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Urinary and plasma fluoride levels in pregnant women from Mexico City

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ABSTRACT

Background: There is need to assess the developmental neurotoxicity of fluoride. Our knowledge of prenatal fluoride exposure is challenged as few population-based studies have been conducted and these generally date back several decades, provide incomplete data on sociodemographic variables, and have methodological limitations.
Objective: To measure urinary and plasma fluoride levels across three time points in pregnant mothers who were enrolled in the Early Life Exposures in Mexico to Environmental Toxicants (ELEMENT) birth cohort study.
Methods: Fluoride levels were characterized in archived urine and plasma from 872 pregnant mothers sampled from the ELEMENT cohort. Various statistical methods were used to analyze the fluoride data with particular consideration for changes across three stages of pregnancy and against sociodemographic variables.
Results: All samples had detectable levels of fluoride. The mean urinary and plasma fluoride levels were 0.91 and 0.0221 mg/L respectively, and these were not statistically different across three stages of pregnancy. Fluoride levels correlated across the stages of pregnancy studied, with stronger correlations between neighboring stages. Urinary fluoride changed as pregnancy progressed with levels increasing until ~23 weeks and then decreasing until the end of pregnancy. For plasma fluoride, there was a decreasing trend but this was not of statistical significance. Creatinine-adjusted urinary fluoride levels did not associate consistently with any of the sociodemographic variables studied.
Conclusions: This study provides the most extensive characterization to date of fluoride exposure throughout pregnancy. These results provide the foundation to explore exposure-related health outcomes in the ELEMENT cohort and other studies.

1. Introduction

Community water fluoridation has been used for nearly 60 years to reduce the prevalence of dental caries. An estimated 210 million people in the United States (CDC, 2013) and millions more across 30+ countries drink water with fluoride levels currently considered optimal for caries prevention (Fawell et al., 2006). In addition to water, population-level exposures in some areas are achieved via the fluoridation of salt (e.g., certain regions of Mexico; Martinez-Mier et al. (2009) and Jimenez-Farfan et al. (2011)) or milk (Petersen et al., 2015; Weitz et al., 2015). Despite clear benefits in relation to dental caries, there remains an intense debate over the safety of fluoridation because of evidence demonstrating that excessive fluoride intake is associated with adverse effects on teeth (DenBesten and Li, 2011), bones (Chachra et al.,

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2008), and childhood cognition (Tang et al., 2008). This controversy, in part, spurred a review of the health literature by the U.S. National Research Council (NRC, 2006). The resulting NRC report called for more research to address the potential impacts of population level fluoride exposure, particularly among vulnerable populations including pregnant women and children.

Based on epidemiological and animal evidence, there is growing concern that fluoride is a developmental neurotoxicant. For example, a systematic review by Choi et al. (2012) that included 27 epidemiological studies found reduced children's intelligence associated with high fluoride exposures. Reports of developmental impacts are plausible given that maternally ingested fluoride has been shown to reach the fetus through the umbilical cord or placenta. Maternal blood fluoride, for example, is moderately correlated with cord blood, indicating that at least some fluoride moves from the maternal compartment to the fetus (Gedalia et al., 1961; Shimonovitz et al., 1995). Fluoride levels in the fetal brain (Du et al., 2008; Narayanaswamy and Piler, 2010) and teeth (Parker and Bawden, 1986) have been shown to increase in parallel with maternal exposures. Taken together, these findings suggest that the fetus is exposed to fluoride, and that maternal fluoride levels can be used as a proxy for fetal exposure. However, very few studies have characterized prenatal exposures to fluoride.

Fluoride exposure in pregnant women, to our knowledge, has been reported in very few population-based studies (Caldera et al., 1988; Gardner et al., 1952; Gedalia et al., 1959; Malhotra et al., 1993). These studies are limited in that, for example, they generally date back several decades, provide incomplete data on socioeconomic or demographic variables that could help interpret exposures, and have methodological limitations (e.g., they lack robust sample sizes, multiple biomarkers, and repeated measures). Given the limitations of the aforementioned studies and a need to better resolve prenatal exposures, the current study aimed to increase our understanding of developmental fluoride exposures by measuring maternal levels of fluoride over the course of pregnancy. To achieve this, we measured urinary and plasma fluoride levels across three time points in pregnant mothers who were enrolled in the Early Life Exposures in Mexico to Environmental Toxicants (ELEMENT) birth cohort study.

