

**Fluoride exposure during pregnancy and its effects on childhood neurobehavior:
a study among mother-child pairs from Mexico City, Mexico**

by

Deena B. Thomas

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of the requirements for the degree of
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Doctoral Committee:

**Professor Karen E. Peterson, Co-Chair
Professor Howard Hu, Co-Chair, University of Toronto
Associate Professor Niladri Basu, University of McGill
Assistant Professor Sung Kyun Park
Associate Professor Brisa N. Sanchez**

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Dedication

This dissertation is dedicated to Dieter Thomas Bouma, P.B. Thomas and Vimala Thomas.

I simply could not have done this work without my husband Dieter by my side. His dedication to justice and peace inspire my work, and his intellect, humor and vitality never fail to brighten my days. He has also been the truest example of selflessness. He has tended to my wellbeing and happiness while I put in long days in the lab, or spent many hours writing. He has encouraged me through the ‘lows,’ cheered with me through the ‘highs,’ and he has been incredibly loving and patient throughout. I look forward to coming home to him everyday, and I can hardly believe I get to spend the rest of my life with him!

My *Amma* Vimala is one of the most courageous people I know. All her life, she fought oppression, sometimes quietly, and sometimes with a certain digit up in the air. Her work on behalf of women has made my life better, and my mother’s love, passion and fearlessness is my greatest example.

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The love, support and encouragement of my husband and parents have sustained me throughout this work.

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Table of Contents

Dedication.....	ii
Acknowledgements.....	iii
List of Figures.....	viii
List of Tables	ix
Abstract.....	x
CHAPTER I Introduction.....	1
I. Background.....	1
A. A brief history of water fluoridation.....	1
B. Adverse health related to fluoride ingestion	1
C. Fluoride exposure in the United States and in Mexico	2
D. Concurrent fluoride and neurobehavior	3
E. Prenatal fluoride exposure.....	4
1. Maternal-fetal transmission of fluoride	4
2. Fluoride in pregnant women	4
3. Prenatal fluoride exposure and the brain	5
II. Methods.....	6
A. Study Population.....	6
1. Participants for prenatal studies.....	7
2. Participants for concurrent exposure study.....	7
B. Biomarkers	8
1. Urinary fluoride	8
2. Plasma fluoride	8
C. Neurobehavioral tests.....	8
1. Wechsler Abbreviated Scales of Intelligence (WASI)	8
2. Bayley Infant Scales of Development-II (BSID-II) and the Mental Development Index (MDI).....	9
III. Thesis overview	9
IV. References.....	12
CHAPTER II Urinary and Plasma Fluoride Levels During Pregnancy and Determinants of Exposure Among Pregnant Women from Mexico City, Mexico	17
I. Abstract.....	17
II. Introduction	19
III. Methods.....	21
IV. Results.....	24
V. Discussion	26
VI. References.....	33
CHAPTER III Prenatal fluoride exposure and neurobehavior: a prospective study.....	37
I. Abstract.....	37
II. Introduction	39

III. Methods.....	41
IV. Results.....	45
V. Discussion	46
VI. References.....	51
CHAPTER IV Concurrent Fluoride and Total WASI in 6-15 year old children from Mexico City, Mexico	54
I. Abstract.....	54
II. Introduction	55
III. Methods.....	57
IV. Results.....	62
V. Discussion	63
CHAPTER V Conclusion	72
I. Overview	72
II. Chapter II.....	72
III. Chapter III.....	75
VI. Chapter IV	77
V. Limitations and future considerations.....	78
VI. References.....	80

List of Figures

Chapter I

Figure I.1: Fluoride metabolism 11

Chapter IV

Figure IV.1: Child's urinary fluoride and total WASI score adjusted for child's Sex, child's age, maternal age, marital status, maternal education, family possessions, and cohort in the entire population..... 68

Figure IV.2: Child's urinary fluoride and total WASI score adjusted for Child's Sex, child's age, maternal age, marital status, maternal education, family possessions, mother's WAIS score and cohort among female children..... 68

List of Tables

Chapter II

Table II.1: Exposures in Mexico to Environmental Toxicants (ELEMENT) cohort recruited in Mexico City, Mexico between 1997 and 2005.	30
Table II.2: Intraclass correlation of urinary fluoride and plasma fluoride over three trimesters of pregnancy and Spearman correlation coefficients of trimester-specific urine and plasma fluoride among pregnant women from Mexico City, Mexico.	31
Table II.3: Generalized Additive Mixed Effects models with maternal urinary fluoride and plasma fluoride as outcome measures in hundreds of pregnant women from Mexico City, Mexico.	32

Chapter III

Table III.1: Descriptive characteristics of mother-infant pairs participating in the ELEMENT birth cohort.	49
study from Mexico City, Mexico.	49
Table III. 2: Generalized Additive Mixed Models of maternal urinary and plasma fluoride during pregnancy and child's MDI at ages one, two and three.	50

Chapter IV

Table IV.1: Descriptive Statistics of Mother-child pairs recruited into the Cholesterol cohort in Mexico City, Mexico.	66
Table IV.2: Generalized Additive Models of Urinary fluoride and WASI among children in the Cholesterol Cohort from Mexico City, Mexico.	67

Abstract

Artificial fluoridation, implemented worldwide to reduce periodontal diseases, was deemed one of the top public health achievements of the 20th century. In recent years, expert reviews by the US National Research Council (NRC) and the World Health Organization (WHO) have concluded children living in high-fluorosis areas may suffer from cognitive declines. However, exposure measures and statistical methods in past studies have been challenged, and, thus, the true relationship between fluoride exposure and cognition remains unclear. In addition, little is known about fluoride exposure during pregnancy.

The objective of this dissertation is to improve understanding of fluoride exposures during pregnancy and to assess whether prenatal and concurrent fluoride exposure is associated with neurobehavioral effects in offspring. The research aims are conducted by using resources from the Early Life Exposure in Mexico to Environmental Toxicants (ELEMENT) study, and a follow-up study called Cholesterol. Chapter 2 is the first population-based study to provide an exposure profile of fluoride during early, mid and late pregnancy, using two different biomarkers, and it is the first to examine whether some periods of pregnancy may be more vulnerable to exposure. Chapter 3 assesses the neurobehavioral effects of prenatal fluoride exposure on the Mental Development Index (MDI) of the children when they are one, two, and three years old. Chapter 4 of this dissertation measures urinary fluoride in children at 6-15 years and examines its association to the total score from the Wechsler Abbreviated Scales of Intelligence Test (WASI.) This work increases our understanding of prenatal and concurrent fluoride exposure and their effects on cognition in children. During pregnancy, we found that

fluoride levels do not change as pregnancy progresses once you adjust for other factors, and children of older mothers may be more susceptible to fluoride exposure. We additionally found that maternal fluoride exposure levels did not have any measurable effects on cognition in early childhood in this cohort. We found that concurrent fluoride exposure in males, but not females, aged 6-15 years, was significantly associated with WASI, and this highlights the need for future research considering gender differences in neurocognitive outcomes using longitudinal data.

CHAPTER I

Introduction

I. Background

A. A brief history of water fluoridation

Artificial fluoridation schemes have been used for nearly 65 years to reduce the prevalence of dental caries, but the relationship between fluoride and dental health stretches back further. At the turn of the 20th century, a handful of dentists in Colorado, Italy and England independently observed fewer dental caries in communities with naturally high fluoride levels in their water supply. The association between the compound and dental health was formally examined in 1945 when the National Institutes of Health (NIH) started an epidemiology study in Grand Rapids, Michigan in 1945. The city had just started fluoridating its drinking water supply, and the study found a 54 percent decrease in decayed, extracted or filled teeth in young children over the next 10 years.^{1,2,3} Following the publication of these promising findings, Sodium Fluoride (NaF) and fluorosilicates were increasingly and steadily added to public drinking water supplies in the United States and in other parts of the world.

B. Adverse health related to fluoride ingestion

Artificial fluoridation, however, has been controversial due, in part, to the adverse health outcomes associated with exposure. Moderate to severe intake of fluoride, for example, affects dental enamel. In some cases, the enamel is hypomineralized and in more severe cases, the

loss of enamel surface leads to brown stains on the surface of the teeth.⁴ Skeletal fluorosis, an abnormality in bone reformation, felt as joint pain and stiffness, is more serious and cases arise in communities where drinking water fluoride exceeds 4 mg/L. It can also be an iatrogenic effect of fluoride therapy for osteoporosis.⁵ Fluoride exposure has also been implicated in lower serum testosterone in men exposed to more than 3 mg/L, in stomach and abdominal pain at levels less than 1 mg/L,^{6,7,8} in spina bifida occulta in high-fluorosis areas,^{9,10} and in lower IQ among children living in high fluorosis areas.¹¹

C. Fluoride exposure in the United States and in Mexico

1. United States: In the United States(U.S.), fluoride is added to water as a preventive measure against dental caries.¹² Naturally fluoridated water, at the EPA's recommendation, is maintained below 4 mg/L, the level above which the risk for skeletal fluorosis increases. Artificially fluoridated water is regulated by state and local governing bodies, but the U.S. Public Health service recommends 0.7 to 1.2 mg/L of fluoride for the prevention of dental caries. In 2012, 74.6% of people with access to public water systems or nearly 211 million people in the United States drank artificially fluoridated water.¹³ Another 13 million were reported to drink naturally fluoridated water in 1993, but no current data is available for this group.¹⁴ The World Health Organization reports either artificial or natural fluoridation in about 30 countries worldwide.¹⁵ No data could be found on the number of pregnant women who are exposed to fluoride in the United States.

2. Fluoride Exposure in Mexico: The participants for this study are from Mexico where fluoride levels in drinking fluids are comparable to fluoride levels in artificially fluoridated water in the U.S. Martinez-Mier et al. (2003)¹⁶ and Jimenez-Farfan et al. (2004)¹⁷ measured 0.07-1.70 mg/L of fluoride in drinking water and other drinks from Mexico. Fluoride

measured in biomarkers like urine and plasma collected in the U.S. are also comparable to the values we found in our analyses of the Mexico City data. Plasma fluoride levels ranged from 0.0035-0.0830 mg/L in our population with a mean of 0.0222 mg/L, which is comparable to the average of 0.019 mg/L found in an U.S. study. (Whitford, Thomas & Adair, 1999) Since the exposure levels in the U.S. and Mexico are comparable, an assessment of prenatal fluoride exposure and neurobehavior and its findings in the Mexican ELEMENT dataset would be applicable to the U.S. population as well.

D. Concurrent fluoride and neurobehavior

The epidemiology studies on fluoride and the brain have primarily come out of China. One study from the Guizhou region of China reported the mean IQ in medium fluorosis-communities as 79.7 ± 12.7 , and the mean IQ in severe fluorosis-areas as 80.3 ± 12.9 . These IQs were significantly lower than the mean IQ of 89.7 ± 12.7 in slight fluorosis communities, and the mean of 89.9 ± 10.4 in non-fluorosis communities ($p < 0.01$.) The urinary fluoride levels in this region ranged from 1.02 to 2.69 mg/L which is comparable to the urinary fluoride concentrations measured in the ELEMENT preliminary analysis.¹⁸ While this and other studies^{19,20,21,22,23} point to a negative association between fluoride exposure and IQ, they are limited in design, size, and power. All of the reported studies use a cross-sectional design, measuring exposure and outcome once, and they do not adjust for critical confounders. Most of the studies also report ecological measures of exposure, an inaccurate indicator of exposure on an individual level and many of these do not have enough power to detect an association. This project, however, uses a large dataset with a prospective study design to assess the association between multiple personal measures of exposure and neurobehavior in hundreds of mother-infant pairs. This study also adjusts for critical confounders like parental education and child's sex to study the effects of concurrent and prenatal exposure. Finally, none of the studies in the literature assess the effects

of exposure during fetal development, a particularly vulnerable period, and early childhood neurobehavior.

