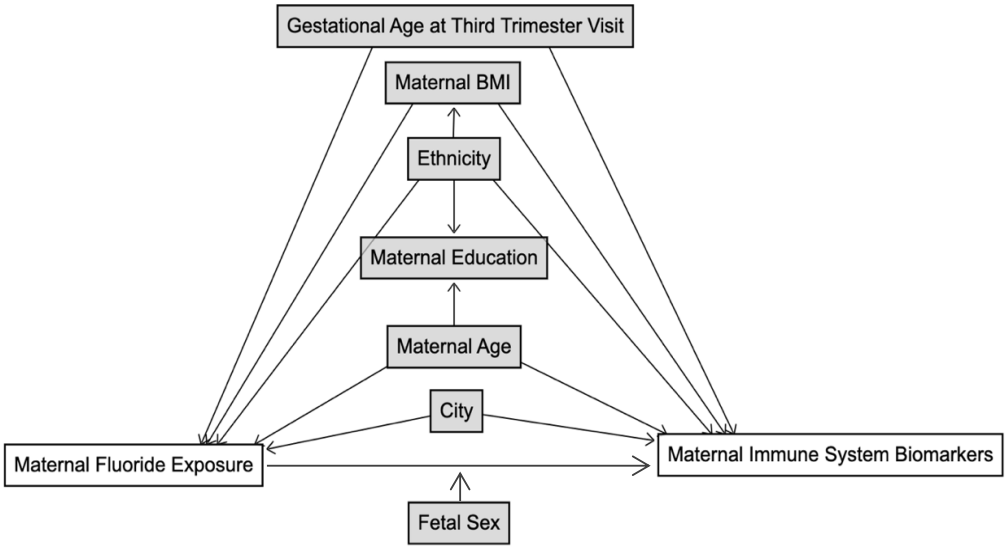
the 4-marker inflammatory index in our primary analyses, but also re-ran the analyses using the PCA-derived 3-marker inflammatory index to confirm the results remained consistent.

Covariates

Potential covariates were identified *a priori* using a directed acyclic graph (DAG; Figure 1) based on the available literature pertaining to associations between fluoride exposure and immune system biomarkers. Maternal covariates previously associated with fluoride exposure include pre-pregnancy body mass index (BMI), maternal age at child's birth, gestational exposure to second-hand smoke, gestational alcohol consumption, education, city, and race/ethnicity (Buzalaf & Whitford, 2011; Till et al., 2018). Maternal cytokine levels may be influenced by several factors, including genetics and lifestyle. Maternal covariates that may be associated with immune system activation include BMI, obesity, smoking status, and age; child covariates that may be relevant include fetal sex and gestational age (Irwin et al., 2019; Jarmund et al., 2021). Many of these associations have been shown to be trimester-specific, highlighting the importance of considering gestational age when interpreting results. Sex differences will also be important to consider as women carrying a female fetus have different cytokine profiles compared to those carrying a male fetus (Jarmund et al., 2021; Ross et al., 2022). The primary model consisted of the following covariates: maternal age, maternal level of education (college diploma or less, or university undergraduate or graduate degree), maternal race/ethnicity (white, other), maternal pre-BMI, gestational age at third trimester visit, fetal sex, and city. A secondary model included the following additional covariates: maternal exposure to second-hand smoke (yes/no) and alcohol consumption (yes/no), given their potential influence on circulating cytokines (Achur et al., 2010; Shiels et al., 2014).

Figure 1

Directed acyclic graph depicting the primary model examining the association between maternal fluoride exposure and maternal immune system biomarkers



Statistical Analysis

Statistical analyses were performed using STATA version 18.0. Descriptive statistics were generated for maternal demographics, and exposure and outcome variables. Distribution plots were examined to assess the distribution of the variables, identify potential outliers, and visualize the association between immune system biomarkers and fluoride exposures. As the immune system biomarkers were positively skewed, variables were transformed by applying a natural log transformation to approximate a normal distribution for these variables. Following a natural log transformation, immune biomarker variables were normally distributed. Spearman's correlation coefficient was used to examine correlations between maternal fluoride exposure and immune system biomarkers. We used the following stratification for interpreting the magnitude of the associations: 0.10-0.39 as representing a weak correlation, 0.40-0.69 as representing a

moderate correlation, and 0.70-0.89 as representing a strong correlation (Schober et al., 2018). We examined visuals (*e.g.*, scatterplots) and explored quadratic models to assess departures from log-linearity. For models with quadratic term *p*-values < .05, margins plots were created to visually inspect curvature and directionality. Associations were further probed by using quartile regression models and a spline analysis restricted at the inflection point.

Linear regression models were first used to examine associations between maternal fluoride exposures (MUF and WFC) and the inflammatory index. In these models, the β coefficient was interpreted as a change in SD units, or change in the z-score unit, in the inflammatory index per 1 mg/L increase in MUF. Next, log-linear regression models were used to examine associations between MUF and $\log_e$-transformed immune system biomarker (*i.e.,* IL-6, IL-8, IL-10, TNF-α, MCP-1) concentrations and the inflammatory index. Beta coefficients were back transformed to yield the percent change (*i.e.,* (e^(β coefficient) – 1) × 100) in immune system biomarker concentration per 1 mg/L increase in MUF. Log-linear regression models were also used to evaluate whether WFC predicted changes in $\log_e$-transformed immune markers (*i.e.,* IL-6, IL-8, IL-10, TNF-α, MCP-1) concentrations and the inflammatory index. Beta coefficients were divided by two and back transformed to yield the percent change (*i.e.,* (e^ (β coefficient/2) – 1) × 100) in immune system biomarker concentration per 0.5 mg/L increase in WFC. Given that the water fluoride range in the sample is less than 1 mg/L, the change is reported per 0.5 mg/L, which corresponds roughly to the difference in water fluoride concentrations between fluoridated and non-fluoridated areas. All regression models were run twice: a primary model adjusting for the core covariates (maternal age, maternal level of education, maternal race/ethnicity, maternal pre-BMI, gestational age at third trimester visit, fetal sex) and a secondary model with adjustment for additional covariates (maternal exposure to second-hand

smoke (yes/no), alcohol consumption(yes/no). Study site (city) was also included as a covariate in the models using MUF, but excluded from the models using WFC due to multicollinearity between city and water fluoride concentration.

As a secondary aim, we tested effect modification by fetal sex through the inclusion of an interaction term in all of the linear regression models described above. Finally, a sensitivity analysis was conducted for models described above excluding women who reported during the first trimester visit taking anti-inflammatory medication, having a chronic condition with known effects of immune system function, and/or those with CRP levels greater than 100 mg/L as these can influence circulating levels of immune system biomarkers during pregnancy (n = 356) . CRP levels greater than 100 mg/L may be indicative of an acute infection at the time of plasma sample collection (Gogna et al., 2021). Conditions included: autoimmune disorders affecting central nervous system or neuromuscular junctions (multiple sclerosis, myasthenia gravis, lupus), thyroid disorders (Grave's disease, Hashimoto's disease, hyperthyroidism, hypothyroidism) or other endocrine-related disorders (diabetes), inflammatory diseases in the gastrointestinal tract (Crohn's disease, colitis, irritable bowel syndrome, celiac disease, ulcerative colitis, gastric ulcer), inflammation in the urinary or reproductive tract (nephropathy, interstitial cystitis, urinary tract infection, endometriosis, polycystic ovary syndrome), dermatological disorders (acne, allergies, rash, psoriasis, rosacea, pityriasis, eczema), other infections (arthritis, Still's disease, iritis, multifocal choroiditis uveitis, sinusitis, pancreatitis, pericarditis, herpes), and other disorders which might affect the immune response (asthma, bronchitis, cystic fibrosis, high blood pressure, sarcoidosis, thalassemia, thrombocytopenia).

