Effect of altitude on urinary, plasma and nail fluoride levels in children and adults in Nepal

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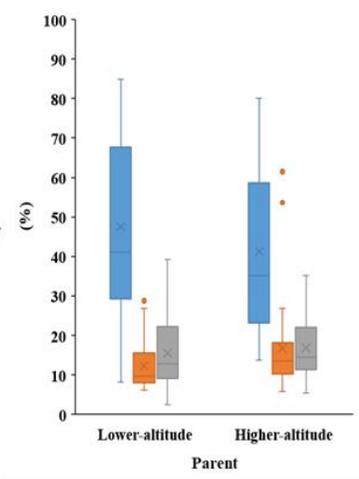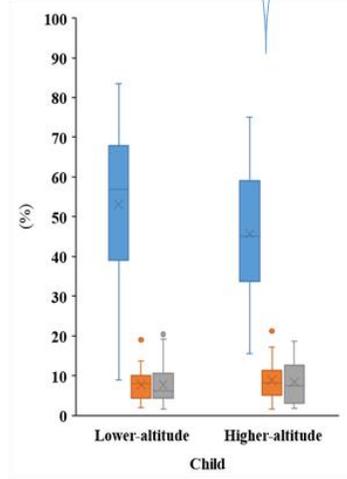Short title: Effect of altitude on fluoride in urine, plasma and nail
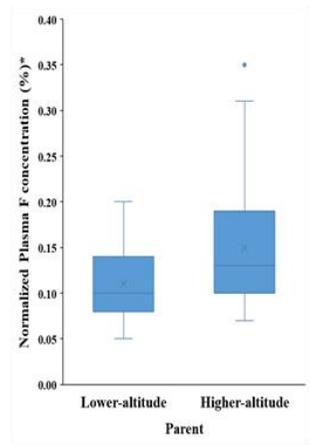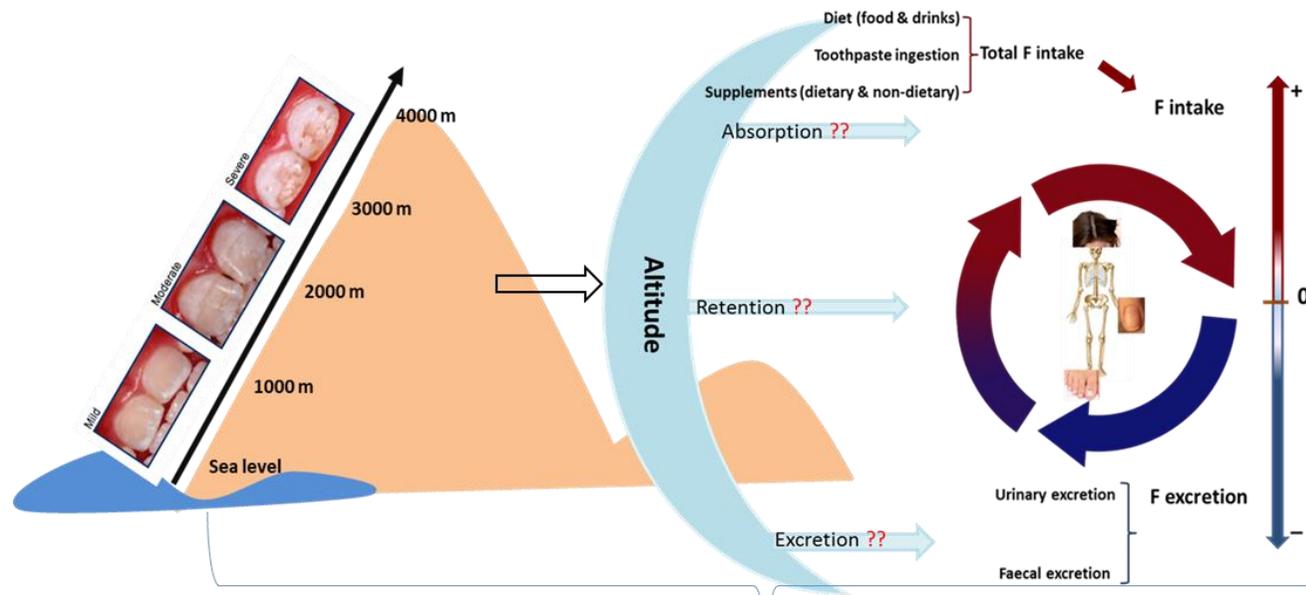
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Abstract:

Introduction

A greater prevalence of dental fluorosis has been reported in higher- versus lower-altitude communities. This study, for the first time, examined several aspects of fluoride metabolism in children, aged 4-5 years, and their parent, living at lower altitude ($\leq$78m) and higher altitude ($\geq$1487) areas in Nepal.

Methods

The study assessed total daily fluoride intake (TDFI), 24h urinary fluoride excretion (UFE), and fluoride concentrations of toe- and finger-nail ($FC_{toenail}$, $FC_{fingernail}$) in children and parents as well as fluoride concentration of plasma (FC_{plasma}) in parents. Fractional urinary fluoride excretion (FUFE) was calculated as the ratio between UFE and TDFI. $FC_{toenail}$, $FC_{fingernail}$ and FC_{plasma} were normalised for TDFI by dividing the variables by TDFI and the ratio was reported as the percentage.

Results

In total, 89 children and 80 parents took part in the study: 42 children and 41 parents from the lower altitude area; 47 children and 39 parents from the higher altitude area. Fluoride concentration of drinking water was significantly ($P<0.001$) higher at lower altitude (0.395 mgF/l) than at higher altitude (0.104 mgF/l). TDFI was significantly ($p<0.001$) higher in both children and parents living in lower altitude than those living at higher altitude.

There was a statistically significant ($p= 0.044$) difference in the mean FUFE of children living at lower altitude (53%) and higher altitude (46%). However, no significant difference in FUFE was found between parents living at lower altitude (47%) compared with higher altitude (41%).