2. Methods

2.1. Study population

The institutional review boards of the National Institute of Public Health of Mexico, University of Michigan, Indiana University, the University of Toronto, and participating clinics approved the study procedures. Study participants were recruited between 1997 and 2006 from three clinics of the Mexican Institute of Social Security in Mexico City (Mexico) as part of the ELEMENT study. Pertinent details of the three cohorts that make up the ELEMENT study, including for example recruitment, collection of demographic information such as maternal age, education, marital status and smoking status during pregnancy, and collection and archival of pregnancy urine and plasma can be found elsewhere (Hu et al., 2006; Afeiche et al., 2011). Of particular note is that all urine samples consisted of early morning 2nd voided specimens collected at our study clinic; and all plasma samples were collected using special methods to prevent contamination and hemolysis (Smith et al., 2002).

The ELEMENT population consists of 2161 mothers (Fig. 1). Here, the study population was drawn from women who had prenatal visits associated with archived prenatal urine or plasma sample with adequate volume for additional analyses (i.e., they were drawn from two of the three ELEMENT cohorts). Of the 997 women in the source population, 872 contributed at least one urine or plasma sample during the early, mid, and late stages of pregnancy; 825 women contributed a total of 1520 urine samples, and 330 women contributed a total of 627 plasma fluoride samples. The gestational week (i.e., self-reported date of last menstrual period) the samples were donated and the child's sex were recorded.

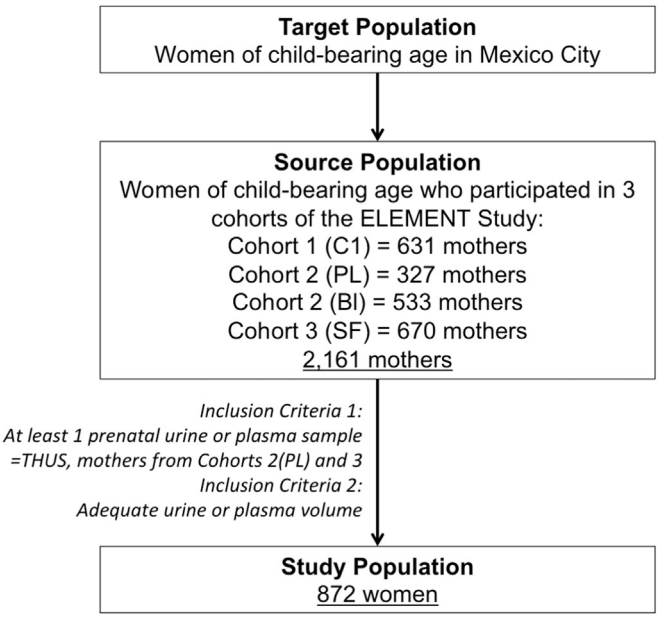

Fig. 1. Flow chart of study population drawn from the Early Life Exposures in Mexico to Environmental Toxicants (ELEMENT) cohort.

2.2. Fluoride in urine samples

The free ionic form of fluoride was measured using a micro diffusion method (Martínez-Mier et al., 2011) at the University of Michigan School of Public Health (UMSPH). Briefly, urine was diluted with equal parts Milli-Q water in a petri dish, and allowed to react for 20–24 h with 3 M sulfuric acid saturated with hexamethyldisiloxane (HMDS). The diffused fluoride was collected in 0.05 M of sodium hydroxide that was spotted on the interior of the petri dish cover. Following neutralization with 0.25 M of acetic acid, the concentration of fluoride in this solution was determined using an ion-selective electrode (Orion Fluoride Combination Electrode).

Urinary reference materials obtained from the Institut National de Santé Publique du Québec (INSPQ) were used to gauge analytical accuracy and precision. In addition, each batch run contained procedural blanks and replicate runs. The average recovery rate (analytical accuracy) for the reference materials was 100%. The mean relative standard deviation (analytical precision) for all samples was below 20% for samples containing less than or equal to 0.2 mg/L of fluoride and below 10% for samples with a fluoride concentration above 0.2 mg/L. Note, samples that had a coefficient of variation $>20\%$ were re-analyzed if there was urine remaining. The analytical detection limit (mean: 0.00656 mg/L) was calculated as the mean blank value plus two times the standard deviation.