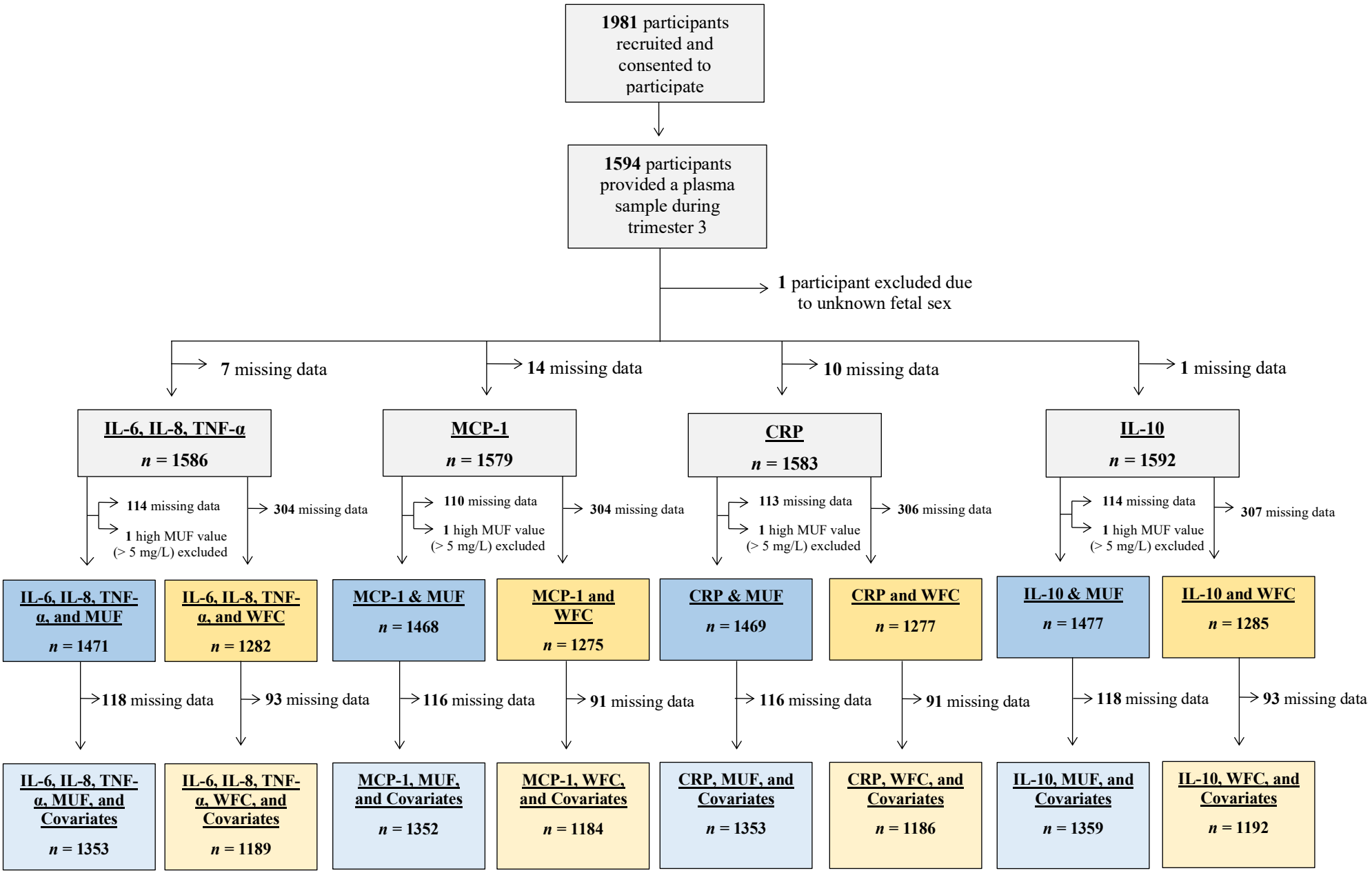
Model diagnostics were examined for all regression models, including collinearity (Variance Inflation Factor (VIF) < 4), influence (Cook's distance), leverage, and

heteroskedasticity (using the Cook-Weisberg test). Plots of residuals versus fitted values were also examined to visualize the distribution of residuals. Statistical significance was evaluated with α set to 0.05. Interaction terms were probed if the *p* value for the interaction term was < .10.

Figure 2 demonstrates the study sample flow chart for participants with WFC, MUF, immune system biomarkers, and covariate data available.

Figure 2

Study sample flow chart for participants with maternal fluoride exposure, maternal immune system biomarkers, and covariate data



Results

Demographics

Of the 1601 participants who provided a plasma sample at trimester 3, 1476-1485 (~92%) and 1282-1292 (~80%) of the participants had MUF and WFC, respectively (Figure 2). Descriptive statistics of samples with data on maternal fluoride exposures (*i.e.,* MUF, WFC), immune system biomarkers (*i.e.,* IL-6, IL-8, TNF-α, MCP-1, CRP, IL-1), and the inflammatory index are presented in Table 3.

Table 3

Descriptive statistics of maternal fluoride exposures and immune system biomarkers

	MIREC participants with:			
	Plasma samples from trimester 3 (n = 1592)[1]	MUF and immune system biomarkers (n = 1478)[2]	WFC and immune system biomarkers (n = 1286)[2]	Missing covariate data (n = 124)
Mean (SD; mg/L)				
MUF_{sg}	0.59 (0.41)	0.59 (0.38)	0.61 (0.42)	0.57 (0.38)
WFC	0.42 (0.25)	0.42 (0.26)	0.42 (0.25)	0.40 (0.27)
GM (95% CI)[3]				
IL-6 (pg/mL)	1.75 (1.67, 1.82)	1.75 (1.67, 1.83)	1.77 (1.68, 1.86)	2.06 (1.73, 2.45)
IL-8 (pg/mL)	2.07 (2.01, 2.14)	2.08 (2.02, 2.15)	2.06 (2.00, 2.13)	2.00 (1.80, 2.23)
TNF-α (pg/mL)	4.38 (4.28, 4.49)	4.37 (4.26, 4.47)	4.37 (4.26, 4.49)	4.09 (3.77, 4.45)
MCP-1 (pg/mL)	38.2 (37.3, 39.1)	38.3 (37.4, 39.3)	38.7 (37.7, 39.8)	37.1 (34.2, 40.2)
CRP (μg/mL)	17.3 (16.4, 18.3)	17.1 (16.1, 18.1)	17.1 (16.1, 18.2)	20.9 (17.3, 25.2)
IL-10 (pg/mL)	21.0 (20.2, 21.9)	21.2 (20.3, 22.1)	21.0 (20.1, 22.0)	22.5 (19.8, 25.5)
Mean z-score (SD)				
Inflammatory index	0.001 (2.54)	0.007 (2.59)	0.02 (2.62)	-0.18 (1.76)

[1] Excludes those with unknown fetal sex (n = 1) and missing data (n = 1)

[2] Includes participants with at least one immune system biomarker analyzed during the third trimester

[3] Immune system biomarker concentrations below the detection limit of the assay were replaced by the LOD divided by $\sqrt{2}$. 0.69% and 0.25% of IL-6 and IL-10 samples fell below the LOD respectively.

Demographic characteristics of the samples are presented in Table 4. Participants with data on IL-6, IL-8, TNF-α, MCP-1, CRP, or IL-10 had a mean (SD) MUF concentration of 0.59 mg/L (0.41) and a mean (SD) WFC of 0.42 mg/L (0.25). Most of the participants with data for MUF and immune system biomarkers were white (86%), married or common-law (96%), had a university degree or higher (64%), had a household income < CA$100,000 (57%), and lived in fluoridated communities (60%). Among the participants with MUF and immune biomarker data, only 81 (5.5%) and 263 (17.8%) endorsed exposure to second-hand smoke and any alcohol consumption during pregnancy. Of those that endorsed alcohol consumption, most (87.8%) participants reported consuming less than one drink per week. Finally, among the participants with data on MUF and immune biomarker data, 265 (17.9%), 55 (3.8%), and 72 (4.9%) reported an inflammatory condition, use of anti-inflammatory medications, or CRP levels greater than 100 mg/L respectively. Similar results were observed for participants with data for WFC and immune system biomarkers.

