EFFECTS OF MATERNAL VITAMIN D SUPPLEMENTATION ON MARKERS OF VITAMIN D STATUS AND RELATED INFANT AND MATERNAL HEALTH OUTCOMES IN SOUTHERN ETHIOPIA

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Vitamin D is crucial for calcium and phosphorous homeostasis and for bone health. Evidence also has linked vitamin D deficiency with impaired immune function. Studies conducted in populations that reside close to the equator show that vitamin D insufficiency is prevalent despite abundant UVB. Response to oral vitamin D supplementation in this population is not known. The aim of this study was to determine effect of vitamin D supplementation on maternal and infant plasma 25(OH)D concentrations, and on markers of bone turnover, infant growth, gross motor development and morbidity. A randomized, placebo-controlled, double-blind trial (NCT02210884) was conducted in Sidama Zone (7^oN, 38^oE), Southern Ethiopia. Lactating women (n=126) enrolled within two weeks postpartum were randomized to vitamin D_3 (15,000 IU/week) or placebo for 12 months. Plasma 25(OH)D, bone-specific alkaline phosphatase (BAP), osteocalcin (OC) and C-telopeptide for type 1 collagen (CTX) were determined. Skin reflectance, dietary intake, and individual UVB exposure were also measured. Moreover, infant health and growth outcomes were also assessed. Median (IQR) 25(OH)D concentrations were not significantly different between vitamin D and control groups at baseline [45 (39, 56) nmol/L vs 47 (37, 57) nmol/L, $p = 0.697$]. 25(OH)D concentrations were significantly higher in the vitamin D group at end-line [109 (93, 121) nmol/L vs 63 (49, 81) nmol/L $p < 0.0001$]. All women in the vitamin D group were vitamin D sufficient (> 50 nmol/L) by the end of the study. Furthermore, 95% had attained 25(OH)D concentrations > 75 nmol/L compared to 39% in the control group. End-line infant 25(OH)D concentrations were not significantly different between groups. Maternal BAP and OC concentrations significantly increased from two weeks post-partum to end-line in both groups ($p < 0.001$). No significant differences by treatment were seen for all bone turnover markers, infant growth, and health outcomes ($p = 0.974$) . Weekly vitamin D supplementation safely eliminated vitamin D insufficiency in the study population. Our results suggest that supplementation can be used to improve vitamin D status in populations with limited sun exposure and low dietary vitamin D and calcium intake.Intervention did not affect bone turnover and infant health outcomes.

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LIST OF ABBREVIATIONS

BAP	Bone-specific alkaline phosphatase
BMI	Body mass index
CTX	C-terminal telopeptides of type 1 collagen
CHV	Community Health Volunteers
CRW	Community Research Workers
ELISA	Enzyme linked immunosorbent assay
HEW	Health Extension Workers
IMNCI	Integrated Management of Childhood Illness
MED	Minimum Erythemal Dose
MUAC	Mid-upper arm circumference
NIST	National Institute of Standards and Technology
OC	Osteocalcin

PTH	Parathyroid hormone
SED	Standard Erythemal Dose
UVB	Ultraviolet light-B
VDSP	Vitamin D Standardization Program
VDR	Vitamin D receptor
WFR	Weighed Food Records
WHO	World Health Organization

CHAPTER I

INTRODUCTION

BACKGROUND

Vitamin D, commonly referred to as the sunshine vitamin, is a pro-hormone that performs critical functions in the body[1,2]. Vitamin D can be produced in the skin by the action of ultraviolet light (UVB) and can also be obtained from a few foods in the diet[3]. The biologically active metabolite of vitamin D, calcitriol, acts as a ligand for the vitamin D receptor (VDR) which in turn regulates gene transcription and signal transduction pathways in target cells[2]. The widely established physiological function of vitamin D is the regulation of calcium and phosphorous homeostasis[1,2]. Through regulation of these nutrients vitamin D ensures proper mineralization of bone[2]. Furthermore, the VDR has been discovered in cells that perform functions beyond calcium homeostasis[4]. Vitamin D influences differentiation and intracellular calcium handling in muscle cell[5]. It also contributes to the regulation of differentiation and proliferation of cells including β cells of the pancreas and hematopoietic cells[2,4]. The VDR is found in activated T-cells, macrophages, monocytes and cytotoxic T cells and modulates immune response[2,6]. Recent evidence also has linked vitamin D with type 1 diabetes, breast and colon cancer, rheumatoid arthritis, infectious diseases and cardiovascular diseases[7-9].

Vitamin D status is defined using plasma or serum level of 25-hydroxyvitamin D (25(OH)D) as a biomarker[10]. According to the cutoff set by the Institute of Medicine (IOM) to

define vitamin D deficiency[11], 25(OH)D concentration less than 30 nmol/L are indicative of deficiency that can result in rickets and osteomalacia. Vitamin D status is considered inadequate for bone and overall health when levels are in the 30-50 nmol/L range. A concentration above 50 nmol/L indicates adequacy[11].

Vitamin D deficiency results in rickets in children and osteomalacia in adults[1,12]. Rickets is a manifestation of chronic vitamin D deficiency and commonly presents in children between the ages of 6 and 29 months[13,14]. Children with rickets suffer from growth retardation and skeletal deformities[12,14]. Sub-clinical vitamin D deficiency in children causes hypocalcemia and bone demineralization and can present as hypocalcemic seizures in infants[14]. Vitamin D deficiency in adults has been associated with muscle weakness, osteoporosis, and increased fracture risk[15,16].

Meeting vitamin D needs is especially important during pregnancy and lactation due to increased demands to support fetal and infant growth and development[17,18]. Stores of vitamin D acquired in utero, and breast milk are the main sources of vitamin D for infants[19]. Maternal vitamin D and 25(OH)D are not readily transferred into breast milk which has been shown to be low in vitamin D[19,20]. Maternal vitamin D deficiency, exclusive breastfeeding, darker skin pigmentation and limited sun exposure are associated with rickets in infants[18].

Several factors can affect cutaneous synthesis, which is the major source of the vitamin in parts of the world where foods are not fortified with vitamin D[21,22]. Skin pigmentation determines the amount of light that penetrates the skin to produce vitamin D[23]. Individuals with darker skin have a higher level of melanin which acts as a UV filter and are thus at a higher risk of vitamin D deficiency especially if sun exposure is limited[3,24]. Latitude affects the amounts of UVB rays that reach the surface of the skin[1]. Regions located between 37º N and 37º S have sufficient sunlight to maintain optimal concentrations of 25(OH)D throughout the year[21]. However, since other factors such as season, dress habits, sunscreen use and air pollution influence vitamin D synthesis, deficiency and insufficiency is prevalent in these regions. Hypovitaminosis D has been

reported in India[25], Iran[26] and Turkey[27] among pregnant women and newborns and in the United Arab Emirates (UAE) among exclusively breastfed infants and lactating mothers[28]. Vitamin D deficiency has also been reported in other target groups in sunshine abundant countries[29-32]. In populations with low dietary vitamin D and calcium intake these factors can compromise vitamin D status and may contribute to high prevalence of infectious disease and maternal mortality[33].

Ethiopia is located near the equator and has abundant sunshine throughout the year. Consequently, vitamin D status and risk factors for deficiency have not been widely explored. Two hospital-based case-control studies conducted in the late 1990's reported a strong association between limited sun exposure and rickets[34] and a high prevalence of rickets in children with pneumonia[35]. A systematic review identified lack of exposure to sunshine and inadequate intake of vitamin D as major risk factors for rickets[34]. Studies that measured 25(OH)D have reported a higher than anticipated vitamin D deficiency (< 30 nmol/L) and insufficiency (30-50 nmol/L). In 1999 Feleke et al. reported a mean 25(OH)D concentration of 23.5 nmol/L for adults and 25 nmol/L for pregnant women[36]. In a recent study conducted among children (aged 10-18) 42% had 25(OH)D concentrations below 50nmol/L[37]. Furthermore, in an assessment of vitamin D status of rural women of reproductive age in southern Ethiopia, 15.8% had 25(OH)D levels below 30 nmol/L and 69.4% had levels between 30-50 nmol/L[38].

According to the 2016 Ethiopian Demographic and Health Survey, 52% of all children under the age of six months are exclusively breastfed and 98% of mothers at least initiate breastfeeding of their infants[39]. Breastfeeding is widely promoted as the best food for the baby for the first six months of life. However, lactating women and infants have not been assessed for vitamin D status in Ethiopia. The contribution of skin pigmentation, UVB exposure, traditional practices that limit sun exposure and other risk factors for vitamin D deficiency also needed to be explored. Thus the primary aims of this study were:

1) To determine the effects of a weekly vitamin D supplement (15,000 IU) vs. placebo administered to lactating women for 12 months on maternal and infant markers of vitamin D status.

2) To determine variability in skin color and personal ultraviolet light (UVB) exposure and their association with vitamin D status, among lactating women.

Secondary outcome measures include maternal markers of bone turnover, infant growth, infant gross motor development, infant morbidity.

