STUDIES ON THE DIETARY INTAKE OF FLUORIDE AND THE CONCENTRATION OF FLUORIDE IN URINE OVER THE COURSE OF PREGNANCY

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DEDICATION

To my parents: Margarita and Fabio, and my life partner: Pedro Mauricio.

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To my mentor, Angeles.

To the unsung heroines behind this dissertation research: Angeles, Mara, Alejandra and Verenice.

To all the participants of the ELEMENT cohorts, and the ELEMENT teams both in Mexico and Michigan.

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To my family in Colombia and Indiana, and my friends –now living in all places around the world.

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STUDIES ON THE DIETARY INTAKE OF FLUORIDE AND THE CONCENTRATION OF FLUORIDE IN URINE OVER THE COURSE OF PREGNANCY

The use of maternal urinary fluoride as a biomarker of prenatal fluoride exposure in epidemiology studies is increasing. However, the knowledge on maternal exposure to fluoride and its biomarkers, has not increased alongside. The objective of this dissertation was to improve our understanding of the dietary intake of fluoride (a major source of fluoride exposure), and spot urinary fluoride levels during pregnancy.

Two secondary data analyses utilizing data from the Early Life Exposures in Mexico to ENvironmental Toxicants (ELEMENT) project were conducted, in a population of women living in a salt-fluoridated community. The first study estimated the dietary intake of fluoride over the course of pregnancy and assessed the influence of compliance with the dietary recommendations of intake of beneficial nutrients for pregnancy (calcium, iron, folate and protein) on the dietary intake of fluoride. The second study compared spot urinary fluoride in women during pregnancy and non-pregnancy (using one-year postpartum as a proxy for the non-pregnant state) and assessed associations between dietary factors (dietary fluoride intake, addition of table salt, calcium intake from diet and supplements, and dietary acid load) and urinary fluoride levels at each state.

Results revealed that the median dietary intake of fluoride in pregnant women was 0.7 mg/day, increased with gestational age and in women who were moderately and highly compliant with recommendations of intake of beneficial nutrients for pregnancy.

On the other hand, spot urinary fluoride levels during pregnancy did not significantly differ with those of women one-year postpartum, increased with gestational age, and decreased in calcium-supplemented women only during pregnancy. The dietary intake of fluoride, calcium, and dietary acid load were not associated with urinary fluoride in either state. Finally, reporting the addition of table salt to meals was associated with an increase in urinary fluoride only at one-year postpartum.

The studies in this dissertation highlight the need for a deeper understanding of fluoride exposure and its biomarkers in the pregnant population.

E. Angeles Martinez Mier, DDS, MSD, PhD, Chair

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Fluoride and its sources in the environment

Fluoride (F) is the ionic form of fluorine, one of the most abundant elements of the earth's crust and the most electronegative in the periodic table [1]. It is naturally present in the environment or released as a consequence of anthropogenic activities. In nature, rocks and fluoride-bearing minerals dissolve and release fluoride to water and soil [1] and it is specially abundant in volcanic areas [2], geothermal waters [3] and seawater [4]. Examples of anthropogenic activities that release fluoride to the environment include chemical and manufacturing industries (e.g. fertilizer, brick, ceramic, glass, fossil fuel burning) [1].

Fluoridation programs and sources of dietary fluoride intake

An experimental study on the fluoridation of the public water supply that took place over the course of the years 1945-1955 in Grand Rapids, MI, concluded that exposure to fluoridated water at a concentration of 1.0 mg/L reduced the prevalence of dental caries experience, with occurrence of mild forms of enamel fluorosis [5]. Based on the evidence of the Grand Rapids experiment, and with the goal of universal access to fluoride for the prevention and control of dental caries, communities around the world have added hydro fluorosilicate, sodium fluorosilicate or sodium fluoride to their public water supplies to adjust the water fluoride concentrations between 0.7 - 1.2 mg/L [6]. It was later estimated that the fortification of salt with fluoride concentrations of 200-250 mg/kg achieved reductions in dental caries comparable to those reached in the water

fluoridation studies [7,8]. In countries where a lack of water distribution systems is considered a barrier for universal fluoride access, salt fluoridation has been implemented. An estimated 370 million people have access to fluoridated water and another 300 million to fluoridated salt [9]. For example, as of 2018, 63.4% of the population in the United States had access to fluoridated water; and according to a 2009 report, 79.4% of the population in Mexico had access to fluoridated salt [10]. Where fluoridation programs are implemented, either fluoridated water and beverages (for the case of water fluoridation) [11] or solid foods (for the case of salt fluoridation) [7] become the main contributors of fluoride to the human diet. However, regardless of the availability of fluoridation programs, fluoride's ubiquity in the environment make it a trace element of the diet [12], and it is universally available in small quantities in foods and agricultural products that uptake fluoride from soil, water, and pesticides. Fluoride can also be transferred to foods during storage or cooking, via fluoride-coated food packaging [13] and cookware [14]. Lastly, ingestion of fluoride may occur from unintentional swallowing during and/or after the use of fluoride-containing oral hygiene products [15].

Dietary Reference Intakes (DRIs) of fluoride

In 1997, the committee for the Dietary Reference Intakes (DRIs) recommended an Adequate Intake (AI) of 3 mg F/day for all life stages, including pregnancy [16]. This recommendation was given in acknowledgement of the robust body of evidence on the negative association between fluoride intake levels and dental caries experience and was based on studies of usual fluoride intake conducted in children up to 5 years old [17]. The lack of evidence on total fluoride intake and side effects other than dental and skeletal fluorosis for other age groups resulted in the derivation of estimated fluoride intakes from

those of children by means of multiplying the AI (0.05 mg/kg) by the reference weight of a given age-group and sex. For the case of pregnant and lactating women, the reference weight was that of women 14-50 years of age (61 kg), which resulted in an AI of 3 mg/day [16].

Aspects of the urinary excretion of fluoride

Fluoride's pH-dependency, absorption, distribution, and excretion

The movement of fluoride through biological membranes depends on pH [18]: in an acidic environment, the form of fluoride that predominates is a weak acid (HF) that diffuses easily through biological membranes, and moves towards alkaline compartments. In contrast, in an alkaline environment, the predominant form is the fluoride anion (F^-), which lacks the ability to cross biological membranes [19].

When ingested in the absence of inhibitors (such as food and calcium-containing products) [20], ~90% of the fluoride ingested is absorbed in the gastrointestinal tract [21], and the not absorbed fraction (~10%) is excreted in the feces [22]. Approximately 25% of the absorption happens in the stomach through a pH-dependent mechanism [23], while the remaining 75% occurs in the proximal small intestine, through a pH-independent mechanism, via paracellular channels [24]. Blood plasma is the central compartment for fluoride distribution; peak levels can be detected within 20-60 min post-ingestion, and baseline levels after 3-11 hours. These plasma fluoride concentrations are not homeostatically regulated, and vary depending on the amount of fluoride intake, uptake and/or removal from soft and hard tissues and its renal excretion [21]. The distribution of fluoride to soft tissues and body fluids is low [25]: fluids such as gingival crevicular fluid and saliva can reach up to 90% of the concentrations found in plasma [26,27], while

others like cerebrospinal fluid and milk reach around 50% or less of plasma concentrations [28,29]. In contrast to the low distribution in soft tissues, fluoride avidly binds to mineralized tissues, so that 99% of the fluoride retained in the body is found in bones and teeth [18]. If not retained in the body, the absorbed fluoride is removed from plasma via the kidneys. In healthy adults, around 60% of the absorbed fluoride is excreted in the urine in less than 24 h [30].

Renal handling of fluoride

Following filtration by the glomerulus, fluoride enters the renal tubules, where 10-90% is reabsorbed into the systemic circulation [31]. The reabsorption of fluoride by the renal tubules is a pH-dependent process, which determines the efficiency of the renal elimination of fluoride [32]. If the tubular fluid is highly acidic, the weak acid HF predominates and its diffusion through the tubules' cell membranes occurs, taking it back to the systemic circulation. Conversely, a tubular fluid with a higher pH will increase fluoride's efficiency of elimination −as it will be present mainly in its ionic form (F^-), and the reabsorption process will not be favored [18]. Thus, any condition favoring acidity of the urine −acute or chronic, increases fluoride's chances to remain in the body to either participate in biological processes or to be retained by mineralized tissues.

Factors that affect the absorption, distribution, and excretion of fluoride

Composition of the diet**:** the intake of fluoride in combination with food influences the absorption of fluoride; with fluoride being more bioavailable when consumed on an empty stomach and with water as a vehicle (as opposed to in combination with food or as part of a meal prepared with fluoridated salt) [33]. High

intake of divalent cations, such as calcium, magnesium and aluminum also reduce fluoride absorption [34]. Dietary patterns with high meat intake are associated with low urinary pH (which decreases fluoride's rate of elimination), while vegetarian diets are associated with higher urinary pH (which favors fluoride excretion) [35,36].

Acute and chronic acid-base disturbances: the kidneys have a key role in the regulation of the body's acid-base balance. In states of respiratory acidosis, the kidney's collecting ducts secrete hydrogen ions that acidify the tubular fluid; whereas in states of respiratory alkalosis, the ducts secrete bicarbonate, which alkalinizes the fluid [37]. Both states (respiratory acidosis/alkalosis) ultimately affect the urinary excretion of fluoride. Acute and chronic acid-base disturbances that had been described to influence fluoride excretion include: chronic obstructive pulmonary diseases, emotional states, physical activity and, altitude of residence [18].

Impaired kidney function*:* patients with progressive renal functional impairment have decreased urinary excretion [38] and higher retention of fluoride in the body [39].

Exercise: moderate and vigorous exercise may be associated with decreased renal clearance of fluoride in humans [40]. This effect could be the consequence of reduced renal blood flow [19] and increased fluoride reabsorption from the renal tubules as a result of the production of lactic acid [32].

Drugs***:*** drugs that inhibit gastric acid secretion or that alkalinize or acidify the gastric content can modify the gastric absorption of fluoride. Other drugs altering urinary

pH can also affect fluoride's distribution and urinary excretion (e.g. ascorbic acid, ammonium chloride, chlorothiazide diuretics and methenamine mandelate) [19].

Circadian rhythm and hormones: fluoride concentrations in plasma and urine follow a rhythmic pattern and may be correlated with serum parathormone levels [41].

Age*:* there is a positive relationship between age and the concentration of fluoride in plasma [42,43]. This relationship may be the result of the stage of skeletal development (with younger ages associated with fluoride retention in the skeleton) or declining renal functional mass [18].

Fluoride concentration in urine as biomarker of fluoride intake

Studies conducted in children and nonpregnant adults have found a linear, positive relationship between total fluoride intake and the urinary excretion of fluoride: the higher the total fluoride intake, the higher the urinary excretion of fluoride [30]. For the assessment of such relationship, the aforementioned studies used short-term dietary intake assessments (duplicate plate, experimental diets or 24 h dietary recalls), together with an estimation of fluoridated toothpaste intake and the collection of urine samples over a 24 h period [30]. This biomarker is a better predictor of fluoride exposure in populations compared to individuals [44].

For practical reasons, the concentration of fluoride in spot urine samples is currently being used as biomarker of fluoride exposure in epidemiological studies. It is however worth mentioning a distinction between measuring urinary fluoride excretion (the process of elimination of certain estimated amount fluoride from the body via the urine in a defined period of time) and the measurement of the concentration of fluoride in spot urine samples. Although fluoride concentrations in spot urine samples may be

associated with sources of fluoride exposure [45] −which validates their use as biomarkers in exposure and health outcome studies; they do not reflect *per se* the process of the urinary excretion of fluoride and should be interpreted with this distinction in mind.

The dietary intake of fluoride and its urinary excretion during pregnancy

Current evidence on the dietary intake of fluoride in pregnant women

Although evidence on the dietary intake of fluoride in pregnant women is scarce, two recent reports on women living in communities exposed to fluoridated water are available. A study conducted in 260 pregnant women living in Spain estimated a mean±SD daily fluoride intake from tap water of 0.1±0.4 mg/day [46]. Another study conducted in Canada estimated fluoride intake from tap water and tea in a sample of 400 pregnant women and found a mean±SD estimate of 0.9±0.4 mg/day [47]. Even though both reports likely underestimated total fluoride intake, it can be inferred that fluoride intake from tap water and fluoride-containing beverages constitutes less than half of the current total fluoride intake recommendation for pregnant women (3 mg/day).

Adaptations of pregnancy with the potential to impact the absorption, distribution, or the urinary excretion of fluoride

Calcium metabolism: during early- and mid-pregnancy, the intestinal absorption of calcium is doubled. This increased absorption allows for the buildup of maternal skeletal calcium stores to meet fetal demands during the third trimester [48]. Therefore, as pregnancy progresses, bone metabolism transitions from a state of predominantly

maternal bone formation, increased bone density and calcium storage to increased bone turnover for the transfer of calcium to the fetus towards the end of gestation [49].