E. Prenatal fluoride exposure

1. Maternal-fetal transmission of fluoride

Although the mechanisms of maternal fetal transmission of fluoride are poorly understood, there is considerable research showing maternally ingested fluoride can reach the fetus through the umbilical cord and placenta and ultimately increase fluoride levels in fetal tissue. Fluoride concentration of cord blood delivered to the fetus is moderately correlated with maternal blood fluoride, indicating that at least some fluoride is leaving the maternal compartment and reaching the fetal compartment.^{24,25} Placental tissue has also provided evidence of fluoride transmission. The correlation between maternal blood fluoride and placental tissue fluoride is low, meaning the placenta is not trapping fluoride but allowing it to pass through to the fetus.²⁶ Fluoride levels in fetal tissue further supports the hypothesis that fluoride traverses the umbilical cord and the placenta. In studies where women and animals take-in fluoride during pregnancy, concentrations in fetal tissue increase as intake increases. Higher concentrations of fluoride were found in fetal brain tissue^{27,28} and dental enamel²⁹ from animals fed higher amounts of fluoride than control animals. Even though maternal intake is the safest and easiest proxy for fetal exposure to fluoride, there exist very few timely and reliable studies on fluoride exposure in pregnant women.

2. Fluoride in pregnant women

There are a handful of studies that report on exposure in pregnant women, but most of them date back several decades, and they lack rigorous study methods, longitudinal measures and multiple biomarkers.^{30,24,31,32,33,34} The studies reviewed were published primarily in the 1960s and 70s and the largest of them recruited 32 people so, they do not provide a current or

reliable representation of fluoride levels in pregnant women. The cross-sectional measures taken from these women are also not representative of fetal exposure for all stages of the pregnancy. Most of the reported fluoride measures are from the third trimester, but research on other contaminants like lead^{35, 36} and mercury^{37,38} demonstrate that contaminant levels can change as pregnancy progresses with different implications for health. The only known study to take multiple fluoride measures was published in 1959 in Jerusalem so it is not timely or relevant to the U.S. population.³⁹ The fluoride studies listed above also only report on a single biomarker of exposure, blood fluoride, but multiple biomarkers can provide more data on acute and chronic exposure. Lastly, these studies provide no data on socioeconomic or demographic factors that may affect exposure in women. The *second* chapter of this dissertation, however, is the first exposure study to report fluoride status in hundreds of pregnant women by using two biomarkers, urine and plasma, that were collected multiple times during pregnancy. It also reports on the maternal and demographic factors that can affect pregnancy fluoride status.

3. Prenatal fluoride exposure and the brain

Following the assessment of prenatal exposure, the *third* chapter of this dissertation presents a novel study on the neurobehavioral effects of exposure in the womb. There are currently no reports on this association, but previous studies report structural damage to fetal brain tissue after fluoride exposure and this begs the question, what if any, lingering effects remain from this exposure. In two human studies from China, brains were collected from five to eight month old fetuses that were aborted in high fluorosis and low fluorosis areas. The brains from the high fluorosis areas contained higher fluoride ($0.28 \pm 0.14 \mu\text{g/g}$) than brains from low fluorosis areas ($0.19 \pm \mu\text{g/g}$) ($p < 0.05$) Brain tissue from the fluorosis endemic area also contained fewer neurons in the brain cortex with smaller volumes. In the cerebellum, the Purkinje fibers of the control brains were organized, but they were disorganized in the brains from the endemic

region.⁴⁰ In another similar study in the same region of China, fewer neurotransmitters like norepinephrine, 5-hydroxytryptamine, and α_1 -receptor were measured in brains from endemic areas compared to controls.⁴¹ In one rat study, pregnant dams subcutaneously injected with sodium fluoride (NaF) birthed male pups that were more hyperactive than the control pups.⁴² In two other rat studies, fluoride was measured in brain tissue and histopathological changes were noted in the brains of rats exposed to fluoride during gestation and lactation. Pups exposed to 100 ppm and 200 ppm of fluoride had significantly more fluoride in their cerebral cortex, medulla and cerebellum than controls rats⁴³ In another paper, Shivarajashankara et al. (2002)⁴⁴ published photomicrographs of shrunken neurons and decreased cell numbers in the hippocampus, amygdala, motor cortex and cerebellum of pups exposed to 100 ppm fluoride during gestation and lactation. The implications of this damage on postnatal neurobehavior remain unstudied, however, so, the *third* chapter of this dissertation reports on the effects of this exposure on childhood cognition. It is the first study to do so, and it measures maternal exposure and reports its association to child cognition during the first three years of life.

II. Methods

A. Study Population

The mother-infants pairs for this study were recruited from the Early Life Exposures in Mexico to Environmental Toxicants (ELEMENT) study, a long-running, award-winning, NIEHS-funded series of birth cohorts that were established to study the developmental effects of toxicants. The study is based out of Mexico City, Mexico, and participants were recruited during their prenatal visits to one of three clinics of the Mexican Institute of Social Security. Women who had a positive pregnancy test, who lived in Mexico City and who were willing to participate in the study were recruited after they were informed of the study procedures and after they

provided written consent. Women in the following categories were excluded from the study: those with psychiatric disorders, those who consumed alcohol daily or were addicted to illegal drugs, those who continuously used prescription drugs and those who were at risk for high-risk pregnancies, pre-eclampsia, renal or circulatory diseases including hypertension, those with gestational diabetes, those who suffered from seizures that required medical attention and those who were more than 14 weeks pregnant.³⁵

1. Participants for prenatal studies

Participants for the prenatal exposure portion of this project were recruited between 1997 and 2005. Each woman contributed between one and three urine samples and between one and three plasma samples during the course of her pregnancy. These samples were frozen at the field site and then transferred to Harvard University and the University of Michigan. After the delivery, the infants were administered neurobehavioral tests at ages one, two and three to continually assess their development.

2. Participants for concurrent exposure study

Participants for the concurrent fluoride and neurobehavior portion of this project were a subset of children recruited from ELEMENT for a follow-up study on lead and neurobehavior. 1272 of the mother-infant pairs eligible to participate in the follow-up were approached between 2008 and 2010, urine samples were available for 653 pairs. The mothers consented to the study, children gave assent, and IRB approval was obtained at the University of Michigan and at the National Institute of Public Health of Mexico.

B. Biomarkers

1. Urinary fluoride

Urinary fluoride, an indicator of acute exposure to fluoride, was measured in urine samples collected from pregnant subjects. The Agency for Toxic Substances and Disease Registry (ATSDR) manual on fluoride reports that urine is the most commonly measured marker in fluoride studies.⁴⁵ A number of studies have found a high correlation between fluoride concentrations in ingested water and urinary fluoride, with one study finding a 98% correlation between daily fluoride intake in water and fluoride excreted in urine.⁴⁶ There are no studies measuring the association between chronic fluoride exposure and urinary fluoride, but urine fluoride levels are closely associated with acute fluoride intake.⁴⁵ (Figure 1)⁴⁷

2. Plasma fluoride

Plasma fluoride, a measure of chronic exposure to fluoride, on the other hand, increases drastically within the first hour of ingestion of fluoride, and starts decreasing within 20 minutes of fluoride ingestion because some fluoride is excreted in the urine and some is incorporated into the bone. (Figure 1)⁴⁷ Overtime, some of the bone fluoride is also released from the bone, and back into the plasma, making plasma a good measure of chronic exposure to fluoride.⁴⁷

C. Neurobehavioral tests

1. Wechsler Abbreviated Scales of Intelligence (WASI)

A Spanish-version of the Wechsler Abbreviate Scale of Intelligence (WASI) was administered to the children during their baseline visit. Trained members of the Mexico team, to whom the fluoride status of the children was unknown, administered the test. Total WASI score, the measure assessed in this study, was calculated by combining sub-scores that measured verbal and executive function.

2. Bayley Infant Scales of Development-II (BSID-II) and the Mental Development Index (MDI)

The existing studies on fluoride and neurobehavior primarily use IQ as the outcome of interest^{11,48,49} but, as the NRC highlights, there are no studies reporting on the effect of fluoride on other critical neurobehavioral outcomes like memory or problem solving.¹⁴ In ELEMENT, one, two and three-year olds were tested on multiple outcomes when we administered a Spanish-version of the BSID-II test to measure mental, motor and behavioral skills. The BSID-II is a validated and widely used test for children between the ages of six and 42 months.

In this fluoride study, the Mental Development Index (MDI) subscale, which measures problem solving, language and social skills, was used.⁵⁰ Research on other environmental agents like phthalates, lead and manganese has found MDI to be sensitive enough to detect the effect of prenatal and postnatal toxicant-exposure on mental development in children in our age range of interest. In the phthalate study, maternal urinary levels of the substance were inversely associated with MDI at age three.⁵¹ The lead and manganese studies detected an association in the ELEMENT dataset; MDI decreased as lead levels increased in two year olds,⁵² and MDI was adversely affected at lower and higher manganese concentrations in one year old children.⁵³ There are no published studies of prenatal or postnatal exposure to fluoride and its effects on MDI, but in one animal study, mice drinking fluoridated water lagged in learning and memory, components of the MDI scale.⁵⁴ This project uses this measure to assess the effects of fluoride on mental function in young children.

III. Thesis overview

The objective of this dissertation is to improve understanding of fluoride exposures during pregnancy and to assess whether prenatal and concurrent fluoride exposure is associated with neurobehavioral effects in offspring. The research aims are conducted by using resources

from the Early Life Exposure in Mexico to Environmental Toxicants (ELEMENT) study, and a follow-up study called Cholesterol. Chapter 2 is the first population-based study to provide an exposure profile of fluoride during early, mid and late pregnancy, using two different biomarkers, and it is the first to examine whether some periods of pregnancy may be more vulnerable to exposure. Chapter 3 assesses the neurobehavioral effects of prenatal fluoride exposure on the Mental Development Index (MDI) of the children when they are one, two, and three years old. Chapter 4 of this dissertation measures urinary fluoride in children at 6-15 years and examines its association to the total score from the Wechsler Abbreviated Scales of Intelligence Test (WASI.) This work increases our understanding of prenatal and concurrent fluoride exposure and their effects on cognition in children. During pregnancy, we found that fluoride levels do not change as pregnancy progresses once you adjust for other factors, and children of older mothers may be more susceptible to fluoride exposure. We additionally found that maternal fluoride exposure levels did not have any measurable effects on cognition in early childhood in this cohort. We found that concurrent fluoride exposure in males, but not females, aged 6-15 years, was significantly associated with WASI, and this highlights the need for future research considering gender differences in neurocognitive outcomes using longitudinal data.

Figures and Tables

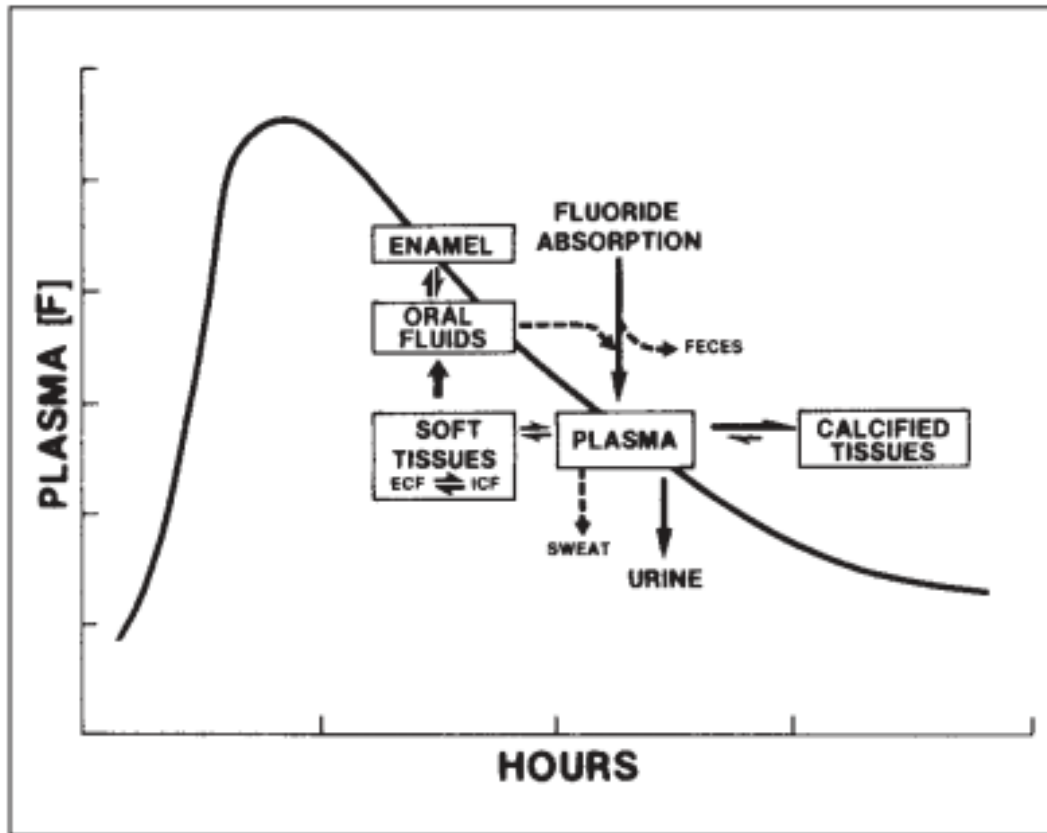


Figure I.1: Fluoride metabolism

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CHAPTER II

Urinary and Plasma Fluoride Levels During Pregnancy and Determinants of Exposure Among Pregnant Women from Mexico City, Mexico

I. Abstract

Background: Maternal fluoride exposure can be used as a proxy for fetal exposure but there is limited knowledge of fluoride exposure during pregnancy.