Table 4

Demographic characteristics of samples used in analyses

	MIREC participants with[1]:			
	Plasma samples from trimester 3 (n = 1592)[2]	MUF and immune system biomarkers (n = 1478)[3]	WFC and immune system biomarkers (n = 1286)[3]	Missing covariate data (n = 124)
Maternal age (years; mean (SD))	32.2 (5.0)	32.3 (5.0)	32.4 (5.1)	30.8 (5.4)
Pre-pregnancy BMI (kg/m^2; mean (SD))	24.9 (5.5)	24.8 (5.4)	24.8 (5.4)	22.9 (3.9)

Ethnicity (%)				
White	85.9	86.2	83.7	82.4
Other	14.1	13.8	16.3	17.6
Marital status (%)				
Married or common law	95.4	95.7	94.9	94.4
Single	4.6	4.3	5.1	5.6
Level of education (%)				
College diploma or less	36.5	36.2	34.3	44.0
University degree	63.5	63.8	65.7	56.0
Household income (%)[4]				
< $100,000	57.2	57.2	56.3	64.8
> $100,000	38.9	38.8	39.4	29.6
Second-hand smoke in trimester 1 (%)				
Yes	5.6	5.5	5.4	92.0
No	94.4	94.5	94.6	8.0
Alcohol consumption (%)				
Yes	17.6	17.8	17.6	12.8
No	82.4	82.2	82.4	87.2
Living in a fluoridated community (%)				
Yes	61.7	60.3	61.7	57.0
No	38.3	39.7	38.3	43.0
Chronic immune condition (%)				
Yes	17.6	17.9	17.2	78.4
No	82.4	82.1	82.8	21.6
Anti-inflammatory medications (%)				
Yes	3.7	3.7	3.7	4.8
No	96.3	96.3	96.3	95.2
Gestational age at third trimester visit (weeks; mean (SD))	33.1 (1.5)	33.0 (1.4)	33.1 (1.5)	33.2 (1.5)
Fetal sex (%)				
Male	52.8	52.2	52.7	56.9
Female	47.2	47.8	47.3	43.1

[1] Sample size may be lower for some of the characteristics due to missing data

[2] Excludes those with unknown fetal sex (n = 1) and missing data (n = 1)

[3] Includes participants with at least one immune system biomarker analyzed during the third trimester

[4] Percentages do not add up to 100% as some participants did not know the answer or preferred not to answer

Spearman correlations indicated moderate positive associations between IL-6 and IL-8 ($\rho = .42$, $p < .05$), IL-6 and IL-10 ($\rho = .42$, $p < .05$), and IL-8 and TNF-α ($\rho = .43$, $p < .05$) and weak positive associations between IL-6 and TNF-α ($\rho = .33$, $p < .05$), IL-6 and CRP ($\rho = .20$, $p < .05$), IL-8 and IL-10 ($\rho = .39$, $p < .05$), TNF-α and IL-10 ($\rho = .32$, $p < .05$), and TNF-α and CRP ($\rho = .16$, $p < .05$; Table 5). Significant correlations were not identified between MUF concentration and any of the maternal immune biomarkers. Likewise, WFC was not associated with any of the maternal immune biomarkers. MUF was moderately correlated with WFC ($\rho = .48$, $p < .05$).

Table 5

Spearman correlations between maternal fluoride exposures and log-transformed immune system biomarkers measured in third trimester plasma samples

	MUF	**WFC**	**IL-6**	**IL-8**	**TNF-α**	**MCP-1**	**CRP**	**IL-10**
MUF	1.00							
WFC	.48*	1.00						
IL-6	.035	.003	1.00					
IL-8	-.002	.005	.42*	1.00				
TNF-α	.038	.009	.33*	.43*	1.00			
MCP-1	.030	.018	.073*	.11*	.063*	1.00		
CRP	.041	.080*	.20*	.027	.16*	.081*	1.00	
IL-10	.011	-.007	.42*	.39*	.32*	.032	.061*	1.00

Abbreviations: MUF = maternal urinary fluoride; WFC = water fluoride concentration; IL-6 = interleukin-6; IL-8 = interleukin-8; TNF-α = tumor necrosis factor alpha; MCP-1 = monocyte chemoattractant protein-1 ; CRP = C-reactive protein; IL-10 = interleukin-10

* $p < .05$

Associations Between Maternal Urinary Fluoride Concentration and Immune System Biomarkers and the Inflammatory Index

In both the non-adjusted and covariate-adjusted linear regression, MUF was not significantly associated with the inflammatory index (non-adjusted $\beta = 0.16$, 95% CI: -0.18, 0.51; adjusted $\beta = 0.098$, 95% CI: -0.31, 0.50). Results remained null using the PCA-derived index.

Non-adjusted log-linear regression models revealed no significant associations between MUF and log-transformed immune biomarkers (Table 6). Likewise, results of covariate-adjusted log-linear regression models assessing the relationship between MUF and log-transformed immune biomarkers were all null in both the primary and secondary models (Table 6; Supplemental Figure 2). Log-linear associations were detected for IL-6, IL-8, TNF-α, MCP-1, and CRP, whereas a departure from linearity was detected in the association between MUF and IL-10 (Figure 3). To probe this J-shaped nonlinear relationship further, a log-linear regression model was conducted restricting to MUF levels greater than 0.80 mg/L and results remained non-significant ($\beta = 0.19$; 95% CI: -0.08, 0.46). Results from the quartile regression model corroborated these nonsignificant findings at higher MUF levels (Supplemental Table 3). Percent changes for the null associations between MUF and immune system biomarkers are presented in Figure 4.

Table 6

Non-adjusted, primary, and secondary log-linear regression models assessing the association between MUF and log-transformed immune system biomarkers

Immune system biomarkers	Non-adjusted				Primary[1]				Secondary[2]			
	n	β	95% CI	*p*	*n*	β	95% CI	*p*	*n*	β	95% CI	*p*
IL-6	1471	0.066	-0.052, 0.18	.27	1353	0.038	-0.095, 0.17	.58	1349	0.040	-0.094, 0.17	.56
IL-8	1471	0.030	-0.049, 0.11	.46	1353	0.022	-0.068, 0.11	.63	1349	0.022	-0.068, 0.11	.63
TNF-α	1471	0.061	-0.003, 0.13	.06	1353	0.038	-0.036, 0.11	.31	1349	0.038	-0.035, 0.11	.31
MCP-1	1468	0.016	-0.048, 0.080	.62	1352	0.0060	-0.067, 0.079	.87	1348	0.006	-0.068, 0.079	.88
CRP	1469	0.048	-0.100, 0.20	.53	1353	-0.031	-0.20, 0.13	.71	1349	-0.025	-0.19, 0.14	.77
IL-10[3]	1477	0.029	-0.081, 0.14	.61	1359	0.032	-0.096, 0.16	.63	1355	0.033	-0.095, 0.16	.61

[1]Primary model adjusted for gestational age at third trimester visit, maternal age, pre-pregnancy BMI, level of education, race and ethnicity, fetal sex, and city centre.

[2] Secondary model adjusted for the covariates above and gestational exposure to second-hand smoke and alcohol consumption.

[3] Departure from linearity detected.

Figure 3

Association between MUF and log-transformed maternal IL-10

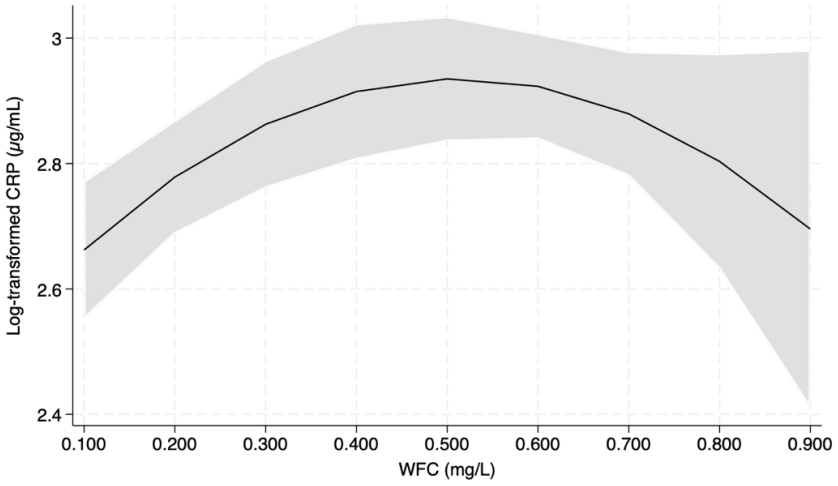
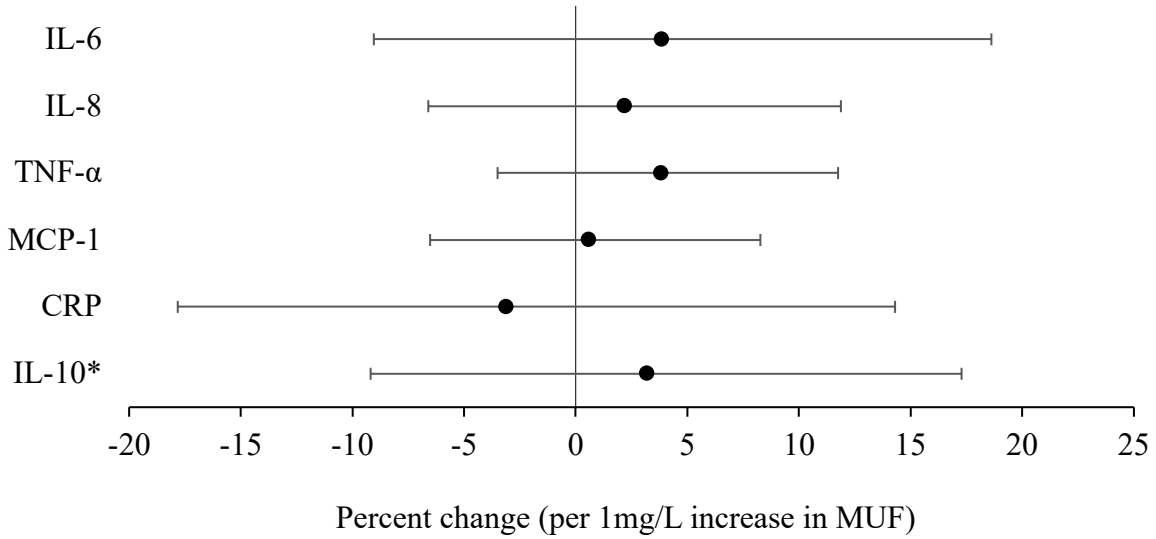


