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Prenatal fluoride exposure, offspring visual acuity and autonomic nervous system function in 6-month-old infants

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Abstract

Background: Prenatal fluoride exposure can have adverse effects on children's development; however, associations with visual and cardiac autonomic nervous system functioning are unknown. We examined associations between prenatal fluoride exposure and visual acuity and heart rate variability (HRV) in 6-month-old infants.

Methods: We used data from Canadian mother-infant pairs participating in the Maternal-Infant Research on Environmental Chemicals (MIREC) cohort. We estimated prenatal fluoride exposure using: i) fluoride concentration in drinking water (mg/L), ii) maternal urinary fluoride adjusted for specific gravity (MUF_{SG}; mg/L) and averaged across pregnancy, and iii) maternal fluoride intake (µg/kg/day) from consumption of water, tea, and coffee, adjusted for maternal body weight (kg). We used multivariable linear regression to examine associations between each measure of fluoride exposure and Teller Acuity Card visual acuity scores (n=435) and assessed HRV (n=400) using two measures: root mean square of successive differences (RMSSD) and the standard deviation of N-N intervals (SDNN) measured at 6-months of age.

Results: Median (IQR) values for water fluoride, MUF_{SG}, and daily fluoride intake were 0.20 (IQR: 0.13-0.56) mg/L; 0.44 (0.28-0.70) mg/L and 4.82 (2.58-10.83) µg/kg/day, respectively. After adjustment for confounding variables, water fluoride concentration was associated with poorer infant visual acuity (B = -1.51; 95% CI: -2.14,-0.88) and HRV as indicated by lower RMSSD (B = -1.60; 95% CI: -2.74,-0.46) but not SDNN. Maternal fluoride intake was also associated with poorer visual acuity (B = -0.82; 95% CI: -1.35,-0.29) and lower RMSSD (B = -1.22; 95% CI: -2.15,-0.30). No significant associations were observed between MUF_{SG} and visual acuity or HRV.

Conclusion: Fluoride in drinking water was associated with reduced visual acuity and alterations in cardiac autonomic function in infancy, adding to the growing body of evidence suggesting fluoride's developmental neurotoxicity.

Keywords: Neurotoxicity; visual acuity; autonomic nervous system; infancy; prenatal

1. Introduction

Approximately 73% of Americans and 39% of Canadians have fluoride added to municipal drinking water supplies at a concentration of 0.7 mg/L to prevent tooth decay (*The State of Community Water Fluoridation across Canada. 2022 Report.*, 2022; *Water fluoridation Data & Statistics*, 2023). While the benefits of water fluoridation are cited widely, a growing body of evidence has linked early-life exposure to fluoride with adverse neurodevelopmental outcomes, even at optimal exposure levels (i.e., 0.7 mg/L) (Dewey et al., 2023; Farmus et al., 2021; Grandjean, 2019; Grandjean et al., 2023; Green et al., 2019; National Toxicology Program. Office of Health Assessment and Translation, 2022). However, some of the findings are mixed (Do et al., 2023; Ibarluzea et al., 2022), possibly reflecting differences in sources, cofactors, doses, and timing of exposure.

The fetus is particularly vulnerable to neurotoxicants, which can cross the placenta and interfere with the developing nervous system (Lanphear, 2015). To date, five pregnancy cohort studies have examined the impact prenatal fluoride exposure on offspring neurodevelopmental outcomes in populations receiving optimally fluoridated water or salt (Bashash et al., 2017; Cantoral et al., 2021; Dewey et al., 2023; Green et al., 2019; Ibarluzea et al., 2022). One study conducted in Mexico, where salt is fluoridated, showed that higher dietary fluoride intake in pregnancy was associated with lower nonverbal abilities in offspring at 24-months of age, but only in boys (Cantoral et al., 2021). Among preschool-aged children, studies conducted in Mexico City (Bashash et al., 2017) and Canada (Green et al., 2019) found that children born to women with higher levels of urinary fluoride in pregnancy had lower intelligence quotient (IQ) scores; this association was only significant among boys in the Canadian study (Green et al., 2019). Another Canadian cohort study reported links between exposure to fluoridated drinking water and lower cognitive flexibility at 3-5 years of age, particularly in girls (Dewey et al., 2023). In contrast, a study conducted in Spain found that higher urinary fluoride levels in pregnancy were positively associated with IQ in boys at age 4; however, this association was driven by

those living in non-fluoridated communities and was attenuated when adjusting for other neurotoxicants (Ibarluzea et al., 2022).

Use of multimethod approaches to assess the developing nervous system may provide the sensitivity needed to detect subtle yet important effects of prenatal fluoride exposure on the developing infant brain. Past fluoride studies conducted in human infants assessed cognitive and motor skills using the Bayley Scales of Infant Development (Cantoral et al., 2021; Valdez-Jimenz et al., 2017; Ibarluzea et al., 2022). Examining markers of both central and peripheral nervous system development, such as visual acuity and cardiac autonomic nervous system (ANS) function, could expand our understanding of the impact of prenatal fluoride exposure on offspring neurodevelopment beyond cognitive and behavioural outcomes. Indeed, markers of sensory system development and ANS function have been used to index aspects of infant neurodevelopment following exposure to prenatal adversity (Gilman et al., 2017). Additionally, assessing infants in the first 6-months of life is advantageous given that postnatal exposure to fluoride sources is limited, especially among breastfed infants. Breastmilk contains extremely low concentrations of fluoride (<0.02 μg/L) due to the limited transfer of plasma fluoride into breastmilk (Dabeka et al., 1986; Ekstrand et al., 1981; Ekstrand et al., 1984; Zohoori et al., 2019). Exposure to fluoride in the first 6-months of life is mainly limited to intake from infant formula, especially if fluoridated water is used to reconstitute formula (Zohoori et al., 2014). Exposure from other sources, such as fluoridated toothpaste and fluoride-containing foods, does not typically occur until after 6-months of age.

Visual acuity, which refers to the ability to detect small visual details with precision (Teller et al., 1986), can be reliably assessed within the first postnatal year and can be used to detect toxicity to the developing central nervous system (Brémond-Gignac et al., 2011). Indeed, prenatal exposure to various toxic chemicals, such as methylmercury (Murata et al., 2006; Yorifuji et al., 2013), chlorpyrifos (Silver et al., 2018), lead (Silver et al., 2016), molybdenum (Wang et al., 2023) and organic solvents (Till et al., 2005; Till et al., 2001) during gestation have been linked to poorer offspring visual acuity.

Poorer visual acuity early in life has been associated with cognitive dysfunction and may be linked to further visual problems over the lifespan (Brémond-Gignac et al., 2011). To our knowledge, no studies have examined links between prenatal fluoride exposure and visual acuity in infancy.