Objective: We utilize The Early Life Exposure in Mexico to Environmental Toxicants (ELEMENT) multi-year birth cohort to measure urine and plasma fluoride in pregnant women (N=810). We assess biomarker stability across pregnancy and the effects of maternal variables on exposure.

Methods: Samples from trimester 1 (471 urine, 231 plasma), trimester 2 (441 urine, 268 plasma) and trimester 3 (261 urine, 158 urine) were analyzed using an ion-selective electrode. Intra-class correlation coefficient (ICC) was calculated for urine and plasma samples across trimesters. Linear mixed models, adjusted for maternal education, age, marital status and smoking during pregnancy, were run to assess their effect on maternal urine and plasma levels during pregnancy. Plasma fluoride was included as a covariate in the urine model and vice-versa.

Results: Urinary fluoride averaged 0.869mg/L and ranged from 0.099 to 4.042 mg/L. Plasma fluoride averaged 0.0222mg/L and ranged from 0.0035 to 0.0830mg/L. No significant

correlation was found between average urinary fluoride and average plasma fluoride ($R = -0.046$, $p = 0.48$) Urinary fluoride was not stable across pregnancy ($ICC = 0.167$) and the same was found for plasma fluoride across the trimesters of pregnancy ($ICC = 0.344$). Women who smoke had higher urinary fluoride than non-smokers, older women had higher plasma fluoride, and women carrying female children had a lower plasma fluoride.

Conclusion: This is the first large-scale epidemiology study to measure urine and plasma fluoride in hundreds of pregnant women. These measures could be proxies for fetal exposure, and they have implications for future health outcomes research. The low ICC values suggest that fluoride values are not stable across trimesters. This is significant for future research which will examine windows susceptible to fluoride exposure during pregnancy. We also found that smokers have higher urinary fluoride, and older mothers have higher plasma fluoride, potentially making them more vulnerable to exposure.

II. Introduction

Artificial fluoridation has been used for nearly 60 years to reduce the prevalence of periodontal disease. A number of publications recommend prenatal supplements as a measure to prevent childhood caries,^{1,2} and some studies recommend fluoride supplements during pregnancy.³ An estimated 210 million people in the United States⁴ and millions more across 30+ countries drink water that is artificially fluoridated.⁵ Despite clear benefits, there remains continued debate over fluoride supplementation because fluoride intake has been shown to cause adverse effects on teeth,⁶ bones,^{7,8} and cognition.⁹ Given the controversy, a committee of experts was convened by the US National Research Council (NRC) to address the potential impacts of population levels of fluoride exposure on a number of adverse health outcomes, such as dental and skeletal fluorosis, carcinogenicity, and reproductive, developmental and endocrine effects.¹⁰ The postnatal effects of fluoride have been studied in children and adults, but few studies have examined health outcomes associated with prenatal exposures. This despite some epidemiological and animal evidence suggesting that fluoride is a developmental neurotoxicant¹¹ Developmental exposures to fluoride are possible as studies have shown that fluoride ingested by the mother reaches the fetus through the umbilical cord and placenta. Maternal blood fluoride, for example, is moderately correlated with cord blood, indicating that at least some fluoride leaves the maternal compartment and reaches the fetal compartment.^{12,13} Placental tissue also provides evidence of this transmission through a lack of significant correlation between maternal blood fluoride and placental tissue. Once in the fetus, fluoride levels in the brain^{14,15} and teeth¹⁶ increase in accordance with maternal exposures. The aforementioned studies suggest that the fetus is exposed to fluoride, and that maternal fluoride levels can be used as a proxy for fetal exposure.¹²

There exist a handful of studies that have reported upon fluoride exposure in pregnant women, however these studies generally date back several decades, provide limited data on socioeconomic or demographic variables that could modify exposure or risk, and lack rigorous methods like robust sample sizes, multiple biomarkers, repeated measures, or demographic information;^{5,13, 17,18,19,20,21} For instance, the largest of the aforementioned papers recruited less than 100 women, and most of the fluoride measures were obtained from single biomarker taken from the third trimester and at delivery. However, research on other contaminants like mercury^{22,23} demonstrate that exposure levels can change as pregnancy progresses (i.e., that developmental windows of susceptibility exist), and that different biomarkers can give distinct information on the source, duration and severity of exposure.²⁴

The general objective of the current study was to increase understanding of developmental exposure to fluoride by establishing a profile of prenatal exposure. To achieve this we characterized fluoride levels in archived urine and plasma samples (which indicate acute and chronic exposure, respectively) from pregnant mothers from three trimesters. The mothers were enrolled in the Early Life Exposure to Environmental Toxicants (ELEMENT) study which is a sequentially enrolled epidemiologic birth cohort series running since 1994.

III. Methods

Study Population

Study participants were recruited between 1997 and 2005 from three clinics of the Mexican Institute of Social Security (IMSS) in Mexico City, Mexico as part of the ELEMENT study. Pertinent details of ELEMENT, such as inclusion and exclusion criteria, collection methods, and demographics can be found elsewhere.²⁵ Briefly, each woman in the study contributed between one and three urine and plasma samples during pregnancy, and these were stored frozen. For this particular study, a subset of available samples from trimester one (471 urine, 239 plasma), two (441 urine, 278 plasma), and three (261 urine, 163 plasma) were studied. Corresponding gestational month values and a range of demographic variables were available for these participants. The institutional review boards of the National Institute of Public Health of Mexico, University of Michigan, the University of Toronto, the Harvard School of Public Health and participating clinics approved the study procedures.

Fluoride in Urine Samples

The free ionic form of fluoride was measured in urine samples using an ion-selective electrode-based assay²⁶ at the University of Michigan School of Public Health. Briefly, urine was diluted with equal parts Milli-Q water in a petri dish, and allowed to react for 20-24 hours with 3 M sulfuric acid saturated with hexamethyldisiloxane (HMDS). The diffused fluoride was collected in 0.05 M of sodium hydroxide that was spotted on the interior of the petri dish cover. Following neutralization with 0.25 M of acetic acid, the concentration of fluoride in this solution was determined using an ion-selective electrode (Orion Fluoride Combination Electrode).

Urinary reference standards obtained from the Institut National de Santé Publique du Québec (INSPQ) were used to gauge analytical accuracy and precision. The average recovery rate for the standards was 100%. In addition, each batch run contained procedural blanks and replicate runs. The mean percent relative percent standard deviation for all samples was below 20%. The analytical detection limit (mean: 0.00656mg/L; range: 2.30×10^{-6} – 0.10mg/L) was calculated as the mean blank value plus two times the standard deviation. A subset of urine samples was also measured at the Indiana University Oral Health Research Institute (OHRI). The correlation coefficient between the two laboratories was 0.873.

Fluoride in Plasma Samples

Plasma samples were measured for fluoride at the Indiana University Oral Health Research Institute (OHRI) using the hexamethyldisiloxane (HMDS) microdiffusion method of Taves (1968) as modified by Martinez-Mier et al. (2011). Briefly, plasma sample diluted 2:1 with diH₂O was pipetted into a plastic Petri dish, with a trap solution of 0.075 N sodium hydroxide (NaOH) placed on each dish lid. Each dish was immediately sealed. Next, HMDS-saturated 3 N SO₄ was introduced into each sample via a small hole burned into each lid. The hole was sealed immediately with petroleum jelly. During overnight diffusion, fluoride was released and trapped in the NaOH. The trap was recovered and buffered to pH 5.2 with acetic acid (CH₃COOH). The recovered solution was adjusted to a final volume of 20 ml with diH₂O. A standard fluoride curve was prepared from similarly diffused fluoride standard solutions and used to determine fluoride content of each plasma sample. Millivolt readings were measured using a fluoride micro-electrode (Microelectrodes, Inc MI-SO) and pH/ISE meter (Orion Dual Star or equivalent). Duplicate analyses were done in sets of approximately 30-40 samples.

Statistical Analysis

Histograms of urinary and plasma fluoride were generated to view the distribution of exposure levels, and summary measures like the mean and median were calculated. Intraclass correlation coefficients (ICC) were calculated to assess the stability of the two biomarkers throughout pregnancy, and Spearman correlation coefficients were generated to test the correlation between the biomarkers of exposure and other demographic variables. The differences in exposure levels across maternal age and maternal smoking status were compared using t-tests and boxplots.

Linear mixed models, adjusted for maternal education, age, marital status, and smoking during pregnancy, were run to assess their effect on maternal urine and plasma levels during pregnancy.

Data were analyzed using R 0.98.507 (RStudio, Inc.) and SAS 9.2 (SAS Institute Inc. Cary, NC).

IV. Results

Fluoride measurements were drawn from ELEMENT mothers (N=810) for whom we had at least one prenatal biomarker. Characteristics of these mothers (age: 26.6 ± 5.4 years; maternal education: 10.7 ± 3 years; 89.36% married or cohabitating) were not statistically different from mothers excluded due to not having a prenatal biomarker measurement. The number of mothers for whom we have prenatal fluoride biomarker measurements from early, mid, or late trimester ($N_{\text{urine}}=471, 441, 261$; $N_{\text{plasma}} = 231, 268, 158$, respectively), and those for whom we have one, two, or all three of the measurements ($N_{\text{urine}}=341, 230, 124$; $N_{\text{plasma}} = 120, 144, 83$ respectively) varies.

Every urine and plasma sample investigated had detectable levels of fluoride. Urinary fluoride levels in this population of pregnant women ranged from 0.099 to 4.042 mg/L with a mean of 0.869. Plasma fluoride levels were significantly ($p<0.001$, Student t-test $t= 23.47$) lower than urinary fluoride levels with a mean of 0.0222 mg/L, and they ranged from 0.0035 to 0.0830 mg/L (Table 1).

No significant spearman correlations were found between urinary and plasma fluoride during trimester 1 ($\rho=0.066$, $p=0.612$), 2 ($\rho= -0.011$, $p=0.895$) or 3 ($\rho=-0.078$, $p=0.616$), but significant correlations were found between urine across trimesters and plasma across trimesters. Correlation values for urine showed that fluoride levels in prior trimesters predicted significantly higher urinary fluoride in subsequent trimesters ($\rho_{1-2} = 0.200$, $p<0.01$; $\rho_{1-3} = 0.201$; $p<0.01$; $\rho_{2-3} = 0.284$, $p<0.01$.) A similar association was found between trimester 1 and 2 plasma ($\rho = 0.453$; $p<0.01$) and trimester 2 and 3 plasma ($\rho = 0.382$; $p<0.01$.) Across the three trimesters, maternal urinary fluoride values were not significantly different and remained fairly stable as

pregnancy progressed. As a summary measure of correlation across trimesters, the ICC for urine was 0.166 and for plasma was 0.347.