Figure 4

Percent change in immune system biomarker concentrations per 1 mg/L increase in MUF with 95% CI



[1] Models adjusted for gestational age at third trimester visit, maternal age, pre-pregnancy BMI, level of education, race and ethnicity, fetal sex, and city centre.

* As departure from linearity was detected, further interpretation of this association is needed.

Water Fluoride Concentration and Immune System Biomarkers and the Inflammatory Index

In both the non-adjusted and covariate-adjusted linear regression, WFC was not significantly associated with the inflammatory index (non-adjusted β = -0.23, 95% CI: -0.80, 0.33; adjusted β = -0.43, 95% CI: -1.03, 0.17). Results remained null using the PCA-derived index.

Non-adjusted log-linear regression models revealed a significant positive association between WFC and CRP (β = 0.38, 95% CI: 0.14, 0.63) and this association remained significant after adjusting for covariates (β = 0.35, 95% CI: 0.10, 0.60). This effect estimate would correspond to a 19.4% increase per 0.5 mg/L increase WFC. However, a departure from log-

linearity was detected in the covariate-adjusted association between WFC and CRP. Specifically, an inverted U-shape was observed (*i.e.,* CRP levels increased and then decreased with increasing WFC; Figure 5). To probe this relationship further, we used a quartile regression model. Results showed that the WFC and CRP association was significantly higher (*i.e.,* more positive) in quartiles 2 and 3 than quartile 1 (Table 7).

Figure 5

Association between WFC and log-transformed CRP

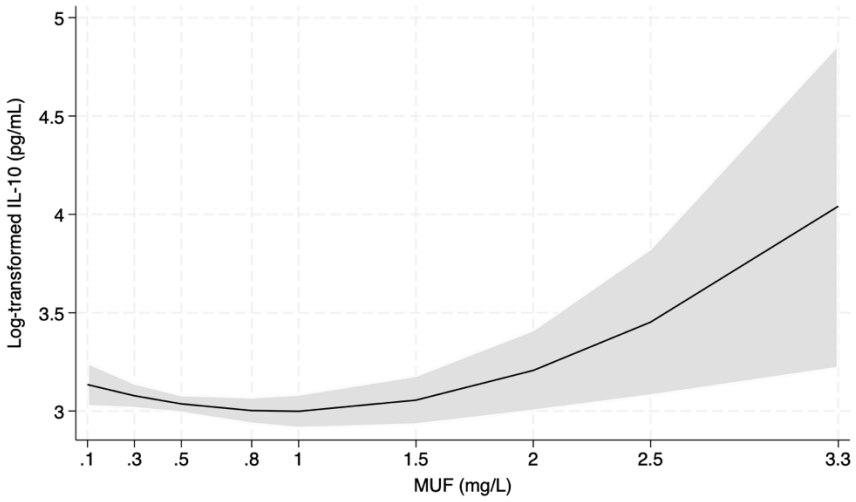


Table 7

Quartile regression effect estimates (95% CI) for the association between WFC and log-transformed CRP and IL-6 in pregnant women in the primary model and sensitivity analysis

		Primary			Sensitivity	
WFC Quartiles[1]	β	95% CI	*p*	β	95% CI	*p*
CRP						
Q2	0.24	0.063, 0.41	.008	0.23	0.051, 0.40	.012
Q3	0.29	0.102, 0.48	.002	0.31	0.12, 0.40	.001
Q4	0.15	-0.032, 0.33	.11	0.16	-0.017, 0.35	.076
IL-6						
Q2	0.21	0.070, 0.35	.003	0.21	0.062, 0.37	0.006
Q3	0.11	-0.035, 0.26	.136	0.18	0.013, 0.34	0.034
Q4	-0.08	-0.22, 0.066	.294	-0.05	-0.21, 0.11	0.533

[1] Q2 corresponds to results for women with WFC and immune biomarkers in the second quartile (*i.e.*, between the 25th and 50th percentiles; 0.14 – 0.52 mg/L), relative to those with levels in the first quartile (*i.e.*, below the 25th percentile, < 0.14 mg/L)

Q3 corresponds to results for women with WFC and immune biomarkers in the third quartile (*i.e.*, between the 50th and 75th percentiles; 0.53– 0.62 mg/L), relative to those with levels in the first quartile (*i.e.,* below the 25th percentile, < 0.14 mg/L).

Q4 corresponds to results for women with WFC and immune biomarkers in the fourth quartile (*i.e.*, above the 75th percentile; 0.63 – 0.87 mg/L), relative to those with levels in the first quartile (*i.e.*, below the 25th percentile, < 0.14 mg/L).

With the exception of CRP, all other non-adjusted models between WFC and immune biomarkers were null (Table 8). However, after adjusting for covariates, a significant negative association was observed between WFC and IL-6 (β = -0.21, 95% CI: -0.41, -0.015) whereas all other covariate-adjusted associations between WFC and immune system biomarkers were null (Table 8; Supplemental Figure 2). However, a departure from log-linearity was detected in the association between WFC and IL-6. As shown in Figure 6, the association gradually increases and then decreases at levels above 0.4 mg/L. Restricting the sample to WFC above 0.40 mg/L, IL-6 levels decreased with increasing WFC (β = -0.64; 95% CI: -1.20, -0.08). Probing this relationship further with a quartile regression model showed a significant positive association in the second quartile (*i.e.*, WFC: 0.14-0.52 mg/L) relative to the first quartile (*i.e.,* WFC: < 0.13 mg/L); consistent with the inverted U-shape, the effect estimates at the highest and lowest WFC levels did not differ (Table 7). Percent changes for the associations between WFC and immune system biomarkers are presented in Figure 7.

