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Biomarkers of chronic fluoride exposure in groundwater in a highly exposed population

Tewodros Rango^{a,*}, Avner Vengosh^a, Marc Jeuland^{b,e}, Gary M. Whitford^c, and Redda Tekle-Haimanot^d

^aNicholas School of the Environment, Duke University, Durham, NC, USA

^bSanford School of Public Policy and Duke Global Health Institute, Duke University, Durham, NC, USA

^cDepartment of Oral Biology, College of Dental Medicine, Augusta University, Augusta, Ga. USA

^dFaculty of Medicine, Addis Ababa University, Addis Ababa, Ethiopia

^eInstitute of Water Policy, National University of Singapore, Singapore

Graphical abstract

Introduction

An estimated 200 million people worldwide are at risk of adverse health effects from exposure to high concentrations of naturally occurring fluoride (F^-) in drinking water sources (Edmunds and Smedley, 2005; WHO 2006). As a result, F^- has been named one of the top ten chemicals of public health concern (WHO, 2016). Low concentrations (0.5-1 mg/L) of F^- in drinking water are anticariogenic and fluoridation of drinking water has been shown to be a safe and sound public health intervention (CDC, 1999; Ayoob and Gupta, 2006; WHO, 2006; U.S. DHHS, 2011). Chronic ingestion of drinking water with a F^-

Corresponding Author: Tewodros Rango Godebo, PhD., Nicholas School of the Environment, 205 Old Chemistry Building; Box 90227, Duke University, Durham, NC 27708, tg67@duke.edu.

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concentration higher than the optimum level, however, has been shown to increase the risk of mild enamel fluorosis (EF), a pre-eruptive, developmental disorder that results in hypomineralized enamel. The stage of EF progresses in dose-response manner from mild (scattered white spots and mottled enamel), to moderate (discolored enamel), to severe EF (discolored enamel with discrete or confluent pitting, fractures, or loss of enamel in the severest cases) (Thylstrup and Fejerskov, 1978; Rozier 1994; Den Besten and Li, 2011). The first 6–8 years of life is the only period during which EF can occur because by that time the enamel has completed its development (Pendrys, 1990; 1999). Skeletal fluorosis (SF) is a bone disorder that manifests as osteosclerosis, ligament calcification, bone deformity, osteoporosis, susceptibility to bone fractures, and chronic joint and bone pain (Boivin et al., 1989; Tekle-Haimanot, 1990; Wang, 1994; Teotia et al., 1998).

A recent U.S. National Research Council (U.S. NRC, 2006) review report also raised several other potential health concerns related to high F^- exposure including altered biochemical and physiological processes, cardiovascular, reproductive, endocrine, gastrointestinal, neurological consequences, and bone fractures. Furthermore, excessive F^- exposure is emerging as a potential neurotoxic agent in that it has been linked to lower intelligence quotient (IQ) in children (Grandjean et al., 2014; Choi et al., 2012; Tang et al., 2008). The research on these various potential health effects is fairly new and the findings require validation through well-designed and well-conducted population-based studies to better understand these potential effects of F^- on human health.

Over 25 countries worldwide have endemic environmental contamination with high levels of naturally-occurring F^- (Ayoob and Gupta, 2006). Ethiopia, is one of several countries with substantial population living in the East African Rift System (EARS), a region that is extensively covered by volcanic materials, and that is known to be one of the global hotspots for high F^- contamination in groundwater (Rango et al., 2013, 2014; Žá ek et al., 2015). Unlike surface water, groundwater supply to the local population is relatively safe from a microbiological perspective, and yet F^- leaching from volcanic aquifers that are characterized by complex lithology, and variation in depth coupled with unique geochemical processes, result in a wide range of F^- levels in groundwater (Rango et al., 2013). Concentrations in the Rift Valley wells typically range from ~1 to 10 times higher than the WHO standard of 1.5 mg/L, putting an estimated 10 million Ethiopians at risk of high F^- exposure in the region (Tekle-Haimanot, 2005; Rango et al., 2014).

The Rift Valley communities investigated in this study are mostly rural and predominantly rely on community-based groundwater wells used for drinking and cooking. This nearly exclusive sourcing, and limited intake of F^- from other sources, provides a rare opportunity to generate a new and comprehensive dataset from a comparably homogenous population. Unlike previous work in the region, we have collected comprehensive data for a large sample of individuals that covers demographic variables, water quality measures, and multiple biomarkers, using well-designed methodologies, and sampling over a wide range of ages and exposures levels.

Biomarkers of F^- exposure can be evaluated using several biological tissues or fluids (i.e., teeth, bone, nail, hair, plasma, urine, and saliva) (Whitford, 2005; Rugg-Gunn et al., 2011).

Urine is the main pathway of F^- elimination from the body, and excretion is proportional to total F^- intake (Whitford, 1996). Fluoride in plasma and other extracellular fluids is removed via uptake in bones and urinary excretion. In young children roughly 80% of absorbed F^- is retained within 24 hours by calcified tissues; whereas in young to middle-aged adults, the retention is 30-50%, with the remainder being excreted in urine (Ekstrand et al., 1994; Whitford, 1996). In contrast to urine, F^- in fingernail clippings reflect the average plasma concentration of F^- that occurred over a protracted (1-2 week) period that represents past exposure roughly 3 to 4 months prior to collection (Levy et al., 2004; Whitford 2005; Buzalaf et al., 2006; Buzalaf et al., 2012). Furthermore, once F^- is incorporated into nail at growth end, it will be unaffected by more recent F^- intake and physiological variables (Whitford, 2005). Fingernail analysis of F^- has previously been used to assess low-level exposures in communities with fluoridation of water, tooth paste, salt, and milk use (e.g., Buzalaf et al., 2011; Lima-Arsati et al., 2010; de Almeida et al., 2007; Buzalaf et al., 2006; Pessan et al., 2005; Levy et al., 2004; Whitford et al., 1999), but epidemiological studies that evaluate the suitability and sensitivity of this biomarker in populations with endemic fluorosis are virtually absent.

Our study aims to assess the concentrations of F^- in fingernail clippings and urine and relate them to the degrees or stages of EF in individuals of different ages with large differences in their chronic exposures to F^- . Since the timing of F^- contaminated well installations has varied across communities (10-56 years), as well as prior use of low F^- surface waters before the wells, the study also offers suggestive evidence on the importance of timing of exposure (early F^- exposure in childhood or later) in influencing EF severity and prevalence. We also investigated the relationship between F^- and Ca (calcium), Mg (magnesium), and Al (aluminum) in water and urine, given that these are known to influence exposure by binding with F^- in the digestive system and body fluids (Whitford 1994; Sauerheber 2013) and since these minerals play a significant role in tooth and bone formation. This work lays a foundation for follow-up studies in developing biomarkers to better understand a range of F^- -related disorders that affect bones, joints, cartilage, and other non-mineralized tissues. There is a great need to better understand such potential effects because these could add to already significant public health challenges that are facing affected populations in this and many other similar low-income communities.