Two linear mixed effects models, with average urine or average plasma as the outcome for each, were run after adjusting for education, age, marital status and smoking during pregnancy. Lower urinary fluoride was detected in those who do not smoke during pregnancy ($\beta = -0.517$, 95% CI: -0.879, -0.154), higher plasma fluoride in older mothers ($\beta = 0.0005$, 95% CI: 8.7×10^{-5} , 0.0008) and lower plasma fluoride in mother's carrying female children ($\beta = -0.0030$, 95% CI: 7.1×10^{-3} , 0.0011). No other significant associations were found. (Table 3) In another analysis, plasma fluoride was included as a dependent variable in the urinary fluoride model, and urinary fluoride as a dependent variable in the plasma model, but no significant associations were detected between the two biomarkers.

V. Discussion

Here we report upon urinary and plasma biomarkers of fluoride exposure from pregnant women of Mexico City. We believe that this is the first large epidemiology study to report fluoride exposure during multiple timepoints of pregnancy using two different biomarkers. Where other studies have only provided exposure data for the last trimester and delivery, our study also examined exposure trends from the first month through delivery and found that fluoride levels do not change as pregnancy progresses once you adjust for other factors. Finally, we showed that women who smoke and older women maybe more susceptible to fluoride exposure.

This is the first study to report urinary and plasma fluoride levels in hundreds of pregnant women from a population drinking fluoridated water at levels recommended by the U.S. Public Health Service. Urinary fluoride ranged from 0.902-3.439 mg/L and plasma fluoride range from 0.004-0.077 mg/L. The levels of fluoride in maternal urine and plasma reported within this study are similar in range to other reports. In a study where women drank 1 mg/L of fluoridated water, urinary fluoride ranged from 0.22 to 0.53 mg/L.²⁰ In a controlled clinical study, pregnant women taking fluoride supplements had a mean urinary fluoride of 1.03 ± 0.14 mg/day in early pregnancy.²⁷ The wider range of urinary fluoride found in this study could be explained by higher levels of exposure in our population. Similarly, the plasma levels we report in this study are comparable to the levels reported in other studies on pregnant women. The most recent study, from Poland, measured 0.0329-0.0373 mg/L of plasma fluoride in 35 women during the third trimester of pregnancy,³ while other studies measured a range of 0.011-0.050 mg/L of plasma fluoride in five women,²⁸ a mean of 0.033 ± 0.003 mg/L in 50 women²⁹ and 0.97 ± 0.22 (first trimester), 0.90 ± 0.25 (second trimester), 0.86 ± 0.18 micromol/liters in 68 women.³⁰

An issue of increasing importance is determining whether there are time-related exposure windows of susceptibility to toxicant exposures especially during the prenatal period. Many lines of evidence now indicate such, including previous work by us concerning lead exposure in the ELEMENT cohort.^{31,32} Here we were able to study whether fluoride levels vary in urine and plasma as pregnancy progressed, however we only saw changes on the individual level while the population averages remained stable throughout pregnancy. This was somewhat surprising to us as a number of physiological and metabolic changes occur during pregnancy that could affect fluoride toxicokinetics. Previous studies reported significantly different levels of exposure between different periods of gestation. One study from Jerusalem, for example, reports an U-shaped distribution with the highest concentration of urinary fluoride measured in the fourth and ninth months of pregnancy.²⁰ The other study on plasma pregnancy did not report any significant differences in plasma fluoride between the 28th and the 33rd week of pregnancy.³

Here we were also able to study urinary and plasma fluoride values in relation to key demographic variables. The strongest changes in plasma fluoride were in relation to maternal age, with older mothers reporting significantly higher levels of plasma fluoride. This may be explained by the exchange of fluoride between plasma and bone during the aging process. Ingested fluoride is absorbed through the gut, taken into the plasma, and then deposited into the bone. As the organism ages, the hydroxyapatite mineral in bone becomes saturated with fluoride, and further deposition of the ion from plasma is prevented.^{7,33} In animal studies, older dogs³⁴ and rats³⁵ had more fluoride in their plasma than their younger counterparts, further supporting the theory that fluoride remains in the plasma or is excreted through the urine than deposited in the bone. There are, however, no such studies on plasma clearance in pregnant animals or humans so, this warrants more study.

Finally, we have limited evidence that suggests that urinary fluoride may be higher in women who smoke during pregnancy. This increase may be explained by the mobilization of fluoride during bone resorption. Fetal demands for calcium, particularly during the third trimester, increase the rate of bone breakdown to release the mineral into the bloodstream.^{36,37,38} To our knowledge, the effect of smoking on this process during pregnancy has not been studied; however, the catalytic effect of smoking on bone resorption in non-pregnant adults has been well documented.³⁹ Higher concentrations of the deoxypyridinoline, C-telopeptide,⁴⁰ and N-telopeptide,⁴¹ markers of bone breakdown, were measured in smokers. When bone resorption exceeds bone formation, as is the case in smokers, the amount of fluoride released from the bone increases without an increase of incorporation of fluoride into the bone.⁷ We hypothesize that urinary fluoride levels, in turn, increase as the body attempts to clear the release of excess fluoride from the bones.

Despite a number of strengths (e.g., large sample size, multiple biomarkers, demographic factors), there are potential limitations of our study that need mention. The spot urine samples we measured cannot account for diurnal variations in urinary fluoride. Previous studies report fluctuations in concentration by time-of-day⁴² which means that the fluoride levels in our samples could be influenced by the time-of-collection or by diet. Future studies should either provide 24-hour measures or measure samples that have been collected at a standard time. This study was also not initially designed to address fluoride exposure, but we have strong biomarkers and the fluoride levels we measured were similar to previous reports.

Regardless, our work provides the most reliable characterization to date of urinary and plasma fluoride throughout the duration of pregnancy. Our values were similar to the ones reported in previous studies, but contrary to other reports, our analysis did not show a significant

trend in urine or plasma fluoride once we adjusted for maternal and demographic factors. The two biomarkers were largely stable from the first to the last month of pregnancy. Interestingly, mothers who smoke seem to have the highest amount of urinary fluoride, and older mothers have the highest plasma fluoride, indicating that fetal exposure could be higher among smokers and older women. These results will be used in future studies related to health outcomes research such as prenatal fluoride exposure and neurobehavior.

Tables and Figures

Table II.1: Exposures in Mexico to Environmental Toxicants (ELEMENT) cohort recruited in Mexico City, Mexico between 1997 and 2005.

		N	Mean	SD	Min	10%	25%	50%	75%	90%	Max
Urinary Fluoride	Early pregnancy	471	0.838	0.500	0.073	0.302	0.446	0.761	1.099	1.450	4.042
	Mid pregnancy	441	0.865	0.540	0.110	0.308	0.463	0.778	1.096	1.566	3.439
	Late pregnancy	261	0.883	0.573	0.057	0.306	0.460	0.787	1.161	1.547	4.042
	Average Urinary Fluoride	695	0.869	0.483	0.099	0.351	0.527	0.814	1.089	1.450	4.042
Plasma Fluoride	Early pregnancy	231	0.0228	0.0221	0.0021	0.0045	0.0070	0.0144	0.0312	0.0559	0.1052
	Mid pregnancy	268	0.0196	0.0169	0.0022	0.0052	0.0077	0.0139	0.0241	0.0426	0.0830
	Late pregnancy	158	0.0180	0.0170	0.0028	0.0045	0.0066	0.0115	0.0234	0.0426	0.0819
	Average Plasma Fluoride	347	0.0222	0.0167	0.0035	0.0066	0.0090	0.0171	0.0296	0.0464	0.0830

Table II.2: Intraclass correlation of urinary fluoride and plasma fluoride over three trimesters of pregnancy and Spearman correlation coefficients of trimester-specific urine and plasma fluoride among pregnant women from Mexico City, Mexico.

		Intraclass Correlation Coefficient	Urine			Plasma		
			Trimester 1	Trimester 2	Trimester 3	Trimester 1	Trimester 2	Trimester 3
Urine	Trimester 1		1.00	0.200**	0.201**	0.066	-0.225*	0.074
				0.0028	0.0067	0.612	0.0713	0.619
			471	246	181	61	65	47
	Trimester 2			1.00	0.284**	-0.093	-0.011	-0.0600
		0.167		441	175	0.329	0.895	0.602
						113	154	79
	Trimester 3			1.00	-0.031	0.160	-0.0778	
					0.806	0.181	0.616	
				261	66	72	44	
	Trimester 1				1.00	0.453**	0.158	
						<0.001	0.150	
					231	169	85	
Plasma	Trimester 2					1.00	0.382**	
		0.344					<0.0001	
						268	139	
	Trimester 3						1.00	
							158	

Table II.3: Generalized Additive Mixed Effects models with maternal urinary fluoride and plasma fluoride as outcome measures in hundreds of pregnant women from Mexico City, Mexico.

	Urinary Fluoride		Plasma Fluoride	
	Parameter Estimate	95% Confidence Interval	Parameter Estimate	95% Confidence Interval
Intercept	1.335	0.898, 1.771	0.0156	-3.50×10^{-3} , 0.0347
Maternal Education	-0.011	-0.026, 0.003	-0.0007	1.4251×10^{-3} , -0.000017
Maternal Age (Continuous)	0.006	-0.002, 0.0137	0.0005	8.667×10^{-5} , 0.0008
Marital Status (ref.=Married)	-0.093	-0.233, 0.048	-0.0030	-1.00×10^{-2} , 0.0040
Maternal Smoking During pregnancy (ref=yes)	-0.517	-0.879, -0.154	0.0039	-1.046×10^{-2} , 0.0184
Child's Sex (ref=male)	-0.009	-0.095, 0.077	-0.0030	7.093×10^{-3} , 0.0011
Gestational Months	0.009	-0.008, 0.026	-0.0003	-1.051×10^{-3} , 0.00037

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CHAPTER III

Prenatal fluoride exposure and neurobehavior: a prospective study

I. Abstract

Background: Recent studies report an inverse association between fluoride exposure and IQ in children, but few utilized personal measures of exposure or assessed associations with prenatal exposure using a prospective study design.

Methods: This study utilizes the Early Life Exposures in Mexico to Environmental Toxicants (ELEMENT) birth cohort and associated archived pregnancy samples to study prenatal fluoride exposure and its association with subsequent child neurobehavioral outcomes at ages 1, 2 and 3. Generalized Additive Mixed Models were used to model the association between urinary fluoride (N=431) and Mental Development Index (MDI), a subscale of the Bayley Scales of Infant Development-II (BSID-II) test. A similar model was estimated for plasma fluoride (N=194). Both models controlled for maternal age, education, marital status, pregnancy smoking status, child's sex, and child's age.

Results: The median for urinary fluoride was 0.831mg/L (0.110, 3.439), and the median for plasma fluoride was 0.01600mg/L (0.0035,0.077.) Neither maternal urinary or plasma fluoride was associated with offspring MDI scores ($\beta_{\text{urine}} = -0.631, p=0.391$; $\beta_{\text{plasma}} = -0.0031, p=0.650$.)

The effect of urinary or plasma fluoride on MDI also did not significantly change between the ages of 1, 2 and 3.

Conclusions: This analysis suggests that maternal intake of fluoride during pregnancy does not have a strong impact on offspring cognitive development in the first three years of life.

II. Introduction

Artificial fluoridation has been used for nearly 60 years to reduce the prevalence of periodontal disease. An estimated 210 million people in the United States¹ and millions in thirty countries drink artificially or naturally fluoridated water.² In the United States and other countries like Poland prenatal fluoride supplements have been administered as a measure to prevent childhood caries.³⁻⁴⁻⁵ Fluoride intake, however, has been connected to adverse outcomes like hypomineralization of dental enamel, skeletal fluorosis, and gastrointestinal pain.⁶⁻⁷⁻⁸

In children, adverse outcomes in relation to fluoride have centered on cognition, with a number of Chinese studies reporting an inverse association between fluoride exposure and intelligence quotient (IQ). These ecological studies found children living in high exposure communities had greater odds of having a lower IQ than their peers living in low exposure areas.⁹ One study measured urinary fluoride in an area with exposure similar to the United States, and found lower IQ correlated with higher urinary fluoride levels.¹⁰ These studies, however, do not use personal measures of exposure or adjust adequately for confounders.