In both children and parents, no statistically significant differences in normalised $FC_{toenail}$, $FC_{fingernail}$ were found between the two altitude areas. However, normalised FC_{plasma} was statistically significantly ($P=0.005$) higher in parents living at higher altitude (0.15%) compared with those living at lower altitude (0.11%).

Conclusion

The results suggest that higher altitude living results in decreased urinary fluoride excretion, and consequently increased fluoride retention in children for a given dose (amount) of fluoride.

1. Introduction

Dental fluorosis refers to a mineralization defect of the enamel as a result of exposure of the developing tooth organ to excessive amounts of fluoride (F). Due to the early commencement of tooth development in the permanent central incisors and first permanent molars, fluorosis in these teeth can start within the first 2 years of life [1]. However in general, the first 8 years of life are the most important for the development of fluorosis in permanent dentition [2]. Although the exact mechanism responsible for developing fluorotic enamel is not fully understood [3], total F exposure and or body F retention has been suggested as the true risk factor for fluorosis [4, 5]. Diet (including drinking water), toothpaste ingestion and F supplements are the main sources of F ingestion. It is estimated that about half of the daily F absorbed by healthy young to middle-aged adults becomes incorporated into calcified tissues, where 99% of the body's F is found, and the other half is excreted in the urine which is the main route for F elimination from the body. In young children, the fractional retention of F (ie the % of ingested F which is retained), is reported to be higher than 50% due to the rich blood supply and relatively large surface area of bone crystallites in the developing skeleton [6, 7]. Many factors can influence the rate of F absorption, excretion and consequently its retention, including total F intake, type of F, renal function, rate of bone metabolism, age as well as genetics, physical activity and altitude of residence [6, 7].

Human epidemiological studies conducted in Kenya [8], Nigeria [9], Tibet [10], Mexico [11, 12], Uganda [13], Tanzania [14] and Nepal [15] have reported a higher prevalence of dental fluorosis in higher altitude communities compared with lower altitude communities, which cannot be explained wholly by differences in F exposure. An increased susceptibility to develop F-induced enamel hypomineralization has also been reported in rats kept in hypobaric conditions, regardless of the levels of ingested F [16, 17]. Compared with laboratory rats kept at sea level, rats residing at a simulated higher altitude (5,486 m) showed a more acidic urine, significantly higher plasma F concentrations and ultimately greater F retention [16-18].

In this study, we aimed to quantify the effects of altitude on the urinary excretion of F, fractional urinary F excretion (FUFE; i.e the % of ingested F which is excreted) and F concentration in finger-

and toe-nail in children aged 4-5 years, and their parent (adults), living in Nepal. We also aimed to explore the influence of altitude on plasma F concentration in parents.

Materials and methods

Ethical approval

This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human participants were approved by the School of Health and Social Care ethics sub-committee, Teesside University, UK (# 077/13), and the Nepal Health Research Council (# 121/13). Written informed consent was obtained from child-parent dyads.

Sample size estimation

A sample size estimation was conducted using the nQuery Advisor 5.0 software package. Since this study was the first of this nature on human participants, the sample-size estimation was informed by effect sizes and standard deviations cited in two previous related studies: a report on the F retention (%) in rats raised in a hypobaric chamber which simulated higher altitude [19], and a study on the prevalence of dental fluorosis in children living at higher and lower altitudes in Nepal [15].

It was estimated that 33 individuals per group would be needed for 80% power. However, 50 paired children and parents (child-parent dyads) in a lower- and higher-altitude area (i.e. 200 participants in total) were recruited to account for possible withdrawal of participants prior to and during data collection.

Study area and participants

The study was carried out in two municipalities in Nepal: Banepa in the Hill region and Rajbiraj in the Tarai region with altitudes of 1,487m and 76 m above sea level, respectively. The initial analysis of drinking water samples collected from both municipalities found a F concentration of less than 0.3 mgF/l for all samples which is regarded as low fluoride water [20].

Healthy children aged 4-5 years were identified for the study through primary schools in both areas after obtaining approval from the head-teachers. Child and parent dyads were then invited to take part in the study. The data collection phase included two home visits. At the first visit (Day 1), the weight

of child and parent, without shoes and jacket, was measured to the nearest 0.1 kg using a portable mechanical personal scale (BR2017; Camry, China), and the height was measured to the nearest 0.5 cm using a portable stadiometer (DE56618903; ADE, Germany). Information about tooth-brushing habits of the child and parent was collected separately using a questionnaire and interview with the parents. At the second home visit, dietary data plus samples of drinking (and cooking) water, drinks and foods, expectorated saliva/toothpaste, nail clippings and 24h urine samples were collected separately for each child-parent dyad.

Assessment of total daily F intake (TDFI)

Total daily dietary F intake was assessed using a three-day food diary, which has been reported in detail elsewhere [21]. In summary, parents were given two 3-day food diary at the first visit, with written and verbal instructions on how to record all food and drink consumed, by themselves and their child, over three days. In addition, parents were given plastic storage containers and requested to collect approximately 50g of drinks and foods consumed. At the second visit, a post-completion interview with parents was conducted to make sure that all dietary data had been recorded as precisely as possible. The accuracy of estimates of food portion sizes made by parents was also checked using common everyday household items.

TDF from diet (µg/day) for each participant was estimated by multiplying the weight of each food/drink sample (g) by its corresponding F concentration (µg/g).

At the second visit, participants were asked to brush their teeth following their customary habits using their normal toothbrush and toothpaste. The amount of toothpaste dispensed onto the toothbrush was weighed using a portable electronic compact balance (A&D Instruments Ltd, Model HL-100, UK). All expectorated saliva, liquids and toothpaste, associated with tooth brushing, were collected in a wide-mouth polystyrene bowl and weighed. The total amount of F (µg) in expectorated saliva/liquid/toothpaste sample was estimated by multiplying the weight of sample (g) by its corresponding F concentration (µg/g). The total amount of F dispensed onto the toothbrush was subtracted from the total amount of F in expectorated saliva/liquid/toothpaste, and the resulting

amount multiplied by the participant's corresponding frequency of daily brushing to calculate the daily F intake from toothpaste ingestion (µg/day).