An additional 390 urine samples were measured for fluoride content at the Indiana University Oral Health Research Institute (OHRI) using a similar procedure detailed in Martínez-Mier et al. (2011) with similar outcomes. Further, a validation study was conducted to determine the degree of similarity between samples measured at UMSPH versus at OHRI. Forty-eight samples were

measured in both labs. A careful statistical assessment of the resulting urinary fluoride levels revealed a correlation coefficient of 0.92 between measures taken by both labs.

2.3. Creatinine in urine samples

Urinary creatinine was measured using a MicroLab AT Plus (Hamilton Co., Reno, NV, USA) and Microplate Spectrophotometer (SpectraMax 340, Molecular Devices, Sunnyvale, CA, USA) at the University of Michigan. To account for variations in urine dilution at the time of measurement, urinary fluoride concentrations were adjusted for creatinine (Petersen et al., 2014). This was done by dividing the concentration of fluoride in the maternally-derived sample (MUF) by the sample's creatinine concentration (MUC), and multiplying by the average creatinine concentration of samples available at each trimester (MUCaverage) using the formula: (MUF/MUC) × MUCaverage. The values (mg/L) for MUCaverage at each trimester were 100.8, 81.6, and 72.4.

2.4. Fluoride in plasma samples

Plasma samples were measured for fluoride at the Indiana University Oral Health Research Institute (OHRI) using the hexamethyldisiloxane (HMDS) microdiffusion method of Taves (1968) as modified by Martínez-Mier et al. (2011). Briefly, plasma sample diluted 2:1 with diH_2O was pipetted into a plastic Petri dish, with a trap solution of 0.075 N sodium hydroxide (NaOH) placed on each dish lid. Each dish was immediately sealed. Next, HMDS-saturated 3 N SO_4 was introduced into each sample via a small hole burned into each lid. The hole was sealed immediately with petroleum jelly. During overnight diffusion, fluoride was released and trapped in the NaOH. The trap was recovered and buffered to pH 5.2 with acetic acid (CH_3COOH). The recovered solution was adjusted to a final volume of 20 ml with diH_2O. A standard fluoride curve was prepared from similarly diffused fluoride standard solutions and used to determine fluoride content of each plasma sample. Millivolt readings were measured using a fluoride microelectrode (Microelectrodes, Inc MI-SO) and pH/ISE meter (Orion Dual Star or equivalent). Duplicate analyses were done in sets of approximately 30–40 samples, and analytical precision of these measurements was less than 10%.

2.5. Data analysis

Summary measures (e.g., mean, median, standard deviation, minimum, maximum, and 10th, 25th, 75th and 90th percentiles) were calculated for urinary fluoride and plasma fluoride samples. Spearman correlation coefficients were generated to show the relationship between the biomarkers of exposure and other demographic variables. To assess the stability of the two biomarkers throughout pregnancy, intra-class correlation coefficients (ICC) were calculated. The ICC calculations excluded the top and bottom 1% of the observations and were calculated using log-transformed concentrations to stabilize the variance components. Bivariate analyses examining the crude associations between fluoride measures at each trimester and covariates were conducted using simple linear regression. Generalized additive mixed models, adjusted for maternal education, age, marital status, smoking status (never smokers, history of smoking but not smoking during pregnancy, or smoking during pregnancy), and child's sex were run to assess their effect on maternal urine and plasma levels during pregnancy, as well as to examine the time trend of the fluoride concentrations during gestation. The selection of model variables was guided through a combination of our review of the literature, an understanding of fluoride's toxicokinetics, and exploration of variables through correlative analyses. Data were analyzed using R 3.0.0 (RStudio, Inc.) and SAS 9.3 (SAS Institute Inc. Cary, NC). Results are shown as means ± standard deviation unless otherwise indicated.

3. Results

Fluoride measurements were made in urine and/or plasma from ELEMENT mothers (N = 872) for whom we had at least one prenatal biomarker (Fig. 1). Characteristics of these mothers (age: 26.6 ± 5.4 years; maternal education: 10.7 ± 3 years; 89.4% married or cohabitating) were not statistically different from mothers (N = 125) excluded due to not having any prenatal biomarkers measured. Pregnant mothers were sampled at 13.5 (± 2.3; range 0–26), 25.3 (± 2.4; range 15–37), and 34.5 (± 2.1; range 22–43) weeks of pregnancy. We acknowledge that some women were sampled at zero weeks of pregnancy as certain women who wanted to get pregnant were enrolled into ELEMENT Cohort 2.