Adverse outcomes earlier in life - specifically during development - have not been studied well, but prenatal studies show that maternally ingested fluoride can traverse the placenta,¹¹ the umbilical cord¹¹⁻¹² and the fetal blood-brain barrier to damage fetal brain tissue. Once in the fetus, the levels of fluoride in the brain increase as maternal ingestion increases.¹³⁻¹⁴ Damage to brain tissue includes fewer or shrunken neurons,¹⁵ more unorganized Purkinje fibers in the cerebellum,¹³ and fewer neurotransmitters such as norepinephrine, 5-hydroxytryptamine, and α_1 -receptor.¹⁶ However, no studies have examined the association between prenatal fluoride exposure and its effects on human neurobehavior in early childhood.

This study reports on this association between maternal urinary and plasma fluoride from pregnancy and offspring neurobehavior at ages 1,2, and 3 for the first time. We use a multi-year birth cohort, the Early Life Exposure to Environmental Toxicants (ELEMENT) study, that measured prenatal fluoride exposure and then assessed neurobehavior when the subjects were aged one, two and three years old. Unlike existing studies that use ecological measures with a cross-sectional study design, we use the more reliable individual level of exposure, maternal urine and plasma, to assess prenatal fluoride exposure. We also examine if the association between fluoride and neurobehavior differs across child's age.

III. Methods

Study Population: Study participants were recruited between 1997 and 2005 from three clinics of the Mexican Institute of Social Security (IMSS) in Mexico City, Mexico. Women with a positive pregnancy test, lived in Mexico City, willing to participate in a 3-year follow-up and planning to live in the area for five years were recruited. The women in the following categories were excluded from the study: those with psychiatric disorders, those who consumed alcohol daily or were addicted to illegal drugs, those who continuously used prescription drugs and those who were at risk for high-risk pregnancies, pre-eclampsia, renal or circulatory diseases including hypertension, those with gestational diabetes, those who suffered from seizures that required medical attention and those who were more than 14 weeks pregnant.¹⁷ The women who were eligible to participate were informed of the study procedures and they were recruited into the study after we obtained their written consent. Each women provided urine and plasma samples during the course of the pregnancy, and the children, once they were born, were administered the Bayley Scales of Infant Development test-II (BSID-II.)

We measured urinary fluoride in 695 women, and of these women, 431 women had a child with at least one MDI score: 355 children had an MDI score at age 1, 397 at age 2, and 358 at age 3. Plasma fluoride was measured in 352 women, and 194 of these women had a child with atleast one MDI score: 194 children had an MDI score at age 1, 184 children at age 2 and 167 children at age 3.

Ethics: The Institutional Review Boards of the National Institute of Public Health of Mexico, University of Michigan and Harvard School of Public Health and participating clinics approved the study procedures.

Collection of Urine and Plasma: Spot urine and venous whole blood was collected once during each trimester of pregnancy. The samples were collected into fluoride-free containers, and the blood was collected into tubes with heparin and centrifuged to extract plasma. After field collection, the urine and plasma samples were immediately frozen at the field site and then shipped and stored at -20°C at the Harvard School of Public Health (HSPH), and then at -80°C at the University of Michigan School of Public Health (UMSPH).

Urinary Fluoride: The fluoride content of the urine samples was measured using ion-selective electrode-based assays. First, 3 M sulfuric acid saturated with Hexamethyldisiloxane (HMDS) was added to the sample to allow fluoride to diffuse from the urine for 20-24 hours. The diffused fluoride was allowed to collect in 0.05 M of sodium hydroxide on the interior of the petri dish cover. Once the diffusion was complete, 0.25 M of acetic acid was added to the sodium hydroxide to neutralize the solution and an ion-selective electrode was used to measure the concentration of fluoride in this solution. The concentrations were calculated using a fluoride standard curve. None of the measured samples were below the average detection limit of 0.00656 mg/L, the percent relative standard deviation for all samples was below 20% and the recovery rate was 100% for an urine standard reference material purchased from the National Public Health Institute of Quebec (INSPQ). The correlation coefficient between our measurements and those done at the Indiana University Oral Health Research Institute (IU-OHRI) was 0.80.

Plasma Fluoride: Samples were selected for analysis in early 2013 and shipped frozen to the IU-OHRI for fluoride analysis. Analysis of duplicate plasma samples was conducted using a modification of the hexamethyldisiloxane (HMDS) microdiffusion method of Taves¹⁸ as modified by Martinez-Mier *et al.*¹⁹ 400 μl of dH_2O were pipetted into plastic Petri dishes (Fisherbrand 35x10-mm disposable Petri dishes), followed by 200 μl of plasma sample or

standard. After applying petroleum jelly to the inside of each Petri dish lid, 10 μl of 0.075 N sodium hydroxide (NaOH, A.R.) trap solution was placed in five equal drops on each dish lid. Each dish was immediately sealed. A small hole was burned into each lid into which will be pipetted 200 μl of HMDS-saturated 3 N SO_4 . The hole was sealed immediately with petroleum jelly. During overnight diffusion, fluoride was released and trapped in the NaOH. The trap was recovered and buffered to pH 5.2 with 5 μl acetic acid (CH_3COOH). The recovered solution was adjusted to a final volume of 20 ml with diH_2O . A standard fluoride curve was prepared from similarly diffused fluoride standard solutions and used to determine fluoride content of each plasma sample. Millivolt readings were measured using a fluoride micro-electrode (Microelectrodes, Inc. MI-SO) and pH/ISE meter (Orion Dual Star or equivalent). Duplicate analyses were done in sets of approximately 30-40 samples. 0.01 ppm F, 0.2 ppm F and 5.0 ppm F standard checks were also be analyzed with each sample set. The percent relative percent standard deviation for all samples was below 20% and 100% of the standard reference material was recovered.

Mental Development Index in Children: A Spanish-version of the Bayley Scales of Infant Development-II (BSID-II), the revised form of the Bayley Scales of Infant Development, was administered to the children at ages 1, 2 and 3 years by trained research staff to whom the prenatal fluoride status of the children was unknown. For quality control, random sessions were videotaped and reviewed by a psychologist.²⁰ The Mental Development Index (MDI), scores were calculated after administering the test and the distributions were normalized for each age group. The mean of the MDI distribution is 100 and the standard deviation is 15.²¹

Statistical Analysis: Summary statistics of urinary fluoride, plasma MDI and other continuous variables were generated to examine the distribution of these variables. Scatterplots and boxplots

were created to visualize the unadjusted relationship between MDI at ages 1, 2 and 3, and fluoride measures, as well as between MDI, fluoride measures and other continuous or categorical variables. The statistical significance of these bivariate associations was tested using t-tests, ANOVA or Spearman correlations, as appropriate. Covariates included in the final model, were selected if they exhibited biological plausibility or if the fluoride association by 10% or more. We ran two Generalized Additive Mixed Models (GAMM) using the average of available trimester-specific plasma or urine measures as the primary predictor. These models controlled for maternal age, education, marital status, pregnancy smoking status, child's sex, and child's age. Effect modification of the fluoride association by lead exposure was examined by adding an interaction between fluoride measures and lead concentration. Similarly, effect modification by child's sex and age was also considered, and interactions between all covariates and child's age and sex were examined to fit models equivalent to models stratified by age and sex. Only the effect of maternal age on child's MDI varied by child's age so, this interaction was retained in the final urine and plasma models. Continuous predictors were initially modeled using splines, and a non-linear relationship was seen between maternal education and child's MDI in both models. The trimester-specific effect of fluoride on MDI was assessed by running GAMM models with trimester-specific fluoride measures as the primary predictors. All statistical analyses were completed on the statistical software R (Version 0.98.501) and the software Statistical Analysis System (Version 9.3)

IV. Results

Fluoride measurements were drawn from ELEMENT mothers ($N_{\text{Urine}}=431$; $N_{\text{plasma}}=194$) for whom we had at least one prenatal biomarker. Characteristics of the mothers in the sample were age: $\sim 26.6 \pm 5.5$ years; maternal education: $\sim 10.9 \pm 3$ years; 89.36% married or cohabitating; child's sex: $\sim 50\%$ female, and did not differ statistically between the urine and the plasma groups.

Each urine and plasma sample analyzed had detectable levels of fluoride. Urinary fluoride levels in this population of pregnant women ranged from 0.110 to 3.439 mg/L with a mean of 0.896. Plasma fluoride levels were significantly lower than urinary fluoride levels with a mean of 0.0208 mg/L, and they ranged from 0.0035 to 0.0770 mg/L (Table 1).

The final two models run to assess the effect of urinary fluoride and plasma fluoride on child's MDI from ages one, two and three were Generalized Additive Mixed Models. Both models were adjusted for maternal age, maternal education, marital status, smoking status during pregnancy and child's sex. Urinary fluoride did not have a significant impact on MDI ($\beta = -0.631$, $p=0.391$), and the same was true for plasma fluoride ($\beta = -0.0031$, $p=0.650$.) The association between maternal exposure and child's MDI at each age was assessed using interaction terms, and none of the associations were significant.

Trimester-specific models with urine or plasma as the main effect did not show any significant impact on the child's MDI score. The effect of urine and plasma on MDI also did not vary by the child's lead scores at ages one, two or three, and there were no significant interactions by child's sex. When urine and plasma were placed in the same model, their associations with MDI did not change.

V. Discussion

Overall, this investigation found no evidence of a detectable adverse outcome on offspring neurobehavioral development associated with maternal fluoride exposure during pregnancy. Fluoride measured in pregnancy urine and plasma was not significantly associated with child's MDI at any age, and it did not differentially affect MDI at ages 1, 2, and 3. Furthermore, trimester-specific measures of maternal fluoride measures were not significantly associated with offspring MDI.

We present these results with the use of personal measures of exposure, and with the use of rigorous statistical methods that have not been reported in previous studies. Most of the studies in this field report bivariate analyses of fluoride and IQ without adjusting for confounders, but our study ran reliable statistical models to account for the effect of these variables. Parental education, for example, is a known confounder of toxicant exposure and cognitive outcomes.²² In addition to parental education, we collected data on maternal marital status, maternal age, child's age, and child's sex and we adjusted for these variables in our models. The inverse trend we saw between fluoride and MDI in bivariate analysis disappeared once we adjusted for these confounders.

There are currently only a few studies of cognitive measures that report personal measures of exposure, but this study uses two personal biomarkers to capture individual exposure to fluoride. Most of the existing studies on fluoride and neurobehavior used ecological measures of exposure and simply report the mean IQ across groups drinking water with different fluoride levels.²³⁻²⁴⁻²⁵ This method is prone to ecological fallacy, where the correlation between two variables at the group level is incorrectly assumed to equal the correlation at the individual

level. Our study, however, collected urine and plasma from each mother, allowing us to more directly assess exposure.

The two biomarkers in this study also capture and assess the effects of acute and chronic exposure to fluoride. Urinary fluoride levels increase in the few hours after ingestion, and subsequently decrease as fluoride is excreted from the body.²⁶ The handful of studies that report biomarkers only use urinary fluoride,¹⁰⁻²⁷ but plasma fluoride, due to an isoionic exchange of fluoride with bone, is a better measure of chronic exposure. Fluoride is deposited into the hydroxyapatite crystals of the bone over time,⁷ and when bone is remodeled some of the fluoride leaves and enters plasma, rendering it a proxy for the bone fluoride and chronic exposure.²⁶ This measure and its potential effect on cognition is being reported for the first time in our study.

Our results, however, should be treated with some degree of caution because we used spot urine and plasma samples. We do not report 24-hour urine measurements, and we do not report specific-gravity or creatinine-adjusted urine. Thus, the urine samples do not account for dilution, a measure that is difficult to adjust for in pregnant women. The spot plasma measurements do not capture the variation due to time of day, fasting vs. eating, and collection site of plasma. A study of nearly 500 individuals found serum fluoride levels dip in the morning and peak in the late evening, and it found the highest serum fluoride concentration 1-1.5 hours after ingestion.²⁸ The plasma measurements used in this study do not capture this variation as the samples were collected at random points during the day, and participants were not required to fast before sample collection. There are also differences in venous and arterial plasma that are not captured by our plasma measurements. Immediately after ingestion, arterial plasma fluoride levels peak, but ten minutes after infusion is stopped, plasma levels are higher than arterial levels and remain that way for long time.²⁶ Our study does not capture these variations.