2. Materials and methods

2.1. Study area and population

The study area in the Ethiopian Rift Valley (Fig. S1) is part of the Great East African Rift Valley, which bisects the northeastern side of the African continent. The rift floor and the plateaus to the east and west that border the Rift floor have average altitudes of 1600m and 2500m above sea level (m.a.s.l), respectively. The climate is semi-arid to arid in the Rift Valley, and the mean annual temperature on the Rift floor exceeds 20°C (Le Turdu et al., 1999).

At the outset of the study (June 2015 and May 2016), a total of 386 individuals (10 to 50 years old) were enrolled from 27 rural communities that were selected to ensure representation across different F^- concentrations found in local groundwater wells in this

region (Group 1: <2 mg/L; Group 2: >2 to 5 mg/L; Group 3: >5 to 8 mg/L; Group 4: >8 to 12 mg/L; and Group 5: >12 to 15 mg/L). Among these 27 communities, 22 (316 individuals) use their respective wells as the primary source for drinking and cooking purposes, while 5 communities—with 6, 9.9, 12.7, 13.7 and 15 mg/L of F^- —use the respective wells for cooking, but not for drinking. Instead, these households primarily drink water that is piped from nearby towns and that is low in F^- (<2 mg/L). In each sample community, households were selected by counting off every 2 or more households in a community, and 1 to 2 subjects from each household were enrolled in the study. Study inclusion criteria for selected individuals were: age between 10 and 50 years; lifelong residence in the community, and consumption of well water since the time of well construction. In each community, we attempted to select individuals from the randomly sampled households so as to achieve equal representation across four age groups: 10-15, >15-25, >25-35, and >35-50 years.

We collected survey data including anthropometric measures, water quality parameters and their urinary and fingernail measures, and nutritional intake for the 386 enrolled individuals (Table 1). Twelve-hour urine samples (overnight, 6pm-6am) were collected from 386 of these, and 327 individuals also provided fingernail samples. The missing samples were due to individuals not having enough nail for clipping or being unwilling to collect overnight urine. Subjects with urinary volume below <150 ml volume ($n=17$) were excluded from the analysis. Urine samples were collected in plastic beakers (each with a capacity of one liter) that were provided to each individual. Nail clippings were collected from all 10 fingers with a stainless steel nail clipper. The study received ethical approval (Protocol No. C0863) from the Institutional Review Board (IRB) at Duke University.

2.2. Measurement of F^- , Ca, Mg and Al in drinking water and urine

The F^- concentration in groundwater and 12-hour urine samples were determined electrochemically using the Thermo Scientific Orion Ion-Selective Electrode (ISE). Samples were buffered with an equal volume of total ionic strength adjustment buffer (TISAB II) solution of pH 5-5.5, which allows for optimal analysis of F^- . Calibration standards (1, 10, and 20 mg/L of F^-) were prepared from a 100 mg/L stock solution. The range of electrode calibration slope for a 10-fold change in F^- concentration was -57 to -60 mV, which is within the acceptable range. Quality control was conducted using freeze-dried urine reference material (SERO210705; LGC Standards). The accuracy of ISE F^- measurements for both urine and water standards ranged from 98% to 102.5% relative to the standard. The detection limit of the F^- electrode is 0.02 mg/L. Calcium, Mg and Al in water and urine were analyzed using inductively coupled plasma mass spectrometer (ICP-MS) as described in Rango et al., 2013. The accuracy of the Ca, Mg and Al measurements for the urine standard was 94.5%, 87%, and 96% relative to the standard, respectively. The detection limit is 0.5 mg/L for Ca, 0.2 mg/L for Mg and 12.5 μ g/L for Al.

2.3. Fingernail pretreatment prior to F^- measurement

Fingernails were first cleaned with double deionized water (DDW), but removal of the visible nail external dirt, which is common in such rural settings with poor hand sanitation, was not wholly successful. To completely clean the loosely adhering external contamination associated with fat, sweat, dirt, and fingernail polish, each nail sample was placed in a

polypropylene tube and washed first with DDW, followed by acetone, a 1% Triton X-100 solution, and DDW, and when necessary any visible dirt on the surface of nails was further removed manually. In each of these steps the samples were sonicated for 20 minutes, and rinsed 3 times with DDW. The washed samples were allowed to dry at 85°C for 3 hours in a drying oven. After cooling, 5 to 20 mg of the dried samples were weighed and used for the analysis. Similar procedures have been used for the preparation of nail samples for other toxic and essential elements (Rodushkin and Axelsson 2000). Here, we have also conducted a washing efficiency experiment for F^- in nails (see Supplemental Material; **nail washing efficiency test**). The results showed a significant difference in F^- content in unwashed nail samples as compared to nails cleaned with acetone, Triton X-100, and DDW. Nail cleaning with one wash versus multiple washes with 1% Triton X-100 solution confirmed that nails cleansed once, twice, or thrice did not vary substantially in F^- content. This helps increase confidence that F^- content in the nail matrix remained intact and unaffected by the cleaning solution.

2.4. Analysis of F^- in fingernails

The F^- content of the fingernail clippings was determined using the hexamethyldisiloxane (HMDS)-facilitated diffusion method (Whitford, 1996). During the diffusion process, the fingernails were allowed to react for 18 to 24 hours with 3M sulfuric acid saturated with HMDS. The solutions in the nonwetable Petri dishes (Falcon, No. 1007) were gently swirled on a rotatory shaker. The diffused F^- was captured quantitatively in 0.05 N NaOH drops on the interior of the petri dish cover. Fluoride standards (0.019, 0.0475, 0.095, and 0.190 μg) were prepared by serial dilution of a stock-standard in triplicate and diffused in the same manner as the samples. In addition, non-diffused F^- standards were prepared with the same solutions (0.05 N NaOH, 0.20 N acetic acid, plus NaF) that were used to prepare the diffused standards and samples. The samples were then buffered with 0.20 N acetic acid, and the concentration of F^- in this solution was determined using the F^- combination ion specific electrode (Orion Research, Cambridge, Mass., USA, model 9609BNWP) coupled to a potentiometer (Orion, model A324). Comparison of the millivolt readings demonstrated that the F^- in the diffused standards had been completely trapped and analyzed. The millivoltage potentials were converted to F^- concentrations using a standard curve with a coefficient of correlation, r^2 0.99. The F^- level in each fingernail clipping was measured twice independently, and the resulting values were averaged.