PRIMARY AND SECONDARY OBJECTIVES

***Primary objectives**:*

1. To assess effects of weekly vitamin D supplementation administered to the mothers on 25(OH)D concentrations of mothers and infants;
2. To assess the association between skin reflectance (color), dress habits, sun-seeking behavior, UVB exposure, and 25(OH)D concentrations.

***Secondary objectives**:*

1. To assess the effect of maternal vitamin D supplementation on maternal markers of bone turnover;
2. To compare differences in attainment of infant motor developmental milestones between treatment groups;
3. To compare differences in infant growth between treatment groups;
4. To compare differences in incidence infant morbidity between treatment groups;
5. To assess dietary vitamin D and calcium intake among lactating women.

HYPOTHESIS

Null Hypothesis for primary objectives

HO_1: There is no significant difference in maternal 25(OH)D concentration between the vitamin D and placebo groups.

HO_2: There is no significant difference in infant 25(OH)D concentration between the vitamin D and placebo groups.

HO_3: Skin reflectance, UVB exposure, dress habits and sun-seeking behaviors will not significantly predict 25(OH)D concentrations.

Null Hypothesis for secondary objectives

HO_4: There is no significant difference in parathyroid hormone (PTH) concentrations between vitamin D and placebo groups.

HO_5: There is no significant difference in maternal and infant bone specific alkaline phosphatase concentrations between vitamin D and placebo groups.

HO_6: There is no significant difference in osteocalcin concentrations between vitamin D and placebo groups.

HO_7: There is no significant difference in C-terminal telopeptides of type 1 collagen concentrations vitamin D and placebo groups.

HO_8: There is no significant difference in infant date of attainment of gross motor milestones by treatment group.

HO_9: There is no significant difference in infant growth by treatment group.

HO_{10}: There is no significant difference in the incidence of infant morbidity by treatment group.

RATIONALE AND SIGNIFICANCE OF STUDY

Vitamin D insufficiency can occur despite the availability of abundant sunlight due to several factors, such as skin pigmentation, dress habits, and sun avoidance, that reduce the efficiency of vitamin D synthesis in the skin[1]. Furthermore, low dietary vitamin D intake and absence of vitamin D fortified foods increase the risk of vitamin D deficiency in UVB sufficient populations[3].

Individuals with a darker skin color have a higher melanin content in their skin. Melanin absorbs UVB light which otherwise starts the synthesis of vitamin D in the body. Melanin can reduce the capacity of the skin to produce vitamin D by 95 to 99% in dark skinned individuals thus increasing the risk of vitamin D deficiency[21]. A high prevalence of vitamin D deficiency has been reported for dark skinned Indians[40], Brazilians[41], and African Americans[42].

A conservative dressing style that only allows exposure at the head, the neck region and hands limits direct sun exposure and significantly reduces vitamin D synthesis. Depending on the type and color of clothing worn, UVB absorption can be reduced by 48-99% [43]. Conservative dress habits and use of a veil for religious reasons have been shown to significantly contribute to the occurrence of vitamin D deficiency in sunshine abundant countries[27,44]. The fact that availability of adequate sunshine alone does not always ensure vitamin D adequacy has been supported by several studies carried out in countries with abundant sunshine that reported vitamin D deficiency and insufficiency[26,27,31,36,38,40,41,44-49].

In Ethiopia studies that assessed levels of circulating vitamin D (25(OH)D) have reported vitamin D deficiency and insufficiency. A preliminary study, conducted in the area where this study was conducted, assessed vitamin D status of women of reproductive age (n=202). The study participants performed several outdoor activities and thus had substantial sunlight exposure. However, despite abundant sunlight exposure, 15.8% of the women were vitamin D deficient, and

69.4% were vitamin D insufficient[38]. Another study reported mean 25(OH)D concentrations indicative of severe vitamin D deficiency (< 25 nmol/L) for adults (n=30) and pregnant women (n=31) residing in Addis Ababa[36]. A study that assessed 25(OH)D levels of lactating women (n=108) in Wondo Genet and Arsi Negele reported 39% vitamin D insufficiency[49]. Study participants had modest clothing habits, did not consume vitamin D rich or fortified foods and covered their babies and themselves from the sun due to religious and cultural reasons[49].

A diet low in calcium and high in phosphorus increases predisposition to vitamin D deficiency through blood calcium and parathyroid hormone (PTH) related mechanisms that cause secondary vitamin D deficiency. Women residing in the study area have been shown to have a low calcium and high phytate intake[50] which increases their risk of vitamin D deficiency. All factors that influence the vitamin D status of women also negatively influence the vitamin D status of newborns and infants and increase the risk of vitamin D deficiency in this target group. Infants are especially vulnerable to vitamin D deficiency if they depend on breastmilk for vitamin D and are not exposed to sunlight or supplemented with vitamin D after birth [19].

This study addressed the knowledge gap regarding vitamin D status of populations at risk of vitamin D deficiency by assessing vitamin D markers in lactating women and infants. Quantitative estimates for factors such as skin pigmentation and sunlight exposure that affect vitamin D status of the individual also were generated. Furthermore, the effect of maternal vitamin D supplementation on maternal and infant vitamin D status and other related outcomes were evaluated.

ASSUMPTIONS

Biological samples were transported from Ethiopia to the US packed in dry ice. It was assumed that the samples were transported without damage. Length of storage of frozen samples was variable due to the different collection time points. It was assumed that analytes are stable after long storage.

LIMITATIONS

- The primary aim of this study was to detect differences in maternal 25(OH)D concentration between the groups based on vitamin D supplementation. Sample size estimations were based on this objective. Hence this study had limited power for subgroup analyses or to test infant outcome differences.
- Becasue our study participants were lactating women, we were not able to ask that they provide a fasting blood specimen. Thus biomarker analysis was not performed on fasting blood samples.
- Serum C-terminal telopeptides of type 1 collagen (CTX) values can be influenced by time of day (diurnal variation). Due to logistical challenges, we were not able to collect all blood samples at the same time of day.
- Infant blood samples were collected only at end-line. Samples collected at baseline and at an interim point would have enabled a more comprehensive depiction of the impact of the intervention.
- This study did not measure bone mineral content (BMC) or bone mineral density (BMD) which are stronger indicators of bone health compared to bone turnover markers.

CHAPTER II

LITERATURE REVIEW

History of Vitamin D

"Vitamin D" is the name commonly used to refer to the two different forms of the vitamin, D_2 (ergocalciferol) and D_3 (cholecalciferol). D_2 is synthesized by plants through the irradiation of ergosterol by yeast. D_3 is produced in the skin through the UVB conversion of 7-dehydrocholesterol to pre-vitamin D3, which then isomerizes to D_3[1]. Vitamin D was first discovered through the work of McCollum and Mellanby [2]. Discovery of vitamin D was tied to its ability to heal rickets that could not be cured by Vitamin A. McCollum performed an experiment in which he removed vitamin A in cod liver oil to determine if it caused rickets. When cod liver oil that did not contain vitamin A cured rickets, he named the unknown vitamin factor vitamin D[2]. Mellanyby hypothesized that rickets was due to a deficiency in the diet. He fed dogs that were kept indoors oatmeal to test this hypothesis. When the dogs developed rickets, he then fed them cod liver oil and treated rickets. He identified the unknown nutrient in cod liver oil as being able to cure rickets[2].

Food Sources and Forms

Vitamin D is naturally found in very limited food sources [15]. Fatty fish, fish liver oil, egg yolk and sun-dried mushrooms are the main foods that contain vitamin D [11]. Non-endogenous vitamin

D can be either in the form of D_3 or D_2. D_3 is obtained from animal sources and D_2 is mainly obtained from plant sources or dietary supplements [1]. In the US and Canada milk and other foods such as margarine, yogurt, ready-to-eat cereal, and orange juice are fortified with vitamin D. Also vitamin D supplements are widely available[1]. However, in most countries in Europe, Asia, and Africa's foods are not fortified with vitamin D [51]. There has been debate over whether what form of vitamin D, D_2 or D_3, is more metabolically active. Although some researchers have argued that D_2 is more potent, a recent meta- analysis reported that a high dose of vitamin D3 raises 25(OH) D proportional to that of vitamin D_2 [52]. Breast milk is a poor source of vitamin D. How much vitamin D is in breast milk will depend on vitamin D status of mothers. Breast milk contains about 20-70 IU/L of vitamin D. An infant consumes 0.78kg (750 mL) of breastmilk in a day[53].