The number of mothers for whom we have prenatal fluoride biomarker measurements from early, mid and late pregnancy (N_{urine} = 505, 454, 335; $N_{urine\ creatinine\text{-}adjusted}$ = 414, 186, 231; N_{plasma} = 220, 255, 152, respectively), and those for whom we have one, two, or all three of the measurements (N_{urine} = 314, 327, 184; $N_{urine\ creatinine\text{-}adjusted}$ = 220, 224, 71; N_{plasma} = 113, 137, 80 respectively) varied.

Every urine and plasma sample investigated had detectable levels of fluoride (Table 1). The overall mean urinary fluoride level was 0.91 mg/L, and the mean values across the three stages of pregnancy that were studied here did not differ (Fig. 2a). The creatinine adjusted urinary fluoride results are not different from

Table 1
Fluoride concentrations (mg/L) in urine and plasma of pregnant women from the Early Life Exposures in Mexico to Environmental Toxicants (ELEMENT) cohort. The "Average" represents data drawn from individuals who provided at least one sample.

	Stage of Pregnancy	N	Mean	SD	Min	10%	25%	50%	75%	90%	Max
Creatinine-adjusted urinary fluoride	Early	436	0.92	0.46	0.15	0.47	0.63	0.82	1.14	1.40	4.43
	Mid	199	0.95	0.47	0.25	0.49	0.65	0.88	1.13	1.55	3.50
	Late	246	0.87	0.48	0.00	0.40	0.57	0.78	1.02	1.45	3.67
	Average	515	0.91	0.40	0.02	0.51	0.65	0.83	1.09	1.37	3.67
Urinary fluoride	Early	637	0.83	0.50	0.07	0.28	0.44	0.74	1.10	1.55	3.22
	Mid	514	0.83	0.55	0.02	0.28	0.42	0.72	1.08	1.55	3.44
	Late	369	0.78	0.52	0.01	0.23	0.40	0.69	1.05	1.44	2.87
	Average	825	0.83	0.45	0.01	0.33	0.51	0.76	1.06	1.41	3.22
Plasma fluoride	Early	220	0.0228	0.0222	0.0021	0.0045	0.0071	0.0145	0.0310	0.0558	0.1052
	Mid	255	0.0194	0.0166	0.0022	0.0052	0.0077	0.0140	0.0240	0.0422	0.0777
	Late	152	0.0184	0.0171	0.0028	0.0047	0.0067	0.0121	0.0242	0.0462	0.0819
	Average	330	0.0221	0.0164	0.0035	0.0066	0.0091	0.0172	0.0299	0.0456	0.0786