TDFI (µg/day) was then calculated by summing the F intakes from diet and toothpaste ingestion.

Assessment of 24h urinary F excretion (UFE)

Urine samples were collected from both child and parent. A urine collection kit, including disposable cups, jugs, potty, funnels and screw top plastic bottles, was given to parents with written and verbal instruction on how to collect their own and their child's urine sample over a 24h period. The resultant 24h urine samples of the child and parent were collected at the second visit and their total volumes were separately measured. UFE (µg/day) for each child and parent was estimated by multiplying the volume of the 24h urine sample (ml/day) by its corresponding F concentration (µg/ml).

The urinary flow rate (ml/h) was calculated by dividing the urine volume by 24. A child or adult urine sample with a flow rate of < 5ml/h or < 9ml/h, respectively, was suspected as incomplete, as suggested by the World Health Organization, and discarded from data analysis [22].

Assessment of F concentration (µg/g) in nail

Samples of nail were collected from both child and parent. Parents were asked to wash their own and child's hands and feet with water and dry them thoroughly before clipping the nails. They were instructed to place samples of finger- and toe-nail separately in labelled zip-locked plastic bags. The samples were weighed and then cleaned at the fluoride laboratory at Teesside University using deionised water with interdental brush and sonicated for 1 minute. The samples were weighed again after drying them at $95 \pm 5°C$ [23].

Assessment of F concentration (µg/g) in plasma

A sample of venous blood was collected from each parent (but not the child) by the study nurse. Plasma was separated from the whole blood by centrifugation (Remi R-8C, India) at 15,000 rpm for 15 minutes at room temperature and stored in a refrigerator (5°C) for immediate F analysis.

F analysis of samples

The concentrations of F in urine, water and drink samples were measured in triplicate at room temperature directly using a F-ion-selective electrode (Thermo Scientific Orion, Model 9609BNWP,

USA) coupled to a potentiometer (Thermo Scientific Orion, Model 720A, USA) after adding total ionic adjustment buffer (TISAB) III [24]. The F concentration of each expectorated saliva/liquid/toothpaste, toothpaste, food, milk-based drink, plasma and nail sample was measured using the overnight micro-diffusion method as detailed previously [24, 25]. In summary, a measured weight (or volume) of the sample was placed into the bottom of a polystyrene Petri dish (14.2 MM, VWR, UK). A sodium hydroxide (NaOH, A.R. Sigma-Aldrich, UK) trap solution was placed on the Petri dish lid and after the addition of sulphuric acid (H_2SO_4, Sigma-Aldrich) saturated with hexamethyldisiloxane (HMDS, Sigma-Aldrich), each dish was sealed very tightly. During an overnight diffusion, the released fluoride (as a result of acid hydrolysis) was trapped in the NaOH-trap. The trap was then recovered, perchloric acid ($HClO_4$, Sigma-Aldrich) added and its final volume adjusted to 75 µL by the addition of deionised distilled water. The F concentration of each sample was then obtained by comparison of the millivolt reading of the sample to standard curves.

The reliability of the F analytical methods was examined by reanalysing 10% of the samples, and the results confirmed no statistically significant differences in the means between test and retest for all the samples.

Data handling

To normalise by body weight, each TDFI (µg/day) and UFE (µg/day) value for each individual participant was divided by the participant's weight and the values were reported as mg per kg body weight per day (mg/kg bw/d).

To normalise for total F intake:

Fractional Urinary Fluoride Excretion (FUFE %) for each participant was calculated from the following equation: (UFE/TDFI) x 100;

Normalised fingernail F concentration (NFFC %) was calculated as: (Fingernail F concentration/TDFI) x 100;

Normalised toenail F concentration (NTFC %) was calculated as: (Toenail F concentration/TDFI) x 100; and,

Normalised plasma F concentration (NPFC %) was calculated as: (plasma F concentration/TDFI) x 100

Statistical analysis

Data were descriptively analysed using SPSS software (IBM Statistics, version 23). An Independent t-test was used to quantify, for each response variable, the mean differences (and associated 95%-confidence intervals) between the two altitudes as well as between the two age groups. Relationships between TDFI and UFE, by altitude and age group, were evaluated by standard linear regressions and Pearson's correlation coefficients.

Results

Number of participants and anthropometric data

In total, 89 children and 80 parents completed the three-day food diary and provided samples of 24h urine and expectorated saliva, liquids and toothpaste: 42 children and 41 parents in Rajbiraj (lower altitude area – LAA); 47 children and 39 parents in Banepa (higher altitude area – HAA). F concentration of drinking water was statistically significantly ($P<0.001$) higher in the LAA (0.395 mgF/l) than the HAA (0.104 mgF/l).

The mean (SD) age of the children in the LAA and HAA was 4.5 (0.5) and 4.7 (0.5) years respectively and their weight was 16.6 (2.9) kg and 15.4 (1.8) kg, respectively.

The mean (SD) age of parents in the LAA and HAA was 28.0 (3.7) and 29.1 (3.8) years respectively and their weight was 55.7 (9.7) and 56.1 (7.7) kg, respectively.

TDFI, UFE and F concentration in nail and plasma in children and parents by altitude

Tables 1 and 2 present mean (SD) urinary flow rate (ml/h), F intake (mg/kgbw/d), UFE (mg/kgbw/d) and F concentrations in nail (µg/g) and plasma (µg/ml) in children and parents, respectively, by altitude.

All children and parents met the inclusion criterion of a urinary flow rate of 5 ml/h and < 9ml/h, respectively. Comparison between the two altitude areas showed no statistically significant differences in mean urinary flow rate in either children (Table 1) or parents (Table 2).

No child or parent who participated in this study took any F tablets or supplements; diet and inadvertent toothpaste ingestion were their only sources of F intake. In children, the overall mean (SD) contribution of diet to TDFI was 75 (19)%: 77 (19)% and 74 (20)% in the LAA and HAA respectively. The overall mean (SD) contribution of diet to TDFI, in parents, was 87 (13)%: 90 (11)% and 84 (14)% in parents living in the LAA and HAA respectively. No statistically significant differences in the F intake from toothpaste ingestion was found between the two altitudes for either group of study participants (Tables 1 and 2), whereas F intake from diet, TDFI and UFE were statistically significantly higher in both children and parents living in the LAA compared with those in the HAA.