Table 2.1. Food sources of vitamin D

Food	**IU per 100g**	**IU per serving**
Cod liver oil (1 tsp)	10000	450
Swordfish, raw (1 Oz)	558	158
Salmon, sockeye, raw (1 Oz)	563	160
Tuna fish canned in water (1Oz)	80	23
Beef liver, raw (Oz)	49	14
Egg whole raw (1 large)	82	41
Mushrooms, shiitake, dried (100 g)	154	154
Mushroom, white, exposed to ultraviolet light, raw (100g)	1046	1046
Whole milk vitamin D fortified (1 cup)	51	124

Holic 2003 [54]

Metabolism of Vitamin D

Role of Sunlight in Endogenous Vitamin D Synthesis

UVB, a component of ultraviolet light with a wavelength range of 280-315 nanomole (nm), initiates the synthesis of vitamin D in the skin[21]. The ozone layer and atmospheric gasses (oxygen, Carbon dioxide) absorb most UVB light released from the sun before it reaches the earth's surface. The amount of UVB light that reaches the ground surface of the earth varies by latitude, time of day and season. The angle of the sun is directly overhead near the equator and at noon. Hence, ambient UVB is more abundant near the equator, during summer and is at peak intensity closer to mid-day[55]. Other environmental factors that influence UVB availability include could cover, aerosols such as dust, smoke and air pollutant, altitude and ground surface reflectivity. Aerosols absorb UVB light. More UVB is available at higher elevations since it travels a shorter distance before reaching the ground, and chemicals in the atmosphere do not absorb it. Personal factors such as skin pigmentation, dress style, and sun-seeking behavior determine how much UVB reaches the skin; consequently leading to variable individual UVB exposure[56].

Cutaneous Production of Vitamin D

Endogenous vitamin D (D_3) synthesis takes place in the skin through the conversion of 7-dehydrocholestrol, found in the dermis and epidermis of the skin, to pervitamin D_3 by the action of UVB light (wavelength 390-450 nm) from the sun[1,2,57]. Per-vitamin D_3 is formed when carbon bonds in 7-dehydrocholestrol are broken by UVB light resulting in a change in structure and chemical property (Figure 1) [2]. Pre-vitamin D_3 then undergoes thermal isomerization to form vitamin D_3 (Cholecalciferol), which is further metabolized to produce active forms of vitamin D[1,2,57]. Excess vitamin D_3 produced due to prolonged sun exposure is photo-isomerized to the non-vitamin D substances lumisterol and tachysterol which are biologically inactive [58].

Consequently, excessive exposure to sunlight does not cause vitamin D intoxication[1]. Furthermore, lumisterol and tachysterol can be converted back to pre-vitamin D_3. Vitamin D3 synthesis in the skin is affected by factors that reduce sun exposure such as pigmentation, latitude, and dress habits [1].

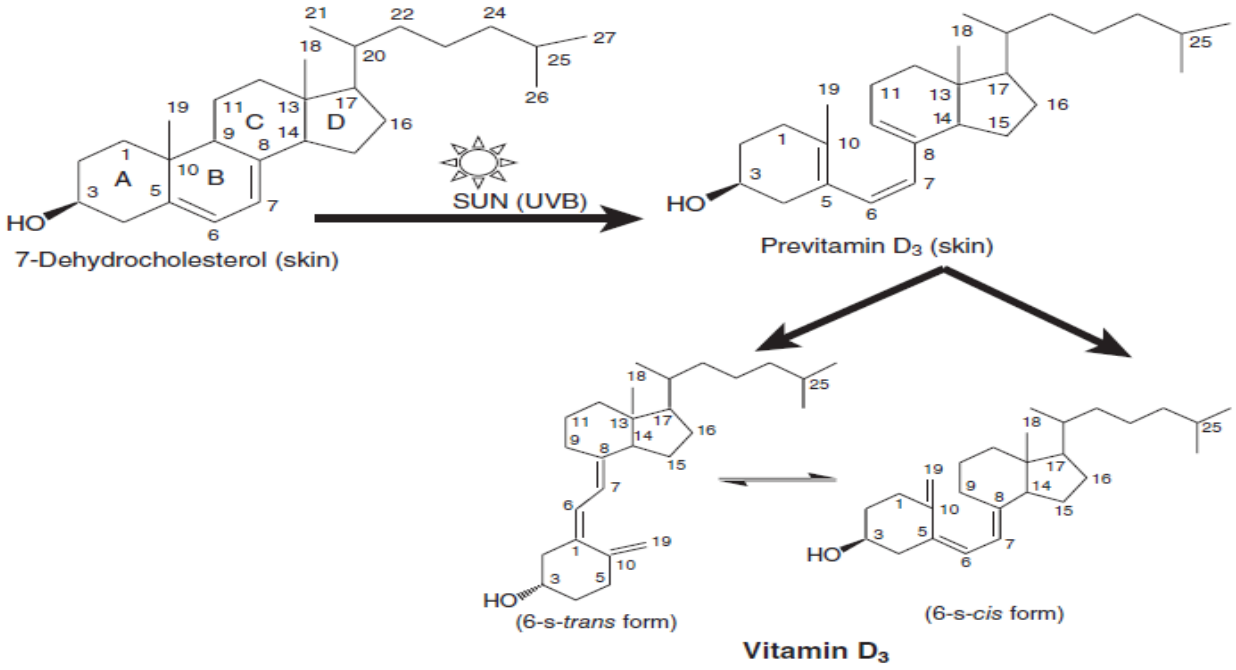

Figure 1 Cutaneous synthesis of vitamin D. Source [2]

Response to Serum 25(OH)D to UVB exposure

Ambient UVB is commonly expressed as the minimum erythemal dose (MED) and standard erythemal dose (SED). MED refers to dose required to produce a barely perceptible erythema in people with skin type 1 (white) (200 J/m^2 of biologically effective UV radiation)[59]. MED is subjective since the determination of reddening of the skin might differ from person to person. Furthermore, it depends on individual sensitivity to UV radiation, site of radiation and skin pigmentation. Hence, values have to be defined for different skin types[59]. Holick showed that

full body exposure with 1MED could produce 10,000 -15, 000 IU vitamin in individuals with type 1 skin. However, those with skin type V will require more exposure to produce the same results[55]. One standard erythema dose (SED) is equivalent to an erythemal effective radiant exposure of 100 J/m^2 (1 SED = 10 mJ/cm^2). It is independent of skin color. Exposure dose in SED may cause erythema in fair skin but none in darker skin.

Armas et.al.[60] used participants with various skin tones to develop a formula below to predict change in serum 25(OH)D in response to a UVB dose and depending on skin lightness (L*).

Z = b *X*Y, Where

Z is Change in serum 25(OH)D in response to a UVB dose;

X is UB dose in MJ/cm^2 ans

Y is skin lightness measures as L*

Using this formula to attain a 30 nmol/L increase in serum 25(OH)D, a northern European person with an L* value of 70 would need 39 mJ/cm2 UV radiation. An African American with and L* score of 50 would require 55(mJ/cm^2)[60].

Absorption and Transport

Endogenous vitamin D is transported in the blood stream bound to vitamin D binding protein (DBP) [61]. Dietary vitamin D is absorbed in the same manner as dietary fats. It is emulsified in bile acids and is transported to the enterocyte as part of micelles. Vitamin D is then packaged as part of chylomicrons and enters systemic circulation through the lymphatic system [2]. Once vitamin D reaches the blood stream, it is transported to the liver and other tissue bound to vitamin D binding protein (DBP)[2,61].

Hydroxylation of Vitamin D

Hydroxylation of vitamin D, first in the liver and then in the kidney, converts the biologically inactive vitamin to its active metabolite 1,25-dihydroxyvitamin D (1,25,$(OH)_2$D), which is also referred to as Calcitriol[2]. Once vitamin D is transported into the liver by DBP, the 25th carbon molecule is hydroxylated by hepatic 25-hydroxylase [2,57]. Microsomal and mitochondrial forms this enzyme have been characterized [57]. The product of the hydroxylation reaction is 25(OH)D (calcidiol). 25(OH)D is the major circulating form of vitamin D in the body. It is bound to the DBP in the blood stream and is transported to the kidney for the second hydroxylation step[2,57]. In the kidney, 25(OH)D is converted to 1,25,$(OH)_2$D by the action of the enzyme 1α-hydroxylase [57].

1, 25$(OH)_2$D enters the circulation from the kidney and acts on peripheral target tissue. It acts as a ligand for the Vitamin D receptor (VDR) and regulates target cell genomic and rapid response generated through the activation of signal transduction pathways[2]. Another hydroxylase enzyme, 24-hydroxylase (CYP24), adds an hydroxyl group at carbon number 24 of both 25(OH)D and 1,25,$(OH)_2$D [57]. This reaction converts the metabolites to less active, 24-hydroxylated, products which are then excreted with bile through feces [57]. Unlike that of 25-hydroxylase, the activity of the enzyme 1α-hydroxylase is tightly regulated[2,57]. This tight control allows the body to regulate the production of 1,25,$(OH)_2$D based on needs. The activity of 1α-hydroxylase is elevated during vitamin D deficiency [2]. Furthermore, when blood calcium levels are low Parathyroid hormone (PTH) increases the activity of 1α-hydroxylase[2,57]. The product of the enzymatic action of 1α-hydroxylase, 1, 25$(OH)_2$D, negatively regulates the activity of PTH and the production of 1α-hydroxylase. Moreover, 1, 25$(OH)_2$D increases activation of 24-hydroxylase their by facilitating degradation of excess 25(OH)D and 1,25,$(OH)_2$D [2,57]. Fibroblast growth factor 23 (FGF23), which regulates phosphate metabolism and, is secreted when serum

phosphate levels are high and suppresses 1 1α-hydroxylase activity in the kidney [2]. 1, 25$(OH)_2$D exerts its function

Physiological Functions of Vitamin D

Skeletal Functions

Vitamin D is important for the absorption of calcium and phosphorus. The action of 1,25$(OH)_2$D increases the efficiency of calcium absorption by up to 40% and that of phosphorous absorption by 80% [57]. Two mechanisms have been proposed for regulation of calcium homeostasis by 1,25$(OH)_2$D. By acting on the nuclear VDR 1,25$(OH),_2$D up-regulates the expression of the calcium channel (TRPV6) and calcium binding protein (calbindin). Another mechanism is that 1,25$(OH)_2$D promotes intestinal absorption of calcium by acting on a plasma membrane VDR which triggers a cascade of signal events that produces a second messenger that facilitates absorption[1,57]. Vitamin D also stimulates the Ca2+-Mg2+ ATPase pump though which calcium is released form the enterocyte to the extracellular fluid [62].