Figure 6

Association between WFC and log-transformed IL-6

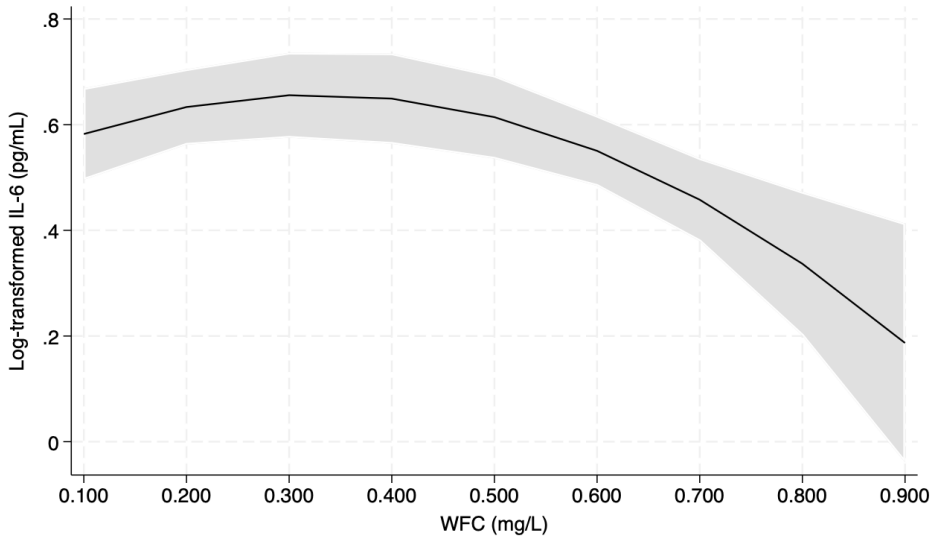


Table 8

Non-adjusted, primary, and secondary log-linear regression models assessing the association between WFC and log-transformed immune system biomarkers

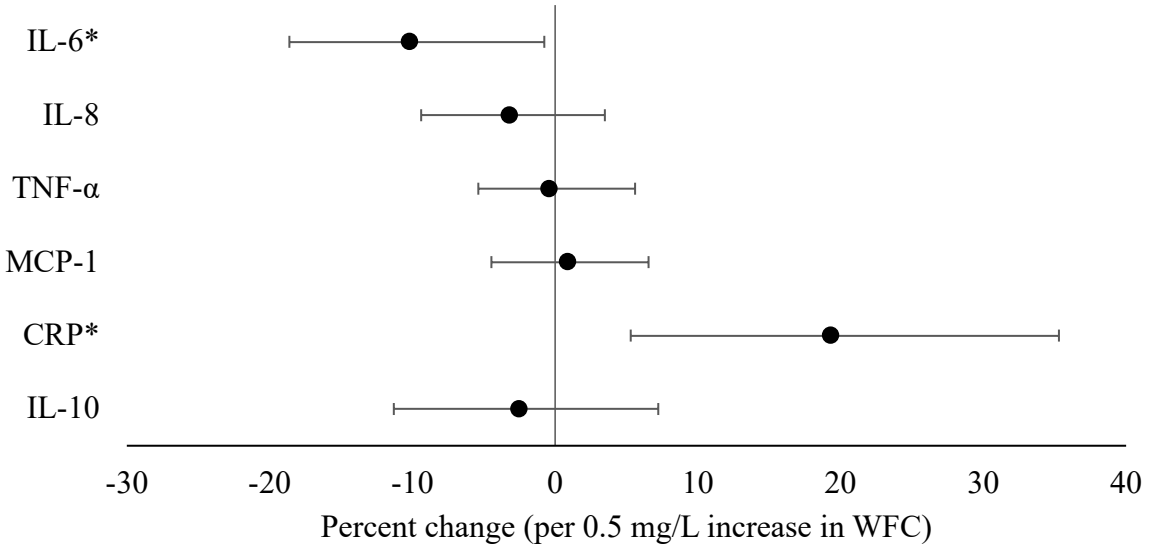
Immune system biomarkers	Non-adjusted				Primary[1]				Secondary[2]			
	n	β	95% CI	*p*	*n*	B	95% CI	*p*	*n*	β	95% CI	*p*
IL-6	1282	-0.070	-0.26, 0.13	.48	1189	-0.21	-0.41, -0.015	.035	1185	-0.22	-0.42, -0.020	.031
IL-8	1282	-0.033	-0.16, 0.09	.61	1189	-0.064	-0.20, 0.069	.34	1185	-0.062	-0.20, 0.072	.37
TNF-α	1282	0.022	-0.083, 0.13	.68	1189	- 0.007	-0.11, 0.11	.99	1185	0.0004	-0.11, 0.11	1.00
MCP-1	1275	0.057	-0.047, 0.16	.28	1184	0.018	-0.091, 0.13	.75	1180	0.020	-0.090, 0.13	.72
CRP	1277	0.38	0.14, 0.63	.002	1186	0.35	0.10, 0.60	.006	1182	0.34	0.087, 0.59	.008
IL-10	1285	-0.031	-0.21, 0.15	.74	1192	-0.050	-0.24, 0.14	.60	1188	-0.055	-0.25, 0.14	.57

[1] Primary model adjusted for gestational age at third trimester visit, maternal age, pre-pregnancy BMI, level of education, race and ethnicity, fetal sex, and city centre.

[2] Secondary model adjusted for the covariates above and gestational exposure to second-hand smoke and alcohol consumption.

Figure 7

Percent change in immune system biomarker concentrations per 0.5 mg/L increase in WFC with 95% CI



[1] Models were adjusted for gestational age at third trimester visit, maternal age, pre-pregnancy BMI, level of education, race and ethnicity, and fetal sex.

* As departure from linearity was detected, further interpretation of this association is needed.

Effect Modification by Fetal Sex

There was no evidence of effect modification by fetal sex for any of the associations between fluoride exposures and log-transformed maternal immune system biomarkers (*p*-interaction terms >.10).

Sensitivity Analysis

Excluding participants with CRP levels above 100 mg/L and those who reported inflammatory conditions and use of anti-inflammatory medications did not alter the results (see

Supplemental Table 4) with one exception: the association between WFC (at levels >0.4 mg/L) and IL-6 was attenuated and no longer significant (original model with 729 participants: β = -0.64; 95% CI: -1.20, -0.08 versus sensitivity model with 560 participants: β = -0.53; 95% CI: -1.17, 0.12).

Discussion

To the best of our knowledge, this is the first cohort study to investigate the association between maternal fluoride exposures and immunotoxicity during pregnancy. In this Canadian pregnancy cohort, maternal urinary fluoride concentrations were not significantly associated with any of the immune system biomarkers that were examined. In contrast, we observed a significant nonlinear association between WFC and CRP. Specifically, CRP levels increased with increasing WFC with quartiles 2 and 3 being significantly higher relative to quartile 1. Per 0.5 mg/L increase in water fluoride, we observed a 19.4% increase in CRP concentrations in the linear regression model. This pattern was consistently observed across multiple analyses, including Spearman correlations, linear regression, and our sensitivity analysis excluding participants who may have had elevated concentrations of inflammatory biomarkers due to other factors (*e.g.,* chronic inflammatory condition, regular anti-inflammatory medication use, acute infection). However, given that the association between WFC and CRP begins to decrease at water fluoride levels greater than 0.63 mg/L (*i.e.,* fourth quartile) and did not reach significance with our 'gold-standard' exposure biomarker (*i.e.,* MUF), results should be interpreted with caution. In addition, we observed a J-shaped association between MUF and IL-10 where the quadratic effect was significant. However, when restricting the sample to MUF levels greater than 0.80 mg/L, the association was no longer significant. As few participants had MUF values greater than 0.80 mg/L (n = 291), there may have been insufficient statistical power to detect an association.

Finally, there was a significant log-linear association between maternal WFC and IL-6; specifically, the association was negative at levels above 0.40 mg/L, albeit only when including participants who may have had elevated concentrations of inflammatory biomarkers due to other factors (*e.g.,* chronic inflammatory condition, regular anti-inflammatory medication use, acute infection). Moreover, the quartile regression model revealed that IL-6 concentration levels increase in quartile 2 (WFC: 0.14-0.52 mg/L) relative to quartile 1 (WFC: < 0.13 mg/L), followed by a decrease at water fluoride levels greater than 0.63 mg/L.

The moderate correlation between our exposure measures ($\rho = .48$) suggests that these metrics may be capturing different aspects of fluoride exposure (Krzeczkowski et al., 2024). Another study conducted with the MIREC cohort found an increased risk of hypothyroidism and with increasing levels of fluoride in drinking water, but not with MUF (Hall et al., 2023). Similar results have also been reported in other cohorts, showing stronger exposure-outcome associations with measures of water fluoride, compared to urinary fluoride (Cunningham et al., 2021; Riddell et al., 2019). Water fluoride concentrations may be more representative of long-term exposure to fluoride as levels remain relatively stable over time (Till et al., 2018), whereas urinary fluoride concentrations capture more recent systemic fluoride exposure (Krzeczkowski et al., 2024). Additionally, water fluoride concentrations do not account for the amount of tap water consumed or other important dietary sources (*e.g*., black tea). Indeed, MUF is considered a high-quality biomarker of fluoride exposure as it facilitates more precise estimates of an individual's fluoride intake across multiple sources.