2.4. Sampling and analysis of F^- in cereals

A total of 44 cereal samples including 14 maize, 14 wheat, and 16 'indigenous' teff (12 white and 4 red varieties) samples were collected from farms located in 14 study communities (corresponding to 212 study individuals). These cereals are the dominant crops grown locally, and all three are regularly consumed by local populations. The samples were collected and kept in plastic bags for further analysis. Samples were washed three times with DDW and dried completely and then samples were ground for F^- analysis with a procedure similar to the fingernails using HMDS- facilitated diffusion method.

2.5. Enamel fluorosis examination

The dental outcomes were evaluated using the TF Index (Thylstrup and Fejerskov, 1978; Fejerskov et al., 1996). TF scores are grouped into the following categories: healthy translucent teeth (score of 0), mild EF (scores of 1 and 2), moderate EF (scores of 3 and 4), and severe EF (scores between 5 and 9). Scores of 1-4 correspond to increasing white opaque areas on the enamel. The severe forms begin with focal pitting, followed by increasing pitting on the white opaque enamel that progresses to confluent pitting, and then total loss of enamel and tooth deformation. Prior to EF examination, the vestibular (buccal) surfaces of teeth were cleaned and dried with sterile gauze, and the teeth were examined under natural light. A total of ~11,000 teeth of the 386 individuals in the study communities were classified according to the TF Index.

2.6. Survey

The survey questionnaire was completed in face-to-face interviews, and included questions about age, place of birth, duration of exposure to the community well, information on other drinking water sources, water intake per day (estimated from the number of standard cups common and known in the region), the drilling year of groundwater well being used, toothpaste use, and basic health survey data on nutritional status. For the nutritional assessments, we used anthropometric and dietary methods. The anthropometric method involves measurement of height and weight to calculate body mass index (BMI; $\text{weight(kg)}/\text{height(m}^2\text{)}$), mid-upper arm circumference (MUAC), and subscapular skinfold thickness (SST) measured for the non-dominant arm.

2.7. Statistical Analysis

Descriptive analyses were carried out using quartiles, means, and standard deviations for continuous variables. The data were not normally distributed, and required log-transformation prior to statistical analysis. The relationships between severity of EF and F^- concentrations in water, urine, and fingernails were evaluated using Pearson's correlation coefficient. Comparison of urinary and fingernail F^- concentrations in individuals in communities with different drinking water F^- concentration groups was done by ANOVA after logarithmic transformation of the data using IBM SPSS statistical package. Multivariable regression analysis was conducted using STATA to further examine the relationships between F^- concentrations in drinking water, and biomarkers or EF outcomes. A basic model of ordinary least squares (OLS) regression was employed, controlling for age, sex and a continuous measure of BMI; community fixed effects were then added to absorb any additional village level differences that may confound these measures (e.g., systemic differences in diet). To proxy for cumulative exposure, an interaction of the duration of water consumption (computed based on the smaller of individual's age or the age of the well) and the F^- concentration in drinking water was included. We also controlled for nutritional variables, including for the frequency of cereals consumption (though F^- concentrations in these were found to be low) and milk consumption. Finally, we explored the correlations between EF outcomes and biomarker concentrations of F^- , as well as other potentially-related elements (Ca, Mg, and Al). The latter analyses used logistic regression to

determine the odds of having EF as a function of water and biomarker concentrations of F^- and these same related elements. The statistical significance was set at $p < 0.05$.

3. Results

3.1. Individuals' daily F^- intake through drinking water

Total daily F^- intake through drinking water was evaluated, taking into account individuals' body weight (bw) and reported drinking water intake. The data from the survey indicated that inhabitants (10-50 years old) consume between 0.3 and 5L of water per day from groundwater sources (an average 1.5 L of water per day). The results from daily F^- intake were compared with the No-Observed-Adverse-Effects-Level (NOAEL) for F^- (0.06 mg/kg bw/day) published by US EPA (2002). The comparison of the calculated dose from drinking water to the NOAEL of 0.06 mg/kg bw/day shows that the majority (80%) of individuals F^- intake exceeded the NOAEL value. The ranges and mean values of the F^- dose in mg/kg bw/day for the four age groups of 10-15, >15-25, >25-35, and >35-50 years old were 0.17 (0.014-0.67), 0.15 (0.021-0.66), 0.16 (0.009-0.43) and 0.15 (0.005-0.72) respectively, whereas the overall mean value across all ages was 0.16 mg/kg bw/day. In terms of per-person exposure from drinking water sources, the residents in the area are exposed to F^- intake ranging from 0.18 to 35.3 (mean: 7.7 ± 6.2) mg/person/day. An estimated 56% ($n=176$) of the individuals had F^- intake from drinking water that exceeded 5.0 mg/person/day, whereas 26% ($n=82$) and 4.8% ($n=15$) had F^- intake exceeding 10 and 20 mg/person/day, respectively.

3.2. Individuals' daily Ca, and Mg intake through drinking water

The mean Ca and Mg concentration in the water samples ($n=21$) was 18 (range: 0.4-38.8), and 4.4 (range: <0.01-13.8) mg/L, respectively. The mean daily per-person Ca and Mg intake from drinking waters was 27 (range: 0.12-123) and 6.7 (range: <0.22-32) mg/person/day. The estimated average dietary Ca requirement is 800 mg/day for young children and 1100 mg/day for adults (IOM 2011). Considering the mean Ca level of 18 mg/L in the drinking water, and an average water intake of 1.5 L, drinking water would provide less than 4% of these requirements. Reflecting the fact that drinking water is not the main source of these essential elements, no correlation was observed between Ca and Mg concentrations in drinking water and the respective Ca and Mg levels in urine. In fact, the geochemistry that produces groundwater with high F^- concentrations is typically found in Ca (and Mg)-poor aquifers (Rango et al., 2012); our water quality data shows such an inverse relation between F^- and Ca (or Mg) ($r=0.45$, $p < 0.001$, $n=21$).

3.3. Fluoride content of major cereal crops in the region

In rural communities of the MER staple foods consist of locally grown cereals (i.e., maize, wheat, and white and red varieties of teff). All cereal samples that were analyzed from the study area contain F^- level below 1 mg/kg ($n=43$), except for one white teff sample (1.73 mg/Kg) (Fig. S2). Red Teff samples contained the highest levels of F^- —on average 0.57 (0.38 to 0.89) mg/Kg. The lowest levels were found in maize 0.15 (0.13 to 0.23) mg/Kg. These values suggest that cereals are not the major contributor to F^- exposure in the population. Similarly, there is limited consumption among study households of other

industrial (e.g., processed diet and soft drinks) or dental products (e.g., toothpaste) that may contribute F^- . In this study, only 5.5% ($n=21$) of 386 of individuals reported using toothpaste. Considering that the groundwater is also used for cooking, water use during food preparation may contribute considerable amounts of F^- , as shown in previous studies from the region (Malde et al., 2003, 2004, 2011).