In children, no statistically significant differences in F concentration of either fingernail or toenail were found between the two altitudes (Table 1). In contrast, in parents, there was no statistically significant difference in F concentration of fingernail, while the mean toenail F concentration was statistically significantly (P=0.027) higher in LAA -living parents than those living in the HAA (Table 2). The mean plasma F concentration was almost similar for parents living in the LAA and HAA (Table 2).

Comparison between children and parents (adults)

Mean and 95% confidence intervals for differences in F intake (mg/kgbw/d), UFE (mg/kgbw/d), and F concentrations in finger- and toe-nail in child-parent dyads by altitude are presented in Table 3. At both altitudes, mean TDFI, F concentration in fingernail and UFE were statistically significantly higher in parents than children, whereas mean F concentration in toenail was slightly, but not statistically significantly, higher in parents than children.

Relationship between TDFI and UFE

The linear relationships between TDFI and UFE, by altitude, for children are presented in Figure 1 and for parents in Figure 2. In children (Figure 1), the statistically significant positive correlation between TDFI and UFE was very strong ($\rho = 0.85$, P<0.001) in the LAA, and strong ($\rho = 0.74$, P<0.001) in the HAA. In parents (Figure 2), the statistically significant positive correlation between

TDFI and UFE was moderate (ρ = 0.52, P=0.001) in the LAA, and strong (ρ = 0.67, P<0.001) in the HAA.

FUFE

In children, the mean (SD) FUFE (i.e. UFE normalised for F-intake) was 53 (18)% and 46 (16)% in the LAA and HAA, respectively, while for parents the FUFE was 47 (23)% and 41 (20)% in the LAA and HAA respectively. The mean FUFE was statistically significantly (p= 0.044) higher in children living in the LAA than in the HAA, but there was no difference for parents.

Although the overall mean FUFE was slightly, but not statistically significantly, higher in children than parents, the range was wider in parents as presented in Figure 3.

F concentration in plasma, finger- and toe-nail, normalised for F-intake

Box and whisker plots of F concentration in finger- and toe-nail, normalised for F-intake, in children and parents living in the LAA and HAA are presented in Figure 3.

No statistically significant differences in finger- and toe-nail F concentrations, normalised for F-intake, were found between the two altitude areas for children or for parents.

As Figure 4 presents, in box and whisker plots of plasma F concentration, normalised for F-intake, the F-intake normalised plasma F concentration was statistically significantly (P=0.005) higher in parents living in the HAA (0.15%) than those living in the LAA (0.11%).

Discussion

This study provides the first data on the effect of altitude on different aspects of F metabolism including urinary F excretion and the proportion of ingested F excreted in the urine in humans, as well as F concentration in plasma and nail. The results suggest that higher altitude leads to decreased urinary F excretion, and consequently increased F retention in children when given the same dose (amount) of F.

The study found diet as the main source of F intake for both children and parents (Tables 1 and 2), with water as the key contributor to dietary F intake. Compared with the HAA, total F intake of children and parents living in the LAA was significantly higher, primarily due to the higher F

concentration of water in the LAA. F concentration of drinking water in the LAA (0.395 mgF/l), in the present study, was almost four times higher than that in the HAA (0.104 mgF/l). The impact of F concentration of water on TDFI, which was clearly demonstrated in this study, reinforces water as the primary route for F exposure in some communities.

The overall mean contribution of diet (75%) to TDFI in the children of the present study corresponds to the 75% reported for 5-year-olds in Iowa [26], and the 71% reported for the Nigerian 4-year-olds [27]. However, a wide variation in the contribution of diet to TDFI has been reported for children, ranging from 88% for Iowan 6-year-olds [26] to 31% for Puerto Rican 4-5-year-olds [28]. The variation in the contribution of diet to total F intake could be explained by differences in the age of children and their dietary habits and composition. In the present study, the contribution of toothpaste ingestion to TDFI was insignificant, in children and parents. However, the literature shows that toothbrushing with a fluoridated toothpaste could, on average, account for up to 69% of TDFI in 4-5 year olds [29].

When normalised by body weight, the present study also showed a higher mean F intake from diet and toothpaste ingestion in children compared with parents (Tables 1-3). This could be explained by the fact that children, generally, consume more food, on a body weight basis, compared with parents due to their higher energy requirements (90 kcal/kgbw/d for children vs 40 kcal/kgbw/d for parents [30]) and require more water to keep hydrated (65-85 ml/kgbw/d for children vs 30-50 ml/kgbw/d in parents [31]). Children also tend to swallow toothpaste as their control of their swallowing reflex is not as fully developed as adults [32].

The mean TDFI of children as well as their parents in the present study was lower than the upper tolerable intake level (UL) of 0.1 mgF/kg bw/d [33] – a value suggested based on a prevalence of less than 5% of moderate dental fluorosis in children.

Even though dental fluorosis has been well-documented to occur as a consequence of excess F ingestion during tooth formation, a lack of F dose-response effect in the occurrence of dental fluorosis has been reported in populations living at high altitude. A survey of 12-year-old Nepalese children reported a dental fluorosis prevalence of 53% in children living at an altitude of 3700m, even though the water F concentration was very low at 0.06 mg/l [15]. A study in Kenya reported a dental fluorosis

prevalence of 78.0% in children aged 11-15 years living at an altitude of 1500m with a water F

concentration of <0.5 mgF/l compared with a prevalence of 36.4% in children at sea levels receiving

similar F concentration in water [8]. A higher prevalence of dental fluorosis has also been reported in

other communities with water F concentrations of <0.8 situated at altitudes of 1700m in Nigeria [9],

2000m and 4300m in Tibet [10], >2000m in Mexico [11], and 1463m in Tanzania [14]. However,

none of these studies assessed total F intake (i.e. the true risk factor for fluorosis).

