Calcium-containing Eggshell Powder Supplementation to Mitigate Toxic Effects of Excess Fluoride Intake among Women in Ethiopian Rift Valley:

Efficacy, association, safety and acceptability

By

Demmelash Mulualem Zewdie

Dissertation for the degree of doctor of philosophy (PhD),

School of Public Health, College of Medicine and Health Sciences,

Hawassa University,

Ethiopia

October, 2024

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Efficacy, association, safety and acceptability

Dissertation Submitted to the School of Public Health, College of Medicine and Health Science, Hawassa University in Partial Fulfilment of the Requirements for the Degree of Doctor of Philosophy (PhD) in Public Health.

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October, 2024

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Declaration

I, Demmelash Mulualem Zewdie, hereby declare that this PhD dissertation is my original work and that to the best of my knowledge and belief, it has not been submitted or presented for the award of any other degree or diploma of the university or other institute of higher learning, and all source materials used in this PhD dissertation are duly acknowledged and cited appropriately.

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Date: **27/10/2024**

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Year : 2024

Title: Calcium-containing Eggshell Powder Supplementation to Mitigate Toxic Effects of Excess Fluoride Intake among Women in Ethiopian Rift Valley: Efficacy, Association, Safety and acceptability

Name: Demmelash Mulualem Zewdie

Print: Hawassa University, Ethioipa

DEDICATION

THIS PHD STUDY IS DEDICATED

TO

ALL MY BELOVED FAMILIES!

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(Submission sheet -1)

This is to certify that the dissertation entitled *"Calcium-containing Eggshell Powder Supplementation to mitigate toxic effects of excess fluoride intake among women in Ethiopian Rift Valley: Efficacy, association and safety and acceptability"* submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy (PhD) with specialization in public health, the Graduate Program of the Department/School of public health, and has been carried out by Demmelash Mulualem Zewdie (ID. No. PhDPh/0010/09), under my/our supervision. Therefore, I/we recommend that the student has fulfilled the requirements and hence hereby can submit the dissertation to the school.

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BY

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Publications and Conference presentations

These are the publications and scientific conference presentations related to the original PhD research.

Publications:

Paper1: Association of Dietary Calcium Intake with Dental, Skeletal and Non-Skeletal Fluorosis among Women in the Ethiopian Rift Valley. *Int. J. Environ. Res. Public Health* **2022**, *19*, 2119. https://doi.org/10.3390/ ijerph19042119

Paper2: Efficacy of Calcium-Containing Eggshell Powder Supplementation on Urinary Fluoride and Fluorosis Symptoms in Women in the Ethiopian Rift Valley. *Nutrients* **2021**, *13*, 1052. https://doi.org/ 10.3390/nu13041052

Paper3: Safety and acceptability of calcium-containing eggshell powder supplementation in the Phase 2 clinical trial among women in Ethiopian Rift Valley *(in manuscript)*.

Scientific Conference Presentations:

Poster presentation: Will eggshell calcium consumption decrease body fluoride load and mitigate dental and skeletal fluorosis among mothers in Ethiopian Rift Valley? The 8th Africa Nutrition Conference (ANEC VIII), Addis Ababa, Ethiopia, October 1-5, 2018.

Poster presentation: Using Eggshell calcium to mitigate fluorosis in Ethiopian Rift Valley. The International Nutrition and Food Industries Conference entitled "Partnership for Food and Nutrition Training and Sustainable Development" Hosted by the Academic Center of Excellence for Human Nutrition, College of Agriculture, Hawassa, University at Haile Resort, Hawassa, Ethiopia, 15th to 18th August 2018.

Oral (PPT) presentation: Dental, skeletal and non-skeletal fluorosis and association with dietary calcium intake among women in the Ethiopian Rift Valley. The 4th Federation of African Nutrition Societies (FANUS) conference entitled "Nutrition in Africa for Sustainable Development in Africa" held in Kigali Rwanda at LEMIGO Hotel, 26th to 30th August 2019.

Abbreviations and Acronyms

BMD	Bone Mineral Density
Ca	Calcium
$CaCO_3$	Calcium Carbonate
CSA	Central Statistical Agency
EPHI	Ethiopian Public health Institute
ESP	Eggshell Powder
F	Fluorine/Fluoride
Fe	Iron
FFQ	Food Frequency Question
Hct	Hematocrit
Hgb	Hemoglobin
IAEA	International Atomic Energy Agency
IOM	Institute of Medicine
KAP	Knowledge, Attitude and Practice
MDD-W	Minimum Dietary Diversity of Women
MoWR	Ministry of Water Resources
NNP	National Nutrition Program
NRC	National Research Council
PAHO	Pan American Health Organization
PTH	Parathyroid Hormone
RDA	Recommended Daily Allowance
SCHER	Scientific Committee on Health and Environmental Risks
SD	Standard Deviation
UL	Upper tolerable intake Level
WHO	World Health Organization
Zn	Zinc

Operational Definitions

Acceptability: The degree to which participants are willing to engage with and adhere to the treatment regimen (in this case, calcium-containing eggshell powder supplementation, assessed through checklist and empty bottle counts, as well as any reported difficulties or discomfort associated with the intervention).

Baseline: The initial set of measurements or observations collected from participants prior to the commencement of an intervention, used as a reference point for comparison with later data.

Calcium-Containing Eggshell Powder: A powdered supplement made from crushed eggshells that provides calcium, typically standardized to a specific amount of elemental calcium per serving (e.g., approximately 1000 mg of calcium per 2.4g of ESP).

Child-bearing Women: Women of reproductive age, generally defined as those between 15 and 49 years old, who may potentially conceive and bear children.

Clinical Symptoms: Observable or reported signs of a condition, such as pain, rigidity, or weakness, assessed through participant interviews, physical examinations, or questionnaires.

Dean's Index: A standardized method for classifying dental fluorosis based on visual examination of teeth, ranging from normal (no fluorosis) to severe (marked changes in enamel), often used in epidemiological studies.

Dental Fluorosis: A type of fluorosis affecting teeth, assessed through clinical examination using indices like Dean’s Index, characterized by discoloration, mottling, or pitting of tooth enamel due to excessive fluoride exposure during childhood.

Diet Diversity: A measure of the variety of different foods and food groups consumed over a specified time period, typically assessed using a dietary diversity score based on the number of unique food items or food groups included in an individual's diet within a given timeframe.

Dietary Calcium Source: Foods or supplements that provide calcium, measured in milligrams (mg), contributing to an individual's daily calcium intake, including dairy products, leafy greens, and calcium-fortified foods.

Dietary Calcium: The amount of calcium obtained through food and drink sources in a person’s diet, measured in milligrams per day using dietary recall or food frequency questionnaires.

Efficacy: The ability of a treatment (in this case, calcium-containing eggshell powder supplementation) to produce the desired therapeutic effect in reducing fluoride absorption and alleviating symptoms of fluorosis, measured through specific outcomes such as urinary fluoride excretion levels and improvements in clinical assessments of fluorosis.

Endline: The final set of measurements or observations collected from participants after the completion of an intervention, used to assess changes or outcomes resulting from the intervention.

ESP Supplementation: The daily intake of calcium-containing eggshell powder (ESP) as a dietary supplement, specified in grams per day, aimed at increasing calcium levels and potentially mitigating the effects of excessive fluoride exposure.

Fluoride Intake: The amount of fluoride consumed from all sources (e.g., drinking water, food, dental products), quantified in milligrams per day (mg/day), and usually assessed through dietary recall or food frequency questionnaires.

Fluoride: A naturally occurring mineral measured in milligrams per liter (mg/L) in water or food sources, known for its role in dental health but potentially harmful in excessive amounts, particularly related to fluorosis.

Fluorosis: A condition caused by excessive fluoride exposure, characterized by changes in tooth enamel (dental fluorosis) and potentially affecting bones (skeletal fluorosis), assessed through clinical evaluations, symptom reporting and diagnostic criteria

Hematocrit: The proportion of blood volume that is occupied by red blood cells, expressed as a percentage (%), determined through centrifugation of blood samples and used to assess overall blood health.

Hemoglobin: The concentration of hemoglobin in the blood, measured in grams per deciliter (g/dL), used as an indicator of oxygen-carrying capacity and overall health, assessed through blood tests.

Mitigation: The process of reducing the severity, impact, or prevalence of a condition or symptom, in this context referring to strategies aimed at decreasing the effects of fluoride exposure.

Non-Skeletal Fluorosis: Symptoms associated with fluoride exposure that do not directly involve bone health, such as muscular weakness and gastrointestinal issues, assessed through participant self-reports and clinical evaluations.

Physical Exercise Testing: Assessments designed to evaluate physical performance and functional capacity, often involving standardized exercises or activities, and measuring parameters such as endurance, flexibility, and strength.

Safety: The assessment of adverse effects or risks associated with the treatment, determined by monitoring the occurrence of side effects (e.g., nausea, vomiting, constipation) during the supplementation period, as well as measuring relevant biomarkers (e.g., iron and zinc levels in blood) to ensure no harmful impacts on participants' health.

Serum Iron: The concentration of iron in the blood, measured in micrograms per deciliter (μg/dL), assessed through blood tests to evaluate iron status and potential deficiencies in participants.

Serum Zinc: The concentration of zinc in the blood, measured in micrograms per deciliter (μg/dL), determined through blood tests to assess zinc status and nutritional adequacy in participants.

Skeletal Fluorosis: A bone condition resulting from chronic fluoride exposure, characterized by symptoms such as joint pain, stiffness, and skeletal deformities, assessed through physical tests and clinical symptoms.

Statistically Significant: A result that is unlikely to have occurred by chance alone, typically determined by a p-value threshold (e.g., $p < 0.05$) in statistical analyses, indicating that the observed effect or difference is likely to be real and not due to random variation.

Symptom Indices: Quantitative scales or scores used to evaluate the severity and frequency of symptoms associated with fluorosis, such as pain, rigidity, or weakness, based on participant reports or clinical assessments.

Urinary Calcium: The concentration of calcium excreted in urine, measured in milligrams per liter (mg/L), obtained through urine samples to assess calcium metabolism and dietary intake.

Urinary Creatinine: The concentration of creatinine in urine, measured in milligrams per liter (mg/L), used to evaluate kidney function and normalize other urinary measurements, such as calcium and fluoride excretion.

Urinary Fluoride Excretion: The amount of fluoride excreted in urine, measured in milligrams per liter (mg/L), used as an indicator of fluoride exposure and body burden, assessed through urine samples.

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The Scientific Environment

In 2015/16, a pilot study on egg and eggshell powder (ESP) consumption was carried out in two kebeles of *Halaba zone* (the former 'Special Woreda'), south Ethiopian Rift Valley, one intervention and one control kebele. The research was financially supported by ''One Health Grant'' University of Saskatchewan Canada. An MSc student from Hawassa University, **Anteneh Omer** was attached to this pilot study and did his master thesis on "Promotion of egg and eggshell powder consumption improve nutritional status of children of under two years of age, 2015/16", supervised by **Susan Whiting and Demmelash Mulualem**. The aim of the pilot study was to test whether an egg-a-day and ESP providing calcium would improve nutritional status of young children. Chickens were given to families who guaranteed that eggs would be fed to the child in the intervention kebele, along with education on poultry production and promotion of eggs for children. ESP was encouraged for use as a calcium supplement to children ≥ 1 year and knowledge, attitude and practice (KAP) of caregivers on preparation and feeding of egg and ESP to their children were assessed. The control kebeles continued with existing nutrition education for the 6-month trial.

Poultry production and egg consumption by children were significantly improved only in the intervention kebele. ESP consumption also got community acceptance (Omer et al., 2018). Egg and ESP consumption among children in the intervention kebele averaged 17 days/month for six months. The KAP assessment of mothers on egg and ESP preparation and feeding of their children improved only in the intervention group. Linear regression analysis showed the egg and ESP intervention increased weight-for-age z-score and reduced underweight (Omer *et al.,* 2019). Therefore, we concluded that promotion of egg consumption with poultry intervention as an

ideal animal source food needed by young children (6-24 months) significantly improved their nutritional status. In our previous study, we promoted chickens to rural farmers as eggs are culturally acceptable food. And with this promotion, eggshells were available. Thus, mothers were taught how to use ground eggshell and mixed with food or drink. They have been accepting it (Omer et al., 2019). Calcium is a limiting nutrient in most of the country, especially for women of reproductive age (Tesfaye et al., 2018). Diet diversity is poor and sources of calcium such as millet, milk, and green vegetables are not consumed frequently, and thus ESP was viable source of calcium.

We learnt from a PhD dissertation entitled "Assessment of Fluoride Intake in Endemic Areas of Ethiopia and Potential Use of Calcium Rich Foods in Mitigating Ingested Fluoride" by **Aweke Kebede, Addis Ababa University**, supervised by **Marian (Malde) Kjellevold of Bergen, Norway** that calcium is necessary to mitigate fluorosis. The authors observed excess daily intake of fluoride and high prevalence of dental, skeletal and non-skeletal fluorosis among school age children in rural Ethiopian Rift Valley (Halaba, Adamitulu and Fentale) (Kebede *et al.,* 2016a). The authors also demonstrated that supplementation of calcium-magnesium or *Moringa stenopetala* leaf in reduced urinary fluoride and increased fecal fluoride levels in rats, indicating less intestinal absorption of fluoride which provides evidence of fluoride-calcium binding as a dietary approach in reducing fluorosis (Kebede *et al.,* 2016b).

To our knowledge, the findings by Kebede et al (2016b) are the first to show dietary calcium reduces urine fluoride and increases fecal fluoride in a rat model, indicating poor fluoride uptake. A small trial has shown calcium reduces urine fluoride in humans using calcium pills (Mehta and

Shah, 2013). Instead of pills or expensive dairy, we were planning a local food-based solution. Our innovation is to use an age-old source of calcium, eggshell (2000 mg/egg), as dietary calcium to bind fluoride from food and beverages, preventing its absorption. Sustainability of eggshell use would occur with introduction of chickens into communities, as this has financial benefits. The impact of eggshell use could be measured in fluorosis-affected areas as decreased body fluoride load (reflected in less urine fluoride excretion) and in mitigation of fluorosis symptoms

Consequently, in 2017, we applied for and got a grant from Grand Challenges Canada, GCC (R-ST-POC-1707-05521). The project short title was "Using eggshell calcium to mitigate fluorosis in Ethiopia". The amount was $100,000 CND with a strict deadline of not exceeding December 31, 2018. In this project Demmelash Mulualem and Anteneh Omer were designated PhD students; Susan J Whiting was the project lead, and Eskindir Loha Shumbullo (local PI), Aweke Kebede (later replace by Masresha Tesema), Carol J. Henry and Wendy Dahl were also the project collaborators.

The project thematic area was "Effects of egg and eggshell calcium consumption in improving nutritional status and mitigation of dental and skeletal fluorosis among children and mothers in Ethiopian Rift Valley, 2017/18". This project theme was sub-divided into three sub-themes: 1) Effects of calcium-containing eggshell powder consumption to mitigate toxic effects of excess fluoride intake among women in *Halaba* district, Ethiopian Rift Valley, the PhD research of Demmelash Mulualem at Hawassa University 2) Promotion of egg consumption in improving nutritional status and developmental milestones among young children in *Halaba* district,

Ethiopian Rift Valley, the PhD research of Anteneh Omer at Hawassa University 3) Dietary fluoride and calcium intake, dental and skeletal fluorosis level and associated factors among school age children in *Halaba* district, Ethiopian Rift Valley, the MSc research of Nahom Tefera at Addis Ababa University (who, with Masresha Tessema, was responsible for biological, food and water samples analysis at Ethiopian Public Health Institute, EPHI). The three concurrent studies were conducted at the same time and place but on different study subjects. This document is only about the PhD research of Demmelash Mulualem.

Therefore, this research work is a result of collaboration between different institutions and funding organizations. The study involved different researchers from the following institutions:

1) College of Medicine and Health Sciences, Hawassa University, Hawassa, Ethiopia
2) College of Pharmacy and Nutrition, University of Saskatchewan, Saskatoon, Canada
3) College of Agriculture, School of Human Nutrition, Hawassa University, Hawassa, Ethiopia
4) Ethiopian Public Health Institute (EPHI), Addis Ababa, Ethiopia

Summary

Background: Worldwide, 50 million people suffer from fluorosis, which affects not only teeth, but also bones, joints, gut and brain functions. In Ethiopia, where defluoridation requires costly infrastructure, more than 14 million individuals, mainly in the Rift Valley, are affected by fluorosis. Drinking water sources, in the Rift Valley of Ethiopia, contain fluoride (F) levels exceeding the World Health Organization (WHO) limit of 1.5mg/L. F exposure may be an added concern for women's bone and dental health where there is low Ca intake. Studies suggest that the adverse effects of F can be reversed or lessened by providing sufficient food intake of protein, calcium (Ca), anti-oxidants and vitamin D. Of these, Ca is the most studied by ecological studies. However, there have been no intervention studies of Ca to mitigate fluorosis at the community level in Ethiopia. We therefore hypothesized that supplementation of an age-old, sustainable and low cost source of Ca, i.e., eggshell, as a dietary Ca source would mitigate the toxic effects of excess F intake in women. Thus, the aim of this study was to test the efficacy of calcium-containing ESP supplementation, as a proof of concept, to reduce F absorption as measured by urinary F excretion (a primary outcome measure) and mitigation of fluorosis symptoms (secondary outcomes) in women living in a fluorosis-affected area. The overall objective of this PhD research was to assess efficacy, association and safety and acceptability of calcium-containing eggshell powder supplementation to mitigate toxic effects of excess fluoride intake among women in Ethiopian Rift Valley.

Methods: Women (n=270) from two villages provided clinical and questionnaire data for the cross-sectional survey. Dental fluorosis examination was done using Dean's Index, and skeletal and non-skeletal fluorosis assessment was carried out using physical tests and clinical symptoms.

Daily Ca intake was estimated by a food frequency questionnaire. Food, drinking water and beverage samples were analyzed for F level. Eighty-two women (41 Intervention Group, IG; 41 Control Group, CG) were recruited for the Phase II clinical trial; 39 in each group completed the trial. Morning spot urine was collected for testing fluoride (F), calcium (Ca) and creatinine concentrations, before (baseline, BL) and after (endline, EL) the intervention that was 6-months daily supplementation with 2.4 gram calcium-containing eggshell powder/ESP (providing ~1000 mg of calcium). Dental, skeletal and non-skeletal fluorosis assessments were carried out at BL and, except for dental, at EL. For the purpose of safety and acceptability assessment, body retention of iron and zinc were measured using blood levels, prior to and after the calcium-containing ESP supplementation among the study subjects. Occurrence of side effects such as nausea, vomiting, constipation, and abdominal bloating and gas related to excess calcium intake in the Phase II trial were assessed using checklists on a monthly period. In addition, blood tests for malaria, hemoglobin and hematocrit were done immediately in the field. Descriptive statistics, bivariate and multivariable logistic regression, relative risk (RR), paired samples t-test and two independent samples t-test, linear generalized estimating equation (GEE) and multivariate analysis of the GEE model were used to analyze and compare outcomes between groups.

Results: Many subjects (56.3%) exhibited dental fluorosis. Women having ≤ 400 mg/day Ca intake had ~3 times greater odds of developing skeletal rigidity with joint pains [AOR=2.8, 95%CI: 1.6, 5.0] and muscular weakness [AOR=2.9, 95%CI: 1.3, 6.3] compared to those with higher intakes. At EL, women in the IG had about six-fold lower urinary F excretion [β = - 6.1 (95% CI: -7.1, -5.1)] compared to women in the CG. The risk of developing skeletal fluorosis

tested using the ability to bend body and touch floor or toe [RR = 0.21 (95% CI: 0.07, 0.69)], and stretch and fold arms to touch back of head [RR = 0.18 (95% CI: 0.04, 0.77)] were significantly reduced in the intervention group by 79% and 82% respectively compared with the control. Majority of the women in IG reported mitigation of pain and muscular symptoms of non-skeletal fluorosis ranging from lowest RR = 0.17 (95% CI: 0.05, 0.52) to highest RR = 0.59 (95% CI: 0.39, 0.88) after the calcium-containing ESP supplementation than in CG.

Conclusion: Signs and symptoms of dental, skeletal and non-skeletal fluorosis were prevalent in women of child-bearing age in this area of the Rift Valley of Ethiopia. As low dietary Ca intake was significantly associated with symptoms related to skeletal and non-skeletal fluorosis, this warrants nutritional intervention on calcium intakes in this setting. A significant reduction in urinary F excretion and reduction in many fluorosis symptoms were observed among women supplemented with calcium-containing ESP, thus providing evidence for using this dietary calcium (ESP) source for mitigation of fluorosis. Safety and acceptability assessments conducted as part of the study indicated that calcium-containing eggshell powder supplementation was generally accepted, well-tolerated and did not pose significant risks to the health of participants. The potential feasibility, sustainability and safety of using home prepared crushed eggshells in areas where fluorosis is endemic need to be studied.

Clinical trials registration: NCT03355222

Keywords: Calcium; Fluoride; Fluorosis; Eggshell powder; Safety; Acceptibility; Women; Ethiopia; Rift Valley.

1. Introduction

1.1. Background

Worldwide, 50 million people suffer from fluorosis, which affects not only teeth, but also bones, joints, gut and brain functions (Tandon *et al.,* 2015). In Ethiopia, where defluoridation requires costly infrastructure, more than 14 million individuals, mainly in the Rift Valley, are affected by fluorosis (Dahi, 1996; Fawell *et al.,* 2006; Tekle-Haimanot *et al.,* 2006; Demelash *et al.,* 2019).

Drinking water sources, in the Rift Valley of Ethiopia, contain fluoride (F) levels exceeding the World Health Organization (WHO) limit of 1.5mg/L (WHO, 2008; Kebede *et al.,* 2016a). As a consequence, many people living in the region are severely affected by fluorosis (Kebede *et al.,* 2016a; Assefa *et al.,* 2004; Kravchenko *et al.,* 2013; Melaku *et al.,* 2012; Tekle-Haimanot and Haile, 2014; Demelash *et al.,* 2019; Wondwossen *et al.,* 2004). In serious cases, dental fluorosis is manifested as brown mottling of the enamel, and results in overall yellowing of the teeth with erosion of the enamel (Kebede *et al.,* 2016a; Wondwossen *et al.,* 2004). Skeletal fluorosis occurs when there is a high degree of bone brittleness due to excess deposition of F in bone that leads to osteosclerosis (Assefa *et al.,* 2004; Tekle-Haimanot and Haile, 2014; Tandon *et al.,* 2015; Dhuna *et al.,* 1992; McDonagh *et al.,* 2006; Shashi *et al.,* 2008). Non-skeletal fluorosis includes muscle weakness manifestations such as stiffness of the back and neck muscles and pain leading to inability to carry out routine domestic activities (Kebede *et al.,* 2016a; Tandon *et al.,* 2015). Research suggests that the adverse effects of fluoride (F) can be reversed or lessened by providing sufficient food intake of protein, calcium (Ca), anti-oxidants and vitamin D (Susheela and Bhatnagar, 2002; Vasant and Amaravadi, 2013; Rango *et al.,* 2012). Of these, Ca is the most

studied by ecological studies (Rango *et al.,* 2012; Teotia *et al.,* 1998; Malde *et al.,* 2004; Kebede *et al.,* 2016a).

Epidemiological studies of communities with similar F exposures have shown a relationship between calcium (Ca) intake and reduction in severity of dental fluorosis (Kebede *et al.,* 2016a; Malde *et al.,* 2004; Teotia *et al.,* 1998; Susheela and Bhatnagar, 2002). The Ca binds with F forming an insoluble Ca fluoride complex in the gastrointestinal tract, preventing absorption and reducing the extent of F exposure (Kebede *et al.,* 2016b). In this way the adverse effects of F are decreased. Two animal studies have shown proof of principle (Kebede *et al.,* 2016b; Pius and Viswanathan, 2008). To our knowledge, only one study has reported an inverse association between dietary Ca (milk) and severity of dental fluorosis in individuals in Ethiopia (Assefa *et al.,* 2004) and no one has reported the association between Ca intake and the severity of skeletal and non-skeletal fluorosis.

Only a few studies have provided evidence of a reduction of F absorption with dietary Ca supplementation. A total of two animal studies (Pius and Viswanathan, 2008; Kebede *et al.,* 2016b) examined aspects of the mechanism and dose-responsiveness of Ca intake to reduce urinary F. A small human trial of 10 per group (Mehta and Shah, 2013) found decreased urinary F excretion. Ca binds with F forming calcium-fluoride, which is insoluble in the gastrointestinal tract, preventing absorption (measured as a fall in urinary F) and therefore reducing the extent of F exposure. In this way it is postulated that the adverse effects of F are decreased and/or do not worsen with Ca intake. However, there have been no intervention studies of Ca to mitigate fluorosis at the community level in Ethiopia.

Defluoridation technologies have been implemented in pocket areas however such technologies require costly infrastructure and the techniques did not remove fluoride as much as intended (Dahi, 1999). In addition, ever increasing number of the population, financial constraints, and pollution of surface water and lack of appropriate technology for defluoridation is posing pressure on water quality management in Ethiopia (Reimann *et al.,* 2002 and 2003). So we need to find ways to reduce the impact of environmental fluoride in a country where malnutrition is a huge problem and livelihoods are dependent on manual labour. Nutritional intervention is a new approach to mitigate ingested fluoride and to reduce the detrimental effect of fluorosis. For example, locally available calcium and anti-oxidant rich foods such as moringa leaf (Babu, 2000), calcium carbonate (Pius and Viswanathan, 2008) and calcium and magnesium-containing vegetable (Kebede *et al*., 2016b) might help in mitigating ingested fluoride.

However, calcium is a limiting nutrient in most of the country, especially for children and women of reproductive age. In addition, diet diversity is poor and sources of calcium such as milk, millet and green vegetables are not consumed frequently (Kebede *et al*., 2016a; Tezera *et al*., 2017). Studies also showed that many micronutrients including calcium are low in Ethiopian diet with children and women are affected the most (Abebe *et al*., 2007; Owino *et al*., 2007). Our innovation is to use an age-old, sustainable, acceptable and low cost source of calcium, eggshell (2000 mg/egg), as dietary calcium source. The impact of eggshell calcium use can be measured in fluorosis-affected areas as decreased body fluoride load and in mitigation of fluorosis symptoms.

We therefore hypothesized that supplementation of an age-old, sustainable and low cost source of Ca, i.e., eggshell, as a dietary Ca source (Bartter *et al.,* 2018) would mitigate the toxic effects of excess F intake in child-bearing women. High F exposure may be an added concern on women's bone, dental and overall health where there is low Ca intake such as in Ethiopia (Tesfaye *et al.,* 2018) and where water F is greater than the WHO limit of 1.5 mg/L (Demelash *et al.,* 2019; Kebedeet *et al.,* 2016a; WHO, 2008). Thus, the aim of this study was to assess the dental, skeletal and non-skeletal fluorosis symptoms and associations with dietary Ca intake; and to test the efficacy of calcium-containing ESP supplementation, as a proof of concept, to reduce F absorption as measured by urinary F excretion (a primary outcome measure) and mitigation of fluorosis symptoms (secondary outcomes) in women living in a fluorosis-affected area.

1.2. Statement of the Problem

Fluorosis is a major public health concern particularly for developing countries including Ethiopia, as the infrastructure needed to remove the excess F ions is lacking or not widely accepted (Wondwossen *et al.,* 2004; Almebo *et al.,* 2021). It can affect both children and adults (Wondwossen *et al.,* 2004). Over 85% of Ethiopians living in the Rift Valley have been exposed to excess levels of F intake (Demelash *et al.,* 2019; Fawell *et al.,* 2006; Tekle-Haimanot *et al.,* 2006). Although drinking water is the dominant pathway of F exposure in Ethiopian Rift Valley areas, food stuffs prepared with high F cooking water is also an additional pathway (Malde *et al.,* 2004). Foods added an average of 2.3 to 4.8 mg/kg F depending on village location and type of foods consumed, both plant and animal foods are locally grown in this high F environment (Kebede *et al.,* 2016a; Malde *et al.,* 2004).

Fluorosis is a significant public health issue globally, characterized by the excessive accumulation of fluoride in the body, which can lead to severe health complications. The impact of fluorosis varies by region, largely influenced by local fluoride exposure sources, water quality, and public health infrastructure.

In Ethiopia, particularly in the Rift Valley region, fluorosis is a major concern due to high fluoride concentrations in groundwater. The region's geological formations lead to elevated fluoride levels in drinking water, resulting in both dental and skeletal fluorosis among the population (Girma *et al.,* 2014). The problem is exacerbated by limited access to alternative water sources and inadequate public awareness about the risks of fluoride (Tadesse *et al.,* 2017). Fluorosis in Ethiopia has significant health implications, including dental discoloration, enamel damage, and in severe cases, skeletal deformities. These health issues not only affect individuals'

quality of life but also place a burden on the healthcare system due to the need for treatment and management of chronic conditions (Wondwosen *et al.,* 2020).

India is one of the most fluorosis-affected countries in the world, with widespread issues in states such as Uttar Pradesh, Rajasthan, and Andhra Pradesh. In these areas, natural fluoride levels in groundwater often exceed the recommended limits, leading to high incidences of both dental and skeletal fluorosis (Susheela, 2015). Like Ethiopia, the problem is exacerbated by reliance on groundwater and insufficient water treatment infrastructure. India faces severe health consequences from fluorosis, including widespread dental and skeletal problems. The Indian government has implemented various mitigation strategies, including defluoridation of drinking water and promotion of alternative water sources, yet challenges remain in effectively managing and reducing fluoride exposure (Nair *et al.,* 2017).

China has a significant burden of fluorosis, particularly in the provinces of Henan, Shanxi, and Shaanxi. The sources of fluoride contamination in China include both natural sources and industrial emissions. The Chinese government has made substantial efforts to control fluoride levels in drinking water, but areas with high natural fluoride concentrations still experience high rates of fluorosis (Zhao *et al.,* 2018). Fluorosis in China has led to considerable public health concerns, with numerous cases of dental and skeletal fluorosis reported. The Chinese government has implemented extensive public health programs to address the issue, including water treatment initiatives and community health education (Li *et al.,* 2016).

In contrast to developing countries, the United States experiences fluorosis mainly in the context of fluoride added to drinking water for dental health benefits. While the levels are generally controlled and regulated, excessive fluoride intake due to overfluoridation has led to cases of mild dental fluorosis (Beltrán-Aguilar *et al.,* 2013). This is less severe compared to the systemic and skeletal effects seen in countries with high natural fluoride concentrations. The public health impact in the U.S. is relatively limited, with most cases of fluorosis being mild and primarily affecting dental appearance. Public health strategies in the U.S. focus on balancing fluoride benefits for dental health while minimizing the risk of fluorosis (CDC, 2019).

In Sub-Saharan Africa including Ethiopia, widespread malnutrition is compounded by fluorosis. About 14 million Ethiopians have fluorosis (Feleke and MOWR, 2012; UNICEF, 2015). More than 90% of the drinking water sources in Ethiopian rift valley contained fluoride above the WHO recommended value (1.5mg/L) (WHO, 2008) .The daily dietary fluoride intake in these areas was above tolerable daily intake. On the other hand, dietary calcium intake is below the RDA. For example, 30% of children in *Halaba* district, southern Ethiopia had dietary calcium intake below the RDA and the prevalence of severe and moderate dental fluorosis was 20% (Kebede *et al*., 2016a).

Dental caries due to dental fluorosis is one of the most common diseases in humans, and is mediated by bacteria, mainly Streptococcus mutans (S. mutans). It has been estimated to affect between 60 and 90% of school children in the developed world and the majority of adults (Petersen and Lennon, 2004), and is consequently a major public health concern. In addition to the developed world, it is the most widespread oral disease in many Asian and Latin American

countries, though it currently appears to be less of a problem in Africa (Wondwosen *et al*., 2004). However, exposure to fluoride, changes in living conditions and increases in sugar consumption within a changing diet suggest that, in Africa too, the incidence of caries will increase, and that it will become a major public health issue (Tressaud and Haufe, 2008).

Ecological studies show diet calcium is related to less severe fluorosis but no one has conducted an intervention of calcium at the community level. An animal study found calcium equivalent to human RDA levels reduces fluoride absorption by 30% (Pius and Viswanathan, 2008; Kebede *et al*., 2016b). But calcium is one of the nutrients that is often lacking in diets in many areas of Africa including Ethiopia (Thacher *et al*., 1997; EPHI, 2013; Tezera *et al*., 2017).

Many researchers contend that the toxic effects of fluoride can be reversed by withdrawing the fluoride source and by providing a diet adequate in protein, calcium, anti-oxidants and vitamin D (Susheela and Bhatnagar, 2002; Rupal and Narasimhacharya, 2011). However, this remains to be demonstrated. In addition, evidence suggests that increased intake of dietary calcium or fluoride reducing treatments are key strategies to address widespread dental and skeletal fluorosis faced by millions of poverty-stricken rural residents in Ethiopian rift valley (Tewodros *et al*., 2012). So we have a sustainable, acceptable and low cost dietary calcium source in a developing country which is Eggshell. It is probably the best natural source of calcium and it is well-absorbed by the human body (Bee, 2011).

Eggshells have been used to provide calcium in other settings (Schaafsma *et al*., 2000; Rovenský *et al*., 2003; Brun *et al*., 2013). A chicken eggshell has 2.07 ± 0.18g of calcium which is a much better source of calcium than limestone or coral sources (Brun *et al*., 2013). The mineral profile

of eggshell represents a unique combination of essential nutrients for bone and dental health. It provides not only a highly concentrated source of calcium, but also provides relevant levels of other trace minerals including strontium, magnesium, zinc and copper and organic matrix consisting mainly of glycoprotein and proteoglycans that contribute to bone and dental health. In addition, unlike eggshell calcium, other calcium products sold in the market are mostly either mined or chemically produced from mined calcium carbonate. Such products have the potential to cause digestive problems such as constipation, nausea and bloating (Schaafsma *et al*., 2000).

Cultural practices surrounding food and dietary supplements can influence how easily eggshell powder can be integrated into daily routines. In cultures where eggs are not a staple part of the diet, or where there are specific dietary restrictions, incorporating eggshell powder might be challenging (Martin *et al.,* 2016). In many cultures, women are primarily responsible for household nutrition and health decisions. Therefore, understanding the role of women in dietary practices is crucial. If eggshell powder is to be promoted as a supplement, it must align with women's cultural practices and preferences. Educational programs should be designed to address the specific needs and concerns of women, ensuring that the benefits and preparation methods of eggshell powder are communicated effectively (Rashid *et al.,* 2020).

This study is essential due to several pressing health concerns. Fluoride exposure, particularly in endemic areas, is linked to significant health issues, including dental and skeletal fluorosis, which are particularly concerning in vulnerable populations such as women (Kumar et al., 2019). The Ethiopian Rift Valley is known for high fluoride levels in drinking water, necessitating effective mitigation strategies (Aklilu et al., 2020).

Calcium supplementation has been proposed as a potential protective mechanism against fluoride toxicity, as calcium can compete with fluoride for absorption in the body (Choubisa, 2018). Eggshell powder, a readily available source of calcium, presents a cost-effective and sustainable solution that could improve dietary calcium intake among women in the region. Furthermore, understanding the efficacy, safety, and acceptability of this supplementation is critical for public health interventions and could inform policies aimed at reducing fluoride exposure and its associated health risks.

The need for this research is underscored by the potential for significant health improvements among affected populations, contributing to broader efforts in nutritional and environmental health. Therefore, this study aims not only to address immediate health concerns but also to provide a framework for future interventions in similar contexts.

1.3. Significance of the Study

Studying the potential of eggshell powder supplementation to mitigate fluorosis in women is of significant importance due to the prevalence of fluorosis in this area with high fluoride concentrations in water sources. Fluorosis is a chronic condition caused by excessive fluoride intake, leading to dental, skeletal, and systemic health issues. Eggshell powder, rich in calcium carbonate, presents a promising avenue for combating fluorosis by binding with fluoride ions and reducing their absorption in the body (Khandare *et al.,* 2010). Research in this area is essential for several reasons. Firstly, it addresses a pressing public health concern, particularly in areas where water sources contain high levels of fluoride. Secondly, it provides an accessible and cost-effective intervention that could benefit populations at risk of fluorosis, especially women who may be more susceptible due to factors such as pregnancy and menopause.

Eggshell powder supplementation offers additional benefits beyond fluoride binding. It provides a source of bioavailable calcium, which may help counteract the demineralizing effects of fluoride on bones and teeth, thus promoting skeletal health. Moreover, studying eggshell powder supplementation in women holds significant importance for health and nutrition:

Calcium Supplementation: Eggshell powder is a rich source of calcium carbonate, which is easily absorbed by the body. Calcium is crucial for maintaining bone health, especially in women who are more prone to osteoporosis and bone fractures, particularly post-menopause. Studies have shown that adequate calcium intake can reduce the risk of osteoporosis and related fractures (Harinarayan *et al.,* 2008; Lopez, 2017).

Bone Health: Research suggests that eggshell powder supplementation can improve bone mineral density (BMD) and bone strength (Genaro and Martini, 2014). A study by Lopez et al. (2008) demonstrated that eggshell powder supplementation in postmenopausal women increased BMD, reducing the risk of fractures and osteoporosis.

Nutritional Benefits: Eggshell powder contains not only calcium but also other essential minerals such as magnesium, phosphorus and trace elements, which contribute to overall bone health and metabolic functions (Rovenský *et al.,* 2003). These minerals play vital roles in bone health and overall wellbeing. The inclusion of these nutrients in eggshell powder supplementation can contribute to better nutrient absorption and utilization in the body (Kwak *et al.,* 2018). Studies have suggested that incorporating eggshell powder into the diet can improve calcium absorption and retention compared to synthetic calcium supplements, indicating its potential as a natural and effective alternative (Itokawa *et al.,* 1999). The bioavailability of calcium in eggshell powder has been demonstrated to be comparable to or even better than traditional calcium supplements, making it a promising option for addressing calcium deficiency in women, especially those at risk of osteoporosis (Ceglia, 2009).

Natural and Sustainable Source: Eggshell powder is a natural byproduct of egg processing, making it a sustainable and cost-effective source of calcium supplementation. Utilizing eggshell waste in this manner reduces environmental pollution and promotes sustainability in food production (Tavafi *et al.,* 2013).

Potential for Disease Prevention: Besides bone health, calcium supplementation through eggshell powder may offer additional health benefits. Adequate calcium intake has been linked to a reduced risk of various health conditions, including hypertension, cardiovascular disease, and certain types of cancer. Therefore, studying the effects of eggshell powder supplementation may provide insights into its potential role in preventing these diseases (Zhu *et al.,* 2015; García-González *et al.,* 2015).

In conclusion, eggshell powder supplementation in women has crucial significance for public health interventions, particularly in areas where fluorosis prevalence is high. By providing a cost-effective and accessible intervention, eggshell powder supplementation has the potential to improve the health outcomes of individuals affected by fluorosis, especially women who may be more susceptible to its adverse effects. In addition, it offers promising approaches for improving bone health, enhancing nutrient intake, and potentially preventing various health conditions. Therefore, the main rationale of this study is to use eggshells as dietary calcium source to bind fluoride from food and beverages, preventing its absorption. This will then help in decreasing body fluoride load which in turn will mitigate fluorosis symptoms.

2. Literature Review

Fluoride is a naturally occurring mineral found in various sources, including water, soil, air, and some foods. The primary route of fluoride intake for humans is through drinking water (CDC, 2001), although it can also be obtained from dental products, certain foods, and environmental exposure. Excessive fluoride intake, especially during tooth development, can lead to a condition known as dental fluorosis, characterized by the mottling or discoloration of tooth enamel (CDC, 2001). The severity of fluorosis depends on the level and duration of fluoride exposure during tooth development, with higher levels of fluoride intake associated with more severe forms of fluorosis (Marthaler, 2004; Choi *et al.,* 2012). To mitigate the effects of fluorosis, various interventions have been explored, including the use of eggshell powder (Khan *et al.,* 2015). Eggshell powder is rich in calcium and magnesium, which have been shown to bind to fluoride ions and reduce their absorption in the body. Studies have demonstrated the potential of eggshell powder to mitigate the severity of fluorosis by reducing fluoride absorption and improving bone health (Peckham and Awofeso, 2014).

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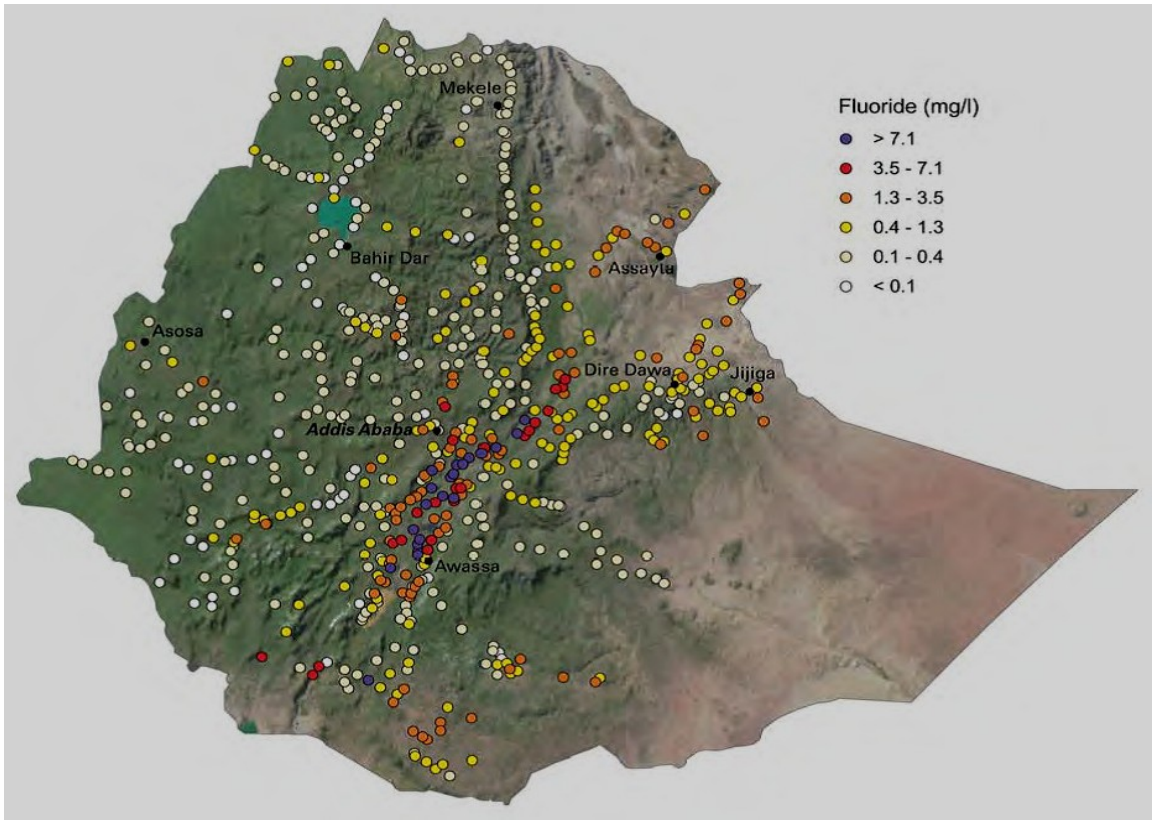
2.1. Fluoride Source and Intake

Fluorine is a nonmetallic univalent element belonging to the halogens family. It is usually a pale yellow green, irritating toxic flammable gas; a powerful oxidizing agent recovered from fluorite (CaF_2), cryolite ($3NaF.AlF_3$) or fluorapatite ($3Ca_3(PO_4).2Ca(F,OH,Cl)_2$ (WHO, 2004; Rappoport and Liebman, 2004). Elemental fluorine has a sharp odour and atomic mass of 18.998. The term ‘fluorine’ is used to denote the element in any of its forms and ‘fluoride’ to denote free inorganic fluoride to which a fluoride ion-selective electrode (ISE) responds during fluoride analysis (Greenwood and Earnshaw, 1997; Emsley, 2011).