Acid-base balance: the gradual size-increase of the uterus pushes up the diaphragm up to 4 cm above its usual position, diminishing total lung capacity [48]. To compensate for the lower lung capacity, progesterone acts as a respiratory stimulant, increasing the volume of air inhaled per minute and leading to a state of *hyperventilation* −breathing faster and deeper [50]. The increased ventilation responds to the fetal demands of oxygen, but the increased exhalation of carbon dioxide leads to an unbalance of the blood's bicarbonate buffering system: as more carbon dioxide is lost, hydrogen ions are removed and blood acidity decreases, leading to *chronic respiratory alkalosis* [51]. To compensate for the decrease in hydrogen ions, the kidneys excrete bicarbonate through the urine and retain hydrogen ions to maintain the pH at physiological levels [48]. Therefore, the renal-compensated respiratory alkalosis of pregnancy tends to increase urinary pH by means of the increased renal loss of bicarbonate [52]. Although pregnancy is a state of chronic respiratory alkalosis, it has not yet been described in the literature as a potential factor influencing the renal excretion of fluoride.

Glomerular filtration rate: during the first trimester of pregnancy, plasma volume increases approximately 40 to 60% and reaches its maximum during the third trimester, at about 32 weeks [53]. Consequently, the volume of blood delivered to the kidneys increases 50-80% by mid-second trimester, raising glomerular filtration rate by 40-60%

[54]. As a result, there is an increase in urine flow and volume and the filtration and excretion of water and solutes [48].

Dietary modifications and drugs: pregnant women modify their usual dietary intake of foods and nutrients for different reasons, such as concern for their own and their babies' health, aversions and cravings [55]. For instance, multivitamin use is very common in pregnancy, compared to the nonpregnant state [56]. On the other hand, most pregnant women experience gastrointestinal diseases at some point, such as nausea, vomiting, gastroesophageal reflux, constipation and diarrhea [57]. The alleviation of symptoms typically include dietary modifications, usually the decreased consumption of triggers (highly acidic foods such as coffee, tomatoes and carbonated beverages) and increase in the intake of healthier, less acidic alternatives [55]. The pharmacological management of gastrointestinal symptoms include antiacids (e.g. calcium carbonate, aluminum or magnesium hydroxide) and acid reducers (e.g. famotinide, ranitidine, omeprazole) [57].

The physiological adaptations of the major systems (cardiovascular, renal and respiratory) return to nonpregnant levels about 2 to 3 months postpartum [48]. However, in lactating women, changes in calcium absorption, distribution and excretion, continue to take place for the production of breastmilk [48]. Markers of bone turnover increase during early lactation − when the infant depends mostly on breastfeeding to meet

nutritional demands. But after 6-12 months of lactation (which naturally decreases frequency and supply after weaning), markers of bone turnover decrease [58].

Studies on the urinary excretion of fluoride during pregnancy

Only six studies have previously investigated the urinary excretion of fluoride or urinary fluoride levels at some point during, or over the course of pregnancy [36,59–63], three of them conducted between 1959 and 1983 [36,59,60]. The six available studies aimed at answering one or two research questions: the first one, whether urinary fluoride levels change with the progression of pregnancy; and the second one, whether these levels change compared to nonpregnant women. From the five studies reporting urinary fluoride levels during pregnancy, three concluded an increase with the progression of pregnancy [36,60,61]. On the other hand, from the four studies that compared urinary fluoride of pregnant women vs. that of nonpregnant women, two concluded lower levels during pregnancy [59,61] and the other two concluded no differences [36,60]. The evidence in this area is contradictory, all six studies have heterogeneous designs and most of them have a small sample sizes [36,60,61] or inadequate methods for the measurement of fluoride in urine using spot urine samples [59,61]. Even though four studies discuss and compare the urinary excretion of fluoride, only two of them truly evaluated excretion [36,60]; and the other two evaluated urinary fluoride levels in spot urine samples [61,64]. What all studies have in common, is a discussion of findings centered on the uptake of fluoride by the fetus as a potential explanation of the results; none discussed or controlled

for the physiological and dietary changes of pregnancy that also have the potential to influence urinary fluoride levels.

Study population and data collection

The study population for this dissertation was pregnant women participating in the Early Life Exposures in Mexico to ENvironmental Toxicants (ELEMENT) project [65]. ELEMENT comprises three mother-child pregnancy and birth cohorts, initiated in the 1990s to study early life exposures and health outcomes in Mexico City − a city with an implemented salt fluoridation program (250 ppm of F/kg salt) [66]. For this dissertation research, only pregnant women from Cohort 3 were included. These women were recruited between 2001 and 2003 (N=670). Pregnant women attending three clinics of the Mexican Institute of Social Security (IMSS) were invited to participate, but only those with gestational age <14 weeks, a healthy singleton pregnancy, no history of systemic diseases (hypertension, diabetes) and intention to stay in Mexico City who agreed to participate through informed consent were included and followed thereafter. Details and demographics of the entire project can be found in the ELEMENT project's profile publication [65]. The project has a dedicated research facility next to Mexico City's ABC Medical Center and participating women were invited to attend at three time-points over the course of their pregnancy and for several months after delivery [65]. Cohort 3 was originally designed as a double-blind Randomized Clinical Trial (RCT) to examine the effects of calcium supplementation on blood lead levels during pregnancy and up to 12 months postpartum [67]. Women were randomized to receive either the

calcium supplement (1200 mg calcium/day) or placebo, and received a bottle containing a known number of pills of the assigned treatment.

Among the information that was collected from study participants by trained research assistants at each study visit, data relevant to this dissertation research were:

- Sociodemographic questionnaires
- Anthropometry
- Food Frequency Questionnaires (FFQ)
- Early morning second-void spot urine samples, which were archived in the project's biorepository at -70°C until analysis
- Registry of adherence to the treatment assigned for the calcium RCT (supplement/placebo). Participants were asked to bring back the bottle containing the pills of the assigned treatment, so that a research assistant could register adherence by counting the remaining pills in the bottle.

Dissertation rationale

Community fluoridation is an effective measure for the reduction of dental caries experience in children [68]. The study of the safety of chronic fluoride exposure at doses relevant for community fluoridation programs has focused on side effects affecting teeth and bones, with children up to 5 years of age and older adults as the populations at risk [69]. More recently, pregnant women are being considered a new potential group at risk of side-effects of fluoride exposure [70]. Epidemiological studies conducted in populations exposed to low levels of fluoride (such as the ones used for community fluoridation), have found associations between maternal urinary fluoride (used as a proxy of prenatal fluoride exposure) and negative cognitive and neurodevelopmental outcomes in children [47,71–73]. However, the availability of data on fluoride exposure collected from pregnant women is very limited. A major source of exposure to fluoride is the diet, and a knowledge-gap in population-based research on the current levels of dietary fluoride intake in pregnant women was identified.

The epidemiological studies addressing the aforementioned associations between prenatal fluoride exposure and cognitive and neurodevelopmental outcomes in children have been using spot urinary fluoride as a biomarker of prenatal fluoride exposure [47,71–73]. The biomarker is used under the rationale that the urinary excretion of fluoride is directly proportional to dietary fluoride intake [30] and that maternal urinary fluoride is positively associated with fluoride levels in maternal serum and amniotic fluid [74]. However, the renal handling of fluoride in a state such as pregnancy has the potential to modify the relationship that is observed in the general population. Specifically, changes in urinary fluoride levels during pregnancy might not exclusively

reflect exposure, but also changes in the renal handling of fluoride and its rate of elimination − which responds to physiological and dietary factors. A second knowledge-gap was identified: the dietary factors that affect spot urinary fluoride during pregnancy − other than dietary fluoride exposure – have not been identified.

Closing the knowledge-gaps on levels of fluoride intake during pregnancy and the effect of dietary factors on urinary fluoride during pregnancy advances our knowledge on urinary fluoride as a biomarker of exposure and informs 1) future dietary recommendations of fluoride intake; and 2) epidemiological studies validating the use of spot urinary fluoride as a biomarker of prenatal fluoride exposure.

Dissertation outline

This dissertation consists of two secondary data analyses that utilized data from the the Early Life Exposures in Mexico to ENvironmental Toxicants (ELEMENT) project. The overall hypothesis was that dietary factors during pregnancy affect the intake of fluoride and the concentration of fluoride in urine.

The first study estimated dietary fluoride intake over the course of pregnancy and estimated the overall adjusted difference of dietary fluoride intake by pregnancy stage and levels of compliance with Mexican dietary recommendations. It was hypothesized that Mexican pregnant women who meet the dietary recommendations of beneficial nutrients for pregnancy also increase their dietary intake of fluoride.

The second study compared spot urinary fluoride levels in the pregnant and non-pregnant states and assessed the association between spot urinary fluoride levels and dietary factors. It was hypothesized that dietary changes during pregnancy affect the concentration of fluoride in urine.

The last part of this dissertation (general discussion) summarizes the overall results, strengths, limitations, and provides recommendations for future research based on the findings.

STUDY 1:

DIETARY FLUORIDE INTAKE OVER THE COURSE OF PREGNANCY IN MEXICAN WOMEN

Summary

A secondary data analysis in a Mexican longitudinal pregnancy cohort exposed to fluoridated salt was conducted. The objectives were: 1) to estimate the dietary intake of fluoride over the course of pregnancy, and 2) the overall adjusted difference of dietary fluoride intake by pregnancy stage and levels of compliance with Mexican dietary recommendations. It was hypothesized that Mexican pregnant women who meet dietary recommendations also increase their dietary intake of fluoride.

A total of 568 women with data available for two or three pregnancy time-points were included in the analytical sample. The dietary intake of recommended prenatal nutrients and fluoride intake (mg/day) were estimated using a validated Food Frequency Questionnaire. Data were summarized with descriptive statistics. Levels of F intake were compared to the USA's IOM Adequate Intake (AI) of 3 mg/day for pregnancy. Adjusted differences of fluoride intake over time and levels of compliance with recommendations were estimated using longitudinal random effects models.

Median (IQR) estimated fluoride intake ranged from 0.64 mg/day (0.38) in the 1st, 0.70 mg/day (0.42) in the 2nd and 0.72 mg/day (0.44) in the 3rd pregnancy visit. Adjusted average intakes of fluoride [95% CI] were 0.72 mg/day [0.70-0.74], 0.76 mg/day [0.74-0.77], and 0.80 mg/day [0.78-0.82]. Women who were moderately and highly compliant with Mexican dietary recommendations ingested, on average, 0.04 and 0.14 mg F/day more than non-compliant women ($p<0.005$).

In conclusion, dietary fluoride intake was below current AI, increased with gestational age and was higher in women who were moderately and highly compliant with dietary recommendations.

Introduction

Fluoride (F) is present in small amounts in the soil, water, plants, and animals; therefore it is naturally present as a trace element in the diet [12]. An extensive body of evidence has proven a clear reduction in dental caries prevalence in fluoride-exposed communities and, as part of community fluoridation programs to prevent dental caries, fluoride has been added to the diet via water or salt for human consumption [6]. Like other nutrients and trace elements in the diet, fluoride has both beneficial and detrimental effects: with low exposures, it prevents and controls dental caries, while higher exposures can lead to hard-tissue changes such as dental fluorosis [75]. Given that the risk for developing dental fluorosis is present only during critical periods of tooth development, dietary fluoride intake has been extensively and traditionally monitored in children [76], but rarely in other age groups. There is, however, emerging evidence on potential adverse effects of prenatal fluoride exposure [70]. Associations between fluoride concentration in urine during pregnancy and poor neurodevelopmental outcomes in the offspring have been reported; not only in populations with endemic fluorosis and exposed to high levels of fluoride [73], but also in populations exposed to low levels, such as the ones considered optimal for community water and salt fluoridation programs [47,71,72]. This newly emerging evidence on the potential side effects of prenatal fluoride exposure suggests monitoring of fluoride intake in other susceptible groups, such as pregnant women, may be warranted.

In 1997 and based on data collected mainly in children and nonpregnant adults, the USA's Institute of Medicine (USA-IOM) recommended an Adequate Intake (AI) for fluoride of 3 mg/day (0.05 mg/kg/day). Using a pre-pregnancy body weight for women >19 yrs of ~61 kg as a reference, the recommendation for both pregnant and non-pregnant women was also set as 3 mg fluoride/day [16]. Given the existing knowledge-gap on fluoride intake in the context of the dietary and physiological changes of pregnancy, there is a need for observational studies of fluoride intake in populations of pregnant women. For instance, pregnant women are encouraged to increase the dietary intake of foods and supplements containing nutrients that are beneficial for maternal and fetal health, such as calcium, iron and folate [16]. In contrast, the dietary intake of fluoride is not particularly encouraged or discouraged during pregnancy and has no reported benefits for fetal health. In a previous investigation on the concentration of fluoride in foods and beverages available in Mexico City [77], small amounts of fluoride in Mexican dietary staples such as cereals, legumes and animal products, were reported. These foods are rich sources of folate, calcium, iron and protein [78], and their frequent consumption may increase the total daily dietary intake of fluoride. It was therefore hypothesized that Mexican pregnant women, attempting to meet dietary recommendations, increase their dietary intake of fluoride. Understanding how fluoride intake changes over the course of pregnancy, and whether meeting the requirements of key beneficial nutrients also increases the dietary intake of fluoride, can serve the purpose of informing future dietary recommendations for pregnancy.

The objectives of this study were to estimate dietary fluoride intake over the course of pregnancy and the overall adjusted difference of dietary fluoride intake by pregnancy stage and levels of compliance with Mexican dietary recommendations.