Since F metabolism can be affected by genetic and environmental factors, it is fundamental to

quantify F excretion and retention rather than only the TDFI when health effects of F are concerned.

Since age (i.e. skeletal development and growth) could be another variable influencing F metabolism,

the current study compared UFE and FUFE between children and their parents subjected to similar

environmental influences including altitude as well as their close genetic profile.

The composition of diet can influence the pH of urine and consequently the magnitude of F excretion

and retention. A vegetarian diet, which promotes an alkaline urine, results in relatively higher F

excretion compared with a meat-based diet which results in a more acidic urine (and therefore more F

retention) [34]. Nepalese people have a vegetarian-based diet with rice and lentils being the staple

food commodities in the Tarai region (i.e. LAA), and maize and millet in the Hill region (i.e. HAA).

Therefore, any differences in UFE between the two areas cannot be explained by the type of diet (i.e.

vegetarian- vs meat-based diet).

The overall mean UFE of both children and parents was higher in the LAA compared with the HAA

(Tables 1 and 2), which could be related to the effect of the higher F concentration of water (and

consequently higher total F intake) in the LAA. Figures 1 and 2 describe the linear association

between total daily F intake and urinary F excretion in both parents and children at each altitude. This

highlights the process by which the F incorporated into bone is steadily released by continuous bone

remodelling and then excreted through urine, even when the F intake is nil or negligible. The

estimated graph intercepts, in the present study, clearly suggest that in the absence of any F exposure,

children living at a higher altitude excrete less F in their urine than those living at a lower altitude

(Figure 1), although this difference was not observed in parents (Figure 2). This finding suggests that

either less F is released from bones or more F is reabsorbed from the renal tubules in children at higher altitudes.

When urinary F excretion was normalised for TDFI (i.e. FUFE), the current study also found a statistically significantly (p=0.044) lower FUFE in children residing at higher altitude (46%) compared with those living at lower altitude (53%). These findings indicate that at a given level of F intake, urinary excretion of F is lower in children living at higher altitudes. However, no significant differences in FUFE were found between parents living at lower- and higher altitudes in the present study. This latter result is in agreement with the reported slightly, but not statistically significantly, lower urinary F excretion seen in 7-week female rats housed in an environmental chamber set at a stimulated altitude of 5486m (18000 ft) compared with those residing at sea level [34]. Considering that one human year equals 3.3 rat days in the pre-pubertal phase [35], the rats in the latter study were equivalent to almost 15 years of age in human terms.

The differences in UFE and FUFE between children and parents may be explained by better adaptation to higher altitudes in adults as well as the differences in the growth rate (a lower growth rate in adults and therefore less F retention in calcified tissues) and the type and form of ingested F and its bioavailability (e.g. a F bioavailability of 100% from water vs 65% from infant milk formula reconstituted with water [36]).

It has been reported that native Tibetans living at high altitude (3,800–4,200 m) have a steady increase in mean arterial haemoglobin oxygen saturation (SaO2) during the first decade of life, followed by a stabilisation during the second decade [37]. Therefore, during the first 3 years of life, when the window of susceptibility to the occurrence of fluorosis is the highest for the permanent incisors [2], the SaO2 is lower compared with older children and young adults.

Therefore, the observed dental fluorosis at high altitudes seen in young children might be explained by a reduced urinary excretion of F, due to the alteration in acid-base balance caused by hypoxia (due to low SaO2) of high altitude, leading to a decrease in pH of urine and ultimately an increase in renal tubule reabsorption of F which results in more F retention [16, 17].

The present study found a very wide variation in FUFE, within the range of 30 to 80% [38-45] reported in the literature for children and adults. The wide variation in FUFE could be explained by

between-individual physiological and dietary/oral hygiene habit differences and/or variations in the patterns of dietary/oral hygiene habits within-individual.

The study [34] with two groups of female rats, residing at sea level and an environmental chamber set at a stimulated altitude of 5486m (18000 ft) for 5 weeks, reported a statistically significantly lower faecal F excretion in the groups at high altitude, indicating greater F absorption at high altitude. In the present study faecal F excretion was not measured but the significantly higher plasma F concentration, as a function of TDFI, detected in the parents at the HAA suggests that a higher proportion of ingested F may be absorbed from gastrointestinal tract at higher altitudes. The plasma F concentrations (0.026 and 0.027 µg/ml in LAA and HAA, respectively) found in the present study were higher than the range of 0.009 to 0.020 µg/ml reported in the literature for adults living in low water F areas (<0.30 µgF/ml) [46]. A plasma F concentration as low as 0.028 µg/ml has been shown to be still capable of inducing mild enamel fluorosis in the rat incisor [18].

Some studies have reported nail as a possible biomarker for chronic F exposure [25, 47-50] and enamel fluorosis [51, 52]. However, the present study found no significant differences in F concentration of finger-nail in both children and parents living at lower- and higher-altitudes despite a significant difference in TDFI between the two locations. The study also showed a statistically significant difference in toe-nail F concentration in parents living at lower- and higher-altitude but not in children. Therefore, more epidemiological studies are required to assess the suitability, acceptability and sensitivity of nail as a F biomarker in different populations, with different dietary habits, and geographical situations.

In conclusion; the results of this study suggest that urinary F excretion, in children, is decreased with chronic residence at higher altitudes which results in increased F retention in their body. This could therefore explain the observed dental fluorosis at high altitudes seen in young children receiving relatively low F water.

Limitations of the study and future recommendations

There are several limitations to the present study which could be addressed by future studies to address the knowledge gaps regarding the impact of altitude on metabolism of F.

The first limitation is that the blood collection was performed exclusively on adults because of the ethical restraint related to collecting blood from healthy children for the purpose of research. Due to the possible age differences in F metabolism, the results for adults may not necessarily apply to children.

The other limitation is that no faecal sample was collected from the study participants due to the practicality issues for both participants and the research team. The only available study with rats [34] showed that in rats on low-F diet (0.032 mgF/day), their faecal F corresponded to 20% and 16% of the F intake when housed at sea level and a stimulated altitude of 5486m, respectively. However, human studies have reported that, on average, almost 10% and 6% of total daily F intake is excreted through faeces in children [53] and adults [54], respectively. Human studies are therefore needed to assess the effect of altitude on faecal F excretion.