Fluoride is the most electronegative and reactive of all the elements and as a result, elemental fluorine does not occur in nature, but is found as fluoride mineral complexes (WHO, 2004). Fluorine is widely distributed in the lithosphere mainly as fluorspar, fluorapatite and cryolite, and is recognized as the 13th most common element in the earth's crust. It is found in seawater at a concentration of around 1.2 - 1.4 mg/liter, in ground water at concentrations up to 67 mg/liter, and in most surface water at concentrations less than 0.1 mg/liter (Burgos *et al.,* 2001; Kharaka and Hanor, 2007; Amini *et al.,* 2008). Fluoride is also found in foods particularly fish and tea (IPCS, 2002). Whilst almost all foodstuffs contain at least traces of fluoride, water and non-dairy beverages are the main sources of ingested fluoride. Fluorides are ubiquitous in nature and are present in rocks, soil, water, plants, foods and air (Fawell *et al*., 2006; NRC, 2006).



(**Source:** Dr Aweke Kebede's PhD Dissertation)

Figure 1: Water fluoride distribution in areas of Ethiopia (RiPPLE, 2008)

2.1.1. Dietary Sources of Fluoride

Drinking water is typically the largest single contributor to daily fluoride intake (WHO, 2002; Fawell *et al*., 2006; NRC, 2006; Iheozor-Ejiofor *et al.,* 2015). However, fluoride intake from other sources is also considerable, especially from food (Toyoda and Taira, 2000; Malde *et al*., 1997; Moynihan and Petersen, 2004). Han and his colleagues (1995) found that food is the main causative factor for fluorosis in areas with low fluoride concentration in the drinking water. Although the fluoride content of most foods is low (less than 0.05 mg/100 grams or 0.5 ppm), high fluoride concentration is often found in food ingredients rich in minerals and trace elements. Teff flour, for instance, contains high level of fluoride, iron, zinc and calcium (Maage *et al*., 1996; Moynihan and Petersen, 2004). Rich sources of fluoride include tea, which concentrates fluoride in its leaves, and marine fish that are consumed with their bones (e.g., sardines). Foods made with mechanically separated (boned) chicken, such as canned meats, hot dogs, and infant foods, also add fluoride to the diet (Fein *et al*., 2001; Kanduti *et al.,* 2016). In addition, certain fruit juices, particularly grape juices, often have relatively high fluoride concentrations (Kiritsy *et al*., 1996; Levy *et al.,* 2000; Choubisa, and Choubisa, 2014).

Tea plants are found to have high fluoride uptake and 97% of it gets accumulated in leaves (Shu *et al*., 2003). The fluoride content of tea leaves is about 1,000 times the soluble fluoride content of soil and 2 to7 times the total fluoride content in soil (Fung *et al*., 1999). Zerabruk and his colleagues (2010) in their assessment of tea leaves brewed in low fluoride containing water found enormous amount of fluoride level in local market in Ethiopia.

According to the weight of evidence obtained from WHO (2004) for children, total fluoride intake via food and water is decreased because of lower consumption. An adult male residing in a community with fluoridated water has an intake range from 1-3 mg/day. Intake is less than 1 mg/day in non-fluoridated areas (Nielsen, 1999). In fluoride contaminated areas estimation of total dietary fluoride intake in individuals may be based either on analysis of raw food ingredients (Malde *et al*., 1997) or analysis of selected prepared food dishes (Zohouri and Rugg-Gunn, 2000). The major sources of fluoride for human exposure are water, food, beverage, tooth paste, fluoride supplements and infant formulas (Moynihan and Petersen, 2004; Fawell *et al*., 2006; EFSA, 2008a; Choubisa, and Choubisa, 2014; Iheozor-Ejiofor *et al.,* 2015).

2.1.2. Fluoride Intake of Children

Small amounts of fluoride have been proven to be effective in preventing dental caries, but excessive, chronic intake by young children can result in the development of dental fluorosis; the critical period of exposure for all permanent teeth being between 11 months and 8 years of age (Do and Spencer, 2007; Fejerskov *et al*.,2008). The main sources of fluoride intake in children are diet (food, beverage and water), fluoride-containing dentifrices and fluoride-containing supplements. Intake from fluoridated dental products can be substantial, particularly in young children who have poor control of swallowing (Wong *et al.,* 2010; Awadia and Awooda, 2013). Intake estimates were generally lower than expected in the studies where duplicate diet technique, which is the most accurate technique of estimating actual daily intake, was used. Fluoride ingestion from toothpastes is also common and often relatively substantial. Children (7 years and under) ingest, on average, 25–38% of toothpaste per brushing (Levy *et al*., 1995; Levy and Warren, 2001; Buzalaf and Pessan, 2011). The amount of toothpaste used by children per

brushing was determined to be 0.7 g on average. The amount was considerably higher than the commonly recommended 'pea size' of 0.25 g (EFSA, 2005).

2.1.3. Fluoride Intake of Adults

Accurate estimates of fluoride intake are important in order to resolve potential problems of too low or too high exposure. Drinking water, food, beverages and fluoride-containing dentifrices are regarded as the main contributors to human fluoride intake (WHO, 2002; Erdal and Buchanan, 2005; Malde *et al.,* 2004). For a given individual, water consumption increases with temperature, humidity, exercise and state of health. As a result of this total daily fluoride exposure can vary markedly from one region to another. However, from several studies, a rough estimate of total daily fluoride exposure in a temperate climate would be approximately 0.6 mg per adult per day in an area in which no fluoride is added to the drinking-water and 2 mg per adult per day in a fluoridated area (WHO, 2008; Opydo-Szymaczek and Borysewicz-Lewicka, 2017a). Different studies indicated that although drinking water is epidemiologically the most important source of fluoride in most areas, considerable exposure risk is also associated with food and drinks, especially tea (WHO, 2002; Malde *et al*., 2004).

The major source of fluoride intake in adults is diet (water, food and beverage). Wide variations in the total intake of fluoride within and between studies are reported (Warren and Levy, 2003; Ponikvar *et al.,* 2007; Malde *et al*., 2011). This can be ascribed to (i) considerable differences in the amounts of fluoride in similar food items, (ii) large variation in the quantities consumed, (iii) differences between the age groups and genders studied and (iv) differences in the analytical techniques used.

It is also important to note that estimates of quantities of foods consumed, such as standard food tables and market basket surveys, were used, rather than actual quantities of food consumed. Several studies reported that the intake of fluoride was higher in areas with fluoridated than non-fluoridated water. The average daily total fluoride intake in non-fluoridated areas ranged from 0.56 to 1.50 mg. The daily intake of fluoride in fluoridated areas was almost two fold higher (being 0.9–3.8 mg) and even much higher in naturally fluoride contaminated areas (Ponikvar *et al*., 2007; Malde *et al*., 2011). In some areas where the fluoride content of the water is low, but the intake from food and tea is sufficiently high for the incidence of dental fluorosis to exceed 80% (Han *et al*., 1995; Fejerskov *et al.,* 2008; Opydo-Szymaczek and Borysewicz-Lewicka, 2017a).

2.2. Fluorosis

Fluorosis is a pathological condition that occurs as the result of excessive consumption of fluoride above optimum level (Whitford, 1997; Fejerskov *et al.,* 2008). It is regarded as the most serious adverse effect of exposure to relatively low doses of fluoride. In extreme cases, fluorosis manifests itself as brown mottling of the enamel, and also results in overall yellowing of the teeth, and a greater degree of brittleness (McDonagh *et al.,* 2000; DenBesten and Li, 2011; Akarslan, *et al.,* 2015).

An upper tolerable intake level (UL) of 0.1 mg/kg BW/day for fluoride has been derived by the EFSA Panel on Dietetic Products, Nutrition and Allergies (EFSA, 2005) based on a prevalence of less than 5% of moderate dental fluorosis in children up to the age of 8 years as the critical

endpoint, i.e. 1.5 mg/day for children1-3 years of age, and 2.5 mg/day for children aged 4-8 years. For adults, an UL of 0.12 mg/kg BW/day was based on a risk of bone fracture, which converts on a body weight basis into 7 mg/day for populations aged 15 years and older, and 5 mg/day for children 9-14 years of age. Tolerable upper intake levels for fluoride have not been established for infants. For infants up to 6 months old, the UK Department of Health (UK DoH, 1994) concluded that 0.22 mg F/kg BW/day was safe.

Individual exposures to fluoride vary considerably and depend on the high variability in the levels of fluoride in waters, and on individual dietary and oral hygiene habits and practices. The emerging picture from all risk assessments conducted on fluoride is that there exists a narrow margin between the recommended intakes for the prevention of dental caries and the upper limits of exposure. All assessments to-date call for continued monitoring of the exposure of humans to fluoride from all sources and an evaluation of new scientific developments on its hazard profile. Exposure assessment has been conducted by the European Food Safety Authority (EFSA) for setting upper tolerable intake levels (UL) related to concentration limits for fluoride in water (EFSA, 2005; EFSA 2008a; EFSA 2008b) and dental products (SCCP, 2009). A similar approach was taken by the United States National Academies of Science in its 2006 review of the United States Environmental Protection Agency's water standards for fluoride (NRC, 2006).

A body of scientific literature seems to suggest that fluoride intake may be associated with a number of adverse health effects. The primary adverse effects associated with chronic, excess fluoride intake range from mild dental fluorosis to crippling skeletal fluorosis as the level and period of exposure increases. Other adverse effects, including hypersensitivity reactions, renal

insufficiency, immunological effects, and possible association with repetitive strain injury, birth defects and cancer have been observed and discussed (Marshould *et al*., 2004; WHO, 2002; Harrison, 2005). There is more of a threat, however, to the higher concentrations due to the severity of the disease posed by such levels within the water (Table 1). The severity depends upon the amount ingested and the duration of intake (WHO, 2004).

Table 1: The effects of too little-and too much-fluoride in drinking water

Level in water	**Effects**
0.8–1.2 mg/l	Prevention of tooth decay, strengthening of skeleton
Above 1.5 mg/l	Fluorosis: pitting of tooth enamel and deposits in bones
Above about 10 mg/l	Crippling skeletal fluorosis

Source: (Choi *et al.,* 2012)

2.2.1. Dental fluorosis

The most sensitive effect of fluoride exposure in humans is dental fluorosis. Dental or enamel fluorosis is an irreversible dose–response effect caused by fluoride ingestion during the pre-eruptive development of teeth (Dean, 1942). Dental fluorosis in a mild form is a cosmetic effect that ranges in appearance from scarcely discernible to a marked staining or pitting of the teeth in severe forms (Whitford, 1997). The pre-eruptive maturation of crowns of the anterior permanent teeth, which are of most concern aesthetically, is complete and, together with the risk of fluorosis, is over by the age of 7–8 years (Fejerskov *et al.,* 2008; Beltrán-Aguilar *et al.,* 2010). Therefore, the most sensitive population to dental fluorosis is children under the age of eight particularly during the pre-eruptive formation and maturation of enamel in teeth (Liang *et al*., 2006). Excessive fluoride intake by people older than seven years will not cause dental fluorosis

(Getaneh *et al.,* 2003; Stefanie *et al*., 2009). Therefore, fluoride intake up to the age of 7–8 years is of most interest. Although it is usually the permanent teeth that are affected, occasionally the deciduous teeth may be also involved. Dental fluorosis is more prevalent in children fed formula than in breast-fed children. The earliest signs of dental fluorosis can be seen as small white lines across the entire enamel surface (WHO, 2003).

In its mildest form, fluorosis appears on the enamel surface as chalky, lace-like marks, and these may not be apparent to the casual observer (Beltrán-Aguilar *et al.,* 2010). As severity increases, over half of the enamel surface may appear white and opaque. In its most severe form, fluorosis causes the enamel to become pitted and brittle. Both moderate and severe fluorosis may be accompanied by the development of a brown discoloration (Bowen, 2002). The NRC analysis determined that severe dental fluorosis appears to occur at a lower dose than stage II skeletal fluorosis and/or bone fractures (NRC, 2006).

The element fluorine in the form of the fluoride ion is well known to be associated with sound dental health (Featherstone, 1999; Fejerskov and Kidd, 2008). Dean and his colleagues (1942) showed an inverse relationship between mottled enamel and the occurrence of dental caries. Mottling had previously been shown to be caused by the presence of fluoride. But Dean and his colleagues came up with the conclusion that fluorine was responsible for reducing the incidence of dental caries. They demonstrated that 1 part per million (1ppm) of fluoride in drinking water was optimal, as it gave the greatest reduction in dental caries without also causing mottling of the teeth. As a result of this study, the condition of tooth mottling was renamed ‘dental fluorosis’, and the study paved the way for extensive fluoridation of domestic water supplies throughout the developed world (Featherstone, 1999; Fejerskov *et al.,* 2008; Martinez-Mier *et al.,* 2011).

Due to ubiquitous exposure to fluoride sources other than drinking water, it is difficult to draw firm conclusions regarding the independent effects of fluoride in drinking water on dental caries and its prevention. Both deficiency and excess of fluoride is associated with human health (WHO, 2002; NRC; 2006). There is a narrow range between intakes which are beneficial and those which begin to be detrimental (IPCS, 2002; WHO, 2011). It has been estimated that moderate dental fluorosis occurs in 1–2% of the population exposed to fluoride at 1 mg/L in drinking water and in about 10% of the population at 2 mg/L; moderate/severe fluorosis occurs in variable percentages ranging up to 33% of the population exposed to fluoride at 2.4 – 4.1 mg/L (WHO, 2002, WHO, 2011). According to Dean *et al* (1942), at a fluoride concentration of 1 mg/L about 20 per cent of children have evidence of dental fluorosis but this fluorosis is of a mild degree and would not be cosmetically obvious to the children or their parents. Thus the evidence suggested that, at least for fluoride naturally present in water, the optimal level of fluoride for a temperate climate was around 1 mg/L; this concentration was associated with a substantial resistance to tooth decay but with only a small and cosmetically insignificant increase in the prevalence of dental fluorosis (Dean, 1942; Susheela, 1999; Fejerskov and Kidd, 2008; Beltrán-Aguilar *et al.,* 2010; Gupta *et al.,* 2016).

2.2.2. Skeletal Fluorosis

Skeletal fluorosis can be defined as excessive deposition of fluoride in bone. This is a pathological condition that is by far the most important aspect of chronic exposure to elevated levels of fluoride, either by inhalation or by ingestion (Shashi *et al*., 2008). In adults; stiffness of the back and neck muscles, unable to bend forward and to stand straight are some of the

indicators of skeletal fluorosis. On the other hand, signs and symptoms of skeletal fluorosis in children include; pain in the lower limbs, knock knee, bow leg and anterior bowing of the lower limb bones (Tekle-Haimanot, 1990; Wang *et al.,* 2008; Tekle-Haimanot and Haile, 2014; Giday *et al.,* 2015).

The skeletal deformities may be associated with or accentuated by nutritional deficiencies or even malnutrition and hard manual work or, possibly, other conditions found in areas of long-term social and nutritional deprivation (WHO, 1994).The situation is specific also for populations consuming large volumes of water, such as athletes or people with certain medical conditions or in some tropical areas where, due to higher temperatures, water consumption is increased. Skeletal fluorosis exhibits several stages. According to NRC (2006), skeletal fluorosis is categorized into one of four stages: a preclinical stage and three clinical stages that increase in severity. Two pre-clinical asymptomatic changes are characterized by slight, radiographically detectable increases in bone mass. An early symptomatic stage is characterized by sporadic pain and stiffness of joints, arthritic symptoms, and slight calcification of ligaments and increased osteosclerosis of cancellous bones, sometimes accompanied by osteoporosis of long bones. The most severe stage (clinical stage III) historically has been referred to as the "crippling" stage. Crippling skeletal fluorosis is characterized by marked limitation of joint movements, considerable calcification of ligaments, crippling deformities of the spine and major joints, muscle wasting and neurological defects associated with compression of the spinal cord (Susheela, 1999; Krishnamachari, 2002, Giday *et al.,* 2015). NRC (2006) concluded that "the weight of evidence supports the conclusion that lifetime exposure to fluoride at drinking water concentrations of 4 mg/L and higher is likely to increase fracture rates in the population.

Most epidemiological research has indicated that an intake of at least 10 mg/day for 10 or more years is needed to produce clinical signs of the milder forms of osteosclerosis. Water fluoride concentrations of 4 – 8 mg/L in temperate climates have not been found to be associated with any signs or symptoms of skeletal fluorosis (WHO, 1994). This data should be regarded with scepticism in view of reports from a number of developing countries that endemic skeletal fluorosis occurs in individuals whose drinking water contains more than 6 mg/L of fluoride (WHO, 2011).

In an epidemiological study in China the relationship between fluoride intake via drinking-water and all other sources, followed a U-shaped dose response with higher rates of fracture at very low intakes below 0.34 mg/L and high intakes above 4.32 mg/L (total intake 14 mg per day) (Li *et al*., 2001). It was concluded by the IPCS (2002) that for a total intake of 14 mg per day there is a clear excess risk of skeletal adverse effects and there is suggestive evidence of an increased risk of effects on the skeleton at total fluoride intakes above about 6 mg per day. However, skeletal fluorosis has been reported in India in a village with average fluoride concentration in drinking water as low as 0.7 mg/L, with range 0.4–1.4 mg/L (Susheela, 1999, NRC, 2006). Although the disease is uncommon at such low concentrations, evidence of skeletal fluorosis, with severe clinical manifestations, has been also reported in at least 9 studies from 5 countries, where water supplies contain fluoride naturally in the range 0.7–2.5 mg/L (Cao *et al.,* 2003; Long *et al.,* 2003; Grandjean and Landrigan, 2014). Recent study in Ethiopia conducted on fluoride intake from locally brewed alcoholic beverages also indicated accelerated development of skeletal fluorosis (Tekle-Haimanot and Haile, 2014).

2.2.3. Fluorosis in Ethiopia

Ethiopian Rift Valley Region covers all or parts of Afar, Oromia and the Southern Nations, Nationalities and Peoples Regional state (SNNPRs). As a result of the long-term use of high-fluoride drinking water, both dental and skeletal fluorosis is common in Ethiopian Rift Valley. The severe cases of skeletal fluorosis (32%) were reported from study at Wonji (Tekle-Haimanot *et al*., 1990). Same study reported that the maximum prevalence is seen in the 10 –14 year old age-groups. Kloos and Tekle-Haimanot (1999) found dental fluorosis in some highland communities where the water is abstracted from volcanic rocks. Regionally 42% of ground water sources tested in Oromia, 30% in SNNPR and 12% in Afar Region have excessive fluoride concentrations (Tekle-Haimanot *et al*., 2006).

On the other hand, although drinking water is likely to be the dominant pathway of fluoride exposure in Ethiopian Rift Valley areas, food stuffs prepared with high fluoride cooking water is also an additional pathway (Malde *et al*., 2004). The fluoride content of a dish depends on the fluoride content of the food ingredients, the fluoride concentration of the water, and the amount of water used and retained in the food during preparation (Malde *et al*., 2004). Recent study by Cao *et al* (2006) also identified food as a potential hazard and states that food consumption may increase the risk of fluorosis. Khan *et al* (2004) on the other hand, proposed that each country should calculate its own optimal level of fluoride in drinking water based on the dose-response relationship of fluoride in drinking water and the levels of caries and fluorosis. It is plausible to estimate the amount of fluoride ingested from all environmental and dietary sources so that

rational and scientifically sound decisions can be made when guidelines for the use of fluorides are established or periodically reviewed and modified (NRC, 2006; WHO, 2008).

Unfortunately, there is no study made on total daily fluoride intake value in Ethiopia, except the study made by Malde *et al* (2004), on dietary fluoride intake in children below 5 years of age, living in a high-fluoride area of Wonji Shoa Sugar Estate. As a result of this, there is no adequate scientific based information on the magnitude of fluoride exposure through dietary sources in the Ethiopian Rift Valley Region. The problem is further aggravated by limited budgets which restricted the feasibility of defluoridation technologies, running cost of those established ones and inability of provision of alternative water sources. In addition, since the economic cost of endemic fluorosis to human beings is largely indirect and the disease is not acute, it is unlikely that fluorosis would be recognized as an area of immediate need by the government and stakeholders in developing countries. In the presence of infectious and acute diseases it is impossible to consider fluorosis as a priority public health problem in the areas. In some places in Ethiopian rift valley, whatever the chemical content is, the presence of water is considered as a blessing (Getaneh *et al.,* 2003; Tekle-Haimanot *et al.,* 2006; Hussen, *et al.,* 2015).

2.3. Mitigation of Fluorosis

2.3.1. Risk Groups for Fluorosis

As ever, the dose critically determines whether there will be any adverse effects of fluorosis, and this depends inter alia on the amount taken in, the proportion absorbed by the body and the weight of the individual. In response to these considerations, the medical profession tends to advise mothers not to make up infant feeds from fluoridated water, though there is as much

concern with the fact that ingested fluoride promotes removal of calcium from the body as CaF_2 in the feces as with any adverse effects of retained fluoride. Other groups who are advised against consuming fluoridated water are patients on kidney dialysis (McDonagh *et al*., 2000; Macek *et al.,* 2006; Møller and Poulsen, 2015). According to Susheela (1999) some groups of population namely pregnant and lactating women, young children, malnourished children, and people with low intake of calcium, people with cardio-vascular and kidney problems and those who involved in hard manual labor in hot climates are more susceptible to the poisonous effects of fluoride than others (Jackson *et al.,* 2002; Silva and Leite, 2003).

Due to the suspected negative health effect of fluoride its exposure has been closely regulated. The current estimates of optimum level of exposure to fluoride are between 0.05 and 0.07 mg/kg of body weight (Burt, 1992). Fluoride is especially important for children to prevent caries. However, it is important to regulate intake, as excessive exposure to fluoride can result in fluorosis. There are several sources of fluoride available to typical children in the developed world, including dentifrices, milk and other foodstuffs, as well as drinking water (Clarkson and McLoughlin, 2000). All these sources need to be taken into consideration when setting the level of fluoride to be added to public drinking water supplies (WHO, 2011).

2.3.2. Water Defluoridation

The primary preferred option is to find a supply of safe drinking-water with safe fluoride levels. Where access to safe water is already limited, de-fluoridation may be sought as a solution. But removal of excessive fluoride from drinking-water is difficult and expensive. Since the 1930s most research efforts have been directed at defluoridation of high fluoride containing water as

fluorosis prevention strategies. Although several fluorosis prevention methods are technically feasible and can routinely be carried out in central water distribution systems, only few of the defluoridation methods are successfully applied on a routine basis (Dahi, 1999; Dakshinamurty and Sarma, 2009; Dahi, 2016). Most defluoridation methods are complicated and/or expensive, unproven and unreliable under field conditions in developing countries because of use of materials of questionable supply and inappropriate technology, ineffective at community level, their social acceptability and lack of sustained government and community commitments. Since all methods produce sludge with a very high concentration of fluoride that has to be disposed of, only water for drinking and cooking purposes should be treated.

The more promising water defluoridation methods which have been field-tested include the granulated bone media and heat-activated bone char, clay and clay pot chip (Dahi, 1999; Meenakshi and Maheshwari, 2006; Upadhyaya and Mishra, 2009; Raju *et al.,* 2009), activated alumina adsorption (Shifera and Tekle-Haimanot, 1999; Kumar *et al.,* 2013) and Nalgonda methods (WHO, 1994). WHO established a guidance value for naturally occurring fluoride in drinking water of 1.5 mg/L based on a consumption of 2 L water/day, and recommended that artificial fluoridation of water supplies should not exceed the optimal fluoride levels of 1.0 mg/L (WHO, 2006). In Europe, only Ireland and selected regions in the UK and Spain currently fluoridate drinking water at concentrations ranging from 0.8 to 1.2 mg/L (Mullen, 2005; Dakshinamurty and Sarma, 2009; Dahi, 2016).

2.3.3. Nutritional Approaches

The management of fluorosis in the community can be effective if nutritional approaches focusing on adequate intake of calcium, vitamins (C and E) and other antioxidants (Balabolkin *et al.,* 2004; Nair and Gupta, 2005), is advocated and practiced simultaneously with consuming safe drinking water (Susheela and Bhatnagar, 2002; Gupta *et al.,* 2016). Nutrient supplementation through a dietary regime has been found to be the best approach and it is sustainablate to mitigate fluorosis in endemic areas. During counseling for nutritional supplementation, it is necessary to emphasize avoiding of food items with high fluoride content and those foods which could be used for mitigation of ingested fluoride. Foods that should be avoided in endemic areas include:, black tea (tea without milk), chewing tobacco, fluoride-containing toothpastes, mouth rinses, varnishes, black rock salt / magadi and other items that are commercially marketed (Ogawa *et al.,* 2004; Niu *et al.,* 2006; Gupta *et al.,* 2012).

Fluoride is not essential for human growth and development but it is considered to be beneficial in the prevention of dental caries (tooth decay). As a result, intentional fluoridation of drinking water and the development of fluoride containing oral care products (toothpastes and mouth rinses), foods (fluoridated salts) and supplements (fluoride tablets) have been employed in several parts of the world as a public health protective measure against tooth decay (SCHER, 2010). However, in many African and Asian countries, exposure to fluoride comes from naturally occurring water, beverages, food, and to a lesser extent, from other environmental sources (Malde *et al*., 2004). Fourteen countries in Africa, eight in Asia and the Middle East and six in the Americas face the problem of fluoride concentration above 1.5 mg F-/L in drinking water. Many of these countries are confronted with the problems of endemic dental and skeletal

fluorosis (Frenckens *et al*., 1990; Fejerskov and Baelum, 2002; WHO, 2008). Therefore, it is appropriate to ensure that parents or guardians of children continue to receive sound advice on safe levels of fluoride for those in their care to be exposed to and, since the cariostatic effect of fluoride is known to occur well after enamel formation during tooth development, treatment to reduce caries should concentrate on those measures that carry the lowest possible risks of fluorosis in countries which have fluoridation program (Fejerskov *et al*., 2008; Krishnamachari, 2002; Burgstahler and Margolis, 2011).

2.4. Eggshell Powder Consumption in Human Nutrition

2.4.1. Mineral Contents of Eggshell

Chicken eggshells, which first serve to protect and provide nutrients to the enclosed embryo, have been used by humans for a long period as a food additive, but on a very modest scale. On average, one egg has about 5.43 $\pm$ 0.79 g of shell. A complete eggshell contains 2.07 + 0.18g of calcium (381$\pm$ 89mg Ca/g eggshell). This means that half of an eggshell can provide all the amount of calcium (Ca) needed per day in adult human beings. Moreover, eggshell contains not only Ca but also other elements (Table 2), such as phosphate and strontium which, despite the low concentration, may contribute to P and Sr requirements and have a positive effect on bone and dental metabolism (Shaafsma *et al*., 2000; Brun *et al*., 2013). In addition, levels of potentially toxic heavy metals (Pb, Al, Cd and Hg) are very low unlike the commercially available CaCO3 products and even natural sources of calcium such as oyster shell (Schaafsma *et al*., 2000; Narahari *et al.,* 2010).

Table 2: Organic and inorganic content of chicken eggshell (mg/100g eggshell)

	Mean	SD
Organic matter	16.1	4.6
Inorganic matter	83.9	5.0
Calcium	38.2	3.5
Carbonate	44.3	3.2
Sodium	0.51	0.09
Phoshapte	0.44	0.06
Sulfate	0.32	0.07
Potassium	0.14	0.05
Strontium	0.14	0.02

Source: (Brun *et al*., 2013)

2.4.2. Eggshell as a Dietary Calcium Source

Calcium is a very important building block of bone and often seen as key element in bone mineralisation and anti-demineralisation strategies. Deficiency of calcium in the diet is a common problem for all age groups of individuals, particularly in developing countries. Calcium intake from dairy products is an appropriate way to fulfill calcium requirements. However, people do not usually consume them in the amounts established by dietary or clinical guidelines. Supplementation with tablets is costly and sometimes involves difficulties of adherence to treatment. Chicken eggshell is a source of calcium, which is available at home that can be used as dietary calcium (Compston, 1995; Devine *et al*., 1997; Schaafsma and Pakan, 1999; Ibrahim *et al.,* 2013; Khan *et al.,* 2016).

Chicken eggshell powder is an excellent dietary calcium source. It contains about 39 % (w/w) elemental calcium with a bioavailability as high as CaCO3 (Schaafsma and Beelen, 1999). Since it is widely available, it may be a cheap and effective solution for many calcium-deficient populations. It is of interest to know that chicken eggshell powder also contains Strontium (on average 380mg/g). This micronutrient may have an anabolic effect on bone (Reginster *et al*., 1999). In small studies, eggshell powder showed positive effects on bone mineral density of the lumbar spine and hip in post-menopausal women with osteoporosis (Makai and Chudacek, 1991; Schaafsma and Pakan, 1999; Khan *et al.,* 2016)

Studies have reported results about the use of eggshells as calcium supplement. It has been demonstrated that for ovariectomized rats' eggshells are an effective calcium source with no significant differences regarding body weight gain, food intake or food efficiency (Omi and Ezawa, 1998). The addition of vitamin D improves bone mineral density of the lumbar spine and the proximal tibia in rats treated with calcium carbonate (CaCO3) or eggshell (Hirasawa *et al*., 2001). In piglets, there were no differences in Ca absorption from CaCO3 and eggshells in casein-based diets and soy protein isolate-based diets (Schaafsma and Beelen, 1999). Moreover, it has been demonstrated that eggshell proteins improve calcium absorption. The total calcium transport across Caco-2 monolayers showed an increase of 64% in the presence of soluble eggshell matrix proteins (Daengprok *et al*., 2003).

There are few earlier studies describing the use of chicken eggshell as calcium supplement in human beings. Schaafsma and Pakan (1999) showed an increase in lumbar spine, total proximal femur and trochanter bone mineral density in osteoporotic postmenopausal women who received

eggshell powder with vitamin D3 and magnesium supplementation. Another study reported a highly positive effect of eggshell calcium supplementation (with added magnesium and vitamin D) on Bone Mineral Density. In this study, the eggshell supplemented group had measurable increases in bone density in their hip bones, after one year. The findings indicate that healthy late post-menopausal women with an adequate calcium intake at baseline may increase bone mineral density of the hip within 12 months following supplementation with the chicken eggshell powder- enriched supplement (Schaafsma *et al*., 2002).

A recent study conducted on analyzing chicken eggshell as suitable calcium source at home reported that a single eggshell contains 2.07g (0.18g) of calcium; therefore half an eggshell could provide the amount of calcium needed by adult human beings per day. In addition, this study concluded that eggshell is an appropriate and cheap source of calcium for human nutrition easily prepared at home. Eggshell obtained with a rolling pin plus a sieve did not produce important changes in flavor and texture in different foods evaluated in this paper. Therefore, the best way to use chicken eggshell as calcium dietary supplement is powdered to add to bread, pizza or spaghetti as there were small changes in texture and no changes in flavor (Lucas *et al*., 2013). Another study found 26.0, 35.4 and 41.4% calcium bioavailability of biscuits containing chicken eggshell powder at 3, 6 and 9% respectively. With respect to the calcium content, texture, sensory properties and calcium bioavailability, the authors suggested that the best way to use chicken eggshell as dietary calcium supplement is powdered to biscuit until 6% eggshell supplementation (Nahla, 2015).

2.4.3. Calcium and Fluoride Amelioration

Epidemiological studies conducted during 1963 -1997 in 45,725 children exposed to high intake of endemic fluoride in the drinking water since their birth reported higher toxic-effects of fluoride among children with inadequate calcium nutrition. In this study children with adequate (dietary calcium > 800 mg/d) and inadequate (dietary calcium < 300 mg/d) calcium nutrition and with comparable intakes of fluoride (mean 9.5 +/- 1.9 mg/d) were compared. The toxic-effects of fluoride were severe and more complex and the incidence of metabolic bone disease (rickets, osteoporosis. PTH bone disease) and bony leg deformities (genu valgum, genu varum, bowing, rotational and wind-swept) was greater (> 90%) in children with calcium deficiency as compared to < 25% in children with adequate calcium who largely had osteosclerotic form of skeletal fluorosis with minimal secondary hyperparathyroidism.The findings strongly suggested that deep bore drinking water supply with fluoride < 0.5 ppm and improvement of calcium nutrition provide 100% protection against the toxic effects of fluoride and are recommended as the cost effective and practical public health measures for the prevention and control of endemic fluorosis (Teotia *et al*., 1998; Bhattacharya and Misra, 2004).

Earlier studies indicated that calcium binds with fluoride forming an insoluble calcium fluoride in the gastrointestinal tract; thus the adverse effect of fluorosis may decrease. A study conducted on quantification of calcium needed to reduce excess fluoride absorption for minimizing fluoride bioavailability in rabbits recommended that a dose of 1200 - 3500 mg of calcium are required for 70 kg body weight human beings to mitigate the adverse effects of fluorosis. In this study different doses of calcium in the form of calcium carbonate were administered to albino rabbits and it was observed that significant decrease in fluoride bioavailability was observed in the

group of animals administered with 11.4, 14.3, 17.2, 21.4 and 50 mg calcium per kg body weight. This calcium dose level is equivalent to 1200, 1500 and 3500 mg of calcium required for a 70 kg body weight human (Anitha and Viswanathan, 2008).

A study conducted in Ethiopian children by Wondwossen with NRC (2006) indicated that second molars were the teeth type most frequently affected by dental fluorosis and there is direct relationship between the severities of dental fluorosis with that of dental caries. In general, caries prevalence was lowest in groups showing no flu and there was a progressive increase in caries with increasing severity of dental fluorosis. Some of the factors which might account for differences in the fluorosis/caries relationship between the US and other countries include differences in dent practices, dietary habits (i.e., consumption of sugars), and nutrient intakes (i.e., calcium balance) (US EPA, 2010).

2.5. Eggshell Powder Consumption Intervention Studies

Experimental and clinical studies performed to date have shown a number of positive properties of eggshell powder, such as antirachitic effects in rats and humans and in vitro stimulation of chondrocyte differentiation and cartilage growth (Rovenský *et al*., 2003). Human clinical studies conducted so far demonstrated that this natural calcium of eggshell significantly increased bone mineral density (Hirasawa *et al*., 2001; Schaafsma *et al*., 2002; Daengprok *et al*., 2003).

In a randomized, double-blind, placebo-controlled human clinical trial, eighty-five healthy Dutch women, 50-70 years of age that were at least 5 years post-menopausal were studied. The subjects were given either purified calcium carbonate or chicken eggshell powder enriched vitamin-

mineral supplement and the effects on bone mineral density (BMD) of the femoral neck were evaluated. After 12 months of supplementation, women given eggshell powder enriched supplement demonstrated the highest and significant increase in BMD by 1.75% in the femoral neck. Similar supplementation with purified calcium carbonate resulted in 1.16% increase in BMD, while the control group actually lost BMD. Based on changes from baseline and compared with placebo, the study concluded that eggshell powder may be preferred over purified CaCO3 as a source of Ca (Schaafsma *et al*., 2002).

Similarly, another clinical study was conducted on 10 persons with osteoporosis or osteopenia to determine the short-term effects of chicken eggshell powder enriched dairy-based supplements on BMD of the lumbar spine and hip over a period of 4-8 months. Supplementation with eggshell powder enriched products increased BMDs of the lumbar spine, proximal femur and trochanter by 5.7%, 2.2%, and 1.8%, respectively, over 8 months. Conversely, BMDs in the control group either stayed the same or decreased over the same period. Within the first 4 months, a reduction in pain was reported and in turn, an improvement in general well-being. The study concluded that eggshell powder is a bioavailable source of calcium that can increase bone mineral density of subjects with osteoporosis or osteopenia in the short term and delays bone demineralization for a longer period of time (Schaafsma and Pakan, 1999; Dogan, 2016).

2.6. Summary of the reviewed literature

Excess fluoride intake primarily results from high levels in drinking water, excessive use of fluoride-containing dental products, and consumption of fluoride-rich foods such as tea (Whitford, 2013). The primary consequence of chronic fluoride exposure is dental and skeletal

fluorosis. Dental fluorosis manifests as discoloration and mottling of teeth, while skeletal fluorosis leads to joint stiffness and bone pain (Gupta *et al.,* 2016). The severity of these conditions correlates with fluoride levels and duration of exposure. To mitigate the adverse effects of fluoride, dietary interventions have been suggested. Increasing calcium, magnesium, and iron intake may reduce fluoride absorption and toxicity. For example, calcium and magnesium can inhibit fluoride absorption in the gastrointestinal tract, while iron deficiency has been linked to increased fluoride uptake (Marinho *et al.,* 2003; Liu *et al.,* 2013; Reddy *et al.,* 2018). Additionally, vitamin C has antioxidant properties that may counteract oxidative stress caused by fluoride (Singh *et al.,* 2012). Limiting fluoride-rich foods and beverages, such as tea, also helps reduce overall fluoride intake (Whitford, 2013). These strategies collectively offer a multifaceted approach to managing fluoride exposure and minimizing its detrimental health effects.

The reviewed literature generally supports the potential of calcium-containing eggshell powder, due to its high bioavailability of calcium, as a feasible intervention to mitigate the toxic effects of excess fluoride intake among women. It suggests that while eggshell powder can effectively reduce fluoride toxicity and improve health outcomes related to fluoride exposure, ongoing monitoring and further research are crucial to confirm its long-term safety and efficacy. The practical application of this intervention in public health strategies appears promising, particularly in regions with high fluoride exposure and calcium deficiency.

2.7. Conceptual framework

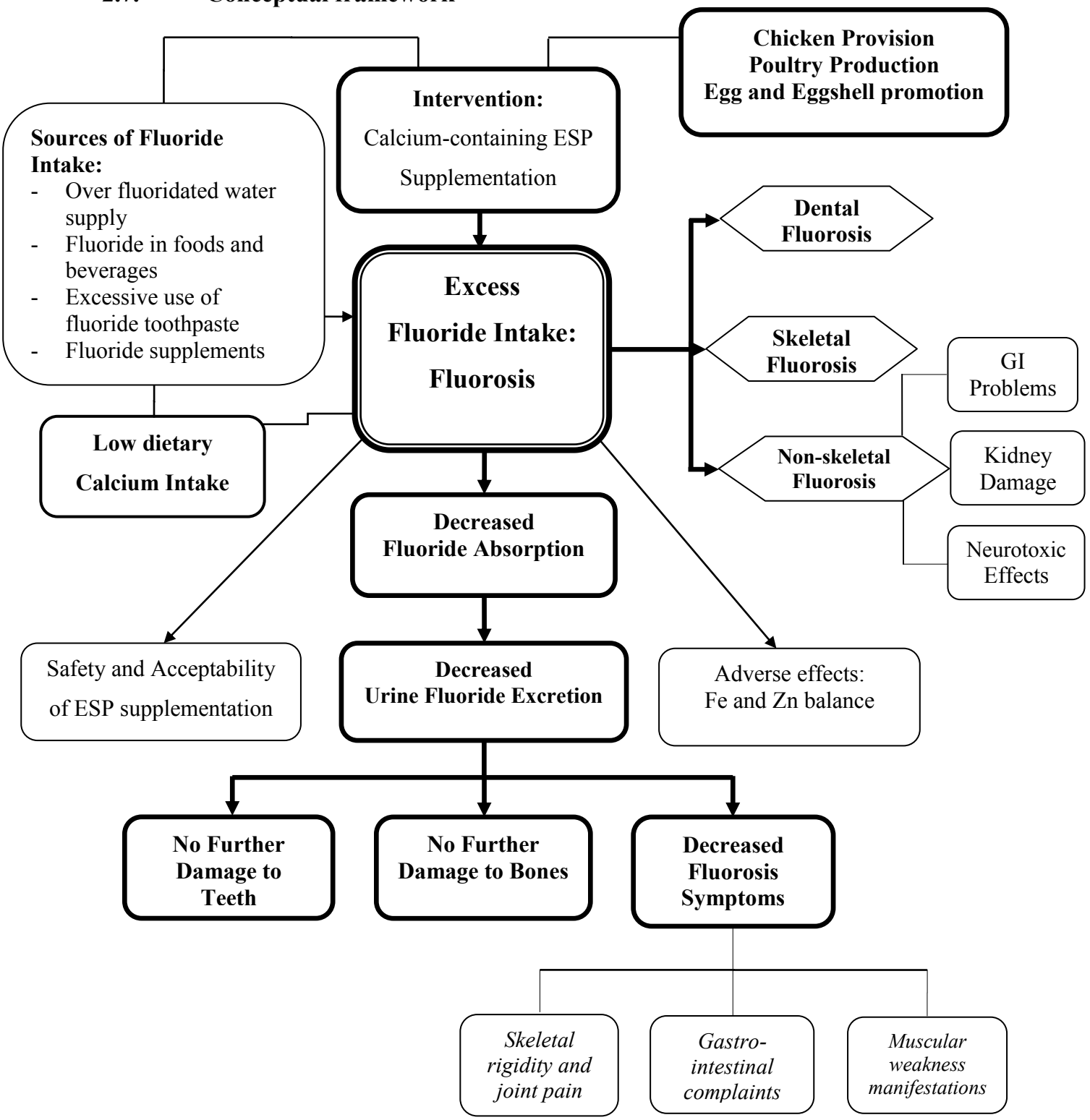


Figure 2: Conceptual framework on causes, consequences and mitigation (dietay calcium supplementation) of excess fluoride intake (Sources: Different literatures).

3. Objective of the study

3.1. General Objective

The overall aim of this PhD research was to assess efficacy, association, safety and acceptability of calcium-containing eggshell powder supplementation to mitigate toxic effects of excess fluoride intake among women in Ethiopian Rift Valley.

3.2. Specific Objectives

Paper 1

To assess dental, skeletal and non-skeletal fluorosis and association with dietary calcium intake among women in Ethiopian Rift Valley: A cross-sectionl study design.

Paper 2

To determine the efficacy of calcium-containing eggshell powder supplementation on urinary fluoride excretion and fluorosis symptoms among women in Ethiopian Rift Valley: An Efficacy trial/ a Phase II clinical trial.

Paper 3

To assess safety and acceptability of calcium-containing eggshell powder supplementation in the Phase 2 clinical trial among women in Ethiopian Rift Valley: A longitudinal study within the Phase IIclinical trial

4. Materials and Methods

4.1. Study Area

This study was conducted in two kebeles (*Guba and Lay Arsho*) of *Halaba* Zone *(the former* Special *Woreda*/district), 85km south west of *Sidama* regional state Capital of, *Hawassa*. The zone is situated at an average altitude of 1800m above sea level with an average 750 mm annual rain fall which is classified as dry mid land. The total population is estimated to be 305,555, of which 85% of the population lives in 79 rural and the remaining in five urban kebeles (CSA, 2007). Livelihood of the people mainly depends on rain fed agriculture producing crops like maize, sorghum, teff, millet, wheat, haricot bean, red pepper, sorghum and potato. The major livestock species in the zone are cattle, goats, sheep, donkeys and poultry. Rainfall is the major limiting factor of agricultural production of the area, which has made it notable as one of the zones in southern Ethiopia where drought recurrently occurs and affects many households.

Figure 3: Geographical location of the study site

Halaba zone is located in the main Rift Valley of South Ethiopia and known for having high fluoride levels in drinking water sources (Kebede et al., 2016a**).** *Halaba* has one zonal hospital, 10 health centers, and supported by 79 satellite health posts functioning in each rural Kebeles. Guba and Lay Arsho kebeles have population sizes of 5475 and 5444 respectively with a total of about 1400 households (Annex X). There are currently 10,271 women health development army (1672 development team leaders and 8599 leaders of 1 to 5 network) working in Halaba to support the health extension program by mobilizing the community, passing health and nutrition messages and positively influencing households to exercise proper health and nutrition care particularly for childbearing women and children (FMoH, 2011; RHB, 2015).