The ANS, which plays a critical role in maintaining homeostasis across organ systems (Thayer & Sternberg, 2006), can be reliably assessed during the first postnatal year using measures of heart rate variability (HRV) (Laborde et al., 2017). Lower HRV is thought to reflect poorer ANS capacity to coordinate adaptive responses to situational demands/environmental challenges. Exposure to environmental toxins, including Bisphenol A (Bae et al., 2012) and particulate matter (Gold et al., 2000; Saenen et al., 2019) are inversely associated with HRV in adults and children. Prenatal environmental toxicant exposures may also adversely impact offspring HRV. For instance, some studies have reported lower HRV in offspring prenatally exposed to methylmercury (Chan et al., 2021; Grandjean et al., 2004; Murata et al., 2006; Sørensen et al., 1999), however, two other studies did not report links (Periard et al., 2015; Zareba et al., 2019). Prenatal exposure to nicotine, opioids, cocaine, and alcohol have also been linked to adverse ANS function in offspring (Fifer et al., 2009; Nordenstam et al., 2017; Sania et al., 2023; Schlatterer & du Plessis, 2021; Schuetze et al., 2007). Despite links to neurotoxicants and substances, as well as evidence suggesting that infant ANS function is sensitive to exposure to prenatal adversity (Chiera et al., 2020; Schlatterer & du Plessis, 2021; Van den Bergh et al., 2020), no studies have investigated associations between prenatal fluoride exposure and offspring HRV. Investigating these links is critical given that adverse ANS development is associated with poorer socioemotional functioning (Porges & Furman, 2011), psychiatric risk (Beauchaine & Thayer, 2015) and risk for adverse cardiometabolic outcomes (Thayer et al., 2010) across the lifespan.

Using data from the Maternal-Infant Research on Environmental Chemicals (MIREC) cohort, we examined associations between prenatal fluoride exposure and infant visual acuity and ANS function measured in 6-month-old infants. Considering findings from previous studies showing sex-specific associations related to prenatal fluoride exposure (Dewey et al., 2023; Green et al., 2019;

Malin & Till, 2015), and the importance of examining sex-specific effects at different developmental stages (Bellinger et al., 2016; Gade et al., 2021; Goodman, In Press), we also explored the potential for sex-specific associations in this study.

2. Methods

Between 2008 and 2011, 2001 pregnant women were recruited from prenatal clinics within the first trimester (11.99, SD=1.5 weeks gestation) across ten Canadian cities (Arbuckle et al., 2013). Eligibility criteria included fluency in English or French, planning to deliver locally, agreement to provide a cord blood sample, and being 18 years or older at enrollment. Participants were excluded at the time of recruitment if there were known fetal abnormalities, history of severe disease, or illegal drug use. A subsample of 525 mothers were invited to participate in the MIREC infant development (MIREC-ID) follow-up study, which involved the assessment of infant health at 6-months of age. Infants were assessed at seven sites across the following Canadian cities: Vancouver, Toronto, Hamilton, Kingston, Ottawa, Montreal, and Halifax (Figure 1). Mother-infant dyads were eligible to participate in MIREC-ID if the infant was born from a singleton pregnancy and was free of birth defects and/or neurological disorders. Among the 525 mother-infant pairs, 90 (16.8%) did not complete the visual assessment due to lack of time or co-operation by the infant. Of the 435 infant who that completed visual testing, another six (1.1%) were excluded due to suspected ocular abnormality, including congenital cataract or retinoblastoma, leaving a final sample of 429 infants with visual acuity data. For the HRV data, 400 of 525 (76%) infants completed the electrocardiogram (ECG) procedure, (125 did not complete HRV testing again due to lack of time/co-operation by the infant). Among the 400 infants with data on HRV, 10 were excluded due to excess noise in >2% of the ECG trace.

Overall, 398 (91.5%) of the 435 participants in the visual acuity sample overlapped with the HRV sample whereas 398 (99.5%) of the 400 participants in the HRV sample overlapped with the visual acuity sample. Comparison of the visual acuity and HRV subsamples against each other and the full MIREC sample can be found in Table S1. All study procedures were approved by ethics boards at

each of the recruitment sites, Health Canada, and Public Health Agency of Canada (MIREC: REB 2006-027H).

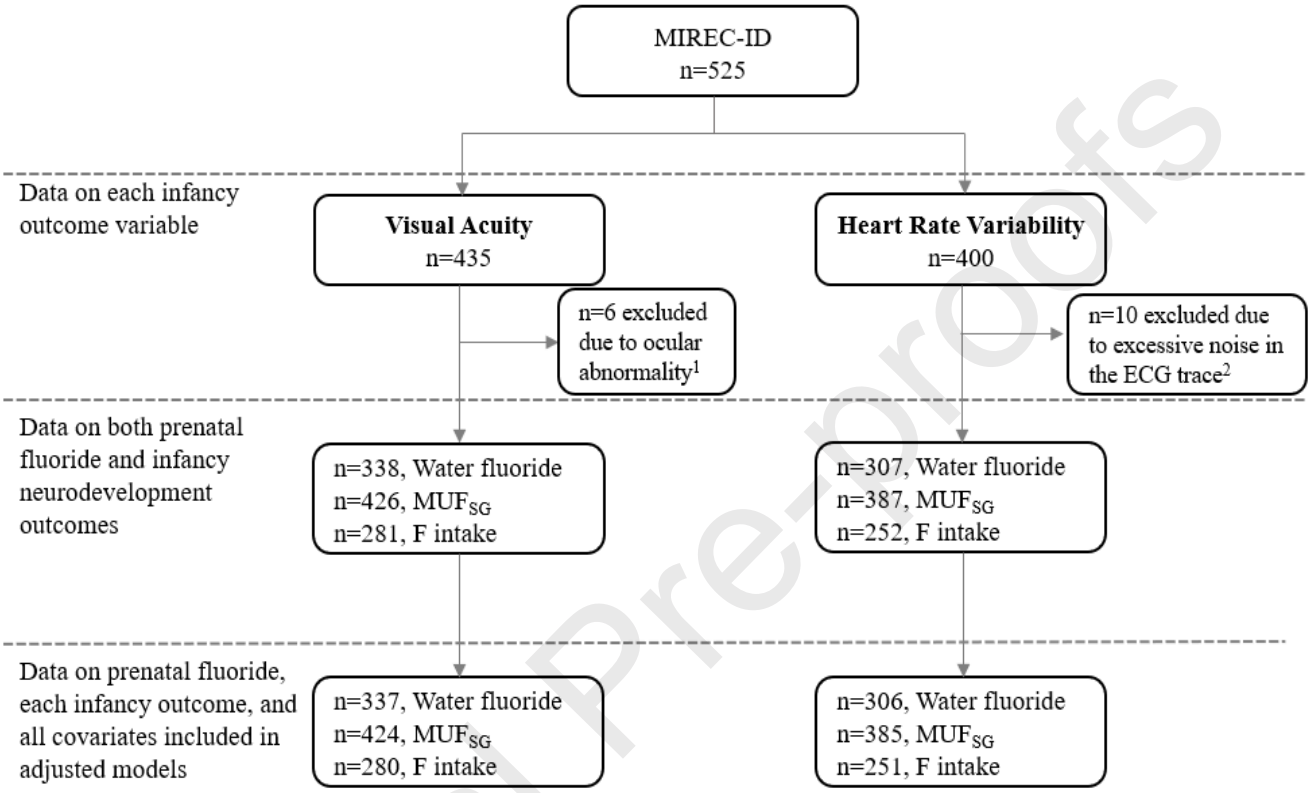

Figure 1: Participant flow chart.