Methods

Study sample

For this study, only pregnant women from the ELEMENT project's Cohort 3 were included (recruited between 2001 and 2003; N=670). This cohort was originally designed as a double-blind Randomized Clinical Trial (RCT) to examine the effects of calcium supplementation on blood lead levels during pregnancy and up to one year postpartum [67]. Women were randomized to receive either the calcium supplement (1200 mg calcium/day) or placebo. During each pregnancy visit, each woman was interviewed by a social worker, who performed anthropometry (weight and height) using calibrated instruments, applied a general demographic questionnaire and a Food Frequency Questionnaire (FFQ). Only women from Cohort 3 with FFQ and all variables of interest available in the database, were included for the analyses (N=568). All participants who were included had data available for at least two pregnancy stages; and 511 participants had data for all three.

Measurement of fluoride in foods, water and other beverages

Mexico City has naturally-occurring water fluoride levels <0.7 ppm [79] and does not have community water fluoridation as used in other countries. Instead, since 1981 a salt fluoridation program was implemented as a method to deliver fluoride for caries prevention (250 ppm of F/kg) in regions with water fluoride levels <0.7 ppm [66,80].

Therefore, the main sources of dietary fluoride intake in Mexico City are foods with intrinsic fluoride content and those containing fluoridated salt added either during and/or after the cooking process. A database was developed specifically for the ELEMENT project by analyzing the fluoride content of typical foods and beverages in the Mexican diet [77]. Details on the methodology for the collection of food and beverages and the analysis of fluoride can be found in the publication by Cantoral et al. [77]. Briefly, fruits and vegetables were bought from three different major markets in Mexico City. Meat products, processed foods, juices, beverages and industrialized foods were bought from four large supermarket chains. Natural juices were purchased from street vendors, flavored waters (a traditional Mexican beverage prepared with water, fruit and sugar) were bought from ice cream parlors and dairy was purchased in creameries. To measure the concentration of fluoride in water drank by participating women, a trained research assistant visited the household of study participants of the ELEMENT project (n=552) and collected water samples (~5 mL). To standardize the concentration of fluoride in the water and salt in foods that are consumed cooked (meats, rice, pasta, legumes), these were boiled using water containing negligible amounts of fluoride (<0.01 mg/L) and fluoridated salt (250 ppm of F/kg), following standardized recipes from the National Health and Nutrition Survey. Traditional foods (e.g. tamales and corn-based foods) were purchased cooked from street vendors. Fluoride analyses were conducted at the Fluoride Research Laboratory at the Oral Health Research Institute (OHRI), Indiana University School of Dentistry using a modification of the hexamethyldisiloxane (HMDS) method [81] as modified by Martinez-Mier et al.[82]. Participants reported the intake of mainly bottled and tap water, which had mean (±SD) fluoride levels of 0.16±0.13 ppm and

0.14±0.09 ppm, respectively. These two average values were the ones included in the database for FFQ-derived estimations of fluoride intake from water.

Estimation of dietary intake of fluoride, macro- and micronutrients

Dietary fluoride intake (mg F/day) was assessed through a semiquantitative questionnaire consisting of 104 items, adapted from the Willett semi-quantitative FFQ [83] to include foods and beverages commonly consumed in the 1983 Dietary Survey of the Mexican National Institute of Nutrition [84], and validated to estimate dietary intake over the previous month in Mexican women of child-bearing age (15-44 years) [85]. The FFQ was applied by a trained social worker at each study visit once per trimester, using visual and measuring aids (spoons, cups) for the identification of foods and portion sizes. Estimates of dietary intake of macro- and micronutrients (in µg or mg/day) were calculated through software developed at the National Institute of Public Health (INSP) using methods for the analysis of dietary data from the Mexican National Health and Nutrition Survey [86]. To generate estimates of daily dietary intake, the software utilizes the following data: 1) the average content of nutrients and trace elements (including fluoride) of each food/beverage item reported the INSP-compiled nutrient composition database [87] and the ELEMENT fluoride database; and 2) the reported frequencies and portion sizes of foods and beverages from the FFQ. Further details on the development, validation and calculations derived from the FFQ are available in the publication by Hernandez-Avila et al. [85]. Although the FFQ did not include quantitative estimations of table salt added after cooking, it did include a dichotomous question on whether table salt was added to foods right before eating them (yes/no), which we used for this secondary data analysis. Given that this cohort was originally designed as a calcium RCT and

women may had chosen to ingest other dietary supplements as well, a variable with group-assignment to the RCT (placebo/supplemented) and another one specifying intake of other dietary supplements (yes/no) was available to control for potential confounding.

Assessment of compliance with dietary recommendations

Dietary fluoride intake in Mexican women is neither contraindicated nor encouraged in Mexican pregnancy dietary recommendations. The Mexican Official Norms regulating health services for pregnant women that were current for the cohort highly encouraged an increase in the dietary intake of key nutrients [78] in order to meet the Adequate Intake (AI) or Estimated Average Requirement (EAR) as recommended by the Dietary Reference Intakes (DRIs) by the USA-IOM [16]. Recommendations on dietary intake during pregnancy are made according to pre-pregnancy BMI and pregnancy stage [88]. The four key nutrients encouraged in the dietary recommendations were calcium (AI: 1000 mg/day), iron (EAR: 22 mg/day), folate (EAR:520 µg/day) and protein (EAR: 0.88 g/kg/day). Intake was recommended from various dietary sources for all nutrients [89]; and, in the case of calcium, folate and iron, the recommendation included intake from both diet and supplements. The dietary intake of each nutrient was estimated only from the intake reported in the FFQ. Since one of the objectives of this study was focused on the relationship between compliance with nutrients from dietary sources and dietary fluoride intake, supplement sources were excluded from the calculation.

Women were classified as compliant with an individual nutrient if their estimated daily dietary intake was equal to or above the AI or EAR for that particular nutrient during pregnancy. Overall compliance at each stage of pregnancy was categorized

according to individual-nutrient compliance as follows: *noncompliance* if noncompliant with all the nutrients (0/4); *moderate compliance* if compliant with one or two nutrients (1/4 or 2/4); and *high compliance* if compliant with three or all the four nutrients (3/4 or 4/4).

Covariates

The selection of covariates was based on bivariate analyses and a review of the literature on the factors that may influence dietary fluoride intake. These included: pregnancy stage, compliance with dietary recommendations, group allocation in the calcium supplementation RCT, intake of other supplements, addition of table salt after cooking, total daily energy intake, pre-pregnancy BMI and educational attainment.

Statistical analyses

Study participants were stratified by pregnancy visit. Differences in key dietary variables across pregnancy visits were assessed with Friedman test (for continuous, non-normally distributed repeated measures variables) or chi-square tests (for categorical variables). Differences in dietary fluoride intake between the RCT allocation groups and according to table salt use were tested with the non-parametric Mann-Whitney test. To estimate the overall adjusted difference of dietary fluoride intake by pregnancy visit (early, middle, late) and levels of compliance with Mexican dietary recommendations (none, moderate, high), covariate-adjusted regression models for panel data were fitted using the *xtreg* command in STATA. Panel data models were chosen because they examine both individual- and time-specific effects to deal with unobserved heterogeneity or individual effects. Regression diagnostics revealed significant differences in the individual- and time-specific variance components (panel effect; Breusch-Pagan LM test

$p<0.05$) and no correlation between individual effects and the regressors (Hausman test, $p> 0.05$); therefore, one-way random effects GLS regressions provided the best fit. After the model's estimates were obtained, adjusted predictions for dietary fluoride intake were calculated for each pregnancy visit using the *margins* command. The association between dietary fluoride intake and individual intake of nutrients (calcium, iron, folate, protein) was assessed following the same approach. All analyses were conducted with STATA v16.0 (StataCorp LP, College Station, TX, USA).

Results

Characteristics of the sample and dietary fluoride intake by pregnancy visit

Table 1 summarizes and compares the characteristics of women included in the analytical sample (N=568) and those who were excluded (N=102). Overall, the median age of the study participants at the time of recruitment was 26.4 years and the median gestational age for women who completed FFQs at the 1st, 2nd or 3rd pregnancy visits was 13.6, 25.4 and 34.3 weeks, respectively. Median weight ranged between 60.5 - 69.4 kg during the three pregnancy stages and most women (57.7%) had a pre-pregnancy BMI categorized as normal according to the guidelines that were current at the time of data collection [78]. Most women (64.3%) had been pregnant more than once; and their highest educational level was secondary school (67.8%). Finally, in the analytical sample, 289 women were allocated to the treatment group of the calcium supplementation RCT, whereas 279 were allocated to the placebo group. The characteristics of women who were excluded were not significantly different from those who were included.

Detailed descriptive statistics of dietary fluoride intake by pregnancy visits are provided in **Table 2.** This sample of pregnant women living in Mexico City had a median

dietary fluoride intake of 0.69 mg/day, ranging between a minimum of 0.11 and a maximum of 3.73 mg/day (for a total of 1649 observations at all pregnancy visits).

Bivariate statistics for key dietary variables by pregnancy visits

Table 3 summarizes bivariate statistics of key dietary variables by pregnancy visit. Variability in both dietary fluoride intake and total energy intake over the course of pregnancy was found. Women had a median dietary fluoride intake (IQR) that ranged from 0.64 mg/day (0.38) in visit 1 to 0.72 mg/day (0.44) in visit 3, and the tendency to increase was statistically significant. Considering that the median weight across pregnancy was 65 kg, this range of median fluoride^{-} intake throughout pregnancy corresponds to 0.01 mg/kg/day. Estimates of dietary fluoride intake between those who reported adding table salt after cooking their meals and those who did not, were not significantly different (0.67 mg/day IQR 0.47 vs 0.70 mg/day, IQR 0.40, respectively) (data not shown in Table 3). In contrast to dietary fluoride, median total energy intake significantly decreased for the second pregnancy visit (median gestational age: 25.4 weeks). There were also variations in overall compliance and individual-nutrient compliance across pregnancy visits. Compliance with both calcium and iron were associated with pregnancy visit. For calcium, compliance rose from 51.2% in the 1st, 58.6% in the 2nd and, 62.0% in the 3rd pregnancy visit, whereas for iron compliance was 6.0% in the 1st, 7.1% in the 2nd and 5.5% in 3rd visit. In contrast, although compliance with protein intake was also associated with pregnancy visit, a tendency to decrease was observed (68.7, 62.6 and 56.8% for the 1st, 2nd, and 3rd pregnancy visits, respectively). Compliance with folate intake from dietary sources experienced a slight increase towards the middle stage, followed by a decrease towards the end of gestation (5.8, 6.3 and 3.8%

for the 1st, 2nd and 3rd pregnancy visits, respectively), and had no statistically significant association with pregnancy visit. Finally, women were mostly moderately compliant with dietary recommendations, and this trend was held across time. Only a slight increase in the proportion of women who were noncompliant towards the end of pregnancy was observed, although this was not statistically significant. Towards the end of pregnancy (3rd visit), the proportion of women reporting adding table salt after cooking was lower (30.3% in 1st vs. 22.6% in 3rd visit); whereas the proportion of women reporting the use of dietary supplements was higher (23.4% in 1st visit vs. 37.9% in the 3rd) (**Table 3**). Median fluoride intake from dietary sources for women allocated to the calcium supplementation group in the RCT was significantly higher than that of women allocated to the placebo group (overall median 0.71 vs 0.67, p=0.03; not shown in Table 3), therefore RCT allocation group was also included as a covariate in the models.

Adjusted dietary fluoride intake by pregnancy visits and compliance with dietary recommendations

After adjustment for covariates, the association between dietary fluoride intake and pregnancy visit was significant (p<0.001, **Table 4**). Compared to the 1st visit (~13.6 weeks gestation), women who attended the 2nd and 3rd visits (~25.4 and ~34.3 weeks, respectively) ingested on average 0.04 and 0.08 mg of fluoride per day. The adjusted predictions of dietary fluoride intake from foods and beverages during pregnancy therefore increased from 0.72 [CI: 0.70-0.74] in the 1st, to 0.76 [CI: 0.74-0.77] in the 2nd, and 0.80 [CI: 0.78-0.82] in the 3rd pregnancy visit (**Figure 1**). Women who reported intake of dietary supplements other than the one provided for the RCT ingested on average 0.03 mg F/day less compared to women who did not take other supplements

(p=0.027). Furthermore, compared to non-compliant women, those who were moderately and highly compliant with dietary recommendations ingested on average 0.04 and 0.14 more mg of fluoride per day, respectively (**Table 4**). The covariates included in the model were total energy intake, allocation group in the calcium supplementation RCT, intake of other supplements and pre-pregnancy BMI.

In order to understand which nutrients were associated with changes in dietary fluoride intake, the association between compliance with individual nutrients and dietary fluoride intake was also assessed. Compared to women who did not meet calcium and iron recommendations, those who were compliant with calcium and iron recommendations ingested on average 0.12 and 0.18 more mg F/day, respectively (p<0.001). In contrast, women who were compliant with folate recommendations ingested on average 0.12 less mg F/day, compared to those who did not meet recommendations (p=0.009) (**Table 5**). Compliance with protein intake recommendations was not associated with dietary fluoride intake.