The other limitation of this study was the sample size. Given the number of participants providing urine samples, we had to detect a 3-point difference in MDI scores per unit increase in urinary fluoride, to report an effect estimate with 80% power. This value is higher than the magnitude of 0.5 of MDI that we detected. This association between sample size and power was also true for the plasma analyses. Despite the low sample size, this is the only study to report personal measures of exposure to study the effect of fluoride on cognition in children.

Regardless of these limitations, this study provides critical answers regarding prenatal fluoride exposure in children. Maternal intake of fluoride during pregnancy does not have any measurable effects on cognition in early life. This maybe a case where the postnatal neuro-system is more susceptible than the prenatal neuronal system, and whether this means, that the nervous system recovers, or the effects may have disappeared over time, is not known. In addition to timing of exposure, a number of questions related to range of exposure, child's age and type of neurobehavioral tests remain. We restricted our analysis to a lower range of exposure among children and we use one cognitive measure. The use of a higher sample size, the inclusion of concurrent exposure and wider ranges of exposure and ages, and more cognitive outcomes, therefore, remain considerations for future work in this area.

Tables and Figures

Table III.1: Descriptive characteristics of mother-infant pairs participating in the ELEMENT birth cohort study from Mexico City, Mexico.

Variable	Urine Analysis (N=431)			Plasma Analysis (N=194)		
	Mean ± SD	Median	Minimum, Maximum	Mean ± SD	Median	Minimum, Maximum
Maternal Characteristics						
Average Maternal Urinary Fluoride(mg/L)	0.896 ± 0.478	0.831	0.110, 3.439			
Average Maternal Plasma Fluoride (mg/L)				0.02081 ± 0.01573	0.01600	0.00350, 0.07700
Maternal Age (years)	26.6 ± 5.5	26	14, 44	26.8 ± 6	26	14, 44
Maternal Education (years)	10.9 ± 2.8	11	2, 21	10.9 ± 2.9	11	3, 21
Married/Cohabiting(%)		88.9			90	
Child Characteristics						
Child's MDI at Age Two	86.71 ± 11.59	86	52, 122	84.88 ± 10.96	86	52, 114
Female Sex (%)		50.4			52.5	

Table III. 2: Generalized Additive Mixed Models of maternal urinary and plasma fluoride during pregnancy and child's MDI at ages one, two and three.

Variable	Urine Analysis (N=431)		Plasma Analysis (N=194)	
	β	p-value	β	p-value
Average urinary fluoride (mg/L)	-0.631	0.391		
Average plasma fluoride (mg/L)			-0.0031	0.651
Maternal Age*Child's Age				
1 year	0.0532	0.579	-0.113	0.347
2 years	0.364	<0.01	0.181	0.108
3 years	0.078	<0.01	0.039	0.664
Child's Sex (ref=male)	2.738	<0.01	3.001	<0.01
Smoking during pregnancy (Ref=No)	-1.645	0.600	2.081	0.570
Marital Status (Ref=Married/cohabitating)	1.440	0.257	-2.555	0.151

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CHAPTER IV

Concurrent Fluoride and Total WASI in 6-15 year old children from Mexico City, Mexico

I. Abstract

Background: Recent studies report an inverse association between fluoride exposure and IQ in children, but few of these studies report personal measures of exposure or adjust for confounders.

Methods: This study utilizes the Cholesterol Cohort and its associated archived urine samples to study concurrent fluoride exposure and its association with subsequent child neurobehavioral outcomes in 6-15 year old children. Generalized Additive Mixed Models were used to model the association between urinary fluoride (N=550) and total score from the Wechsler Abbreviated Scale of Intelligence (WASI), adjusting for child's sex, child's age, maternal age, marital status, maternal education, family possessions, and cohort. GAM models were also run after stratifying by gender ($N_{\text{Males}} = 289$, $N_{\text{Females}} = 261$.)

Results: Urinary fluoride predicted higher WASI scores in children, but the association was only significant in males after stratifying by gender. ($\beta_{\text{males}} = 3.98$, $p=0.05$)

Conclusion: This analysis suggests concurrent urinary fluoride exposure has a strong positive impact on cognitive development among males aged 6-15 years.

II. Introduction

Artificial fluoridation has been used for nearly 60 years to reduce the prevalence of periodontal disease. An estimated 210 million people in the United States¹ and millions in thirty countries drink artificially or naturally fluoridated water.² Additionally, a number of publications recommend prenatal supplements as a measure to prevent childhood caries,^{3,4} and in some countries like Poland, women are given supplements during pregnancy.⁵ Fluoride intake, however, has also been connected to adverse outcomes.^{6,7,8}

Among children and young people, the neurobehavioral studies, though weak in methodology, have received the most attention. They report an inverse association between fluoride exposure and IQ in school-age children.^{9,10,11, 12, 13, 14, 15,16} The exposures in these studies, however, are ecological measures, where fluoride concentration of drinking water is assigned to individuals, or they are recall measures, where participants must remember the frequency of exposure. These methods are prone to a great amount of error and exposure misclassification, and they do not allow for accurate assessment of the neurobehavioral effects of fluoride exposure. Most of these studies also fail to adequately adjust for confounders. Parental, familial and environmental factors such as maternal education, maternal age, family socioeconomic status and child's age and education have well-known associations with cognitive function, but most studies report the neurobehavioral effects of fluoride without controlling for these factors. A review of the available literature, therefore, shows that the relationship between fluoride intake and cognitive function in children must be assessed using stronger, and more reliable methods.

This study reports on the association between fluoride intake and child cognitive function using a cohort that measured concurrent urinary fluoride and neurobehavior in children aged 6-

15 years old. Unlike existing studies that use ecological and recall measures, we use the more reliable individual level of exposure, urinary fluoride, to assess fluoride status. We also adjust for confounders that control for the effect of familial and environmental factors.

III. Methods

Study Population: Cholesterol and ELEMENT Cohorts

The study participants for this project were 6-15 year old children who were recruited from the Early Life Exposure to ENvironmental Toxicants in Mexico City (ELEMENT) study for a follow-up study called Cholesterol. ELEMENT is a long-running, NIH-funded set of birth cohorts which recruited pregnant women with the original aim of studying the effects of prenatal lead exposure on developmental outcomes in early childhood. The first set of participants (Cohort 1) were approached and recruited between 1994 and 1997, the second set (Cohort 2) between 1997 and 2000, and the third set (Cohort 3) was approached and recruited between 2001 and 2005. All of the women were recruited from the Mexican Institute of Social Security (IMSS) in Mexico City, Mexico during early pregnancy; those with a positive pregnancy test, who lived in Mexico City and who were willing to participate in the study were recruited. The women in the following categories were excluded from the study: those with psychiatric disorders, those who consumed alcohol daily or were addicted to illegal drugs, those who continuously used prescription drugs and those who were at risk for high-risk pregnancies, pre-eclampsia, renal or circulatory diseases including hypertension, those with gestational diabetes, those who suffered from seizures that required medical attention and those who were more than 14 weeks pregnant.¹⁷ Of the women-infant pairs recruited into the ELEMET cohorts, 827 pairs were eligible to participate in the Cholesterol study.

Ethics

Mother's consented to the study, children gave assent, and Institutional Review Board approval was granted at the University of Michigan and at National Institute of Public Health of Mexico.

Urinary Fluoride

For this analysis, 827 urine samples were collected in 5mL Fisherbrand containers when the children visited the National Institute of Public Health of Mexico (INSP) for their baseline visit. The samples were frozen at the field site before they were packaged on dry ice and shipped to the University of Michigan for storage in a -80°C freezer. In mid-2013, 654 children's urine samples remained in the freezers. These were thawed and measured for the free ionic form of fluoride using a gold-standard ion-selective electrode-based assay.¹⁸ Briefly, in this procedure, urine was diluted with equal parts Milli-Q water and 1 mL of the diluted urine was placed into a petri dish. It was mixed with 3 M sulfuric acid saturated with hexamethyldisiloxane (HMDS), and allowed to react for 20-24 hours. The diffused fluoride was collected in 0.05 M of sodium hydroxide that was spotted on the interior of the petri dish cover. Following neutralization with 0.25 M of acetic acid, the concentration of fluoride in this solution was determined using an ion-selective electrode.

Several analytical quality control measures were used. All laboratory glassware and plastic was acid-washed (cleaned, soaked overnight in 6M nitric acid followed by another overnight soak with 2M nitric acid) prior to use. Accuracy and precision were measured by use of urinary reference standards obtained from the Institut National de Santé Publique du Québec (INSPQ). The average recovery rate for the standards was 100%. In addition, each batch contained procedural blanks and duplicate runs. The mean percent relative percent standard

deviation for all samples was below 20%. The analytical detection limit, calculated as the sum of the average and two times the standard deviation of duplicate blank values, was calculated for each batch. Samples that fell below the limit were excluded. The average analytical detection limit was 0.075 mg/L and the range was 0.034 mg/L-0.381mg/L. A subset of samples from the ELEMENT birth-cohort studies was also measured at the Indiana University Oral Health Research Institute (OHRI). The correlation coefficient between our measurements and those done at the Indiana University Oral Health Research Institute (OHRI) was 0.87.

Wechsler Abbreviated Scale of Intelligence (WASI)

A Spanish-version of the Wechsler Abbreviate Scale of Intelligence (WASI) was administered to the children during their baseline visit. Trained members of the Mexico team, to whom the fluoride status of the children was unknown, administered the test. Total WASI score, the measure assessed in this study, was calculated by combining sub-scores that measured verbal and executive function.

Demographic and Socioeconomic Information

Demographic and socioeconomic information was collected during the baseline visit when the mothers were administered a questionnaire. The following variables were pulled from the questionnaire: child's age, child's sex, mother's age, mother's WAIS scores, mother's current marital status and education of the head of the household. Data on family income level, however, is unavailable because it is culturally unacceptable to directly ask about earnings. The questionnaire instead approximated family income by asking about possessions such automobiles and electronics. The ownership of a computer, microwave, or car was awarded one point each,

and a new variable called “family possessions,” which is the sum total of these points was then generated. Those with the lowest score, owned the fewest items, and those with highest scores, conversely, owned the most items. The variable was not weighted. The covariates listed above were available for 550 mother-children pairs, of which 289 were male children, and 261 were female children.

Statistical Analysis

Summary variables such as mean, median, and range, and histograms of urinary fluoride, total WASI score and other continuous variables were generated to examine the distribution of these variables. A scatterplot was created to visualize the unadjusted relationship between the total WASI score and urinary fluoride, and the relationship, at the outset, appeared linear. Scatterplots and boxplots were also created to see if the relationship between the exposure variable and potential continuous covariates such child’s age, child’s sex, child’s BMI, maternal age, maternal marital status, maternal smoking status 1-month post-partum, mother’s WAIS Score, and ELEMENT cohort were linear. The same was done for the outcome variable and the potential covariates. Mean urinary fluoride and total WASI score was also tested across child’s sex, maternal marital status and maternal smoking status using the Student’s t-test, and the difference in means was tested across ELEMENT cohorts using the ANOVA test. The Spearman correlation coefficient test was run to test the correlation between all variables. The covariates included in the final model were selected either on the basis of biological plausibility or if they changed the main effect by 10%. The final Generalized Additive Model (GAM) was as follows:

$$\text{Total WASI}_{\text{All}} = \beta_{\text{Urinary Fluoride}} + \beta_{\text{Child's Sex}} + \beta_{\text{Maternal Age}} + \beta_{\text{Marital Status}} + S(\beta_{\text{Maternal Education}}) + S(\beta_{\text{Family Possessions}}) + \beta_{\text{Cohort}} + \beta_{\text{Mother's WAIS Score}}$$

GAM models were also run after stratifying by the child's gender. In the final models, splines were added to maternal age, family possessions, and mother's WAIS score among males, and on maternal education and family possessions among females. The final models were as follows:

$$\text{Total WASI}_{\text{Males}} = \beta_{\text{Urinary Fluoride}} + \beta_{\text{Child's Sex}} + s(\beta_{\text{Maternal Age}}) + \beta_{\text{Marital Status}} + \beta_{\text{Maternal Education}} + s(\beta_{\text{Family Possessions}}) + \beta_{\text{Cohort}} + s(\beta_{\text{Mother's WAIS Score}})$$

$$\text{Total WASI}_{\text{Females}} = \beta_{\text{Urinary Fluoride}} + \beta_{\text{Child's Sex}} + \beta_{\text{Maternal Age}} + \beta_{\text{Marital Status}} + s(\beta_{\text{Maternal Education}}) + s(\beta_{\text{Family Possessions}}) + \beta_{\text{Maternal WAIS score}} + \beta_{\text{Cohort}}$$

All statistical analyses were completed on the statistical software R (Version 0.98.501) and on the software Statistical Analysis Systems (Version: 9.3.)