[1]Congenital cataract, retinoblastoma
[2]Excessive motor movements, unable to identify QRS complex in ECG trace
Abbreviations: ECG: electrocardiogram; MIREC-ID: Maternal infant research on environmental chemicals cohort-Infant Development; MUF$_{SG}$: Maternal urinary fluoride adjusted for specific gravity; F intake: maternal fluoride intake

2.1 Measures

2.1.1 Maternal Fluoride Exposure

2.1.1.1 Water fluoride concentration (mg/L)

We solicited municipal drinking-water reports from water treatment plants for the period spanning 2008 to 2012. Daily water fluoride measurements were provided by treatment plants that add

fluoride to municipal drinking water. Weekly or monthly water fluoride measurements were provided by treatment plants that do not add fluoride to municipal drinking water. The first three letters of participant postal codes were used to match participants' residences to the boundary regions serviced by each water treatment plant. We estimated the average water fluoride concentration (i.e., geometric mean; mg/L) in the municipal drinking water for each woman across the duration of her pregnancy. Women were included if their postal code could be linked to a water treatment plant and if they reported drinking tap water in pregnancy, as described previously (Till et al., 2018).

2.1.1.2 Maternal urinary fluoride (MUF) concentration (mg/L)

Maternal urinary fluoride (MUF) was analyzed in urine spot samples collected in trimesters 1 (mean=11.57, SD=1.57 weeks gestation), 2 (mean=19.11, SD=2.39 weeks gestation) and 3 (mean=33.11, SD=1.50 weeks gestation). Spot urine samples were collected in Nalgene containers and aliquoted samples into smaller cryovials. Urinary fluoride concentration was measured at the Indiana University School of Dentistry using a modification of the dexamethyldisilonane (Sigma Chemical Co) microdiffusion procedure (Martinez-Mier et al., 2011). The LoD for analyses was 0.02 mg/L, and none of the values fell below the LoD (see (Till et al., 2018) for further details). Given that urinary fluoride concentration can vary across pregnancy, we only included women who had a valid measure of urinary fluoride measured in each trimester. MUF was standardized for specific gravity (SG) to account for urine dilution using the following equation (MacPherson et al., 2018).

$$MUF_{SG} = MUF_i \left(\frac{SG_M - 1}{SG_i - 1}\right)$$

Here, MUF_{SG} is the fluoride concentration standardized by SG (mg/L), MUF_i is the unadjusted fluoride concentration (mg/L) and SG_i and SG_m are the SG of the individual urine sample, and median sample SG for the cohort, respectively. We used the average dilution-adjusted MUF_{SG} concentration by taking the average across all three trimesters for each woman.

2.1.1.3 Maternal fluoride intake (µg/kg/day)

Given that fluoride can be ingested from sources other than water, including black tea (Waugh et al., 2016), we estimated a measure of daily fluoride intake. We derived maternal fluoride intake by multiplying water fluoride concentration (mg/L) by the total volume (L) of water, tea, and coffee consumed per day, and adding fluoride content that would be expected from each 200 mL cup of black tea or green tea consumed. We estimated a fluoride content of 0.326 mg per cup of black tea, and 0.260 mg per cup of green tea (Krishnankutty et al., 2022). Intake of water-based beverages was obtained from self-reported questionnaires completed in trimesters 1 and 3. Our estimate of maternal fluoride intake was adjusted for trimester-specific body weight (BW) using the formula:

$$\frac{\frac{[(WaterF_{(T1)} * TotalCups_{(T1)}] + BlackTeaF_{(T1)} + GreenTeaF_{(T1)}}{BW_{(T1)}} + \frac{[(WaterF_{(T3)} * TotalCups_{(T3)}] + BlackTeaF_{(T3)} + GreenTeaF_{(T3)}}{BW_{(T3)}}}{2}$$

Here, WaterF is based on each woman's tap water fluoride concentration and indicates the level of fluoride in a 200 mL cup. TotalCups indicates a women's self-reported number of cups of water-based beverages (i.e., cups of water and cups of coffee and/or tea consumed). BlackTeaF and GreenTeaF reflect the additional fluoride content of black and green teas, respectively; for example, if the woman reported drinking two cups of black tea, the BlackTeaF value would be 0.652. BW reflects the woman's body weight in kilograms. T1 and T3 reflect the first and third trimesters, respectively.

2.1.2 Infant Outcomes

2.1.2.1 Grating Visual Acuity

We measured visual acuity using the Teller Acuity Cards II (TAC-II) test, which is the gold standard vision test for infants as young as one-month of age (Teller et al., 1986). The TAC test is based on the preferential-looking phenomenon in infants, whereby the infant prefers to look at a stimulus (i.e., vertical black and white grating) as opposed to a blank area when both are presented simultaneously(Teller et al., 1986). Infants were shown laminated cards (25.5 x 55.5 cm) with the stimulus on one side (12 x 12 cm square-wave grating, with a 60-70% contrast) and a blank grey area

on the opposite side. The TAC test was administered by trained research nurses who were blinded to the mother's exposure information. Testing lasted 5 minutes (Polevoy et al., 2020). Infants were seated on their parent's lap approximately 55 cm from the cards. Infants viewed cards sequentially, and each card included progressively narrower gratings, from 1.3 to 38 cycles per degree (cpd). Visual acuity was assessed as the finest grating (i.e., highest spatial frequency or highest cpd) eliciting the infant's visual preference as judged by longer stimulus viewing relative to the blank page; a higher score indicates better visual acuity. Nurses judged the infant's visual preference by looking through a small hole in the middle of the card. At each site, all visual acuity tests were performed by the same nurse, and all of the nurses administering the TAC were trained by the same professional.

2.1.2.2 Infant heart rate variability (HRV)

We used an electrocardiogram (ECG) to examine infant heart rate variability (HRV), a measure of heart rhythm that is under autonomic nervous system control. HRV reflects the variability of the N-N intervals of a traditional heart-beat waveform; the N-N interval is also referred to as the distance between two adjacent R-peaks on the ECG waveform (Laborde et al., 2017; Sassi et al., 2015). Seven electrodes were placed on the infant at the following positions: i) right and ii) left clavicles, bilateral to the sternum; iii) left clavicle, at the mid-clavicular line; iv) at the lower right chest wall; 5) at the 4th intercostal space (right sternal edge); 6) sixth rib at the left mid-clavicular line; and 7) fifth intercostal space, at the left axillary line. Infant HRV was acquired throughout the study visit, which included an anthropometric assessment as well as participation in two validated tasks used to induce fluctuations in HRV. This included the tilt procedure (Zygmunt & Stanczyk, 2010) and the arm restraint task (Goldsmith & Rothbart, 1996). Both tasks are thought to produce changes in HRV. HRV was collected continuously across all the assessments, and averaged across the short anthropometric procedure, the tilt procedure, and the arm restraint task to obtain an overall measure of HRV reactivity. Lower HRV indicates an inflexible system that may exhibit difficulties responding to situational demands; conversely, greater HRV indicates a system capable of flexibly altering activity to better adjust to

situational demands (Appelhans & Luecken, 2006; Kim et al., 2018). A cardiologist blinded to maternal fluoride status examined each ECG trace for artifacts, and data were excluded if more than 2% of the recording exhibited noise.