3.4. Fluoride in groundwater, urine, and fingernails

The concentration of F^- in study subjects' drinking water ranged from 0.6 to 15 mg/L (mean: 6.5 mg/L). All of the water samples ($n=27$)—except one with 0.6 mg/L—were above the WHO drinking water standard of 1.5 mg/L. Fluoride was also detected in the urine and fingernails of all tested individuals (Fig. 1). The F^- concentration in sample individuals' 12-hour urine ranged from 0.4 to 34 mg/L (mean: 8.9 ± 6.2 mg/L). The corresponding range of urinary F^- excretion was 0.2 to 30 mg/12-hour (mean: 5.7 ± 4.2 mg/12-hour urine). Multiplying this 12-hour F^- excretion value by 2 approximates the 24-hour F^- excretion (0.4 to 60 mg), as discussed by Pessan et al. (2005). The 24-hour urinary F^- excretion exceeded 10 mg/day in 43.6% ($n=137$) individuals. The fingernail F^- content ranged from 0.5 to 34 mg/Kg (mean: 5.1 ± 4.3 mg/Kg). One fingernail sample had unusual growth (nail thickening) and a very high F^- value of 104 mg/Kg, and was excluded from the analysis. To our knowledge 34 mg/Kg is the highest F^- level ever reported in human nails. An estimated 62.5% ($n=167$) and 8.5% ($n=22$) of individuals had fingernail F^- below 5 mg/Kg and above 10 mg/Kg, respectively, with 29% ($n=78$) between 5-10 mg/Kg.

We stratified the urinary F^- levels from participants into four groups based on the Biological Exposure Index (BEI) of 2 mg/L, which is used by industrial hygienists as a guideline for occupational exposure to F^- (ACGIH, 2012). Urinary F^- was evaluated using four concentration ranges defined by $1\times$, $>1-5\times$, $>5-10\times$, and $>10\times$ of the BEI value (Fig. S3). More than 96% of the urine samples contained F^- at levels higher than the BEI value, and only 3.7% of the urine samples were below BEI values for urinary F^- . The mean fingernail F^- level in individuals with urinary F^- concentration below BEI values was ~ 1 mg/Kg ($n=14$; Fig. 2). This value can be equivalently considered as the reference concentration or safe exposure value.

The drinking water wells with F^- concentrations of 6, 9.9, 12.7, 13.7 and 15 mg/L were excluded only from the exposure-biomarker analysis, as the individuals from these communities use additional sources of water from nearby towns having low F^- levels (<2 mg/L). Our data show a significant and positive correlation between F^- concentration in drinking water and individuals' 12-hour urine ($r=0.78$, $p<0.001$, $n=286$), and 12-hour urinary F^- excretion ($r=0.75$, $p<0.001$, $n=286$) (Fig. 2). Lower correlation was observed between F^- concentrations in drinking water and individuals' fingernail F^- content ($r=0.59$, $p<0.001$, $n=267$) (Fig. 3). Thus, urine is slightly better biomarker of F^- exposure than fingernails in these communities. Unsurprisingly, the mean community-level correlations between the various biomarkers and groundwater concentrations were stronger ($r=0.9$, $p<0.001$, $n=287$) than the individual correlations (Fig. 4). In Fig. 4, communities relying only on wells for drinking and cooking are identified by the diamond symbols, whereas the triangle symbols indicate communities that also consume piped water that is low in F^- from the nearby towns

(and whose individuals were excluded from the analyses shown in Fig. 3 and 4). The data confirm higher correlations for communities that solely use groundwater as their major drinking and cooking water source.

A two-way ANOVA analysis showed no interactions between the effects of F^- exposure and age on F^- content of fingernails ($p>0.05$). While there was a statistically significant difference in mean F^- in fingernails between the different F^- exposure groups ($p<0.001$), there was no significant difference between age groups ($p>0.05$) (Table 2a). Still, there is a gradual decrease in mean F^- in fingernails across age groups. The younger age group (10-15 years) had higher mean F^- in fingernails (see Table 2a; C: 8.01 mg/Kg, D: 9.13 mg/Kg, and E: 6.02 mg/Kg) than the older age groups. This may be explained by the high intake of F^- from water per body weight in children as compared to adults (see section 3.1).

The mean F^- in fingernails was significantly ($p<0.05$) higher in the drinking water F^- exposure groups D (7.3 ± 4.4 mg/Kg; CI: 6.2-8.4 mg/Kg) and C (6.8 ± 5.8 mg/Kg; CI: 5.34-8.23 mg/Kg) as compared to the lower F^- exposure groups, B (3.8 ± 2.3 mg/Kg; CI: 3.3-4.2) and A (2.6 ± 1.6 mg/Kg; CI: 2.2-3.1). However, there were no significant differences in fingernail F^- between groups A and B, or between high F^- groups of C and D ($p>0.05$) (Table 2a).

The mean F^- levels measured in 12-hour urine were significantly ($p<0.001$) higher in F^- exposure groups C (13.2 ± 5.3 mg/L) and B (5.7 ± 3.0 mg/L) compared to the lower exposure group, A (4.3 ± 3.1 mg/L). However, there were no differences ($p>0.05$) in urinary F^- between groups C (13.2 ± 5.3 mg/L) and D (13.8 ± 6.0 mg/L) (Table 2b). Similarly, no statistically significant difference in F^- in urine or fingernails was found between males and females (Fig. S4). In contrast to fingernail measures of F^- , the urinary F^- levels in older individuals (>25-35 and >35-50 years) were higher than in younger ones (10-15 and >15-25 years).

3.5. Fluoride in drinking water and enamel fluorosis

The relationship between F^- in drinking water (range: 0.6 to 15 mg/L) and the prevalence of severe EF in individuals (10 to 50 years old) follows a positive linear relationship, whereas inverse relationships were observed with mild-to-moderate EF (Fig. 5). None of the individuals from the low- F^- area (0.6 mg/L) showed any signs of fluorosis. Yet, severe EF occurred even at drinking water concentrations close to 1.5 mg/L (i.e., the WHO drinking water standard). Communities with groundwater concentration of F^- between 1.4 to 2 mg/L ($n=4$) had an average prevalence of 4.3%. At 4 mg/L, the prevalence of severe fluorosis rose to 16%, and about 52% of individuals had mild-to-moderate fluorosis. At a water F^- concentration close to 15 mg/L, the prevalence of severe fluorosis reached 64%.