4.2. Study Design

This study was designed in three phases. In the first phase, a cross-sectional survey was conducted to assess the association of dietary calcium intake with dental, skeletal and non-skeletal fluorosis (objective/paper one). In the second phase, an efficacy trial (a phase II clinical trial) was carried out to determine the efficacy of calcium-containing eggshell powder supplementation on urinary fluoride excretion and fluorosis symptoms (objective/paper two). In the last phase, as part of the Phase II clinical trial, a longitudinal study design was employed to assess safety and acceptability of ESP consumption during the phase II clinical trial (objective/paper three). The activities conducted in each phase are outlined in Table 3.

Table 3: Summary of study design, size, outcome variables, collected samples/data and analysis

	Study design	Sample size	Collected samples	Collected data	Outcome variables	Statistical data analyses
Phase one (Paper/Objective 1): Fluorosis assessment	Cross-sectional survey	270	16 staple food samples 3 drinking water sample 1 beverage sample	Socio- demographic and economic characteristics, Data on water, hygiene and environmental sanitation practices Obstetric histories, Dental fluorosis exam Skeletal and non-skeletal fluorosis assessment Usual dietary calcium intake using FFQ Diet diversity using 24hr recall	Magnitude of dental, skeletal and non-skeletal fluorosis	Descriptive statistics Bivariate and multivariable logistic regression
Phase two[$] (Paper/Objective 2): Efficacy of ESP supplmentation	An efficacy trial (a phase II clinical trial)	78	Urine (for fluoride, creatinne and calcium excretions)	Skeletal and non-skeletal fluorosis assessment Usual dietary calcium intake using FFQ Diet diversity using 24hr recall	Urine fluoride concentration (primary ouctome) Magnitude of skeletal and non-skeletal fluorosis symptoms (secondary outcome)	Descriptive statistics Chi-square (X^2) test Paired samples t-test Two independent samples t-test Bivariate and multivariable logistic regression Generalized estimating equation (GEE) Multivariate analysis of the GEE model

Phase three[$] (Paper/Objective 3): Safety and acceptability of ESP	A longitudinal study	78	Venous blood (Fe and Zn) Capillary (finger prick) blood (Hgb, Hct and malaria tests	Side effects related to excess Ca intake including nausea, vomiting, constipation, abdominal bloating and gas Data on adherence/ compliance to the ESP supplementation (acceptability)	Blood levels of Hgb, Hct, Fe and zinc (primary) Side effects related to excess Ca intake (secondary) Adherence/compliance to supplementation (secondary)	Descriptive statistics Chi-square (X^2) test Paired samples t-test Two independent samples t-tests Generalized estimating equation (GEE) Multivariate analysis of the GEE model

[$]All the samples and data in phase two and three were collected at baseline and endline

4.3. Study Period

The actual data collection for the cross-sectional survey was conducted from January to February, 2018. Participant recruitment for the trial study took place after the cross-sectional survey based on the eligibility criteria. Thereafter the baseline (BL) biological (urine and blood) sample collections were carried out. The implementation of the intervention was conducted for six consecutive months with weekly follow up and supervision. Setting this duration would allow for a thorough evaluation of the supplementation's efficacy and safety. This duration facilitated biological adaptation and captured both immediate and long-term physiological changes, while also enabling the monitoring of potential adverse effects. Additionally, it provided insights into participant compliance and acceptability in real-world settings. The trial ended when the study subjects had received the intervention for the intended period of time. At the end, the second round (endline, EL) data collection, measurements, flurosis assessment and biological specimen collection were done for the purpose of the trial study (Figure 4).

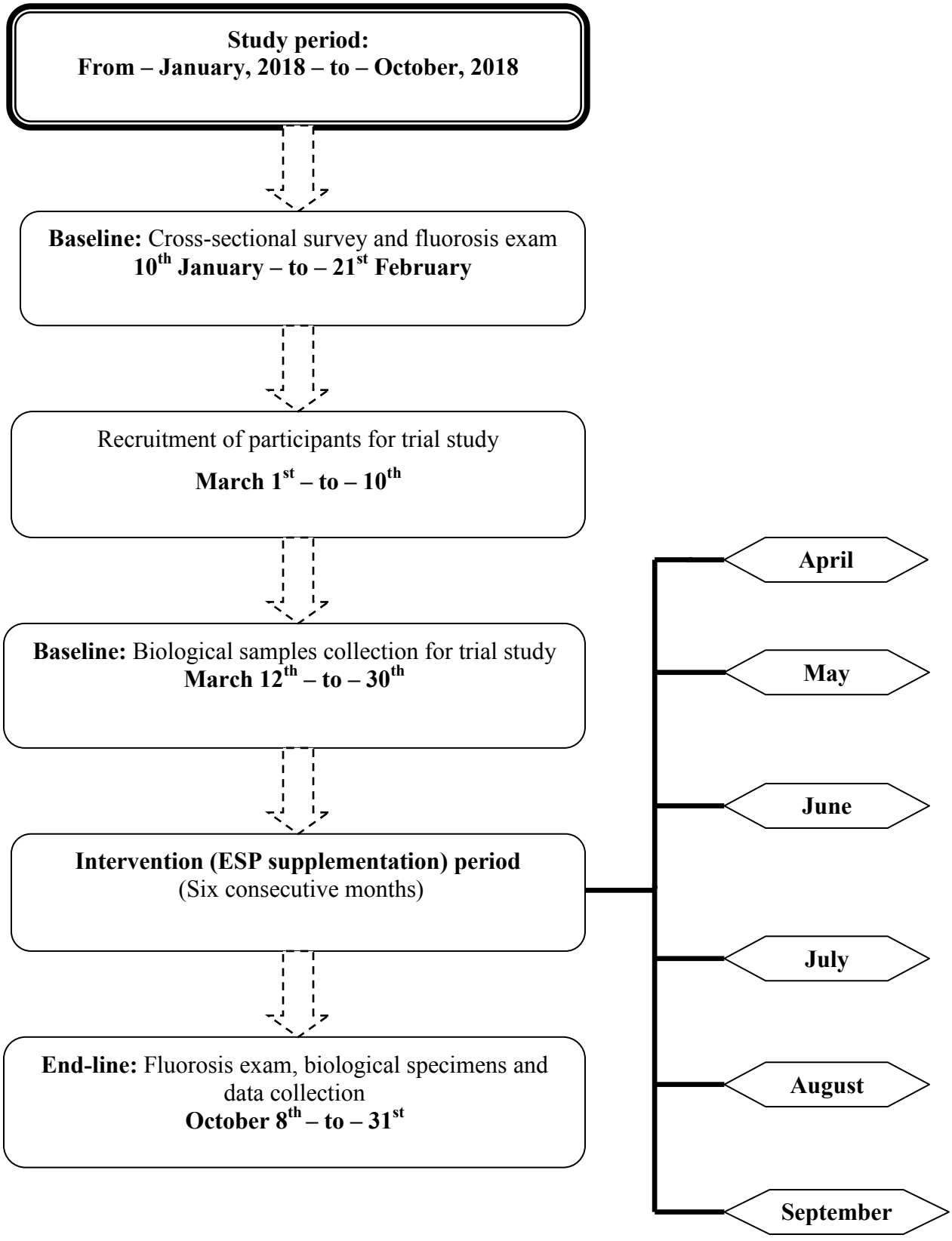


Figure 4: Flow diagram showing the chronological order of the study period

4.4. Population and Sampling

4.4.1. Source and Study Population

The source population for this study was all women in reproductive age who were permanent residents in the study area that was known (Kebede *et al.,* 2016a) to have a fluorosis problem. The study population encompasses: i) for the cross-sectional survey, all eligible biological mothers of young children who were permanent residents in the study area and which would be the target population for a trial study. ii) the study population for the trial was all randomly selected biological mothers of young children who had participated in the cross-section survey.

4.4.2. Eligibility Criteria

Eligibility for women to be included in this study was defined in terms of having a child (age 6-18 months) eligible for a parallel study of child growth. The participant inclusion criteria were being a biological mother of an index child aged 6 to 18 months and permanent resident in the study area. Those who were taking drugs for any type of illness had any apparent signs and symptoms of diseases such as fever and cough at the time of enrollment and those enrolled in any other health and nutrition intervention program were excluded from participation (Figure 5).

4.4.3. Sample Size Estimation

The sample size estimation for the cross-sectional survey (objective one) was done using EpiInfo Version 7.0.8.3 for descriptive study by considering 12% prevalence of clinical symptoms of skeletal and non-skeletal fluorosis among school-age children, assuming the prevalence of fluorosis is similar among women and school-age children as they lived in the same study area (Kebede *et al.,* 2016a) at 95% level of confidence (an absolute precision, d = 5%). Then, the calculated sample size was multiplied by 1.5 to consider the design effect (*DE* = 1.5) as the

sampling was carried out at kebele (cluster) level. Finally, based on the above assumptions and considering 10% non-response rate, a minimum sample size of 267 subjects was estimated to determine the level of skeletal and non-skeletal fluorosis among women.

In estimation of the trial size as a Phase II trial (objective two and three), our primary aim was to detect an effect of calcium-containing ESP on fluoride excretion. Mehta and Shah (2013), using 10 subjects per group, found that calcium supplementation significantly reduced urinary F excretion. As we also evaluated fluorosis symptoms, the sample size estimation to detect the effect of intervention on fluorosis was done using EpiInfo Version 7.0.8.3 software with double population proportion formula by considering the percentage outcome in intervention arm (P_1=90%, percent of pain and rigidity in the joints before intervention which is a major skeletal and non-skeletal fluorosis symptoms) and control arm (P_2=60%, percentage recovery during intervention) from previous similar published studies (Kebede *et al.,* 2016a; Susheela and Bhatnagar,2002). Thus, a minimum sample size of 76 women (38 for each arm) was estimated to detect the effect of intervention on fluorosis symptoms using power (1- β) of 80% at 95% two-sided confidence level for a one-to-one intervention/control arms ratio. Therefore, we increased the sample size to 41 from each kebele to account for variability in symptom prevalence and loss to follow up.

4.4.4. Sampling Procedure

First, family folder reviewing was done at the health post to identify the eligible study subjects within a family (i.e. to identify the number and age of family members in a household and the location of the households in the subdivisions of the kebele called 'Got'). The family folder reviewing was a base to conduct the house-to-house registration. Then, using a list of mothers of

young children aged 6-18 months old obtained from the kebeles health post, the house to house registration was conducted to identify eligible households which would be the target population for the parallel interventional (child growth) and trial (ESP supplementation) studies. Upon the initial sample size calculation, a total of 267 subjects were estimated to determine the level of skeletal and non-skeletal fluorosis during the cross-sectional survey. However, for the two studies, since cluster (kebele) randomization was planned to be done over individual-level randomization to minimize information contamination, all eligible subjects (young children 6-18 months of age and their mothers) in the selected kebeles were included. Consequently, a total of 280 study subjects were included for participation in the cross-sectional survey from a total of 301 mothers of young children aged 6-18 months identified from the family folder. The total population and number of households in the two kebeles were 10919 and 1363 (**Annex X**) respectively.

The two kebeles were purposively selected for the trial on the basis of having high fluoride levels in the drinking water sources (Kebede *et al.,* 2016a) and for feasibility reasons. The study subjects were selected within the respective selected kebeles. First, the exclusive code numbers assigned to each household (the target households for the trial study) during the cross-sectional survey were used as a sampling frame to select the study subjects. The code numbers were 001 to 140 in each kebele by excluding those who refused to participate in the cross-sectional survey. Then, the code numbers were entered into two separate SPSS data templates. Finally, the trial subjects (41 women from each kebele) were randomly selected using the function Random Sample of Cases selection in IBM SPSS version 25 (Chicago, IL, USA) statistical software.

4.4.5. Randomization and Masking in Trial

The trial study randomized two purposely selected clusters that were characterized during the baseline cross-sectional survey. To assign the two kebeles (clusters) either to the intervention or control arm, a lottery method (flipping of a coin) was done by the health extension worker under the supervision of the PI and research/field supervisors. The clusters were distant rural kebeles (villages) of Halaba district having fluorosis, located in the main Rift Valley of South Ethiopia. The physiotherapist who did the skeletal and non-skeletal fluorosis assessment, the laboratory technologists who collected blood and urine specimens, and the data collectors who did the interviews were kept uninformed with respect to the intervention allocation. In addition, the experts who performed the biochemical specimen analyses at the Ethiopian Public Health Institute (EPHI) were masked. All biochemical samples could only be identified with exclusive codes assigned for each participant.

4.4.6. Trial Profile Diagram

The overall progress of subjects through the phases of the clinical trial is depicted in Figure 5.

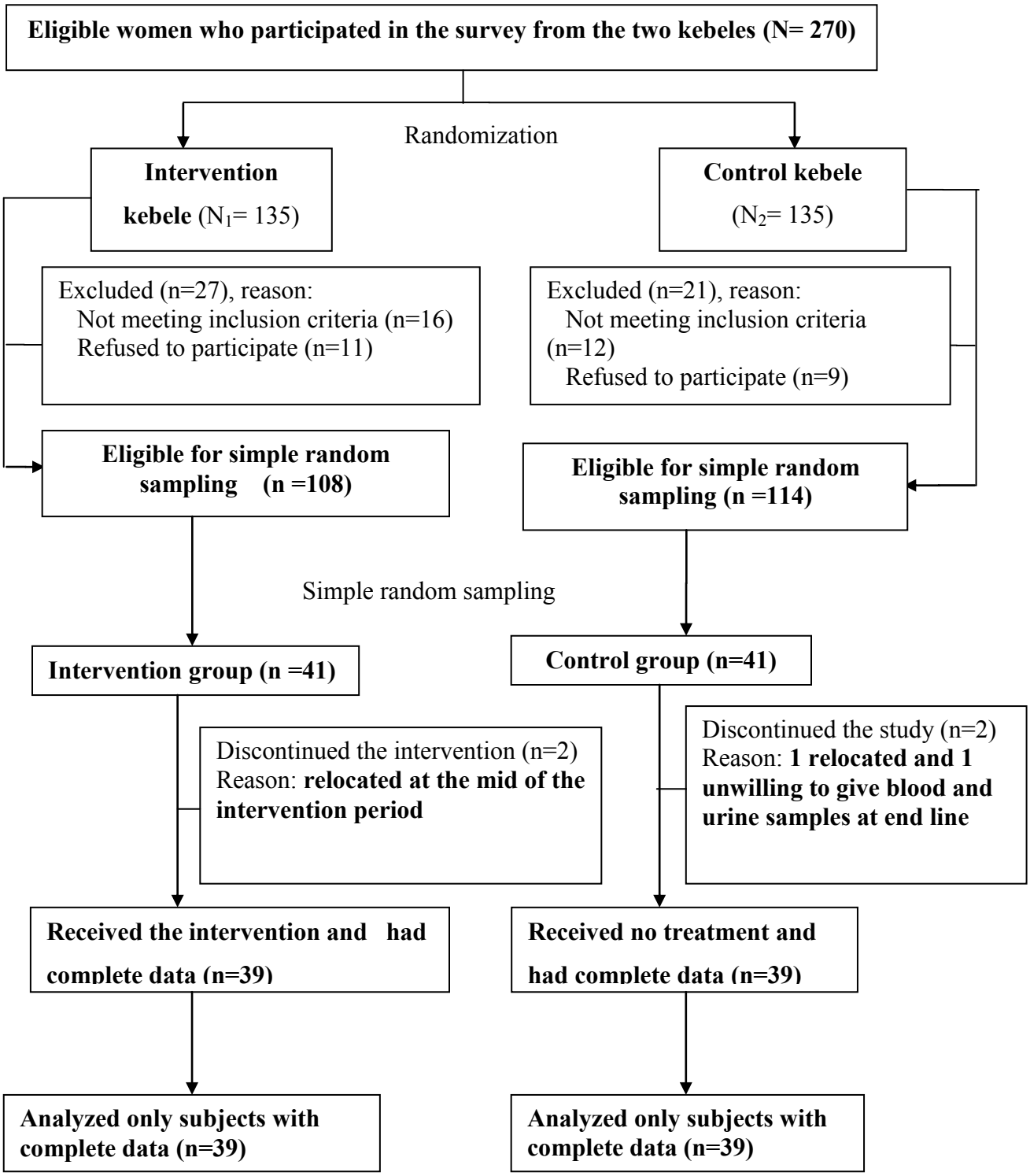


Figure 5: Flow diagram of the progress through the Phase II clinical trial

4.5. Study Variables

4.5.1. Outcome Variables

The dependent variable for the cross-sectional survey was magnitude of dental, skeletal and non-skeletal fluorosis and their association with dietary calcium intake. For the trial study, a decreased urine fluoride excretion concentration was the primary outcome measure. Skeletal and non-skeletal fluorosis levels (i.e. mitigation of fluorosis symptoms) were secondary outcome measures. Urine creatinne and calcium excretion concentrations were also measured. Then, calcium-creatinine ratio was computed to assess calcium status and to measure consistency of the spot urine collection. For the purpose of safety assessment of the Phase 2 trial, the primary outcome measures were blood levels of hemoglobin, hematocrit, iron and zinc prior to and after the ESP supplementation among the study subjects. Side effects related to excess calcium intake (i.e. nausea, vomiting, constipation and abdominal bloating and gas) were assessed as secondary outcome measures.

4.5.2. Explanatory Variables

The main exposure variable was calcium intake (from diet and supplementation). Other predictor variables included: socio-demographic and economic characteristics such as age, family size, educational status, occupation and years of residency; maternal obstetric history such as parity and gravidity; dietary practices such as diet diversity, frequency of calcium rich foods consumption and fluoride intake from drinking water and staple foods; water, hygiene and environmental sanitation practices such as hand washing, household latrine type, drinking water source and water treatment practice.

4.6. The Intervention (Exposure) in Trial

The intervention (exposure) in the trial study was daily supplementation of 2.4 grams of calcium-containing ESP for six months measured out by the women using a premeasured plastic cap from a plastic soft drink bottle. Women were instructed to take three half caps daily where each half cap provided ≈ 800 mg ESP which is equivalent to 320 mg calcium. Each dose was to be taken at different times over the day, not all at once. This daily dose of ESP was provided approximately 960 mg of calcium.

Eggshells were originally planned to be available to families through provision of chickens; however, to maintain consistent supply and quality eggshells were purchased from local restaurants. When families later began accumulating eggshells from their donated chickens, we purchased eggshells from women in the community. White eggshells were washed, dried, and then crushed before being processed into a fine powder using High–speed Multi-functional Grinder (®RIRIHONG, RRH–A1000, Shanghai Yuan wo Industrial and Trade Co. Ltd. Manufacture: Hongtaiyang Electrical and Mechanical Services Co. Ltd of Yongkang City Zhejiang Province). After sieving, the ESP was packed in 100 mL screw-capped polyethylene bottle before it was distributed to subjects in the intervention group. Two local coordinators, four health extension workers and eight health development agents were organized as a team to distribute packaged ESP in the intervention kebele, and conduct the regular follow-ups, monitoring and supervision. There was no placebo distributed in the control kebele. At every fourth week time point, new ESP bottles were distributed and old and empty bottles were returned. Participants were instructed to add the ESP to foods. Compliance with the supplementation regimen was tracked through regular monitoring and supervision, self-reports

and ESP bottles counts. Regular follow-ups were scheduled to ensure adherence and address any issues.

4.7. Data Collection and Measurement

During the cross-sectional survey data on socio-demographic and economic characteristics, water, hygiene and environmental sanitation practices and obstetric history of mothers were collected using a semi-structured questionnaire (Annex I-III) that was to *Hallabigna,* the language of the district. The interview was conducted face-to-face with the selected women in their living home by trained data collectors. We conducted pretest to validate the effectiveness of the questionnaire in capturing accurate data. In addition, we included questions that can cross-validate responses, such as asking about food frequency and consumption patterns in different formats. The checklists used to assess skeletal and non-skeletal fluorosis were developed by Susheela (2002) and Shashi et al (2008) and described by Kebede et al (2016a).

For the trial study, only the baseline biological (urine and blood) samples were collected after the cross-sectional survey carried out. The remaining data which were collected during the cross-sectional survey served as the baseline data for the trial subjects. At endline, both the biological samples and other data collection were carried out.

The team of data collectors who were hired for purposes of this study included qualified dentist (MD, DMD), physiotherapist (BSc, MSc), laboratory technologists (BSc), public health expert (BSc), nutritionist (BSc), clinical nurses, other bachelor degree holders and high school graduate enumerators (Annex XI).

4.7.1. Diet Diversity using 24 hour Dietary Recall

In order to determine the Minimum Dietary Diversity of Women (MDD-W), we collected data on specific foods consumed by women in the previous day or night using 24-hour dietary recall method (FAO and FHI, 2016). The recall was conducted on all women during the cross-sectional survey and only women included in the trial at EL. The enumerators asked a series of standard probing questions to help the women recall all foods and beverages consumed the previous day and night. Then, each food or beverage that the respondent mentioned was circled on the predefined questionnaire (Annex I-III). During the recall, the amount of food consumed by the respondents was not considered as the type of food alone would be sufficient to determine the diet diversity. MDD-W is a dichotomous indicator of whether or not women 15-49 years of age have consumed at least five out of ten defined food groups in the previous 24 hours. The ten food groups were 1) grains, white roots and tubers, and plantains 2) pulses (beans, peas and lentils) 3) nuts and seeds 4) dairy (milk and milk products) 5) meat, poultry and fish 6) eggs 7) dark green leafy vegetables 8) other vitamin A-rich fruits and vegetables 9) other vegetables 10) other fruits. Women who consumed five or more food groups in that day were considered to have fulfilled the minimum diet diversity. Hence, the proportion of women 15-49 years of age who reached this minimum in a population could be used as a proxy indicator for higher micronutrient adequacy which is one of the important dimensions of diet quality (FAO and FHI, 2016).

4.7.2. Estimation of Habitual Dietary Calcium Intake

A structured food frequency questionnaire (Annex I-III) that asked daily and weekly frequencies of consumption of specific calcium-rich foods common in this district (milk, yogurt, millet, fruits, kale, other vegetables, and moringa) was used to assess the usual dietary calcium intake of women. This was done for all women during the cross-sectional survey and at EL for women

included in the trial. The assessment was done just after completing the diet diversity recall of each woman at their living home. Portion size estimation was made using bowls and plates available in the household of each woman to help them visualize and serve the amount of each food consumed. These frequencies were converted to daily dietary calcium intakes, as described by Gozdzik et al (2009), using published values for calcium content (EPHI, 2013), and recipes were used to identify the amount of calcium in high-calcium foods such as millet injera (Mesfin, 2006). The assumptions used to convert frequencies of consumption to daily dietary calcium intake included: if the frequency of consumption of a particular food more than once a day assumed to be twice a day; if once per day, assumed to be once a day; if more than once per week (i.e 3.5 days on average, thus, 3.5/7 = 0.5), so assumed as to be 0.5 times per day; if once per week (1/7 = 0.15), so assumed as to be 0.15 times per day; if once per month (1/30 = 0.033), so assumed as to be 0.033 times per day and if not consumed over that month, assumed as not consumed daily during that month period (Gozdzik *et al.,* 2009).

4.7.3. Determination of Dental Fluorosis

The women were assessed for dental fluorosis using Dean's index (Dean, 1934) which was used previously by Kebede et al (2016a). This Index classifies fluorosis on a scale of 0 to 4 (Annex VII) as: **Class 0,** No Fluorosis; **Class 1,** Very Mild Fluorosis (opaque white areas irregularly covering 25% of the tooth surface); **Class 2,** Mild Fluorosis (white areas covering 25–50% of the tooth surface); **Class 3,** Moderate Fluorosis (all surfaces affected, with some brown spots and marked wear on surfaces subject to attrition); and **Class 4**: Severe Fluorosis (widespread brown stains and pitting). The average score of the two most severely affected teeth was used to derive this classification. The women were instructed to thoroughly clean their teeth prior to the dental

fluorosis examination. The dental assessment was done on all women only during the cross-sectional survey by an experienced dentist (MD, DDS) who was hired for this purpose.

4.7.4. Assessment of Skeletal and Non-Skeletal Fluorosis

Skeletal and non-skeletal fluorosis were determined using clinical symptoms and physical exercises (Annex VIII) as developed by Susheela (2002) and Shashi et al (2008) and as described by Kebede et al (2016a). Individuals who could not perform the physical exercises were categorized as having skeletal fluorosis (Shashi *et al.,* 2008). The assessment was carried out on all women (IG and CG) during the cross-sectional survey (BL) and at EL on women included in the trial by an experienced physiotherapist (BSc, MSc) who was hired to perform the physical examination. All study subjects were asked to perform three physical exercises which indicate skeletal fluorosis: (i) bending the body and touching floor or toe, (ii) touching chest with chin, and (iii) folding arms to touch back of head. The physiotherapist showed each mother how to perform the exercise. The same physiotherapist assessed the women for the presence or absence of thirteen symptoms of skeletal and non-skeletal fluorosis (Annex VIII).

4.7.5. Assessment of Side Effects related to Excess Calcium Intake

A checklist (Annex IX) was developed to assess the occurrence of possible side effects related to excess calcium intake during ESP supplementation in the trial. The assessment was done at BL and EL on women in both groups, and every month (April to September 2018, during the monthly monitoring period) only on each woman getting ESP at their living home after the commencement of the supplementation. The clinical symptoms assessed for side effects of excess calcium intake include nausea, vomiting, constipation, abdominal bloating and gas.

4.7.6. Food and Water Samples Collection and Analysis

The food samples (Table 7) which are collected from different households during the cross-sectional survey (March 2018) were dried, grounded, and homogenized using drying oven, miller, and homogenizer respectively for fluoride analysis. Aliquots of 5 ml were taken out for analysis after the ash had settled and the solutions were clear. Care was taken to avoid the settled ash. Before analyzing, 0.5 ml TISABIII was added to obtain a pH of 5.2-5.4, which is the optimal pH-range for fluoride determination. Reagent blanks were always prepared together with the samples and were brought through the whole procedure. The fluoride level of standard solutions and sample solutions were measured in ppm using a fluoride ion-selective electrode. For all food samples, 1 ml of 100 ppm fluoride standard was spiked to determine % recovery. Water fluoride was determined by using ES ISO 10359-1: 2001 method, whereas food fluoride level was measured by food fluoride test method (Malde and Bjorvatn, 2001). The electrode was calibrated by working standards starting from most diluted to concentrated standard. The concentrations of the solutions were directly measured in ppm by using fluoride ion selective electrode.

4.7.7. Urine Specimen Collection and Analysis

At BL and EL collections in both clusters, women were instructed to provide morning spot urine specimens using a screw capped plastic bottle (without using additives). No one provided urine specimen of less than 30 mL. The samples were labeled with a unique code and kept in an ice box until they were brought to Hawassa University. The ice box was cooled with ice packs which were changed at 12 hours intervals to prevent the samples from decomposition. The samples were stored at -20ºC in the Hawassa University, Nutrition and Food Science Laboratory

until it was transported to Ethiopian Public Health Institute (EPHI) for analysis fluoride, creatinine and calcium concentration.

The fluoride concentration in urine samples was analyzed using PerfectION^{TM} combination fluoride ion selective electrode (Mettler Toledo, Germany) coupled with bench top dual channel ion-meter (Jenway, model 3345, England), using facilities at EPHI as described previously (Kebede et al., 2016a and 2016b). The samples were determined potentiometrically using a fluoride ion selective electrode after calibrating the electrode with five point calibration standards. The methods used were that of Orion (Orion, 1991).

The calcium and creatinine concentrations in urine samples obtained on a Roche/ Hitachi cobas® c 501 analyzer (Roche Diagnostics International AG, Rotkreuz, Switzerland) using the Roche Calcium Gen.2 and Creatinine plus ver.2 reagents, respectively. These concentration values were compared with those determined using the Roche Calcium reagent on a Roche/Hitachi MODULAR P analyzer for urine calcium and using the corresponding reagent on a Roche/Hitachi 917 analyzer for urine creatinine. The urine sample was analyzed in duplicate at the EPHI. All the analyses (F, Ca, and creatinine) were analyzed against blank samples, and along with standards for quality control. Moreover, Certified Reference Materials were analyzed along the samples to check the accuracy and sensitivity of the method.

4.7.8. Hemoglobin (Hematocrit) and Malaria Testing

At BL and EL, a drop of blood (finger prick) was taken from all women in both groups prior to and after the supplementation to analyze hemoglobin (and hematocrit) immediately in the field using HemoSmart^{TM} GOLD Hemoglobin Screening Meter, APEX BIOTECHNOLOGY CORP,

No.7, Li Hsin Rd. V, Hsinchu Science Park Hsinchu, Taiwan, R.O.C. Disposable blood lancet, alcohol and swabs were used to collect capillary blood by finger pricking. The HemoSmart™ GOLD Hemoglobin Screening Meter uses HemoSmart™ GOLD Hemoglobin Test Strips designed specifically for use with fresh capillary whole blood taken from a fingertip and a venous whole blood to test both hemoglobin and hematocrit simultaneously. Plasma or serum samples cannot be used. Testing must be performed immediately after the sample is obtained.

In addition, while we assessed hemoglobin, malaria testing (which is useful for making decision about anemia status) was also done for all women in both groups at baseline and end of the intervention. The women appreciated this additional health information. First, a finger prick was done using disposable blood lancet after thoroughly cleaning with alcohol and swab. Thereafter the malaria testing was done immediately in the field using rapid diagnostic test (RDT) kit. A one Step Rapid Test for Malaria Pf/Pv Ag, MERISCREEN Malaria Pf/Pv Ag, Meril Diagnostic Pvt, Ltd, India

4.7.9. Venous Blood Specimen Collection and Analysis

A sample of 10 mL venous blood was collected from all trial women at BL and EL in each group by qualified laboratory technologists. This was collected to analyze for serum iron, zinc and calcium levels. The blood samples were collected at the local health center. After taking the blood sample, the spot was covered by a band-aid to protect from infection. The samples were collected into serum tubes lacking coagulant (Vacutainer tubes). The tubes were stored in an ice box for 30 minutes, and then centrifuged at room temperature for 10 minutes at 2000 x g (WHO, 2011). The serum was transferred to plastic trace mineral vials and stored at -20ºC in the Hawassa University, Nutrition and Food Science Laboratory until it was transported to Ethiopian

Public Health Institute for analysis. There, serum iron and zinc were analyzed using Avarian Spectr AA® Flame Atomic Absorption Spectrometry, Shimadzu. Disposable 10 mL syringes, alcohol and swabs, trance mineral-free gloves and polyethene pipettes were used to collect blood specimens.

4.8. Data Quality Assurance

Three days (3rd to 5th of January 2018) training was prepared by principal investigator on interviewing and other data collection techniques. The training was intensively delivered to all data collectors. A practice survey day was undertaken on 5% of the sample size (i.e. a group of 14) in people who were outside of the chosen study area. Data collection forms were modified after this practice survey. All the questionnaires were translated from English to local languages (Amharic and *Hallebigna*) then re-translated back to English to check their consistencies. In addition, supervision, checking for data completeness and data cleaning were done before the data collection period completed and corrective measures were taken accordingly.

4.9. Dropouts in Trial

A total of 82 women (41 in each arm) were recruited into this trial (Figure 2). Two women from the treatment group and one woman from the control group moved away from their homes during the middle of the intervention. At the end of the intervention, one woman from the control group was unwilling to provide urine and blood specimens and thus excluded from the study. Efforts were made to minimize study participants' attrition in both groups during the intervention period by close follow-up and providing incentives such as soap and iodized salt.

4.10. Data Processing

Data were double entered into EpiData version 3.1 (Odense, Denmark) and then exported into IBM SPSS version 25 (Chicago, IL, USA) for analyses. All continuous data were checked for normality using Kolmogorov-Smirnov and Shapiro-Wilk Tests of Normality. Data on skeletal and non-skeletal fluorosis symptoms were categorized into three symptoms indices according to Tandon et al (2015). Index-1 is named *Skeletal rigidity and joint pain*, Index-2 is named *Muscular weakness manifestations*, and Index-3 is named *Gastro-intestinal complaints*. Index-1 included: skeletal rigidities and pain of the neck region, back, knee and shoulder joints. Index-2 included: stiffness of the back and neck muscles, unable to bend forward and to stand straight, and inability to carry out routine activities. Index-2 included: early signs of fluoride toxicity such as loss of appetite, nausea, pain in the stomach, constipation, and gas formation in the stomach with bloated feeling. When a woman complained of ≥ 2 symptoms of skeletal and non-skeletal fluorosis, she got a score of 1 and if not, scored 0. This was done for each fluorosis index. Data on dental fluorosis was analyzed as 1= having any level of dental fluorosis severity (very mild through severe) and 0= if questionable or normal. The dichotomized value for each index as well as for dental fluorosis was used in the regression analysis. The main hypothesized exposure variable and other predictors were computed in the bivariate logistic regression to check their association with fluorosis symptoms indices. The variables that emerged as predictor for each fluorosis symptoms index at $P < 0.2$ in the bivariate analysis were recruited for further multivariate logistic regression analysis. These included: age of women, educational status, occupation, parity, DDS, dietary calcium intake, family size and years of residency. Finally, all the enrolled independent variables from the bivariate logistic regression were entered into the multivariate logistic regression model to see their association with the outcome variable and to

control possible confounders. Collinearity diagnostics test was done using linear regression to check if the independent variables were independent from each another or not. In the collinearity diagnostic test statistics, the variance inflation factors (VIF) for each independent variable varied from 1.033 to 1.13 and the tolerance varied from 0.888 to 0.968. Therefore, in the regression model there was no multicollinearity between the independent variables. Data restructuring was done to carry out the generalized estimating equation (GEE) analysis.

4.11. Statistical Methods

The Chi-square (X^2) and relative risk (RR) test were used to compare baseline characteristics and clinical symptoms between the intervention and control groups. Bivariate and multivariate logistic regression was done to assess the association of explanatory variables with the outcome variable, to control possible confounders and to compare the dichotomized outcome variables (skeletal and non-skeletal fluorosis symptoms) before and after the intervention. Paired samples t-test was used to compute the 'within' mean differences of endline continuous outcome variables from the baseline. The independent samples t-test was used to compute the mean differences of continuous outcome variables between the IG and CG at BL and EL. In this study, urine fluoride, hemoglobin and hematocrit levels were normally distributed but serum iron, serum zinc, urine calcium and urine creatinine levels were not normally distributed. Therefore, a linear generalized estimating equation (GEE) was used to allow the repeated measures of continuous variables, non- homogeneity of variance and the non-normally distributed data (Heck *et al.,* 2012). Maternal age and parity, family size (number of children, parents and relatives living together in the household), year of residence in the area, diet diversity and dietary calcium intake were included in the multivariate analysis of GEE model. Then, the multivariate linear regression analysis was carried out and beta coefficient at 95% confidence interval (CI) reported. Complete case analyses were done for each statistical test at baseline and endline as there were only four dropouts (two women in each arm).

4.12. Ethical Consideration

Ethics approvals were obtained from Hawassa University College of Medicine and Health Science (Ref. No: *IRB/019/10*) and University of Saskatchewan Biomedical Research Ethics, Saskatoon, Canada (Ref. No: *Bio# 17-150*) (Annex XII). Letters of support were also obtained from Regional Health Office, Zonal Health Desk and District Health Offices. Confidentiality of personal information has been kept. Women's personal information would only be used for the purpose of the study. The participants would not be personally identified in the study report. Furthermore, women's information would be coded so that their identity was not disclosed. After the purpose and methods of the study were fully explained, and their right to refuse was explained, informed verbal and written consents were obtained from all study participants prior to their participation in the study.

5. Results

5.1. Paper I: Association of Dietary Calcium Intake with Dental, Skeletal and Non-Skeletal Fluorosis

5.1.1. Socio-Demographic and Economic Characteristics

The mean (SD) age of the women in completed years was 29.5 ± 4.2 years. Most of the women (96.5%) were married, the majority (67.4%) had not attended school (were illiterate) and just more than half (51.9%) of them had no paid job outside their home. The main source of income for most (87.8%) of the households was agriculture. The average family size (parents and children together) was approximately 7 ± 2 (Table 4).

Table 4: Socio-demographic and economic characteristics of study participants in Halaba district, southern Ethiopian Rift Valley, 2018.

Characteristics (n=270)		N	(%)
Mother's educational status	Illiterate (hadn't attended school)	182	67.4
	Primary education (1st to 8th grade)	74	27.4
	Secondary education (9th to 12th grade)	11	4.1
	College (above 12th grade)	3	1.1
Mother's main occupation	No outside job/housewife/	140	51.9
	Agriculture	96	35.6
	Petty trading	29	10.7
	Employed	5	1.9
Main source of household income	Agriculture	237	87.8
	Petty trading	24	8.9
	Employed	9	3.3
Residency	> 15 years	138	51.1
	11– 15 years	46	17.0
	5 – 10 years	62	23.0
	< 5 years	24	8.9

¥ETB = Ethiopian birr. 1 ETB = $0.02USD in 2018.

5.1.2. Water Supply, Hygiene and Environmental Sanitation

The main source of drinking water for all households was a tap at a common public stand pipe, and the majority (63.7%) of the study participants walked about an hour to get the drinking water from the public stand pipe. Most of the study participants (82.2%) were not filtrating or treating the water before drinking it. Pit latrine was available in most (88.5%) households among the study women and most (89.3%) of them used a bar of soap while washing their hands (Table 5). There was no attempt by study participants or health workers in these kebeles to take any measure (including dietary) to lessen the toxic effects of excess F intake. Study participants also did not receive health education about excess intake of F in foods or drinks, its consequences on health (fluorosis), and measures needed to be taken to reduce F exposure, prior to this study. None of the households used rainwater for drinking as it was scarce and due to fearing of dusts contamination from the roof. Bed nets were available in less than half (44.4%) of the study participants' households.

Table 5: Water, hygiene and environmental sanitation of study participants in Halaba district, southern Ethiopian Rift Valley, 2018

Characteristics (n=270)		**N**	**%**
Time to bring water from public stand pipe	1 hour or less	172	63.7
	> 1 hour	98	36.3
Equipment used to store drinking water	Jerrican	215	79.6
	Bucket	55	20.4
Filtrating/treating the water before drinking	No	222	82.2
	Yes	48	17.8
If yes, method of filtration (n= 48)	Water treatment pills	29	60.4
	Filtering cloths	17	35.4
	Boiling and removing sediment	2	4.2
Use same water source for drinking and cooking	No	230	85.2
	Yes	40	14.8
Use same water source for drinking and bathing	No	220	81.5
	Yes	50	18.5

Knowledge on hand washing (affirmative)	Before preparing food	42	15.6
	Before feeding the child	226	83.7
	After using toilet	215	79.6
	After cleaning child (defecated)	201	74.4
Substance used for hand washing	Soap bar	241	89.3
	Just only water	20	7.4
	Liquid soap	9	3.3
Household latrine type	Outside home latrine	239	88.5
	Protected open field	20	7.4
	No latrine (open field)	11	4.1

5.1.3. Obstetric History of Women

Regarding the history of childbirth of the women, 23.3% of mothers had a history of spontaneous abortion (i.e. miscarriage) and 15.2% had a history of stillbirth. The average number of live-born children by the study mothers was approximately four (Table 6).

Table 6: Obstetric history of women in *Halaba* district, southern Ethiopian Rift Valley, 2018

Characteristics (n=270)		**N**	**%**
History of miscarriage	No	207	76.7
	Yes	63	23.3
History of stillbirth	No	229	84.8
	Yes	41	15.2
History of giving birth of a child with congenital anomaly	No	259	95.9
	Yes	11	4.1
If congenital anomaly present, type of birth defect (n = 11)	Paralysis of upper or lower extremity	7	63.6
	Any defect of the trunk (e.g. spinal bifida)	4	36.4
Gravidity (mean ± SD)	4.7 ± 1.8		
Parity (mean ± SD)	4.3 ± 1.5		

SD = Standard Deviation

5.1.4. Fluoride Level in Food, Beverage and Water Samples

The F content in staple food samples varied between 0.8 – 13.6 mg/kg. The foods which were prepared from maize and millet had higher F levels whereas vegetable food sources such as cabbage and potato had low concentration of F. For all food items percent recovery varied between 90.4% – 107.9% which falls into the acceptable range of percent recovery. All common drinking water sources in the study area had F level exceeding the WHO guidelines for drinking water (1.5mg/L). The average F level in drinking water sources was approximately 5mg/L (Table 7).

Table 7: Fluoride level in staple foods, beverage and drinking water consumed by the study subjects in Halaba district, southern Ethiopian Rift Valley, 2018

Sample Type	Fluoride	Recovery (%)
Food	**mg/kg**	
Injera (unknown ingredients)	10.6	100.6
Unleavened bread (unknown ingredients)	13.6	97.2
Unleavened bread, maize	12.5	102.5
Taro, Colacasia antiquorum:, boiled	4.6	96.2
Injera (Maize, Millet (1:1))	8.3	102.5
Unleavened bread, millet	4.9	93.7
Boiled cabbage	0.8	103.2
Boiled potato and carrot	1.6	92.2
Stew without tomato	3.9	103.6
Banana	5.3	107.9
Boiled beetroot	3.2	102.7
Cabbage and potato stew	2.4	95.9
Rice with carrot	3.2	103.6
Injera (sorghum, maize, millet (1:1:1))	4.9	95.4
Injera (millet)	7.8	105.7
Bread (maize)	10.5	99.0
Beverage	**mg/kg**	
Coffee (prepared from coffee leaf)	3.5	104.5
Drinking water:	**mg/L**	
Drilled tap water	4.7	
Hand pump water	6.2	
Well water	3.3	

5.1.5. Dietary Calcium Intake of Women

The average daily dietary Ca intake of women was 406 ± 97 mg (Table 8). Only 37.4% of women consumed one or more locally available Ca-rich foods daily. More than half (55.2%) of the women also did not consume yogurt and approximately one-quarter (23.3%) did not drink milk during the last month prior to this survey. No participant reported consuming moringa. The mean (±SD) dietary diversity score of women based on ten food groups was 4.92 (±1.91).

Table 8: Frequency of calcium-rich food consumption by women in Halaba district, southern Ethiopian Rift Valley, 2018

Frequency of consumption (n=270)	Milk N (%)	Yogurt N (%)	Millet N (%)	Kale N (%)	Fruits* N (%)	Other Vegetables# N (%)	Moringa N (%)
> Once per day	29 (10.7)	21 (7.8)	114 (42.2)	125 (46.3)	22 (8.1)	25 (9.3)	0
Once a day	27 (10.0)	19 (7.0)	66 (24.4)	61 (22.6)	9 (3.3)	12 (4.4)	0
> Once per week	32 (11.9)	15 (5.6)	55 (20.4)	49 (18.1)	39 (14.4)	38 (14.1)	0
Once a week	67 (24.8)	30 (11.1)	26 (9.6)	35 (13.0)	147 (54.4)	122(45.2)	0
Once a month	52 (19.3)	36 (13.3)	9 (3.3)	0	16 (5.9)	25 (9.3)	0
Not consumed	63 (23.3)	149 (55.2)	0	0	37 (13.3)	48 (17.8)	270 (100)
Mean (SD) daily dietary calcium (mg): 406.4 (96.8)							

5.1.6. Fluorosis Assessment of Women

The percentage of severe dental fluorosis was 9.3%; and the percentage of women with moderate dental fluorosis was 25.2%. The overall percentage of women who were unable to perform the physical exercises which indicate skeletal fluorosis was 34.4%. During the skeletal fluorosis examination, 40.4% of women were unable to touch chest with chin and 34.8% of women were unable to touch back of head by stretching and folding their hands. Overall, 124 (45.9%) of women complained of more than one symptom listed in Table 9. The majority of the women felt lower back pain (70.7%) and had constipation (79.3%). More than half (54.8%) of them also had

muscle weakness. In addition, around half of the women had neck pain (46.3%) or had been complaining of abdominal pain (48.5%). Overall, more than half (56.3%) of the study women had very mild to severe dental fluorosis (Table 9).