The present study focused on two narrow age groups (4-5- and 20-35 year-olds) and did not explore any possible effect of gender on F metabolism. Since the study by Beall [37] in native Tibetans living at high altitude reported age- and gender-differences in SaO2, more studies are needed to look at the effect of altitude on F metabolism in different age groups and genders.

Acknowledgments

Authors' Contributions

FVZ, AM, OS conceived and designed the study; OS collected and analysed the samples; FVZ supervised the project with help from AM; FVZ and OS analysed the data and AM contributed to the interpretation of the results; FVZ took the lead in writing the manuscript. All authors read, provided critical feedback and approved the submitted paper.

Declaration of interests:

The authors have no conflicts of interest to disclose. The funder had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript

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Table 1. Estimated Mean (SD) urinary flow rate (ml/h), fluoride intake (mg/kgbw/day), urinary fluoride excretion (mg/kgbw/d), and fractional urinary fluoride excretion (%) in <u>children</u> by altitude.

Variables	Altitude		Differences		P value
	Lower (LAA)	Higher (HAA)	Mean	95% CI	
Urinary flow rate (ml/h)	29 (9)[a]	20 (6)[b]	9	6, 12	NS
Daily F intake (mg/kgbw/d)					
– Diet	0.050 (0.028)[a]	0.034 (0.021)[b]	0.016	0.005, 0.026	0.003
– Toothpaste ingestion	0.015 (0.016)[a]	0.012 (0.011)[b]	0.004	-0.002, 0.010	NS
– Total	0.065 (0.029)[a]	0.046 (0.020)[b]	0.019	0.009, 0.030	<0.001
24h urinary F excretion (mg/kgbw/d)	0.036 (0.024)[a]	0.021 (0.014)[b]	0.015	0.007, 0.023	<0.001
F concentration in					
– Fingernail (µg/g)	4.526 (2.266)[c]	3.989 (1.884)[d]	0.537	-0.634, 1.707	NS
– Toenail (µg/g)	4.346 (2.268)[e]	3.399 (1.772)[f]	0.947	-0.252, 2.147	NS
Fractional Urinary F excretion (%)	53 (18)	46 (16)	7	0.2, 15	0.044

Number of samples (n): [a] (n=42), [b] (n=47), [c] (n=25), [d] (n=26), [e] (n=26), [f] (n=22)

Table 2. Estimated Mean (SD) urinary flow rate (ml/h), fluoride intake (mg/kgbw/day), urinary fluoride excretion (mg/kgbw/d) and fractional urinary fluoride excretion (%) in <u>parents</u> by altitude.

	Altitude		Differences		P value
Variables	Lower (LAA)	Higher (HAA)	Mean	95% CI	
Urinary flow rate (ml/h)	44 (15) [a]	43 (20) [b]	1	-6, 9	NS
Daily F intake (mg/kgbw/d)					
– Diet	0.022 (0.006) [a]	0.017 (0.006) [b]	0.005	0.003, 0.008	P<0.001
– Toothpaste ingestion	0.003 (0.003) [a]	0.003 (0.003) [b]	-0.1	0.001, 0.001	NS
– Total	0.025 (0.006) [a]	0.020 (0.007) [b]	0.005	0.002, 0.008	0.001
Daily urinary F excretion (mg/kgbw/d)	0.012 (0.007) [a]	0.008 (0.006) [b]	0.004	0, 0.006	0.012
F concentration in					
– Fingernail (µg/g)	2.989 (1.370) [c]	2.962 (1.656) [d]	0.027	-0.795, 0.849	NS
– Toenail (µg/g)	3.712 (1.932) [e]	2.860 (0.758) [f]	0.851	0.101, 1.601	0.027
– Plasma (µg/g)	0.026 (0.011) [g]	0.027 (0.008) [g]	-0.0005	-0.005, 0.004	NS
Fractional Urinary F excretion (%)	47 (23)	41 (20)	6	-3, 16	NS

Number of samples (n): [a] (n=41), [b] (n=39), [c] (n=26), [d] (n=30), [e] (n=24), [f] (n=32), [g] (n=37)

Table 3. Mean (95% confidence interval (CI)) difference in fluoride intake (mg/kgbw/day), urinary fluoride excretion (mg/kgbw/d) and fractional urinary fluoride excretion (%) between children and parents by altitude.