IV. Results

Fluoride measurements were drawn from 6-15 year old children (N= 550) during their baseline visit in the Cholesterol study. Characteristics of these children and their mothers (Child's WASI Score: 94.6 ± 11.5 ; child's Age: 10.3 ± 2.5 years; 47.5% female children; maternal age: 36.3 ± 5.8 years; maternal education: 10.7 ± 2.8 years; family possession: 7.6 ± 3.0 score and 79.6% married or cohabitating) were not statistically different from mother-children pairs for whom we did not have urinary fluoride.

Of the urine samples we measured, all of them contained detectable levels of fluoride, and the mean concentration in this population was 0.640 ± 0.34 mg/L with a median of 0.597 mg/L and a range of 0.123-2.812mg/L.

To assess the adjusted effect of child's fluoride levels on cognitive function, we ran GAM models, first in the overall population and then stratified by gender. In the overall population, urinary fluoride appears to have no significant impact on total WASI scores ($\beta = 1.32$, $p=0.33$), but this association changes once the models are separated by male and female children. Male children showed a significantly positive trend ($\beta=3.81$, $p=0.05$), and females showing a negative trend that was not significant ($\beta= -1.57$, $p=0.39$).

V. Discussion

Overall, this investigation found a significant positive impact on neurobehavioral development due to ingestion of fluoride in male children but no association was detected in female children. Fluoride measured in urine was significantly associated with total WASI score in male children ages 6 to 15 years old. We present these results with the use personal measures of exposure, and with the use of rigorous statistical methods that have not been reported in previous studies.

Most of the studies in this field report bivariate analyses of fluoride and IQ without adjusting for confounders, but our study ran reliable statistical models to account for the effect of these variables. Parental education, for example, is a known confounder of toxicant exposure and cognitive outcomes.¹⁹ In addition to parental education, we collected data on maternal marital status, maternal age, child's age, and child's sex and we adjusted for these variables in our models.

Most of the studies on fluoride exposure and IQ use either ecological measures of exposure or recall measures, leaving these studies prone to a lot of error. Most of the existing studies on fluoride and neurobehavior used ecological measures of exposure and simply report the mean IQ across groups drinking water with different fluoride levels.^{20,21,22} This method is prone to ecological fallacy, an incorrect interpretation of the statistical data where correlation between two variables at the group level is assumed to equally correlate at the individual level. Our study, however, has directly measured fluoride in each individual by collection urine samples from all participants.

Despite a number of strengths (e.g., large sample size, adjusting for confounders and personal measures of exposure), there are potential limitations of our study that need mention.

The spot urine samples we measured cannot account for diurnal variations in urinary fluoride. Previous studies report fluctuations in concentration by time-of-day²³ which means that the fluoride levels in our samples could be influenced by the time-of-collection or by diet. Additionally, we lack information on family socioeconomic status (SES), and there may be residual confounding from this factor that cannot be adjusted for in this study. SES, for example, is known to be associated with food intake, which in turn can affect fluoride levels. SES is also highly correlated with our outcome variable, cognition. To address these limitations, future studies should either provide 24-hour measures or measure samples that have been collected at a standard time, and they should provide information on SES.

Additionally, we do not have plasma fluoride measures, which may account for the differences in association found between males and female children. In this study, urinary fluoride had a significant positive impact on male children but not on female children, but this association could change once plasma fluoride is added in the model. First, plasma fluoride is correlated to urinary fluoride, and second, plasma fluoride could provide a measurement of the fluoride released from bone during the drastic changes in bone growth and metabolism during puberty. Male children have higher rates of bone resorption during puberty than their female counterparts²⁴, which could release more fluoride stored in bone and increase circulating levels of plasma fluoride, which could affect cognition. So, future studies should account for this critical confounder.

Despite these limitations, this work provides the most reliable assessment of urinary fluoride and cognition in children, and we found that WASI scores increase in male children as fluoride intake increases. These results were surprising in that they show opposite trends to what has been reported in the literature so, more studies with similar reliable methodology, which

account for plasma fluoride, diurnal variations in urinary fluoride and children's SES, are needed. If these results are substantiated, different fluoride interventions may be needed for male children versus female children.

Table IV.1: Descriptive Statistics of Mother-child pairs recruited into the Cholesterol cohort in Mexico City, Mexico.

Variable	ALL				MALES				FEMALES			
	N	Mean \pm SD	Median	Range	N	Mean \pm SD	Median	Range	N	Mean \pm SD	Median	Range
Child variables												
Urinary Fluoride	550	0.640 \pm 0.34	0.597	0.123, 2.812	289	0.67 \pm 0.34	0.62	0.123, 2.81	261	0.61 \pm 0.33	0.55	0.14, 2.41
Total WASI Score	550	94.6 \pm 11.5	94.5	66, 133	289	95.1 \pm 12.0	95	66,128	261	94.1 \pm 10.9	94	66, 133
Age	550	10.3 \pm 2.5	9.9	6.6, 15.7	289	10.5 \pm 2.5	9.9	6.9, 15.5	261	10.1 \pm 2.5	9.7	6.6, 15.7
Female Sex (%)			47.5				---				---	
Maternal Variables												
WAIS Total Score	550	87.5 \pm 12.7	87	51, 130	289	86.8 \pm 12.8	86	51, 130	261	88.3 \pm 12.5	88	53, 117
Age	550	36.3 \pm 5.8	36	24, 56	289	36.3 \pm 5.8	36	24, 56	261	36.4 \pm 5.7	36	24, 53
Family Possessions	550	7.6 \pm 3.0	7	1, 16	289	7.8 \pm 3.1	8	1, 16	261	7.4 \pm 2.9	7	1, 16
Maternal Education	550	10.7 \pm 2.8	11	1,18	289	10.6 \pm 2.7	11	1, 17	261	10.6 \pm 2.8	11	2,18
Married/Cohabiting(%)			79.6				82.4				76.6	

Table IV.2: Generalized Additive Models of Urinary fluoride and WASI among children in the Cholesterol Cohort from Mexico City, Mexico.

Variable	ALL ^s		MALES (N=289)		FEMALES* (N=261)	
	β	p-value	β	p-value	β	p-value
Urinary fluoride	1.32	0.33	3.81	0.05	-1.57	0.39
Child's Sex (ref=female)	1.53	0.09				
Maternal Age	0.05	0.57	*	*	-0.10	0.38
Marital Status (Ref=married/cohabitating)	0.44	0.71	-1.69	0.36	1.56	0.29
Maternal Education	*	0.07	0.58	0.05	*	0.18
Family Possessions	*	0.40	*	0.70	*	0.17
Cohort 2 BL (Ref=Cohort 1)	2.61	0.05	2.53	0.19	3.44	0.05
Cohort 2 PL (Ref= Cohort 1)	3.20	0.02	1.28	0.53	6.28	<0.01
SF Cohort (Ref=Cohort 1)	3.61	<0.01	3.82	0.07	4.33	0.02
Mother's WAIS Score	0.30	<0.01	*	*	0.34	<0.01

*Asterisk denotes splines

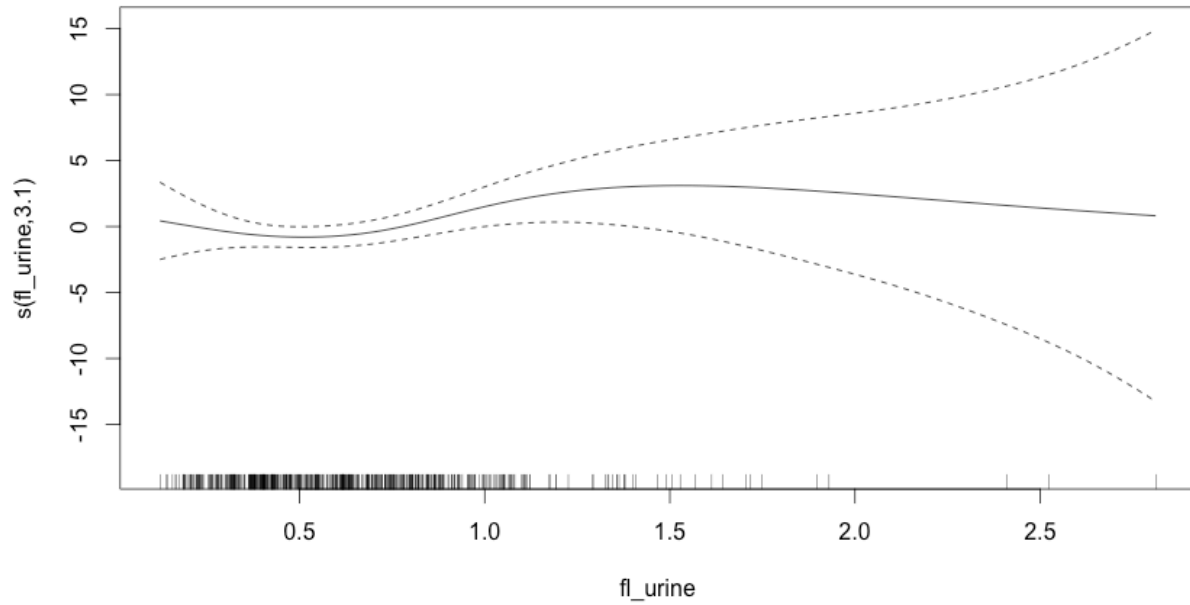


Figure IV.1: Child's urinary fluoride and total WASI score adjusted for child's Sex, child's age, maternal age, marital status, maternal education, family possessions, and cohort in the entire population.

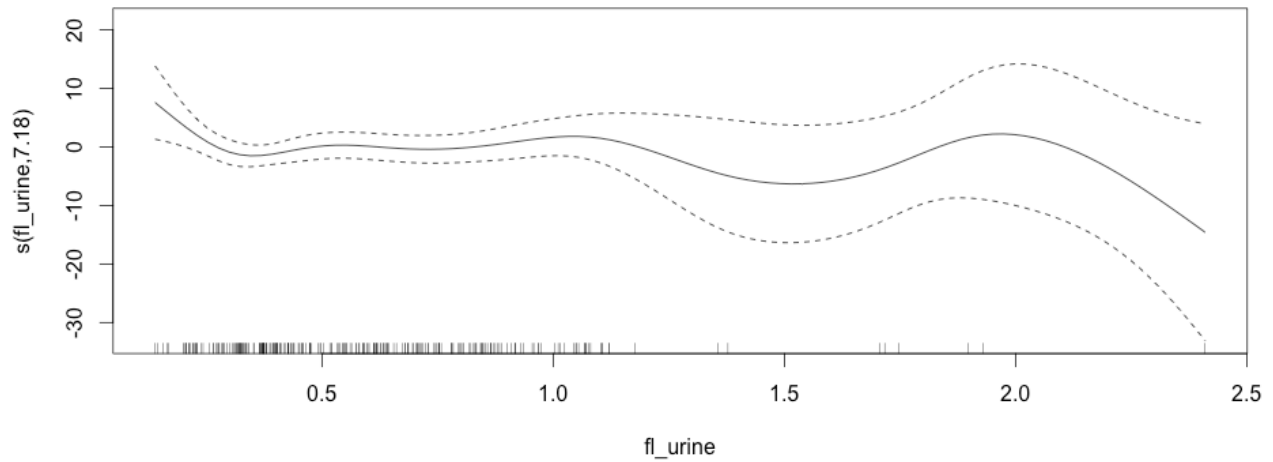


Figure IV.2: Child's urinary fluoride and total WASI score adjusted for Child's Sex, child's age, maternal age, marital status, maternal education, family possessions, mother's WAIS score and cohort among female children.