Furthermore, 1,25$(OH)_2$D stimulates the production of fibroblast growth factor 23 (FGF 23) which intern increases phosphate excretion by decreasing the expression of phosphate transporters in the kidneys. When blood calcium concentrations are low 1,25$(OH)_2$D acts on the nuclear VDR receptor found in osteoblasts (bone forming cells) and increases expression of RANKL (receptor activator of nuclear factor-κB ligand). RANKL binds to the RANK receptor located on preosteoclasts and promotes maturation to osteoclasts (bone resorption cells). Osteoclasts then release calcium and phosphorus from the bone to the blood maintaining serum concentrations[1,57]. Figure 1 presents a pictorial representation of skeletal functions of vitamin D.

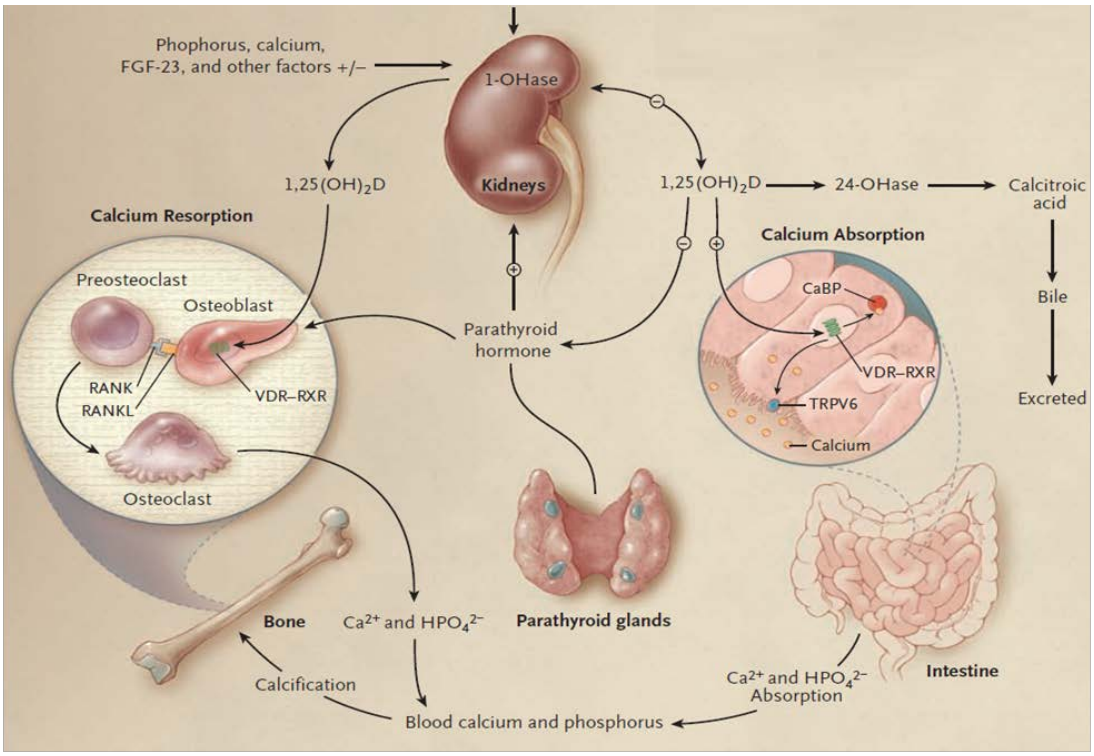

Figure 2: Skeletal functions of vitamin D. Adapted from [1]

Non-skeletal Functions

The active metabolite $1,25(OH)_2D$ is involved in many processes that extend beyond bone health. Most cells in the body have VDR and can perform the second hydroxylation step in vitamin D metabolism and convert 25(OH)D to 1,25(OH)D[1]. This discovery provides a mechanism for proposed non-skeletal functions of vitamin D such as reduction of risk of cancer, cardiovascular diseases, autoimmune diseases and infectious diseases[1].

Calcitriol is proposed to suppress proliferation and differentiation of cancer cells[1,9]. In vitro studies have shown that cancer cells have the VDR receptor and hydroxylase activity and can produce both $1,25,(OH)_2D$ and calcitriolic acid. By acting on VDR $1,25,(OH)_2D$ is proposed to inhibit angiogenesis and induce apoptosis in cancer cells[1]. Vitamin D also plays a role in the

innate immune system through enhancing of chemotaxis and the phagocytic capabilities of macrophages. It also promotes the production of antibacterial peptides cathelicidins which act destroy microbial agents such as Mycobacterium tuberculosis [1]. Moreover, vitamin D decreases the Th1 and Th17 cell response and causes an increase in T regulatory cells. Thus it regulates cytokine and immunoglobulin synthesis. VDR is present in both the nucleus and plasma membrane of mammalian skeletal muscle cells and is proposed to play a role in muscle function[1,9].

Dietary Reference Intakes (DRI)

The fact that vitamin D can be produced in the body aside from being obtained in the diet makes the establishment of DRI for the nutrient challenging [1]. In 2011 the US Institute of Medicine's (IOM) Committee to Review Dietary Reference Intakes for Vitamin D and Calcium-set Estimates Average Requirements (EAR), Recommended Dietary Intake (RDA), Adequate Intake (AI) and Tolerable Upper Intake (UI) recommendations for the US and Canada. The RDA for vitamin D was set at 600 IU (15µg). However, due to increased need, the RDA for older adults was set at 800 IU (20 µg). The EAR is 400 IU (10 µg) for all age groups. The AI for infants is 400 IU, and an RDA has not been set for this age group [11]. The UI for vitamin D is 4000IU/day.

The 2004 World Health Organization (WHO) report on Vitamin and Mineral Requirements for Human Nutrition recommended a daily intake of 200IU as adequate to meet the needs of 98% of the general population. The report identified limited accuracy in estimating dietary intake and skin synthesis as major challenges for setting requirements for the general population [63]. The WHO vitamin and mineral recommended dietary intake recommendations have not been updated since 2004.

Vitamin D Deficiency

Estimation of global prevalence of vitamin D deficiency is difficult, since different investigators use different 25(OH) D concentration cutoffs to define deficiency. However, if 25(OH)D concentration below 75nmol/L is used as indicative of deficiency (insufficiency), one billion people globally can be considered as vitamin D deficient or insufficient [1] .

Calcium and vitamin D deficiency during pregnancy reduces fetal skeletal mineralization [57]. Prolonged vitamin D deficiency can cause growth retardation and skeletal deformities in the fetus and rickets during childhood [12]. Among adults, deficiency can result in muscle weakness and osteomalacia. Furthermore, it can increase the risk of osteoporosis and fractures [1]. Pregnant and lactating women and infants are considered to be at risk of vitamin D deficiency due to a number of reasons. Skeletal effects of vitamin D deficiency seen during pregnancy (after birth) persist in to early childhood (45). Non-supplemented infants are highly dependent on prenatal and postnatal maternal vitamin D status to meet their needs [12]. Infants born to vitamin D-deficient mothers have lower levels of 25(OH)D at birth and are likely to be vitamin D deficient [64]. Risk of deficiency in heightened in infants that are exclusively breastfed and do not receive vitamin D supplementation or sufficient sun exposure [19].

Wagner et al. described three stages of vitamin D deficiency. In the first stage serum concentrations of 25(OH)D decrease, hypocalcemia and euphosphatemia are observed. However, 1,25(OH) $_2$D might be increased or remain unchanged. In the second stage serum concentrations of 25(OH)D continue to decrease. PTH acts to maintain calcium through demineralization of bone. Alkaline Phosphatase (AP) increases slightly, and eucalcemia and hypophosphatemia are observed. In the last stage of deficiency serum concentrations of 25(OH)D are very low, and there is a marked increase in AP. Overt signs of bone demineralization, such as rickets, are observed. And finally, both hypocalcemia and hypophosphatemia are observed [13]. Clinical signs of vitamin

D deficiency include; decrease in gut calcium absorption, low concentrations of serum 25(OH)D, increase in PTH, reduction in bone mass, increased risk of fracture, rickets, osteomalacia and osteopenia [1,13,15]

Rickets was first described by Daniel Whistler in 1645 and Francis Glisson in 1650 [14]. Rickets is a preventable condition and is caused by inadequate intake of vitamin D and limited exposure to sunlight [13]. Enlargement of the skull, enlargement of joints of long bones, curvature of spine and femur, and muscle weakness are classic signs of rickets [13,14]. Rickets is a manifestation of extreme vitamin D deficiency. Thus deficiency exists before clinical signs of rickets are observed [13]. The clinical symptoms of nutritional rickets are commonly seen between 6 to 29 months. However symptomatic rickets is observed early in infancy. Before the development of overt clinical signs of rickets, hypocalcaemia and bone demineralization are observed in infants. Vitamin D deficiency presents as hypocalcemic seizures in infants younger infants[13]. Rickets is usually observed in two forms. In the first form, symptomatic hypocalcemia, seizures during rapid periods of growth, and increased vitamin D need are present. However no physical or radiological signs deficiency are observed. The second form of rickets is more chronic and is accompanied by decreased bone mineralization [13, 14].