Table 9: Prevalence (%) of fluorosis among women (n = 270) in Halaba district, southern Ethiopian Rift Valley, 2018

Measurement	N	%
Dental fluorosis category		
Normal	96	35.6
Questionable	22	8.1
Very mild: opaque white areas covering 25% of the tooth surface	38	14.1
Mild: white areas covering 25%–50% of the tooth surface	21	7.8
Moderate: all surfaces affected, with some brown spots and marked wear on surfaces subject to attrition	68	25.2
Severe: widespread brown stains and pitting	25	9.3
Skeletal Fluorosis Exercise Testing		
Unable to bend body and touch floor or toe	76	28.1
Unable to touch chest with chin	109	40.4
Unable to stretch and fold arms to touch back of head	94	34.8
Symptoms of Non-skeletal Fluorosis		
Feel lower back pain	191	70.7
Feel leg pain, joints	182	67.4
Feel arm pain, joints	143	53.0
Feel tingling in the hands and feet	172	63.7
Feel neck pain with movement	125	46.3
Feel muscle weakness	148	54.8
Feel loss of appetite	100	37.0
Have nausea	101	37.4
Have abdominal pain	131	48.5
Have bloating in stomach	60	22.2
Experience polydipsia (excessive thirst)	22	8.1

Experience polyuria (excess urine volume)	16	5.9
Experience constipation	214	79.3

5.1.7. Association of Fluorosis Indices with Dietary Ca Intake

As shown in Table 10, we tested the association of skeletal fluorosis with subject characteristics and environmental factors. For Skeletal rigidity and joint pains, Index-1, age, diet diversity, and dietary Ca intake were significantly associated with having two or more symptoms. For having Muscular weakness, Index-2, age, parity, and dietary Ca intake were significantly associated, in the adjusted model. For both Index-1 and Index-2, only age and dietary calcium contributed to worsening of this condition. This study revealed that women whose dietary Ca intake was less than 400 mg/day, which was the average intake (Table 8), had about three times greater odds of developing Index-1 Skeletal rigidity with joint pains (AOR=2.8, 95%CI: 1.6, 5.0) and Muscular weakness, index-2 (AOR=2.9, 95%CI: 1.3, 6.3) than those who had higher intake.

The odds of developing both index-1 and index-2 fluorosis symptoms was 1.2 (95%CI: 1.0, 1.2) and 1.7 (95%CI: 1.5, 2.0), respectively, with each passing year of age (Table 10). No variables were significantly associated (P>0.05) with either Index-3 (Gastro-intestinal complaints) (Annex XIV Supplementary Table I) or dental fluorosis (Annex XIV Supplementary Table II).

Table 10: Factors associated with muscular-skeletal symptoms of fluorosis Index-1 and Index-2 among women: logistic regression model

Variable (n=270)	Category	Skeletal rigidity and joint pains Index-1		COR (95%CI)[#]	AOR (95%CI)	Muscular weakness Index-2		COR (95%CI)[#]	AOR (95%CI)
		No N (%)	Yes N (%)			No N (%)	Yes N (%)		
Age (y)	Mean (SD)	29.5 (4.2)		1.2 (1.1, 1.2)	1.2 (1.0, 1.2)*	29.5 (4.2)		1.8 (1.6, 2.0)	1.7 (1.5, 2.0)*
Education	Unable to read and write	47 (33.8)	92 (66.2)	1.6 (0.9, 2.5)	1.6 (0.9, 2.7)	65 (46.8)	74 (53.2)	1.7 (1.0, 2.7)	1.2 (0.6, 2.5)
	Able to read and write	58 (44.3)	73 (55.7)	1		78 (59.5)	53 (40.5)	1	
Occupation	No job	40 (39.6)	61 (60.4)	1		47 (46.5)	54 (53.5)	1	
	Have job	65 (38.5)	104 (61.5)	1.1 (0.6, 1.7)	1.3 (0.8, 2.4)	96 (56.8)	66 (42.3)	1.4 (0.9, 2.3)	1.5 (0.7, 3.0)
Parity	≤ 4 live births	32 (37.2)	54 (62.8)	1		90 (57.7)	66 (42.3)	1	
	> 4 live births	73 (39.7)	111(60.3)	1.1 (0.7, 1.9)	1.2 (0.6, 2.2)	53 (46.5)	61(53.5)	1.6 (1.0, 2.6)	2.5 (1.1, 5.6)*
Family size[$]	≤ 5 members	32 (45.1)	39 (54.9)	1		46 (64.8)	25 (35.2)	1	
	> 5 members	73 (36.7)	126 (63.3)	1.4 (0.8, 2.4)	1.3 (0.7, 2.4)	97(48.7)	102 (51.3)	1.9 (1.1, 3.4)	1.1 (0.4, 2.3)
Diet diversity	≤ 5 food groups	53 (32.5)	110 (67.5)	1.9 (1.19, 3.2)	2. 0 (1.1, 3.4)*	81 (49.7)	82 (50.3)	1.4 (0.9, 2.3)	1. 5 (0.7, 3.0)
	> 5 food groups	52 (48.6)	55 (51.4)	1		62 (57.9)	45 (42.1)	1	
Dietary Ca	≤ 400mg/day	35 (25.4)	103 (74.6)	3.3 (2.0, 5.6)	2.8 (1.6, 5.0)*	54 (39.1)	84 (60.9)	3.2 (1.9, 5.3)	2.9 (1.3, 6.3)*
	> 400mg/day	70 (53.0)	62 (47.0)	1		89 (67.4)	43 (32.6)	1	
Residency	≤ 10 years	40 (46.5)	46 (53.5)	1		57 (66.3)	29 (33.7)	1	
	> 10 years	65(35.3)	119 (64.7)	1.6 (0.9, 2.7)	1.1 (0.6, 2.0)	86 (46.7)	98 (53.3)	2.2 (1.3, 3.8)	1.8 (0.8, 4.0)

[$]Number of children and parents living together; [#]in the bivariate logistic regression, all these variables were significant predictors at P < 0.2; *statistically significant association observed at P <0.05. COR = Crude Odds Ratio AOR = Adjusted Odds Ratio

5.2. Paper II: Efficacy of Calcium-containing ESP Supplementation on Urinary Fluoride and Fluorosis Symptoms

5.2.1. Socio-Demographic and Nutritional Characteristics

At BL, the mean (±SD) age of women in completed years was 29.6 ± 3.8 years. There was no significant difference (t = -0.91, DF=76, P= 0. 368) in the distribution of age between the two arms. Most of the women (93.6%) were married and more than half of them were illiterate (56.4%) and had no job outside their household (53.8%). The main source of income for most (88.5%) of the households was agriculture (Table 4). The mothers' average number of live-born children was approximately four. The average family size (parents, children and relatives living together) was approximately 7 ± 2, in which at least two of them were children under five years old. Overall, the groups were comparable at baseline and the differences were small (Table 11).

About two-thirds of the women did not fulfill the minimum daily diet diversity score (consuming at least from 5 food groups out of the 10 food groups). The mean (SD) dietary diversity score (DDS) of women based on ten food groups was approximately 5 ± 2. The average dietary Ca intake was 403 ± 124 mg, and the independent two samples t-test showed a small and non-significant mean difference in the DDS (t = 0.74 (DF=76), P = 0.459) and in the mean difference of dietary Ca intake (t = 0.65(DF=76), P = 0.516) between the intervention and control groups at BL (Table 11).

Table 11: Baseline socio-demographic, economic and nutritional characteristics of study participants, Halaba district, southern Ethiopian Rift Valley, 2018.

Characteristics		Intervention Group (n=39)	Control Group (n=39)	P-value¥
Socio-demographic and economic				
Age (years)	Mean (SD)	29.9 (3.9)	30.5 (3.8)	0.368
		N (%)	N (%)	
Educational status	Illiterate	24 (61.5)	20 (51.3)	0.361
	Literate	15 (38.5)	19 (48.7)	
Main occupation	No outside job	22 (56.4)	20 (51.3)	0.650
	Outside job	17 (43.6)	19 (48.7)	
Main source of income	Agricultural	35 (89.7)	34 (87.2)	0.723
	Non-agricultural	4 (10.3)	5 (12.8)	
Parity	≤ 4 live births	21 (53.8)	20 (51.3)	0.821
	> 4 live births	18 (46.2)	19 (48.7)	
Family size	≤ 5 members	10 (25.6)	13 (33.3)	0.456
	> 5 members	29 (74.4)	26 (66.7)	
Residency	< 10 years	12 (30.8)	9 (23.1)	0.444
	≥ 10 years	27 (69.2)	30 (76.9)	
Nutritional				
Diet diversity score	Mean (SD)	5.1 (1.99)	4.7 (1.96)	0.459
Dietary calcium intake	Mean (SD)	394 (120)	412 (129)	0.516
		N (%)	N (%)	
Average dietary calcium intake	≤ 400 mg/day	22 (56.4)	21 (53.8)	0.820
	> 400 mg/day	17 (43.6)	18 (46.2)	
Dietary diversity score	≤ 5 food groups	24 (61.5)	27 (69.2)	0.475
	> 5 food groups	15 (38.5)	12 (30.8)	

ETB = Ethiopian Birr; IG = Intervention Group; CG = Control Group. ¥For values with mean (SD) comparison was done using independent samples t test; for prevalence values, comparison was done using Chi-square test.

5.2.2. Baseline Dental Fluorosis

The proportion of severe dental fluorosis among all women included in this trial was 10.3%; and the proportion of women with moderate dental fluorosis was 29.5% (30.8% in the intervention and 28.2% in the control groups). Overall, more than half, 57.7% (64.1% in the intervention and 51.3% in the control groups) of the study women had very mild to severe dental fluorosis level. There was no statistically significant difference in the proportion of dental fluorosis categories between the treatment and control groups during the baseline assessment (P>0.05) (Table 12). As

dental fluorosis is assumed to be an irreversible process, the dental examination was not repeated after the end of the trial.

Table 12: Comparison of prevalence of dental fluorosis at the baseline between the intervention and control groups in Halaba district, southern Ethiopian Rift Valley, 2018

Dental Fluorosis Category[¥]	IG (n=39)	CG (n=39)	Total (n=78)	P-value#
	N (%)	N (%)	N (%)	
Normal	11 (28.2)	14 (35.9)	25 (32.1)	0.628
Questionable	3 (7.7)	5 (12.8)	8 (10.3)	0.712
Very mild	4 (10.3)	3 (7.7)	7 (9.0)	0.999
Mild	5 (12.8)	2 (5.1)	7 (9.0)	0.431
Moderate	12 (30.8)	11 (28.2)	23 (29.5)	0.999
Severe	4 (10.3)	4 (10.3)	8 (10.3)	0.999

IG = Intervention Group; CG = Control Group; [¥]Assessment was based on criteria of Dean's index (Dean, 1934).

P-values were generated by chi-square test using OpenEpi Version 3.03.

5.2.3. Urinary Fluoride, Calcium and Creatinine

At BL, the independent two samples t-test showed that the average urinary F (~ 10 mg/L) excretion by the women between the intervention and control groups was similar [t = 0.11 (DF=76, 95% CI: -1.31, 1.47)]. After the calcium-containing ESP supplementation, there was more than 50% (Table 3) significant reduction in the urinary F excretion of women in the intervention group compared with their baseline measurements using paired samples t-test; [t = 5.7 (DF=76, 95% CI: 4.77, 6.49)]. At endline, women in the intervention group had about six-fold lower urinary F excretion [β = - 6.1 (95% CI: -7.1, -5.1)] compared to women in the control group (Table 13). Urinary creatinine excretion was used as a measure of the spot urine collection. As shown in Table 13, all four collections of creatinine showed similar average concentrations of creatinine. There was no significant difference between the intervention and control groups in creatinine concentration at BL (P=0.785) and EL (P=0.571). In addition, there

was no significant difference in urinary Ca excretion among the groups prior (P=0.945) and after (P=0.247) the supplementation of calcium- containing ESP (Table 13).

Table 13: Urinary excretion of fluoride (F), calcium (Ca), and creatinine of women in the intervention and control groups in Halaba district, Ethiopian Rift Valley, 2018.

Measure	Baseline	Endline	Within mean difference (95%CI)†	Beta coefficient (95%CI)¥	P-value†
	Mean (95%CI) (n = 39)	Mean (95%CI) (n = 39)			
F (mg/L)					
IG	10.3 (9.2, 11.3)	4.6 (4.1, 5.2)	5.7 (4.77, 6.49)#	- 6.1 (-7.1, -5.1)	<0.001
CG	10.4 (9.4, 11.3)	10.8 (9.9, 11.7)	-0.5 (-0.96, 0.05)	1	
Ca (mmol/L)					
IG	0.52 (0.24, 0.80)	0.82 (0.50, 1.13)	-0.3 (-0.74, 0.15)	0.3 (-0.27, 0.78)	0.346
CG	0.53 (0.34, 0.73)	0.57 (0.30, 0.85)	-0.04 (-0.38, 0.29)	1	
Creatinine (mmol/L)					
IG	4.8 (3.8, 5.7)	5.0 (4.2, 5.7)	-0.2 (-1.2, 0.9)	0.5 (-1.1, 2.2)	0.542
CG	5.0 (4.0, 5.9)	4.6 (3.7, 5.6)	0.4 (-1.0, 1.7)	1	

†The 'within' mean difference of endline from baseline computed using paired samples t-test. #statistically significant difference observed at P<0.05; ¥Beta coefficient at 95% Wald confidence interval and P-value analyzed using GEE linear model. ¥ Urinary fluoride, calcium and creatinine were adjusted for age, parity, family size, residency, diet diversity and dietary Ca intake. IG = Intervention Group; CG = Control Group.

5.2.4. Skeletal Fluorosis using Physical Exercise Testing

At baseline, the overall proportion of physical signs of skeletal fluorosis among women in this trial was 41.3% (43.6% in treatment and 38.5% in the control group). The two groups were similar (P > 0.05) in all skeletal fluorosis exercise testing (Table 14). After the six months supplementation of calcium-containing ESP, the overall proportion of physical signs of skeletal fluorosis significantly decreased from 43.6% to 17.9% in the treatment group (P < 0.001), while the control group did not change. The risk of developing skeletal fluorosis tested using the ability to bend body and touch floor or toe [RR = 0.21 (95% CI: 0.07, 0.69)], and stretch and fold arms

to touch back of head [RR = 0.18 (95% CI: 0.04, 0.77)] were significantly reduced in the intervention group by 79% and 82% respectively compared with the control (Table 14).

Table 14: Skeletal fluorosis (SF) exercise testing in a 6-month ESP intervention between intervention and control groups in Halaba district, Ethiopian Rift Valley, 2018

	Group	Skeletal Fluorosis Exercise Testing§, N (%)					
		Bend body and touch floor or toe		Stretch and fold arms to touch back of head		Touch chest with chin	
		SF	No SF	SF	No SF	SF	No SF
Baseline	IG (n=39)	15 (38.5)	24 (61.5)	17 (43.6)	22 (56.4)	19 (48.7)	20 (51.3)
	GC (n=39)	13 (33.3)	26 (66.7)	15 (43.6)	24 (56.4)	17 (43.6)	22 (56.4)
	RR (95%CI)	1.15 (0.64, 2.10)		1.13 (0.66, 1.93)		1.12 (0.69, 1.81)	
Endline	IG (n=39)	3 (7.7)	36 (92.3)	2 (5.1)	37 (94.9)	12 (30.8)	27 (69.2)
	CG (n=39)	14 (35.9)	25 (36.1)	11 (28.2)	28 (71.8)	15 (38.5)	24 (61.5)
	RR (95%CI)	0.21 (0.07, 0.69)*		0.18 (0.04, 0.77)*		0.80 (0.43, 1.48)	

SF = Skeletal Fluorosis (cannot do exercise); §Testing was done according to Shashi et al [22]. *Statistically significant association observed at p-value <0.05. IG = Intervention Group; CG = Control Group.

5.2.5. Pain and Muscular Symptoms of Non-skeletal Fluorosis

At baseline, all pain and muscular symptoms of non-skeletal fluorosis were similar (P>0.05) among the intervention and control group (Table 15). The majority of the women had lower back pain (71.8%, 76.9% in the intervention group and 66.7% in the control group) and tingling sensation in the hands and feet (70.5%, 69.2% in the intervention group and 71.8% in the control group). About two-third of them had also leg pain (64.1%, 69.2% in the intervention group and 59% in the control group) and muscle weakness (62.8%, 64.1% in the intervention group and 61.5% in the control group), which were not statistically different between the two groups (P>0.05) (Table 15). Majority of the women in IG reported mitigation of pain and muscular symptoms of non-skeletal fluorosis ranging from lowest RR = 0.17 (95% CI: 0.05, 0.52) to

highest RR = 0.59 (95% CI: 0.39, 0.88) after the calcium-containing ESP supplementation than in CG (Table 15).

Table 15: Pain and muscular symptoms of non-skeletal fluorosis in a 6-month ESP intervention between IG and CG in Halaba district, Ethiopian Rift Valley, 2018

		Pain and muscular symptoms§ of non-skeletal fluorosis, N (%)													
		Lower back pain		Leg joints pain		Arm joint pain		Neck pain with movement		Tingling, hands and feet		Muscle weakness		Abdominal pain	
	Group	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No
Baseline	IG (n=39)	30 (76.9)	9 (23.1)	27 (69.2)	12 (30.8)	21 (53.8)	18 (46.2)	20 (51.3)	19 (48.7)	27 (69.2)	12 (30.8)	25 (64.1)	14 (35.9)	22 (56.4)	17 (43.6)
	CG (n=39)	26 (66.7)	13 (33.3)	23 (59.0)	16 (41.0)	16 (41.0)	23 (59.0)	22 (56.4)	17 (43.6)	28 (71.8)	11 (28.2)	24 (61.5)	15 (38.5)	24 (61.5)	15 (38.5)
	RR (95%CI)	1.15 (0.87, 2.53)		1.17 (0.84, 1.64)		1.31 (0.82, 2.11)		0.91 (0.60, 1.37)		0.96 (0.72, 1.28)		1.04 (0.74, 1.47)		0.92 (0.63, 1.33)	
Endline	IG (n=39)	17 (43.6)	22 (56.4)	9 (23.1)	30 (76.9)	5 (12.8)	34 (87.2)	3 (7.7)	36 (92.3)	5 (12.8)	34 (87.2)	8 (20.5)	31 (79.5)	8 (20.5)	31 (79.5)
	CG (n=39)	29 (74.4)	10 (25.6)	27 (69.2)	12 (30.8)	21 (53.8)	18 (46.2)	18 (46.2)	21 (53.8)	20 (51.3)	19 (48.7)	26 (66.7)	13 (33.3)	27 (69.2)	12 (30.8)
	RR (95%CI)	0.59 (0.39, 0.88)*		0.33 (0.18, 0.61)*		0.24 (0.10, 0.57)*		0.17 (0.05, 0.52)*		0.25 (0.10, 0.60)*		0.31 (0.16, 0.59)*		0.30 (0.15, 0.57)*	

§Testing was done according to Shashi et al [22]; *Statistically significant association observed at p-value <0.05. IG – Intervention group, CG = Control group.

5.2.6. Gastrointestinal and Urinary Symptoms of Non-Skeletal Fluorosis

At baseline, the two groups were comparable ($p > 0.05$) in all gastrointestinal and urinary symptoms of non-skeletal fluorosis (Table 16). The majority of the women complained of constipation (79.5% in the intervention group and 87.2% in the control group). Just half of the women had nausea (53.8% in the intervention group and 48.7% in the control group). Only very few women reported gastrointestinal or urinary symptom of non-skeletal fluorosis. After the six months supplementation of calcium-containing ESP, the risk of developing gastrointestinal symptoms such as loss of appetite and nausea, but not constipation or bloating were significantly reduced in women in the IG compared with those in

the CG. The risk of developing bladder-related symptoms such as polyuria and polydipsia was not different from baseline after six months of calcium-containing ESP supplementation and not different from that in women who received no ESP (Table 16).

Table 16: Gastrointestinal and urinary symptoms of non-skeletal fluorosis in a 6-month ESP intervention between IG and CG in Halaba zone, Ethiopian Rift Valley, 2018

		Gastrointestinal and urinary symptoms§ of non-skeletal fluorosis, N (%)											
		Loss of appetite		Nausea		Bloating in stomach		Polydipsia		Polyuria		Constipation	
	Group	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No
Baseline	IG (n=39)	14 (35.9)	25 (64.1)	21 (53.8)	18 (46.2)	7 (17.9)	32 (82.1)	6 (15.4)	33 (84.6)	2 (5.1)	37 (94.9)	31 (79.5)	8 (20.5)
	CG (n=39)	12 (30.8)	27 (69.2)	19 (48.7)	20 (51.3)	8 (20.5)	31 (79.5)	3 (7.7)	36 (92.3)	3 (7.7)	36 (92.3)	34 (87.2)	5 (12.8)
	RR (95%CI)	1.15 (0.87, 2.53)		1.17 (0.84, 1.64)		1.31 (0.82, 2.11)		0.91 (0.60, 1.37)		0.96 (0.72, 1.28)		1.04 (0.74, 1.47)	
Endline	IG (n=39)	4 (10.3)	35 (89.7)	5 (12.8)	34 (87.2)	7 (17.9)	32 (82.1)	5 (12.8)	34 (87.2)	2 (5.1)	37 (94.9)	10 (25.6)	29 (74.4)
	CG (n=39)	17 (43.6)	22 (56.4)	21 (53.8)	18 (46.2)	9 (23.1)	30 (76.9)	7 (17.9)	32 (82.1)	4 (10.3)	35 (89.7)	13 (33.3)	26 (66.7)
	RR (95%CI)	0.59 (0.39, 0.88)*		0.33 (0.18, 0.61)*		0.24 (0.10, 0.57)*		0.17 (0.05, 0.52)*		0.25 (0.10, 0.60)*		0.31 (0.16, 0.59)*	

§Testing was done according to Shashi et al [22]); *statistically significant association observed at p-value <0.05. IG = Intervention group, CG = Control group.

5.3. Paper III: Safety and Acceptability of Calcium-containing ESP Supplementation in the Phase II Clinical Trial

5.3.1. Socio-Demographic and Economic Characteristics

The mean (±SD) age of women in completed years was 29.6 ± 3.8 years. Most (93.6%) of the women were married and had an average of four live-born children. More than half of the women were illiterate (56.4%) and had no job (53.8%) outside their household. The average (±SD) number of family members (parents, children and relatives) living together in same household was approximately 7 ± 2, in which at least two of them were children under five years old. Overall, there was no significant difference in common baseline characteristics of study subjects between the two groups (Table 11).

5.3.2. Calcium Intake prior to and after Supplementation in Comparison with the Recommended Intake and Relative to Upper Intake Level

Overall, the average daily dietary calcium intake of women in both groups was below the recommended adequate intake (<1000mg/day), ~ 403mg/day (Table 17). The independent two samples t-test showed non-significant differences in mean dietary calcium intake between the treatment and control arms at baseline (t = -0.65 (76), *P = 0.516*) and endline (t = 0.10 (76), *P = 0.922*) assessments. The daily dietary calcium intake of the women in the intervention group was increased with 960mg calcium using the daily supplementation of calcium-containing eggshell powder (ESP). With this supplementation, all the women in the intervention group fulfilled the recommended daily calcium intake. None of the women's daily calcium intake exceeded the upper intake level (Table 17).

Table 17: Comparison of daily calcium intake of women with the recommended intake and relative to upper intake level in *Halaba* district, southern Ethiopian Rift Valley, 2018

Categories of daily dietary Ca intake	Intervention		Control	
	Baseline (39) N (%)	Endline (39) N (%)	Baseline (39) N (%)	Endline (39) N (%)
Adequate intake (1000mg/day)	0		0	0
Below the recommendation (<1000mg/day)	39 (100)	39 (100)	39 (100)	39 (100)
Above the UL (≥ 2500mg/day)	0	0	0	0
Mean (±SD)	394 ± 120	409 ± 93	412 ± 129	407 ± 84
Ca from daily ESP supplementation (mg/day)	0	960	0	0
Total intake (average + supplement) (mg/day)	274 - 514	1276 - 1462	283 -541	323 - 491
Total intake relative to adequate intake (mg/day)	486 – 726[#]	276 - 462[¥]	459 - 717[#]	509 - 677[#]
Total intake relative to UL (mg/day) [†]				

Ca = Calcium
UL = Upper intake Level
[#]The women's total daily calcium intake was below the adequate intake within these ranges
[¥]The women's total daily calcium intake was above the adequate intake within by this range
[†]All the women's total daily calcium intakes were below the upper intake level

5.3.3. Side Effects related to Excess Calcium Intake in the Phase II Trial

At baseline, about 50% of the women in both groups were complaining all of the symptoms related to excess calcium intake. These symptoms were similar (*P>0.05*) in women between the intervention and control group both in the unadjusted and adjusted analyses. Three of the symptoms (loss of appetite, abdominal pain and nausea or vomiting) were significantly decreased only in the treatment group compared with the control group at the end of the trial (Table 18).

Table 18: Comparison of symptoms related to excess calcium intake in women between the two arms prior to and after supplementation in *Halaba* district, Ethiopian Rift Valley, 2018

Period	Symptoms		IG (n=39) N (%)	CG (n=39) N (%)	Unadjusted OR (95%CI)	Adjusted[£] OR (95%CI)	*P-value*[¥]
Baseline	Have nausea or vomiting	Yes	21 (53.8)	19 (48.7)	0.81 (0.34, 1.98)	0.81 (0.32, 2.05)	0.657
		No	18 (46.2)	20 (51.3)	1	1	
	Feel loss of appetite	Yes	14 (35.9)	12 (30.8)	0.79 (0.31, 2.04)	0.70 (0.25, 1.94)	0.488
		No	25 (64.1)	27 (69.2)	1	1	
	Have abdominal pain	Yes	22 (56.4)	24 (61.5)	1.24 (0.50, 3.05)	1.16 (0.46, 2.91)	0.753
		No	17 (43.6)	15 (38.5)	1	1	
	Have belching (excess gas)	Yes	7 (17.9)	8 (20.5)	1.18 (0.38, 3.65)	1.18 (0.38, 3.74)	0.773
		No	32 (82.1)	31 (79.5)	1	1	
	Experience constipation	Yes	31 (79.5)	34 (87.2)	1.76 (0.52, 5.94)	2.05 (0.54, 7.79)	0.291
		No	8 (20.5)	5 (12.8)	1	1	
Endline	Have nausea or vomiting	Yes	5 (12.8)	21 (53.8)	0.13 (0.04, 0.39)	0.08 (0.02, 0.31)	<0.001
		No	34 (87.2)	18 (46.2)	1	1	
	Feel loss of appetite	Yes	4 (10.3)	17 (43.6)	0.15 (0.05, 0.50)	0.15 (0.04, 0.51)	0.003
		No	35 (89.7)	22 (56.4)	1	1	
	Have abdominal pain	Yes	8 (20.5)	27 (69.2)	0.12 (0.04, 0.32)	0.10 (0.03, 0.31)	<0.001
		No	31 (79.5)	12 (30.8)	1	1	
	Have belching (excess gas)	Yes	7 (17.9)	9 (23.1)	0.73 (0.24, 2.20)	0.75 (0.24, 2.33)	0.617
		No	32 (82.1)	30 (76.9)	1	1	
	Experience constipation	Yes	10 (25.6)	13 (33.3)	0.69 (0.26, 1.84)	0.65 (0.23, 1.79)	0.404
		No	29 (74.4)	26 (66.7)	1	1	

IG = Intervention Group; CG = Control Group

[¥]*P-Values are for the adjusted OR*

[£]*Adjusted for age, parity, family size, year of residence, diet diversity and dietary calcium intake*

In the treatment group, about half (48.7%) of the women experienced constipation at the end of the third month supplementation. Overall, one-quarter (~25%) of the women complained most of the symptoms related to excess calcium intake from the second through the fourth month of the

supplementation period. However, these symptoms showed a decreasing trend until the end of the sixth month supplementation period (Figure 6).

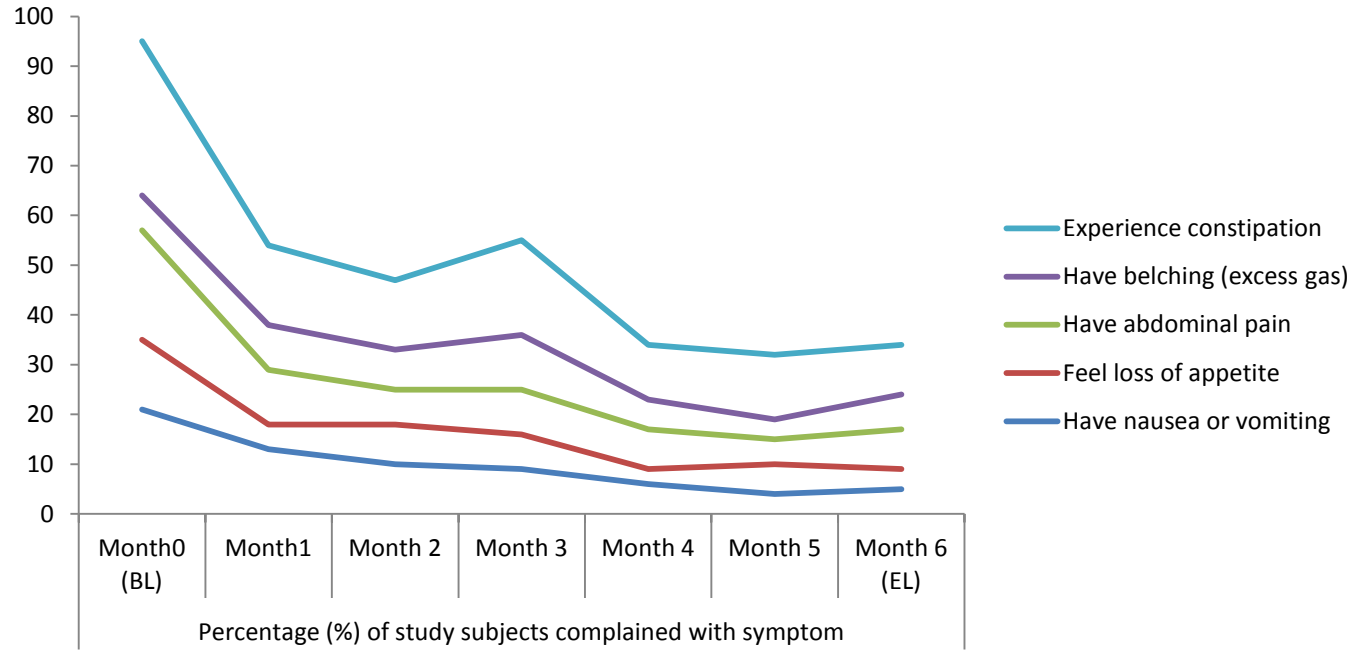


Figure 6: Symptoms related to excess calcium intake through the course of supplementation period among women in the treatment arm in *Halaba* district, Ethiopian Rift Valley, 2018

5.3.4. Hemoglobin and Malaria Test Results

The average hemoglobin (hematocrit) level of women was similar, ~13.6g/dL (41.2%) at baseline and endline assessment. There was no significant difference [$t_{(df=76)} = 1.29$ (95% CI: -0.25, 1.14)] in mean hemoglobin level of mothers between the treatment and control group at baseline. Post-intervention, the independent two samples t-test also did not show significant difference [$t_{(df=76)} = 0.24$ (-0.48, 0.60)] in mean hemoglobin level between the two groups (Table 19). In addition, the overall prevalence of anemia (Hgb <12.5g/dL) among women was 15.4% at baseline assessment. This statistically remained unchanged (Pearson $X^2 = 0.83$, $P=0.362$) after the six months calcium-containing ESP supplementation.

Malaria tests were done at baseline and end of the trial using RDT in both groups. Overall, malaria positive results were observed on 7 (9.0%) and 9 (11.5%) women at baseline and endline tests respectively (Table 19)

Table 19: Comparison of malaria and hemoglobin (hematocrit) test results of women between the two groups in *Halaba* district, Ethiopian Rift Valley, 2018

Test results		**IG (n=39) N (%)**	**CG (n=39) N (%)**	***Unadjusted OR (95%CI)***	***Adjusted*[£] *OR (95%CI)***	***P-value*[†]**
Baseline[#]						
Malaria positive	Yes	4 (10.3)	3 (7.7)	1.4 (0. 29, 6.58)	1.1 (0.20, 6.38)	0.899
	No	35 (89.7)	36 (92.3)	1		
Anemia (Hgb <12.5 g/dL)	Yes	5 (12.8)	7 (17.9)	0.67 (0.19, 2.34)	0.29 (0.05, 1.70)	0.171
	No	34 (87.2)	32 (82.1)	1		
Hemoglobin (g/dL)	Mean ±SD	13.7 ± 1.5	13.3 ± 1.6	$t_{(df=76)}$ = 1.29(95%CI:-0.25, 1.14)		0.201[¥]
Hematocrit (%)	Mean ±SD	41.7 ± 4.8	40.0 ± 4.8	$t_{(df=76)}$ = 1.54(95%CI:-0.49, 3.86)		0.127[¥]
Endline[†]						
Malaria positive	Yes	3 (7.7)	6 (15.4)	0.46 (0.11, 1.98)	0.39 (0.07, 2.18)	0.282
	No	36 (92.3)	33 (84.6)	1		
Anemia (Hgb <12.5 g/dL)	Yes	5 (12.8)	8 (20.5)	0.57 (0.17, 1.93)	0.55 (0.13, 2.26)	0.404
	No	34 (87.2)	31 (79.5)	1		
Hemoglobin (g/dL)	Mean ±SD	13.8 ± 1.2	13.7 ± 1.2	$t_{(df=76)}$ = 0.24(95%CI:-0.48, 0.60)		0.814[¥]
Hematocrit (%)	Mean ±SD	41.6 ± 3.6	41.4 ± 3.7	$t_{(df=76)}$ = 0.31(95%CI:-1.38, 1.89)		0.758[¥]

[£]*Adjusted for age, parity, family size, diet diversity, and baseline hemoglobin (hematcorit) or malaria status, iron and zinc levels*

[†]*P-Values are for the adjusted OR*

[¥]*P-values were computed using independent samples t test*

5.3.5. Biochemical Outcome Measures prior to and after ESP Supplementation in both Groups

At endline, there were no significant differences (P>0.05) in all biochemical outcome measures of women in both groups compared with their baseline measurements (Table 20). The GEE analysis also revealed similar hemoglobin, hematocrit and zinc levels of women in the

intervention group compared with the control women. But after the calcium-containing ESP supplementation, women in the intervention group had statistically significantly lower (by ~20%) serum iron level [β = - 0.21 (95% CI: (-0.4, -0.1)] compared to women in the control group (Table 20).

Table 20: Comparison of biochemical levels of women between the treatment and control arms prior and after the supplementation in *Halaba* district, Ethiopian Rift Valley, 2018

Measurements	**Baseline**	**Endline**	**Within mean difference (95%CI)**[†]	**Beta coefficient (Wald 95%CI)**[¥]	***P-value***[¥]
	Mean (95%CI) (n = 39)	**Mean (95%CI) (n = 39)**			
Hemoglobin (g/dL)					
Treatment	13.7 (13.2, 14.2)	13.8 (13.4, 14.2)	-0.1 (-0.7, 0.5)	-0.4 (-1.3, 0.5)	0.402
Control	13.3 (12.8, 13.8)	13.8 (13.4, 14.2)	-0.5 (-1.2, 0.2)	1	
Hematocrit (%)					
Treatment	41.7 (40.1, 43.2)	41.6 (40.5, 42.8)	0.1 (-1.9, 2.0)	-1.4 (-4.2, 1.3)	0.311
Control	40.0 (38.4, 41.5)	41.4 (40.2, 42.6)	-1.4 (-3.5, 0.7)	1	
Iron (mg/L)					
Treatment	0.82 (0.7, 0.9)	0.72 (0.6, 0.8)	0.1 (-0.1, 0.2)	-0.21 (-0.4, -0.1)	0.036
Control	0.61 (0.5, 0.7)	0.73 (0.6, 0.8)	-0.1 (-0.3, 0.1)	1	
Zinc (mg/L)					
Treatment	1.96 (1.7, 2.2)	1.87 (1.7, 2.0)	0.1 (-0.2, 0.3)	0.23 (0.56, 1.87)	0.171
Control	2.33 (2.2, 2.5)	2.0 (1.8, 2.2)	0.3 (0.1, 0.6)	1	

[†]The 'within' mean difference of endline from baseline computed using paired samples t test

[#]Statistically significant difference observed at $P<0.05$

[¥]Beta coefficient at 95% Wald confidence interval and *P*-value analyzed using GEE linear model. Hemoglobin, hematocrit, serum iron and zinc were adjusted for age, parity, family size, residency, diet diversity, dietary calcium intake and malaria status.

6. Discussion

6.1. Discussion of the Main Findings

Fluorosis is a global concern, significantly impacting dental, skeletal, and overall health, particularly in Ethiopia's Rift Valley, where millions of inhabitants are affected by high fluoride levels in drinking water. This study hypothesized that supplementing calcium from eggshell powder (ESP) could mitigate the toxic effects of excess fluoride, particularly for women with low dietary calcium intake. The research involved a cross-sectional survey of 280 women and a clinical trial with 82 participants, assessing urinary fluoride excretion, serum levels of iron and zinc, and fluorosis symptoms before and after a 6-month supplementation of 2.4 grams of calcium-containing eggshell powder (providing approximately 1000 mg of calcium) daily. Results showed that many subjects (56.3%) had dental fluorosis, and those with lower calcium intake faced higher odds of skeletal and non-skeletal fluorosis symptoms. Women in the intervention group had significantly lower urinary fluoride excretion and reduced symptoms of fluorosis compared to the control group, indicating the effectiveness of calcium supplementation. Thus, this study concluded that low dietary calcium is linked to fluorosis symptoms, and ESP supplementation is a promising, safe, and acceptable intervention for mitigating fluorosis in affected populations. Further research is needed to explore the feasibility of using crushed eggshells as a sustainable calcium source in fluoride-endemic areas.

6.1.1. Prevalence of Fluorosis and Associated Factors (Paper I)

The F level in drinking water sources in this area averaged ~ 5mg/L and in staple foods ranged from 0.8 – 13.6 mg/kg. Similarly, the F level of the drinking water in this area was previously reported as 4.6 ± 1.7 mg/L (Kebede *et al.,* 2016a). The primary preferred option may be to find a

supply of safe drinking-water with safe F levels. In such instances, de-fluoridation may be sought as a solution (Shifera and Tekle Haimanot, 1999; Dahi, 1999). However, there was no defluoridation of high F containing water as well as no fluorosis prevention strategies attempted in the current study area. In this area, rainwater, a source of low F drinking and cooking water, was not used for drinking as it was scarce and due to fear of dust contamination from the roof of the house when available. Rainwater is not viewed as clean in many parts of the world (Ahimed *et al.,* 2016).

Consequently, more than half of the women in this study had dental fluorosis as diagnosed using Dean's index criteria (Dean, 1934). This is in line with the findings of a recent systematic review which reported high prevalence of dental fluorosis in Ethiopian Rift Valley (Demelash *et al.,* 2019). In addition, a high prevalence of dental fluorosis was reported among children living in moderate F (24.1%) and high-fluoride (75.9%) areas of the Ethiopian Rift Valley (Wondwossen *et al.,* 2006). Another study also reported higher prevalence of dental fluorosis (62%) among adult inhabitants in the main Rift Valley of Ethiopia (Kravchenko *et al.,* 2014). Our data show that women can be affected by high F, but dental fluorosis develops mainly during early childhood (Beltrán-Aguilar *et al.,* 2005). In contrast with Kravchenko et al (Kravchenko *et al.,* 2014) who reported an inverse association with milk intake in young adults living in the Rift Valley, we found no predictors of dental fluorosis. This could be due to the fact that dental fluorosis is an irreversible process that occurred as a result of long-term exposure to excess fluoride during childhood period.

Few studies have reported on non-dental fluorosis symptoms in women. We classified symptoms according to skeletal rigidity and pain (skeletal fluorosis), muscular weakness (a major outcome

of non-skeletal fluorosis) and gastro-intestinal complaints (other symptoms of non-skeletal fluorosis (Tandon *et al.,* 2015). Skeletal rigidity accompanied by joint pains was common among women. Muscular weakness manifestations were also observed in many of the women. In addition, early signs of F toxicity, such as gastro-intestinal complaints manifested as loss of appetite, nausea and constipation, were observed in some of the women.

The women in the present study had a low intake of dietary Ca, averaging close to 400 mg per day. This is in agreement with the findings of the survey conducted in Ethiopia for applying international guidelines for calcium supplementation to prevent pre-eclampsia (Tesfaye *et al.,* 2018) which assessed national Ca intakes of Ethiopian women in the reproductive age using a single 24-hour dietary recall, and found the national average to be 478 mg per day. For women in the same state as our study, the Southern Nations, Nationalities and People Regional state (SNNPR), these authors found average Ca intake of 622 mg per day. In contrast, the Ca intakes of women in this study were estimated from daily and weekly consumption frequencies of Ca-rich foods during a one month period. Differences may be due to different methodologies or actual differences in our kebeles, however, both sets of results show a lack of adequate dietary Ca (RDA of 1000 mg/day) in most women.

In our regression analysis, study mothers who had inadequate Ca intake (≤ 400 mg/d) had three times increased odds of developing skeletal and non-skeletal fluorosis symptoms, namely skeletal rigidity with joint pains and muscular weakness manifestations, compared with those with higher Ca intake. To our knowledge no study has been done to assess Ca intakes as predictors of skeletal or non-skeletal fluorosis symptoms in areas when dietary F intakes are

high. Kravchenko and colleagues (2014) found a significant correlation between milk intake and dental fluorosis severity among men and women in the Ethiopian Rift Valley. As milk was the main source of Ca this presumably measured calcium intake and represented usual practice. Not many women in our study ingested dairy products as a source of Ca.

A decrease in F absorption by dietary Ca has only been demonstrated in a small number of animal studies (Kebede et al., 2016b; Pius and Viswanathan, 2008). In these experiments, Ca added to the diet reduced urinary F excretion, indicating the gastrointestinal binding of F ions by Ca. Kebede et al. (2016b) directly verified this finding by showing a decrease in fecal F levels which represented a lack of F absorption. However, most support for the Ca effect on F has been shown in observational studies. An earlier epidemiological study of children in India reported greater prevalence of fluorosis symptoms among children with inadequate dietary Ca intake (Teotia *et al.,* 1998). In that study, children with adequate dietary Ca (> 800 mg/d) and deficient dietary Ca (< 300 mg/d) having comparable intakes of F (mean 9.5 ± 1.9 mg/d) were compared; and severe toxic effects of F was observed in children with Ca deficiency (Teotia et al., 1998).

Similarly, our recent efficacy trial provided a proof of concept on calcium intake and fluoride. In this trial the study subjects in the treatment group were supplemented with an approximate of 1000mg of calcium (using calcium-containing eggshell powder) on a daily base for six consecutive months. The urinary F excretion in the supplemented group was six-fold lower (β = -6.1 (95% CI: -7.1, -5.1)) compared to the control group that received no extra cacium. The risk of developing skeletal and non-skeletal fluorosis were also significantly ($p < 0.001$) reduced in the treatment group (Mulualem *et al.,* 2021).