We examined infant ANS cardiac function using time domain assessments of HRV. This included the root mean square of successive differences (RMSSD) and the standard deviation of N-N intervals (SDNN) (Laborde et al., 2017). RMSSD is a measure of the beat-to-beat heart rate variance and indicates the level of parasympathetic (PSNS) influence on the heart (Laborde et al., 2017). It is correlated with high frequency power measures of HRV, but is thought to be less affected by respiration than other high frequency HRV metrics, such as respiratory sinus arrhythmia (Hill & Siebenbrock, 2009). SDNN is considered an index of both sympathetic and PSNS influence, is correlated to multiple frequency domain measures including ultra low frequency, very low frequency and low frequency bands on the heart (Shaffer & Ginsberg, 2017) and is thought to reflect the brain's influence on the heart in response to changing situational/environmental demands (Shaffer & Ginsberg, 2017).

2.1.3 Covariates

We examined the potential confounding effects of infant age at testing (months), birthweight (g), and sex, and maternal age (years), pre-pregnancy body mass index (BMI), smoking in trimester 1 (never, former, quit during pregnancy/current), education (dichotomized as 'bachelor's degree or higher vs. trade school diploma or lower), race (white vs. other), birth country (Canada vs elsewhere), parity, family income before tax (defined as <$50,000 CAD/year, between $50,000-$100,000, and >$100,000), marital status, and self-reported ratings of warmth/affection (i.e., mother's pleasure and affection displayed when interacting with their infant from the Parental Cognitions and Conduct Toward the Infant Scale (Boivin et al., 2005); see supplement for further information on all confounding variables). Covariates were included in the models if previous research examining links between prenatal exposure to neurotoxicants (including fluoride) and offspring neurodevelopmental

outcomes supported their inclusion as confounding variables (e.g., infant age, infant sex, maternal education) (Cantoral et al., 2021; Farmus et al., 2021; Green et al., 2019). Additional confounding variables were retained in our models if their p-value was <0.2 or if they altered the regression coefficient of the main predictor by more than 10% (e.g., maternal race, infant birthweight), and do not constitute a mediator or potential collider. Our final covariate-adjusted models included the following variables: infant age, sex, birth weight, maternal education, and race. We also adjusted for city in models examining MUF_{SG}. City was not included in water fluoride models owing to collinearity between city and water fluoride levels. Finally, because data on exclusive breastfeeding were missing for 23% of the sample, breastfeeding was only considered as a covariate in our sensitivity analyses.

2.2 Statistical analyses

We first examined the distributions and frequencies for all fluoride exposure and infant outcome variables to ensure normality. We summarized descriptive and exposure characteristics of mother-infant pairs using means and standard deviations for continuous variables and frequencies for categorical variables. We used Spearman correlations to examine associations between each of our fluoride exposure variables. We used separate linear regression models to assess associations between prenatal fluoride exposure and measures of visual acuity, RMSSD and SDNN before and after adjusting for confounding variables. We also examined potential effect modification by testing the interaction between infant sex and each of our fluoride measures. We applied a $\log_{10}$-transformation given that RMSSD and SDNN values were positively skewed; however, these variables remained skewed even after log transformation. However, regression diagnostics, including kdensity plots, pnorm, and qnorm plots, indicated normally distributed residuals of the regression models using both log and non-log transformed variables and results remained consistent using log and non-logged data. Therefore, to facilitate interpretations of associations between fluoride exposure and our HRV variables, we present associations using the non-transformed values.

In a sensitivity analysis, we re-ran each model separately controlling for first trimester log-transformed maternal blood lead concentration (Pb, μg/dL), blood mercury concentration (Hg, μg/L). Pb and Hg exposure have been previously associated with infant visual acuity and ANS function (Grandjean et al., 2004; Silver et al., 2016; Yorifuji et al., 2013). We also controlled for duration of exclusive breastfeeding (reported at the MIREC-ID visit), given links between breastfeeding and brain development (Brown Belfort, 2017). Given evidence suggesting that birth weight may be associated with fluoride exposure (Arun et al., 2022; Kampouri et al., 2022; Ortiz-Garcia et al., 2022), as well as visual acuity (Molloy et al., 2013) and ANS function (Souza et al., 2017), we re-ran the models without adjusting for birth weight to avoid the possibility that birth weight is on the causal pathway (Kim et al., 2015). We also examined associations excluding those born <37 weeks' gestation, given that visual acuity can be affected in infants born premature. Finally, to examine whether potential postnatal fluoride exposure via formula reconstituted with fluoridated water modified out results, we examined the interaction between water fluoride concentration and a variable that indicated if infants were i) never breastfed/not exclusively breastfed for the first 3 months of life or ii) were exclusively breastfed for 3 months or more.

For this study, we report beta coefficients for both water fluoride and MUF_{SG} concentrations per 0.5 mg/L; 0.5 mg/L corresponds to the approximate difference in water fluoride concentration between a fluoridated and non-fluoridated community as assessed in our prior work conducted in the MIREC cohort (Till et al., 2018). For our fluoride intake variable, we report unstandardized beta coefficient per 10 μg/kg/day. Statistical tests were two-tailed, and an alpha of 0.05 was the criteria for statistical significance for main effects; interactions were probed if the *P* value for the interaction term was <0.10. We applied a Holm-Bonferroni correction to account for the family-wise error rate across our three fluoride measures. Each family of infant outcomes (visual acuity, and then the HRV outcomes, RMSSD and SDNN) was probed within each "family" of exposure measures. We also probed our data for outliers that could bias associations. For linear regression models, we examined Cook's distance

values to identify potential influential observations (all observations had Cook's distance value <0.5, indicating no evidence of influential observations), and variance inflation factors were <4 for variables in adjusted models. We used STATA version 17.0 (STATA corporation) for all statistical analyses.

3. Results

Table 1 shows the demographic characteristics of participants with fluoride exposure and complete covariate data who were included in adjusted models for mother-infant pairs with visual acuity and HRV data. Women were, on average, 31.6 (SD=4.81) years at enrollment; the majority were married (94.6%), had a bachelor's degree or higher (66.1%), and reported being White (91.3%). At the time of testing, infants were, on average, 6.81 months of age (SD=0.89; range: 4.93 to 10.09), and 48% were female; 22 of 424 (5.2%) infants were born moderately-to-late preterm (i.e., <37 weeks of gestation), and 26 (6.1%) had a low birth weight (<2500 g).

Relative to the full MIREC sample (n=1983), participants with data on MUF_{SG}, complete covariates (used in adjusted models), and visual acuity (n=424) or HRV (n=385) were less likely to be current or recent smokers, were more likely to be White, and had a greater gestational age. Those with visual acuity data were also less likely to have an income >$100,000, while those with HRV data were less likely to be married relative to the full MIREC sample. No differences were found between either the visual acuity or the HRV sub-samples compared to the MIREC-ID sample (n=525) (Table S1). Additionally, Table S2 compares the 398 participants who had data on both infant outcomes against those who were recruited for MIREC-ID, but did not have data on both infant outcomes (n=127). Participants with data on both infant outcomes had mothers who were more educated, more likely to be married, have white race, and had slightly higher birthweights ($p \leq .05$). Finally, in Table S3, we compared characteristics that may impact child development between those living in fluoridated vs. non-fluoridated regions. Infants born in fluoridated regions had a higher birthweight (3527.22 g; SD=498.52) than those born in non-fluoridated regions (3419.72 g; SD=519.62). No other differences were observed.