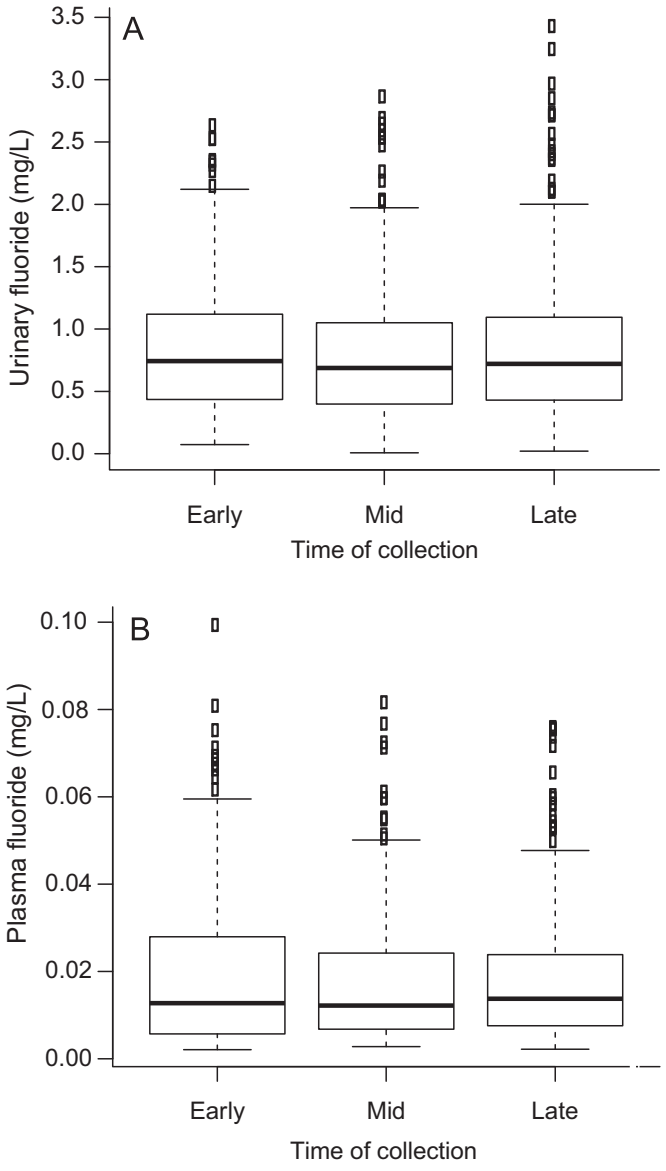

Fig. 2. Box and whisker plots of creatinine-adjusted urinary (plot 2A) and plasma (plot 2B) fluoride levels in pregnant women from the Early Life Exposures in Mexico to Environmental Toxicants (ELEMENT) cohort. Values are shown across three stages of pregnancy corresponding to approximately 13.5, 25.3, and 34 weeks of pregnancy.

the unadjusted values (matched results are summarized in Supplemental Table 1). Plasma fluoride levels were ~40-times lower than urinary fluoride levels. The overall mean plasma fluoride value was 0.0221 mg/L. Like in the urine, the mean plasma fluoride level did not differ across the three stages of pregnancy that were studied here (Fig. 2b).

The consistency of urinary and plasma fluoride concentrations across the three stages of pregnancy was studied (Table 2). In urine, fluoride levels were correlated across the three stages of pregnancy with Spearman coefficients ranging from 0.21 to 0.39. For plasma, fluoride levels correlated between neighboring time periods (i.e., between early and mid stages, and mid and late stages) but were not correlated between early and late stages of pregnancy. Analyses of the correlation between urinary and plasma fluoride levels was explored but were limited during certain stages of pregnancy owing to low sample sizes. In general there was a lack of correlation between these two biomarkers, though a significant correlation was found in fluoride levels between urine

Table 2
Spearman correlation coefficients relating fluoride levels in urine (creatinine-adjusted) and plasma at three different stages of pregnancy among participating mothers in the Early Life Exposures in Mexico to Environmental Toxicants (ELEMENT) cohort. In each cell, the first line is the correlation coefficient, followed by the p-value (line 2) and the sample size (line 3).

	Stage of pregnancy	Urine			Plasma		
		Early	Mid	Late	Early	Mid	Late
Urine	Early	1.00 – 436	0.39 <0.001 145	0.21 <0.001 202	0.29 0.004 97	0.03 0.72 136	0.09 0.42 90
	Mid		1.00 – 199	0.29 0.01 90	Only N=1 subject had both plasma and urine from mid pregnancy		
	Late			1.00 – 246	0.11 0.40 59	−0.1 0.39 76	−0.24 0.07 56
Plasma	Early				1.00 – 220	0.44 <0.001 162	0.14 0.21 82
	Mid					1.00 – 255	0.38 <0.001 133
	Late						1.00 – 152

and plasma of mothers sampled during early stages of pregnancy. Across the three stages of pregnancy, maternal urinary fluoride and plasma values (Fig. 2) were not different and remained fairly stable as pregnancy progressed. As a summary measure of consistency in fluoride concentrations across the three stages of pregnancy from which we sampled biomarkers, the ICC for urine was 0.25 and for plasma was 0.39.

To increase understanding of the fluoride biomarkers, biomarker values were stratified according to key sociodemographic variables (Table 3). For simplicity here we present fluoride biomarker values that have been averaged across the three sampling periods. The analyses shows that urinary fluoride was associated with the cohort from which the mothers were drawn and their level of education, but these differences did not remain once the urinary fluoride was adjusted for creatinine.

Two linear mixed effects models, with repeated measures of either creatinine-adjusted urine fluoride or plasma fluoride as the outcome, were run after adjusting for maternal education, age, marital status, smoking status (never, during pregnancy or smoking history but no during pregnancy), and child's sex. Urinary fluoride changed as pregnancy progressed with levels increasing until 22–23 weeks and then decreasing until the end of pregnancy (Fig. 3a and b). For plasma fluoride (Fig. 3c and d), there was a decreasing trend but this was not of statistical significance. In another analysis, plasma fluoride was included as a dependent variable in the urinary fluoride model, and urinary fluoride as a dependent variable in the plasma model, but no significant associations were detected between the two biomarkers.