Interpretation of our findings in relation to the existing literature is shaped by the dynamic physiological and metabolic adaptations intrinsic to pregnancy as few, if any, studies have investigated the association between fluoride exposures and immune system biomarkers

during gestation. Several studies examining longitudinal changes in cytokine profiles throughout pregnancy have presented conflicting findings, underscoring the importance of trimester- and assay-specific considerations when interpreting inflammatory markers (Bohn & Adeli, 2022; Ku et al., 2003; Stokkeland et al., 2019; Szarka et al., 2010; Velez et al., 2008). Some studies have reported associations between elevated maternal inflammation during the first and second trimester and altered neurodevelopmental outcomes (Mac Giollabhui et al., 2019; Meyer et al., 2007b), while others have noted changes associated with late gestation (Spann et al., 2023). For example, during the third trimester (34-37 weeks' gestation), maternal IL-6 and CRP levels were significantly associated with the strength of connectivity in the salience network in the medial prefrontal cortex, temporoparietal junction, and basal ganglia among infants (40-44 weeks; Spann et al., 2023). In particular, IL-6 has been identified as a critical mediator of MIA-associated behavioral and transcriptional changes (Smith et al., 2007).

In response to inflammation, IL-6 is secreted by neutrophils and macrophages and stimulates production of acute phase proteins and neutrophils in the liver and bone marrow respectively (Del Giudice & Gangestad, 2018). IL-6 may also have anti-inflammatory and immunosuppressive properties (Trapero & Cauli, 2014), regulate neuronal survival and function (Guzmán et al., 2010), and maintain integrity of the blood-brain barrier (Spooren et al., 2011). One proposed mechanism by which fluoride alters immune function may be by markedly reducing the synthesis of pro-inflammatory mediators, such as IL-6 and IL-8 (Zhu & Wei, 2024). In an animal model, IL-6 gene expression decreased slightly in the high NaF level group after 5 months of NaF (≥ 25 mg/L) administration, but was not statistically significant compared to the control group (Zhou et al., 2014). In contrast, increased IL-6 levels were observed in liver and kidney tissues following exposure to fluoride (100 mg/L) in another animal model (Yu et al.,

2022). In humans, increased levels of pro-inflammatory cytokines, including TNF-α and IL-6, were elevated among participants in low (0.1-0.9 mg/L) and high (≥ 1 mg/L) exposure groups in Pakistan (Bibi et al., 2023). Variations across these studies could be attributed to the differences in signaling pathways that regulate IL-6 production, secretion, and response. Although IL-6 has been considered a reliable biomarker of inflammation, it can also exert anti-inflammatory, regenerative, and protective functions (Schaper & Rose-John, 2015). In the absence of inflammation, IL-6 may function as a signaling molecule and regulate several physiological processes (Del Giudice & Gangestad, 2018). Further characterization of circulating IL-6 receptors may help elucidate the mechanism by which IL-6 exerts long-term health effects (Del Giudice & Gangestad, 2018).

While several epidemiological studies have demonstrated associations between high levels of fluoride exposure and elevated levels of CRP, few have examined this relationship at levels consistent with CWF. A cross-sectional analysis completed in fluoride endemic regions in China revealed that increased CRP levels were associated with higher drinking water fluoride exposure (1.95 ± 1.13 mg/L) among adult participants (Liu et al., 2014a). Similarly, in Turkey, plasma high-sensitivity CRP – a marker of inflammation and significant predictor of health risks (Bassuk et al., 2004) – levels were also significantly higher among participants with endemic fluorosis (urinary fluoride: 1.9 ± 0.1 mg/L; Varol et al., 2012). Among children with low-to-moderate fluoride exposure (urinary fluoride: 1.36 ± 1.31 mg/L; WFC: 1.42 ± 1.00 mg/L), each 1 mg/L WFC increase was associated with a 0.58 mg/L increase in CRP (Tang et al., 2023). Studies exploring exposures to other environmental chemicals and CRP have demonstrated similar results. For example, gestational exposure to ambient air pollution was positively associated with CRP, but not IL-6, IL-8, or TNF-α, measured in third trimester plasma samples

among MIREC participants (Gogna et al., 2021). Similar to the current study, this association persisted when excluding women with inflammatory conditions, including preeclampsia, impaired glucose tolerance, and gestational diabetes (Gogna et al., 2021). As previously noted, studies have reported various changes in CRP levels across pregnancy (Belo et al., 2015; Jarmund et al., 2021; Ferguson et al., 2014; Yu et al., 2019). Inconsistencies in results may be attributed to cohort characteristics, trimester-specific changes, or differences in CRP isoforms and their associated functions (Del Giudice & Gangestad, 2018). CRP is originally arranged in its pentameric isoform (pCRP) comprised of five identical subunits; however, when pCRP encounters signals of damage or ongoing inflammation, the five subunits irreversibly dissociate and lead to the formation of the monomeric isoform of CRP (mCRP; Del Giudice & Gangestad, 2018). In the absence of active inflammation, the net effects of pCRP are largely anti-inflammatory, whereas mCRP exerts pro-inflammatory functions, including the secretion of pro-inflammatory cytokines and release of reactive oxygen species (Thiele et al., 2014; Trial et al. 2016). Most behavioural and biomedical studies only reflect levels of pCRP as mCRP is not freely soluble in plasma and cannot be detected with standard assays (Del Giudice & Gangestad, 2014; Trial et al., 2016).

Although several studies have reported that male offspring are more sensitive to prenatal exposure to neurotoxicants (Green et al., 2020; Goodman et al., 2023), we did not observe any evidence of effect modification by fetal sex in our analyses. Differences between male and female placentas and fetuses have previously been reported in response to infection and inflammation, where pregnancies carrying male fetuses have stronger inflammatory responses following an immune challenge (Baines & West, 2023). A review of MIA papers from 2000 to 2018 analyzing offspring cytokine levels revealed that no studies had been conducted using only

female offspring and, of those that did include female offspring in their experimental paradigms, many combined them with males without reporting whether or not sex effects had been analyzed (Cioro & Pollak, 2019). Given the evidence of sex differences in susceptibility to both environmental chemicals and inflammation, it is critical that effect modification by fetal sex continue to be explored and reported in MIA studies.

Strengths and Limitations

To our knowledge, the present study is the first to evaluate how maternal exposure to fluoride may impact immune system function during pregnancy. The MIREC birth cohort included sensitive biomarkers of both exposure and outcome, in addition to relevant maternal covariates. As a result, we were able to account for several potential covariates in our analyses, including chronic inflammatory conditions and regular use of anti-inflammatory medications, both of which could influence circulating levels of immune system biomarkers during pregnancy. Given that most of the participants included in our analyses were exposed to water fluoride levels below 0.7 mg/L, our findings could be applicable to other populations living in communities with CWF.

Although this study has notable strengths, it also presents potential limitations. As maternal immune system biomarkers were measured once in the third trimester, we were not able to establish participants' baseline immune system biomarker levels or evaluate how fluoride exposures predict changes in these biomarkers across pregnancy. As outstanding questions remain regarding the timing and severity of exposures on MIA-associated phenotype changes (O'Connor & Ciesla, 2022), future studies could endeavour to capture exposures across pregnancy. Previous studies have demonstrated associations between heightened maternal inflammation in the first and second trimesters and neurodevelopmental outcomes (Mac

Giollabhui et al., 2019; Meyer et al., 2007b). The present study contributes insights into how fluoride may contribute to inflammatory processes in later stages of pregnancy.

Participants who did not provide third trimester plasma samples were also excluded from this study, which precluded examination of the association between maternal fluoride exposures and immune system biomarkers among a group that may be especially vulnerable to inflammation during gestation, including those who experienced a miscarriage or delivered extremely or very preterm. While the sensitivity analysis offers insights into the relationship between fluoride exposures and immune system biomarkers in a "healthy" group, the exclusion of participants with elevated markers of inflammation due to other factors may miss those who are the greatest risk of fluoride-induced health effects.

More broadly, generalizability of our findings may be limited due to the characteristics of the study cohort. Compared to Canadian women, MIREC participants tended to be older, married or common law, less likely to smoke, have a university degree, and have higher household income (Arbuckle et al., 2013). Moreover, a previous study identified that most participants in the MIREC cohort had adequate vitamin D status, which authors ascribed to the high adherence to multivitamin supplement use early during pregnancy (Weiler et al., 2021). High maternal iron, zinc, and vitamin D status has been identified as a factor promoting resilience to MIA, while gestational diabetes and maternal stress have been identified as factors promoting susceptibility (Meyer, 2019). Taken together, these results suggest that MIREC participants may possess protective factors that could mitigate the association between fluoride exposures and inflammation, limiting the generalizability of our findings to the broader Canadian population.