Vitamin D Toxicity

Vitamin D toxicity usually occurs from excessive intake of supplements. Toxicity results in hypercalcemia and hypercalciuria which is associated with renal and kidney stones. Symptoms include anorexia, nausea, vomiting, thirst, polyuria, muscular weakness, joint pains, and general disorientation[2,65]. Studies have supplemented with 10, 000 IU/day for up to 5 months with no serious adverse reactions [66-68].

Factors That Affect Vitamin D Status

Skin pigmentation, latitude, dress habits, time of day, season, urbanization, air pollution, lack of vitamin D fortified foods, and prenatal and postnatal maternal vitamin D deficiency are some of the factors that have been documented to affect vitamin D status [18].

Skin Pigmentation

A higher prevalence of vitamin D deficiency has consistently been reported for individuals that have darker skin pigmentation[69]. Individuals with a higher skin pigmentation have a higher melanin content in the skin which absorbs photons of light which are otherwise absorbed by 7-dehydrocholesterol to produce Vitamin D_3[70].

Hall et al. assessed the effect of skin pigmentation, UVB exposure, vitamin D intake, and other factors on serum 25(OH)D concentrations of young adults in the US, and reported need for a higher vitamin D intake for the maintenance of sufficient serum 25(OH)D concentrations in those with darker skin pigmentation [42]. Furthermore, a high prevalence of vitamin D deficiency has been reported in dark skinned individuals that have migrated to areas with limited sun availability compared to those with lighter skin pigmentation [71]. However, the extent of reduction in 25(OH)D concentrations due to skin pigmentation in areas with abundant sun exposure has not been widely explored.

Latitude and Season

It has been proposed that amount of UVB light available for the endogenous production of vitamin D is limited in latitudes below 37^{O} N, in the early morning and late evening and in winter [21]. However, hypovitaminosis D has been reported in countries that are located well below

37^{O} N and have abundant sunshine throughout the year [18-25]. Binkley et al.[47] assessed changes in serum 25(OH)D concentrations among 93 adults in Honolulu, Hawaii (latitude 21°). The study participants similar sun exposure but variable serum 25(OH)D concentration values. The authors noted variable responsiveness to UVB radiation despite abundant sun exposure stressing the need for consideration of other factors such as skin pigmentation together with sun exposure.

Sun-Seeking Behavior

Social, cultural and religious practices that affect dress habits limit sun exposure and can affect 25(OH)D concentrations[43]. Andiran et al., assessed vitamin D status of newborns (n=54) and mothers in Turkey, and found that 46% of mothers and 80% of newborns had 25(OH)D levels < 25 nmol/L. Maternal dress habit (covering hair all the time and face sometimes) was found to be a major predictor of both maternal and infant vitamin D status. These effects were seen despite the availability of abundant sunlight (20). A high prevalence of vitamin D deficiency was also reported for veiled immigrant women [72].

Markers of Vitamin D Status

25-Hydroxyvitamin D (25(OH)D)

Although 1,25-OH2-D is the metabolically active form of the vitamin, 25-hydroxyvitamin D (25(OH)D) is the currently accepted marker of vitamin D status [73]. This is so because 1,25-OH2-D levels can be elevated despite vitamin D deficiency because of secondary hyperparathyroidism. Serum concentration of 25(OH)D reflects dietary and cutaneous vitamin D and body stores. Thus total circulating 25(OH)D is composed of $25(OH)D_2$ and $25(OH)D_3$, has a half-life of about 3 weeks and is the best reflection of vitamin D concentrations in the body [10]. Furthermore, serum 25(OH)D has been shown to be very stable and unaffected by repeated freeze-thaw cycles [74]. The reference categories (cutoffs) used for the interpretation optimal concentrations of 25(OH)D vary widely in the literature [75]. Most researchers have used

concentrations below 50 nmol/L as indicative of vitamin D deficiency [75]. In 2011 the US Institute of Medicine's (IOM) Committee to Review Dietary Reference Intakes for Vitamin D and Calcium concluded that the scientific evidence that supports the use of this cutoff was not adequate [11]. The committee recommends the use of < 30 nmol/L as indicative of risk of deficiency-related to bone health. Intakes above 50 nmol/L are indicative of vitamin D sufficiency (Table 1) [11] .

Serum concentrations of 25(OH)D are affected by sun exposure, skin pigmentation, season, latitude, dietary intake and other factors such us adiposity [17,76] . Different types of assays are used to assess serum 25(OH)D. Radioimmunoassay (RIA), high-performance liquid chromatography (HPLC) and liquid chromatography coupled with mass spectrometry (LC/MS) are some of the widely used techniques for the quantification of serum 25(OH)D [77]. The radioimmunoassay method uses a synthetic antigen that generates an antibody specific for 25(OH)D2 and 25(OH)D3 [77]. HPLC and LC/MS are direct detection methods for the quantification of 25(OH)D_2 and 25(OH)D D_3 [77].

Table 2.2. Classification of 25(OH)D concentrations

Concentration	**Health status**
<30 nmol/L	Vitamin D deficiency leading to rickets and osteomalacia
30–50 nmol/L	Inadequate for bone and overall health in healthy individuals
≥50 nmol/L	Considered adequate for bone and overall health in healthy individuals

Source [11]

Calcium (Ca)

The duodenum and jejunum (proxaima1) are primary sites of calcium absorption. Calcium absorption is regulated by the action of calcitriol and PTH. During lactation calcium requirements are high and absorption is increased to meet these requirements. When dietary calcium intakes are low (< 400 mg/day) intestinal calcium absorption is stimulated through the action of PTH. Hence PTH levels will be elevated[2]. Dietary calcium bound to fiber, phytate, oxalate and fatty acids is excreted in feces. Hence these substances inhibit calcium absorption (although some of the Ca bound to fiber can be hydrolyzed and absorbed) [62] . Some of the Ca bound to insoluble fiber is absorbed in the colon (4-10%) when its liberated when colonic microorganisms ferment fiber[2,78]. Overall 30% of calcium from the diet is absorbed. Blood ionized calcium concentration is tightly maintained between 1.1-1.3mM for physiological process that depend on calcium, including muscular function and bone mineralization to be performed [79] .

Phytate (Phytic acid) which is the storage form of phosphorus, chelates Ca and inhibits its absorption[2]. Plant-based diets are usually high in phytate. A Dietary Phytate: Ca ratio greater than 0.2 is indicative of reduced ca bio-availability in the diet (critical value)[80,81]. Phytic acid (IP6) is hydrolyzed to produce degradation products IP5 to IP1 during digestion. These degradation products are formed when phosphate groups are removed from phytic acid (IP6). With the removal of phosphate groups, chelation potential is reduced (IP6 a more potent chelateor than IP5)[80].

Parathyroid Hormone (PTH)

Blood calcium concentration is tightly controlled through the action of PTH, calcitriol, and calcitonin. A decrease in blood ionized calcium and an increase in phosphate triggers release of PTH by chief cells of the Parathyroid gland [11,79]. PTH directly increases blood calcium concentration by promoting calcium breakdown in the bone and subsequent release to the blood

and by reducing urinary calcium loss. PTH exerts an indirect influence by stimulating the production of calcitriol in the kidneys[11]. Calcitriol increases calcium absorption in the enterocyte and together with PTH acts on the bone and kidney to increase the release of calcium to the extracellular fluid [11,79]. When blood calcium levels are elevated free calcium binds to Ca-sensing receptors (CaSR) on the parathyroid cells and triggers cellular changes that increase intracellular calcium and suppress secretion of PTH. Elevation of intracellular calcium levels intern activates calcium sensitive proteases which degrade PTH in the secretary vesicles of chief cells [82].