4. Discussion

To our understanding, this is the first large exposure assessment of fluoride during multiple time points of pregnancy using two different biomarkers (urine, plasma). Where other studies have provided exposure data for the last trimester and delivery, our work examined exposure trends from the first month of pregnancy through delivery and found that levels in urine and plasma are relatively stable. Specifically, the population-average pattern of fluoride levels over time were fairly stable (see Fig. 3)

Table 3
Fluoride levels in urine (creatinine-adjusted) and plasma (values averaged across three different stages of pregnancy) among participating mothers in the Early Life Exposures in Mexico to Environmental Toxicants (ELEMENT) cohort according to key sociodemographic variables. Further details on the cohorts can be found in Hu et al. (2006) and Afeiche et al. (2011).

		Urinary fluoride, mg/L			Urinary fluoride (creatinine-adjusted), mg/L			Plasma fluoride, mg/L		
		N (%)	Mean (SE)	p-value	N (%)	Mean (SE)	p-value	N (%)	Mean (SE)	p-value
Married	No	241(29.2)	0.79(0.03)	0.50	155(30.1)	0.88(0.03)	0.84	96(29.1)	0.024(0.002)	0.07
	Yes	584(70.8)	0.82(0.02)		360(69.9)	0.88(0.02)		234(70.9)	0.020(0.001)	
Maternal age	18–23 years	261(31.6)	0.80(0.03)	0.47	169(32.8)	0.86(0.03)	0.31	98(29.7)	0.021(0.002)	0.67
	24–28 years	285(34.5)	0.79(0.03)		172(33.4)	0.87(0.03)		116(35.2)	0.020(0.002)	
	29–44 years	279(33.8)	0.84(0.03)		174(33.8)	0.91(0.03)		116(35.2)	0.022(0.002)	
Cohort	PL	232(28.1)	0.93(0.03)	<0.0001	150(29.1)	0.90(0.03)	0.42	53(16.1)	0.024(0.004)	0.35
	SF	593(71.9)	0.77(0.02)		365(70.9)	0.87(0.02)		277(83.9)	0.021(0.001)	
Education	0–9 years	371(45.0)	0.75(0.02)	0.0007	227(44.1)	0.86(0.02)	0.29	139(42.1)	0.021(0.001)	0.15
	10–12 years	336(40.7)	0.87(0.02)		217(42.1)	0.91(0.02)		138(41.8)	0.022(0.001)	
	>12 years	118(14.3)	0.85(0.04)		71(13.8)	0.86(0.04)		53(16.1)	0.017(0.002)	
Child sex	Male	342(49.6)	0.83(0.02)	0.32	239(48.0)	0.90(0.02)	0.18	123(49.2)	0.022(0.001)	0.15
	Female	347(50.4)	0.79(0.02)		259(52.0)	0.86(0.02)		127(50.8)	0.020(0.001)	
Firstborn	No	551(66.8)	0.82(0.02)	0.22	340(66.0)	0.88(0.02)	0.83	232(70.3)	0.022(0.001)	0.25
	Yes	274(33.2)	0.78(0.03)		175(34.0)	0.88(0.03)		98(29.7)	0.019(0.002)	
Smoking status	Never	407(49.9)	0.83(0.02)	0.10	257(50.3)	0.87(0.02)	0.73	163(50.5)	0.021(0.001)	0.84
	History of smoking	390(47.9)	0.78(0.02)		243(47.6)	0.89(0.02)		149(46.1)	0.021(0.001)	
	Smoked during pregnancy	18(2.2)	0.95(0.10)		11(2.2)	0.91(0.10)		11(3.4)	0.018(0.005)	

though within women there was less consistency of the measurements throughout pregnancy (e.g., ICC of 0.25 for urine). The work also shows that key sociodemographic variables are not associated with fluoride biomarkers in this population. In addition, our study extends upon previous fluoride biomarker ones in that we extensively document analytical quality control parameters (e.g., accuracy and precision), have measures of both plasma and urine, provide relatively larger sample sizes that are drawn from across multiple time points of pregnancy.