Gastrointestinal complaints (Index-3) were not associated with Ca intake or any other factors related to development of fluorosis, in contrast to skeletal rigidity and pains (Index-1) and muscular weakness (Index-2). Assefa et al. (2004) used four clinical signs of non-dental fluorosis and compared these to radiological confirmation of skeletal fluorosis in 180 men and 5 women in Ethiopia from a high F area, mean age 55 years. Skeletal fluorosis was present in 70% of subjects while clinical prevalence using kyphosis, impaired squatting, impaired neck mobility and impaired lumbar mobility, averaged 20%. This indicates clinical signs have low sensitivity; however, all signs except kyphosis were in high agreement ($P < 0.001$) with radiological skeletal fluorosis, indicating good specificity.

In the current study, older age was significantly associated with greater prevalence of skeletal and non-skeletal fluorosis symptoms. This is in line with the findings of a community-based survey on epidemiology of skeletal fluorosis by Melaku and his colleagues (2012). These authors reported that older people of age 55 years and above had 20 times higher risk of developing skeletal fluorosis than adolescents and young adults of 15 to 24 years of age (Melaku *et al.,* 2012). This could be due to the fact that in an area where F intake is higher, more F would be accumulated in bones and soft tissues of individuals who consumed it throughout their longer lifetime.

Our findings showed that women that have a high parity had greater odds of developing skeletal and non-skeletal fluorosis symptoms compared with those with lower parity. Older women were found to have a greater number of children, and hence parity may reflect the older age of women with more children and additionally, the stress of repeated pregnancy and lactation that may have

depleted the Ca stores of these women (More *et al.,* 2001; Imdad and Bhutta, 2012). Consequently, fluorosis symptoms would be manifested more among the women with high parity level. Therefore, this finding may have implications for recommendations of Ca during pregnancy and lactation in high F areas.

6.1.2. Efficacy of ESP supplementation (Paper II)

At baseline, we found urinary F levels to be high, reflecting the F exposure of those living in this district of the Rift Valley. The F excretion by the women, averaging 10.2 mg/L in urine, implies that women were exposed to about 20 mg per day, assuming urine excretion to be at least 2 L per day. Our data on F exposure were twice the Upper Level of 10 mg per day set by the Institute of Medicine (1997). The primary preferred option for fluorosis is to find a supply of safe drinking water using de-fluoridation (Dahi, 1996; Ayenew, 2008; Kloos and Tekle Haimanot, 1999). However, in these communities there was no defluoridation of the high fluoride-containing water. As well, there were no fluorosis prevention strategies being attempted. In this area, rainwater, a potential source of low F drinking and cooking water, was not used for these purposes as it was scarce and due to fear of dust contamination from the roof of the house.

Currently in Ethiopia, eggs are promoted as an excellent animal source food (NNPII, 2016). Our previous research had shown that the use of eggshell powder by young children in Ethiopia was acceptable (Omer *et al.,* 2018). However, we had not linked its use to fluorosis. Therefore, this study assessed the effects of ESP supplementation as a source of Ca on fluorosis symptoms and body F handling in women using urinary F as a biomarker. Eggshells have been used to provide Ca in other settings (Bartter *et al.,* 2018; Schaafsma *et al.,* 2000; Rovenský *et al.,* 2003; Brun *et*

al., 2013; Hassen, 2015; Bee, 2011). They contain predominantly Ca carbonate and trace amounts of other micro-minerals such as iron, zinc, magnesium and copper (Schaafsma *et al.,* 2000; Bee, 2011). Chicken eggshells are reported to contain 2.07 ± 0.18 g of Ca and studies show that this source of Ca was equivalent to other types of Ca supplement sources (Brun *et al.,* 2013). In addition, adding eggshell to foods or beverages did not produce important changes in flavor and texture. Similar Ca absorption was found comparing diets fed to rats containing chicken eggshell powder to one with a calcium carbonate, both with equal Ca content (Hirasawa, 2001). Those authors, therefore, recommended chicken eggshell as a bioavailable Ca dietary supplement, as others have done (Bartter *et al.,* 2018).

At EL, the urinary F excretion was significantly decreased among women supplemented with ESP compared with the control group. This is in line with a clinical study conducted in India to evaluate the clinical reversal of dental fluorosis in adolescents (age 8 to 17 years) with various combinations of Ca, vitamin D3, and ascorbic acid. That study also measured serum and spot urine F levels, and no change in clinical grades of dental fluorosis was noted, however, a significant reduction in serum and urine F levels was observed among subjects either given daily Ca (250 mg) with once-weekly vitamin D supplements (60,000 IU) for three months (Mehta and Shah, 2013, Opydo-Szymaczek and Borysewicz-Lewicka, 2017a)

That Ca reduces F absorption was studied in two animal studies. One study examined different doses of Ca (provided as calcium carbonate) that were administered to rabbits. Significant decreases in F excretion were observed, in a dose-response manner. The higher doses of 17.2, 21.4 and 50 mg Ca/kg body weight were equivalent to 1200, 1500 and 3500 mg of Ca in a 70 kg

human. The authors predicted doses of 1200 - 3500 mg of Ca would be required in humans to mitigate the adverse effects of fluorosis (Pius and Viswanathan, 2008). In a second animal study (Kebede *et al.,* 2016b), total daily F excretion, both urine and fecal, by rats given additional Ca and magnesium was monitored. This study supported the hypothesis that gastrointestinal binding of F ions by Ca occurred, as fecal F excretion increased in concert with a decrease in urinary excretion.

Calcium has been identified as a potential agent to reduce fluoride absorption. Research suggests that calcium can inhibit fluoride absorption in the gastrointestinal tract. A study by Marinho et al. (2003) highlighted that calcium intake might play a role in reducing fluoride's bioavailability, potentially mitigating its harmful effects on the body (Marinho et al., 2003). High-calcium foods such as dairy products, leafy greens, and fortified plant-based milk can contribute to this protective effect. Magnesium has also been suggested to influence fluoride metabolism. Magnesium competes with fluoride for absorption and can affect its systemic availability. A study by Liu et al. (2013) found that adequate magnesium intake might reduce the severity of skeletal fluorosis by altering fluoride's metabolism and distribution (Liu et al., 2013). Foods rich in magnesium include nuts, seeds, whole grains, and green leafy vegetables. Vitamin C is another dietary component that may counteract fluoride toxicity. It has been shown to have antioxidant properties and can potentially reduce oxidative stress associated with fluoride exposure. A study by Singh et al. (2012) demonstrated that vitamin C supplementation could alleviate some of the oxidative damage caused by fluoride (Singh et al., 2012). Citrus fruits, strawberries, and bell peppers are excellent sources of vitamin C. Iron deficiency has been linked to increased fluoride absorption and toxicity. Adequate iron intake can help in reducing fluoride

absorption and its harmful effects. A study by Reddy et al. (2018) found that iron supplementation could lower the fluoride burden in the body, highlighting the importance of iron in mitigating fluoride toxicity (Reddy *et al.,* 2018). Foods such as lean meats, legumes, and fortified cereals are good sources of iron. Furthermore, reducing the intake of fluoride-rich foods and beverages is a direct approach to mitigating fluoride exposure. This includes limiting consumption of tea, which can be high in fluoride, and certain processed foods that may contain added fluoride (Whitford, 2013). These dietary strategies provide a multifaceted approach to managing fluoride exposure, leveraging the potential of various nutrients to mitigate fluoride's adverse effects. Further research and clinical trials are needed to validate these interventions and optimize recommendations for fluoride reduction and management.

All the four urine collections showed similar average concentrations of creatinine which indicated that spot urines were collected in each group in a similar manner at BL and EL. A large but non-significant increase in urinary Ca excretion was observed among intervention women after supplementation with calcium-containing ESP. As these women had a low mean intake of dietary Ca of approximately 400 mg/day consistent with other women in Ethiopia (Tesfaye *et al.,* 2018), the additional Ca intake from ESP supplementation might contribute to fulfill the Recommended Daily Allowance (RDA) for many of the subjects receiving it. There was no evidence of hypercalciuria.

A significant mitigation of skeletal and non-skeletal fluorosis symptoms was observed among women who received additional Ca from ESP over 6 months in the present study. Women and children in this part of Ethiopia had been reported as having high prevalence of fluorosis

symptoms (Demelash *et al.,* 2019; Kebede *et al.,* 2016a) and low intake of dietary Ca (Tesfaye *et al.,* 2018). These symptoms of pain and poor mobility are also reported in osteomalacia due to low calcium and low vitamin D (Uday and Högler, 2019). Traditional sources of Ca such as dairy and green leafy vegetables are not widely available in Ethiopian communities. Further, eurxperimental and clinical studies conducted to date have shown that vitamin D levels in Ethiopian women are low (Gebreegziabher and Stoecker, 2013). Thus, it is possible Ethiopian women are at risk of osteomalacia. However, by including non-skeletal fluorosis measurements and documentation of dental fluorosis we measured mitigation of fluorosis.

6.1.3. Safety and Acceptability of ESP Supplementation (Paper III)

Previous studies have demonstrated the safety of calcium supplementation in various populations (Smith *et al.,* 2018; Jones and Wang, 2019). Consistent with these findings, our research found that calcium-containing eggshell powder supplementation was well-tolerated among participants, with no reports of serious adverse effects or safety concerns. The safety assessment through follow-up and monitoring checklists revealed no significant alterations in the reported side effects prior and after supplementation of calcium-containing eggshell powder among women. Furthermore, the calcium-containing eggshell powder supplementation was generally accepted by the study participants.

Calcium supplement intake has long been associated with other side effects such as constipation, abdominal bloating and gas (Jackson *et al.,* 2006; Prince *et al.,* 2006), which varies greatly from person to person and dose of the calcium supplement. Likewise, one-quarter of the women complained most of the symptoms (such as loss of appetite, abdominal pain, nausea or vomiting)

related to excess calcium intake from the second through the fourth month of the supplementation period albeit these symptoms showed a decreasing trend until the end of the supplementation period.

Calcium interaction with other essential micronutrients such as iron and zinc in the diet has been considered a potential risk related to high calcium intakes, albeit it is important to consider the habitual dietary calcium intake prior to provide the calcium supplement. There have been concerns related to the effects of calcium supplements on iron absorption based on short-term study reporting that calcium supplements inhibit iron absorption by 28 to 55% depending on the dose, type of salt used, time of supplementation and the presence in the food of hem or non-hem iron (Cook *et al.,* 1991). Moreover, an earlier study had measured the effect of calcium carbonate and hydroxyapatite on whole-body retention of iron and zinc and reported significant reduction of iron retention in the treatment groups compared with the placebo (control) group (Ilich-Ernst *et al,* 1998).

Similarly, women in the current intervention group had statistically significantly lower (by ~20%) serum iron level at the end of calcium-containing ESP supplementation compared to women in the control group. A significant decrease in serum iron levels may suggest that calcium-containing ESP supplementation is negatively impacting iron absorption, potentially exacerbating iron deficiency or iron deficiency anemia in women (McLean *et al.,* 2009). This is particularly relevant for populations at higher risk of iron deficiency, such as premenopausal women and pregnant women (Huffman *et al.,* 2017). Monitoring iron levels in women receiving calcium-containing ESP supplementation is essential to prevent iron deficiency. Adjustments in

supplementation regimens or dietary recommendations may be needed to ensure adequate iron intake and avoid anemia (Harrison *et al.,* 2020).

In contrast, other short-term absorption studies with consumption of 1000–1200 mg/day supplemental calcium for 12 - 26 weeks had no impact on iron status (Sokoll and Dawson-Hughes, 1992; Minihane and Fairweather-Tait, 1998). Another earlier placebo controlled randomized trials suggested that calcium intakes of 1,500 to 1,700 mg/day did not interfere with zinc (McKenna *et al.,* 1997) or iron (Ilich-Ernst *et al,* 1998) absorption. Earlier and recent studies also show no effect on iron status of prolonged calcium supplementation taken at the same time or separate of meals (Yan *et al.,* 1996; Kalkwarf and Harrast, 1998; Abrams, 2001; Harris, 2002; Mølgaard et al., 2005; Gaitan et al., 2011). Moreover, calcium interaction with other essential micronutrients such as zinc in the diet has been considered a potential risk related to high calcium intakes. However, different earlier studies assessing the effect of calcium on zinc absorption or zinc balance seem to indicate that supplemental calcium have no effect (Lönnerdal *et al.,* 1984; Dawson-Hughes *et al.,* 1986; Spencer *et al.,* 1984; McKenna *et al.,* 1997).

While our study provided promising evidence of the safety and acceptability of calcium-containing eggshell powder supplementation among women, several limitations should be acknowledged. Firstly, the sample size was relatively small, limiting the generalizability of the findings. Future studies with larger and more diverse participant cohorts are needed to confirm the acceptability of supplementation across different populations and settings. Additionally, individual adherence to the supplementation protocol would vary, influenced by factors such as

taste preferences, cultural beliefs, and logistical challenges. Qualitative assessments of acceptability and adherence may provide valuable insights into the barriers and facilitators of supplementation uptake in real-world settings. Addressing these factors is essential for maximizing the effectiveness and sustainability of calcium-containing eggshell powder supplementation intervention.

The limitations of this study include using some non-specific clinical symptoms and physical exercise tests to assess skeletal and non-skeletal fluorosis that may be inaccurate and may introduce mis-classification bias. However, we employed the same dentist and physiotherapist to do all measurements which reduce measurement bias. We also did not include women with only one symptom as being fluorotic to minimize the false positives arising from counting one nonspecific symptom. For skeletal fluorosis we did not have access to x-ray measurements. We combined symptoms to create unique indices in order to analyze presence of multiple symptoms. Daily dietary calcium intake of women was estimated from frequencies of calcium-rich foods consumption which might not have estimated the usual calcium intake of women beyond that month. During sample size estimation for the cross-sectional survey, we used a wider marginal error ($d = 5\%$) which might underestimate the sample size that adequately required for cross-sectional studies. In addition, we assumed similar prevalence of fluorosis among women and school-age children based on previously published study conducted in the same study area. This also might not properly estimate the desired number of women to be included in the cross-sectional survey. Moreover, this study includes lack of access to x-ray confirmation of skeletal fluorosis and thus, we used several non-specific clinical symptoms and physical exercise tests to assess skeletal and non-skeletal fluorosis. The effect of Ca on body F load was measured using

urinary F excretion biomarker which is an indirect measure of F absorption. Dietary Ca intake of women was estimated from frequencies of calcium-rich food consumption which might not have accurately estimated the usual Ca intake of women beyond that month. As the trial was conducted at the community level, due to fear of information contamination, the randomization was done only at the kebele level. In addition, there was no placebo treatment for the women in the control group.

6.2. Methodological Discussion

6.2.1. Study Deign

In this dissertation, three different research designs were employed: a cross-sectional study design (Paper I); a Phase II clinical trial/ an efficacy trial (Paper II) and a longitudinal study design in the Phase2 clinical trial (Paper III). Cross-sectional studies are observational research designs that provide a snapshot of a population at a single point in time. These studies are valuable for examining prevalence, associations between variables, and patterns of disease or behavior within a population (Schoenborn and Heyman, 1994). They are often used to generate hypotheses or explore associations between variable (Aschengrau and Seage, 2019). They are conducted as prevalence studies that are useful for planning purposes in public health (Levin, 2006). They are helpful in identifying associations that can be further examined using studies with stronger design such as cohort studies and randomized controlled trials. They can be also used by clinicians to make diagnoses and estimate the predictive value of investigations (Mann, 2003).

However, a cross-sectional study design has some limitations in identifying causation and the sequence of exposure and outcome (Mann, 2003; Grimes and Schulz, 2002). Nevertheless, there are conditions such as a stationary population, non-selective survivorship, similarity in duration of exposure between exposure groups, and no reverse causality (Reichenheim and Coutinho, 2010) that make a cross-sectional study appropriate for making causal inferences. We used a stable population with similar duration of exposure; and due to the absence of reverse causality and selective survivorship, the associations observed between dental, skeletal and non-skeletal fluorosis with dietary calcium intake can suggest causal relationships. However, since data

collected at a single time point, the temporal relationships or causality between the variables cannot be established. In addition, sampling biases or non-response biases may affect the generalizability of findings to the broader population.

A Phase II clinical trial, often termed an efficacy trial, is a pivotal stage in drug development where the focus shifts from primarily assessing safety (as in Phase I trials) to evaluating the drug's efficacy in treating a specific condition or disease in a larger cohort of subjects or patients. Phase II trials expand the testing to a larger group of subjects or patients afflicted with the target condition to evaluate the drug's efficacy, safety profile, and further determine the optimal dosage (ICH, 1996). The primary objective of Phase II trial is to determine whether the drug demonstrates efficacy in treating the targeted condition while continuing to assess safety profiles (Chow and Liu, 2012). In Paper II, we carried out a Phase II clinical trial because the effect of calcium, in this case derived from eggshell powder, had never been tested (to our knwoldge) on mitigating fluorosis signs and symptoms in women in Ethiopia. Previous studies (IOM, 2011) have demonstrated safety of calcium supplementation when given below the UL of 2500 mg for adults. Eggshell powder as a source of calcium has demonstrated safety (Mehta and Shah, 2013). In this trial, we employed randomized controlled trial, by utilizing control arm as comparator to assess the efficacy of ESP supplementation in the treatment arm. The data on efficacy of ESP supplementation were collected through various measures, including clinical outcomes (urine fluoride excretion) and study participants'- reported outcomes (fluorosis symptoms), and were analyzed statistically to evaluate the potential benefits of ESP supplementation. Therefore, the findings in this trial provided crucial evidence about the efficacy of ESP supplementation, supporting further development and progression to a Phase III trial.

In Paper III, a longitudinal study design was used to assess safety and acceptability of the ESP supplementation in the Phase2 clincal trial. Longitudinal studies are essential in medical research for understanding changes over time in individuals or populations. This design allows researchers to track participants over an extended period, providing valuable insights into disease progression, risk factors, and treatment outcomes (Cook and Campbell, 1979). In longitudinal studies, data collection occurs at multiple time points, allowing researchers to capture changes and trends over time. Various methods, including surveys, medical examinations, and biomarker measurements, may be employed (Little and Rubin, 2014). Longitudinal studies require consistent follow-up of participants over the study period. Retention strategies are crucial to minimize loss to follow-up and maintain the integrity of the data (Shrout and Bolger, 2002). Researchers use longitudinal designs to evaluate the effectiveness of interventions over time. By comparing outcomes between intervention and control groups, they can assess the impact of treatments, preventive measures, or health promotion efforts (Rowe and Kahn, 1997). Similarly, in this research, study subjects were followed for six consecutive months with weekly follow-up and supervision. The valuable changes were investigated to identify any signs and symptoms associated with ESP supplementation. Moreover during the weekly follow-up, checklist (Annex IX) was used to assess participants' adherence to the ESP supplementation.

6.2.2. Sample Size

Sample size refers to the number of participants or other units that should be included in a study which is sufficient to address the research questions (Pourhoseingholi *et al.,* 2013). Insufficient sample size leads to a wide confidence interval (Rothman *et al.,* 2008; Christensen *et al.,* 2021) which reduces power, resulting in likelihood of committing a Type 2 error (Nayak, 2010). We can reduce the random error, the distortion of an observed estimate from the true

population value, by increasing the sample size. Thus, the role of chance will be minimized (Bonita *et al.,* 2006). We need an optimum sample size to estimate the population prevalence or associations between variables with fair precision in cross-sectional study (Pourhoseingholi *et al*., 2013).

The determination of sample size in cross-sectional studies involves consideration of several factors, including the desired level of precision, expected prevalence of the outcome, significance level, and statistical power. According to Katz et al. (2016), a larger sample size increases the precision of estimates and enhances the study's ability to detect associations between variables. However, excessively large samples may result in unnecessary costs and resource burdens.

In this cross-sectional study (Paper I), we aimed to assess the prevalence of fluorosis and associations between ESP supplementation and fluorosis symptoms in women at a single point in time. Adequate sample size determination is paramount to ensure the reliability and generalizability of findings. Thus, we included a sufficient sample size to represent the target population accurately and to provide precise estimates of prevalence and associations between variables. Moreover, the determination of the sample size involved considerations such as the desired level of precision, the expected prevalence or effect size, the desired level of confidence, and the potential for subgroup analysis.

According to Chow and Liu (2004), Phase II trials often aim to provide preliminary evidence of a treatment's efficacy, guiding decisions about whether to proceed to larger and more definitive

Phase III trials. The sample size in Phase II trials is typically smaller compared to Phase III trials, as the primary objectives are to assess safety, explore dosing regimens, and gather initial efficacy data.

Thus, in this Phase II clinical trial (Papers II and III), we typically aimed to evaluate the efficacy and safety of calcium-containing ESP supplementation in a relatively small group of volunteer participants, following promising results from previous studies. Therefore, we determined an adequate and appropriate sample size by ensuring the study was adequately powered to detect meaningful treatment effects while balancing practical considerations such as budget, time, and study participant availability.

Furthermore, ethical considerations play a crucial role in sample size determination in clinical trials. It is essential to ensure that the number of participants exposed to experimental treatments is minimized while still obtaining adequate data to make informed decisions about the intervention's safety and efficacy (Friedman *et al.,* 2010). This involves carefully weighing the potential risks and benefits to participants and adhering to ethical principles of minimizing harm and maximizing benefit. Consequently, we were allowed to study on only 30% of the subjects involved in the cross-sectional study (Paper I). Therefore, we followed the recommendation given by IRB of Hawassa University College of Medicine and Health Science (**Annex XII**), and attempted to involve considerations such as the anticipated effect size, desired level of statistical power, acceptable type I error rate and contingency for dropout rates in determining the sample size in this Phase II trial.

6.2.3. Validity of the Study Findings

Paper I has findings obtained from the cross-sectional study. In cross-sectional studies, ensuring validity is crucial to accurately characterize the relationship between variables of interest within a population at a single point in time. Validity in cross-sectional studies encompasses various dimensions including internal, external, construct, and statistical validity.

Internal validity refers to the extent to which the study design, sampling methods, and measurement tools accurately measure the variables under investigation while minimizing biases and confounding factors (Babbie, 2015). In this study, in order to enhance internal validity and strengthen the study's findings, we applied proper sampling techniques, used standardized measurement instruments, and attempted to control for potential confounders.

External validity, also known as **generalizability**, pertains to the extent to which the findings of a cross-sectional study can be extrapolated to other populations or contexts beyond the study sample (Trochim, 2006). For maximizing external validity and ensuring the findings are applicable to broader populations, we included representative sample size and diverse study participants.

Construct validity involves the degree to which the measurements used in a cross-sectional study accurately reflect the underlying constructs or concepts being studied (Trochim, 2006). For establishing construct validity and interpreting the study results accurately, we also used valid and reliable measurement instruments that were able to capture the intended constructs.

Statistical validity, in cross-sectional studies, encompasses the appropriateness and rigor of the statistical methods used to analyze the data and draw conclusions (Babbie, 2015). Furthermore, for ensuring that the reported associations are robust and trustworthy, valid statistical techniques were employed by taking into account for factors such as sampling variability, measurement error, and potential confounding variables during the statistical analyses.

Therefore, we attempted to maintain validity in this cross-sectional study (Paper I) by emphasizing careful attention to study design, sampling methodology, measurement tools, and statistical analysis techniques to minimize bias, enhance generalizability, accurately measure constructs, and draw valid inferences about the relationships between variables (ESP supplementation and fluorosis symptoms) within the population.

In Phase II clinical trial, ensuring validity is crucial to accurately assess the efficacy and safety of investigational interventions. Validity refers to the degree to which the trial measures what it intends to measure and accurately reflects the true effects of the intervention on the target population. According to Temple (2010), validity in Phase II trials is primarily concerned with two aspects: internal validity and external validity. **Internal validity** pertains to the trial's ability to accurately measure the effects of the intervention on the study population, without bias or confounding factors. Thus, to enhance internal validity in this Phase II trial (Paper II and III), we employed randomized controlled design and blinding techniques which help to minimize biases and ensure that observed outcomes are attributable to the intervention under investigation.

Furthermore, **external validity**, also known as **generalizability**, refers to the extent to which the findings of the trial can be extrapolated to broader patient populations or real-world clinical settings. While Phase II trial typically involve relatively homogeneous patient populations and controlled settings, efforts to enhance external validity include recruiting diverse patient populations representative of the target patient population, employing pragmatic trial designs, and incorporating real-world clinical endpoints (Ioannidis *et al.,* 2014).

Moreover, ensuring **construct validity**, which involves measuring the intended treatment effects accurately, is critical in Phase II trial. This includes using appropriate outcome measures that capture relevant clinical endpoints and biomarkers, as well as selecting patient populations that are likely to benefit from the intervention based on preclinical evidence and prior clinical experience (Trochim and Donnelly, 2006). In addition to these considerations, addressing threats to validity such as attrition bias, measurement bias, and confounding factors is essential to maintain the integrity and reliability of Phase II trial results (Kraemer *et al.,* 2008).

For Paper II and III, we employed Phase II clinical trial, with a longitudinal study design. Thus, to ensure validity in this trial, we involved comprehensive efforts to minimize biases, enhance the generalizability of findings, and accurately measure treatment effects, thereby providing reliable evidence to inform subsequent phases of clinical development.

7. Conclusions and Recommendations

7.1. Conclusions

Signs and symptoms of dental, skeletal and non-skeletal fluorosis were prevalent in women of child-bearing age living in *Halaba* zone located in the Rift Valley of Ethiopia, where water and food sources of fluoride were high. Dietary calcium intake was low relative to requirements, averaging only 400 mg per day. The low intake of dietary calcium, less than 400 mg per day, was significantly associated with Musculo-skeletal fluorosis symptoms but not with non-skeletal fluorosis or dental fluorosis. The presence of Musculo-skeletal fluorosis impairs everyday life for the women, who have a very labor-intensive life of raising children and having farm and home responsibilities. Our findings also showed that women that have a high parity had greater odds of developing skeletal and non-skeletal fluorosis symptoms compared with those with lower parity. (Objective I/Paper I). This suggests the need for further investigation on improving calcium intakes to mitigate the toxic effects of high fluoride intake in Ethiopian Rift Valley.

Mitigation of many of the symptoms related to skeletal and non-skeletal fluorosis and reduction in urinary F excretion were observed among women supplemented with calcium-containing ESP in a fluorosis prone area of Ethiopia. Therefore, this study provides a proof of concept on using a traditional source of dietary Ca, eggshell, for mitigation of fluorosis in an area where there is endemic fluorosis and low intake of dietary Ca (Objective II/Paper II). The potential feasibility, sustainability and safety of using home prepared crushed eggshells in areas where fluorosis is endemic need to be studied.

Safety and acceptability assessments conducted as part of the study indicated that calcium-containing eggshell powder supplementation was well-tolerated and did not pose significant risks to the health of participants. The research findings also suggested that calcium-containing eggshell powder supplementation was generally acceptable among women in the Ethiopian Rift Valley (Objective III/Paper III). However, the study recognizes the importance of cultural relevance and contextual factors in shaping the acceptability and effectiveness of supplementation interventions. Tailoring supplementation programs to the local context, addressing cultural beliefs and preferences, and involving community stakeholders might be additional key factors contributing to the success of the intervention.

In conclusion, this research has potentially important findings for public health policy and practice in the Ethiopian Rift Valley and similar regions facing fluoride-related health challenges. The findings support the integration of calcium supplementation into existing fluoride mitigation strategies and nutrition programs targeting women's health and nutrition. Moreover, while the study provides valuable insights into the efficacy, association, safety, and acceptability of calcium-containing eggshell powder supplementation, further research is warranted to address remaining knowledge gaps and optimize intervention strategies. Future studies may focus on validating this study findings, exploring optimal dosage regimens, refining supplementation protocols, exploring alternative delivery methods, and evaluating long-term sustainability and scalability of supplementation programs.

7.2. Recommendations

Based on the findings of this research, we rendered the following recommendations:

7.2.1. Operational Recommendations:

Community engagement: Engage with local communities, community leaders, and healthcare providers to raise awareness about the link between dietary calcium intake and fluorosis. Foster community participation in designing and implementing interventions aimed at promoting calcium-containing eggshell powder supplementation and reducing fluoride exposure. Provide nutritional counseling and education to women in the Ethiopian Rift Valley regarding the safety and benefits of calcium-containing eggshell powder supplementation where dietary calcium intake is low. Address any concerns or misconceptions about supplementation and emphasize its role as a complementary strategy to support overall health.

Capacity building: Build capacity among healthcare professionals, nutritionists, and community health workers to screen for and manage fluorosis among women in the Ethiopian Rift Valley. Develop culturally appropriate behavior change communication strategies to promote the acceptability and uptake of calcium-containing eggshell powder supplementation among women in the Ethiopian Rift Valley where ESP would be beneficial. Address misconceptions, beliefs, and barriers to adoption through targeted messaging and community engagement. Provide health education and training to healthcare providers, community health workers, and other stakeholders on the benefits and safety of calcium-containing eggshell powder supplementation. Equip them with the knowledge and skills to support women in making informed decisions about supplementation.

Integration with existing programs: Explore opportunities to integrate calcium-containing eggshell powder supplementation into existing public health programs targeting women's health, nutrition, or water quality management in the Ethiopian Rift Valley as needed. Leverage existing infrastructure and resources to maximize reach and impact.

Cultural sensitivity and participant feedback: Tailor calcium-containing eggshell powder supplementation intervention to the cultural context of the Ethiopian Rift Valley to enhance acceptability among women who have low dietary calcium intake. Respect cultural beliefs, dietary preferences, and traditional practices related to health and nutrition when designing and implementing supplementation programs. Solicit feedback from participants regarding their experiences with calcium-containing eggshell powder supplementation, including perceptions of safety, tolerability, and acceptability. Incorporate participant perspectives into program design and implementation to improve adherence and satisfaction.

Monitoring and continuous evaluation framework: Conduct an assessment and monitoring about F exposure and dietary calcium intake of residents of the Rift Valley of Ethiopia. Establish a robust monitoring framework to track the implementation and impact of calcium-containing eggshell powder supplementation programs. Collect data on supplementation coverage, adherence, health outcomes, and fluoride levels to assess program effectiveness and inform future interventions. Establish mechanisms for continuous evaluation and quality assurance of calcium-containing eggshell powder supplementation programs. Regularly review safety data, conduct risk assessments, and make adjustments to supplementation protocols as needed to ensure ongoing safety and acceptability among women in the Ethiopian Rift Valley.

7.2.2. Policy Advocacy, Collaboration and Scaling Up:

Policy advocacy: Advocate for policy changes at the national and regional levels to promote dietary calcium intake (including calcium-containing eggshell powder supplementation) as a preventive measure against fluorosis in the Ethiopian Rift Valley. Support the integration of calcium-rich foods into government nutrition programs and policies targeting women's health and well-being.

Collaboration: Foster collaboration between researchers, healthcare professionals, policymakers, and community stakeholders to address knowledge gaps, share best practices, and facilitate the translation of research findings into policy and practice. This multidisciplinary approach can enhance the effectiveness and sustainability of calcium-containing eggshell powder supplementation to mitigate the toxic effects of excess fluoride intake among women in the Ethiopian Rift Valley.

Scaling-up: Look for funding from government agencies, non-governmental organizations, and international donors to scale up calcium-containing eggshell powder supplementation programs in the Ethiopian Rift Valley in areas requiring intervention. Highlight the potential health benefits and cost-effectiveness of calcium-containing eggshell powder supplementation as a public health intervention.

7.2.3. Future Research:

A Phase III clinical trials: Conduct randomized controlled trials (RCTs) to rigorously evaluate the effectiveness of calcium-containing eggshell powder supplementation in reducing urinary fluoride levels and alleviating fluorosis symptoms among women (as well men) in the Ethiopian Rift Valley. Design the trials with appropriate control groups and sample sizes to ensure statistical power and validity of the findings.

Dose optimization studies: Explore different dosages of calcium-containing eggshell powder supplementation to identify the most effective dose in reducing urinary fluoride levels and improving fluorosis symptoms. Conduct dose-response studies to determine the optimal dosage range that balances efficacy and safety. This should take into account factors such as age, body weight, and baseline fluoride levels.

Longitudinal follow-up: Implement longitudinal follow-up studies to assess the long-term effects of calcium-containing eggshell powder supplementation on urinary fluoride excretion and fluorosis symptoms over time. Monitor participants for changes in urinary fluoride levels and clinical manifestations of fluorosis at regular intervals. Conduct a more detailed longitudinal studies focusing on the interaction between dietary calcium and other factors such as genetic, environmental or nutritional factors that might contribute to the development of fluorosis.

Biomarker analysis and bioavailability studies: Analyze biomarkers of fluoride exposure and metabolism, such as urinary fluoride concentration, serum fluoride levels, and bone fluoride content, to provide objective measures of the efficacy of supplementation. Correlate changes in biomarker levels with clinical outcomes to elucidate the mechanisms underlying the effects of

supplementation. Assess the bioavailability of calcium and other nutrients present in eggshell powder to ensure adequate absorption and utilization in the body. Compare different supplementation methods (e.g., capsules, fortified foods) to identify the most bioavailable delivery system.

Quality control and standardization: Ensure quality control and standardization of calcium-containing eggshell powder supplements used in the studies to minimize variability and ensure consistency in efficacy. Conduct thorough characterization of the supplement, including calcium content, purity, and bioavailability, to verify its suitability for supplementation.

Long-term safety studies: Evaluate the long-term safety of calcium-containing eggshell powder supplementation through comprehensive longitudinal safety studies and follow-up assessments. Monitor for potential adverse effects associated with calcium-containing eggshell powder supplementation such as gastrointestinal discomfort, renal function impairment, mineral imbalances and any adverse effects associated with the supplementation over an extended period.

Adherence monitoring: Monitor adherence to calcium-containing eggshell powder supplementation protocols through regular assessments and participant surveys. Identify factors influencing adherence, such as taste, convenience, and perceived benefits, to optimize intervention delivery and maximize participant compliance.

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9. Appendices

Annex I: English version Questionnaires, 24hr Dietary Recall and FFQ used for Interviewing

IDENTIFICATION			
01	Date /Month/Year of Interview:_____	02	Name of interviewer:_____
03	Got/Village:_____	04	Kebele::_____
05	Mother's Name: _____	06	Mother's Age :_____
07	Child's Name:_____	08	Sex of the child : 1 Male 2 Female
09	Age of child in months :_____ (Check using vaccination card if the child is 6-18 months old)	10	Child's Date of birth /D/M/Y/ _____/_____/_____

SECTION-I: Socio-economic and demographic characteristics		
Instruction:-Ask the question as written. Please, don't read the alternative response unless you're instructed to do so. If you instructed to skip question, please skip it accordingly. Write a ring on the given answer by the respondent.		
101	Family size (individuals living in the household)	1. Males _____ 2. Females_____
102	Number of under five children	1. Males _____ 2. Females_____
103	Year of residency in this area	1. Below 5 years 2. 5 – 10 years 3. 11 to 15 years 4. Above 15 years
104	Your educational background	0. Illiterate 1. Elementary school (Grade 1st - 8th) 2. Secondary school (Grade 9th - 12th) 3. College/University (Above12th grade) 99. Other (Specify)_____

105	Husband's educational background	0. Illiterate 1. Elementary school (Grade 1st - 8th) 2. Secondary school (Grade 9th - 12th) 3. College/University (Above12th grade) 98. Husband was died 99. Other (Specify)_____
106	Marital status	1. Married 2. Divorced 3. Widowed 99. Other (Specify)_____
107	Your main occupation	1. Farmer 2. Government employed 3. Private employed 4. Petty trader 5. None/Housewife 99. Other (Specify)_____
108	Your husband main occupation	1. Farmer 2. Government employed 3. Private employed 4. Petty trader 5. None 99. Other (Specify)_____
109	Main source of income of the household	1. Farming 2. Animal husbandry 3. Daily laborer 4. Petty trader 5. Government employed 99. Other (Specify)_____
210	Average monthly income of the household in birr	1. Below 500birr 2. 501-1000 birr 3. 1001-1500 birr 4. 1501-2000 birr 5. Above 2000 birr
SECTION 2: Drinking water, Personal hygiene and Environmental sanitation		
201	What is the **main** source of drinking water for members of your household? *(Do not read options aloud. Record only one option).*	1. Piped water 2. Water from an open well 3. Covered well/ borehole 4. Surface water (spring, river/stream, pond/lake)

		5. Rainwater 99. Other (Specify): _____
202	How many hours does it take to bring water on foot from the source? *(Ask this question only for those who don't have pipe and/or open well water source in their living compound).*	1. Below an hour 2. Above an hour 3. Don't know
203	How do you store your drinking water?	1. Closed container/ Jerry can 2. Open container/ Bucket 99. Other (Specify): _____
204	Do you treat your water in any way before drinking?	0. No ➔ Skip to Q206 1. Yes
205	*If yes:* what do you usually do to the water to make it safer to drink? *(Probe:* Anything else? *Mark all that apply by recording the number in the corresponding answer box).*	1. Strain It Through Cloth 2. Boil 3. Add Bleach eg. wuha agar 4. Water Filter (Ceramic, Sand, Composite) 99. Other (Specify): _____
206	Is the water used for drinking and preparing food from same or different source?	0. No, it is the same 1. Yes, it is different
207	Is the water used for drinking and hygiene/bathing from same or different source?	2. No, it is the same 0. Yes, it is different
208	When do you wash your hands? *(Probe:* Any other time? *Mark all that apply by recording the number in the corresponding answer box).*	0. Not washing at all 1. Before food preparation 2. Before feeding child 3. After defecation/ visiting the toilet 4. After attending to child who has defecated/soiled
209	**<u>Usually</u>** what you use to wash your hands with water? **(***Record only one option. Please observe the cleaning agent, if possible***)**	0. None 1. Soap 2. Detergent 3. Ash 4. Mud/sand 99. Other (Specify): _____
210	What kind of toilet facility does your	0. No toilet—bush, field 1. Flush toilet /septic tank

	household use? *(Record only one observation)*	2. Traditional Pit latrine 3. Latrine with shade 99. Other (Specify): _____
211	Do you receive education about fluoride/fluorosis from the *Kebele's* HEW and/or any health professional?	0. No, I didn't 1. Yes, I do
212	*If yes to Q211:* what education do you receive about fluoride/ fluorosis? *Probe:* Anything else? *(Mark all that apply by circling the number in the corresponding response alternatives. More than one response is possible).*	1. About fluoride source of food and beverage types. 2. About the toxic effects of excess fluoride intake 3. About measures to be taken to lessen or mitigate the toxic effect of fluoride 4. Consumption of calcium and/or vitamin C rich foods to mitigate the toxic effects of excess fluoride 99. Other (Specify): _____
213	Is there any means/measure taken to lessen the fluoride content of your drinking water source?	0. No 1. Yes 2. I don't know **If yes, please mention** _____ _____
214	Does your household have any mosquito nets that can be used while sleeping?	0. No 1. Yes
215	Did you sleep under the mosquito net last night?	0. No 1. Yes

SECTION 3: Women obstetric history		
301	Number of gravidity	_____
302	Number of parity	_____
303	History of miscarriage	0. No 1. Yes
304	History of stillbirth	0. No 1. Yes
305	Did you give a child with any kind of birth defect?	0. No 1. Yes
306	If yes to Q 305, mention the kind of birth	1 Paralyses of extremities

	defect you had. (*More than one response is possible. Please don't read the alternatives. Circle the given response from the list of options given*).	5. Spinal bifida 6. Hydrocephalus 7. Missed body part (s) 8. Presence of extra body part (s) 99. Other (Specify)_____

SECTION 4: 24hr dietary recall

Instruction: Now I would like to ask you about foods that you may have had yesterday during the day or the night. Please also include foods consumed outside your home. Did you eat (Name the food) yesterday during the day or night? **(Read each item aloud and record responses before moving to the next item).**

	Food item/group	**Response**
401	Maize, bread, sorghum, teff, wheat, barley, rice, noodles/indomi, porridge, or other foods made from cereals?	0. No 1. Yes
402	Pumpkin, carrots, squash, or sweet potatoes that are yellow or orange inside?	0. No 1. Yes
403	White potatoes, sweet potato, kocho/bulla, white yams, cassava, or other foods made from roots/tubers?	0. No 1. Yes
404	Any dark green leafy vegetables (eg. kale)?	0. No 1. Yes
405	Ripe mangoes, papaya, or other orange colored fruits?	0. No 1. Yes
406	Any other vegetables?	0. No 1. Yes
407	Any other fruits? Fore-example Avocado	0. No 1. Yes
408	Any foods made from beans, peas, lentils, or nuts?	0. No 1. Yes
409	Cheese or other food made from milk (eg. yogurt)?	0. No 1. Yes
410	Eggs? Any kind of egg	0. No 1. Yes
411	Liver, kidney, heart or other organ meat	0. No 1. Yes
412	Any meat, such as beef, pork, lamb, goat, chicken?	0. No 1. Yes
413	Fish? Any kind of fish	0. No

		1. Yes
414	Other fats or oils (eg. vegetable oil, ghee, sheno, kibe) or foods made with them?	0. No 1. Yes
415	Sugar and other sugary products (eg. chocolates, sweets, candies, soda)	0. No 1. Yes
416	Any other semi-solid, solid, or soft/mashed foods that I have not mentioned?	0. No 1. Yes

SECTION 5: Food frequency on calcium and/or vitamin C rich food consumption

Instruction:- Put (√) mark if the women consume the food items based on the given frequency of consumption.