Discussion

Median dietary fluoride intake in this sample of pregnant women living in Mexico City was 0.69 (min - max: 0.11 – 3.73) mg F/day, or 0.01 mg/kg/day [90]. To date, only two other studies have reported dietary fluoride intake during pregnancy in large samples of pregnant women. In Canada, fluoride intake from beverages was assessed in 162 pregnant women living in communities with access to fluoridated water, with a reported intake of (mean ± SD) 0.93 ± 0.43 mg F/day [47]. Another study, conducted in Spain in 575 pregnant women also living in a community with access to fluoridated water, reported a median fluoride intake from beverages of 0.02 mg/kg/day (min - max: 0.005 – 0.043) [46]. While the median dietary fluoride intake reported in our study is lower than in other study populations, there are differences that limit the ability to make direct comparisons between these three studies. First, the study samples were drawn from populations exposed to different vehicles for community fluoridation. In Mexico City, community-wide fluoridation of salt is used, while in the Canadian and Spanish studies, women had access to fluoridated drinking water. Second, the studies from Canada and Spain only report fluoride intake from beverages, while we report fluoride intake from both foods and beverages, including bottled and tap water. Nonetheless all three study populations found dietary fluoride intakes in pregnancy below the USA-IOM's AI recommendation of from all sources.

Our report has several strengths in the area of fluoride exposure assessment, including repeated measures reported over the course of pregnancy, use of a validated instrument of dietary assessment and, a fluoride database specific to the population under study. Limitations of this study include those inherent to FFQ-derived estimates and

secondary data analyses. First, low fluoride water (<0.01 mg/L) and fluoridated salt with standard F levels (250 ppm/kg) [66] were used to prepare foods that are consumed cooked. There may be variation in the natural levels of fluoride in water and even among and within brands of salt [79], with the potential to add uncertainty to the estimates. And second, we lack quantitative estimates of table salt added after cooking. We believe, however, that as fluoride levels in Mexico City are low (<0.7 ppm) [79] and levels of fluoride in salt are regulated by the government [66,80], the lack of a quantitative estimate of fluoride in salt after cooking is the main source of uncertainty in the present study, which most likely underestimates the true dietary fluoride intake. To control for potential differences that could be explained by the habit of table salt use, we used a dichotomous question on the practice of adding table salt to meals after the cooking process (yes/no) that was available in the FFQ. This variable allowed us to make a rough calculation of what total fluoride intake would look like in women who add salt to their meals. Using a quantitative report of added table salt by Mexican non-pregnant women aged 23-50 years in the State of Mexico (5.4 g of salt/day) [91] and assuming all salt gets ingested at a concentration of 250 ppm fluoride, women in our study who reported adding table salt to their meals (median dietary fluoride intake of 0.67 mg F/day) would be ingesting about 1.31 mg F/day (0.02 mg F/kg/day) −approximately double the amount of those who reported not adding extra salt. This approximation, however, should be interpreted with caution, as quantitative estimations of salt intake after cooking were not made in the sample under study. We recommend the inclusion of quantitative measures of table salt intake for future studies in populations of pregnant women exposed to fluoridated salt. Furthermore, the median dietary intake found for this population (0.69

mg F/day) constitutes the contribution of only intrinsic fluoride in foods and fluoride from salt added to foods during the cooking process. To provide a broader perspective of fluoride exposure, future research should also consider the contribution of other sources of fluoride, such as occupational exposures and the unintentional intake of fluoridated oral hygiene products.

Dietary fluoride intake in this sample of pregnant women increased with the progression of pregnancy, suggesting that dietary fluoride intake does change during gestation − as would be expected given increased food consumption by pregnant women to meet the nutrient demands of the growing fetus. We were interested in testing whether the increase observed in fluoride intake throughout pregnancy remained after controlling for covariates that explain dietary fluoride intake such us total energy intake, the practice of addition of table salt after cooking, and compliance with dietary recommendations. We chose these variables because most nutrients, including fluoride, have a positive linear relationship with total energy intake [92]; pregnant women increase their total energy intake towards the end of gestation responding to an increased resting metabolic rate [93]; and there is tendency to change dietary behaviors with the progression of pregnancy [94]. In fact, controlling for total energy intake, women who attended the 2nd and 3rd pregnancy visits ingested more fluoride compared to those attending the 1st, 0.14 mg F/day (~0.2 mg/day total) if they were compliant with pregnancy dietary recommendations. The biological significance of an increase of ~0.2 mg F/day during the third trimester of pregnancy (~7% of the current recommendation) is, however, still unknown and should be considered for future research. Studies on the association between maternal dietary fluoride intake and health outcomes in the offspring are needed

to investigate the biological significance of the current levels of intake. Only one study has reported that a 1-mg increase in maternal fluoride intake from beverages was associated with a 3.7 decrease in intelligence scores among boys and girls [47]; which would be of public health significance for about 10% of the women in the present study (90^{th} percentile, **Table 1**). Further research on the effect of lower prenatal fluoride exposure levels on neurodevelopment −such as the median dietary intake found in this study (0.69 mg/day) [90], is needed to inform future dietary fluoride intake recommendations for pregnant women.

We were interested in understanding whether Mexican women, attempting to meet dietary recommendations, increase their dietary intake of fluoride. We found an association between women who were compliant with recommendations for both calcium and iron and increased fluoride intake levels (**Table 5**). This observation could be explained by the frequent consumption of calcium- and iron-rich foods with low-to-moderate amounts of fluoride, which can lead to an overall increase in fluoride intake. In Mexico, foods rich in both calcium and iron with a moderate content of fluoride include milk, corn-based products, and legumes [77]. We also found that women who reported to consume dietary supplements had lower dietary fluoride intakes. A plausible explanation for this negative association is that individuals who proactively take supplements tend to eat lower amounts of nutrient-dense foods [95]. Therefore, it is possible that pregnant women in this sample who reported supplement intake relied on supplementation to meet their dietary goals instead of choosing more nutrient-dense foods. Sociodemographic factors have also been reported to influence dietary fluoride intake [96]; however, although we found no association, we cannot entirely tease out their effect on dietary

intakes of this study's population given that it included a relatively homogeneous group of women attending the clinics of the Social Security System in Mexico (IMSS) that serves a low-to-middle income population.

Conclusion

Within the limitations of this study, we conclude that the levels of dietary fluoride intake in the present study population were below the current AI, were greater towards the end of gestation and in women who were moderately and highly compliant with Mexican dietary recommendations [90]. Given the mounting evidence of potential adverse effects [47,71,72] and multiple sources of exposure to fluoride, additional assessment and monitoring of dietary intakes and exposures from other community sources, especially in vulnerable populations such as pregnant women and children, should be considered in future dietary recommendations for fluoride intake.

Table 1. Characteristics of women with complete data for all covariates of interest that were included in the analytical sample and women who were excluded because they had incomplete data [90].

	All*	Included			Excluded			*p-value*
		n	Median	IQR	n	Median	IQR	
Age at study recruitment (years)	670	568	26.4	8.0	102	27.1	8.1	*0.840*
Gestational age (weeks)								
Visit 1	635	568	13.6	2.3	67	13.4	2.0	*0.775*
Visit 2	557	554	25.4	2.3	3	24.7	2.3	*0.503*
Visit 3	528	527	34.3	2.5	1	33.7	0.0	*0.726*
Weight (kg)								
Visit 1	668	568	60.5	13.5	100	61.75	15.5	*0.798*
Visit 2	555	554	65.5	13.1	1	77	0.0	*0.462*
Visit 3	528	527	69.4	13.5	1	58.2	0.0	*0.163*
	All*	Included			Excluded			*p-value*
		n	Freq	%	n	Freq	%	
Pre-pregnancy BMI								
Underweight	670	568	35	6.2	102	15	14.7	*0.082*
Normal			328	57.7		50	49.0	
Overweight			102	18.0		20	19.7	
Obese			103	18.1		17	16.6	
Educational Attainment								
None	670	568	1	0.2	102	0	0.0	*0.707*
Elementary			129	22.7		21	20.6	
Middle School			385	67.8		68	66.7	
Highschool			53	9.3		13	12.7	
Marital Status								
Married	670	568	387	68.1	102	73	71.6	*0.451*
Common-law marriage			111	19.5		22	21.6	
Single			69	12.2		7	6.8	
Divorced			1	0.2		0	0.0	
First pregnancy, yes	670	568	203	35.7	102	34	32.1	*0.640*
Assigned to Ca supl., yes†	670	568	289	50.8	102	45	44.1	*0.208*

Note: comparisons between included and excluded women were performed with Mann-Whitney tests for continuous variables and Chi squared tests for categorical variables. *Total number of women with data available for each variable. †Number of women assigned to the calcium supplementation group in the Randomized Clinical Trial

Table 2. Dietary fluoride intake (mg F/day) by pregnancy visit. Median gestational age for each visit is specified in parenthesis. The "All visits" category represents data for all observations available during pregnancy [90].

Pregnancy visit	N	Mean	SD	Min	10%	25%	50%	75%	90%	Max
Visit 1 (13.6)	568	0.72	0.35	0.11	0.35	0.49	0.64	0.87	1.18	2.05
Visit 2 (25.4)	554	0.76	0.36	0.14	0.38	0.52	0.70	0.94	1.20	3.73
Visit 3 (34.3)	527	0.79	0.39	0.11	0.40	0.52	0.72	0.96	1.24	3.08
All visits	1649	0.76	0.37	0.11	0.38	0.51	0.69	0.93	1.21	3.73

Table 3. Bivariate statistics for key dietary variables by pregnancy visits [90].

	Pregnancy Visit						
	Visit 1 (n=568)		Visit 2 (n=554)		Visit 3 (n=527)		
	Median	IQR	Median	IQR	Median	IQR	*p-value**
Fluoride intake (unadjusted, mg/day)	0.64	0.38	0.70	0.42	0.72	0.44	*<0.001*
Total Energy Intake (Kcal)	1811.5	802.5	1785.7	828.2	1802.2	706.0	*<0.001*
	%		%		%		*p-value*†
Compliance with individual nutrients (% above AI or EAR)							
Calcium (AI: 1000 mg/day)	51.2		58.6		62.0		*<0.001*
Iron (EAR: 22 mg/day)	6.0		7.1		5.5		*0.571*
Folate (EAR: 520 µg/day)	5.8		6.3		3.8		*0.151*
Protein (EAR: 0.88 g/kg/day)	68.7		62.6		56.8		*<0.001*
Overall compliance							
None	25.4		27.1		30.4		
Moderate	66.9		64.7		62.7		*0.412*
High	7.8		8.1		6.8		
Adds table salt after cooking, yes	30.3		28.6		22.6		*0.012*
Use of other supplements, yes	23.4		32.0		38.0		*<0.001*

IQR: Interquartile range
* Friedman test (n=511)
† Chi-square test
‡ *None:* noncompliant with all of the nutrients (0/4); *moderate:* compliant with one or two nutrients (1/4 or 2/4); and *high:* compliant with three or all the four nutrients (3/4 or 4/4).

Table 4. Associations between daily fluoride intake (mg/day), pregnancy visit and compliance with dietary recommendations [90].

n=568	β*	SE	95% CI		*p-value*
Pregnancy Visit					
Visit 1	Ref	Ref	Ref	Ref	Ref
Visit 2	0.04	0.01	0.01	0.06	*<0.001*
Visit 3	0.08	0.01	0.06	0.10	*<0.001*
Compliance †					
None	Ref	Ref	Ref	Ref	Ref
Moderate	0.04	0.02	0.01	0.08	*0.004*
High	0.14	0.03	0.08	0.20	*<0.001*
Intake of other supplements‡					
Yes	-0.03	0.01	-0.05	0.00	*0.027*
Use of table salt after cooking					
Yes	-0.01	0.01	-0.04	0.01	*0.289*

Note: Estimates from a one-way random effects GLS regression model adjusted for total energy intake, allocation group in the calcium supplementation RCT, intake of other supplements and pre-pregnancy BMI.

† *None:* noncompliant with all of the nutrients (0/4); *moderate:* compliant with one or two nutrients (1/4 or 2/4); and *high:* compliant with three or all the four nutrients (3/4 or 4/4).