Mean infant TAC score was 5.75 cpd (SD=3.00; range: 0.43-19.00), which is in the normal range for infants between 5 and 10 months of age (Courage & Adams, 1990; Leone et al., 2014; Polevoy et al., 2017; Salomao & Ventura, 1995; Teller et al., 1986). Mean infant RMSSD was 15.25 (SD=4.89; range: 8.00-39.00), and SDNN was 39.10 (SD 15.12; range: 16.00-133.00), consistent with age-normative scores reported in the literature (Harteveld et al., 2021).

3.1 Measures of prenatal fluoride exposure

For the visual acuity subsample, the median (IQR) values for water fluoride (n=337), MUF_{SG} concentration (n=424), and daily fluoride intake (n=280) were 0.20 mg/L (IQR: 0.13-0.56 mg/L), 0.44 mg/L (0.28-0.70 mg/L), and 4.82 µg/kg/day (IQR: 2.58-10.83 µg/kg/day), respectively. These exposure levels were similar to those observed in the HRV subsample.

In the visual acuity subsample, Spearman rank correlations showed that MUF_{SG} was moderately correlated with both water fluoride concentration (n=335, r_s = 0.47, p<0.001) and fluoride intake (n=280, r_s = 0.49, p<0.001), and water fluoride concentration was strongly correlated with fluoride intake (n=280, r_s = 0.75, p<0.001). These correlations were highly similar to those observed in the HRV subsample: MUF_{SG} and water fluoride concentration: n=304, r_s =0.46, p<0.001; MUF_{SG} and fluoride intake: n=251, r_s =0.47, p<0.001; water fluoride concentration and fluoride intake: n=251, r_s=0.75, p<0.001.

3.2 Associations between prenatal fluoride exposure and infant outcomes

Figure 2 shows a significant negative association between water fluoride concentration and visual acuity in both the unadjusted (B= -1.44; 95% CI: -2.07, -0.82, p<0.001) and covariate-adjusted models (B = -1.51; 95% CI: -2.14, -0.88, p<0.001). We also observed a significant association with maternal fluoride intake (unadjusted: B= -0.85; 95% CI: -1.39, -0.32, p=0.002; adjusted: B= -0.82; 95% CI: -1.35, -0.29, p=0.003). In contrast, no statistically significant association was observed between MUF_{SG} and visual acuity (adjusted: B= 0.11; 95% CI: -0.30, 0.51, p=0.60).

We found a significant negative association between water fluoride concentration and infant RMSSD in both the unadjusted (B= -1.63; 95% CI: -2.74, -0.52, *p*=0.004) and covariate-adjusted models (B = -1.60; 95% CI: -2.74, -0.46, *p*=0.006). For maternal fluoride intake, significant associations were observed (unadjusted: B= -1.24; 95% CI: -2.16, -0.32, p=0.008; adjusted: B= -1.22; 95% CI: -2.15, -0.30, *p*=0.01). In contrast, no significant association was observed between MUF_{SG} and RMSSD (adjusted: B=0.22; 95% CI: -0.47, 0.92, *p*=0.53). Finally, there were no associations between any of the fluoride exposure variables and SDNN (water fluoride: B= -1.31, 95% CI: -4.70, 2.09, *p*=0.45; $MUF_{SG:}$ B= 0.10, 95% CI: -0.20, 2.20, *p*=0.92; fluoride intake: B= -2.13, 95% CI: -4.98, 0.72, *p*=0.14) (Figure 2).

Table 1: Demographic characteristics[1]

Participant	Visual acuity sub-sample			HRV sub-sample		
	Water fluoride (n = 337)	MUF_{SG} (n = 424)	Fluoride intake (n = 280)	Water fluoride (n = 306)	MUF_{SG} (n = 385)	Fluoride intake (n = 251)
Mothers						
Age (years); mean (SD)	31.90 (4.87)	31.85 (4.70)	31.71 (4.84)	31.78 (4.92)	31.81 (4.70)	31.55 (4.87)
Education; *n* (%)						
Less than bachelor's degree	117 (34.72)	145 (34.20)	93 (33.21)	106 (34.64)	132 (34.29)	81 (32.27)
Bachelor's degree or higher	220 (65.28)	279 (65.80)	187 (66.79)	200 (65.36)	253 (65.71)	170 (67.73)
Household income (CDN$); *n* (%)						
<$50000	65 (19.82)	72 (17.43)	55 (20.15)	63 (21.00)	69 (18.30)	52 (21.14)
$50000 -$100000	153 (46.65)	199 (48.18)	128 (46.89)	137 (45.67)	180 (47.75)	115 (46.75)
>$100000	110 (33.54)	142 (34.38)	90 (32.97)	100 (33.33)	128 (33.95)	79 (32.11)
Marital status; *n* (%)						
Married or common law[2]	318 (94.36)	403 (95.05)	262 (93.57)	290 (94.77)	368 (95.58)	236 (94.02)
Separated, other	19 (5.64)	21 (4.95)	18 (6.43)	16 (5.23)	17 (4.42)	15 (5.98)
Smoking in T1; *n* (%)						
Never	222 (65.88)	282 (66.51)	182 (65.00)	202 (66.01)	255 (66.23)	163 (64.94)
Former	85 (25.23)	105 (24.76)	72 (25.71)	78 (25.49)	98 (25.45)	66 (26.29)
Current/Quit in pregnancy	30 (8.90)	37 (8.73)	26 (9.29)	26 (8.50)	32 (8.31)	22 (8.76)
Maternal warmth[3]; mean (SD)	9.33 (0.79)	9.38 (0.74)	9.34 (0.81)	9.35 (0.78)	9.40 (0.73)	9.36 (0.79)
Parity (n; %)						
1	161 (47.77)	199 (46.93)	138 (49.29)	147 (48.04)	180 (46.75)	125 (49.80)
2	133 (39.47)	163 (38.44)	106 (37.86)	122 (39.87)	149 (38.70)	96 (38.25)
≥3	43 (12.76)	62 (14.62)	36 (12.86)	37 (12.09)	56 (14.55)	30 (11.95)
White race; *n* (%)						
Yes	305 (90.50)	390 (91.98)	253 (90.36)	279 (91.18)	356 (92.47)	229 (91.24)
No	32 (9.50)	34 (8.02)	27 (9.64)	27 (8.82)	29 (7.53)	22 (8.76)
T1 maternal blood lead (Pb, µg/dL); mean (SD)[4]	0.64 (0.60; 0.67)	0.61 (0.58;0.64)	0.66 (0.62;0.69)	0.64 (0.61;0.67)	0.61 (0.58,0.64)	0.66 (0.62,0.70)