Food item	Once a day	More than once a day	Once a week	More than once a week	Once in a month	Never eaten
Milk						
Yogurts						
Millet						
Kale						
Fruit (Banana)						
Non-Kale vegetables						
Moringa leaf						

Annex II: Amharic version Questionnaires, 24hr Dietary recall and FFQ used for Interviewing

በሀላባ ልዩ ወረዳ ከእንቁላል ቅርፊት የተዘጋጀ የካልሲየም ዱቄትን በመጠቀም በፍሎራይድ አማካኝነት በጥርስና አጥንት ላይ የሚደርሰውን ጉዳት ለመቀነስ በሚካሄደው ጥናት ዙሪያ የዳሰሳ መጠይቅ::

የመለያ መረጃዎች			
01	መጠይቁ የተሞላበት ቀን:_____	02	የጠያቂ ስም:_____
03	ጎጥ/ሰፈር:_____	04	ቀበሌ:_____
05	የእናት ስም፡ _____	06	የእናት ዕድሜ:_____
07	የህፃኑ ስም:_____	08	የህፃኑ ፆታ፡ 1 ወንድ 2 ሴት
09	የህፃኑ ዕድሜ በወር:_____ (ከ6-18 ወር የሆነ ህፃን ልጅ መሆኑን የክትባት ካርድ በማየት ያረጋግጡ)	10	ህፃኑ የተወለደበት፡ ቀን_____ ወር_____ ዓ.ም_____
ክፍል 1:ማህበራዊና ኢኮኖሚያዊ ጥያቄዎች			
<u>መመሪያ</u>:-ጥያቄዎቹን በተፃፈዉ መሰረት ይጠይቁ፡፡ የተዘረዘሩ መልሶችን እንዲያነቡ ካልታዘዙ በስተቀር እንዳያነቧቸዉ፡፡ እንዲዘሉ የሚታዘዙበት ቦታ ላይ በታዘዙት መሰረት ይዝለሉ፡፡ የጥያቄዎቹን መልስ መልሱ ላይ በማክበብ ያመልክቱ፡፡አንድ ምላሽ ብቻ ለሚያስፈልጋቸው ጥያቄዎች አንድ ምላሽ ብቻ ፃፍ፡፡			
101	እርስዎን ጨምሮ በቤት ዉስጥ ምን ያህል ሰዉ ይኖራል?		1. ወንድ _____ 2. ሴት _____
102	ከ5ዓመት በታች የሆኑ ስንት ልጆች አለዎት?		1. ወንድ _____ 2. ሴት _____
103	በዚህ አካባቢ/ወረዳ/ቀበሌ ለምን ያህል ጊዜ ወይም ዓመት ኖሩ/ኖራችሁ?		5. ከ5ዓመት በታች 6. ከ5-10ዓመት 7. ከ11-15ዓመት 8. ከ15ዓመት በላይ
104	የእናት የትምህርት ደረጃ		0. ያልተማረች 1. አንደኛ ደረጃ (1ኛ-8ኛ ክፍል) 2. ሁለተኛ ደረጃ (9ኛ-12ኛ ክፍል) 3. ኮሌጅ/ዩኒቨርሲቲ (ከ12ኛ ክፍል በላይ) 99. ሌላ ካለ ይግለጹ_____
105	የአባት የትምህርት ደረጃ		0. ያልተማረ 1. አንደኛ ደረጃ 1ኛ-8ኛ ክፍል) 2. ሁለተኛ ደረጃ (9ኛ-12ኛ ክፍል) 3. ኮሌጅ/ዩኒቨርሲቲ (ከ12ኛ ክፍል በላይ) 98. አባት በህይወት የለም 99. ሌላ ካለ ይግለጹ _____
			1. በጋብቻ ውስጥያሉ

106	አሁን ያለዎት የጋብቻ ሁኔታ ምንድን ነው?	2. አግብተው የፈቱ 3. ባል የሞተባቸው 99. ሌላ ካለ ይግለጹ_____
107	የእርስዎ ዋነኛሥራ ምንድን ነው?	0. ገበሬ/የግብርና ስራ 1. የመንግስት ስራ ተቀጣሪ 2. የግል ድርጅት ተቀጣሪ 3. አነስተኛ የንድግ ስራ 4. ስራየሌላት 99. ሌላካለ ይግለጹ _____
108	የባልዎ ዋነኛሥራ ምንድን ነው?	1. ገበሬ/የግብርና ስራ 2. የመንግስት ስራ ተቀጣሪ 3. የግል ድርጅት ተቀጣሪ 4. አነስተኛ የንድግ ስራ 5. ስራየሌላት 99. ሌላካለ ይግለጹ _____
109	የቤተሰብዎ ዋነኛ የገቢ ምንጭ ምንድን ነዉ?	1. እርሻ 2. ከብት እርባታ 3. የቀን ስራ 4. አነስተኛ የንድግ ስራ 5. የመንግስትስራ 99. ሌላ ካለ ይግለጹ_____
210	አማካኝ የቤተሰቡ ጠቅላላ ወርሃዊ ገቢ በብር ስንት ነው?	6. ከ 500ብር በታች 7. ከ 501-1000ብር 8. ከ 1001-1500ብር 9. ከ 1501-2000ብር 10. ከ 2000ብር በላይ

ክፍል 2፡ የመጠጥ ውሃ፣የአካባቢና የግል ንጽህና በተመለከተ።።

201	የቤተሰቡ ዋነኛ የመጠጥ ዉሃ ምንጭ ምንድነዉ?	1. ከቧንቧ 2. ያልተከለለ/ያልተጠበቀ የጉድጓድ ዉሃ 3. የተከለለ/የተጠበቀ የጉድጓድ ዉሃ 4. ከወንዝ፤ ከምንጭ፤ ቆሬ 5. ከተጠራቀመ የዝናብ ውሃ 99. ሌላ ካለ ይግለጹ_____
202	ውሃ ቀድቶ ለመመለስ ምን ያህል ጊዜ ይወስዳል? **(በግቢያቸው ውስጥ ቧንቧ ወይም የጉድጓድ ዉሃ ለሌላቸው የሚጠየቅ ነው)**	4. ከ1ሠዓት በታች 5. ከ1ሠዓት በላይ 6. አይታወቅም
203	የመጠጥ ዉሃ እነዴት ነዉ የሚያስቀምጡት?	1. በጀሪካን 2. በባሊዲ/እንስራ 99. ሌላ ካለ ይግለጹ_____
204	የመጠጥ ዉሃ ከመጠጣቱ በፊት ንጽህናዉን ለመጠበቅ ያጣሩታል?	0. አላጣራም 1. አጣራለሁ **አላጣራም ካሉ ወደ ጥያቄ 206 ይለፉ**
205	የሚያጣሩ ከሆነ ለማጣራት ምንድነዉ የሚጠቀሙት?	1. በጨርቅ ማጥለል 2. ዉሃዉን በማፍላት

	እንዲያስታዉ እርዳቸዉ። (ከአንድ በላይ መልስ መመለስ ይችላሉ።)	3. ዉሃ ማከሚያ መጨመር (ለምሳሌ፤ ዉሃ አጋር) 4. ቆሻሻዉ እንዲዘቅጥ በማድረግ (ለምሳሌ፤ አሸዋ በመጠቀም) 99. ሌላ ካለ ይግለጹ_____
206	ምግብ ለማዘጋጀት የሚጠቀሙበት ዉሃ ከመጠጥ ዉሃ ይለያል?	3. አይለይም/አንደ አይነት ነው/ 4. አዎ ይለያል
207	ለንፅህና/ሠውነት ለመታጠብ የሚጠቀሙበት ዉሃ ከመጠጥ ዉሃ ይለያል?	9. አይለይም/አንደ አይነት ነው/ 10. አዎ ይለያል
208	እጅዎን መቼ መቼ ነዉ የሚታጠቡት? **እንዲያስታዉ እርዳቸዉ።** **(ከአንድ በላይ መልስ መመለስ ይችላሉ።)**	0. ታጥቤ አላዉቅም 1. ምግብ ከማብሰለዎ በፊት 2. ልጅ ከመመገብዎ በፊት 3. ሽንት ቤት ከተጠቀሙ በኋላ 4. የህፃኑን ሰገራ ካፀዱ በኋላ
209	እጅዎን በአብዛኛው የሚታጠቡት በምንድን ነዉ? **(አንድ መልስ ብቻ የመዝግቡ።)**	0. ምንም አልጠቀምም 1. ሳሙና 2. ፈሳሽ ወይም ዱቄት ሳሙና 3. አመድ 4. ጭቃ ወይም አሸዋ 99. ሌላ ካለ ይግለጹ_____
210	ቤተሰቡ በዋናነት የሚጠቀምበት የሽንት ቤት አይነት ምንድን ነው? **አንድ መልስ ብቻ ይፃፉ።**	0. ሽንት ቤት የለም - ጓሮ እርሻ ዉስጥ ነው የምንጠቀመዉ 1. ክለላያለዉ ሽንት ቤት 2. የደረቅ ጉድጓድ ሽንት ቤት 3. በዉሃ የሚሰራ ሽንት ቤት 99. ሌላ ካለ ይግለጹ_____
211	ከቀበሌአችሁ የጤና ኤክስቴሽን ወይም ከሌላ የጤና ባለሙያስለፍሎራይድ ትምህርት አግኝተውዋል?	2. አይ አላገኘሁም 3. አዎአግኝቻለሁ
212	ለጥያቄ ቁጥር 211 መልስዎ‹**አዎ አግኝቻለሁ**› ከሁነ፤ ያገኙት ትምህርት በምን ዙሪያ ነው **(ከአንድ በላይ መልስ መመለስ ይቻላል።)**	4. ፍሎራይድ በብዛት የሚገኝባቸው የምግብና የመጠጥ አይነቶች 5. ፍሎራይድ ስለሚያስከትለው ጉዳት 6. ፍሎራይድ የሚያስከትለውን ጉዳት ለመቀነስ ስለሚወሰዱ እርምጃዎች 7. የፍሎራይድ መጠን ከሠውነት ለመቀነስ በካልሲየምና በቫይታሚን ሲ የበለፀጉ ምግቦችን አዘውትሮ ስለመመገብ/ስለመጠቀም 99. ሌላ ካለ ይግለጹ_____
213	ፍሎራይድን ከምትጠቀሙበት ዉሃ ለመቀነስ ወይም ለማጥፋት የምታደርጉት ጥረት አለ?	3. አይ፤የለም 4. አዎ፤አለ ‹**አዎ**› ካሉ ምን እንደሆነ ይግለጹ_____ _____
		0. አይ፤ደለም

214	ቤተሰቡ አጎበር ዉስጥ ነዉ የሚተኛዉ?	1. አዎ
215	በትላንትናዉ ዕለት አጎበር ዉስጥ ነበር የተኙት?	0. አይ፤ደለም 1. አዎ

ክፍል 3፡ የልጅ አወላለድን የተመለከቱ ጥያቄዎች፡፡

301	ስንት ጊዜ አርግዘሽ/ው ያውቃሉ?	_____
302	ስንት ልጅ አሉሽ/ሎት?	_____
303	ውርጃ አጋጥሞሽ/ዎት ያውቃል?	0. አያውቅም 1. አዎ
304	በማህፀን ውስጥ/ሳይወለድ/ የልጅ መሞት ችግር ገጥሞዎት ያውቃል?	0. አያውቅም 1. አዎ
305	በተፈጥሮ ሲወለድ የአካል ችግር ያለበት ልጅ ወልደው ያውቃሉ?	0. አልወለድኩም 1. አዎ
306	ለጥያቄ ቁጥር 305 መልስዎ **አዎ** ከሆነ በልጅዎ ላይ የደረሰውን የአካል ችግር አይነት ምን እንደሆነ ይግለፁ? **(ከአንድ በላይ መመለስ ይቻላል፡፡ ምርጫዎችንአያንቡላቸው፡፡መልሳቸውን ከተዘረዘሩት አማራጮች ውስጥ በሚቀረበው መሠረት ያክብቡ፡፡)**	11. የእጅ ወይም የእግር መስለል/ሸባ መሆን 12. ወገብ ላይ የተከሰተ የተፈጥሮ ችግር 13. ጭንቅላት ከጤንነት በላይ ትልቅ መሁን 14. የአካል ክፍል መጉደል 15. ትረፍ የሠውነት አካል መኖር 16. ሌላ ካለ ይግለጹ_____

ክፍል 4፡ በትላንትናው እለት በ24 ሠዓት ውስጥ የተመገቡት የምግብ አይነት/ክፍል በተመለከተ

መመሪያ፡ የተዘረዘሩትን የምግብ አይነቶች ያንብቡላቸዉ፡፡ ከተዘረዘሩት የምግብ አይነት አንዱን እንኳን ከጠቀሱ እንደተመገቡ በመቁጠር ምልክት ያርጉበት፡፡ (አሁን የምጠየቅዎት እርሰዎ ትናንት ጧት፤ቀን፤ ማታና ሌሊት ቤት ውስጥ ወይም ከቤት ውጭ የተመገቡትን የምግብ አይነቶች እንዲነግሩኝ ነዉ፡፡)

	የምግቡ አይነት/ክፍል	**ምላሽ**
401	ከበቆሎ፣ ማሽላ፣ጤፍ፣ ስንዴ፣ገብስ፣ሩዝ የተዘጋጀ (ገንፎ፣አጥሚት፤እንጆራ፣ዳቦ፣ቂጣ፣ወዘተ) ፓስታ፣መኮሮኒ፣ብስኩት፣ኩኪስ?	0. አልበላሁም 1. በልቻለሁ
402	ዱባ፤ ካሮት፤ ስኳር ድንች፤ ወይም መልካቸዉ ቀይ፣ ቡርቱካማ ወይም ቢጫ መልክ ያላቸዉ አትክልቶች	0. አልበላሁም 1. በልቻለሁ
403	ነጭ ድንች፤ቆጮ፤ ቡላ፤ ቦይና፤ ካሳቫ፤ ወይም ሌላ የስራስር ምግቦች	0. አልበላሁም 1. በልቻለሁ
404	መልካቸዉ ጠቆር ያለ አረንጓዴ ቅጠል ያላቸው አትክልቶች ለምሳሌ ያበሻ ጎመን፣ የሽፈረው ቅጥል	0. አልበላሁም 1. በልቻለሁ
405	ማንጎ፤ ፓፓያ፤ ወይም ሌላ ቢጫ መልክ ያላቸዉ ፍራፍሬ፣ የፍራፍሬ ጭማቂ	0. አልበላሁም 1. በልቻለሁ
406	ሌላ የተመገቡት የአትክልት ዘር አለ?	0. አልበላሁም 1. በልቻለሁ
407	ሌላ የተመገቡት የፍራፍሬ ዘር ለምሳሌ አቮካዶ?	0. አልበላሁም 1. በልቻለሁ
408	ጥራጥሬዎች፡ ከባቄላ፤ ሽምብራ፤ አተር፤ ምስር፤ አደንጓሬ፣ ቦሎቄ፣ ኦቾሎኒ የተዘጋጀ ምግብ	0. አልበላሁም 1. በልቻለሁ
409	ወተትና የወተት ተዋፆዎች፡- አይብ፣እርጎ አሬራ፣አንት	0. አልበላሁም 1. በልቻለሁ

410	እንቁላል፡- የማንኛውም አእዋፍት እንቁላል	0. አልበላሁም 1. በልቻለሁ
411	በብረት ማዕድን የበለፀጉ የእንሰሳት ስጋ፡- ጉበት፤ ኩላሊት፤ ልብ፣ ዱለት	0. አልበላሁም 1. በልቻለሁ
412	ማንኛዉንም አይነት የእንሰሳት ስጋ የበሬ፤ የዶሮ፤ በግ፤ የፍየል፤ የአሳማ፣የሌሎች አእዋፍት ስጋ፣ ወዘተ	0. አልበላሁም 1. በልቻለሁ
413	አሳ፡-የማንኛውም አይነት አሳ ስጋ	0. አልበላሁም 1. በልቻለሁ
414	ከዘይት፣ከቅቤ ወይም ከስብ የተዘገጀ ምግብ	0. አልበላሁም 1. በልቻለሁ
415	ማነኛውም ስኳራማ/ጣፋጪ ምግብ ወይም መጠጥ ለምሳሌ፡- ቸኮሌት፣ከረሜላ፤ ለስላሳ መጠጦች እንደ ሚሪንዳ፤ ፔፕሲ፤ ኮካ	0. አልበላሁም 1. በልቻለሁ
416	በትላንትናዉ ዕለት ከቤት ውጭ ምግብ በልተዋል/ተመግበዋል?	0. አልበላሁም 1. በልቻለሁ
417	ከላይ እኔ ያልጠቀስኩት ሌላ የተመገቡት የምግብ አይነት ካለ ይንገሩኝ	

ክፍል 5፡ በካልሲየም ወይም በቫይታሚን ሲ የበለፀጉ የምግብ አይነቶችን የአመጋገብ ድግግሞሽ ለማወቅ የተዘጋጀ

መመሪያ፡- ለተዘረዱት የምግብ ዓይነቶች አዘውትረው የሚመገበቡትን የምግብ አይነት በተሠጠው አማራጭ መሠረት በማክበብ ይህን የኤክስ (√) ምልክት ያስቀምጡ፡፡

የምግቡ አይነት	**በቀን1 ጊዜ**		**በቀን ከ1 ጊዜ በላይ**		**በሳምንት 1 ጊዜ**		**በሳምንት ከ1 ጊዜ በላይ**		**በወር 1 ጊዜ**		**በልቼ አላዉቅም**	
ወተት												
እርጎ												
ዳጉሳ												
ያበሻ ጎመን												
ፍራፍሬ												
አትክልት												
ሞሪንጋ /ሽፈራው/												

ስለነበረን ቆይታ ከልብ አመሰግናለሁ!

Annex III: *Hallebigna* version (local language) Questionnaires, 24hr Dietary recall and FFQ used for Interviewing

ሀለቀመንችመለለንሽ ኮዱ_____

ሀዋሲ ዩንቨሪስትታ ኢቻስሪአት ት/ሚን

ቀበሊት_____ክልሉ_____ዞኑ_____ወረዱ_____
ጠሜኖበሩ፡___/___/____
ኢገራንችስሙ_____በሩ_____
ፍሪሙስ_____

ክፍሉማቱ፡ሀለቆመኖመንለገልቴሰ - ሀብትስሀለቱ			
ቁጡሩ	ጣምናም	ማልስስምርጫ	ሕሳቀጠለኖጥየቄበ ይጎን
01	ጩልስዛማኑመኡሀ?	_____ ዘመኒ	
02	ምንክኔማንቁጡር?	ነ.ጎንኡሱት_____ 2.ሜአንኡሱት_____ 3.አመትአኑ_____ 4.ሚንአዜንበሪገንትሄአንመነከት___ 5.ሆሩአበሮስልኩ_____	
03	ምክኔማንሀይማምቱ?	ነ.ኦርቶኮክለሀ 2.ሙስሊመሀ 3.ፕሮቴስተንታሀ 4.ካቶልከሀ 5.ገለሀ_____	
04	ቴሱየንታብተሪክኔ?	ነ.ብተሪሀእኮሀ 2.ብተሪእሄነፋገሬመ 3.ሚንሰአትሬየእ 4.መሰለሜንሄደሩ 5.ግለ	
05	አነበቡወይንፃፉአተልቴነን?	0.አተለምእ ነ.አተለም	
06	ሜእቅክፍልእለ ተመሪቴንተእ?	ነ.አነበቡወይንፃፉ 2.ዎኔአራከንትምህርት 3.ለምቅደረጃ /እርከን /ት/ት 4.ዲፕሎማስእሰችአሎ.	
07	ማንስአመትትምህርት ተመሪቶእ?	0.ተመሪቶበእ ነ.አተመሪቶእ	

08	ማንስአመትሚእቅክፍል እለተመሪትእ?	1.አነበቡለጻፉ 2.ዋኔእርከንት/ት 3.መሬርለምቅደረጃ/እረከነ 4.ለምቅደረጃ 5.ዳኘሎማለእናእሰችአሌ	
09	ሚንስገሸንቹ አዬት?	1.እሰት 2.ሚንስተአማ 3.አበኑ /አበማት 4.ግለዮጌሬኩሎን	
10	አበሮስህስአቤራኡለት/ቀሪምት ዮስ?	1.ዮበእ 2.ኤዮኤ	
11	ቀሪሚስጌስከንከህ? ቀግሰንቹ፡ 1 አህቱ፡1/4 ሄክተራህ	----------ሄክተረህ ----------አህቱ	
12	ጉመሰአትስሆጉትማህን?	1.ሚንሆጉታ 2.መንግስቴሆጉታ 3.ገበሬታ 4.ቀቀዉንግከ 5.ግሉዮጎሬኩሌ.	
13	ሚንክአንሆጉትማህን?	1.ሆጉትዮስበእ2.ገበሬታ3.ቀበንቾህ. 4. መንግስቴህሰራተኛ 5.ቀሪሜሆጉተአሰኖሰራተኛ 6.ሆሸሰራተኛ 7.ገግስቡከገለንቹ 8.ቀጠራምአሰንቹ 9.ቀርሜችግለ 10.ግሉዮጎራተ	
14	አበሮሰስአገንገቢስ ከንከህ?	1.<500 በረህ 2.500-1000 ከረህ 3.1001-1500 ከረህ 4.>1500 ከረህ 5.ግሉዮጎሬኩሌ	
15	ሚንግዙ /ፎለሙ/ዮስ? ፎለሙ ቦሩ ሰት ፊለኡ ሆለትህርት ፋሹ/በቁለት ግሉ	ዮኔበእዮኔቁጠሩስ 0 ---------- 0 /---------- 0 /---------- 0 /----------0 /----- ------0 /---------- 0 /----------	

16	ቀጠልትዮእሀኑኙአተእራ እከምንከአዜንዮእሩ	1.ሬድዮን/ቴፕ 2.ቴሌቪዥንሀከኑኡተን? 3.ሰይከልታ 4.በትሬተን 5.ምበዩለሀን 6.ፋሪስ/ሀሬገሪት 7.ግለሀ 8.ጠቀስንቶሩጉሙንኩዮበእ	
17	አበሮስክተጠቀመሮማአይነት ሹመምንህት?	0.ሹመምኑዮበእ 1.ምለሹመኤሎ 2.ሀሎ/ደንጉዮስኤሌሹመምን 3.በከሩአገኖስቆሀቀምሹመምን 4.ዘመነስ/ዊንኢሰኖሹመምን 5.ግሉአይነቱዮጎሬተኩሌ----------	
18	ምጌስሄኤመእ?	-----ዘማኒ	
19	አከበቤንዮእፋይመ ኤከስቴንሽነሠራተኛከች ፋይመለእቻተእቴኖ ትምህርትደቅቴንተእ?	0.ደቅዮምበእ 1.ዲቅዮም	
20	ቅጥየቁተትምህርት ደቅዮምይቴንተችደቅቴንተ ስህኑስመሪችዙርየንት (መትችበተአኖመልስፍንቀሹአተሌሎከልሰምለ ቆሀቀመኖአመራጫከበቤስወንገሴተቅጥዬቤቾንጻፌ::) -----	1.ፍሎራይዱኤበኖጉዳትከመቀነስወቀ3.ፍሎሪዱደገመኖቤ ቹ4.ፍሎሪዳሆፍሰሀ5.ስለነፃመተ6.አበሮስልከወሰኑ 7.ግሉዮጎሬተኩሌ----------	
ለምቅከፍል፡አገኖዋደገንተእቤቹለፋይመስአገሩ			
21	አበሮስአጌኑዉዋኑቤቹስማሀን?	1.ምንአዜንዘንሞዚዋሀ 2.ጊቤአዜንዮእሞዚዋሀ 3.ሜተመዋዚዋ/ህዝበሀ 4.ከለሌመኤሌዋ 5.ከለለምበኤሌዋ 6.ከለሌመቡቁተ 7.ከለለምበቡቁተ 8.ጤንዋሀ 9.ዘዘኖዋዘጉኤለእርከንዋ 10.ግሉዮጌሬ----------	
22	ዋእንክልሀምጌሰስአት መስኖ? (ኢንከሌንፈንቀልሀ)	1.ለምጽመደቂቀተ 2.ሸለዉደቂቀተ 3.መቱሰአት 4.ለሙሰአትለእሰችአሌ	
23	አበሰአትዋእንክሉአዬታ ሆጉ ? 2.ሚኒአኑ/አመት	1.እሰገጉሰ 2.ሚኒአኑ/አመት 3.አሱት 4.ምንሠራተኛ 5.ግሉዮጎሬታ	
24	እቻተአሰሀደግለሸቀሜኖ ዉአግሀየጠቀሜዬዊችዳገመኖ ቤቹገለሀንደ?		

25	አልሀደግለሽቀንቄናተ ው አግሀ ደግለሽቀንቴናተሀንችውስደገመኖሁ ግለሀንደ?		
26	አጌዮ ዋ መሪችንትኢፋሴኖሁ? ምደምን:ጀሪከንንግለ?		
27	ፍሎራይደሆፍሰሀአሴሮርቹ(ወከቱት) ዮጎሬተኩሌመእቀ?		
28	ጤን ዋ ደግሰሽቀሜኖ?(አግሀ)		
30	መልሱከኔ ኤ እኮችጤን ዋ ደግለሽቀሜኖ (አግሀ) ደግለሽቀሜኖአገኙ?		

ለምቅከፍል፡- አመኖኤንኬኑጠመንቹ		
ተጠሩ	ጠመንቹ	መልሱስ
አስከኔስሙአዬት?	_____	
ጩሌከዕድምሴሜአሀ?	___ /	
ፍሎራይደዬኖርቾማደገን?	ነ. ኤ 2.እሰበእ	
ፍሎራይደዬኖርቹሆከኔደገመኖ?	ነ. ኤ 2.እሰበእ	
ኤይትንትችፍሎራይድ ም አዜን ደገመኖ?		
ፍሎራይድበትሴንመሱኤበኖጉዳትደገን?	ነ.ኤ2.እሰበእ	
ፍሎይድልኩስምቅ ቀ ጭመተኤበምንተገን?	ነ. ኤ2.እሰበእ.	
ፍሎራይድልኩስበተአምራእንኮበሸመተ ኤበኖንተገን?		
ከልሰየምጠዋ መ ደገን?	ነ.ኤ2.እሰበእ.	
ከልሰየምጠዋ መ ደገን?	ነ.ኤ 2.እሰበእ.	
ከልሰየምንዊንቶእቻተ ደገን?		
ኤ ይቶንት ች ከልሰየምንዋንቶ እቻተኩል?		
ከልሰየሙፋይመተአሰኖደግለሸተ ደገን?	ነ.ኤ2.እስበእ.	
.ኤይቶንትችደግለሸስግሀን?	ነ.ኤ2.እስበእ.	
ቡጴጎኑከልስየምዊምን/ዮስገተዳን?	ነ.ኤ. 2.እስበእ	
ዘንገዱከልስየምንዊምንተገን?	ነ.ኤ2.እስበእ	
አዙትከልስምንዊምንተገን?	ነ.ኤ2.እስበእ	
ቄሱከልሰየምንዊምንተገን?	ነ.ኤ2.እስበእ	
ሽፊራዉከልሰየምንዊምንተገን?	ነ.ኤ2.እስበእ	
ከልሰየሙፍሎራይድኤበኖ ጉዳትቀነሰኖንተገን?		
ከልሰየምፋየእንኩትሄኢጋአስኖገ ደገን?	ነ.ኤ2.እስበእ	
ከልሰየሙፋዩምቁሄአኖጋ አስኖጋደገን	ነ.ኤ2.እስበእ.	

ስቅከፍል፡- አመኑታለኤኖጥየቅተ			
ነ	ከልሰየምንዊንቶእቻትእንኮበሸመተከመኖንደ?	ነ.ኤ በጠምተሰመመአም 2.ተስማማ አም. 3.ሜሬረን ቾሀ 4.ተስማመአምበእ 5. በጣምተሰመመአምበእ	
2	ከልሰየምንዊንቶእቻትምቅቀጭመተከንተእ?	ነ.ኤ በጠምተሰመመአም 2.ተስማማ አም. 3.ሜሬረን ቾሀ 4.ተስማመአምበእ 5. በጣምተሰመመአምበእ	
3	ፍሎራይድበትሴንኢቱእንኮበሸእሀምክንያትእሀኖዶ?	ነ.ኤ በጠምተሰመመአም 2.ተስማማ አም. 3.ሜሬረን ቾሀ 4.ተስማመአምበእ 5. በጣምተሰመመአምበእ	
4	ፍሎራይድበትሴንኢቱምቅቀጭመተ ምክንያትእሀኖ?	ነ.ኤ በጠምተሰመመአም 2.ተስማማ አም. 3.ሜሬረን ቾሀ 4.ተስማመአምበእ 5. በጣምተሰመመአምበእ	
5	ከስሰያማዬኖሁፍሎራይድተቆጠጠሪደግስለኖ?	ነ.ኤ በጠምተሰመመአም 2.ተስማማ አም. 3.ሜሬረን ቾሀ 4.ተስማመአምበእ 5. በጣምተሰመመአምበእ	

ሸልቅከፍል፡ ከልሰዬምንዊንቶ/ያሰእቻትኢቴኖስአይነቱ			
ነ	ቤሬተጨልክአዙተአጎእ?	ነ.ኤ	2.እሰበእ
2	ቤሬተጨልክቄስእቶእ?	ነ.ኤ	2.እሰበእ
3	ቤሬተጨልክዞኬትዮስእቻተ እቶእ?	ነ.ኤ	2.እሰበእ
4	ቤሬተጨልክተልቡዮስእቻተእቶእ?	ነ.ኤ	2.እሰበእ
5	ቤሬተጨልክኑጉዮስእቻተእቶእ?	ነ.ኤ	2.እሰበእ
6	ቤሬተጨልክሰልጡዮስእቸተእቶእ?	ነ.ኤ	2.እሰበእ
7	ቤሬተጨልክሀምሱዮስእቸተእቶእ	ነ.ኤ	2.እሰበእ
8	ቤሬተጨልክቡዲንገ/ቡዲአምሱ ዮስእአተእቶእ?	ነ.ኤ	2.እሰበእ
9	ቤሬተጨልክሸፈራዉዮስእቻተ እቶእ?	ነ.ኤ	2.እሰበእ

አንትቅከፍል፡- እቻበርገቀንሸጠመንቹ

ኢቻአይነቱ	ኢተሞ 0.ኤ ነ.እሰበእ*	ኢተመበርገቀንሸ**			አበሰአት ኢተምወቅቱ***	ግምትስልኩግራምን
ጠፊት						
አለሱ						

በቆሉ						
መሹሉ						
ዘኬት						
ሰአ						
አጀ						
ድንቻ						
ሰከሪዶንቻ						
ዌሴተ						
በቄለ						
ሹምቡራ						
ምሸራ						
ካሮጽ						
ትማትመ						
ጥቀልነምን						
ህምሉ						
ሸፈራዉ						
ጡርጉ						
ደበቄለ						
ሎምታ						
ቡርቱከነ						
ዘይቶነ						
ማለ						
አዙተ						
ቁርጪሜተ						
ሰነፍጨ						
ተልበ						
ኑገ						
ሰልጠ						
ቡጴነገ						
ጠፌት						

ኢኖእቻተ

*0= ኢተምበእ
**0=ቁገ
1.፡ ጉመራን(በየበር)እተምሞ
2.፡ ለምች-ሎመራእለስምነት አዜን
3.፡ስምንት አዜንመቶራ
4.፡አገነን
5.፡ሰሶ አገነንመቶራ

**0=ቁገ
1,ሬዮእሀ
2.ጠበሰምሀ3.ጠሮሸን/ዳቦ መልከን
4.ጠብትን መልከ
5.መርቅ መልከን

***
1 =
2 =
3 =
4 = Mar-May

ጨል

ሜዘኑስ፡

ወቅቱስ	ኢተምቤቹ	ኢቸት/ዋፎዝርዘርሰለሬእሰመሰዘድተ	ኢተምጌሱ(እቴምሰአደተ)	ኢተምጌሰግራምን

አልከለአጌንተ?ኤ/አይእሰበእ			ጪልከጥዘእቅ?ኤ/እሰበእ	
ኩ እቱጉመሰአትእትቸከኔ፣ ሜጠሃአይነት?ደ? ኤ/እስበእ			ኤ እኮችጥዘንሰእቻተእቱከምሰቅ(ቀነሾእቅ)? ኤ/እሰበእ እስአሌንእቸሰፍልመንተጨልከመሰዮእ? ኤ/እሰበእ	

ሜጠመጠመቀንሸ

ነ. ፍሎራይድማህን?

2. እንኩተቢሸእሸቸለምቀቀጭሸቸበርገምፍሎራይድሃ 5ሄቤቹ፡ኤንኬኑ?

3. ፍሎራድጌሱበተኡኤበኖጉዳቱቀነሰሀ/ሆፍሰሀአሰምአበንሸ (መሴመእርምጃ)ዮችአብራራዬኤ?

4.ገልቴስእቹሁኔቱመአጉደኖ፡ለምሳሌ፡- አሰተ/ሜአተፈቀዴመሰእችትዮእ?

5. ገልቴሰንአዜንተገንተእከልሰየምንዊንትእእቸትመሀመሀን?

6. ከልሰየምደግለሸስማህን?

Annex IV: English version Consent form for Volunteer Women to Consume ESP

This is a study being conducted by a PhD student: Demmelash Mulualem Zewdie at Hawassa University. The local study coordinator and data collectors are being paid to conduct this research study. You are invited to take part in this research study because you live in this *kebele*. Your participation is voluntary. It is up to you to decide whether or not you wish to take part. If you decide to participate, you are still free to withdraw at any time and without giving any reasons for your decision. Please take time to read {… listen to …} the following information carefully. You can ask the study staff to explain any information that you do not clearly understand. You may ask as many questions as you need. Please feel free to discuss this with your family, friends before you decide.

WHAT DOES THE STUDY INVOLVE?

You will be asked to answer different questions about you. Questions from questionnaires will be read to you in your language. Some questions ask about the living standard of the household. Other questions concern the safety and acceptability of providing eggshell powder for you. You may refuse to answer questions that you are uncomfortable with. This will take around 30 minutes.

Hemoglobin levels give a broad picture of health, as it tells us whether there is dietary deficiency or underlying illness. You will be asked to have blood taken from your vein and by finger prick to assess the serum iron, zinc, hemoglobin levels and test malaria at the beginning and end of the study. The blood will be collected in a sterile cuvette that will be disposed of after measurement. No further tests will be done on the blood.

We will collect a urine sample from you at the beginning and end of the study to measure fluoride. Only a small amount is needed and collection will be arranged at the health center/post.

At the beginning of the study, a dentist will assess your teeth. And also a physiotherapist (health professional) will assess your ability to move and ask questions about some symptoms related to excess fluoride ingestion.

WHAT HAPPENS IF I DECIDE TO WITHDRAW?

You can stop participating at any time without penalty, and without having to provide any explanation for doing so. We will retain any data collected up to the point of your withdrawal from the study.

WHAT HAPPENS IF SOMETHING GOES WRONG?

- That in the event of an adverse event, the research participant should seek immediate attention. There is a study coordinator available to contact and you will get the appropriate health care at the nearby health facility free of charge.
- These services will be provided at no cost to the research participant.
- You are not waiving any legal rights to seek compensation for damages by signing

WHAT WILL THE STUDY COST ME?

There are no costs associated with this study.

WILL MY PARTICIPATION BE KEPT CONFIDENTIAL?

Your personal information will only be used for the purpose of the study. You will not be personally identified in the study report. Your information will be coded so that your identity is not disclosed.

PARTICIPANT CONSENT TO PARTICIPATE

- I have read (or someone has read to me) the information in this consent form.
- I understand the purpose and procedures and the possible risks and benefits of the study.
- I was given sufficient time to think about it.

- I had the opportunity to ask questions and have received satisfactory answers.
- I am free to withdraw from this study at any time for any reason and the decision to stop taking part will not affect my future medical care.
- I agree to follow the study doctor's instructions and will tell the study doctor at once if I feel I have had any unexpected or unusual symptoms.
- I give permission for the use and disclosure of my de-identified personal health information collected for the research purposes described in this form.
- I understand that by signing this document I do not waive any of my legal rights.
- I will be given a signed and dated copy of this consent form.

I agree to participate in this study:

Printed name of participant: Signature Date

Printed name of person obtaining consent: Signature Date

Annex V: Amharic version Consent form for Volunteer Women to Consume ESP

በሙሉ ፈቃደኝነት ከእንቁላል ቅርፊት የተዘጋጀ ዱቄት ለመመገብ በተመረጡ እናቶች የሚሞላ ስምምነት ቅጽ

ጤና ይስጥልኝ! እኔ የመጣሁት ከሃዋሳ ዩኒቨርሲቲ ጥናት ለማካሄድ ነው፡፡የመጣሁበት ዋናዉ የጥናቱ አላማም ከ5ሚሊግራም ያልበለጠ ከእንቁላል ቅርፊት የተዘጋጀ ዱቄት በየቀኑ ከሚመገቡት ማንኛውም ምግብ ወይም መጠጥ ጋር በመቀላቀል ለተከታታይ ስድስት ወራት እንዲጠቀሙ በማድረግ ፍሎራይድ በተባለ ንጥረ-ነገር አማካኝነት በዋናነት በጥርስና አጥንት ላይ የሚከሰተውን የጤና ጉዳት የሚቀነስበትን ዘዴ ለማጥናት ነው፡፡ የእንቁላል ቅርፊት ዱቄት በውስጡ ካልሲየምና ሌሎች ጠቃሚ ንጥረ-ነገሮች የያዘ ሲሆን ከማንኛውም ምግብ ሆነ መጠጥ ጋር ቀላቅለን መጠቀም የምንችለው ነው፡፡ ይህን ከእንቁላል ቅርፊት የተዘጋጀ ዱቄት የጥናቱ ቡድን በየወሩ የሚያስፈልገዎትን መጠን በማዘገጀት ያቀርብለዎታል፡፡
ለዚህ ጥናት ስኬታማነት የእርስዎ ተሳትፎ እጅግ አስፈላጊ ነዉ፡፡ በተጨማሪም የዚህ ምርምር ዉጤት ለቀጣይ ስራዎች ከፍተኛ አስተዋጽኦ ያደርጋል፡፡ በዚህ ምርምር ላይ እንዲሳተፉ የተጠየቁት በዚህ ቀበሌ ስለሚኖሩ ነዉ፡፡

ተሳትፎዎ የሚወሰነዉ ፈቃደኛ ሆነዉ ሲገኙ ብቻ ነዉ፡፡ በጥናቱ ላይ ለመሳተፍ የሚወስኑት እረስዎ ኖት፡፡ ለመሳተፍ ከፈቀዱ በኋላም እንኳን በማንኛዉም ጊዜ ምክንያት ማቅረብ ሳይኖርብዎ ከጥናቱ አቋርጠዉ መዉጣት ይችላሉ፡፡

ጥያቄ ካለዎት ማንኘዉንም የምርምሩ ቡድን ዉስጥ አባል የሆኑትን ሰዎች ማብራሪያ መጠየቅ ይችላሉ፡፡ የፈለጉትን ያህል ጥያቄ መጠየቅ ይችላሉ፡፡ በጥናቱ ለመሳተፍ ከመወሰንዎ በፊት ከቤተሰብዎ እና ከወዳጅዎ ጋር መመካከር ይችላሉ፡፡

በዚህ ጥናት ለመሳተፍ ፈቃደኛ ከሆኑ ከክንድዎ ላይ 5 ሚሊ ሊትር (አንድ የሻይ ማንኪያ) የሚሆን ደም እና የሽንት ናሙና ጥናቱ ሲጀምርና ሲያበቃ እንወስዳለን፡፡ ከዚህ ናሙና ከጥናቱ አላማ በቀር ሌላ የሚደረግ ምርመራ አይኖርም፡፡ ይህን ምርመራ ማድረግ የፍሎራይድ፣ ካለሲየም፣ አይረን እና የዚነክ መጠን ሁኔታ ምን ያህል እነደሆነ ለመረዳትና ተገቢዉን የማስተካከያ መፍትሄ ለመጠቆም ያግዛል፡፡

በዚህ ጥናት በመሳተፍዎ የሚደርስብዎ ችግር አይኖርም፡፡ ነገር ግን በጥናቱ ወቅት ምንም አይነት ከጥናቱ ጋር የተያያዘ ችግር ቢገጥምዎ በቶሎ ለጥናት ቡድኑ ሃላፊ ያሳዉቁ፡፡ ለሚደረገዎ እርዳታ ምንም አይነት ክፍያ አይጠየቁም፡፡ የሚሰጡን መረጃ በሚስጥር ይጠበቃል፡፡ መረጃዉ ለዚህ ምርምር ብቻ ይዉላል፡፡

የስምምነቱ ማረጋገጫ
ይህን የስምምነት ቅጽ ያነበብኩኝ (የተነበበልኝ) ሲሆን የጥናቱን አላማና በጥናቱ ብሳተፍ የማደርጋቸዉን ነገሮች በሚገባ ተገንዝቤያለሁ፡፡ በዚህ ጥናት የመሳተፍ ግዴታ እንደሌለብኝና ተሳትፎዬ በራሴ ሙሉ ፈቃደኝነት ላይ የተመሰረተ እንደሆነም አዉቃለሁ፡፡
እባክዎን በጥናቱ ለመሳተፍ ፈቃደኛ ነዎት?

አዎ ☐ የተሳታፊ ስምና ፊርማ _____

አይደለሁም ☐ የጠያቂዉ ስምና ፊርማ_____ ቀን_____

ጥያቄ ወይም ጉዳይ ካለዎት፤ እባክዎን በዚህ ስልክ ቁጥር ወይም ኢሜል **ደመላሽ ሙሉዓለም** ብለው ያግኙኝ፡

ስልክ:-+251 911 06 57 28 ኢሜል:- solymico27@gmail.com

በዚህ ምርምር ለመሳተፍ ፍቃደኛ ስለሆኑ ከልብ አመሰግናለሁ፡፡

Annex VI: Conversion Factors used for Dietary Calcium Intake Calculation

The assumption used during daily calcium intake calculation using conversion factors based on the frequency of calcium rich food consumption (FFQ):

Food	mg/100g	Notes
MILK	89	Fresh cow's milk
YOGURT	147	Canadian FCT value
MILLET	353	Black, flour
KALE	215.9	Kale + Salt+ Water: Boiled
FRUIT	8	Banana
		Non-Kale Vegetables:
VEG	12	Mixed
MORINGA	440	Leaves

Amount consumed	Serving size
MILK	1 cup = 200 mL = 200g
YOGURT	1 cup = 200 mL = 200g
MILLET	One Injera/bread baked from Millet: black flour = 32g
KALE	Two table spoonful boiled kale = 100g
FRUIT	Two medium sized bananas = 100g
VEG	Two table spoonful non-Kale Vegetables: Mixed = 100g
MORINGA	Half tablespoon Morniga leaves powder = 25g

Frequency to daily using factors:

> 1 per day = assume 2x
1 per day = 1x
> 1 per week = assume 3.5 per week =
3.5/7 = 0.5x
1 per week =
0.15x
I per month = 1/30 = 0.033x
never = 0x

Annex VII: Dental Fluorosis Assessment Checklist based on Dean's Index

(To be completed by qualified dentist)
Kebele _____

Mother's:- Name _____ Age: _____ years

Instruction: Circle the appropriate dental fluorosis rating

S. No	Clinical assessment	Dental Fluorosis rating score/Category
1	Dental fluorosis rating score/category of the women	**1. Normal (0)** (Enamel represents the usual translucent type of structure)
		2. Questionable (1) (Enamel discloses slight aberrations)
		3. Very Mild (2) (Opaque white areas covering 25% of the tooth surface)
		4. Mild (3) (White areas covering 25% –50% of the tooth surface)
		5. Moderate (4) **(**All surfaces affected and some browning)
		6. Severe (5) **(**Widespread brown stains and pitting)

Note for the assessor (dental fluorosis examiner):

Normal: Enamel represents the usual translucent semivitriform type of structure. The surface is smooth, glossy, and usually of a pale creamy white color.

Questionable: The enamel discloses slight aberrations from the translucency of normal enamel, ranging from a few white flecks to occasional white spots. This classification is utilized in those instances where a definite diagnosis of the mildest form of fluorosis is not warranted and a classification of 'normal' is not justified.

Very mild: Small opaque, paper white areas scattered irregularly over the tooth but not involving as much as25% of the tooth surface. Frequently included in this classification are teeth showing no more than about 1–2 mm of white opacity at the tip of the summit of the cusps of the bicuspids or second molars.

Mild: White opaque areas in the enamel of the teeth are more extensive but do not involve as much as 50% of the tooth.

Moderate: All enamel surfaces of the teeth are affected, and the surfaces subject to attrition, show wear. Brown stain is frequently a disfiguring feature.

Severe: Includes teeth formerly classified as 'moderately severe and severe.' All enamel surfaces are affected and hypoplasia is so marked that the general form of the tooth may be affected. The major diagnostic sign of this classification is discrete or confluent pitting. Brown stains are widespread and teeth often present a corroded-like appearance.

Annex VIII: Skeletal and Non-Skeletal Fluorosis Level Assessment Checklist

Section I: Skeletal fluorosis level assessment
(To be completed by physiotherapist or trained nurse or other health care professionals)

Kebele _____

Mother's:- Name _____ Age: _____ years.

Instruction: Write (**1**) if able to perform the exercise or (**0**) if unable to perform the exercise for each, separately.