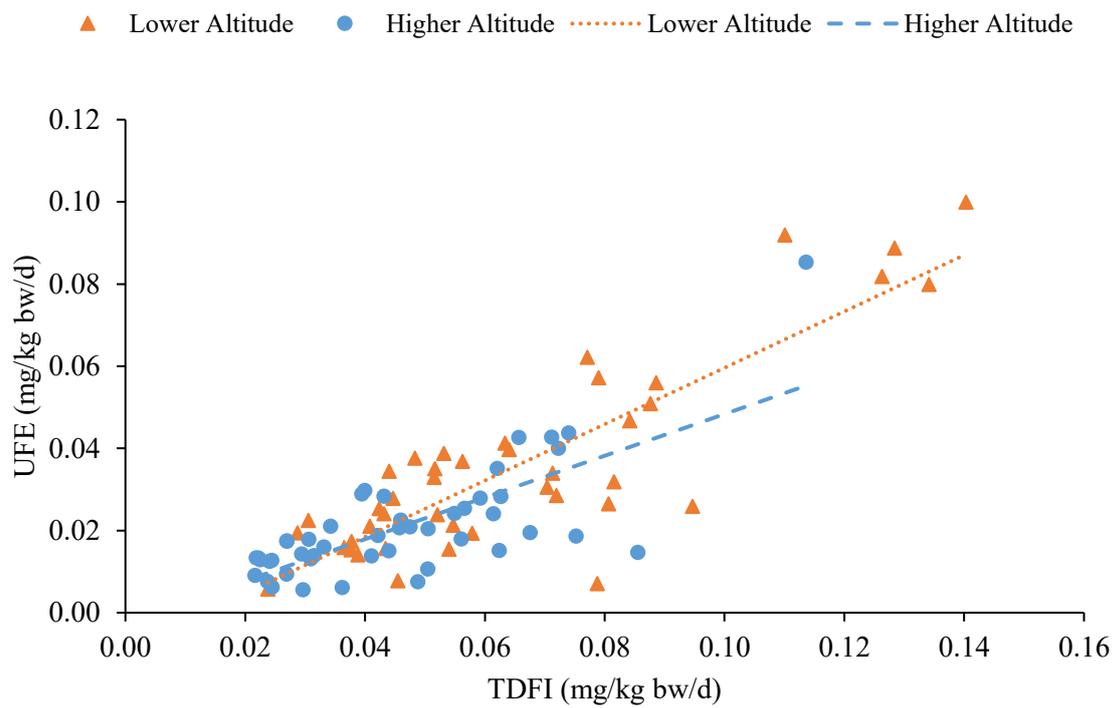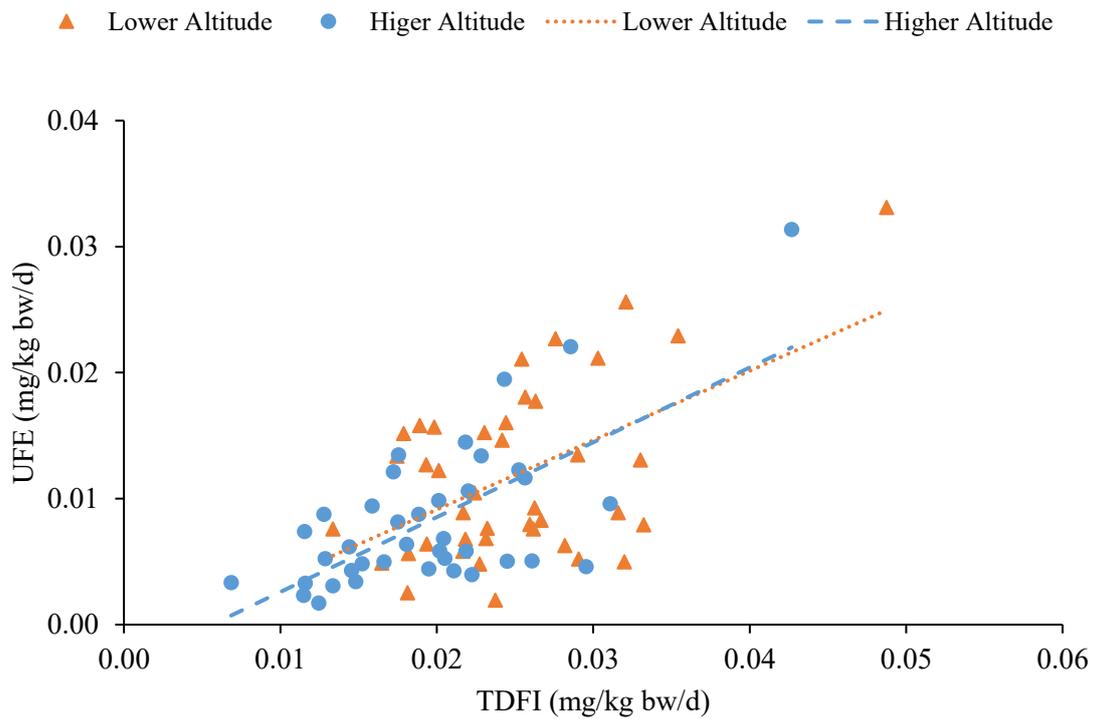|	Lower altitude (LAA)		Higher altitude (HAA)	
	Mean (95% CI) of difference	P value	Mean (95% CI) difference	P value
Daily F intake (mg/kgbw/d)				
– Diet	-0.028 (0.037, -0.018)	<0.001	-0.018 (-0.024, -0.011)	<0.001
– Toothpaste ingestion	-0.012 (-0.018, -0.007)	<0.001	-0.009 (-0.012, -0.005)	<0.001
– Total	-0.040 (-0.050, -0.031)	<0.001	-0.026 (-0.033, -0.20)	<0.001
Daily urinary F excretion (mg/kgbw/d)	-0.024 (-0.032, -0.06)	<0.001	-0.013 (0.017, -0.008)	<0.001
F concentration in				
– Fingernail (µg/g)	-1.537 (-2.586, -0.488)	0.005	-1.027 (-1.975, -0.78)	0.034
– Toenail (µg/g)	-0.634 (-1.837, 0.568)	NS	-0.538 (-1.244, 0.167)	NS
Fractional Urinary F excretion (%)	-6 (-15, 3)	NS	-4 (-12, 3)	NS

Figure 1. Relationship between total daily F intake (TDFI: mg/kg bw/d) and 24h urinary F excretion (UFE: mg/kg bw/d) for children living at lower- and higher- altitudes

Lower-altitude: UFE (mg/kg bw/d) = -0.009 + 0.686 [TDFI (mg/kg bw/d)]; (ρ = 0.85, P<0.001)

Higher-altitude: UFE (mg/kg bw/d) = -0.002 + 0.508 [TDFI (mg/kg bw/d)]; (ρ = 0.74, P<0.001)

Figure 2. Relationship between total daily F intake (TDFI: mg/kg bw/d) and 24h urinary F excretion (UFE: mg/kg bw/d) for parents living at lower- and higher- altitudes

Lower-altitude: UFE (mg/kg bw/d) = -0.002 + 0.552 [TDFI (mg/kg bw/d)]; (ρ = 0.52, P=0.001)