T1 maternal blood mercury (Hg, µg /L); mean (SD)[5]	0.67 (0.61; 0.73)	0.67 (0.62; 0.72)	0.68 (0.11; 0.14)	0.66 (0.60; 0.73)	0.67 (0.61;0.73)	0.66 (0.59,0.74)
Infants						
Age at testing (months); mean (SD)	6.82 (0.89)	6.81 (0.89)	6.85 (0.92)	6.80 (0.84)	6.77 (0.83)	6.84 (0.87)
Breastfeeding duration (months)[6], *n* (%)						
None to <3	55 (16.32)	70 (16.51)	48 (17.14)	48 (15.69)	62 (16.10)	40 (15.94)
3 to <6	155 (45.99)	190 (44.81)	127 (45.36)	142 (46.41)	174 (45.19)	114 (45.42)
>6	49 (14.54)	63 (14.86)	37 (13.21)	47 (15.36)	60 (15.58)	97 (14.74)
Missing	78 (23.15)	101 (23.83)	68(24.29)	69 (22.55)	89 (23.12)	60 (23.90)
Sex; *n* (%)						
Male	178 (52.82)	220 (51.89)	147 (52.50)	158 (51.63)	196 (50.91)	129 (51.39)
Female	159 (47.18)	204 (48.11)	133 (47.50)	148 (48.37)	189 (49.09)	122 (48.61)
Gestational age (weeks); mean (SD)	39.56 (1.40)	39.56 (1.41)	39.63 (1.36)	39.56 (1.35)	39.55 (1.36)	39.63 (1.32)
Prematurity, *n* (%)						
Late preterm (34 to 37 weeks)	15 (4.45)	18 (4.45)	11 (3.93)	10 (3.27)	12 (3.12)	7 (2.79)
Preterm <34 weeks	0 (0)	1 (0.24)	0 (0)	0 (0)	1 (0.26)	0 (0)
Small for gestational age						
No	315(93.47)	398 (93.87)	261 (93.21)	287(93.79)	364(94.55)	235(93.63)
Yes	22 (6.53)	26 (6.13)	19 (6.79)	19(6.21)	21(5.45)	16(6.37)
Birth weight (g); mean (SD)	3492.84 (522.09)	3491.10 (513.18)	3491.00 (524.92)	3487.92 (505.03)	3489.81 (499.47)	3489.31 (509.44)
Fluoride Exposure						
Water fluoride concentration (mg/L); mean (SD)	0.37 (0.25)	0.37 (0.25)	0.34 (0.25)	0.36 (0.25)	0.36 (0.25)	0.33 (0.25)
MUF_{SG} (mg/L); mean (SD)	0.55 (0.36)	0.55 (0.37)	0.54 (0.35)	0.55 (0.37)	0.54 (0.38)	0.52 (0.34)
Fluoride intake *µg/kg/day*); mean (SD)	7.51 (6.70)	7.51 (6.70)	7.51 (6.70)	7.29 (6.47)	7.29 (6.47)	7.29 (6.47)

Community water fluoridation; *n* (%)						
Yes	167 (49.55)	165 (49.25)	118 (42.14)	147 (48.04)	145 (47.70)	100 (39.84)
No	170 (50.45)	170 (50.75)	162 (57.86)	159 (51.96)	159 (52.30)	151 (60.16)

[1] Sample with complete data on all predictors, outcomes and covariates reported at 6-month visit.

[2] In Canada, common-law partners must have cohabited for at least one year.

[3] Maternal self-reports on the Parental Cognitions and Conduct Toward the Infant Scale-Parental warmth subscale(Boivin et al., 2005)

[4,5]Geometric mean, (95% CI)

[6]Months of exclusive breastfeeding at the MIREC-ID visit. Of the 525 infants in MIREC-ID, 335 (64%) reported on months of exclusive breastfeeding.

Abbreviations: CDN: Canadian dollars, T1: trimester 1; g: grams, µg: micrograms, mg: milligrams, L: litre; MUF_{SG} = maternal urinary fluoride adjusted for specific gravity

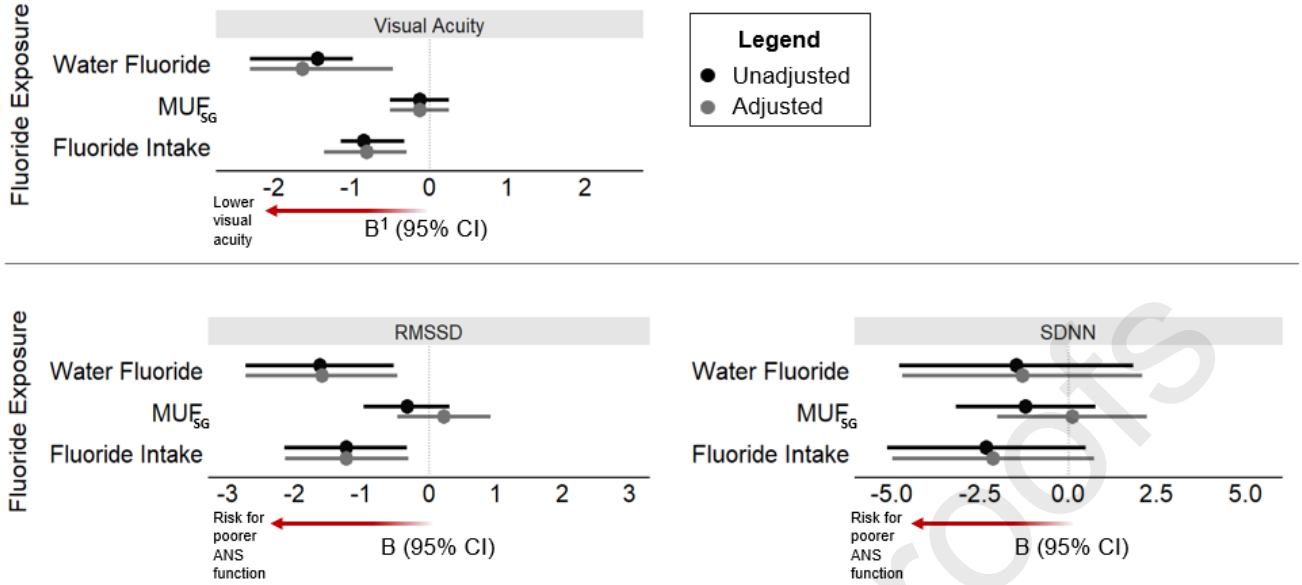

Figure 2: Unadjusted and adjusted associations between fluoride exposure variables and visual acuity and heart rate variability outcomes. Associations adjusted for infant age, infant sex, maternal education, maternal race, and infant birth weight. We also adjusted for city when examining associations with MUF_{SG}.

[1] Unstandardized beta coefficients are shown per 0.5 mg/L water fluoride and MUF_{SG} and per 10 µg/kg/day fluoride intake.
Abbreviations: MUF_{SG}= Maternal urinary fluoride adjusted for specific gravity, B=unstandardized beta

3.3 Effect modification by infant sex

No significant interaction by sex was found for any of our prenatal fluoride exposure measures and infant outcomes. However, in stratified analyses, the association between water fluoride concentration and RMSSD outcome was significant in boys (B = -2.21; 95% CI: -3.84, -0.58), but not girls (B = -0.53; 95% CI:- 2.04, 0.97); thus, we may not have had the statistical power to detect a significant interaction. Associations stratified by infant sex are shown in Table S4.