Individuals developed different degrees of EF when exposed to similar F^- concentrations in drinking water. This heterogeneity suggests that total F^- intake varies across individuals within each community, or that there is differential susceptibility to fluorosis that might related to other factors such as age, duration and magnitude of exposure during critical period of enamel formation or nutritional status within populations. Genetic variability influencing fluorosis (as described in animal studies; Everett et al., 2002; Carvalho et al.,

2009) is expected to be low in the study region, as the study populations are of the same ethnicity, but ethnicity may not capture all components of genetic diversity that would cause different degrees of fluorosis under similar exposure conditions.

A two-way ANOVA analysis shows a statistically significant correlation between the effects of F^- exposure and age on EF ($p < 0.001$) (Table 3). Individuals aged between 10 and 35 years had a much greater proportion (81%) of teeth showing signs of EF than older individuals (36 to 50 years old; 27.5% of teeth), and the mean EF severity decreased with increasing age (Fig. 6). This suggests that older individuals likely had lower F^- exposures in early childhood when the teeth were forming (before high F^- wells were in use), which is the only period for risk of EF. In such cases, EF is not an ideal biomarker of recent F^- exposure.

3.6. Biomarkers and enamel fluorosis

Figure 7 presents a summary of biomarker concentrations and EF outcomes across the groundwater exposure groups for F^- . The asterisk indicates statistically significant differences, and other differences in urinary and fingernail F^- concentrations across groups are not statistically meaningful. Fingernail F^- concentrations were relatively insensitive in differentiating between the low or the high level F^- exposure groups. It is notable that the percentage of teeth with severe EF increased sharply from 8% (below 5 mg/L of F^- in water) to between 33% and 71% (from 5 to 15 mg/L of F^-) across the two groups with significant fingernail F^- differences (Fig. 7, arrow). Further significant differences beyond the third group could not be detected in fingernail or urinary measures despite increasing severity of EF.

3.7. Percentage of individuals in relation to percentage of fluorosed-teeth affected

Important information about EF is displayed in the cumulative frequencies of severity of fluorosis across the study population (Fig. 8). The overall percentage of individuals with at least one tooth showing signs of fluorosis (TF score ≥ 1) was 81%, while the percentage of individuals with at least one tooth exhibiting severe fluorosis (TF score ≥ 5) was 52%. Also, 35% of individuals had at least 50% of their teeth with TF scores ≥ 5 . The prevalence of mild (TF scores of 1 to 2), moderate (TF scores of 3 to 4) and severe (TF scores of 5 to 9) EF was 21.7%, 20.4%, and 34.1%, respectively. Among severely fluorosed teeth, at least 13% showed more than 50% loss in the outermost enamel surface.

Individuals without EF ($n=63$) had a significantly lower mean (SD) fingernail F^- concentration (3.6 ± 2.5 mg/Kg) than those with at least mild fluorosis (5.5 ± 4.6 mg/Kg) ($n=205$) ($p=0.002$). These individuals are largely in the older age groups, mostly past the age of 25 (mean: 35 years), who have been exposed to water with F^- concentrations below 6 mg/L.

3.8. Multivariable regression

The multivariate analyses show that relationships between F^- in water and both fingernail and urinary F^- are very similar (Table 4). That is, a 1 unit increase in water concentration of F^- is associated with a 0.8 unit increase in fingernail and urinary F^- . Importantly, none of

the control factors are statistically significant in explaining the mean TF score, except for age. Older individuals have systematically lower TF scores, presumably due to the expected lower exposure to F^- during their early years of enamel formation. We further show that the cumulative exposure to F^- in water (as proxied by duration of consumption from high F^- wells) is not strongly related to EF or urinary F^- (Table S3). For the former, this likely reflects the fact that EF outcomes are especially due to exposure during a defined childhood window of susceptibility, while for the latter, it is unsurprising since urinary F^- reflects short-term exposure. There is a slightly negative relationship between cumulative exposure and fingernail F^- , likely because higher intake of F^- from water per body weight in children than in adults. The type and frequency of cereal consumptions is not strongly related to EF or urinary, or fingernail F^- (Table S3). We also found that F^- in urine is more closely related to EF outcomes than fingernail F^- (Table S4), but that the odds of having EF at all increase similarly (by 26%) for every additional 1 mg/L of F^- in each of these biomarkers (Table S5). Finally, the concentrations of the other elements (Ca, Mg, and Al) in urine are not statistically related to TF scores or the odds of having EF (Table S6).

4. Discussion

The new cohort study in the Ethiopian Rift Valley that is presented in this paper was initiated with a long-term goal of understanding F^- related mineralized tissue disorders, as well as other poorly explored F^- related health problems such as neurodevelopmental disorders. The design takes advantage of a unique opportunity to study children and adults from a relatively homogeneous population (e.g., ethnicity, diet, socioeconomic) that is nonetheless exposed to a wide range of F^- levels in drinking water. As such, the study documents new information on exposures, biomarkers, and EF outcomes that should help to inform intervention and policy design that aims to minimize the risk of potentially adverse effects of chronic exposure to excessive F^- .

Globally, drinking water continues to be a primary route of exposure to F^- at levels that exceed the optimum F^- concentrations to protecting dental decay and many naturally occurring contaminants of major public health concern (Meacher et al., 2002; Villanueva et al., 2014; WHO, 2016). This study demonstrated that many individuals in this region of the world are exposed to unacceptable and harmful levels of drinking water F^- ranging up to 15 mg/L. Along with individuals' exposures to these high concentrations, we documented a linear relationship between F^- in drinking water and prevalence of severe EF, and a corresponding inverse relationship with mild-to-moderate EF. The children in our study were the most affected by severe EF, with pitting and loss of permanent teeth, and increased tooth sensitivity were widely observed among the young children enrolled in the study. Such effects and increased risk of tooth decay have previously been documented in studies from other locations (Levy 2003; Burt and Eklund, 1999; Wondwossen et al., 2004; Mine et al., 2011; Zhang et al., 2014), and EF has also been linked to psychological and social impacts due to stigma arising from the brownish discoloration of individuals' teeth (Wondwossen et al., 2006). Consistent with these impacts, many individuals that participated in the study reported an inability to chew hard foods (loss of dental function) from a young age, which harms the overall quality of life. In spite of our observation that the prevalence of severe EF primarily increases as a function of F^- in drinking water, younger individuals (aged 10 to 35

years) had a much greater proportion (81%) of teeth showing signs of EF than older individuals in our study (36 to 50 years old; 27.5% of teeth). The lower EF severity observed in older individuals is likely due to low F^- exposure during critical period of teeth formation at young ages (the first 8 years). In fact, the average age of the wells tested in the study was 28 years (a range of 10 to 56 years) and a number of the high F^- concentration wells are younger than the older individuals in the study. Thus, older individuals in the sample were more likely to have relied on alternative, and lower F^- concentration sources mainly from surface waters during their early years (communication with the households). This further emphasizes the fact that EF can be clinically detected only after extended periods of excessive exposure to F^- in early life (Pendrys, 1990, 1999; Hong et al., 2006; Buzalaf and Levy, 2011). Protecting children from early childhood exposure to excessive F^- intake appears to be critical if damage and long-term chronic pain in teeth and bones, and possibly other negative health consequences (such as neurodevelopment and endocrine systems yet to be verified and understood) are to be avoided.