Higher-altitude: UFE (mg/kg bw/d) = -0.003 + 0.594 [TDFI (mg/kg bw/d)]; (ρ = 0.67, P<0.001)
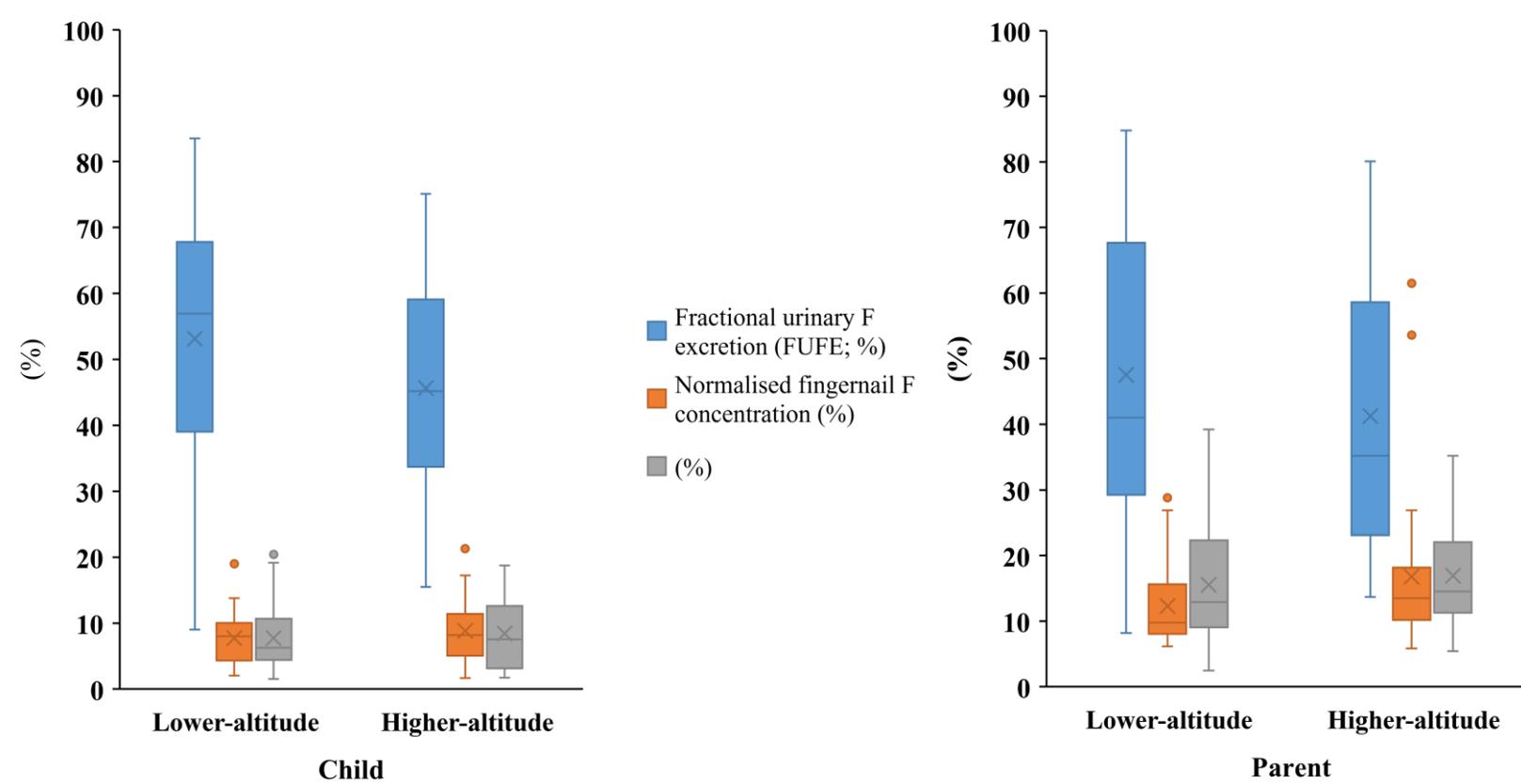
Figure 3. Box and whisker plots of fractional urinary F excretion and normalised F concentration in finger- and toe-nail in children and parents living at lower- and higher-altitudes**.**
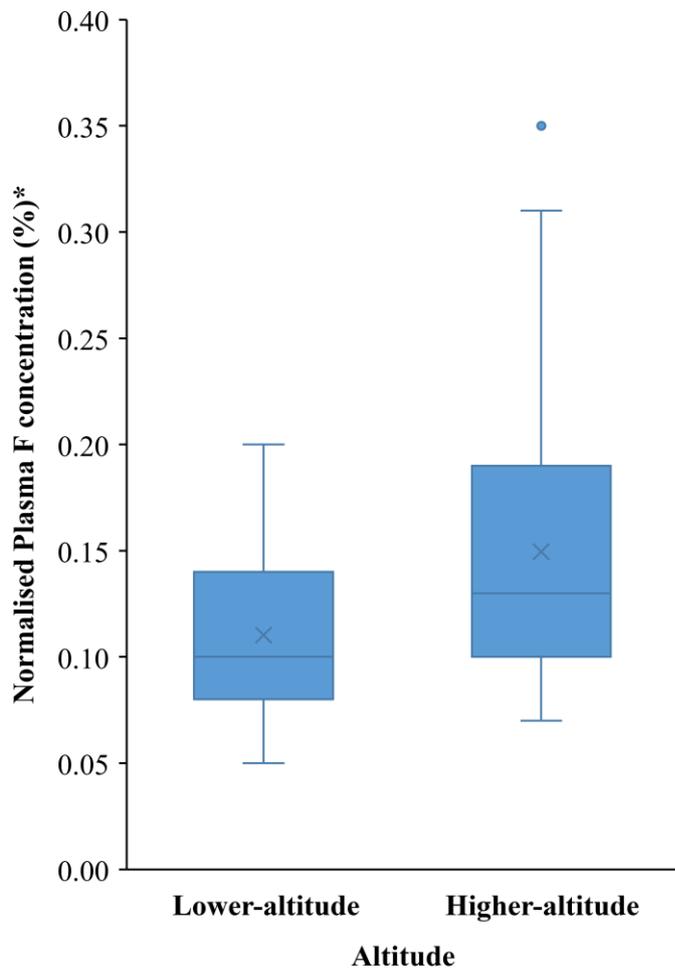
Figure 4. Box and whisker plots of normalised plasma F concentration in parents living in lower- and higher-altitude areas

* [(Plasma F concentration/TDFI) x 100]