3.4 Sensitivity Analyses

The significant associations observed between water fluoride and maternal fluoride intake, and infant visual acuity and RMSSD remained significant when controlling for trimester 1 maternal blood lead (Pb) and mercury (Hg) concentrations. Results also remained significant when removing birth weight as a covariate, excluding those born <37 weeks gestation, and when adjusting for exclusive

breastfeeding duration (Table S5). Finally, there was no significant interaction between water fluoride concentration and our dichotomous breastfeeding variable (none/<3 months vs. >3 months) for visual acuity ($p>0.10$) and RMSSD ($p>0.10$)

4. Discussion

In this Canadian prospective birth cohort study, we examined whether prenatal fluoride exposure was associated with grating visual acuity and cardiac autonomic function in infants using two time domain measures of heart rate variability, RMSSD and SDNN. We found that higher levels of fluoride in drinking water during pregnancy were associated with lower infant visual acuity and risk for ANS problems indexed by lower RMSSD. Relative to the findings with water fluoride concentration, similar, but weaker associations were observed with maternal fluoride intake (which accounts for exposure from water and additional dietary sources of fluoride, such as black tea) and offspring visual acuity and RMSSD. Taken together, these results suggest that water fluoride levels and maternal fluoride intake may be associated with poorer central (visual acuity) and peripheral (cardiac ANS) markers of nervous system functioning in infant offspring. These results are novel given that prior human studies examining prenatal exposure to fluoride have only assessed offspring outcomes using measures of cognitive and behavioral development.

In contrast, maternal urinary fluoride levels were not associated with any of our infant outcomes, consistent with past studies conducted in this cohort (Hall et al., 2023) as well as other samples (Malin et al., 2018; Riddell et al., 2019) showing stronger exposure-outcome associations with water fluoride compared with urinary fluoride levels. The moderate correlation between our fluoride exposure measures suggests that these metrics are capturing different aspects of fluoride exposure and are therefore considered complementary to each other. We consider water fluoride concentration to be a more representative measure of long-term exposure to fluoride, given that levels are relatively stable over months (Castiblanco-Rubio et al., 2022; Till et al., 2018). However, we acknowledge that this exposure metric is also prone to measurement error since a concentration of fluoride in drinking water

does not reflect consumption habits, particularly amount of water consumed. We attempted to address an individual's consumption habits by estimating maternal fluoride intake using questionnaire data reported by women at two time-points during pregnancy. Maternal fluoride intake is therefore thought to reflect a more individualized metric of exposure relative to water fluoride level because it takes into consideration overall volume of water, tea, and coffee consumed as well as additional exposure to fluoride from black tea consumption.

In contrast to our estimated fluoride exposure measures (i.e., water fluoride and fluoride intake), MUF_{SG} measured from urine spot samples captures *recent* systemic fluoride exposure from all sources and is more likely to exhibit variability over time. Our past work comparing MUF_{SG} levels across each trimester found an intraclass correlation coefficient of 0.37 (Till et al., 2018). This moderate consistency across trimesters in the MIREC cohort is likely due to the short (~6 hr) half-life of fluoride in the body (Whitford, 1994) and differences in amount of fluoride that is absorbed and released from long-term accumulation in bones due to continuous bone remodeling (Villa et al., 2010). While we attempted to minimize variability in urinary fluoride by averaging MUF_{SG} across three trimesters, the measure remains impacted by variability in day-to-day fluoride exposures (Castiblanco-Rubio et al., 2022), which may have contributed to null associations.

Another explanation for why we observed associations with our water-based measures, but not with urinary fluoride, may be due to unmeasured confounding. It is possible that differences in water fluoride levels and intake levels (which differ by city) could serve as a proxy for other relevant characteristics that may differ between people living in fluoridated versus non-fluoridated communities. However, comparison of mother-child dyads by CWF status did not reveal any differences that could explain the observed associations over and above the covariates that were used in our models (Table S3). Finally, we note that we do not control for "city" in the models that used water-based fluoride measures due to collinearity between city and water fluoride concentration. To check whether controlling for "city" controls for community characteristics that are associated with the

decision to fluoridate water supplies, we removed 'city' from the MUF_{SG} model. Results remained non-significant, meaning that the difference in the association between the urine biomarker versus our other fluoride measures are not due to differences in covariate adjustment.

Previous studies have reported poorer visual acuity following prenatal exposure to a variety of neurotoxicants, including methylmercury, lead, chlorpyrifos, and organic solvents (Silver et al., 2018; Till et al., 2005; Till et al., 2001; Yorifuji et al., 2013), reflecting the exquisite sensitivity of the developing visual system to early-life exposure to neurotoxicants (Grandjean & Landrigan, 2014; Lanphear, 2015). Furthermore, poorer visual acuity in infancy is an important outcome because it may have prognostic value for potential adverse outcomes later in life, including lower IQ and poorer reading comprehension (Brémond-Gignac et al., 2011).

Studies have also shown that toxins can impact heart rate variability in exposed adults and children (Bae et al., 2012; Gold et al., 2000; Halabicky et al., 2022; Saenen et al., 2019). Prenatal exposures, including methylmercury, nicotine, and alcohol, may also adversely affect offspring ANS development (Chan et al., 2021; Grandjean et al., 2004; Murata et al., 2006; Sørensen et al., 1999). We extend this evidence to prenatal fluoride exposure, albeit only with RMSSD, perhaps suggesting that prenatal fluoride exposure may disrupt the development of HRV mediated by the parasympathetic branch of the ANS, thereby indicating risk for adverse ANS function (Laborde et al., 2017; Shaffer & Ginsberg, 2017). Indeed, the ANS consists of two major branches, the parasympathetic (PSNS) and sympathetic (SNS) branch which work in concert to balance energy mobilization (SNS), and relaxed, restorative functions (PSNS) (Thayer & Sternberg, 2006). Therefore, adverse PSNS development can lead to ANS dysfunction, thereby increasing disease risk across multiple organ systems (Quigley & Moore, 2018; Thayer & Sternberg, 2006; Thayer et al., 2010). Other studies have also found that early adversity, including exposure to neurotoxicants, can negatively impact the PSNS branch of the ANS (Schlatterer & du Plessis, 2021). For instance, lead and methylmercury exposure have been linked to greater sympathetic dominance (i.e., poorer PSNS influence on the heart) (Halabicky et al., 2022) and

lower PSNS activity (Gribble et al., 2015). Furthermore, *prenatal* methylmercury exposure was linked to lower PSNS activity and greater SNS dominance (Chan et al., 2021; Murata et al., 2006). Disruptions in parasympathetic function can impact development of socioemotional functioning (Porges & Furman, 2011), including self-regulation (Holzman & Bridgett, 2017), and increase risk for psychopathology (Beauchaine & Thayer, 2015).

While the mechanisms underlying the current findings are unclear, observing effects across both central (i.e., visual system development) and peripheral (i.e., ANS function) nervous system levels suggest that prenatal fluoride exposure may be associated with more diffuse, rather than focal effects on the developing nervous system. Fluoride readily crosses the blood-brain barrier and can accumulate in multiple brain areas, resulting in damage to myelin and neurons as well as decreases in dendritic arborization (Wang et al., 2018; Whitford et al., 2009). Furthermore, its effects on myelin could adversely impact third trimester myelination of the vague nerve, which is particularly important for parasympathetic influence on the heart (Cerritelli et al., 2021; Porges & Furman, 2011). This may also impact emerging connectivity pathways within the visual system that begin development prenatally and continue into childhood (Dayan et al., 2015).