S. No	Signs of skeletal fluorosis (Based on physical exercise) #	Response 1 = Yes; 0 = No	Notes
1	Cannot bend body and touch floor or toe **(Exercise A)**		
2	Cannot stretch and fold arms to touch back of head **(Exercise B)**		
3	Cannot touch chest with chin **(Exercise C)**		

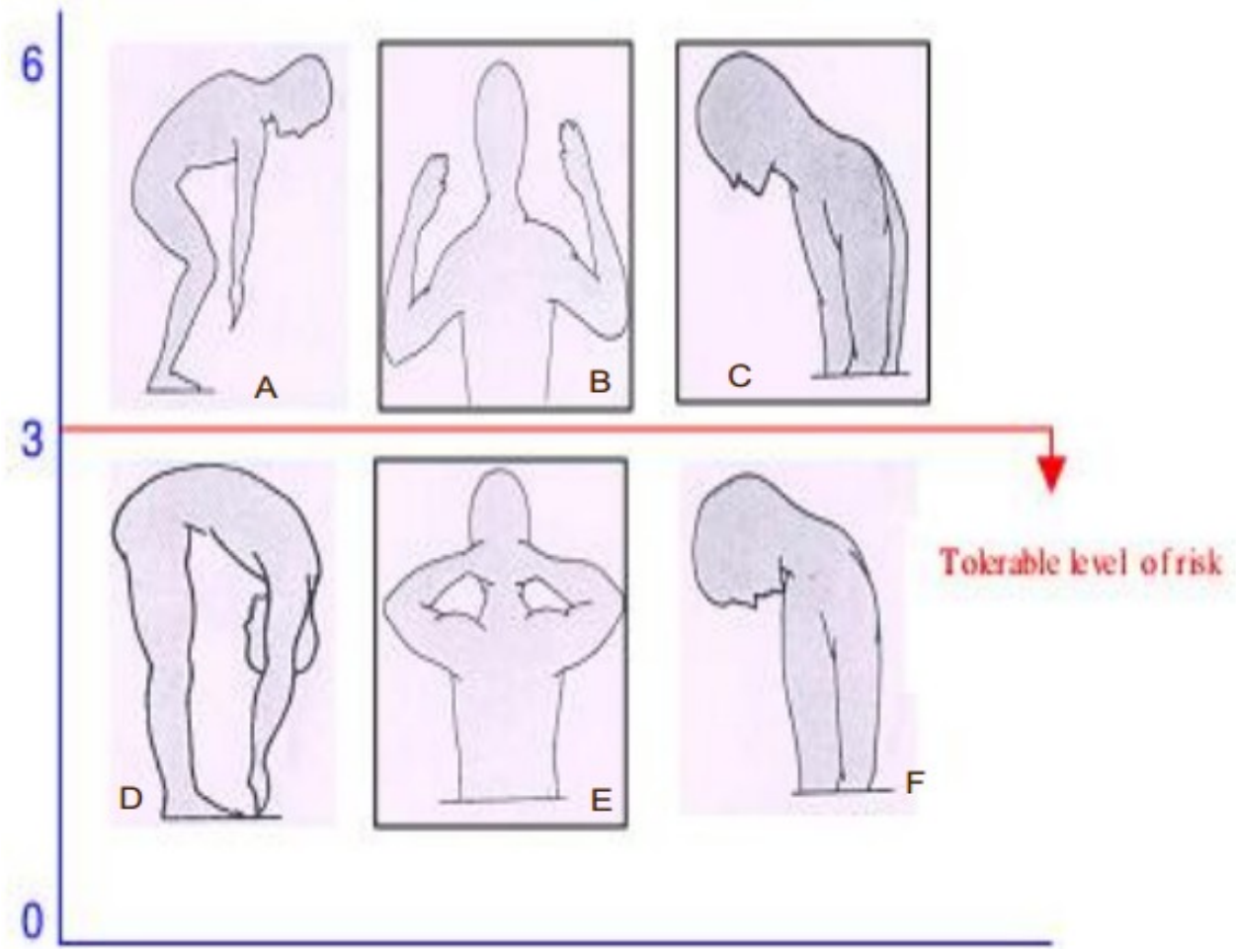
Section II: Non-skeletal fluorosis symptoms level assessment

Instruction: Write (**1**) if symptoms present or (**0**) if the symptoms not present for each, separately.

S. No	Non-skeletal fluorosis symptoms	Response		Notes
		0 = No	1 = Yes	
1	Feel lower back pain			
2	Feel leg pain, joints			
3	Feel arm pain, joints			
4	Feel tingling sensation in the hands and feet			
5	Feel neck pain with movement			
6	Feel muscle weakness			
7	Feel loss of appetite			
8	Have nausea			
9	Have abdominal pain			
10	Have bloating in stomach			
11	Experience polydipsia (excessive thirst)			
12	Experience polyuria (excess urine volume)			
13	Experience constipation			

Note for the assessor:

Physical exercises used to identify skeletal fluorosis and tolerable level of risk:



Notice:-

Fluoride toxicity manifestation (Exercises):

A = *Unable to bend without folding knees*

B = *Unable stretch hands, fold arms and touch back of head*

C = *Unable to bend neck-touching chest with chin not possible*

Normal health individual (Exercise):

D = *Can bend body and touch the floor/toes*

E = *Can stretch hands, fold arms and touch back of head*

F = *Can touch chest with chin*

Annex IX: Checklist used to Assess Safety and Acceptability of ESP

Supplementation

Instruction: *Most of these questions should not be prompted; so do not read from the list but ask as an open question unless you are told to read out loud.*

Kebele: _____ **HH ID:** _____ **Woman's Name:** _____

S. No	Questions and filters	Coding categories	Remarks
1	Are you consuming the ESP since the last time?	1. Yes 2. No	If ''No'' skip to **Q12**
2	Where do you get the eggshells used to prepare the ESP? **(Multiple responses are possible)**	1. My home 2. Neighbors 3. Restaurants 4. Other (Specify) _____	
3	How frequent you consume the ESP?	1. Always (daily) 2. Often (>2 times a week) 3. Sometimes (once a week) 4. Don't know 5. Other (Specify) _____	
4	How much ESP you are consuming at a time? **(Write if she used any household utensil**	1. Half of a bottle cap 2. One bottle cap 3. Two bottle caps 4. Don't know 5. Other (Specify) _____	
5	You are consuming "ESP" since the last time. What is your opinion about "ESP"? **(Multiple responses are possible)**	1. It is good for my health 2. I want to continue to consume 3. I don't want to consume it anymore 4. I didn't notice any difference 5. Other (Specify) _____	
6	Whom else consuming the ESP?	1. Only myself 2. My husband 3. My children 4. My neighbors /relatives 5. Other (Specify) _____	
7	What were the positive effects after consuming "ESP" **Do not read from the list BUT ask as an open question and probe by asking "anything else"!** **(Multiple responses are possible)**	1. Make me healthy (no pains) 2. No abdominal discomforts 3. No positive effects 4. Don't know 5. Other (Specify) _____	
8	What were the negative effects after consuming "ESP" **Do not read from the list BUT ask as an open**	1. Joint pains 2. Nausea and vomiting 3. Constipation, bloating and gas	

	question and probe by asking "anything else"! **(Multiple responses are possible)**	4. No negative effects 5. Don't know 6. Other (Specify) _____	
9	What barriers you faced while consuming the "ESP"? **(Multiple responses are possible)**	0. No barrier at all 1. Unavailability of the eggshells 2. Lack of time to prepare the ESP 3. Difficult to remember consuming ESP 4. Causes negative effects 5. Other (Specify) _____	
10	What are the supports and/or motivators that helped you to consume the "ESP"? **(Multiple responses are possible)**	1. Make me healthy (absence of pains, cramps, discomforts) **2.** I didn't experience side effects **(probe which one)** 3. Support from others (HEWs, HDAs, Neighbors, husband etc) 4. The ESP consumption project 5. Other (Specify) _____	
11	Please show me any remaining "ESP" or collected eggshells or the cup you stored the "ESP" you have in your house right now? **(Describe what you observe)**		
12	When is the last time you stopped consuming the ESP? **(Do not read from the list BUT circle the appropriate option for her response)**	0. Don't (never) consume the ESP at all 1. When the provision interrupted 2. After consuming for 2-3 weeks 3. After consuming for 2-3 months 4. Few days (a week) ago 5. Other (Specify) _____	
13	Why you stopped consuming the ESP? **(Write all reasons she provides)**		
14	Ask at least three questions by probing based on the reasons why she stopped consuming the ESP. (E.g. if her reason is ''don't to get eggshells'' ask her the reasons why she doesn't get eggshells). **(Write all her responses for each probed question separately)**		

15	What do you suggest or what support you need to continue consuming (including your families) the ESP? **(Ask this particular question for all women whether consuming the ESP or not and write all responses)**		

Annex X: Population Size and Number of Households in the Study Area

Summary the total and target population, and the number of households available in the study kebeles at the time of the study.

Pair of Study Clusters (pairs of control and intervention)	Study Cluster	Kebele[#]	Distance from Alaba town[#]	Population, 2017 (2009 E.C.)	Population of children of <2 mths of age (estimation)	Population of children of 6-18 mths of age (obtained from family folder)	Number of household	Remark
Pair 1	Guba	Guba	15	5475	284	154	685	
Pair 2	Arsho	Lay Arsho	10	5444	282	147	678	
		Tach Arsho	11	6241	323	-	850	
		Mirab Gortancho	12	5017	260	-	621	

[#] All the kebeles are more than 20km far from each other, they don't have common market and located in different direction from the woreda capital, Halaba

Sources: Woreda (kebele) administrative office, health center and health post

Annex XI: Profiles of Individuals Involved during Fieldwork

Profiles of individuals (name, qualification and role of individuals) hired in eggshell-calcium project for data and biological samples collection.

S. No	Name	Profession (qulaification)	Role	Remark
1	Dr. Getahun Habitamu	Dentist (DMD)	Dental fluorosis exam	Owner of private special clinic
2	Mr. Shewaneh Alemu	Physiotherapist (BSc, MSc)	Skeletal and non-skeletal fluorosis assessment	
3	Mr. Nasir Hussen	Laboratory tech.	Blood, urine, stool samples collection, Hemoglobin and malaria testing, Centrifuging the blood and separating the serum, labeling, etc in intervention kebele.	Work in IG
4	Miss. Mskerem Teshome	Laboratory tech.		
5	Miss. Keyriya Hussen	Clinical nurse		
6	Miss. Meliha Abdela	Clinical nurse		
7	Mr. Addis Negussie	Laboratory tech.	Blood, urine, stool samples collection, Hemoglobin and malaria testing, Centrifuging the blood and separating the serum, labeling, etc in control kebele	Work in CG
8	Mr. Mohammed Sirbela	Laboratory tech.		
9	Mr. Mundino Woticha	Clinical nurse		
10	Mr. Abebe Awol	Clinical nurse		
11	Mr. Eshetu Lukas	Nutritionist (BSc)	Co-ordination, monitoring, translation, etc	
12	Mr. Alemayehu Misganu	Public health (BSc)	Co-ordination, monitoring, translation, etc	
13	Mr. Sultan Kedir	12th grade completed to BSc in civil engineering (who were non-employed and local in Hallaba)	Data collectors (Interview-based data collection using questionnaires)	
14	Mr. Abdi Sunkamo			
15	Mr. Nigus Kassa			
16	Mr. Behredin Kedir			
17	Mr. Abdilkdir Seid			
18	Mr. Nesradin Awole			
19	Miss. Amarech Hussen	Health extension workers	Community mobilization, co-ordination, etc	
20	Miss. Haysha Abate			
21	Miss. Sadiya Mahidilo			
22	Miss. Ayura Bashir			
23	Miss. Mihiret T/Mariam			
24	Mr. Hussen Kajela			
25	Mr. Zewdneh Shanka Mr.	HU- Van driver	Travel research assistants from Hawassa to Hallaba during data and blood collection	
26	Getachew Zewdie			
27	Mr. Tamirate Shigute	Minibus driver	Shuttle service for data collectors and research groups from Halaba to kebeles	
28	Mr. Zeyinu Sume	Motor-bicyclist	Motor bike service for community mobilization within the kebeles.	
29	Mr. Anteneh Omer	MSc	Research assistant (PhD student)	
30	Mr. Demmelash Mulualem	MSc	Research assistant (PhD student)	

DMD *=Dental Medical Doctor;* ***IG*** *= Intervention group/kebele;* ***CG*** *= Control group/kebele;* ***HU*** *= Hawassa University*

Annex XII: Ethical Clearance Letters

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HAWASSA UNIVERSITY
COLLEGE OF MEDICINE AND
HEALTH SCIENCES
Institutional Review Board

Ref. No: IRB/019/10
Date: 12/12/2017

Name of Researcher(s): ***Demmelash Mulualem Zewdie, Anteneh Omer Ali, Susan J Whiting, Eskindir Loha, Marian Kjellevold and Bernt Lindtjorn***

Topic of Proposal: ***Effects of egg and eggshell calcium consumption in improving nutritional status and mitigation of dental and skeletal fluorosis among children and mothers in Ethiopian Rift Valley***

Dear researcher(s),

The Institutional Review Board (IRB) at the College of Medicine and Health Sciences of Hawassa University has undertaken a scientific, conflict of interest and ethics review of the aforementioned research protocol and endorsed it for implementation.

Note that regarding the eggshell study, only the experimental aspect involving supplementation of eggshell powder to 20-25 volunteer women is approved. No other study or sub-study related to eggshell such as nutrition education and promotion about eggshell use and its benefits, assessment of community knowledge, attitude and practice about eggshell, etc can be conducted as part of this study.

This approval will be valid for a one year period as of its issuance (12th Dec 2017). If the study takes more than one year, renewal of the approval should be sought.

If any amendment is made to this protocol after this approval is given, then the amended version of the protocol should be approved by our IRB before its implementation.

Yours faithfully,

Ayalew Astatkie (PhD),

Institutional Review Board Chairperson.

☎ +046 820 92 90
Fax + 046 2208755
✉ 1560 Hawassa

UNIVERSITY OF SASKATCHEWAN

Biomedical Research Ethics Board (Bio-REB)

Certificate of Approval

PRINCIPAL INVESTIGATOR	DEPARTMENT	Bio #
Susan J. Whiting	Nutrition and Dietetics	17-150

INSTITUTION(S) WHERE RESEARCH WILL BE CARRIED OUT
Hawassa University Ethiopia

STUDENT RESEARCHER(S)
Demmelash Mulualem, Anteneh Omer

FUNDER(S)
GRAND CHALLENGES CANADA

TITLE
Using Eggshell Calcium to Mitigate Fluorosis in Ethiopia

ORIGINAL REVIEW DATE	APPROVED ON	APPROVAL OF	EXPIRY DATE
29-May-2017	25-Jul-2017	Revised Application received July 25,2017 Treatment Group Consent Form Version 3 July 24,2017 Control Group Consent Form Version 3 July 24,2017 Substudy 1 Consent Form for Control Group Version 2 May 31, 2017 Substudy Consent Form for Treatment Group Version 2 July 25,2017 Questionnaire 1a submitted 24-May-2017 Questionnaire 1b,c - Food Frequency submitted 24-May-2017 Questionnaire 2 - Child Illness Report submitted 24-May-2017 Questionnaire 3: Infant Feeding and Knowledge, Attitude and Practice Questions on Eggs and Eggshell submitted 24-May-2017 Questionnaire 4a - Dean's Index to be completed by qualified dentist submitted 24-May-2017 Questionnaire 4b - Skeletal and non-skeletal symptoms submitted 24-May-2017	24-Jul-2018

Delegated Review ☒ Full Board Meeting ☐

IRB 1 Registration #00001471 ☐ IRB 2 Registration #00008358 ☐ Not Applicable ☒

CERTIFICATION
The University of Saskatchewan Biomedical Research Ethics Board (Bio-REB) has reviewed the above-named research study. The study was found to be acceptable on scientific and ethical grounds. The principal investigator has the responsibility for any other administrative or regulatory approvals that may pertain to this research study, and for ensuring that the authorized research is carried out according to governing law. This approval is valid for the specified period provided there is no change to the approved protocol or consent process.

FIRST TIME REVIEW AND CONTINUING APPROVAL
The University of Saskatchewan Biomedical Research Ethics Board reviews above minimal studies at a full-board (face-to-face) meeting. If a protocol has been reviewed at a full board meeting, a subsequent study of the same protocol may be reviewed through the delegated review process. Any research classified as minimal risk is reviewed through the delegated (subcommittee) review process. The initial Certificate of Approval includes the approval period the REB has assigned to a study. The Status Report form

Please send all correspondence to:
Research Services and Ethics Office
University of Saskatchewan
Room 223 Thorvaldson Building
110 Science Place
Saskatoon, SK Canada S7N 5C9

PRINCIPAL INVESTIGATOR	DEPARTMENT	Bio #
Susan J. Whiting	Nutrition and Dietetics	17-150

must be submitted within one month prior to the assigned expiry date. The researcher shall indicate to the REB any specific requirements of the sponsoring organizations (e.g. requirement for full-board review and approval) for the continuing review process deemed necessary for that project. For more information visit http://research.usask.ca/for-researchers/ethics/index.php.

REB ATTESTATION

In respect to clinical trials, the University of Saskatchewan Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Part 4 of the Natural Health Products Regulations and Part C Division 5 of the Food and Drug Regulations and carries out its functions in a manner consistent with Good Clinical Practices. Members of the Bio-REB who are named as investigators, do not participate in the discussion related to, nor vote on such studies when presented to the Bio-REB. This approval and the views of this REB have been documented in writing. The University of Saskatchewan Biomedical Research Ethics Board is constituted and operates in accordance with the current version of the *Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans* (TCPS 2 2014).

Ildiko Badea, Vice-Chair
University of Saskatchewan
Biomedical Research Ethics Board

Please send all correspondence to:

Research Services and Ethics Office
University of Saskatchewan
Room 223 Thorvaldson Building
110 Science Place
Saskatoon, SK Canada S7N 5C9

Annex XIII: Sample Photos Taken during this Research Work

Consent was obtained from all individuals to use their photos related to this research work

Sample photos during ESP preparation

Sample photos during ESP supplementation

Sample photos during dental examination

Sample photos during skeletal flurosis examination

Sample photos during blood and urine specimen collection

Annex XIV: Supplementary Result Tables

Supplementary Table I: Predictors of fluorosis symptoms (Gastro-intestinal complaints) among mothers: logistic regression model, *Halaba* district, South Ethiopian Rift Valley, 2018

Variable (n=270)	Category	Gastro-intestinal complaints index		COR (95%CI)[#]	AOR (95%CI)[*]
		No Number (%)	Yes Number (%)		
Age (year)	Mean (SD)	29.5 (4.2)		0.95 (0.89, 1.01)	0.95 (0.88, 1.01)
Education	Unable to read and write	82 (59.0)	57 (41.0)	1.1 (0.69, 1.84)	1.2 (0.72, 2.01)
	Able to read and write	81 (61.8)	50 (38.2)	1	
Occupation	No job	64 (63.4)	37 (36.6)	1	
	Have job	99 (58.6)	70 (41.4)	1.2 (0.74, 2.03)	1.2 (0.72, 2.04)
Parity	≤ 4 live births	92 (59.0)	64 (41.0)	1	
	> 4 live births	71 (62.3)	43(37.7)	1.2 (0.70, 1.89)	1.1 (0.65, 1.91)
Family size (parents and children)	≤ 5	40 (56.3)	31 (43.7)	1	
	> 5	123(61.8)	76 (38.2)	1.3 (0.72, 2.17)	1.3 (0.73, 2.33)
Diet diversity	≤ 5 food groups	99 (60.7)	64 (39.3)	0.96 (0.59, 1.58)	1. 0 (0.61, 1.70)
	> 5 food groups	64 (59.8)	43 (40.2)	1	
Dietary calcium	≤ 400mg/day	84 (60.9)	54 (39.1)	1.1 (0.64, 1.70)	1.0 (0.58, 1.74)
	> 400mg/day	79 (59.8)	53 (40.2)	1	
Residency	≤ 10 years	56 (65.1)	30 (34.9)	1	
	> 10 years	107(58.2)	77 (41.8)	1.3 (0.79, 2.29)	1.5 (0.84, 2.58)

[#]In the bivariate logistic regression, some of variables were significant predictors at $P < 0.2$.
*There was no statistical significant association observed for any of the variables at $P > 0.05$.

Supplementary Table II: Predictors of dental fluorosis among mothers: Logistic regression model, *Halaba* district, South Ethiopian Rift Valley, 2018

Variable (n=270)	Category	Dental fluorosis[$]		COR (95%CI)[#]	AOR (95%CI)[*]
		Absent Number (%)	Present Number (%)		
Age (year)	Mean (SD)	29.5 (4.2)		1.0 (0.96, 1.07)	1.0 (0.96, 1.08)
Education	Unable to read and write	57 (41.0)	82 (59.0)	1.3 (0.77, 2.03)	1.2 (0.73, 1.99)
	Able to read and write	61 (46.6)	70 (53.4)	1	
Occupation	No job	37 (36.6)	64 (63.4)	1	
	Have job	81 (47.9)	88 (52.1)	1.6 (0.96, 2.64)	1.6 (0.95, 2.68)
Parity	≤ 4 live births	65 (41.7)	91 (58.3)	1	
	> 4 live births	53 (46.5)	61 (53.5)	1.2 (0.75, 1. 98)	1.3 (0.78, 2.02)
Family size (parents and children)	≤ 5	32 (45.1)	39 (54.9)	1	
	> 5	86 (43.2)	113 (56.8)	1.1 (0.63, 1.86)	1.0 (0.78, 2.27)
Diet diversity	≤ 5 food groups	72 (44.2)	91 (55.8)	1.1 (0.64, 1.72)	1. 0 (0.60, 1.66)
	> 5 food groups	46 (43.0)	61 (57.0)	1	
Dietary calcium	≤ 400mg/day	61 (44.2)	77 (55.8)	1.0 (0.64, 1.69)	1.3 (0.76, 2.24)
	> 400mg/day	57 (43.2)	75 (56.8)	1	
Residency	≤ 10 years	40 (46.5)	46 (53.5)	1	
	> 10 years	78 (42.4)	106 (57.6)	1.2 (0.71, 1.98)	1.2 (0.68, 2.26)

[$]*Dental flurosis was 'absent' if the teeth were examined as normal or questionable; 'present' if the teeth were examined as very mild, mild, moderate or severe dental fluorosis.*
[#]*In the bivariate logistic regression, some of variables were significant predictors at P < 0.2.*
*****There was no statistical significant association observed for any of the variables at P >0.05.*

Annex XV: Original Papers (Published and in Manuscript)

These are original papers of the PhD research (Published and Manuscript)

1) Paper I:

nutrients

MDPI

Article

Efficacy of Calcium-Containing Eggshell Powder Supplementation on Urinary Fluoride and Fluorosis Symptoms in Women in the Ethiopian Rift Valley

Demmelash Mulualem [1], Dejene Hailu [2], Masresha Tessema [3] and Susan J. Whiting [4,*]

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[4] College of Pharmacy and Nutrition, University of Saskatchewan, Saskatoon, SK S7N 5E5, Canada
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Abstract: Dietary calcium binds Fluoride (F), thus preventing excess F absorption. We aimed to assess the efficacy of supplementing calcium-containing Eggshell Powder (ESP) on F absorption using urine F excretion and on fluorosis symptoms. In total, 82 women (41 Intervention Group, IG; 41 Control Group, CG) were recruited; overall, 39 in each group completed the trial. Morning spot urine was collected before (baseline, BL) and after (endline, EL) the intervention that was 6-months daily supplementation with 2.4 g ESP (providing ~1000 mg of calcium). Dental, skeletal, and non-skeletal fluorosis assessments was carried out at BL and, except for dental, at EL. Relative risk (RR) and linear generalized estimating equation were used to compare outcomes between groups. At BL, urinary F excretion in the IG and CG groups was similar, ~10 mg/L. At EL, urinary F excretion in IG women was six-fold lower ($\beta = -6.1$ (95% CI: −7.1, −5.1)) compared to CG. The risk of developing skeletal and non-skeletal fluorosis were significantly ($p < 0.001$) reduced in the intervention group. A significant reduction in urinary F excretion and reduction in many fluorosis symptoms were observed among women supplemented with calcium-containing ESP, thus providing evidence for using this dietary calcium source for mitigation of fluorosis. Clinical trials registration: NCT03355222.

Keywords: calcium; fluoride; fluorosis; women; Ethiopia; Rift Valley; eggshell; dental

check for updates

Citation: Mulualem, D.; Hailu, D.; Tessema, M.; Whiting, S.J. Efficacy of Calcium-Containing Eggshell Powder Supplementation on Urinary Fluoride and Fluorosis Symptoms in Women in the Ethiopian Rift Valley. *Nutrients* **2021**, *13*, 1052. https://doi.org/10.3390/nu13041052

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1. Introduction

Worldwide, 50 million people suffer from fluorosis, which affects not only teeth, but also bones, joints, gut, and brain functions [1]. In Ethiopia, where defluoridation requires costly infrastructure, more than 14 million individuals, mainly in the Rift Valley, are affected by fluorosis [2–5]. Research suggests that the adverse effects of fluoride (F) can be reversed or lessened by providing sufficient food intake of protein, calcium (Ca), anti-oxidants, and vitamin D [6–8]. Of these, Ca is the most studied by ecological studies [8–11]. However, there have been no intervention studies of Ca to mitigate fluorosis at the community level in Ethiopia.

Only a few studies have provided evidence of a reduction in F absorption with dietary Ca supplementation. A total of two animal studies [12,13] examined aspects of the mechanism and dose-responsiveness of Ca intake to reduce urinary F. A small human trial of 10 per group [14] found decreased urinary F excretion. Ca binds with F forming calcium-fluoride, which is insoluble in the gastrointestinal tract, preventing absorption (measured as a fall in urinary F) and therefore reducing the extent of F exposure. In this way it is postulated that the adverse effects of F are decreased and/or do not worsen with Ca intake.

We therefore hypothesized that supplementation of an age-old, sustainable and low cost source of Ca, i.e., eggshell, as a dietary Ca source [15] would mitigate the toxic effects

2) Paper II:

International Journal of
Environmental Research and Public Health

MDPI

Article

Association of Dietary Calcium Intake with Dental, Skeletal and Non-Skeletal Fluorosis among Women in the Ethiopian Rift Valley

Demmelash Mulualem [1], Dejene Hailu [2], Masresha Tessema [3] and Susan Joyce Whiting [4,*]

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Abstract: Fluorosis is a major public health problem in the Rift Valley of Ethiopia. Low calcium (Ca) intake may worsen fluorosis symptoms. We assessed the occurrence of fluorosis symptoms among women living in high-fluoride (F) communities in South Ethiopia and their associations with dietary Ca intake. Women (n = 270) from two villages provided clinical and questionnaire data. Dental fluorosis examination was done using Dean's Index, and skeletal and non-skeletal fluorosis assessment was carried out using physical tests and clinical symptoms. Daily Ca intake was estimated by a food frequency questionnaire. Food, drinking water and beverage samples were analyzed for F level. Many subjects (56.3%) exhibited dental fluorosis. One-third of the women were unable to perform the physical exercises indicative of skeletal fluorosis; about half had ≥2 symptoms of skeletal/non-skeletal fluorosis. The average F level in drinking water sources was ~5 mg/L. The F content in staple food samples varied from 0.8–13.6 mg/kg. Average Ca intake was 406 ± 97 mg/day. Women having ≤400 mg/day Ca intake had ~3 times greater odds of developing skeletal rigidity with joint pains [AOR = 2.8, 95%CI: 1.6, 5.0] and muscular weakness [AOR = 2.9, 95%CI: 1.3, 6.3] compared to those with higher intakes. No association of calcium intake was seen with dental fluorosis. As low dietary Ca intake was associated with symptoms related to skeletal and non-skeletal fluorosis, this warrants nutritional intervention on calcium intakes in this setting.

Keywords: calcium intake; fluoride; dental fluorosis; skeletal fluorosis; non-skeletal fluorosis; Ethiopian Rift Valley

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1. Introduction

Drinking water sources in the Rift Valley of Ethiopia contain fluoride (F) levels exceeding the World Health Organization (WHO) limit of 1.5 mg/L [1,2]. As a consequence, many people living in the region are severely affected by fluorosis [2–8]. In serious cases, dental fluorosis is manifested as brown mottling of the enamel and results in overall yellowing of the teeth with erosion of the enamel [2,8]. Skeletal fluorosis occurs when there is a high degree of bone brittleness due to excess deposition of F in bone that leads to osteosclerosis [3,6,9–12]. Non-skeletal fluorosis includes muscle weakness manifestations, such as stiffness of the back and neck muscles and pain leading to inability to carry out routine domestic activities [2,9].

Fluorosis is a major public health concern particularly for developing countries including Ethiopia, as the infrastructure needed to remove the excess F ions is lacking or not widely accepted [13,14]. It can affect both children and adults [13]. Over 85% of Ethiopians living in the Rift Valley have been exposed to excess levels of F intake [7,15,16]. Although drinking water is the dominant pathway of F exposure in Ethiopian Rift Valley areas, food

3) Paper III manuscript:

Safety and Acceptability of Calcium-Containing Eggshell Powder Supplementation in a Phase II Clinical Trial among Women in Ethiopian Rift Valley

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Abstract

Many people living in the Rift Valley of Ethiopia are severely affected by fluorosis as the drinking water sources contain fluoride levels exceeding the World Health Organization limit of 1.5mg/L. Dietary Ca intake in many areas of Ethiopia is well below the recommended dietary allowance. Fluoride exposure may be an added concern for women's bone and dental health where there is low Ca intake. Thus, the aim of this study was to assess safety and acceptability of calcium-containing eggshell powder supplementation in the Phase II clinical trial. Eighty-two women (41 Intervention Group; 41 Control Group) were recruited for the Phase II clinical trial; 39 in each group completed the trial. For the purpose of safety and acceptability assessment, serum iron and zinc were measured using blood levels, prior to and after the calcium-containing ESP supplementation among the study subjects. Occurrence of side effects such as nausea, vomiting, constipation, and abdominal bloating and gas related to excess calcium intake in the Phase II trial were assessed using checklists on a monthly period. Descriptive statistics, bivariate and multivariable logistic regression, paired and two independent samples t-tests were used to analyze and compare outcomes between groups. Overall, one-quarter (~25%) of the women complained most of the symptoms related to excess calcium intake from the second through the fourth month of the supplementation period albeit a decreasing trend was observed until the end of the sixth month supplementation period. The serum zinc level of women in the intervention group was similar (*P = 0.171*) compared with the control women. But women in the IG had statistically significantly lower (by ~20%) serum iron level [β = - 0.21 (95% CI: (-0.4, -0.1)] compared to women in the CG after the calcium-containing ESP supplementation. The calcium-containing eggshell powder supplementation was generally accepted, well-tolerated and did not pose significant risks to the health of participants.

Keywords: Calcium, Eggshell powder, Side effects, Safety, Acceptability, Iron, Zinc, Women.

***Clinical trials registration number**: NCT03355222*

1. Introduction

Excessive intake of fluoride (F) is accompanied by a characteristic sequence of changes in teeth, bone and periarticular tissues (dental, skeletal and non-skeletal fluorosis) (Dean, 1934; Erdal and Buchanan, 2005; WHO, 2006; Rango *et al*., 2012). Calcium (Ca) binds with fluoride forming an insoluble calcium fluoride in the gastrointestinal tract, preventing absorption and therefore reducing the extent of fluoride exposure and its adverse effects (Mehta and Shah, 2013; Kebede *et al.,* 2016b; Mulualem *et al.,* 2021). However, reversing or lessening the adverse effects of F by providing adequate dietary or supplemental Ca might be problematic related to excess intake calcium and deficiencies of other micronutrients, particularly divalent trace minerals such as iron and zinc, which might be affected by a high calcium intake.

Calcium plays a central role in a wide range of the body's essential functions. It is an essential nutrient for all age groups including women of child-bearing age (IOM, 2011). The tolerable daily dietary upper intake level (UL) for calcium is set at 2500 mg for adults 19 through 50 years of age which is more than twice the recommended daily allowance (RDA, 1000 mg/day) for this age group (IOM, 2011). Calcium intakes above the UL are considered to increase the risk of adverse effects including hypercalcemia, vascular and soft tissue calcification, renal stone formation, interactions with zinc and iron absorption (Dawson-Hughes *et al*., 1986; Jackson *et al.,* 2006; Murshed and McKee, 2010; IOM, 2011). Natural food sources of calcium are primarily dairy foods and green leafy vegetables. When intake is not sufficient, calcium may be prescribed as a dietary supplement often consumed in the form of pills of calcium salts including carbonate, phosphate, citrate, and citrate malate (Weaver and Peacock, 2019). Calcium supplement intake also has long been associated with other side effects including constipation and gas (Jackson *et*

al., 2006; Prince *et al.,* 2006), which varies greatly from person to person and dose of the calcium supplement.

In Ethiopia, iron and zinc are among the four main micronutrients that have high public health significance (FMoH, 2008; NNP II, 2016). Calcium's interaction with iron and zinc in the diet has been considered a potential risk related to high calcium intakes. However, results seem inconclusive (McKenna *et al.,* 1997; Ilich-Ernst *et al,* 1998; Abrams, 2001; Harris, 2002; Mølgaard *et al.,* 2005; Gaitan *et al.,* 2011). Nevertheless it was deemed important to consider whether a calcium supplement would affect markers of iron and zinc status as well as side-effects related to excess intake of calcium. Therefore, this study aimed to assess the safety and acceptability of a calcium-containing ESP supplement in the Phase II trial (Mulualem *et al.,* 2021) that was conducted to measure F absorption and fluorosis symptoms in an area of Ethiopia known for endemic fluorosis.

2. Methods

2.1. Study design

This safety study was carried out as part of the Phase II clinical trial which tested efficacy of calcium-containing ESP supplementation on urinary fluoride excretion and fluorosis symptoms compared to no supplementation (Mulualem et al., 2021).

2.2. Setting and participant recruitment

The study participants in the trial were women who were permanent residents in high fluorosis area (Mulualem *et al.,* 2021) located in the main Rift Valley of South Ethiopia. The participants were recruited in March 2018 (i.e. after the cross-sectional survey). The trial subjects were randomly selected among all eligible women who had participated in the cross-sectional survey (Mulualem *et al.,* 2022). Thus, all women who participated in the calcium-containing ESP supplementation trial were in this safety study. The participant eligibility criteria and methods of selection were described elsewhere (Mulualem *et al.,* 2021).

2.3. Sample size in the trial

In the trial, the minimum sample size of 82 women (41 for each arm) was estimated to detect the effect of calcium-containing ESP supplementation on fluoride excretion and fluorosis symptoms using power (1- β) of 80% at 95% two-sided confidence level (Mulualem *et al.,* 2021).

2.4. Supplementation in the trial

The supplement given in the trial (Mulualem *et al.,* 2021) was calcium-containing eggshell powder (ESP) at a daily base. In that trial the study participants were supplemented half cap of ESP weighing ~800 mg three times a day which was equivalent to ~1000 mg of elemental

calcium (Mulualem *et al.,* 2021). The period of supplementation was from 1st April 2018 to 30th September 2018 (for six consecutive six months) with weekly follow up and supervision.

2.5. Outcome variables

For the purpose of this safety assessment, the primary outcome measures were serum iron and zinc as measured using blood levels, prior to and after the calcium-containing ESP supplementation among the study subjects. Occurrence of side effects such as nausea, vomiting, constipation and abdominal bloating and gas related to excess calcium intake in trial were assessed as secondary outcome measures.

2.6. Data Collection and Measurements

2.6.1. Habitual Dietary Calcium Intake

The usual dietary calcium intake of women in both groups at baseline in March and at the end of the trial was assessed. A structured food frequency questionnaire that asked daily and weekly frequencies of consumption of specific calcium-rich foods common in this district was used. Portion size estimation was made using bowls and plates available in the household of each woman to help them visualize and serve the amount of each food consumed. These frequencies were converted to daily dietary calcium intakes, as we previously described (Mulualem *et al.,* 2022), using published values for calcium content.

2.6.2. Assessment of Side Effects related to Excess Calcium Intake in Trial

A checklist was developed to assess the occurrence of possible side effects related to calcium-containing ESP consumption in the trial. The list of the side-effects was extracted from

literatures (Jackson *et al.,* 2006; Prince *et al.,* 2006; IOM, 2011). The assessment was done at baseline (March 2018) and endline (October 2018) in both groups, and every month only on women in the treatment group until the trial ended. The clinical symptoms assessed for side effects of excess calcium intake were nausea or vomiting, loss of appetite, constipation, abdominal pain and belching (excess gas). The women in the control group were not assessed for these symptoms at the monthly base.

2.6.3. Hemoglobin (Hematocrit) and Malaria Testing

At baseline in March and end of the trial in October 2018, a drop of blood (finger prick) was taken from all women in both groups prior to and after the supplementation to analyze hemoglobin (and hematocrit) immediately in the field using HemoSmart™ GOLD Hemoglobin Screening Meter, APEX BIOTECHNOLOGY CORP, No.7, Li Hsin Rd. V, Hsinchu Science Park Hsinchu, Taiwan, R.O.C. The HemoSmart™ GOLD Hemoglobin Screening Meter uses HemoSmart™ GOLD Hemoglobin Test Strips designed specifically for use with fresh capillary whole blood taken from a fingertip and a venous whole blood to test both hemoglobin and hematocrit simultaneously. Disposable blood lancet, alcohol and swabs were used to collect capillary blood by finger pricking which was done after thoroughly cleaning the finger tip with alcohol and swab.

While we assessed hemoglobin, malaria testing was done for all women in both groups at baseline and end of the intervention. The women appreciated this additional health information and it provided information about anemia status. The same finger prick which was done for hemoglobin testing was used. The malaria test was done immediately in the field using Rapid

Diagnostic Test (RDT) kit (a one Step Rapid Test for Malaria (Pf/Pv Ag), MERISCREEN Malaria Pf/Pv Ag, Meril Diagnostic Pvt, Ltd, India.

2.6.4. Venous Blood Specimen Collection and Analysis

A sample of 10 mL venous blood was collected from all trial women at baseline (March 2018) and at the end of the 6-month trial by qualified laboratory technologists. This was collected to analyze for serum iron, zinc and calcium levels. The blood samples were collected at the local health center. After taking the blood sample, the spot was covered by a band-aid to protect from infection. The samples were collected into serum tubes lacking coagulant (Vacutainer tubes). The tubes were stored in an ice box for 30 minutes, and then centrifuged at room temperature for 10 minutes at 2000 x g (WHO, 2011). The serum was transferred to plastic trace mineral vials and stored at -20ºC in the Hawassa University, Nutrition and Food Science Laboratory until it was transported to Ethiopian Public Health Institute for analysis. There, serum iron and zinc were analyzed using Avarian Spectr AA® Flame Atomic Absorption Spectrometry, Shimadzu. Disposable 10 mL syringes, alcohol and swabs, trance mineral-free gloves and polyethene pipettes were used to collect blood specimens.

2.7. Data Processing and Analysis

All data were double entered into EpiData version 3.1 (Odense, Denmark) and then exported into IBM SPSS version 25 (Chicago, IL, USA) for analysis. Continuous data were checked for normality using Kolmogorov-Smirnov and Shapiro-Wilk Tests of Normality. The Chi-square (X^2) test was used to compare baseline characteristics and clinical symptoms between the intervention and control groups. Paired and independent samples t-tests were used, for each

group, to compute the 'within' and 'between' mean differences of continuous outcome variables. Mother age and parity, family size (number of children, parents and relatives living together in the household), year of residence in the area, diet diversity, dietary calcium intake and malaria status of women were included in the multivariate analysis of GEE model. Then, the multivariate linear regression analysis was carried out and beta coefficient at 95% confidence interval (CI) reported. Complete case analyses were done for each statistical test at baseline and endline as there were only four dropouts (two women in each arm).

2.8. Ethical Clearance

Ethics approvals were obtained from Hawassa University College of Medicine and Health Science, University of Saskatchewan Biomedical Research Ethics, Saskatoon, Canada. Letters of support were also obtained from Regional Health Office, Zonal Health Desk and District Health Offices. Confidentiality of personal information has been kept. After the purpose and methods of the study were fully explained, and their right to refuse was explained, informed verbal and written consents were obtained from all study participants prior to their participation in the study.

3. Results

3.1. Socio-Demographic and Economic Characteristics

The mean (±SD) age of women in completed years was 29.6 ± 3.8 years. Most (93.6%) of the women were married and had an average of four live-born children. More than half of the women were illiterate (56.4%) and had no job (53.8%) outside their household. The average number of family members (parents, children and relatives) living together in same household was approximately 7 ± 2, in which at least two of them were children under five years old.

Overall, there was no significant difference in common baseline characteristics of study subjects between the two groups (Table 1).

Table 1: Baseline characteristics of study participants in *Halaba* district, southern Ethiopian Rift Valley, 2018

Characteristics		**IG (n=39)**	**CG (n=39)**	***P-value***
Age	Mean (SD)	29.9 (3.9)	30.5 (3.8)	0.368
Diet diversity score	Mean (SD)	5.1 (1.99)	4.7 (1.96)	0.459
		N (%)	**N (%)**	
Educational status	Illiterate	24 (61.5)	20 (51.3)	0.361
	Literate	15 (38.5)	19 (48.7)	
Main occupation	No outside job	22 (56.4)	20 (51.3)	0.650
	Outside job	17 (43.6)	19 (48.7)	
Main source of income	Agricultural	35 (89.7)	34 (87.2)	0.723
	Non-agricultural	4 (10.3)	5 (12.8)	
Average monthly income	< 1000 ETB	31 (79.5)	34 (87.2)	0.362
	≥ 1000 ETB	8 (20.5)	5 (12.8)	
Diet diversity score	≤ 5 food groups	24 (61.5)	27 (69.2)	0.475
	> 5 food groups	15 (38.5)	12 (30.8)	
Parity	≤ 4 live births	21 (53.8)	20 (51.3)	0.821
	> 4 live births	18 (46.2)	19 (48.7)	
Family size	≤ 5 members	10 (25.6)	13 (33.3)	0.456
	> 5 members	29 (74.4)	26 (66.7)	
Residency	< 10 years	12 (30.8)	9 (23.1)	0.444
	≥ 10 years	27 (69.2)	30 (76.9)	

ETB = Ethiopian Birr; IG = Intervention Group; CG = Control Group
For values with mean (SD) comparison was done using independent samples t test; for prevalence values, comparison was done using Chi-square test.

3.2. Calcium Intake prior to and after Supplementation in Comparison with the Recommended Intake and Relative to Upper Intake Level

Overall, the average daily dietary calcium intake of women in both groups was below the recommended adequate intake (<1000mg/day), ~ 403mg/day (Table 2). The independent two samples t-test showed non-significant differences in mean dietary calcium intake between the treatment and control arms at baseline (t = -0.65 (76), *P = 0.516*) and endline (t = 0.10 (76), *P = 0.922*) assessments. The daily dietary calcium intake of the women in the intervention group was increased with 960mg calcium using the daily supplementation of calcium-containing eggshell powder (ESP). With this supplementation, all the women in the intervention group fulfilled the recommended daily calcium intake. None of the women's daily calcium intake fallen in the upper intake level (Table 2).

Table 2: Comparison of daily calcium intake of women with the recommended intake and relative to upper intake level in *Halaba* district, southern Ethiopian Rift Valley, 2018

Categories of daily dietary Ca intake	Intervention		Control	
	Baseline (39) N (%)	Endline (39) N (%)	Baseline (39) N (%)	Endline (39) N (%)
Adequate intake (1000mg/day)	0		0	0
Below the recommendation (<1000mg/day)	39 (100)	39 (100)	39 (100)	39 (100)
Above the UL (≥ 2500mg/day)	0	0	0	0
Mean (±SD)	394 ± 120	409 ± 93	412 ± 129	407 ± 84
Ca from daily ESP supplementation (mg/day)	0	960	0	0
Total intake (average + supplement) (mg/day)	274 - 514	1276 - 1462	283 -541	323 - 491
Total intake relative to adequate intake (mg/day)	486 – 726[#]	276 - 462[¥]	459 - 717[#]	509 - 677[#]
Total intake relative to UL (mg/day) [†]				

Ca = Calcium; UL = Upper intake Level
[#]The women's total daily calcium intake was below the adequate intake within these ranges
[¥]The women's total daily calcium intake was above the adequate intake within by this range
[†]All the women's total daily calcium intakes did not exceeded the upper intake level

3.3. Side Effects (Symptoms) related to Excess Calcium Intake in the Phase II Trial

At baseline, about half (50%) of the women in both groups were complaining all of the symptoms related to excess calcium intake. These symptoms were similar (*P>0.05*) in women between the intervention and control group both in the unadjusted and adjusted analyses. Three of the symptoms (loss of appetite, abdominal pain and nausea or vomiting) were significantly decreased only in the treatment group compared with the control group at the end of the trial (Table 3).