Differences among our subjects' F^- exposure were further explored by quantifying the concentrations of F^- in urine and fingernail samples and relating the findings to the F^- levels in drinking water. Since the first evaluation on the potential of fingernail clippings as a biomarker for subchronic F^- exposure conducted by Whitford et al., (1999), follow-up studies have focused on how these biomarkers reveal low-level intake from various sources, including fluoridated water and toothpaste (e.g., Rodrigues et al., 2004; Levy et al., 2004; Pessan et al., 2005; Buzalaf et al., 2006 and 2011). Our study is unique since the variations in fingernail and urinary F^- levels, and their sensitivity and suitability were examined over a much larger range of exposures than previous studies. Our result shows that the F^- concentration in water was highly correlated with the F^- in urine and fingernails. However, urinary F^- was a slightly more sensitive and better indicator of recent F^- exposure than fingernail in this population. In spite of the observed gradual increase in the mean F^- in fingernails with increasing F^- exposure in drinking water, significant differences were found only when there was a broad separation in F^- concentrations in the drinking water (Fig. 7). In the younger age groups (10-15 years old), F^- concentrations in the drinking water, urine and fingernail clippings were reliable EF monitoring tools. This is consistent with the occurrence of early life F^- exposure in these children, suggesting that the different degrees of EF severity may be a useful biomarker to use for studies on neurological and other disorders that affect children during a specific window of developmental susceptibility.

There was a lack of significant variation in fingernail F^- content (though gradually decreasing) across age groups (10-15, >15-25, >25-35, and >35-50 years) and sexes. The multivariate analyses also indicated that F^- in nails is not sensitive to age- or sex-related differences. This finding is contrary to the expected increase of F^- in nails as a function of age that has been found in previous work (Fukushima et al., 2009), due to an increase in plasma F^- concentration with age (Whitford, 2005; Buzalaf and Whitford, 2011). In fact, we measured higher F^- in fingernails in younger children (10-15 years) than in other age groups, which could be from the higher dose of F^- exposure in younger individuals enrolled in this study.

The levels of F^- in fingernails measured up to 34 mg/Kg in this study are among the highest reported in the literature, which mostly addressed low- F^- cases of fluoridated water, salt, milk, and toothpaste exposure (e.g., Whitford et al., 1999; Levy et al., 2004; Pessan et al., 2005; Buzalaf et al., 2006 and 2011; de Almeida et al., 2007; Lima-Arsati et al., 2010). To our best knowledge, no population-based studies are available from regions of endemic exposure linking the F^- concentrations in fingernail clippings to EF (or SF). However, we did find relevant medical reports documenting high fingernail F^- levels in patients with SF. In one case, a 48-year-old woman in the US was found to be affected by advanced SF due to decades of consumption of large volumes of brewed tea. This patient had a mean fingernail F^- concentration of 11.8 mg/kg and excreted 12.3 mg/day with the urine (Izuora et al., 2011). In another patient with SF (from excessive tea and toothpaste consumption), the fingernail F^- concentration was 9.1 mg/kg, and F^- excretion was 16.9 mg/day (Joshi et al., 2010). Thus tea is a largely unrecognized source of F^- in the US and several Asian countries where tea is habitually consumed (e.g., Cao et al., 2013; Johnson et al., 2007; Izuora et al., 2011; Fan et al., 2016). The fact that many individuals in our study had biomarker concentrations exceeding these high levels points to a strong possibility of significant SF occurrence in this population. This could also engender a range of associated neurological complications due to bone calcification and mechanical compression of nerve roots and the spinal cord (Hafsa et al., 2008), or hearing loss from sclerosis of the auditory ossicles (Tekle-Haimanot 1990).

Limitation

The biomarkers of F^- exposure were measured at a single point in time, and miss past early child exposures among individuals who were born prior to the year of well installation. However, among children 10 to 15 years old, we are able to capture lifetime exposure very well, which is apparent in the strong correlation between groundwater concentration of F^- and severe EF to both early (incisors and first molars) and late erupting teeth (canines, premolars and second molars). This is consistent with the most important window of susceptibility for early and late erupting teeth, which is before age 2 (Hong et al., 2006), and between age 2 and 8 (Bhagavatula et al., 2016), respectively. The other limitation is that our measures of F^- mainly capture groundwater exposures. Although a complete F^- intake calculation required from all food source including vegetables, the low F^- content of cereals regularly consumed in the study area and the multivariate regression results suggest that groundwater is the principal source of F^- exposure among study individuals.

5. Conclusion

Naturally occurring F^- in drinking water is a public health issue of growing importance that currently affects millions of people worldwide, many in developing regions. Current challenges with such exposures persist because of limited knowledge and investment in interventions to reduce F^- contamination. Our study documented exceptionally high F^- contamination in drinking water and related these concentrations to individuals' biomarker measures in fingernails, urine and EF. The analysis shows that concentrations of F^- in urine and nails can be used to indicate differential F^- exposure; however, urinary F^- was slightly more robust biomarker of F^- exposure and EF as compared to fingernail F^- . Yet, the utility

of fingernail F^- characterizing SF that primarily depends on cumulative F^- exposure requires further studies. This study also shows that enamel loss was most severe in individuals exposed to high F^- during childhood and suggests that such effects could continue to worsen as this population increasingly relies on contaminated sources of groundwater. Only recently (2015-2016) the region was severely affected by extreme drought event (El Niño) attributed to climate change that would cause a drop in groundwater table that could be associated with an increase of F^- concentration in the groundwater, and thus higher- F^- exposure to the local population. Of particular concern are the potential impacts on non-mineralized tissues that have been demonstrated in animal experiments, including neurodevelopmental disorders, anemia, and adverse pregnancy outcomes (Choi et al., 2012; Agalakova et al., 2013; Jiang et al., 2014). Though it has been well established that many toxic elements including Pb, Hg, and As, cause neurodevelopmental disorder in humans (e.g., Grandjean and Landrigan, 2014), F^- is also emerging as an element with potential negative effects on cognition (Choi et al., 2012). Well-designed epidemiological studies in this and in other endemic regions are therefore urgently needed to explore such consequences.