Although we found no statistically significant evidence of effect modification by sex in the association between fluoride exposure and infant visual acuity and ANS functioning, effects were generally stronger among male infants, especially for RMSSD, which is consistent with some previous studies showing stronger effects of prenatal fluoride exposure on males (Cantoral et al., 2021; Farmus et al., 2021; Green et al., 2019). However, a recent study reported adverse cognitive development in females whose mothers were exposed to optimally fluoridated water in pregnancy (Dewey et al., 2023). Sex specific effects are complex and depend on multiple factors including the functional outcome assessed and developmental stage that offspring are assessed (Joel & McCarthy, 2017). Indeed, male and female fetuses use different strategies to respond to placental perturbations, with the male placenta investing more resources in growth relative to females (Sandman et al., 2013). This emphasis on

growth is thought to reduce the male fetus's ability to respond flexibly to prenatal adversities (Sandman et al., 2013), which could explain why males may be at greater risk for poorer PSNS development, a system which mediates adaptation and flexibility in response to internal/external demands (Thayer & Sternberg, 2006). Research also suggests that there is an interaction between sex and thyroid hormones and that certain neurotoxicants, including fluoride, may change thyroid physiology in a sex-specific manner (Batista & Hensch, 2019; Hall et al., 2023), which in turn may disrupt ANS functioning (Brusseau et al., 2022). However, while important to acknowledge mechanisms underlying sex differences following fetal exposure to neurotoxicants, further research is needed to elucidate the role of offspring sex in these links.

Our study must be examined in the context of the following limitations. First, we were unable to assess the magnitude of fluoride that reaches the fetal brain. While fluoride is commonly measured in spot urine or water samples, these exposure metrics do not account for variability in placental transport and metabolism and thus may not accurately reflect fetal exposure. Moreover, our archived data did not have information about day-to-day behaviors that may have occurred, such as morning fasting or use of fluoridated dental products or consumption of distilled bottled water prior to urine sampling. These behaviors would influence urine-fluoride levels given the short half-life of fluoride (~6 hours) and contribute measurement error, which would be more likely to negatively bias our effect estimates towards the null. However, we attempted to mitigate the potential for exposure misclassification by taking the average urinary fluoride concentration across all three trimesters. Future studies should consider alternate biomarkers that can capture chronic fluoride exposure, such as tooth dentin and toenails (Nayak et al., 2021; Vidyadharan et al., 2020)). Tooth dentin can measure serial and cumulative prenatal and postnatal systemic exposure to fluoride (Yu et al., 2021). It is an optimal biomarker because the week-by-week history of fluoride exposure beginning at approximately 14 weeks gestation and continuing through the first 2 to 3 years of life can be reconstructed to test for critical windows of exposure (Davis et al., 2020). This type of biomarker would have the advantage of

assessing fluoride exposures occurring in early infancy through formula-feeding and introduction of solid foods, which we were unable to do in the current study. To focus our analysis on fetal fluoride exposure, we conducted a sensitivity analysis controlling for exclusive breastfeeding. Results remained consistent, suggesting that the fetal period may be a critical exposure window, though this analysis was limited because we were missing infant feeding information at 6-months of age for almost 25% of the sample. Third, while the TAC is considered a reliable measure of grating acuity, it is a behavioral assessment that is based on preferential looking and may not generalize to other aspects of vision. Finally, future studies could also aim to examine other measures of ANS activity using additional HRV measures, including frequency domain and non-linear assessments.

5. Conclusions

This study is the first to report associations between prenatal fluoride exposure and offspring visual acuity and HRV as assessed by RMSSD in 6-month-old infants. Our findings that prenatal fluoride exposure may adversely affect visual acuity and ANS functioning in infants highlights the gestational period as a critical period of susceptibility to fluoride. This work contributes to the growing body of evidence on the potential toxicity of fluoride to the developing brain.

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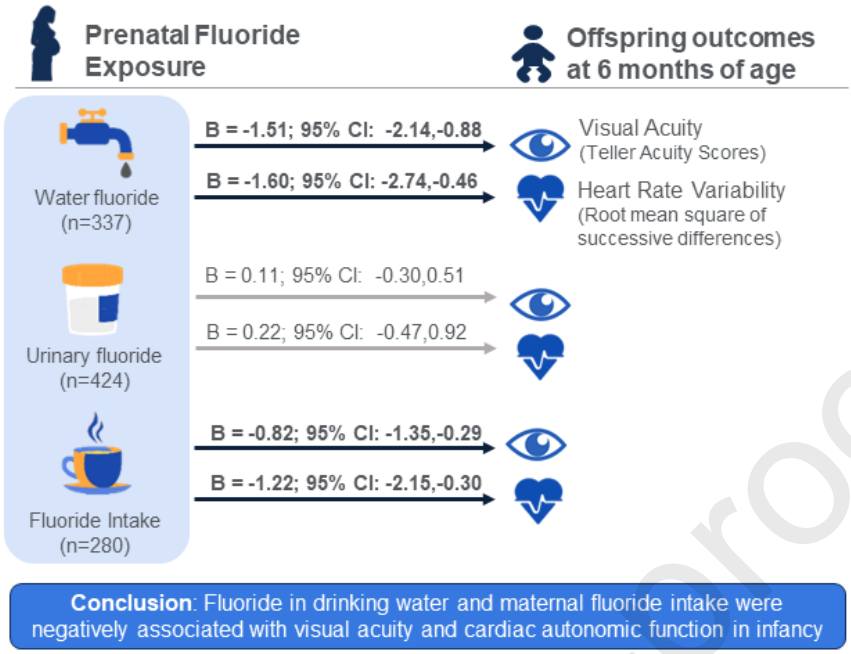

Highlights

- This is the first study to examine associations between prenatal fluoride exposure and disruptions to visual acuity and cardiac autonomic function in infants
- In this Canadian pregnancy cohort, prenatal fluoride exposure was linked to poorer visual acuity
- Prenatal fluoride exposure was linked to lower heart rate variability (root mean square of successive differences)
- Results suggest that prenatal fluoride exposure may be associated with poorer central and peripheral markers of nervous system functioning in infant offspring

Declaration of interests

☐ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☒ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Disclosure: Dr. Lanphear (co-author) served as a non-retained expert witness in the federal fluoride case to describe the results of the fluoride studies using the MIREC cohort (Food & Water Watch, et al. vs. U.S. Environmental Protection Agency, United States District Court for the Northern District of California at San Francisco. He received no payment for his service. All authors report no conflict of interest.

Author statement

J.E.K, C.T. and B.L conceptualized the idea for the study, conducted analyses and wrote the first draft of the manuscript. M.H., C.V.G., R.G., T.M., and Y.O. contributed to the intellectual content of the manuscript, consulted on statistical analyses and the interpretation of results and in writing of the manuscript. D.S. G,M contributed to the design of the study, collection of outcome measures, statistical analyses, interpretation and writing of the manuscript. All authors were involved in discussions to finalize the manuscript.