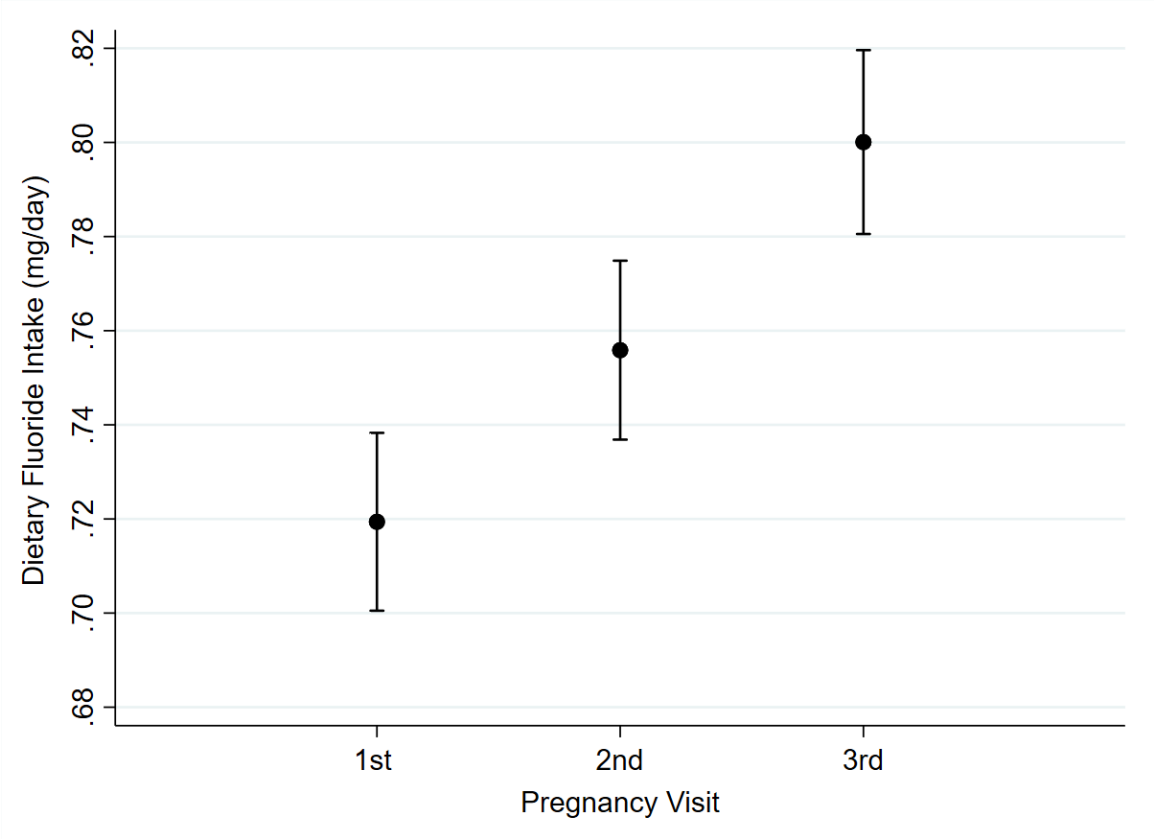
‡ Supplements other than the one provided for the calcium supplementation RCT.

Table 5. Associations between daily fluoride intake and compliance with individual key nutrients [90].

n=568	β*	SE	95% CI		*p-value*
Calcium					
Non compliant	Ref	Ref	Ref	Ref	Ref
Compliant	0.12	0.01	0.09	0.14	*<0.001*
Iron					
Non compliant	Ref	Ref	Ref	Ref	Ref
Compliant	0.18	0.27	0.13	0.24	*<0.001*
Folate					
Non compliant	Ref	Ref	Ref	Ref	Ref
Compliant	-0.12	0.03	-0.18	-0.064	*<0.001*
Protein					
Non compliant	Ref	Ref	Ref	Ref	Ref
Compliant	-0.02	0.02	-0.05	0.01	*0.247*

Note: Estimates from a one-way random effects GLS regression model adjusted for pregnancy stage, allocation group in the calcium supplementation RCT, intake of other supplements, total energy intake, addition of table salt after cooking (yes/no), pre-pregnancy BMI and educational attainment.

Figure 1. Estimated fluoride intake (95% CI) by pregnancy visit [90].



Note: Estimates from a one-way random effects GLS linear regression model adjusted for total energy intake, allocation group in the calcium supplementation RCT, intake of other supplements and pre-pregnancy BMI.

STUDY 2:

ASSOCIATIONS BETWEEN URINARY FLUORIDE AND DIETARY FACTORS DURING PREGNANCY AND ONE-YEAR POSTPARTUM IN MEXICAN WOMEN

Summary

For this study, it was hypothesized that pregnancy and its associated dietary changes affect the concentration of fluoride in urine. During pregnancy and one-year postpartum, this study: 1) compared urinary fluoride levels between each state; and 2) assessed the association between urinary fluoride and dietary factors that are known to influence the renal handling of fluoride, namely, dietary fluoride intake (F), calcium (Ca) intake from both diet and supplements, dietary acid load (AL), and use of table salt (TS) after cooking (yes/no).

A total of 421 women were included in the study sample, of whom 167 attended at least one visit during pregnancy. Dietary factors were estimated from validated FFQs. Urinary fluoride (UF, mg/L) was determined by microdiffusion/fluoride-specific electrode and dilution-corrected with specific gravity (SG). Data were summarized with descriptive statistics. A longitudinal random effects models for pregnancy and a cross-sectional linear regression model for one-year postpartum were generated.

SG-corrected UF levels (median, range) during pregnancy (0.77, 0.01 – 4.73 mg/L) did not significantly differ from 1-yr postpartum levels (0.75, 0.15 – 2.62 mg/L); but did increase every 10 gestational weeks (β= 0.05 [CI: 0.00 – 0.10]). Dietary F and Ca intake, and AL were not associated with UF in either state. Ca supplementation decreased UF only during pregnancy (β= -0.012 mg/L [CI: -0.023 – 0.00]). Reporting the addition

of TS to meals was associated with a 12% increase in UF only at 1-yr postpartum (p=0.026). This research suggests that pregnancy and its associated dietary factors affect urinary fluoride levels in spot urine samples.

Introduction

The urinary excretion of fluoride is a contemporary biomarker of fluoride exposure validated for children and nonpregnant adults [30,97] and is traditionally used for the monitoring of water or salt fluoridation programs for the prevention of dental caries [98]. More recently, this biomarker has been used as a proxy for prenatal fluoride exposure in epidemiological studies in women and children, which have found associations between maternal spot urinary fluoride and neurodevelopmental and cognitive outcomes in children living in communities with implemented water or salt fluoridations programs [47,71,72]. However, our current knowledge of urinary fluoride as a biomarker of prenatal fluoride exposure is based on research mostly conducted in children and nonpregnant adults [30,97].

In healthy non-pregnant adults, around 60% of a given amount of absorbed fluoride is excreted through the urinary system [21]. The renal handling of fluoride involves tubular reabsorption mediated by a pH-dependent process: any acute or chronic condition affecting urinary pH increases fluoride's chance to remain circulating in the body or accelerates its rate of elimination [35]. Urinary fluoride increases proportionally to its dietary intake [30], but there are other dietary factors known to influence the concentrations of fluoride in urine and/or the urinary pH, including calcium intake [99] and the acidity of the dietary pattern [35]. For instance, experimental studies in volunteers consuming diets associated with an acidic urinary pH (high in meat and

animal products), reported lower urinary excretion of fluoride compared to those consuming diets associated with alkaline urinary pH (high in fruits and vegetables) [35,36]. On the other hand, physiological factors that influence the concentration of urinary fluoride of healthy nonpregnant adults include bone metabolism [18] and disturbances of the acid-base balance [100].

In the pregnant state, most of the factors that influence the absorption, distribution, and renal excretion of fluoride converge: dietary changes [55], adjustments in the renal system [48], increased bone turnover [49], and chronic respiratory alkalosis [51]. All of these factors return to non-pregnant levels by about three months postpartum [48]. Available studies on urinary fluoride during pregnancy have compared levels in spot samples between pregnant and nonpregnant (or postpartum) women [36,59–61], with contradictory results and discussions centred in fetal fluoride uptake [59,61]. It is therefore still unknown whether pregnancy and its associated dietary factors affect the concentration of fluoride in spot urine samples. This information is necessary to advance our knowledge on fluoride biomarkers, which are important in assessing prenatal fluoride exposure in human populations.

We hypothesized that pregnancy and its associated dietary changes affect the concentration of fluoride in urine. The aims of this study were: 1) to compare urinary fluoride during pregnancy vs. one-year postpartum, and 2) to assess the association between urinary fluoride and dietary factors that are known to influence the renal excretion of fluoride, over the course of pregnancy and one-year postpartum.

Methods

Study sample

The source population were women from the Early Life Exposures in Mexico to ENvironmental Toxicants (ELEMENT) project. ELEMENT comprises three mother-child pregnancy and birth cohorts, initiated in the 1990s to study early life exposures and health outcomes in Mexico City. Mexico has a salt fluoridation program implemented since 1981 [66]; therefore these women included fluoridated salt in their diets. Details and demographic characteristics of the entire project are published elsewhere [65]. Briefly, pregnant women attending three clinics of the Mexican Institute of Social Security (IMSS), living in Mexico City, and who had the intention to stay during the next 5 years were invited to participate. Included participants in this study were women who were in their first trimester (<14 weeks), had a healthy singleton pregnancy, no history of hypertension or diabetes and agreed to participate through written informed consent. Participating women were aware that their data were fully anonymized and were invited to attend the research facility at different time-points during pregnancy and up to one-year postpartum. During each study visit, a trained social worker applied a general demographic questionnaire and a Food Frequency Questionnaire (FFQ) to each participant, and measured weight and height using calibrated instruments. Participants also provided early morning second-void spot urine samples, which were archived in the project's biorepository at -70°C until analysis. The Institutional Review Boards of the National Institute of Public Health of Mexico, the University of Michigan and Indiana University approved all study procedures.

The source population and inclusion criteria for the study sample are summarized in **Figure 2**. The source population consisted of participants who were recruited between 2001 to 2003 (ELEMENT Cohort 3) and were followed during pregnancy *or* one-year postpartum (n=443). From the source population, only participants who had available the following criteria were included in the study sample: 1) at least one archived urine sample with adequate volume for fluoride and specific gravity analyses (SG), collected at any pregnancy visit *or* one-year postpartum; and 2) complete demographic, anthropometric and dietary (FFQ) data. A total of 421 women were included in the study sample. From these 421 women, 167 and 421 attended visits during pregnancy and one-year postpartum, respectively. From the 167 women with available data at any pregnancy visit, 135 attended the study's 1st pregnancy visit; 101 attended the 2nd and 71 attended the 3rd. The data available one-year postpartum is cross-sectional and corresponds to one study visit.

Dietary variables

Dietary variables were calculated using data from a semiquantitative Food Frequency Questionnaire (FFQ) that was applied during each study visit, adapted from the Willett semi-quantitative FFQ [83]. The questionnaire was validated to estimate dietary intake over the previous month in Mexican women of child-bearing age [85]. It consisted of 104 items, and included foods commonly consumed in the 1983 Dietary Survey of the Mexican National Institute of Nutrition [84] and a questionnaire on intake of dietary supplements and multivitamins. Using the reported frequencies and portion sizes from the FFQ, estimates of dietary intake of macro- and micronutrients, minerals and trace elements were calculated through a software developed at the National Institute

of Public Health (INSP). The software utilized the INSP-compiled nutrient composition database [87] and a fluoride database specifically developed for the ELEMENT project [77].

Dietary fluoride intake: table salt is the major source of dietary fluoride for those living in Mexico City, due to the salt fluoridation program [66]. The estimates derived from the FFQ included fluoride from beverages, tap and bottled water, and fluoride from salt added to foods during the cooking process [77]. Popular dietary supplements distributed in Mexico do not contain fluoride and, therefore, the estimates are only from the diet and do not include supplement use.

Addition of table salt to meals: although the amount of salt that was added to food by participants after cooking is unknown, the questionnaire did include a dichotomous question on whether salt was added to meals at the table, which we used as a proxy for added salt after cooking.

Calcium from RCT: an additional source of calcium for about half of the study participants was a calcium supplementation regimen. ELEMENT's Cohort 3 (the source population) was originally designed as a double-blind Randomized Clinical Trial (RCT) to examine the effects of calcium supplementation on blood lead levels during pregnancy and up to one-year postpartum [101]. For this study, the original RCT design of the cohort was leveraged to assess the association between urinary fluoride and calcium supplementation. Women were randomized to receive either the calcium supplement or placebo and were provided with a bottle containing pills with the assigned treatment at each study visit. Participants were instructed to consume two pills containing 600 mg of calcium as calcium carbonate at bedtime (1200 mg calcium/day total). A dichotomous

variable on RCT group allocation (supplement/placebo) was used to assess the association between urinary fluoride and RCT group allocation. During each visit, participants returned the treatment bottle to a research assistant, who performed pill count since the last visit to register adherence to the treatment. This adherence registry was used to estimate a continuous variable of calcium intake (mg/day) from the RCT (% of the required dose ingested per day*1200 mg) [67] and used to assess the association between urinary fluoride and the actual dose of calcium supplement intake in a sensitivity analysis.

Dietary calcium intake: estimates of dietary calcium intake included both calcium from foods and beverages, and from other dietary supplements consumed by study participants, reported in the FFQ.

Dietary acid load: the excretion of fluoride is modified by the acidity of the diet, which modifies the body's acid-base balance. For this study, we estimated the Net Endogenous Acid Production (NEAP) − the ratio of the estimated dietary intake of protein (acid precursor) and potassium content (base precursor) as a proxy of the acid balance in healthy individuals. The dietary acid load was estimated using the equation determined by Frassetto et al. [102]:

$$NEAP = -10.2 + 54.5 \times (Pro/K) \quad (1)$$

Where NEAP is the net endogenous acid production (mEq/day), Pro is the daily dietary intake of protein (g/day), and K is the daily intake of potassium (mEq/day) from both diet and supplements.