PTH stimulates the production of 1,25(OH)D in the kidneys and bone calcium reabsorption [57] through the activation of osteoblasts which in turn stimulate the maturation of osteoclasts. PTH activated mature osteoclasts stimulate the production of $1,25(OH)_2D$ in the kidneys [57]. As it is with calcium, elevated levels of calcitriol suppress PTH secretion. Both calcium and calcitriol inhibit the transcription of PTH gene and reduce levels of PTH in circulation. When vitamin D deficiency is present, calcium absorption is reduced and PTH is elevated to maintain blood calcium concentrations. Fibroblast growth factor 23(FGF 23) increases excretion of phosphate in the kidneys and consequently inhibits PTH secretion[11]. The action of PTH in target cells (Bone, Kidney) works thorough activation of a signaling pathway in the cells. PTH attaches to the PTH/PTHrP receptor located in the cells. Through conformational changes in the G protein-coupled receptor and subsequent production of the second messenger cAMP, protein kinases that can activate or deactivate enzyme[11]. PTH promotes phosphate excretion in the kidneys and reduces excretion of calcium by increasing reabsorption[2,11] Plasma 25(OH)D concentrations are inversely associated with PTH concentrations until 25(OHOD concentrations are around 75-100 nmol/L. After these concentrations PTH concentrations remain constants and do not decrease with increasing 25(OH)D concentrations [1].

Vitamin D and calcium requirements during Lactation

Calcium needs are high during lactation since a lactating woman needs to meet the calcium need of the growing infant as well as her own. Up to 200-300 mg of calcium in a day is transferred into breastmilk, most of it from maternal bone[83,84]. Lactating women lose up to 10% of their bone mineral density to provide sufficient calcium in breast milk for the rapidly growing infant[83,85]. However due to physiological adaptations that take place in the body to meet this high demand calcium and vitamin D requirements are not higher. Bone loss during lactation is restored to pre-pregnancy and lactation states, and no residual effects on bone health are seen[83,84].

Bone resorption during lactation is mainly directed by increased secretion of parathyroid hormone-related protein (PTHrp) during nursing and reduced estradiol secretion. Elevated PTHrp and low estradiol stimulate bone resorption[83,85]. Intestinal calcium absorption is lower than pregnancy but still higher than non-pregnant women, and renal absorption is elevated. For western European women PTH concentrations are typically low and increase during lactation[85]. Levels are elevated in women with very low calcium or high phytate intake[86]. Calcitriol levels are elevated during pregnancy but return to normal during lactation. 25(OH)D concentrations are stable. Calcitriol does not pass into breastmilk. Vitamin D can penetrate breastmilk relatively easily compared to 25(OH)D[85].

Bone and bone turnover markers

Bone tissue has two components. The organic component contains bone cells, proteoglycans and collagen[87,88]. It is repos bile for flexibility of bone and contributes to a third of dry weight of bone. The inorganic component of bone contains *hydroxyapatite (mineral component) and is important for* weight bearing. It is the source of calcium (99%) and phosphorus (85-95%) for extracellular fluids[89] Bone is comprised of cortical and trabecular bone. Cortical bone is the outer layer, which is a thick, dense, highly calcified matrix. Cortical bone accounts for 75% of skeletal

weight. Trabecular bone, found in the interior of bone, has a structure resembling sponge. Cortical bone serves as mineral reservoir while trabecular bone is metabolic active[88].

Bones in a continual state of change modeling and remodeling. Bone modeling results in linear and circumferential growth during childhood and adolescence. Bone remodeling takes place in response to changes in forces (strains, maintain Ca concentration) applied to bones. In order to maintain stability and integrity of bone, it is constantly undergoing remodeling. About 10% of bone material is renewed each year. During bone, remodeling Osteoblasts lay down new bone and osteoclasts remove old bone. Bone formation and bone resorption typically occur simultaneously. Bone remodeling is uncoupled in diseases such as osteoporosis and also during lactation[89].

Markers of bone turnover

Bone turnover markers are biochemical products that reflect the metabolic activity of bone. They can be measured in blood or urine. Bone turnover markers are categorized in two; markers of bone formation or markers bone resorption[90]. Markers of bone formation are direct or indirect products of active osteoblasts and reflect different aspects of osteoblast function. Bone-specific alkaline phosphatase (BAP) and osteocalcin (OC) are two of the most widely used bone formation markers. OC is a non-collagenous protein in bone matrix and is produced by osteoblasts during bone formation. Some portion of OC leaks into the extracellular compartment, where it can be measured. BAP is released when newly formed osteoid undergoes maturation followed by mineralization[90]. It is secreted by secreted by osteoblasts into the extracellular fluid and can be measured in serum. Alkaline phosphatase has hepatic and bone isoforms. The bone isoform is an indicator of bone formation. C-terminal and N-terminal cross-linking telopeptides (CTX, NTX) of the type I collagen molecule is a commonly used bone resorption markers. It is a degradation product of type I collagen.

Maternal Vitamin D Supplementation during Lactation

Increased vitamin D needs during infancy and the effect of maternal vitamin D status on breastmilk vitamin D makes lactation a critical period for vitamin D deficiency. Studies that analyzed breastmilk vitamin D have mostly been carried out in countries with a temperate climate where season significantly influences vitamin D status. These studies have reported that breastmilk vitamin D content ranges from 5- 80 IU/L and have shown that breastmilk vitamin D is low in mothers that themselves are vitamin D sufficient[91,92]. Maternal vitamin D supplementation during lactation can be a feasible intervention to reduce vitamin D deficiency; especially in counties where foods are not fortified with vitamin D[93].

Several randomized controlled trials have been conducted where lactating mothers with limited sun exposure were supplemented with vitamin D (Table 2.5). These studies showed improvements in maternal and infant 25(OH)D through maternal supplementation. Hollis & Wagner [91] supplemented lactating mothers (n=18) with either 1600 IU D_2 & 400 IU D_3 (G1) or 3600 IU D_2 & 400 IU D_3 (G2) for 3 months. Supplementation increased circulating 25(OH)D concentrations for both mothers and infants. The antirachitic activity of milk from mothers receiving 2000 IU/d vitamin D increased by 34.2 IU/L and by 94.2 IU/L for those in group 2. Infant circulating 25(OH)D2 concentrations reflected maternal intake and the amount contained in the milk.

In another 6 months supplementation study, lactating mothers (n=19) were supplemented with 400 IU vitD3/day (control) or 6000 IU vitD3 + 400 IU vitD3/day (part of a prenatal vitamin). Infants of mothers in the control group were supplemented with 300 IU vitD3/day [92]. 25(OH)D was measured at 7 time points every month after enrollment. In the control group maternal 25(OH)D reduced over visits before increasing (lowest 25.9 ng/ml $\pm$ 9.1). Change in

25(OH)D from baseline to visit 7 was: 32.2 ng/ml to 38.4ng/ml. Infants supplemented with vitamin D, and infants whose mothers were in the high dose group had a similar increase in 25(OH)D. Milk antirachitic activity (IU/L) increased significantly in the high-dose group (82.4 IU/L to 873.5 IU/L) compared to control group (59.6 IU/L to 76.3 IU/L). In a follow-up study they showed that when mothers were supplemented with 6400 IU $VitD_3$/Day for 6 months improvements seen in their infants were similar to infants that received 400IU VitD /day[94].

Saadi et al.[28] conducted a combined maternal and infant supplementation study in Unite Arab Emirates (UAE). In the study lactating women (n = 90) were supplemented with 2000 IU/daily or 60 000 IU/monthly. All infants received 400 IU/ daily for the duration of the study period (3 months). Both daily and monthly maternal supplementation significantly increased 25(OH)D concentrations. Supplementation at this dose also reduced infant vitamin D deficiency and did not result in hypervitaminosis D.

Vitamin D Status Assessment in Ethiopia

Only a limited number of studies have assessed markers of vitamin D status in Ethiopia probably because sunshine is abundant in Ethiopia and vitamin D deficiency is thought to be unlikely to occur. Muhe et al.[35] assessed the prevalence of rickets in children with pneumonia and compared children which did not have pneumonia in a case-control study and reported that the prevalence of rickets was significantly higher in children with pneumonia. Furthermore, duration of exclusive breast feeding and crowding were significantly different between groups (Table 2.6).

Table 2.3 Effects of vitamin D supplementation of lactating women on vitamin D markers

Authors	County	Intervention		Timing	Effect on maternal 25(OH)D	Effect on Infant 25(OH)D	Effect on milk activity
		Mothers	Infants				
Hollis (2004) [92] n =18	US	G1: 2000IU/day (1600 IU $VitD_2$ & 400 IU $VitD_3$) Vs. G2: 4000 IU/day (3600 IU VitD2 & 400 IU VitD3)	—	1 month postpartum to 3 months	G1: 69 to 90 nmol/L G2: 82 to 111 nmol/L	G1: 32 to 70 nmol/L G2: 33 to 77 nmol/L	G1: 36 to 70 IU/L G2: 40 to 135 IU/L
Wagner (2006)[93] n =19	US	G1: 400 IU vitD3/day Vs. G2: 6400 IU vitD3/day	G1: 300 IU vitD3/day	1 months postpartum to 6 months	G1: 80 to 96 nmol/L G 2: 85 to 147 nmol/L	G1: 33 to 108 nmol/L G2: 35 to 115 nmol/L	G1: 59.6 to 76.3 IU/L G2: 82.4 to 873.5 IU/L
Basile (2006)[96] n =25	US	G1: 2000 IU VitD3/ day Vs. G2: 4000 IU VitD3/day	—	1 month postpartum to 3 months	G1: 56 to 100 nmol/L G 2: 71 to 108 nmol/L	—	—
Saadi (2007)[28,97] n =90	UAE	G1: 2000 IU $VitD_2$/day Vs. G2: 60 000 IU VitD2/ Month	G1 and G2: 400 IU vitD3/day	1 month postpartum to 3 months	G1: 27 to 42 nmol/L G 2: 23 to 38 nmol/L	G1: 14 to 50 nmol/L G 2: 14 to 45 nmol/L	—