Furthermore, the outcomes of the current study help to identify communities severely affected by F^- exposure, and can therefore inform targeting of effective F^- exposure mitigation strategies that would provide low fluoride alternative sources or water treatment to remove fluoride and better safeguard the health of populations in the region.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This is part of an ongoing cohort study in the Ethiopia Rift Valley with a long-term goal of understanding the health impacts of F^- in teeth, cartilage, joint, and bone and other poorly explored F^- related health problems in children and adults. We are immensely grateful for the funding from the NIEHS's career development grant (K99ES023472). We acknowledge Dr. Joel Meyer at Nicholas School of the Environment, Duke University and Dr. Michelle Mendez at the University of North Carolina for reviewing and providing valuable comments that improved the manuscript. We thank all the children and parents who participated in this study, and the local water bureaus for their help in recruiting them as well as guiding us during the field work. The content expressed in this paper is the responsibility of the authors and does not necessarily reflect the official views of the NIH.

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Abbreviations

ANOVA	Analysis of Variance
Bw	body weight
BMI	body mass index
BEI	Biological Exposure Index
DDW	Double Deionized Water
EF	enamel fluorosis
EARS	East African Rift System
F⁻	fluoride
IRB	Institutional Review Board
ISE	Ion Selective Electrode
IQ	Intelligence Quotient
mg/L	milligram per liter
mg/kg	milligram per kilogram
mg/kg bw/day	milligram per kilogram body weight per day
HMDS	Hexamethyldisiloxane
MCLG	Maximum-Contaminant-Level Goal
MUAC	mid-upper arm circumference
NOAEL	No-Observed-Adverse-Effects-Level
OLS	Ordinary Least Squares
SMCL	Secondary-Maximum-Contaminant-Level Goal
SST	subscapular skinfold thickness
TISAB	Total Ionic Strength Adjuster Buffer
TF Index	Thylstrup and Fejerskov Index
U.S. NRC	U.S. National Research Institute
U.S. EPA	U.S. Environmental Protection Agency
WHO	World Health Organization

Highlights

- Relationships between F^- in water, fingernails, and urine; and enamel fluorosis (EF) were established.
- Fingernail F^- was assessed as a biomarker for EF in a high- F^- region for the first time.
- Water, and F^- biomarker levels were more strongly linked to children's EF than adults, consistent with their greater exposure to F^- during early childhood.
- Children's EF may be useful as a biomarker for other health effects that depend on early-life F^- exposure

Figure 1. Box and whisker plots of F^- in urine and fingernails of individuals in communities from MER across four groups of F^- exposure from drinking water.

Figure 2. Relationships between F^- concentration in drinking water (mg/L) and urinary F^- excretion in 12-hour urine of individuals in communities that primarily use wells for drinking and cooking.

Figure 3. Relationships between F^- concentration in drinking water (mg/L) and F^- concentration in fingernails of individuals in communities that primarily use wells for drinking and cooking.

Figure 4.

Relationships between F^- in groundwater from community wells with **a)** the mean urinary F^- excretion in 12-hour urine and **b)** the mean F^- in fingernails of individuals in the respective communities. The corresponding F^- in urine and fingernails is also shown as mean and standard deviation. Note that the communities with green and purple triangle symbols also consume drinking water from piped water obtained from nearby towns, which is also reflected in the lower levels of biomarkers.

Figure 5.

Prevalence of mild and moderate (TF scores 4) and severe (TF scores 5) EF among MER individuals (10-50 years; $n=350$), compared to F^- concentration in groundwater. Two communities with F^- concentrations of 1.9 and 5.6 mg/L in groundwater had much more severe EF—deviating from the trend—of 46.3% and 56%, respectively, and were excluded. In these two communities, the urinary and fingernail levels of F^- were also lower than would be expected, given the EF prevalence. More investigation is required to explain the risk factors that caused the higher EF prevalence in those areas.

Figure 6. Percentage of healthy and EF-affected (mild, moderate, and severe) permanent teeth from 386 individuals across the four age groups.

Figure 7. Percentage of healthy and EF-affected (mild, moderate, and severe) permanent teeth from 386 individuals across the five drinking water F^- exposure groups with the biomarkers. The asterisk indicates consecutive statistically significant urinary and fingernail F^- concentrations between the different F^- exposure groups. *: $p < 0.05$.

Figure 8. Cumulative frequency distribution of individuals according to the proportion of teeth in individuals exhibiting various degree of severity (TF scores 1, 2, 3, 4, 5, 6, 7, 8 and =9).

Table 1

Statistical descriptions of the characteristics and F⁻ exposures of subjects' in the survey.

	Percentiles						
	N	Mean±SD	Min	25th	50th	75th	Max
Anthropometric measures							
Age	386	24.5±11.1	10.0	15.0	21.5	34.0	50.0
Weight (kg)	386	50.3±11.9	19.5	43.7	51.9	58.5	83.0
Height (m)	386	1.60±0.11	1.30	1.53	1.59	1.65	1.80
BMI (kg/m ²)	386	19.6±3.2	10.9	17.7	19.4	21.3	30.7
MUAC (cm)	380	23.8±3.6	9.0	22.0	24.0	26.0	36.5
SST (mm)	377	11.3±3.5	6.0	9.0	10.0	12.0	30.0
Years of well use (years)	384	28±13.4	10	18	26	37	56
Groundwater intake (liter/day)	384	1.5±0.7	0.3	1.0	1.3	2.0	5.0
Drinking water F⁻ concentrations							
F ⁻ in groundwater (mg/L)	386	6.4±4.2	0.6	2.5	5.6	9.9	15.0
F ⁻ intake (mg/day)	384	9.8±8.3	0.2	3.6	6.9	14.1	49.5
F ⁻ dose from water (mg/kg bw/day)	384	0.2±0.16	0.005	0.08	0.15	0.28	0.94
Drinking water Ca, Mg, and Al levels							
Ca (mg/L)	384	17.6±10.5	3.0	10.4	16.4	22.8	41.7
Ca intake (mg/day)	384	27.0±21.7	0.98	11.3	21.6	34.3	138
Mg (mg/L)	384	3.8±3.3	bd	1.1	3.7	5.1	13.8
Mg intake (mg/day)	384	5.9±5.8	bd	1.26	4.2	9.12	31.9
Al (µg/L)	384	43.6±70	2.3	5.9	18.2	52.2	310
Al intake (µg/day)	384	63.8±109	0.9	9.8	26.3	63.8	818
Urinary F⁻, Ca, Mg, and Al levels (12-hour urine)							