Urinary fluoride and specific gravity (SG)

Urinary fluoride was determined with the hexamethyldisiloxane (HMDS) microdiffusion and fluoride-specific electrode method [81], as modified by Martinez-Mier [82]. Each urine sample was thawed and vortexed at room temperature. A 1.0 mL aliquot was reserved for fluoride analyses and a second aliquot (~0.8 mL) was used to measure urinary SG. The 1.0 mL urine aliquot was pipetted into a disposable petri dish. A trap of 0.05N sodium hydroxide solution was placed in the form of five equal drops on the inside of the lid. After a tight sealing with petroleum jelly and burning a small hole into each lid, HMDS-saturated 3N sulfuric acid was pipetted and the fluoride was allowed to diffuse overnight at room temperature. The following day, the petri dish was opened, and the solution of diffused fluoride contained in the lid was recovered, buffered to pH 5.2 with 0.1 M acetic acid and then diluted in deionized water. Most analyses were performed in duplicate (providing the availability of a sufficient volume of urine). The concentration of fluoride was then measured using a fluoride ion selective electrode coupled to a pH/ISE meter (Orion™ Fluoride Electrode and Dual Star™ pH-meter, Thermo Scientific, Waltham, MA). A calibration curve using fluoride standards (Orion™ ISE calibration standards) was constructed following the same procedure. Millivolt readings from the samples were recorded and the unknown concentrations of fluoride from urine samples were determined using the equation that explained the relationship between the log of the fluoride concentration of the standards, and their corresponding millivolt readings ($R^2 > 0.9$). Testing included a daily standard check using a fluoride standard traceable to NIST (National Institute of Standards and Technology, U.S Department of Commerce) after sample analysis.

The measurement of urinary SG took place in a dark room using a pen refractometer (ATAGO®). The prism head of the refractometer was immersed into the 0.8 mL urine aliquot, and the sample's reading was recorded. SG-corrected fluoride in urine was calculated using a standard equation [103] as modified by Till et al. [63]:

$$UF_{SG} = UFi \times (SG_M-1)/(SG_i-1) \quad (2)$$

Where UF_{SG} is the SG-corrected fluoride concentration (mg/L), UF_i is the observed fluoride concentration, SG_i is the SG of the individual urine sample, and SG_M is the median SG for all available urine samples for either pregnancy or one-year postpartum, depending on the case.

Statistical analyses

Exploratory data analyses were conducted using univariate and bivariate graphical summaries. Summary statistics used for continuous variables included means, medians, range, standard deviation (SD) and interquartile range (IQR); whereas categorical variables were summarized with frequencies and percentages. Comparisons were performed using Mann-Whitney and Chi-squared tests. Given that each physiological state is associated with unique variables (e.g gestational age during pregnancy and breastfeeding in the postpartum); two different models to assess the relationship between urinary fluoride and dietary factors were generated: a longitudinal model for pregnancy (with repeated measures at different gestational ages) and a cross-sectional linear regression model for the one-year postpartum data.

Pregnancy model (longitudinal): to account for time and individual-specific effects during pregnancy, regression models for panel data were generated. Given that individual effects were uncorrelated with the regressors (Hausman test) and the presence of

groupwise heteroscedasticity (Modified Wald test), pregnancy data were fit to a random effects Generalized Least Squares (GLS) regression model with heteroscedasticity-robust standard errors. The outcome variable was the uncorrected concentration of fluoride in urine (mg/L) and the explanatory variables of interest were dietary fluoride intake (mg/day), adding table salt after cooking (yes/no), dietary calcium intake, calcium from RCT (supplement/placebo), dietary acid load and gestational age. Based on our review of the literature, covariates that were included as potential confounders were total energy intake and pre-pregnancy BMI. SG was included as a covariate in the model, so the significance of other variables could be independent of its effects [104].

One-year postpartum model (cross-sectional): linear regression models for one-year postpartum were generated. The outcome variable was the natural log-transformed uncorrected urine fluoride concentrations in urine (mg/L) and the explanatory variables of interest were dietary fluoride intake, reporting adding salt to meals, dietary calcium intake, calcium from RCT (supplement/placebo) and dietary acid load. Covariates included: urinary SG, total energy intake, age, months since delivery, breastfeeding (yes/no) and BMI at the "one-year postpartum" visit. Studentized residuals displayed a normal distribution and were homoscedastic.

For both models (pregnancy/one-year postpartum) sensitivity analyses were performed using the continuous variable of calcium intake (mg/day) from the RCT that was estimated from the treatment's adherence registry, instead of the dichotomous variable (supplement/placebo). All analyses were conducted with STATA v16.0 (StataCorp LP, College Station, TX, USA).

Results

Characteristics of the study sample

The characteristics of the study sample are summarized in **Table 6**. Women with available data during pregnancy (n=167) had a median age (IQR) of 26.7 (7.5). Included women completed 11 to 12 years of education − equivalent to high school diploma, and most had low or very low socioeconomic status (~57%). Most women were married or cohabitating with a partner, and most (~65%) had been pregnant at least once before participating in the ELEMENT project. Although most women with data available during pregnancy were classified with a pre-pregnancy BMI in the range of normal, the BMI of women with data available one-year postpartum tended to shift towards the overweight and obese categories, and the difference was statistically significant (p=0.002). Around half of the included women were allocated to the calcium supplementation group, and the other half were allocated to the placebo group of the RCT. The characteristics of the women included vs. excluded during pregnancy and postpartum were similar, although women excluded from the pregnancy analyses for having incomplete data had a higher education level (p=0.011, **Table 7**).

Descriptive statistics for key time-varying variables

The measurement of key dietary variables took place one-to-three times during pregnancy, and once for the one-year postpartum visit and are summarized in **Table 8**. Median gestational age for the 1st, 2nd and 3rd pregnancy visits was 13.1, 25.3 and 33.9 weeks, respectively. On the other hand, the “one-year postpartum” visit occurred at a median time of 12.2 months. As expected, total energy intake tended to increase with the progression of gestation and was higher compared to one-year postpartum (p=<0.001).

The dietary intake of fluoride and calcium followed a similar increasing tendency over the course of gestation and were statistically significantly higher compared to one-year postpartum. In contrast, the acid load of the diet was lower during pregnancy compared to one-year postpartum and the difference was statistically significant. In general, most women reported not adding table salt after cooking, and the voluntary use of dietary supplements (not the RCT supplement) was higher at one-year postpartum compared to pregnancy. Around 30% of women reported to be breastfeeding their children by the time of the one-year postpartum visit.

Urinary fluoride during pregnancy and one-year postpartum

Detailed descriptive statistics of the concentration of fluoride from second-void morning spot urine samples at each pregnancy visit and at one-year postpartum (corrected and uncorrected for dilution effects) are presented in **Table 9**. The distributions of fluoride concentrations corrected and uncorrected for the effects of dilution during pregnancy and one-year postpartum were not significantly different. Urinary SG was significantly higher (urine was more concentrated) at one-year postpartum (p=0.002).

Association between urinary fluoride and dietary factors

The results of a longitudinal, random effects model for pregnancy are summarized in **Table 10**. The association between urinary fluoride and addition of table salt after coking was not significant. Women who were assigned to a calcium supplementation regimen (1200 mg calcium/day) excreted on average 0.12 mg/L less fluoride [95% CI:-0.023, 0.00] compared to women who did not take calcium supplements (**Table 10**). This effect was observed exclusively for calcium from supplements and not for dietary calcium. In sensitivity analyses, when calcium supplementation was included as a

continuous variable (calculated by means of pill-count at each visit as described in the methods section), for each 100 mg of calcium supplement intake, supplemented women excreted 0.02 mg/L less fluoride [95% CI: -0.03, 0.00] compared to non-supplemented women (p=0.014). An association between urinary fluoride and dietary acid load was not found. On the other hand, urinary fluoride slightly increased with the progression of pregnancy: for every 10 weeks of gestation, the concentration of fluoride in urine increased 0.05 mg/L [95% CI:0.00, 0.10]. **Figure 3** displays the relationship between gestational age and urinary fluoride as predicted by the multivariate longitudinal model, according to calcium supplementation groups.

Table 11 summarizes the results for the one-year postpartum model. In contrast to what was observed during pregnancy, urinary fluoride at one-year postpartum was associated only with the addition of table salt after cooking. On average, women who reported adding table salt excreted 12% more fluoride [95% CI: 1.40, 23.87] compared to those who did not, controlling for breastfeeding practices, urinary SG, time since delivery, total energy intake, age, and BMI.

Discussion

The concentration of urinary fluoride in SG-corrected and uncorrected spot urine was not significantly different in pregnancy vs. one-year postpartum (**Table 9**). Two reports, comparing spot urine fluoride between samples of pregnant and nonpregnant women, concluded that lower concentrations of urinary fluoride during pregnancy vs. nonpregnant controls, discussing findings as potential evidence of fetal uptake of fluoride [59,61]. However, those investigations date back several decades and urinary fluoride was not corrected for dilution effects − which are critical in considering the use of spot

urine samples and the challenges that pregnancy presents for water homeostasis and hydration [105]. In fact, in this study we found that urinary SG was significantly lower during pregnancy (urine was more diluted, **Table 9**), which artificially lowers the levels of any analyte in the sample and highlights the importance of performing comparisons considering the effects of urine dilution. The presently observed lack of significant differences in SG-corrected spot urine fluoride levels between pregnancy and one-year postpartum agree with two experimental studies that utilized 24 h urine collections [36,60] (which do not require correction for dilution). There are, however, changes in urinary fluoride levels during the gestation period. When gestational age was included as an independent variable in a model to explain urinary fluoride during pregnancy, we observed a 0.05 mg/L increase in urinary fluoride for every 10 weeks of gestation (**Table 10**). This result is consistent with a recent study conducted in a large sample of pregnant women (n=1566), where maternal urinary fluoride increased from the first to the third trimester [63]. An increase in urinary fluoride over the course of gestation can be explained by the renal-compensated, chronic, mild respiratory alkalosis experienced during pregnancy, which alkalinizes the urinary pH [51,52] with a consequent increase in fluoride's excretion rate. Our results differ, however, from a previous study performed in all cohorts from the ELEMENT project, which found a tendency for fluoride in urine to decrease over the course of gestation [62]. Methodological differences may explain this discrepancy: in the previous ELEMENT study [62], the method for the correction of urinary dilution was creatinine, which can be affected by BMI [105,106], maternal age [107] and gestational age [105]. In the current study, we used urinary SG, which has been reported to be a less variable indicator of hydration status during pregnancy [105].

Collectively, these differences among studies highlight the importance and influence of dilution-correction methods in the assessment of urinary fluoride in spot urine samples. Changes in urinary fluoride levels have been noted in all studies over the course of gestation, highlighting the importance of including gestational age as a covariate in the analysis of urinary fluoride in pregnant women.

Associations between urinary fluoride in second-void spot urine samples and fluoride intake assessed with a FFQ were not found, neither during pregnancy nor one-year postpartum. There is evidence of a positive linear relationship between dietary fluoride intake measured with duplicate-diet approaches and urinary fluoride collected over 24 h in children and nonpregnant adults [108]. However, given that after ingestion fluoride is rapidly eliminated from the body [18], studies using other dietary assessment methods, such as questionnaires and recalls over longer periods of time, paired with urine collected over short periods of time (such as the ones that were available for this study), have failed to find significant associations between the dietary intake of fluoride and its urinary excretion [109,110]. Therefore, the question of whether there is an association between dietary fluoride intake and urinary fluoride excretion in pregnant women remains unanswered and future studies assessing diet over shorter periods of time paired with 24 h urine samples are encouraged.

As expected for a population exposed to fluoridated salt [66], women who reported adding table salt after cooking had 12% higher concentration of fluoride in urine compared to those who did not (p=0.026), and this association was observed only at one-year postpartum. The lack of association between reporting addition of table salt urinary fluoride during pregnancy may be explained by changes in the absorption of fluoride

during the pregnant state. It could be hypothesized that the vitamin D-mediated enhancement of intestinal absorption of calcium during pregnancy − which no longer occurs in nonpregnant women −[111] upregulates the mechanisms favoring the absorption of calcium, in detriment to the intestinal absorption of fluoride [24,112]. We acknowledge that one of the limitations of this study is the lack of quantitative estimates of table salt intake after the cooking process, and its inclusion would be recommended for future studies in populations exposed to fluoridated salt.

This secondary data analysis was conducted in a population of women who participated in a calcium-supplementation RCT. The WHO recommends calcium supplementation during pregnancy (1200-1500 mg/day) for the prevention of pre-eclampsia and its complications [113], and its use for the prevention of fetal lead exposure has also been investigated [114]. In this study, we found that women supplemented with calcium (1200 mg/day) had lower urinary fluoride, compared to women assigned to the placebo group. This effect was observed only during pregnancy (not at one-year postpartum) and only with calcium from supplements. A biologically plausible explanation for this finding is the increased bone turnover that occurs towards the end of gestation, which mobilizes maternal stores of calcium for fetal transfer [49]. In the cohort of women from which this study's sample was drawn, it was previously demonstrated that calcium administered during pregnancy was associated with reduced bone resorption [67]. Since fluoride is stored in bone, it could be hypothesized that the inhibition of bone resorption associated with calcium supplementation during pregnancy [67] could also inhibit the release of fluoride from bones to plasma. In contrast to what is observed during pregnancy, markers of bone turnover decrease in women who continue

lactation one-year postpartum and are not affected by calcium supplementation [58], which would explain why this finding was exclusive to calcium supplementation during pregnancy.