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CHAPTER V

Conclusion

I. Overview

This work increases our understanding of prenatal and concurrent fluoride intake and its effects on cognition in children. In chapter II, we describe, for the first time, urinary and plasma fluoride levels in pregnant women. We also examined exposure trends from the first month through delivery and found that the concentration of fluoride, once adjusted for other factors, does not change as pregnancy progresses. We also show that older women and those who carry male children maybe more susceptible to fluoride exposure. In chapter III, fluoride measured in pregnancy urine and plasma was not significantly associated with child's Mental Development Index (MDI) in early childhood and it did not differentially affect MDI over a three-year period. In Chapter IV, we find no detectable adverse outcomes on offspring neurobehavioral development due to simultaneous intake of fluoride, but a significant positive impact on Wechsler Abbreviated Scales of Intelligence (WASI) scores was seen in male children. We present the results in these three chapters with the use personal measures of exposure, and with the use of rigorous statistical methods that have not been reported in previous studies.

II. Chapter II

This is the first study to report urinary and plasma fluoride levels in hundreds of pregnant women from a population drinking fluoridated water at levels recommended by the U.S. Public

Health Service. Urinary fluoride ranged from 0.902-3.439 mg/L and plasma fluoride range from 0.004-0.077 mg/L. The levels of fluoride in maternal urine and plasma reported within this study are similar in range to other reports. In a study where women drank 1 mg/L of fluoridated water, urinary fluoride ranged from 0.22 to 0.53 mg/L.¹ In a controlled clinical study, pregnant women taking fluoride supplements had a mean urinary fluoride of 1.03 ± 0.14 mg/day in early pregnancy.² The wider range of urinary fluoride found in this study could be explained by higher levels of exposure in our population. Similarly, the plasma levels we report in this study are comparable to the levels reported in other studies on pregnant women. The most recent study, from Poland, measured 0.0329-0.0373 mg/L of plasma fluoride in 35 women during the third trimester of pregnancy,³ while other studies measured a range of 0.011-0.050 mg/L of plasma fluoride in five women,⁴ a mean of 0.033 ± 0.003 mg/L in 50 women⁵ and 0.97 ± 0.22 (first trimester), 0.90 ± 0.25 (second trimester), 0.86 ± 0.18 micromol/liters in 68 women.⁶

An issue of increasing importance is determining whether there are time-related exposure windows of susceptibility to toxicant exposures especially during the prenatal period. Many lines of evidence now indicate such, including previous work by us concerning lead exposure in the ELEMENT cohort.^{7,8} Here we were able to study whether fluoride levels vary in urine and plasma as pregnancy progressed, however we only saw changes on the individual level while the population averages remained stable throughout pregnancy. This was somewhat surprising to us as a number of physiological and metabolic changes occur during pregnancy that could affect fluoride toxicokinetics. Previous studies reported significantly different levels of exposure between different periods of gestation. One study from Jerusalem, for example, reports an U-shaped distribution with the highest concentration of urinary fluoride measured in the fourth and

ninth months of pregnancy.⁹ The other study on plasma pregnancy did not report any significant differences in plasma fluoride between the 28th and the 33rd week of pregnancy.³

Here we were also able to study urinary and plasma fluoride values in relation to key demographic variables. The strongest changes in plasma fluoride were in relation to maternal age, with older mothers reporting significantly higher levels of plasma fluoride. This may be explained by the exchange of fluoride between plasma and bone during the aging process. Ingested fluoride is absorbed through the gut, taken into the plasma, and then deposited into the bone. As the organism ages, the hydroxyapatite mineral in bone becomes saturated with fluoride, and further deposition of the ion from plasma is prevented.^{10,11} In animal studies, older dogs¹² and rats¹³ had more fluoride in their plasma than their younger counterparts, further supporting the theory that fluoride remains in the plasma or is excreted through the urine than deposited in the bone. There are, however, no such studies on plasma clearance in pregnant animals or humans so, this warrants more study.

Finally, we have limited evidence that suggests that urinary fluoride may be higher in women who smoke during pregnancy. This increase may be explained by the mobilization of fluoride during bone resorption. Fetal demands for calcium, particularly during the third trimester, increase the rate of bone breakdown to release the mineral into the bloodstream.^{14,15,16} To our knowledge, the effect of smoking on this process during pregnancy has not been studied; however, the catalytic effect of smoking on bone resorption in non-pregnant adults has been well documented.¹⁷ Higher concentrations of the deoxypyridinoline, C-telopeptide,¹⁸ and N-telopeptide,¹⁹ markers of bone breakdown, were measured in smokers. When bone resorption exceeds bone formation, as is the case in smokers, the amount of fluoride released from the bone increases without an increase of incorporation of fluoride into the bone.¹⁰ We hypothesize that

urinary fluoride levels, in turn, increase as the body attempts to clear the release of excess fluoride from the bones.

This work provides the most reliable characterization to date of urinary and plasma fluoride throughout the duration of pregnancy. Our values were similar to the ones reported in previous studies, but contrary to other reports, our analysis did not show a significant trend in urine or plasma fluoride once we adjusted for maternal and demographic factors. The two biomarkers were largely stable from the first to the last month of pregnancy. Interestingly, mothers who smoke seem to have the highest amount of urinary fluoride, and older mothers have the highest plasma fluoride, indicating that fetal exposure could be higher among smokers and older women. These results will be used in future studies related to health outcomes research such as prenatal fluoride exposure and neurobehavior.

III. Chapter III

Overall, this investigation found no evidence of a detectable adverse outcome on offspring neurobehavioral development associated with maternal fluoride exposure during pregnancy. Fluoride measured in pregnancy urine and plasma was not significantly associated with child's MDI at any age, and it did not differentially affect MDI at ages 1, 2, and 3. Furthermore, trimester-specific measures of maternal fluoride measures were not significantly associated with offspring MDI.

We present these results with the use of personal measures of exposure, and with the use of rigorous statistical methods that have not been reported in previous studies. Most of the studies in this field report bivariate analyses of fluoride and IQ without adjusting for confounders, but our study ran reliable statistical models to account for the effect of these variables. Parental education, for example, is a known confounder of toxicant exposure and

cognitive outcomes.²⁰ In addition to parental education, we collected data on maternal marital status, maternal age, child's age, and child's sex and we adjusted for these variables in our models. The inverse trend we saw between fluoride and MDI in bivariate analysis disappeared once we adjusted for these confounders.

There are currently only a few studies of cognitive measures that report personal measures of exposure, but this study uses two personal biomarkers to capture individual exposure to fluoride. Most of the existing studies on fluoride and neurobehavior used ecological measures of exposure and simply report the mean IQ across groups drinking water with different fluoride levels.^{21,22,23} This method is prone to ecological fallacy, where the correlation between two variables at the group level is incorrectly assumed to equal the correlation at the individual level. Our study, however, collected urine and plasma from each mother, allowing us to more directly assess exposure.

The two biomarkers in this study also capture and assess the effects of acute and chronic exposure to fluoride. Urinary fluoride levels increase in the few hours after ingestion, and subsequently decrease as fluoride is excreted from the body.²⁴ The handful of studies that report biomarkers only use urinary fluoride,²⁵ but plasma fluoride, due to an isoionic exchange of fluoride with bone, is a better measure of chronic exposure. Fluoride is deposited into the hydroxyapatite crystals of the bone over time, and when bone is remodeled some of the fluoride leaves and enters plasma, rendering it a proxy for the bone fluoride and chronic exposure.²⁶ This measure and its potential effect on cognition is being reported for the first time in our study.

In conclusion, the prenatal study found that maternal intake of fluoride during pregnancy does not have any measurable effects on cognition in early life. This maybe a case where the postnatal neuro-system is more susceptible than the prenatal neuronal system, and whether this

means, that the nervous system recovers, or the effects may have disappeared over time, is not known. In addition to timing of exposure, a number of questions related to range of exposure, child's age and type of neurobehavioral tests remain. We restricted our analysis to a lower range of exposure among children and we use one cognitive measure. The use of a higher sample size, the inclusion of concurrent exposure and wider ranges of exposure and ages, and more cognitive outcomes, therefore, remain considerations for future work in this area.

VI. Chapter IV

Overall, this investigation found a significant positive impact on neurobehavioral development due to ingestion of fluoride in male children but no association was detected in female children. Fluoride measured in urine was significantly associated with total WASI score in male children ages 6 to 15 years old. We present these results with the use personal measures of exposure, and with the use of rigorous statistical methods that have not been reported in previous studies.

Most of the studies in this field report bivariate analyses of fluoride and IQ without adjusting for confounders, but our study ran reliable statistical models to account for the effect of these variables. Parental education, for example, is a known confounder of toxicant exposure and cognitive outcomes.²⁶ In addition to parental education, we collected data on maternal marital status, maternal age, child's age, and child's sex and we adjusted for these variables in our models.

Most of the studies on fluoride exposure and IQ use either ecological measures of exposure or recall measures, leaving these studies prone to a lot of error. Most of the existing studies on fluoride and neurobehavior used ecological measures of exposure and simply report the mean IQ across groups drinking water with different fluoride levels.^{27,28,29} This method is

prone to ecological fallacy, an incorrect interpretation of the statistical data where correlation between two variables at the group level is assumed to equally correlate at the individual level. Our study, however, has directly measured fluoride in each individual by collection urine samples from all participants.

This work provides the most reliable assessment of urinary fluoride and cognition in children, and we found that WASI scores increase in male children as fluoride intake increases. These results were surprising in that they show opposite trends to what has been reported in the literature so, more studies with similar reliable methodology, which account for plasma fluoride, diurnal variations in urinary fluoride and children's SES, are needed. If these results are substantiated, different fluoride interventions may be needed for male children versus female children.

V. Limitations and future considerations

Despite a number of strengths (e.g., large sample size, adjusting for confounders and personal measures of exposure), there are potential limitations of our study that need mention. The spot urine samples we measured cannot account for diurnal variations in urinary fluoride. Previous studies report fluctuations in concentration by time-of-day³⁰ which means that the fluoride levels in our samples could be influenced by the time-of-collection or by diet. Future studies should either provide 24-hour measures or measure samples that have been collected at a standard time. This study was also not initially designed to address fluoride exposure, but we have a strong biomarker and the fluoride levels we measured was similar to previous reports. In addition to spot urine samples, in the prenatal study, we restricted our analysis to a lower range of prenatal exposure and we only use one cognitive measure. So, the use of concurrent exposure, with wide range of exposure and more cognitive outcomes in older children, remain

considerations for future work in this area. Also, the concurrent fluoride study is cross-sectional, having only collected exposure and outcome measures from one timepoint. So, questions related to temporality remain, and they can only be resolved once data is collected at multiple timepoints.

In chapter 4, we lack information on family socioeconomic status (SES), and there may be residual confounding from this factor that cannot be adjusted for in this study. SES, for example, is known to be associated with food intake, which in turn can affect fluoride levels. SES is also highly correlated with our outcome variable, cognition. To address these limitations, future studies should either provide 24-hour measures or measure samples that have been collected at a standard time, and they should provide information on SES.

Additionally, in chapter 4, we do not have plasma fluoride measures, which may account for the differences in association found between males and female children. In this study, urinary fluoride had a significant positive impact on male children but not on female children, but this association could change once plasma fluoride is added in the model. First, plasma fluoride is correlated to urinary fluoride, and second, plasma fluoride could provide a measurement of the fluoride released from bone during the drastic changes in bone growth and metabolism during puberty. Male children have higher rates of bone resorption during puberty than their female counterparts³¹, which could release more fluoride stored in bone and increase circulating levels of plasma fluoride, which could affect cognition. So, future studies should account for this critical confounder.

VI. References

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