	Percentiles						
	N	Mean±SD	Min	25th	50th	75th	Max
Volume (liter)	369	0.65±0.36	0.15	0.39	0.63	0.84	3.50
F ⁻ concentration (mg/L)	369	10.2±7.2	0.44	4.6	8.41	13.8	43.6
F ⁻ excretion (mg)	369	5.7±4	0.22	2.7	4.7	7.8	29.9
Ca concentration (mg/L)	369	43.0±50.6	1.73	10.6	26.0	53.4	382
Ca excretion (mg)	369	25.7±31.3	1.12	5.30	13.3	32.7	251
Mg concentration (mg/L)	369	123.0±78	0.57	65.9	103.0	165	499
Mg excretion (mg)	368	67.2±37	0.26	43.1	62.9	84.8	319
Al concentration (µg/L)	369	46.2±157	bd	14.6	23.3	37.7	1851
Al excretion (µg)	369	27.2±97	bd	7.8	13.7	24.3	1600
Urinary pH	386	6.0±0.65	4.8	5.5	5.9	6.4	8.9
Fingernail F ⁻ content (mg/Kg)	323	5.3±4.2	0.5	2.5	4.4	6.8	34.0
F ⁻ in cereal samples (mg/Kg) (Maize, Wheat, Teff)	212	0.28±0.29	0.09	0.13	0.17	0.33	1.73

Notes: This includes study participants living in all 27 sample communities. Twenty-two of these communities use their respective wells as the primary source for drinking and cooking purposes, while 5 communities—with 6, 9, 9, 12, 7, 13, 7 and 15 mg/L of F⁻—use the wells mainly for cooking, but not drinking. Note that urine volume <150 ml were excluded from the analysis (n=17). bd: below detection.

Table 2

Mean F⁻ concentrations (±SD, mg/kg) in fingernails and 12-hour urine (mg/L) by F⁻ exposure in communities and age groups (years).

a) Fluoride exposure groups (mg/L)	Fingernail F ⁻ at each age group (in years).				Total
	10-15 years	>15-25 years	>25-35 years	>35-50 years	
A: <2	2.31±1.45 ^{aA}	2.98±1.63 ^{aA}	3.24±2.18 ^{aA}	2.08±1.17 ^{aA}	2.62±1.60 ^a
B: >2-5	3.70±2.06 ^{aA}	3.21±2.09 ^{aA}	3.39±1.35 ^{aA}	4.79±3.05 ^{bA}	3.76±2.28 ^a
C: >5-8	8.01±7.52 ^{bA}	6.57±3.08 ^{bA}	6.73±6.51 ^{bA}	4.58±2.14 ^{bA}	6.79±5.78 ^b
D: >8-12	9.13±5.43 ^{bA}	7.95±3.78 ^{bA}	5.09±3.61 ^{bB}	5.72±2.49 ^{bB}	7.29±4.42 ^b
E: Total	6.02±5.61 ^A	4.95±3.41 ^A	4.62±3.99 ^A	4.31±2.70 ^A	5.09±4.26

b) Fluoride exposure groups (mg/L)	Urinary F ⁻ at each age group (in years).				Total
	10-15 years	>15-25 years	>25-35 years	>35-50 years	
A: <2	3.4±2.6 ^{aA}	4.8±2.5 ^{aB}	4.9±3.98 ^{aB}	4.1±3.5 ^{aB}	4.32±3.1 ^a
B: >2-5	4.5±1.7 ^{bA}	6.3±3.7 ^{aA}	6.25±2.77 ^{bA}	6.3±3.6 ^{bA}	5.7±3.0 ^b
C: >5-8	9.8±3.26 ^{aA}	13.6±3.9 ^{bA}	15.2±6.6 ^{aA}	16.4±5.9 ^{aA}	13.2±5.34 ^c
D: >8-12	13.1±5.45 ^{aA}	13.9±4.6 ^{bA}	15.5±7.5 ^{aA}	12.6±6.2 ^{aA}	13.8±6.0 ^c
E: Total	7.6±5.2 ^A	8.97±5.5 ^B	10.6±7.4 ^B	9.1±6.5 ^B	8.94±6.2

Different lowercase letters in a column indicate statistically significant differences between the consecutive F⁻ exposure groups. Different uppercase letters in a row indicate statistically significant difference between the consecutive age groups (p<0.05).

Table 3

Mean TF score by F⁻ exposure and age groups (years).

c) Fluoride exposure groups (mg/L)	DF at each age group.				Total
	10-15 years	>15-25 years	>25-35 years	>35-50 years	
A: <2	2.5±1.8 ^{aA}	2.6±1.6 ^{aA}	1.6±2.0 ^{aB}	0.52±0.72 ^{aB}	1.93±1.8 ^a
B: >2-5	2.7±1.4 ^{aA}	2.1±1.55 ^{aB}	0.63±1.2 ^{bC}	0.023±0.09 ^{bD}	1.56±1.62 ^b
C: >5-8	4.6±1.5 ^{aA}	4.1±2.34 ^{aA}	2.7±2.7 ^{cB}	1.1±2.2 ^{cC}	3.5±2.4 ^c
D: >8-12	5.6±1.5 ^{aA}	4.84±1.8 ^{aA}	3.7±1.7 ^{dA}	2.32±2.1 ^{dB}	4.3±2.1 ^d
Total	3.8±2.0 ^A	3.2±2.1 ^A	2.2±2.2 ^B	0.9±1.7 ^C	2.7±2.3

Different lowercase letters in a column indicate statistically significant differences between the F⁻ exposure groups. Different uppercase letters indicate statistically significant differences between the age groups (p<0.05).

Table 4
Multivariate OLS regression analysis for fluorosis and biomarker outcomes

Variable	Mean TF score	Mean TF score	Fingernail F-	Fingernail F-	Urinary F-	Urinary F-
F ⁻ in water (mg/L)	0.35 ^{**} (0.055)	0.37 ^{**} (0.008)	0.61 ^{**} (0.090)	0.82 ^{**} (0.020)	0.79 ^{**} (0.077)	0.76 ^{**} (0.026)
Age	-0.10 ^{**} (0.010)	-0.10 ^{**} (0.012)	-0.041 [*] (0.023)	-0.029 (0.021)	0.057 (0.037)	0.054 (0.039)
Sex	-0.13 (0.19)	-0.22 (0.19)	-0.60 (0.60)	-0.43 (0.54)	0.21 (0.40)	0.20 (0.40)
BMI	0.039 (0.032)	0.039 (0.031)	-0.098 (0.084)	-0.13 (0.11)	0.057 (0.079)	0.080 (0.085)
Constant	2.74 ^{**} (0.62)	2.18 ^{**} (0.48)	5.71 [*] (2.71)	4.77 (3.07)	-1.45 (0.94)	-3.00 ^{**} (1.01)
Village fixed effects	NO	YES	NO	YES	NO	YES
n	316	316	267	267	313	313
R ²	0.469	0.627	0.248	0.321	0.444	0.491

Notes: Standard errors are reported in parentheses; significance is indicated by

^{**} p<0.01,

^{*} p<0.05.