Authors	County	Intervention		Timing	Effect on maternal 25(OH)D	Effect on Infant 25(OH)D	Effect on milk activity
		Mothers	Infants				
Oberhelman (2013)[98] n =40	US	G1: 5000 IU $VitD_2$/day Vs. G2: 150, 000 IU	___	28 days	G1: 73 to 125 nmol/L G 2: 73 to 103 nmol/L	G1: 43 to 100 nmol/L G 2: 40 to 100 nmol/L	Vitamin D3 G1: 18 to 20 nmol/L G2: 18 nmol/L (no change at 28 days)
Czech-Kowalska (2014)[99] n =174	Poland	G1: 400 IU $VitD_2$/day Vs. G2: 1200 IU	G1 and G2: 400 IU vitD3/day	6 months	Mean change G1: 14 + 21 nmol/L G 2: 27 + 24 nmol/L		___
Hollis (2015)[95] n =334	US	G1: 400 IU VitD3/day G3: 6400 IU VitD3/day	G1: 400 IU VitD3/day	4 to 6 weeks postpartum to 6 months	At visit 4 G1: 87 to 80 nmol/L G 2: 99 to 149 nmol/L At visit 7 G1: 89 to 79 nmol/L G 2: 99 to 151 nmol/L	At visit 4 G1: 34 to 109 nmol/L G 2: 40 to 109 nmol/L At visit 7 G1: 36 to 109 nmol/L G 2: 41 to 109 nmol/L	___

In a follow-up study conducted in the same facility, Lulseged & Fitwi [34] identified underweight[OR = 12.7 (95% CI 4.47-11.08)], and lack of exposure to the sunshine [OR = 3.5 (95% CI 1.33-5.84)] as major predictors of rickets. Low calcium intake in children with and without rickets has also been reported [95]. In 1999 Feleke and colleagues[36] compared serum 25(OH)D concentrations of Ethiopian adults and pregnant women with their Norwegian counterparts. Mean 25(OH)D concentrations for Ethiopian participants was 23.5 nmol/L for adults (n=30) and 25 nmol/L for pregnant women (n=31) where as it was 81 nmol/L and 36 nmol/L for Norwegian adults and pregnant women[36]. In a recent assessment of vitamin D status of women of reproductive age, 15.8% had 25(OH)D levels below 30 nmol/L and 69.4% had levels between 30-50 nmol/L[38]. Table 2 presents a summary of studies.

Table 2.4 Vitamin D studies in Ethiopia

Author	Target group	Major finding
Muhe (1997)[35]	Children with Pneumonia (cases) (n=521) Controls (n=500)	Children with Pneumonia were 13.11 more likely to have rickets. Vitamin D might be a predisposing factor for Pneumonia
Lulseged (1999)[34]	Children with rickets (Cases) (n= 157)	Lack of exposure to sunshine and protein energy malnutrition associated with rickets.
Feleke (1999)[36]	Adult men (n=30) & pregnant women (n=31)	Adults : mean 25(OH)D 23.5 nmol/L Pregnant women : mean 25(OH)D 25 nmol/L
Belachew (2005)[95]	Cases (n=30) Children with rickets Controls (n= 104)	Low calcium intake in both cases and controls. Children with rickets had limited sun exposure
Pruitt (2007)[49]	Lactating women (n=108)	39% vitamin D insufficiency
G.Egziabher (2013)[38]	Reproductive age women (n=205)	15.8% < 30 nmol/L 69.4% < 30-50 nmol/L
Wakayo (2013)[37]	Children (aged 11-18) (n=174)	42% < 50 nmol/L 61.8% < 50 nmol/L in urban 21.2% < 50nmol/L in rural

CHAPTER III

MATERIALS AND METHODS

Trial Design

The study was a parallel group, double-blind, randomized, controlled trial conducted among Ethiopian lactating women (Trial registered at clinicaltrials.gov NCT02210884). Participants were randomized to one of the study groups and received 15,000 IU vitamin D_3 or placebo capsules weekly. The study trial profile shows participant enrollment (Figure 3.1). Participants were enrolled in the trial within two weeks after delivery and were followed for 12 months.

Sample Size

The endpoint of this study was to assess mean differences in 25(OH)D concentrations between the two study groups (placebo and treatment). Hence sample size was estimated for a repeated measure, between factors ANOVA test using the software G*Power version 3.0.10 (Franz Faul, Kiel University, Kiel, Germany). A sample size of 108 was calculated to be adequate using a small effect size of 0.25, a 0.05 level of significance and 90% power. After a 15% adjustment for a possible loss to follow-up, the final sample size for the study was 126 (63 in each group).

Study Setting

The study was conducted in four rural communities located in Hawassa Zuria Woreda (Hawassa area district), Southern Ethiopia. Ethiopia has an estimated population of 102,374,044[96]. Approximately 86% of the population reside in rural areas [39]. Agriculture is the primary source of income for the country, and 39% of the population lives below the poverty line of $ l.25 per day [97]. Health care providers widely promote exclusive breastfeeding for the first six months of life. According to the 2016 Ethiopian Demographic and Health Survey, 58% of all children under the age of 6 months are exclusively breastfed [98]. Furthermore, 91% of children aged 12-17 months were currently breastfeeding [98]. Foods are not fortified with vitamin D in Ethiopia.

Ethiopia has two main seasons: dry and rainy. The small rainy season extends from February to May. June is usually dry and is a transition period between the small and main rainy seasons. Heavy rainfall and dense cloud cover characterize the main rainy season, which extends from July to October. The dry season begins in November and ends in January. The country has UV exposure adequate to produce sufficient vitamin D all year round .

The study areas, Alamura, Finchawa, Chefe Koti Jebesa, and Tullo, are located near Hawassa town (7^{o}N, 38^{o}E), which is the capital of the Southern Region. Subsistence farming is the main source of income for the study population. Maize (Zea maysL.) and enset (Enset ventricosum) are the main staple foods [50]. A cross-sectional analysis of vitamin D status of women in the study area reported that 84% of the participants had 25(OH)D concentrations below 50 nmol/L [38].

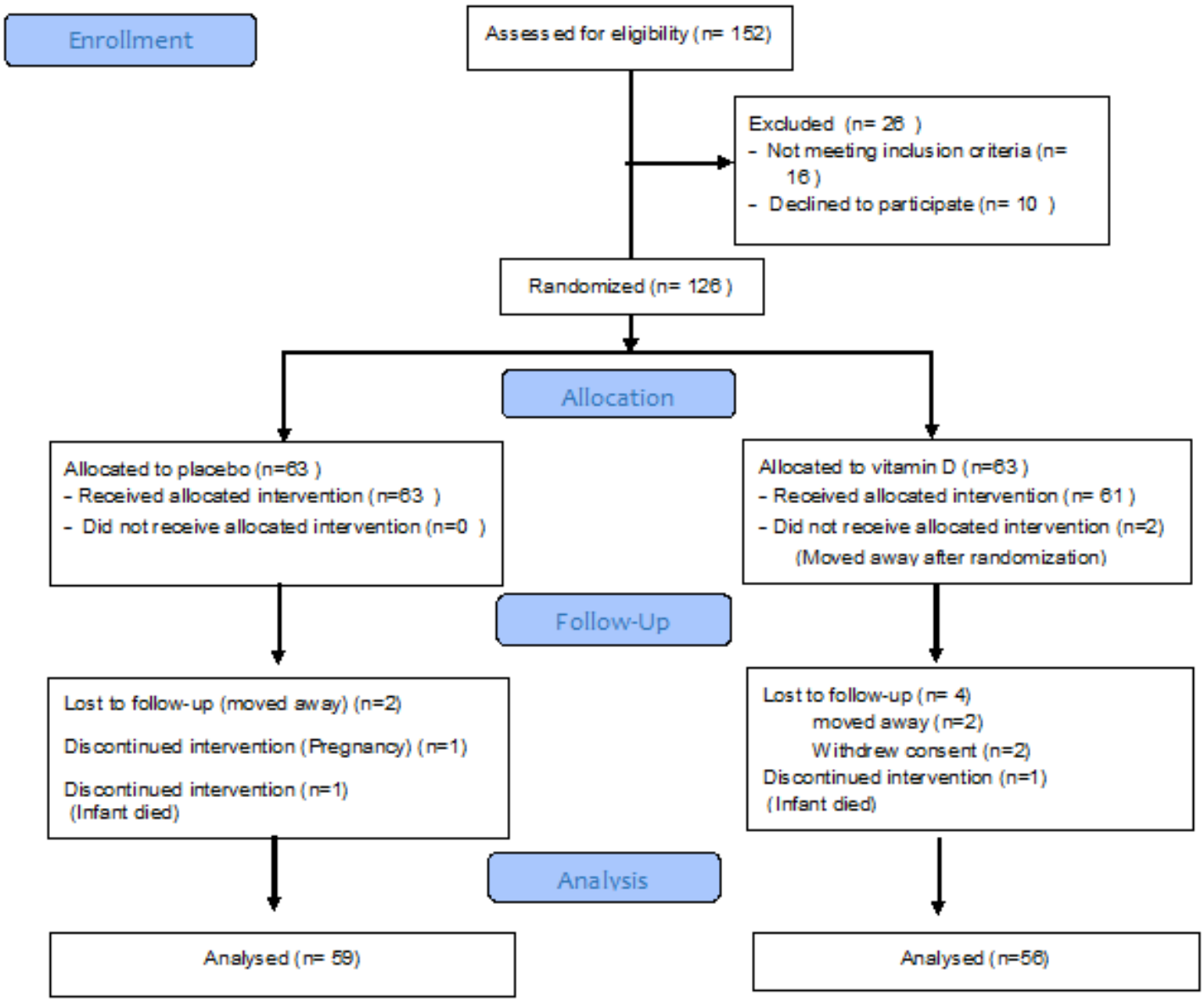

Figure 3.1: Study trial profile

Study Participants

Lactating women who delivered during the enrollment period and met inclusion criteria were recruited. Eligibility was determined using the inclusion and exclusion criteria listed below.