We also assessed the association between urinary fluoride and the dietary acid load, measured with NEAP estimated from the FFQs [102]. Although acidic diets have shown associations with lower urinary fluoride excretion [35], and in this study the acid load of the diet had a negative regression coefficient, the association was not significant in either state. The lack of association may be attributed to the use of spot urine samples which usually have weaker correlations with dietary acid load compared to 24 h samples, as previously discussed by Welch et al. [115].

This study had several limitations, such as the use of spot urine samples instead of 24 h samples and the lack of quantitative estimates of table salt use in a population exposed to fluoridated salt. However, the study also has several strengths compared to currently available research on this topic, including larger sample sizes, correction for the effects of urinary dilution, repeated measures of urinary fluoride during pregnancy, and the incorporation of regression models controlling for important covariates in each state. Furthermore, we had the ability to test calcium supplementation in a population who participated in a calcium supplementation RCT. Given the increasing number of studies using maternal spot urinary fluoride as a biomarker of prenatal fluoride exposure and the limited available knowledge on factors affecting urinary fluoride during pregnancy, the conduct of this analysis leveraging already existing data is well-justified.

Conclusion

In summary, we found that while urinary fluoride during pregnancy vs. one-year postpartum did not significantly differ, levels did increase as pregnancy progressed. Dietary fluoride intake and dietary acid load measured with one-month recalls were not associated with urinary fluoride either during pregnancy or at one-year postpartum. The addition of table salt after cooking was not associated with higher urinary fluoride during pregnancy, but only one-year postpartum. Calcium supplementation during the second and third trimesters of pregnancy affected urinary fluoride concentrations. Specifically, calcium supplementation of 1200 mg/day, decreased urinary fluoride during pregnancy but not at one-year postpartum.

The consideration of the influence of gestational age and calcium supplementation during pregnancy is warranted when using maternal urinary fluoride as a biomarker of prenatal fluoride exposure.

Figure 2. Flow chart of source population and study sample, drawn from the Early Life Exposures in Mexico to Environmental Toxicants (ELEMENT) project.

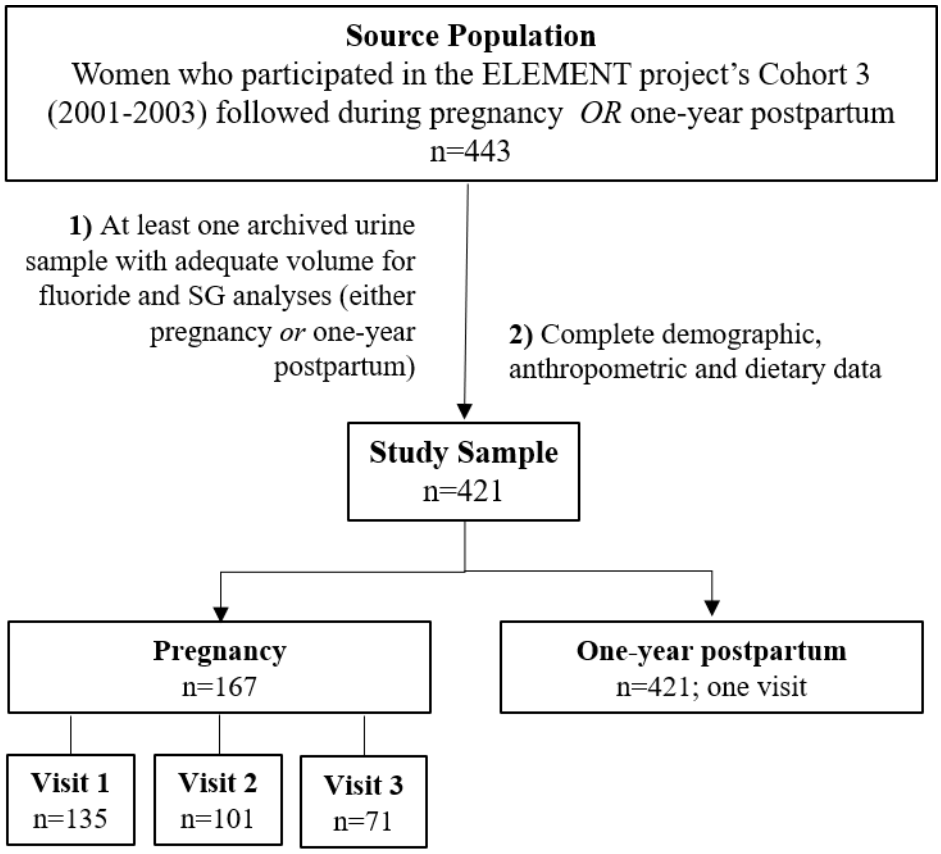


Table 6. Characteristics of the study sample at pregnancy and one-year postpartum (time-invariant).

	Pregnancy n=167		One-year postpartum n=421		*p-value*
	Median	*IQR*	*Median*	*IQR*	
Age* (years)	26.7	7.5	27.8	8.0	–
Completed years of education	12.0	3.0	11.0	3.0	*0.163*
	Freq	*%*	*Freq*	*%*	
Marital Status					
Married/cohabitating	145	87	367	87	*0.422*
Single/divorced	23	13	54	13	
Socioeconomic status					
High	36	23	44	23	
Middle	30	19	38	20	*0.920*
Low	62	39	74	39	
Very low	29	18	33	17	
Primigravida					
Yes	57	34	149	35	*0.953*
No	110	66	272	65	
BMI**					
Underweight	8	5	16	4	*0.002*
Normal	92	55	179	42	
Overweight	32	19	109	26	
Obese	35	21	117	28	
Calcium supplementation					
Placebo	79	47	211	50	*0.611*
Supplemented	88	53	210	50	
Breastfeeding					
Yes	–	–	125	30	–
No	–	–	296	70	

*Age during pregnancy corresponds to the average age at three pregnancy visits.
**Pre-pregnancy BMI for pregnancy and BMI at one-year postpartum

Note*:*Mann-Whitney tests were used for continuous variables and chi-squared tests for categorical variables

Table 7. Characteristics of women with all data of interest (n=167for pregnancy and n=421 one-year postpartum) and women who were excluded due to incomplete data (n=503 for pregnancy and n=22 one-year postpartum).

	Pregnancy					One-year postpartum				
	Included		Excluded		*p-value*	Included		Excluded		*p-value*
	Median	*IQR*	*Median*	*IQR*		*Median*	*IQR*	*Median*	*IQR*	
Age (years)	27.0	7.5	26.9	8.2	0.080	27.8	8	27.7	8.8	*0.653*
Gestational age in weeks	25.1	18.5	24.9	18.4	0.471	–	–	–	–	–
Completed years of education	11.0	3.0	11.0	3.0	0.011	11.0	3	11.0	3	*0.311*
Time since delivery (months)	–	–	–	–	–	12.2	0.8	12.5	1.2	*0.652*
Socioeconomic status	*%*		*%*			*%*		*%*		
High	23		24			23		17		
Middle	19		26		0.359	20		50		*0.125*
Upper-low	39		35			39		–		
Low	18		15			17		33		
Marital Status										
Married or cohabitating	87		89		0.228	87		95		*0.251*
Single or divorced	13		11			13		5		
Primigravida										
Yes	34		36		0.699	35		23		*0.224*
No	66		64			65		77		
Calcium supplementation										
Placebo	47		51		0.238	50		45		*0.670*
Supplemented	53		49			50		55		
BMI										
Underweight	5		7			4		0		
Normal	55		59		0.128	43		58		*0.073*
Overweight	19		17			26		26		
Obese	21		17			26		5		

Table 8. Key variables measured at different time-points during pregnancy and at one-year postpartum (time-varying).

	Pregnancy			One-year postpartum			*p-value**
Gestational Age (weeks)	n	Median	IQR	n	Median	IQR	
Visit 1	135	13.1	2.1	–	–	–	–
Visit 2	101	25.3	2.1	–	–	–	–
Visit 3	71	33.9	2.6	–	–	–	–
All observations	307	23.7	14.3	421	0.63	0.37	*0.008*
Time since delivery (months)	–	–	–	421	12.2	0.8	–
Total Energy Intake (Kcal/day)							
Visit 1	135	1780.2	847.5	–	–	–	–
Visit 2	101	1790.8	903.5	–	–	–	–
Visit 3	71	1814.7	681.2	–	–	–	–
All observations	307	1792.8	803.0	421	1,613.1	678.4	*0.000*
Dietary fluoride intake (mg/day)							
Visit 1	135	0.63	0.39	–	–	–	–
Visit 2	101	0.74	0.49	–	–	–	–
Visit 3	71	0.73	0.42	–	–	–	–
All observations	307	0.70	0.43	421	0.63	0.37	*0.008*
Dietary Calcium Intake (mg/day)							
Visit 1	135	1021.4	695.5	–	–	–	–
Visit 2	101	1183.0	795.1	–	–	–	–
Visit 3	71	1127.1	582.9	–	–	–	–
All observations	307	1102.7	716.8	421	939.5	616.1	*0.000*
Acid Load (NEAP, mEq/day)							
Visit 1	135	33.2	12.6	–	–	–	–
Visit 2	101	33.9	11.9	–	–	–	–
Visit 3	71	34.2	12.7	–	–	–	–
All observations	307	33.7	12.2	421	36.8	10.7	*0.000*
Adds table salt after cooking, yes	n	Freq	%	n	Freq	%	*p-value**
Visit 1	135	35	26	–	–	–	–
Visit 2	101	21	21	–	–	–	–
Visit 3	71	16	23	–	–	–	–
All observations	307	72	23	421	89	21	*0.458*
Use of other supplements, yes							
Visit 1	135	37	27	–	–	–	–
Visit 2	101	33	33	–	–	–	–
Visit 3	71	26	63	–	–	–	–
All observations	307	96	31	421	173	49	*0.007*
Breastfeeding, yes							
All observations	–	–	–	421	125	30	–

*Comparisons among groups using all available observations for each state (Mann-Whitney/chi-squared tests.

Table 9. Urinary fluoride (mg/L) at different visits during pregnancy and at one-year postpartum.

	Pregnancy (n=167)					One-year postpartum (n=421)					*p-value**
	n	Mean	Median	SD	Range	*n*	Mean	Median	SD	Range	
Uncorrected urinary fluoride											
Visit 1	135	0.85	0.76	0.51	0.10 - 2.35	–	–	–	–	–	–
Visit 2	101	0.80	0.65	0.52	0.09 - 2.73	–	–	–	–	–	–
Visit 3	71	0.75	0.64	0.53	0.01 - 2.54	–	–	–	–	–	–
All observations	307	0.81	0.69	0.52	0.01 – 2.73	421	0.77	0.69	0.49	0.07 – 2.98	*0.415*
SG-corrected urinary fluoride											
Visit 1	135	0.82	0.75	0.47	0.08 - 4.73	–	–	–	–	–	–
Visit 2	101	0.83	0.77	0.39	0.28 - 2.12	–	–	–	–	–	–
Visit 3	71	0.86	0.77	0.51	0.01 - 3.02	–	–	–	–	–	–
All observations	307	0.83	0.77	0.46	0.01 - 4.73	421	0.83	0.75	0.37	0.15– 2.62	*0.950*
SG											
Visit 1	135	1.016	1.017	0.007	1.002 - 1.035	–	–	–	–	–	–
Visit 2	101	1.014	1.014	0.007	1.002 - 1.033	–	–	–	–	–	–
Visit 3	71	1.013	1.013	0.005	1.003 - 1.026	–	–	–	–	–	–
All observations	307	1.015	1.015	0.007	1.002 – 1.035	421	1.017	1.017	0.008	1.001– 1.046	*0.002*

Note: SD, Standard Deviation; SG, Specific Gravity
*Mann-Whitney test

Table 10. Associations between dietary factors and urinary fluoride during pregnancy (n=167; 307 observations).