The accuracy of urinary measures of fluoride could have also been impacted by differences in behaviours prior to sample collections, including use of fluoridated toothpaste,

diet, or drinking tap water or black tea. We attempted to minimize this variability by averaging MUF across three trimesters and adjusting for urinary dilution. Future studies may also seek to include other individualized measures of fluoride exposure, such as bone fluoride or dietary fluoride intake across several sources. As the maternal immune system biomarkers fluctuate within and across trimesters, human chorionic gonadotropin levels may have been a more accurate estimate of gestational age (Larsen et al., 2013). Given the multiple comparisons between each of the fluoride exposure variables and individual immune system biomarkers, and the inflammatory index, the probability of making a Type I error may have increased. While setting a more stringent α may decrease the probability of a Type I error, it may also lead to increased probability of a Type II error.

Further characterization of the timing of maternal immune system activation is needed to advance our understanding of critical periods of vulnerability. Longitudinal studies following children born to mothers who experienced an immune challenge during pregnancy would help elucidate factors promoting resilience and susceptibility, along with a greater understanding of long-term neurodevelopmental outcomes associated with MIA. Likewise, collection of plasma samples for immunological analyses during the course of pregnancy would provide valuable insights into the differential outcomes linked to timing and severity of MIA. Further exploration of the mechanistic pathways through which fluoride affects immune organs, cells, and cytokine production is warranted to better understand the relationship between fluoride exposures during gestation and maternal immune system function. Moreover, studies investigating the relationship between co-exposures to environmental chemicals and inflammatory biomarkers could elucidate the molecular mechanisms associated with health and disease outcomes across the lifespan (Liu et al., 2022).

Conclusions

In this Canadian birth cohort, water fluoride concentrations were associated with changes in some maternal immune system biomarkers, suggesting fluoride may be implicated in inflammatory responses during the third trimester. CRP levels increased with increasing WFC; however, these results should be interpreted with caution as the dose-response was observed to be non-monotonic. More studies with exposure levels exceeding "optimal" fluoride levels (*i.e.*, 0.7 mg/L) are necessary to determine the shape of the dose-response.

Given the ubiquitous nature of fluoride exposure, there is an urgent need to identify potential health risks associated with fluoride during critical and sensitive periods of development. Although several mechanisms may underscore MIA-associated phenotype changes, outstanding questions remain regarding the molecular mechanisms by which these processes alter neurodevelopmental processes in utero and across the lifespan. Further research is needed to better understand the mechanism by which fluoride may disrupt immune system function during pregnancy. This research can help inform health policy and improve health equity by mitigating risks among vulnerable populations. In turn, findings from this study may also be shared with engaged stakeholders, such as pregnant women, policy makers, and primary health care providers, to help inform their understanding of the safety of fluoride exposure during critical periods of development.

Appendix

Supplemental Table 1

Principal component analysis

	Eigen value	Difference	Proportion of variance	Cumulative variance
Component 1	1.80	0.80	0.45	0.45
Component 2	1.01	0.18	0.25	0.70
Component 3	0.82	0.46	0.21	0.91
Component 4	0.36	.	0.09	1.00

Supplemental Table 2

Eigenvectors of principal component analysis for inflammatory index

	Component			
	1	2	3	4
IL-6	0.589	-0.139	0.520	0.603
IL-8	0.463	0.162	-0.821	0.293
TNF-α	0.662	-0.032	0.102	-0.742
MCP-1	0.029	0.977	0.213	0.013

Supplemental Table 3

Quartile regression model assessing the association between MUF and log-transformed IL-10

Log-transformed IL-10	β	95% CI	*p*
MUF – 2nd Quartile (.33 - .49 mg/L)[1]	-0.14	-0.27, -0.0035	.044
MUF – 3rd Quartile (.49 - .74 mg/L)	-0.11	-0.26, 0.026	.111
MUF – 4th Quartile (.74 – 5.6 mg/L)	-0.09	-0.24, 0.054	.219

[1] Q2 corresponds to results for women with MUF and IL-10 in the second quartile (*i.e.*, between the 25th and 50th percentiles) relative to those with levels in the first quartile (*i.e.*, below the 25th percentile).

Q3 correspond s to results for women with MUF and IL-10 in the third quartile (*i.e.*, between the 50th and 75th percentiles), relative to those with levels in the first quartile (*i.e*., below the 25th percentile).

Q4 corresponds to results for women with MUF and IL-10 in the fourth quartile (*i.e*., above the 75th percentile), relative to those with levels in the first quartile (*i.e*., below the 25th percentile)

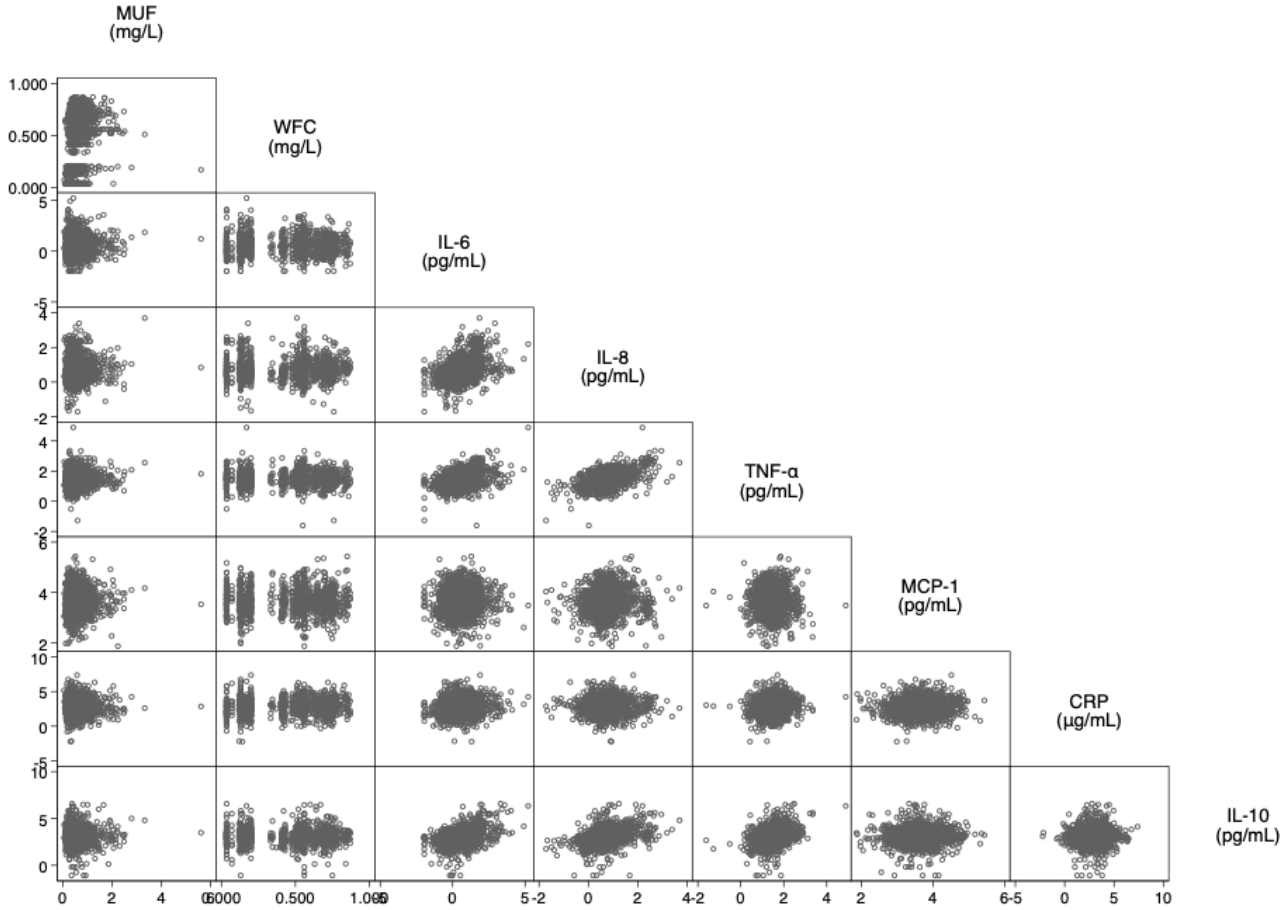
Supplemental Table 4

Log-linear regression models for the sensitivity analysis assessing the association between fluoride exposures and maternal immune system biomarkers excluding women who reported a chronic immune condition, anti-inflammatory medication use, and/or CRP levels greater than 100 mg/L