In the current study, maternal urinary fluoride levels in spot urine ranged upwards of ~3 mg/L with an overall mean value of 0.8 mg/L, and plasma fluoride ranged upwards of ~0.1 mg/L with an overall mean value of 0.02 mg/L. In general, these values were not associated with any of the sociodemographic variables that we investigated. Here we compare these fluoride levels to values reported elsewhere, albeit the number of studies from which we could draw comparisons was quite limited. In a study of 117 healthy pregnant women living in Jerusalem, fluoride levels in spot urine samples from ranged from detection limits to 0.8 mg/L, with mean values that ranged from 0.22 to 0.53 (Gedalia et al., 1959). Interestingly, Gedalia et al. (1959) measured urinary fluoride in these pregnant women monthly between months 4 and 9 of pregnancy and found the mean value to decline (i.e., 0.53, 0.43, 0.34, 0.28, 0.22, and 0.29). In a study of 31 pregnant women living in Poland, fluoride levels in spot urine samples from ranged from 0.15 to 1.69 mg/l, with mean value in women sampled in the 28th and 33rd week of pregnancy being 0.65 and 0.84 mg/L respectively (Opydo-Szymaczek and Borysewicz-Lewicka, 2005). This study from Poland also documented that the urinary fluoride levels in the pregnant participants were lower than measured in a control group of non-pregnant, healthy women (1.3 mg/L), and showed there to be no difference in fluoride measurements between a fasting morning urine sample and a 24-h collected urine sample. For plasma, others have reported similar findings to ours. For example, a study of 50 pregnant women sampled from Jerusalem found a mean plasma fluoride level of 0.03 mg/L (Ron et al., 1986), and a study from Poland involving 35 women sampled during the third trimester of pregnancy measured 0.033–0.037 mg/L of plasma fluoride (Opydo-Szymaczek and Borysewicz-Lewicka, 2006).

Given concerns about the potential neurodevelopmental effects of fluoride exposure (Choi et al., 2012), there is a need to better understand exposures during pregnancy. Further, an issue of increasing importance is determining whether there are time-related exposure windows of susceptibility to toxicant exposures especially during the prenatal period. Many lines of evidence now indicate such, including previous work by us concerning lead exposure in the ELEMENT cohort (Cantonwine et al., 2010; Hu et al., 2006). Here we were able to study fluoride levels in urine and plasma as pregnancy progressed. While no statistically significant changes were observed, creatinine-adjusted urinary fluoride levels increased until 22–23 weeks and then decreased until the end of pregnancy. These trends somewhat matched observations in older studies by Gedalia et al. (1959) and Hanhijarvi (1981) in which they also found non-significant reductions in the mean urinary fluoride in the final trimester. Examination of the un-adjusted urinary fluoride levels revealed no significant changes, with the levels trending downward in a linear manner over the course of pregnancy. The lack of significant changes in urinary or plasma fluoride during pregnancy was somewhat surprising given the number of physiological and metabolic changes occur during pregnancy, such as plasma volume expansion, mineral homeostasis and bone turnover, that could potentially affect fluoride pharmacokinetics and biomarker concentration levels.

An important feature of our study was the ability to relate the fluoride biomarkers with a range of sociodemographic measures. In general there were no consistent associations though we note that statistically significant changes in urinary fluoride were found in relation to cohort (mothers from cohort #3-SF had ~17% lower mean levels than those sampled from cohort #2-PL) and education (mothers with less than 10 years of education had ~13% lower mean levels than those with more education) but these did not remain when the urinary fluoride values were adjusted for creatinine.

Despite a number of strengths (e.g., large sample size, multiple biomarkers, demographic factors, longitudinal design with repeated measures in the same individuals), there are potential limitations of our study that need mention. Upon enrollment women were not screened for renal problems that could potentially affect the urinary fluoride levels. The early morning 2nd voided urine samples and plasma samples we measured were not taken with the requirement that each subject be in the fasting state, which may have introduced variability. However, the early morning 2nd void nature of the urine specimens would have reduced time-of-day variations driven by diurnal metabolic changes,