	Urinary Fluoride (mg/L)				
	β	SE	95% CI		*p-value*
Dietary fluoride intake (x100 mg/day)	-0.43	10.85	-21.70	20.84	*0.968*
Adds table salt after cooking					
No	Ref	Ref	Ref	Ref	Ref
Yes	-0.01	0.07	-0.15	0.13	*0.856*
Calcium from RCT					
Placebo	Ref	Ref	Ref	Ref	Ref
Supplement	-0.12	0.05	-0.23	0.00	*0.021*
Dietary calcium intake (x1000 mg/day)	-0.04	0.10	-0.22	0.15	*0.710*
Dietary acid load (x10 mEq/day)	-0.04	0.03	-0.09	0.02	*0.208*
Gestational Age (x10 weeks)	0.05	0.02	0.00	0.10	*0.050*

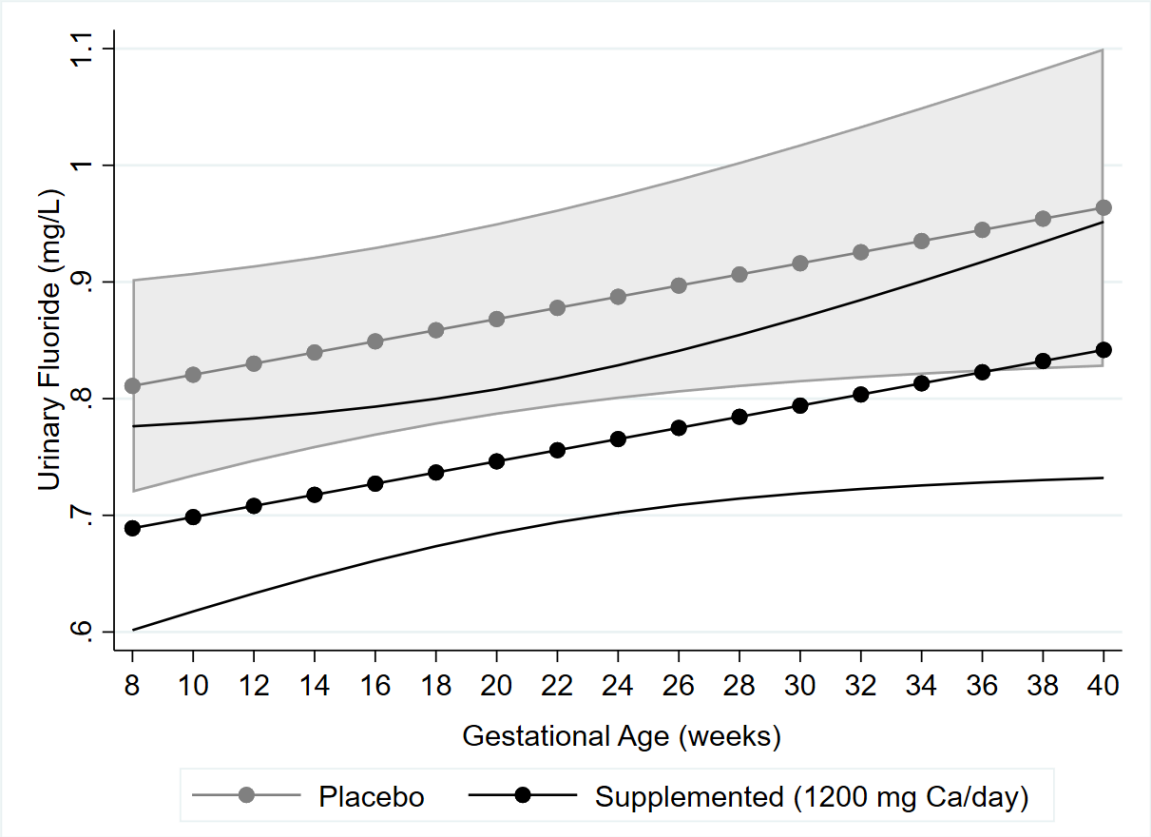
Note: Estimates from a longitudinal random effects GLS regression, adjusted for urinary specific gravity, total energy intake and pre-pregnancy BMI and age at first pregnancy visit

Table 11. Associations between dietary factors and urinary fluoride one-year postpartum (n=421).

	Log_Urinary Fluoride (mg/L)	
	Percent change	*p-value*
Fluoride intake (mg/day)	-8.04	0.475
Adds table salt after cooking		
No	Ref	Ref
Yes	12.01	0.026
Calcium from RCT		
Placebo	Ref	Ref
Supplement	-1.21	0.768
Dietary calcium Intake (mg/day)	-1.07	0.159
Dietary acid load (mEq/day)	-4.51	0.062

Note: Estimates from a cross-sectional OLS linear regression model, adjusted for urinary specific gravity, months since delivery, total energy intake, breastfeeding practices, age, and BMI.

Figure 3. Urinary fluoride (mg/L) and 95% confidence intervals for women allocated to the placebo group and the calcium supplementation group over the course of gestation, adjusting for urinary SG, total energy intake, pre-pregnancy BMI, and age at first pregnancy visit.



GENERAL DISCUSSION

This work improves our understanding of fluoride intake during pregnancy and maternal urinary fluoride as a biomarker of exposure. Through the development of two studies, we investigated the effect of dietary changes associated with pregnancy on both the dietary intake of fluoride and spot urinary fluoride, utilizing data collected from the Early Life Exposures in Mexico to ENvironmental Toxicants (ELEMENT) project.

In the first study, we found that the estimated median dietary intake of fluoride in Mexican pregnant women was 0.7 mg F/day; ~77% lower than the IOM's recommendation of 3 mg/day for pregnant women [16]. This estimate is limited in that it constitutes the contribution of only intrinsic fluoride in foods and fluoride from salt added to foods during the cooking process. To overcome this limitation, future studies specifically designed for the assessment of *total* fluoride exposure, should consider the contribution of other sources, such as individual measures of fluoride concentration in water and salt − including individual estimates of salt added to meals after cooking; occupational and environmental exposures (e.g. cigarette smoke); and the unintentional intake of fluoridated dental products. Only through a more comprehensive assessment of exposure, will we be able to determine how current levels of exposure compare to the IOM's current recommendation.

A secondary finding of our dietary exposure study was that the dietary intake of fluoride was higher in women who follow the recommendations of intake of beneficial nutrients for pregnancy [90]. This finding suggests that the intake of foods in the Mexican diet that are rich in nutrients that are beneficial for pregnancy, indirectly increase the intake of a trace element that can be potentially harmful for the developing

baby. This is, however, another finding that would better inform future recommendations if the relative contribution of each source of exposure could be clearly identified, emphasizing that from fluoridated water or salt, if available.

Future studies on the association between maternal dietary fluoride intake and health outcomes in the offspring are needed to investigate the biological significance of the levels of dietary intake found in this population living in a salt-fluoridated community (median (range): 0.7 (0.1-3.7) mg F/day). A study conducted in women living in water-fluoridated communities in Canada, which had a similar estimated daily intake of fluoride from beverages ([mean ± SD] 0.9 ± 0.4 mg/day) reported that an increase of 1 mg in the intake of fluoride was associated with a 3.7 decrease in intelligence scores among boys and girls [47]. Such a decrease would be of public health significance for the offspring of about 10% of women in the present study [90].

In the second study, we found that although the concentrations of fluoride in urine during pregnancy do not significantly differ with those one-year postpartum, they do increase with the progression of gestation and are affected by dietary factors. In particular, calcium supplementation consumed during the second and third trimesters of pregnancy (1200 mg/day), increased the concentrations of fluoride in urine during pregnancy, but not one-year postpartum. On the other hand, reporting the use of table salt after cooking increases fluoride concentrations in urine one-year postpartum (as it is expected for a population exposed to fluoridated salt),; but not during pregnancy. Such differences in the dietary variables associated with urinary fluoride in each state suggest a potential role for physiological mechanisms, specifically bone metabolism and the

intestinal absorption of fluoride. These hypotheses would be worth exploring in future research.

Currently, research in environmental epidemiology validating the use of maternal urinary fluoride as a biomarker of prenatal fluoride exposure utilizes variables that have been reported to affect urinary fluoride concentrations for the general population [62,63]. The differences in the dietary factors that affect fluoride concentrations in urine at each state (pregnant vs. one-year postpartum) that we found in this study do suggest that the selection of covariates to be considered in future research should be informed by studies conducted specifically in the pregnant population.

Strengths and limitations

Although the number of people exposed to fluoridated water and salt is similar (~300 million people each) [9], exposure research on populations with implemented community salt fluoridation programs is scarce. The study on the estimation of fluoride intake in Mexican pregnant women presented in this dissertation is the first one conducted in a population of pregnant women living in a salt-fluoridated community, setting a precedent in fluoride exposure assessment. The strengths of the dietary estimations reported in this dissertation include the use of a fluoride database specific to the population under study to include both beverages and solid foods [77], a validated instrument of dietary assessment that allows for the estimation of multiple nutrients [85], and repeated measures of dietary intake over the course of pregnancy adjusting for confounders. We also had available repeated measures of fluoride concentrations in urine during pregnancy for larger sample sizes compared to similar studies [36,59,61]; and analyses were performed with more appropriate analytical methods, adjusting for the

effects of urinary dilution and dietary covariates. Furthermore, the original RCT design of ELEMENT's cohort 3 was leveraged to assess the association between calcium supplementation during pregnancy and fluoride concentrations in urine.

Despite several strengths, there are limitations of the research reported in this dissertation that need to be mentioned. The cohort was not originally designed to assess fluoride exposure [65]; therefore, some conditions were not ideal. First, quantitative estimates of salt added to foods after cooking were not available. Instead, a dichotomous variable on added salt intake (yes/no) that was available in the questionnaire was used for bivariate analyses and as a covariate in multivariate models. It is therefore most likely that the true intake of fluoride in this sample of pregnant women is being underestimated. For the second study of this dissertation research, we used fluoride estimates derived from a one-month recall (FFQ) [85] as an explanatory variable, and fluoride from spot urine samples collected during the early morning as the outcome variable in regression analyses. However, after ingestion, any given amount of absorbed fluoride is excreted in the urine in ~24 h. Therefore, the lack of association between fluoride intake and fluoride concentrations in urine in this study can be regarded to the time discrepancy between the dietary assessment (one-month recall) and the measurement of fluoride in urine (second-void morning spot urine). The dataset, however, served well the purpose of measuring the effects on fluoride concentrations in urine of other dietary factors that have longer-lasting effects on the body, such as the intake of calcium supplements during pregnancy (associated with reduced bone resorption [67]) and the habit of addition of table salt to meals after cooking. Future research estimating dietary fluoride intake in populations in relation to urinary fluoride excretion in populations exposed to fluoridated salt should

include quantitative measures of table salt intake and assess diet over shorter periods of time (e.g. 24-h dietary recalls), together with 24-h urine collections.

Conclusion and recommendations for future research

The dietary intake of fluoride in women living in Mexico City increased with the progression of pregnancy and in women who were moderately and highly compliant with the recommendations of intake of beneficial nutrients for pregnancy. Furthermore, the concentrations of fluoride in spot urine samples were affected by gestational age and calcium supplementation. Collectively, the results of this dissertation suggest that the recommendations for the dietary intake of fluoride and the assessment of spot urinary fluoride as a biomarker of prenatal fluoride exposure do require to be studied specifically for the pregnant population.

It is recommended for future studies to perform a comprehensive assessment of the relationship between total daily fluoride intake from multiple sources of exposure (source contribution analysis), using 24-h dietary assessments and urinary fluoride measurements. This, together with the measurement of dietary factors at different time-points over the course of pregnancy will refine and advance our knowledge in prenatal fluoride exposure and inform the design of future epidemiological studies.

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CURRICULUM VITAE

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Education

- Doctor of Philosophy in Dental Sciences (Ph.D.) 06/2021
  Indiana University-Purdue University Indianapolis (IUPUI)
  Indianapolis, IN, USA

- Master in Basic Biomedical Sciences (M.Sc.) 11/2014
  Universidad El Bosque, Bogotá D.C, Colombia

- Doctor in Dental Surgery (D.D.S) 07/2010
  Universidad Nacional de Colombia, Bogotá D.C, Colombia

Honors and awards

- Best Ph.D. Student Research -IUSD Research Day 2021
  King Saud University Travel Award

- IUPUI ELITE 50 Award 2020
  Recognition awarded to the 50 top IUPUI graduate students

- Best PhD Student Research -IUSD Research Day 2020
  King Saud University Travel Award

- Representative of IUSD at the Hinman Dental Student 2019
  Research Symposium
  The Hinman Dental Society Travel Award

- ORCA conference fellowship 2018
  Travel fellowship. The European Organization for Caries Research

- Fellowship for PhD studies 2017
  Indiana University School of Dentistry

- Fellowship for PhD studies 2015
  Colombian Administrative Department of Science,
  Technology and Innovation (COLCIENCIAS)

- Meritorious distinction for master's thesis 2014
  Universidad El Bosque School of Science

- Fellowship to attend the ORCA Caries Research retreat held in Bogota, Colombia. 2013
  The European Organization for Caries Research

- Hatton-Unilever Award 2012
  Colombian Division- International Association for Dental Research

- Young Researchers and Innovators Fellowship 2011
  Full stipend (24 months) to conduct research in a Colombian institution. Awarded by COLCIENCIAS

- Magna Cum Laude. DDS Class 2010 2010
  Universidad Nacional de Colombia, Bogotá, Colombia

Professional Memberships

- International Association of Dental Research (IADR)
- European Organization for Caries Research (ORCA)
- International Society for Children's Health and the Environment (ISCHE)

Scientific Meetings

- Annual IUSD Research Day 2017-2021
  Indiana University School of Dentistry

- 67th Annual ORCA congress 2020
  Virtual meeting

- Expert panel: Enhancing surveillance of fluorosis 2019
  University of North Carolina at Chapel Hill

- 25th Hinman Student Research Symposium 2019
  University of Tennessee

- 65th Annual ORCA congress 2018
  Copenhagen, Denmark

- 63rd Annual ORCA Congress 2016
  Athens, Greece

- 92nd General Session & Exhibition of the IADR 2014
  Cape Town, South Africa

- 90nd General Session & Exhibition of the IADR 2012
  Iguazu Falls, Brasil

Publications

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