Table 3: Comparison of symptoms related to excess calcium intake in women between the two arms prior to and after supplementation in *Halaba* district, Ethiopian Rift Valley, 2018

Period	Symptoms		IG (n=39) N (%)	CG (n=39) N (%)	Unadjusted OR (95%CI)	Adjusted[£] OR (95%CI)	*P-value*[¥]
Baseline	Have nausea or vomiting	Yes	21 (53.8)	19 (48.7)	0.81 (0.34, 1.98)	0.81 (0.32, 2.05)	0.657
		No	18 (46.2)	20 (51.3)	1	1	
	Feel loss of appetite	Yes	14 (35.9)	12 (30.8)	0.79 (0.31, 2.04)	0.70 (0.25, 1.94)	0.488
		No	25 (64.1)	27 (69.2)	1	1	
	Have abdominal pain	Yes	22 (56.4)	24 (61.5)	1.24 (0.50, 3.05)	1.16 (0.46, 2.91)	0.753
		No	17 (43.6)	15 (38.5)	1	1	
	Have belching (excess gas)	Yes	7 (17.9)	8 (20.5)	1.18 (0.38, 3.65)	1.18 (0.38, 3.74)	0.773
		No	32 (82.1)	31 (79.5)	1	1	
	Experience constipation	Yes	31 (79.5)	34 (87.2)	1.76 (0.52, 5.94)	2.05 (0.54, 7.79)	0.291
		No	8 (20.5)	5 (12.8)	1	1	
Endline	Have nausea or vomiting	Yes	5 (12.8)	21 (53.8)	0.13 (0.04, 0.39)	0.08 (0.02, 0.31)	<0.001
		No	34 (87.2)	18 (46.2)	1	1	
	Feel loss of appetite	Yes	4 (10.3)	17 (43.6)	0.15 (0.05, 0.50)	0.15 (0.04, 0.51)	0.003
		No	35 (89.7)	22 (56.4)	1	1	
	Have abdominal pain	Yes	8 (20.5)	27 (69.2)	0.12 (0.04, 0.32)	0.10 (0.03, 0.31)	<0.001
		No	31 (79.5)	12 (30.8)	1	1	
	Have belching (excess gas)	Yes	7 (17.9)	9 (23.1)	0.73 (0.24, 2.20)	0.75 (0.24, 2.33)	0.617
		No	32 (82.1)	30 (76.9)	1	1	

Experience constipation	Yes	10 (25.6)	13 (33.3)	0.69 (0.26, 1.84)	0.65 (0.23, 1.79)	0.404
	No	29 (74.4)	26 (66.7)	1	1	

IG = Intervention Group; CG = Control Group

¥*P-Values are for the adjusted OR*

£*Adjusted for age, parity, family size, year of residence, diet diversity and dietary calcium intake*

Moreover, in the treatment group, about half (48.7%) of the women experienced constipation at the end of the third month supplementation. Overall, one-quarter (~25%) of the women complained most of the symptoms related to excess calcium intake from the second through the fourth month of the supplementation period. However, these symptoms showed a decreasing trend until the end of the sixth month supplementation period (Figure 1).

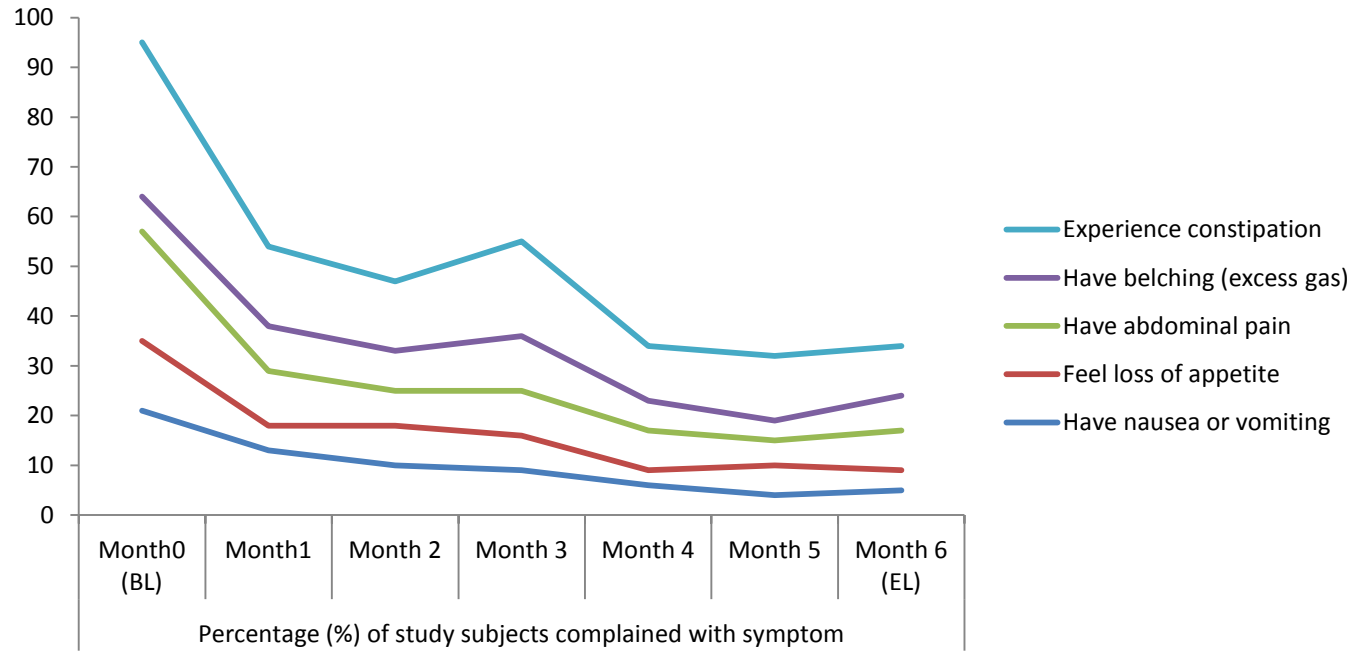

Figure 1: Symptoms related to excess calcium intake through the course of supplementation period

3.4. Hemoglobin (Hematocrit) and Malaria Test Results

The average hemoglobin (hematocrit) level of women was similar, ~13.6g/dL (41.2%) at baseline and endline assessment. There was no significant difference [$t_{(df=76)} = 1.29$ (95% CI: -0.25, 1.14)] in mean hemoglobin level of mothers between the treatment and control group at baseline. Post-intervention, the independent two samples t-test also did not show significant difference [$t_{(df=76)} = 0.24$ (-0.48, 0.60)] in mean hemoglobin level between the two groups (Table 4). In addition, the overall prevalence of anemia (Hgb <12.5g/dL) among women was 15.4% at baseline assessment. This was statistically remained unchanged (Pearson $X^2 = 0.83$, *P=0.362*) after the six months calcium-containing ESP supplementation.

Malaria tests were done at baseline and end of the trial using RDT in both groups. Overall, malaria positive results were observed on 7 (9.0%) and 9 (11.5%) women at baseline and endline tests respectively (Table 4)

Table 4: Comparison of malaria and hemoglobin (hematocrit) test results of women between the two groups in *Halaba* district, Ethiopian Rift Valley, 2018

Test results		IG (n=39) N (%)	CG (n=39) N (%)	*Unadjusted OR (95%CI)*	*Adjusted*[£] *OR (95%CI)*	*P-value*[†]
Baseline[#]						
Malaria positive	Yes	4 (10.3)	3 (7.7)	1.4 (0. 29, 6.58)	1.1 (0.20, 6.38)	0.899
	No	35 (89.7)	36 (92.3)	1		
Anemia (Hgb <12.5 g/dL)	Yes	5 (12.8)	7 (17.9)	0.67 (0.19, 2.34)	0.29 (0.05, 1.70)	0.171
	No	34 (87.2)	32 (82.1)	1		
Hemoglobin (g/dL)	Mean ±SD	13.7 ± 1.5	13.3 ± 1.6	$t_{(df=76)} = 1.29$(95%CI:-0.25, 1.14)		0.201[¥]
Hematocrit (%)	Mean ±SD	41.7 ± 4.8	40.0 ± 4.8	$t_{(df=76)} = 1.54$(95%CI:-0.49, 3.86)		0.127[¥]
Endline[†]						
Malaria positive	Yes	3 (7.7)	6 (15.4)	0.46 (0.11, 1.98)	0.39 (0.07, 2.18)	0.282
	No	36 (92.3)	33 (84.6)	1		
Anemia (Hgb <12.5 g/dL)	Yes	5 (12.8)	8 (20.5)	0.57 (0.17, 1.93)	0.55 (0.13, 2.26)	0.404
	No	34 (87.2)	31 (79.5)	1		
Hemoglobin (g/dL)	Mean ±SD	13.8 ± 1.2	13.7 ± 1.2	$t_{(df=76)} = 0.24$(95%CI:-0.48, 0.60)		0.814[¥]
Hematocrit (%)	Mean ±SD	41.6 ± 3.6	41.4 ± 3.7	$t_{(df=76)} = 0.31$(95%CI:-1.38, 1.89)		0.758[¥]

[£]*Adjusted for age, parity, family size, diet diversity, and baseline hemoglobin (hematcorit) or malaria status, iron and zinc levels*

[†]*P-Values are for the adjusted OR;* [¥]*P-values were computed using independent samples t test*

3.5. Biochemical Outcome Measures: Hemoglobin (Hematocrit), Serum Iron and Zinc Levels prior to and after ESP Supplementation in both Groups

At endline, there were no significant differences (P>0.05) in all biochemical outcome measures of women in both groups compared with their baseline measurements (Table 5). The GEE analysis also revealed similar hemoglobin, hematocrit and zinc levels of women in the intervention group compared with the control women. But after the calcium-containing ESP supplementation, women in the intervention group had statistically significantly lower (by ~20%) serum iron level [β = - 0.21 (95% CI: (-0.4, -0.1)] compared to women in the control group (Table 5).

Table 5: Comparison of biochemical levels of women between the treatment and control arms prior and after the supplementation in *Halaba* district, Ethiopian Rift Valley, 2018

Measurements	Baseline	Endline	Within mean difference (95%CI)[†]	Beta coefficient (Wald 95%CI)[¥]	*P-value*[¥]
	Mean (95%CI) (n = 39)	Mean (95%CI) (n = 39)			
Hemoglobin (g/dL)					
Treatment	13.7 (13.2, 14.2)	13.8 (13.4, 14.2)	-0.1 (-0.7, 0.5)	-0.4 (-1.3, 0.5)	0.402
Control	13.3 (12.8, 13.8)	13.8 (13.4, 14.2)	-0.5 (-1.2, 0.2)	1	
Hematocrit (%)					
Treatment	41.7 (40.1, 43.2)	41.6 (40.5, 42.8)	0.1 (-1.9, 2.0)	-1.4 (-4.2, 1.3)	0.311
Control	40.0 (38.4, 41.5)	41.4 (40.2, 42.6)	-1.4 (-3.5, 0.7)	1	
Iron (mg/L)					
Treatment	0.82 (0.7, 0.9)	0.72 (0.6, 0.8)	0.1 (-0.1, 0.2)	-0.21 (-0.4, -0.1)	0.036
Control	0.61 (0.5, 0.7)	0.73 (0.6, 0.8)	-0.1 (-0.3, 0.1)	1	
Zinc (mg/L)					
Treatment	1.96 (1.7, 2.2)	1.87 (1.7, 2.0)	0.1 (-0.2, 0.3)	0.23 (0.56, 1.87)	0.171
Control	2.33 (2.2, 2.5)	2.0 (1.8, 2.2)	0.3 (0.1, 0.6)	1	

[†]The 'within' mean difference of endline from baseline computed using paired samples t test

[#]Statistically significant difference observed at P<0.05

[¥]Beta coefficient at 95% Wald confidence interval and P-value analyzed using GEE linear model. Hemoglobin, hematocrit, serum iron and zinc were adjusted for age, parity, family size, residency, diet diversity, dietary calcium intake and malaria status.

4. Discussion

The safety assessment through follow-up and monitoring checklists revealed no significant alterations in the reported side effects prior and after supplementation of calcium-containing eggshell powder among women. Furthermore, the calcium-containing eggshell powder supplementation was generally accepted by the study participants. Previous studies have demonstrated the safety of calcium supplementation in various populations (Smith *et al.,* 2018; Jones and Wang, 2019). Consistent with these findings, our research found that calcium-containing eggshell powder supplementation was well-tolerated among participants, with no reports of serious adverse effects or safety concerns.

Calcium supplement intake has long been associated with other side effects such as constipation, abdominal bloating and gas (Jackson *et al.,* 2006; Prince *et al.,* 2006), which varies greatly from person to person and dose of the calcium supplement. Likewise, one-quarter of the women complained most of the symptoms (such as loss of appetite, abdominal pain, nausea or vomiting) related to excess calcium intake from the second through the fourth month of the supplementation period albeit these symptoms showed a decreasing trend until the end of the supplementation period.

Calcium interaction with other essential micronutrients such as iron and zinc in the diet has been considered a potential risk related to high calcium intakes, albeit it is important to consider the habitual dietary calcium intake prior to provide the calcium supplement. There have been concerns related to the effects of calcium supplements on iron absorption based on short-term study reporting that calcium supplements inhibit iron absorption by 28 to 55% depending on the

dose, type of salt used, time of supplementation and the presence in the food of hem or non-hem iron (Cook *et al.,* 1991). Moreover, an earlier study had measured the effect of calcium carbonate and hydroxyapatite on whole-body retention of iron and zinc and reported significant reduction of iron retention in the treatment groups compared with the placebo (control) group (Ilich-Ernst *et al,* 1998). Similarly, women in the current intervention group had statistically significantly lower (by ~20%) serum iron level at the end of calcium-containing ESP supplementation compared to women in the control group.

In contrast, other short-term absorption studies with consumption of 1000–1200 mg/day supplemental calcium for 12 - 26 weeks had no impact on iron status (Sokoll and Dawson-Hughes, 1992; Minihane and Fairweather-Tait, 1998). Another earlier placebo controlled randomized trials suggested that calcium intakes of 1,500 to 1,700 mg/day did not interfere with zinc (McKenna *et al.,* 1997) or iron (Ilich-Ernst *et al,* 1998) absorption. Earlier and recent studies also show no effect on iron status of prolonged calcium supplementation taken at the same time or separate of meals (Yan *et al.,* 1996; Kalkwarf and Harrast, 1998; Abrams, 2001; Harris, 2002; Mølgaard *et al.,* 2005; Gaitan *et al*., 2011). Moreover, calcium interaction with other essential micronutrients such as zinc in the diet has been considered a potential risk related to high calcium intakes. However, different earlier studies assessing the effect of calcium on zinc absorption or zinc balance seem to indicate that supplemental calcium have no effect (Lönnerdal *et al.,* 1984; Dawson-Hughes *et al.,* 1986; Spencer *et al.,* 1984; McKenna *et al.,* 1997).

While our study provided promising evidence of the safety and acceptability of calcium-containing eggshell powder supplementation among women, several limitations should be acknowledged. Firstly, the sample size was relatively small, limiting the generalizability of the findings. Future studies with larger and more diverse participant cohorts are needed to confirm the acceptability of supplementation across different populations and settings. Additionally, individual adherence to the supplementation protocol would vary, influenced by factors such as taste preferences, cultural beliefs, and logistical challenges. Qualitative assessments of acceptability and adherence may provide valuable insights into the barriers and facilitators of supplementation uptake in real-world settings.

5. Conclusion

In conclusion, safety and acceptability assessments conducted as part of the Phase II clinical trial (Mulualem *et al.,* 2021) indicated that calcium-containing eggshell powder supplementation was well-tolerated and did not pose significant risks to the health of participants. This and other research findings (Omer *et al.,* 2019) also suggested that calcium-containing eggshell powder supplementation was generally acceptable among women in the Ethiopian Rift Valley. However, the study recognizes the importance of cultural relevance and contextual factors in shaping the safety and acceptability of calcium-containing eggshell powder supplementation intervention. Tailoring supplementation programs to the local context, addressing cultural beliefs and preferences, and involving community stakeholders might be additional key factors contributing to the success of the supplementation intervention.

Author Contributions: D.M., M.T. and S.J.W. participated in the conception and design. D.M., D.H. and S.J.W., participated in the analyses of the study. Acquisition of data was by D.M. The first draft of the work was by D.M. All authors revised the manuscript critically for important intellectual content. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the University of Saskatchewan's Biomedical Research Ethics Board and Hawassa University's Institutional Review Board.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Data will be made available upon request.

Conflicts of Interest: The authors declare no conflict of interest.

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Annex XVI: Certificate and Published Book of Abstracts for Scientific Conference Presentations

1) Published Book of Abstract for Poster Presentation at ANEC VIII Conference

Published online: September 30, 2018

8th Africa Nutrition Conference

Addis Ababa, Ethiopia, October 1-5, 2018

Abstracts

Co-organized by:
The African Nutrition Society and
The Food and Nutrition Society of Ethiopia

WILL EGGSHELL CALCIUM CONSUMPTION DECREASE BODY FLUORIDE LOAD AND MITIGATE DENTAL AND SKELETAL FLUOROSIS AMONG MOTHERS IN ETHIOPIAN RIFT VALLEY?

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BACKGROUND AND OBJECTIVES:
Fluorosis affects teeth, bones, joints, and brain functions. Diets rich in calcium may reduce the severity of fluorosis by binding fluoride thereby prevent its absorption. With the promotion of eggs and chickens to rural farmers, eggshells are available as sustainable and low cost source of calcium (2000 mg/egg).The aim of the study will be to assess the effects of eggshell calcium consumption on body fluoride load and mitigation of dental and skeletal fluorosis among mothers in Ethiopian rift valley.

METHODS:
At baseline, 41 mothers at the intervention site and 41 at the control site have been assessed, as well as all mothers (n= 135) of under 2 children at each site underwent examination for fluorosis by dentist and physiotherapist. The intervention is six months supplementation of 2.5g eggshell powder/day in three divided doses. Blood, urine, food and water samples were collected from study mothers and sent to Ethiopian Public Health Institute for analyses.

RESULTS:
The mean dietary diversity score of mothers computed using ten food groups was approximately 5 ± 2, with no significant difference (*P=0.572*) between the two groups. Dietary calcium intake was low. Prevalence of anemia (Hgb <12.5g/dL) was 18.3%. More than half (56.3%) of the mothers had mild to severe dental fluorosis; those with moderate and severe dental fluorosis was 68 (25.2%) and 25 (9.3%) respectively. The overall prevalence of skeletal fluorosis among all mothers was 34.4% using a score of 16 signs and symptoms. Majority of the mothers feel lower back pain (70.7%), some had neck pain with movement (46.3%) and abdominal pain (48.5%). There were no significant (*P>0.05*) differences in most signs and symptoms of fluorosis among mothers between the two sites.

CONCLUSIONS:
These suggest the need for innovative approaches to mitigate the toxic effects of excess fluoride in Ethiopian rift valley.

Key words: Eggshell powder, Fluorosis, Mothers.

WILL EGGSHELL CALCIUM CONSUMPTION DECREASE BODY FLUORIDE LOAD AND MITIGATE DENTAL AND SKELETAL FLUOROSIS AMONG MOTHERS IN ETHIOPIAN RIFT VALLEY? PROTOCOL FOR A RANDOMIZED CONTROL TRIAL

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Background

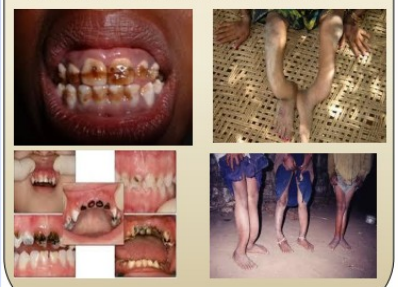
Worldwide, over 50 million people suffer from fluorosis, which affects teeth, bones, joints, and brain functions.

In Sub-Saharan Africa, widespread malnutrition is compounded by fluorosis. More than 14 million Ethiopians have fluorosis but defluoridation requires costly infrastructure.

Diets rich in calcium reduce the severity of fluorosis by binding fluoride. Eggs are promoted as an ideal animal source food and with the promotion of eggs and chickens to rural farmers, as we are doing, eggshells are available.

Our innovation is to use an age-old, sustainable and low cost source of calcium, eggshell (2000 mg/egg), as dietary calcium to bind fluoride from food and beverages, preventing its absorption. Thereby, decrease body fluoride load and mitigate dental and skeletal fluorosis.

Figure 1: Sample photos of dental and skeletal fluorosis



Objectives

General objective

To assess the effects of eggshell calcium consumption on body fluoride load and mitigation of dental and skeletal fluorosis among mothers in Ethiopian rift valley, 2018

Specific objectives

1. To assess dental and skeletal fluorosis levels among school age children and mothers in the study sites
2. To determine mineral composition of local scavenging and common caged-exogenous breed chicken eggshells for human nutrition
3. To estimate mothers' blood levels of calcium, iron, hemoglobin and zinc as a biomarkers for calcium-fluoride amelioration prior and after the intervention
4. To estimate urine fluoride among mothers prior and after the intervention in both groups
5. To determine calcium and fluoride levels of drinking water and staple foodstuffs in the study sites

Method and Materials

Study design:
Reconnaissance survey with descriptive and analytical components, randomized controlled trial and Laboratory-based studies (at EPHI).

Study subjects:
Mothers and school age children

Study size:
135 Mothers-school age children pairs at each site
41 mothers at each site for the RCT study

Method and Materials...cont'd

RCT Profile:

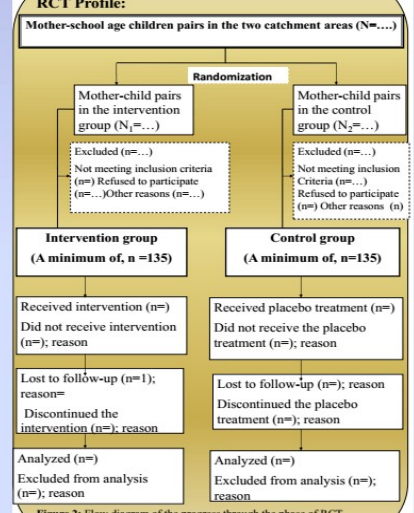


Figure 2: Flow diagram of the progress through the phase of RCT

Method and Materials...cont'd

Intervention:
The exposure is six months supplementation of 2.5g eggshell powder/day in three divided doses.

Implementers: Local coordinators (2) ➔HEWs (4) ➔HDAs (8) ➔Mothers (40). Organized in to two groups.

Monitoring and supervision: weekly, biweekly and monthly.

Intervention package: include the following components:
- ❖Training of health extension workers
- ❖Training of agricultural extension workers
- ❖Cooking demonstration and feeding trials
- ❖Demonstration on eggshell powder preparation
- ❖Feeding a child with one egg a day
- ❖Provision of ESP to encourage use by participants
- ❖Community sensitization/Chicken gift ceremony
- ❖Cages construction to all households.

Method and Materials...cont'd

Data analyses:
Double entry using Epi Data. SPSS analyses include repeated measures ANOVA, logistic regressions, Generalized linear mixed models. *P-Value*<0.05 with 95%CI will be considered significant and reported.

Dental fluorosis:- assessed by dentist using Dean's index (Dean, 1934) on a scale of 0 to 4.

Skeletal fluorosis:- assessed by physiotherapist using physical exercises, signs and symptoms of fuorosis as developed by Susheela (2002) and used by Shashi and Bhardwaj (2008).

Biochemical samples analyses:
All laboratory analyses will be done at Ethiopian Public Health Institute (EPHI). The analyses include serum iron, zinc and calcium ; and fluoride level in urine, water and food samples.

Ethical clearance obtained from:-
- ➢Hawassa University College of Medicine and Health Science Institutional Review Board.
- ➢Ethical committee of Norway
- ➢University of Saskatchewan , Canada

Preliminary results

The overall prevalence of skeletal fluorosis among all mothers was 34.4% using a score of 16 signs and symptoms.

More than half (56.3%) of the mothers had mild to severe dental fluorosis.

Dietary calcium intake was low. Prevalence of anemia (Hgb <12.5g/dL) was 18.3% and mean DDS was ≈ 5 ± 2, with no significant difference (*P=0.572*) between the intervention and control groups.

There were no significant (*P>0.05*) differences in baseline characteristics and signs and symptoms of fluorosis among mothers between the two sites.

These suggest the need for innovative approaches to mitigate the toxic effects of excess fluoride in Ethiopian rift valley.

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- ➢Hawassa University
- ➢University of Bergen , CIH, Norway
- ➢University of Saskatchewan, Canada

2) Certificate for Poster Presentation at HU Internation Conference

Certificate of Achievement

This certificate is awarded to DEMMELASH MULUALEM for presenting a poster during the International Nutrition and Food Industries Conference entitled "Partnership for Food and Nutrition Training and Sustainable Development"

Hosted by the Academic Center of Excellence for Human Nutrition

College of Agriculture, Hawassa University

15 to 18 August 2018, Haile Resort, Hawassa, Ethiopia

Fikadu Reta Alemayehu

(Director, SNFST)

Dr Tarekegn Yoseph

(Dean, College of Agriculture

USING EGGSHELL CALCIUM TO MITIGATE FLUOROSIS IN ETHIOPIAN RIFT VALLEY

Demmelash Mulualem[1], Eskindir Loha[2], Ingunn Engebretsen[3], Bernt Lindtjørn[4], Susan J. Whiting[5]

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[5]PhD, Professor, College of Pharmacy and Nutrition, University of Saskatchewan, Saskatoon SK, Canada S7N 5C9

Why fluoride?

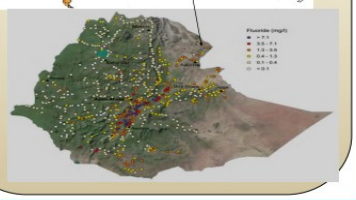
Excess fluoride (F) is a problem globally.

F released through weathering and dissolution of minerals (water in deep wells), in emissions from volcanoes, and in marine aerosols

Problem created through coal, steel, fertilizer, glass, glue industries

Water is scarce

Rift Valley is high F



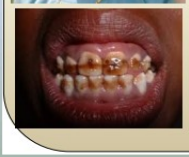
Physiological effects of excess F

$Ca_{10}(PO4)_6X_2$ (X = F, Cl, OH)

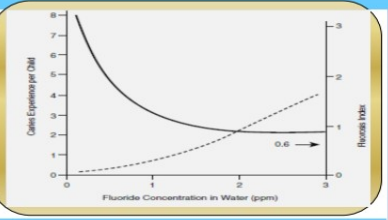
Dental, skeletal and non-skeletal (e.g., joints)

OTHER
Psychological & behavioral

Impedes function in countries such Ethiopia which reliant on physical labour



F benefit and excess (fluorosis)



What does the literature say?

Most studies are population-based, often cross-sectional

Two key intervention studies:
1. In rabbits (2008):
Decreased fluoride bioavailability was observed in +F animals administered with 11.4, 14.3, 17.2, 21.4 and 50 mg Ca/kg bw. Calcium doses ~1200, 1500 and 3500 mg Ca for a 70 kg bw.

2. Reversal of dental fluorosis: Clinical study (2013)
No reversal after 3 months but ↓urine and plasma F with calcium supplementation

Fluorosis research in Ethiopian Rift Valley by Aweke Kebede and Marian Kjellevold:
Thesis: *Assessment of Fluoride Intake in Endemic Areas of Ethiopia and Potential use of Calcium rich foods in Mitigation of Ingested Fluoride*
Defense: *December 2014*
Main findings:
2016 a publication → Rat feeding trial: 6 weeks
Purpose: to show effect on fecal and urine F of diet Ca/Mg or plant Ca/Mg. Fecal F is higher with Mg or Ca
4 groups:
i) FF = fluoride free → little F excretion in urine
ii) FC = +F in water → highest urine F excretion = most absorption
iii) Ca = Ca/Mg supplement → less urine F because less absorption
iv) M = moringa (rich in Ca and Mg) → less urine F because less absorption

Fluoride levels and Health

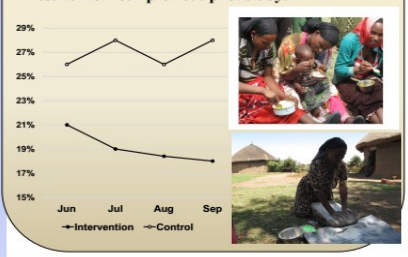
Fluoride	Media	Effects
1 ppm	Water	Dental Caries reduction
2 ppm or <2 ppm	Water	Mottled Enamel (dental fluorosis)
8 ppm	Water	10 % osteosclerosis
20 – 80 mg/day	Water or food	Crippling Skeletal fluorosis
50 ppm	Water or food	Thyroid changes
100 ppm	Water or food	Growth retardation
125 ppm	Water or food	Kidney changes
2.5 – 5 g		Acute dose causing death

What does the literature...cont'd

Intervention Study to mitigate fluorosis: Small pilot of 28 women: urine F (ppm)

Treatment groups	N	Mean	SD	Min	Max
Moringa	8	8.3[a*]	2.1	4.3	10.2
Calcium	8	8.6[a]	2.1	5.8	11.9
Milk	8	10.1[b]	0.9	8.5	11.7
Control	8	12.2[c]	1.5	10.6	14.9

Results from our previous pilot study:



Next Steps!

Use egg shell as source of calcium (Ca)
Have a chicken gift ceremony
Young children to consume egg daily
Women to consume eggshell Ca

Do study in area with high F

Measure urinary F as measure of impact of additional Ca
Measure blood levels of Fe and Zn as safety reason

Add measures of child development, dental, skeletal and non-skeletal health

Overall feasibility of eggshell Ca use by villagers

Publishing data on calcium-fluoride amelioration

Scaling up to a broader RCT

Selected references

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- University of Bergen , CIH, Norway
- University of Saskatchewan, Canada

3) Certificate for Oral Presentation and Published Book of Abstracts at FANUS Conference

Federation of African Nutrition Societies (FANUS)

22nd May 2019

Demmelash Mulualem
School of Nutrition and Food Science
Hawassa University
P.O. Box 05,
Hawassa, Ethiopia
solymico27@gmail.com

Dear Demmelash Mulualem

Re: Invitation to the 4th Federation of African Nutrition Societies (FANUS), conference, Kigali Rwanda 26-29th August 2019

On the behalf of the Scientific Committee of the 4th Federation of African Nutrition Societies (FANUS), conference to be held in Kigali Rwanda 26th to 29th August 2019, I am pleased to inform you that your abstract has been accepted for presentation: ***"Dental, skeletal and non-skeletal fluorosis and association with dietary calcium intake among lactating women in Ethiopian Rift Valley".***

The preliminary programme with mode of presentation will be available at the congress web-site. www.fanus.org by 15th June 2019.

For more information about accommodation and social program, we advice you to visit the conference **.Website: www.fanus.org**

Please use this letter for claiming of the visa from the Immigration at the airport or Rwandan Embassy in your country.

Welcome to Kigali

Robert Fungo (PhD)
On behalf of LOC
Vice President Federation of African Nutrition Societies

Federation of African Nutrition Societies (FANUS)

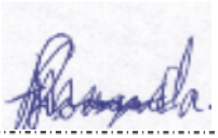
Certificate of Attendance

Awarded to

Demmelash Mulualem

For attending the 4th Federation of African Nutrition Societies (FANUS), Conference that was held in Kigali Rwanda between 26th to 30th August 2019"

Hosted by Rwanda Nutritionist Society and The Federation of African Nutrition Societies (FANUS)



Dr Robert Fungo
Vice President
The Federation of African Nutrition Societies (FANUS)

SY VII.04: DENTAL, SKELETAL AND NON-SKELETAL FLUOROSIS AND ASSOCIATION WITH DIETARY CALCIUM INTAKE AMONG LACTATING WOMEN IN ETHIOPIAN RIFT VALLEY

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Background: Dental and skeletal fluorosis is widely distributed in the Rift Valley of Ethiopia, where drinking water contains fluoride >WHO limit. Adequate calcium intake may mitigate excess fluoride in the body.

Objective: To assess prevalence of dental, skeletal and non-skeletal fluorosis and association with dietary calcium intake among lactating women in the Rift Valley.

Methods: A total of 280 mothers were included with non-response rate of 3.6%. All mothers underwent dental fluorosis examination using Dean's Index by qualified dentist. Skeletal and non-skeletal fluorosis assessment was carried out by qualified physiotherapist using physical exercises and asking about clinical symptoms of fluorosis. Dietary calcium intake was measured by estimating daily calcium intake using food frequency questionnaire. Logistic regression analysis was done to identify predictors of fluorosis symptoms indices. Values of $p<0.05$ with 95% confidence level were considered statistically significant.

Results: Average daily calcium intake was 406mg. More than half (56.3%) of the subjects had very mild to severe dental fluorosis. The prevalence of skeletal fluorosis was 34.4%. Overall, 124 (45.9%) mothers complained of ≥ 2 symptoms of skeletal and non-skeletal fluorosis. Mothers whose dietary calcium intake was $\leq$ 400mg/day had 2.9 times greater risk of developing muscular weakness than those with higher intake [AOR=2.9 (95%CI:1.32, 6.29)]. The study also showed that mothers who had high parity level had 2.5 more odds of developing muscular weakness than those mothers who had low parity level [AOR=2.5 (95%CI:1.12, 5.59)]. Also, the odds of developing muscular weakness was 1.7 with each passing year of age [AOR=1.7 (95%CI: 1.49, 1.96)].

Conclusion and recommendation: Dental and skeletal fluorosis was prevalent in these women. Dietary calcium intake was low and an independent predictor for fluorosis symptoms. This suggests the need for innovative approaches providing additional calcium to mitigate the toxic effects of excess fluoride in Ethiopian Rift Valley.

Keywords: Calcium, Fluorosis, Ethiopian Rift Valley, Women.

Annex XVII: Biography of the PhD Candidate

Demmelash Mulualem Zewdie *was born in 1984 from his mother* **Alemitu Ayehu Worku** *and his father* **Mulualem Zewdie Zeleke** *in a place called* **Woreta** *town, south Gondar. He attended his primary education (grade 1st - 8th) at* ***Woreta*** *primary school. After* successful *completion of his primary education according to the result of the regional exam (*ministry*), he joined* ***Woreta Senior*** *Secondary School for his secondary/high school education (grade 9th - 12th). He had accomplished his grade 12th* with *a very good university entrance exam result in 2002. Thereafter, he was assigned at Hawassa University (the former Debub University) for his undergraduate study. After completing the first year "Fresh man program", Demmelash was assigned at School of Nursing in Hawassa University to study BSc. Nursing. He studied Nursing for four years and successfully graduated on July 12, 2006. By overcoming most of the challenges he faced during the undergraduate study, he became one of the top ten best scorers of the school with very distinction grade. In September 2006, he joined the regional Hawassa Health Science College.*

After serving for six years (from 2006 to 2011) as graduate and lecturer assistant at the regional Hawassa Health Science College, he joined School of Nutrition, Food Science and Technology of Hawassa University in 2012 and he successfully completed his graduate study in Applied Human Nutrition on March 14, 2014.

Up on graduated with MSc degree in Applied Human Nutrition, he got an employment in the school he did his MSc (i.e., School of Nutrition, Food Science and Technology of Hawassa University), and has been working as lecturer and researcher up-to-date. His deep-rooted interest for teaching-learning, doing researches and working for research publications rewarded him with an opportunity to study his terminal degree-PhD in Public Health at the School of Public Health, College of Medicine and Health Science, Hawassa University under the supervision of **Professor Susan J Whiting** *from University of Saskatchewan, Canada, and* **Dr. Dejene Hailu Kassa** *from Hawassa University.*

Today, on October 07/2024, **Demmelash** *is here at the stage in which he is going to present his PhD Dissertation and to share findings from his PhD Research Project to esteemed members of The Board of Examiners, distinguished guests and audiences.*

Annex XVIII: Glossary

Adherence: The extent to which participants follow the prescribed treatment or intervention as outlined in the study protocol.

Attrition Bias: The bias that occurs when participants drop out of a study, potentially skewing the results if the dropouts are related to the outcome.

Bioavailability: The degree to which nutrients or supplements can be absorbed and utilized by the body.

Biomarker Analysis: The measurement of biological indicators (such as urinary fluoride concentration) to assess exposure and health effects related to interventions.

Calcium (Ca): An essential mineral important for bone health; its intake is linked to reduced severity of fluorosis.

Calcium Fluoride: An insoluble compound formed when calcium binds with fluoride, preventing its absorption in the gastrointestinal tract.

Calcium Supplementation: The process of providing additional calcium to meet recommended dietary allowances, especially important for vulnerable populations.

Capacity Building: Efforts to enhance the skills and knowledge of healthcare professionals and community health workers to improve health outcomes.

Causation: The relationship between cause and effect, which is often difficult to establish in cross-sectional studies due to the lack of temporal data.

Clinical Endpoints: Outcomes that are measured in clinical trials to assess the effectiveness of a treatment, such as survival rates or symptom relief.

Collaboration: Working together with various stakeholders, including researchers, policymakers, and community members, to achieve common health goals.

Community Engagement: The process of involving local communities, leaders, and healthcare providers to raise awareness and foster participation in health interventions.

Confidence Interval: A range of values derived from sample data that estimates the uncertainty around a population parameter, reflecting the precision of the sample estimate.

Confounding Factors: Variables that may influence the outcome of a study, potentially leading to incorrect conclusions if not controlled

Construct Validity: The degree to which measurement instruments accurately reflect the underlying constructs or concepts being studied.

Control Arm/Group: The group in a clinical trial that does not receive the experimental treatment, serving as a comparison against the treatment group.

Cross-Sectional Survey: A study design that analyzes data from a population at a specific point in time, often used to assess the prevalence of health-related issues.

Cultural Sensitivity: Respecting and considering cultural beliefs and practices when designing and implementing health interventions.

De-Fluoridation: The process of removing fluoride from drinking water to reduce its concentration to safer levels for consumption.

Dental Fluorosis: A condition resulting from excessive fluoride exposure during childhood, leading to changes in the appearance of teeth.

Dietary Calcium Intake: The amount of calcium consumed through food sources; often assessed by tracking consumption of calcium-rich foods.

Dose Optimization Studies: Research aimed at determining the most effective dosage of a treatment to achieve desired health outcomes safely.

Dropout Rate: The percentage of participants who withdraw or are lost from a study before its completion, which can affect study outcomes and sample size.

Effect Size: A quantitative measure of the magnitude of a phenomenon, used to assess the strength of associations in studies.

Efficacy Trial: A study that tests the effectiveness of an intervention in controlled settings, often part of clinical trials.

Eggshell Powder (ESP): A dietary supplement made from crushed eggshells, rich in calcium carbonate and trace minerals, used to enhance calcium intake.

External Validity: Also known as generalizability, it refers to the extent to which study findings can be applied to broader populations or different contexts beyond the study sample.

Fluoride (F): A naturally occurring mineral found in water, soil, and various foods; essential for dental health but can cause toxicity at high levels.

Fluorosis: A condition caused by excessive fluoride exposure, leading to symptoms affecting teeth and bones.

Generalizability: The extent to which study findings can be applied to broader populations or different contexts beyond the study sample.

Generalized Estimating Equations (GEE): A statistical method used for analyzing correlated data, often in longitudinal studies

Gravidity: It is a term used in obstetrics to denote the number of times a woman has been pregnant, regardless of the outcome (live births, stillbirths, miscarriages, or ectopic pregnancies)

Halaba Zone: A district in southern Ethiopia, known for its dry midland climate and high fluoride levels in drinking water.

Health Development Army: Community health workers in Ethiopia tasked with promoting health and nutrition, especially among women and children.

Hypercalciuria: A condition characterized by excessive calcium in the urine.

Integration with Existing Programs: The incorporation of new health interventions into current public health initiatives to enhance effectiveness and resource use.

Internal Validity: The degree to which a study accurately measures the effects of the intervention without bias or confounding factors.

Iron Deficiency Anemia: A condition resulting from insufficient iron, leading to decreased hemoglobin levels and related health issues.

Kebele: The smallest administrative unit in Ethiopia, similar to a neighborhood or district.

Level of Precision: The degree of certainty associated with an estimate, often influenced by sample size and the variability of the data.

Longitudinal Study: A research design that follows subjects over a period of time to observe changes and developments.

Long-Term Safety Studies: Research conducted to evaluate the safety of a treatment over an extended period, monitoring for potential adverse effects.

Masking: A method used in trials to keep participants and researchers unaware of group assignments to prevent bias

Measurement Bias: Systematic errors in the collection or interpretation of data that lead to incorrect conclusions about the relationships between variables.

Misclassification Bias: An error that occurs when subjects are incorrectly categorized, which can affect the validity of study results.

Non-Skeletal Fluorosis: Symptoms of fluoride toxicity not related to the bones, including muscular weakness and gastrointestinal complaints.

Nutrient Absorption: The process by which the body takes in and utilizes nutrients from food.

Nutritional Counseling: Guidance provided to individuals or groups on dietary choices to improve health and nutritional status.

Nutritional Intervention: Strategies implemented to improve dietary intake and nutritional status to prevent or treat health issues.

Osteomalacia: A condition resulting from inadequate vitamin D or calcium, leading to weak or soft bones; associated symptoms include pain and poor mobility.

Parity: The number of times a woman has given birth, typically categorized as nulliparous (no births), primiparous (one birth), or multiparous (two or more births).

Phase II Trial: A clinical trial stage aimed at evaluating the efficacy and safety of an intervention, typically involving a smaller participant group compared to Phase III trials.

Physical Exercise Tests: Assessments designed to evaluate physical capabilities and health, which may be used to measure effects related to conditions like fluorosis.

Policy Advocacy: Efforts to influence policy decisions at various levels to promote public health initiatives and improve health outcomes.

Prevalence: The proportion of a population found to have a particular condition at a specific point in time.

Quality Control: Processes implemented to ensure that products meet specific standards of quality and consistency, particularly in supplements.

Random Error: The distortion of an observed estimate from the true population value, which can be minimized by increasing the sample size.

Randomization: The process of assigning participants to different treatment groups in a study randomly, to minimize bias.

Recommended Daily Allowance (RDA): The daily intake level of a nutrient considered sufficient to meet the requirements of most healthy individuals.

Retention Strategies: Methods used to keep participants in a study over time to minimize loss to follow-up and ensure data integrity.

Rift Valley: A geographical region in Ethiopia known for high fluoride levels in drinking water, leading to widespread fluorosis.

Safety Assessment: The evaluation of potential adverse effects and overall safety of a supplement, monitored through follow-up and checklists in studies.

Sample Size: The number of participants or units included in a study, determined to adequately address research questions and ensure statistical validity.

Sampling Bias: A bias that occurs when the selected sample does not represent the broader population, potentially skewing study results.

Scaling Up: The process of expanding health programs to reach a larger population or area, often requiring additional resources and funding

Skeletal Fluorosis: A bone condition caused by prolonged exposure to high fluoride levels, resulting in skeletal rigidity and pain.

Statistical Power: The probability that a study will correctly reject a false null hypothesis, often increased with larger sample sizes.

Statistical Validity: The appropriateness of the statistical methods used to analyze data, ensuring that conclusions drawn are based on reliable statistical techniques.

Temporal Relationships: The connections between events or variables that occur over time, crucial for establishing causation in research.

Tolerable Daily Intake: The maximum amount of a substance (like fluoride) that can be ingested daily without adverse health effects.

Treatment Effects: The impact of an intervention on the outcomes of interest in a study, measured through various clinical endpoints and assessments.

Type I Error: It is often denoted as alpha (α), is the probability of rejecting the null hypothesis when it is actually true.

Type II Error: The error that occurs when a study fails to detect an effect that is actually present, often due to insufficient sample size.

Upper Level of Intake: The maximum daily intake level of a nutrient that is unlikely to cause harmful effects.

Validity: The degree to which a study accurately measures what it intends to measure.