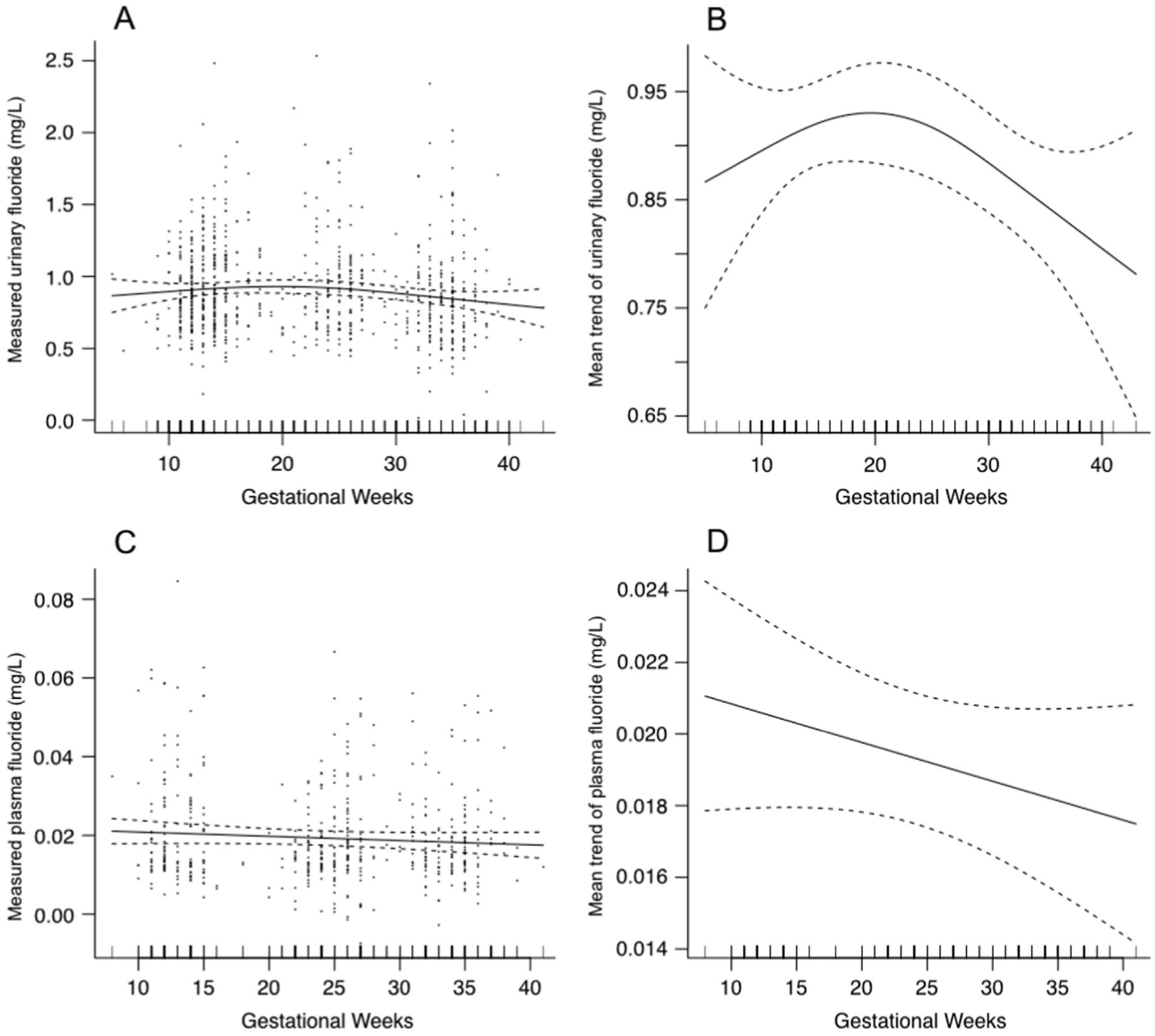

Fig. 3. Creatinine-adjusted urinary (plots 3A and 3B) and plasma (plots 3C and 3D) fluoride levels in pregnant women from the Early Life Exposures in Mexico to Environmental Toxicants (ELEMENT) cohort according to sampling time expressed as gestational weeks. Measured values are shown (plots 3A and 3C) as are the trend lines (plots 3B and 3D).

and the adjustment by creatinine corrected for urinary dilution. Nonetheless, future studies should either provide 24-hour measures or measure samples that have been collected after fasting. This study was also not initially designed to address fluoride exposure, but we had archived urinary and plasma samples that served well to retrospectively investigate fluoride and in doing so, the fluoride levels we measured here were similar to previous reports. In addition, the study was not designed to ascertain specific sources of fluoride exposure. The population from which our study was drawn (i.e., pregnant women residing in Mexico City) is mainly exposed to fluoride through ingestion of salt which is fluoridated to 250 ppm, though here we were not in a position to better characterize this exposure by collecting, for example, food items. Regardless of such limitations, our work provides the largest characterization to date of urinary and plasma fluoride throughout pregnancy. These results can be used in future studies to explore exposure-related health outcomes in the ELEMENT cohort.

Competing financial interests declaration

The authors have no competing financial interests to declare.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.envres.2016.06.046.

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