	n	β	95% CI	*p*
MUF				
IL-6	1054	0.056	-0.100, 0.21	.48
IL-8	1054	0.019	-0.088, 0.13	.73
TNF-α	1054	0.017	-0.071, 0.10	.71
MCP-1	1051	0.035	-0.054, 0.12	.44
CRP	1059	-0.03	-0.21, 0.14	.73
IL-10	1058	-0.03	-0.18, 0.12	.70
WFC				
IL-6	930	-0.16	-0.38, 0.062	.16
IL-8	930	-0.047	-0.20, 0.11	.55
TNF-α	930	0.037	-0.089, 0.16	.56
MCP-1	924	-0.013	-0.14, 0.11	.84
CRP	932	0.36	0.11, 0.62	.005
IL-10	931	0.023	-0.19, 0.24	.83

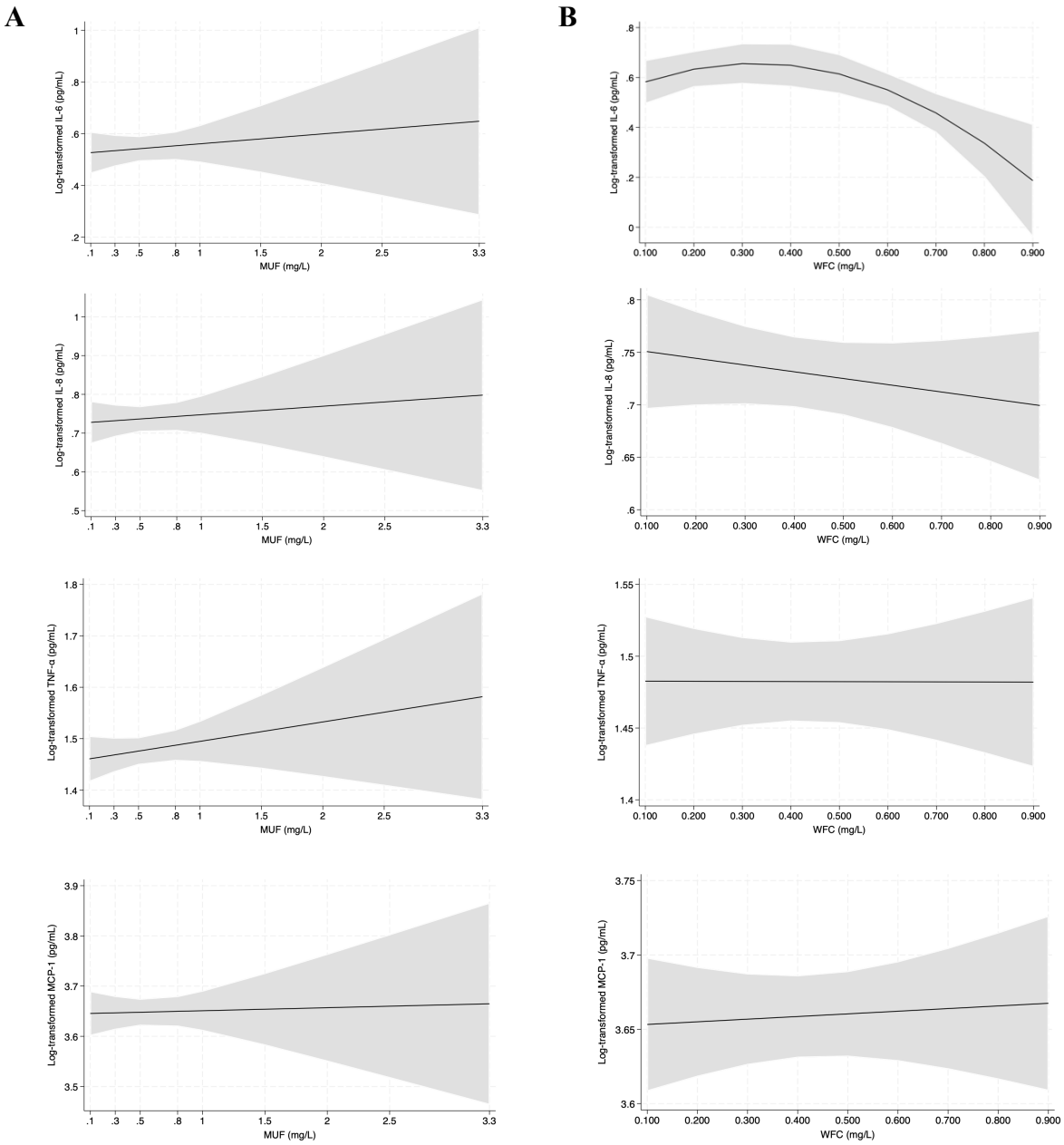
Supplemental Figure 1

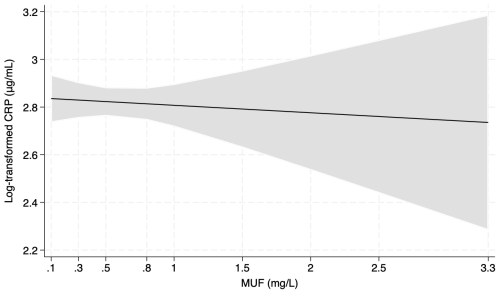
Correlation matrix for maternal fluoride exposures and log-transformed immune system biomarkers

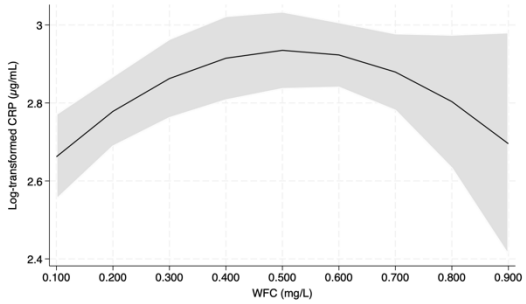


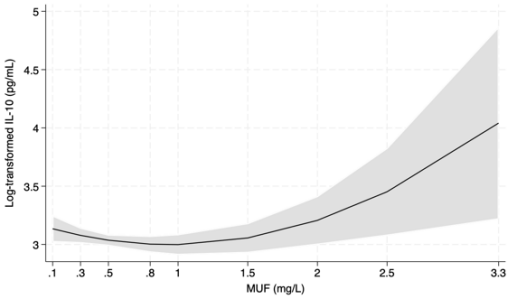
Supplemental Figure 2

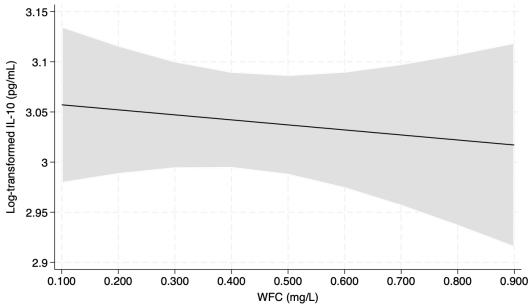
Margins plots for log-linear and non-linear regression models assessing the associations between maternal urinary fluoride (A) and maternal water fluoride (B) concentrations and log-transformed maternal immune system biomarkers




Log-transformed CRP (µg/mL)
3.2
3
2.8
2.6
2.4
2.2
.1
.3
.5
.8
1
1.5
2
2.5
3.3
MUF (mg/L)


Log-transformed CRP (µg/mL)
3
2.8
2.6
2.4
0.100
0.200
0.300
0.400
0.500
0.600
0.700
0.800
0.900
WFC (mg/L)


Log-transformed IL-10 (pg/mL)
5
4.5
4
3.5
3
.1
.3
.5
.8
1
1.5
2
2.5
3.3
MUF (mg/L)


Log-transformed IL-10 (pg/mL)
3.15
3.1
3.05
3
2.95
2.9
0.100
0.200
0.300
0.400
0.500
0.600
0.700
0.800
0.900
WFC (mg/L)

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