Inclusion criteria:

1) Currently breastfeeding and plan to breastfeed for 12 months.

2) Available for enrollment within two weeks after delivery;

3) Intend to reside in the study area for the duration of the study;

4) Apparently healthy.

Exclusion criteria:

1) Any self-reported chronic or acute disease condition such as liver and kidney disease, diabetes, tuberculosis, and HIV;

2) Not planning to breastfeed;

3) Twin births.

Recruitment, Randomization and Enrollment

At the start of the study, Health Extension Workers (HEW) and Community Health Volunteers (CHV) conducted a mini-census to identify all pregnant women in the study areas expected to deliver within three months. Additionally, HEW and CHV compiled a weekly list of all women who delivered in the study area during the enrollment period. Screening and enrollment was carried out from October 4th, 2014 to January 17th, 2015. Enrollment extended over three and half months in order to meet the target sample size from the four study kebeles. The study team assessed eligibility using a screening questionnaire and provided all the

information (in the local language) eligible mothers needed to make an informed decision about participation in the study. Written consent was mandatory for participation.

A non-participating faculty member of the School of Nutrition, Food Science and Technology performed randomization for the study. The website Randomization.com (http://www.randomization.com/) [99] was used to generate a simple randomization allocation sequence, which consisted of a number (1 to 126) with a corresponding intervention code (AB or CD). To conceal the sequences before allocation, they were sealed in opaque envelopes that were numbered from 1 to 126 on the outside. When a mother met the screening criteria and gave informed consent, she was assigned an envelope that contained an allocation sequence. Envelopes were allocated to participants sequentially following the numbering on the outside of the envelopes. After an envelope was assigned to the mother, the principal investigator opened the sealed envelope and recorded the mother's intervention code in a separate master sheet. The mother's name and other personal details were registered on a card and placed in an envelope. All envelopes were kept locked. Assignment to one of the intervention groups was considered as official enrollment in the study.

Provision of Study Intervention

After enrollment, lactating women in the treatment group were given 15,000 1U Vitamin D_3 capsules weekly while those in the placebo group received capsules containing no vitamin D every week for 12 months. Pure Encapsulations (Pure Encapsulations Inc., Sudbury, MA, USA) formulated both the treatment and placebo capsules, which were identical in appearance. Community Research Workers (CRW) made weekly home visits and gave the intervention to the lactating women. To guarantee compliance, mothers took the capsules while the CRW watched. Potential reactions to the intervention were assessed during these weekly home visits.

Compliance and loss to follow-up

Loss to follow-up was calculated by dividing the number of participants that were lost to follow-up by the number that received the intervention [70]. Total study loss to follow-up was 9 %. Loss to follow-up was 8% in the Vitamin D group and 6% in the control group. Overall compliance (proportion of capsules consumed to distributed) was 99%. The vitamin D group had 99.2% compliance, and the control group had 99 % compliance.

Outcome assessment

Data were collected from October 2014 to January 2016 (15 months). Outcome and explanatory variables were measured during four data collection time points: Baseline, three months, six months and end-line (12 months) (Table 3.1).

Table 3.1 Outcome assessment time points

Assessment period	Duration	Season
Baseline assessment	October 8, 2014 – January 17, 2015	Long dry season (Bega)
3 month	January 16, 2015 -- April 22, 2015	Small rainy season (Belg)
6 month	April 23, 2015 – July 23, 2015	Main rainy season (Kiremt)
12 month	October 15, 2015-January 5, 2016	Long dry season (Bega)

In addition to these measurements, infant morbidity and infant gross motor development were assessed during weekly home visits. Because participants were enrolled within two weeks of delivery baseline assessment was conducted in participant's homes. All subsequent assessments were performed in the local central health post. Table 3.2 provides a summary of variables and assessment times.

Table 3.2. Outcome assessment timeline

Maternal Study procedures	**Baseline**	**3 months**	**6 months**	**End-line**
Maternal blood	√	√	√	√
Maternal Urine	-	√	√	√
Breast milk	√	√	√	√
Maternal anthropometry	√	√	√	√
Skin reflectance	√	√	√	√
Dietary assessment: Weighed food records	-	√	-	-
Questionnaires	-	√	-	-
Questionnaires: Infant feeding practices	-	-	√	√
Ultraviolet (UVB) exposure	-	-	-	√
Infant Study procedures	**Baseline**	**3 months**	**6 months**	**End-line**
Infant blood	-	-	-	√
Infant skin reflectance	-	-	-	√
Infant anthropometry	√	√	√	√
Assessment for rickets	-	-	-	√
Motor developmental milestones	*Weekly*			
Infant morbidity	*Weekly*			

Biological sample collection, initial separation, and storage

Maternal venous blood samples were collected at baseline, 3, 6 and 12 months using lithium heparin coated S-Monovette tubes (7.5 ml) (Sarstedt, Inc., Newton, NC.). Infant venous blood samples were collected at end-line using S-Monovette (4.5 ml) neutral tubes. Sterile butterfly needles (Sarstedt, Safety-Multifly, 21-23G) were used to draw blood. A topical numbing anesthetic cream (Lidocaine 2.5% and Prilocaine 2.5% (EMLA) cream) was also applied a few minutes before the draw to reduce discomfort. An experienced laboratory technician collected the blood samples. Samples were stored in a box with ice until initial separation. They were then centrifuged at 2000 x g for 10 minutes at room temperature to separate plasma or serum. Maternal spot urine samples were collected at 3, 6, and 12 months. Urine samples were provided by the

participants in disposable plastic cups and were then transferred by a research assistant into polyethylene vials. All samples were stored at -15 to -20 ^{O}C at Hawassa University before shipment in dry ice to Oklahoma State University for further analysis.

Anthropometric measurements

Research assistants measured maternal height, weight, mid-upper arm circumference (MUAC), wrist circumference and elbow breadth at baseline, 3, 6 and 12 monhts. Infant weight and MUAC were also measured at these time points. Infant recumbent length was measured at 3, 6 and 12 months. Measurements were taken using standardized techniques [100] and calibrated equipment. Height and length were measured to the nearest 0.1 cm using the Shorr measuring board (Shorr Productions, Olney, MD, USA). Weight was measured to the nearest 0.1kg using a digital scale (SECA Uniscale, UNICEF, Copenhagen). At baseline, infant weight was measured to the nearest 0.01kg using a portable baby scale (SECA354, UNICEF, Copenhagen). Subsequent measurements were taken on the 0.1 kg person scale. A non- stretchable Teflon measuring tape was used to measure MUAC and wrist circumference. Elbow breadth was measured using a skinfold caliper. Infant anthropometric indices of length-for-age, weight-for- age and BMI-for-age were calculated from the measurements using the WHO Anthro software (Version 3.2.2.).

Assessment of rickets

At end-line, a pediatrician screened the infants for clinical signs of rickets. Signs that were examined were delayed fontanel closure, wrist widening, rachitic rosary, double malleoli, bow legs, ping-pong sensation on the skull (craniotabes), and delayed teeth eruption or dental abnormalities. The pediatrician made a clinical diagnosis based on the presence of a combination of two or more symptoms.

Infant morbidity

Trained CRWs assessed the incidence of common childhood illnesses weekly during home visits. Illnesses were defined based on the WHO Integrated Management of Childhood Illness (IMNCI) guidelines[101]. CRWs asked the mother the number of times the infant had vomited, coughed, and had diarrhea in the past seven days. Vomiting was defined as the forceful evacuation of stomach contents. Reflux, which is common in breastfeeding infants was not counted as vomiting. Diarrhea was defined as three or more loose stools in a day. The CRWs also observed if the child had a runny nose at the time of the visit and measured temperature to check for fever. Temperature was measured using a non-contact forehead thermometer. A stopwatch with a countdown timer was used to record breathing rate (The number of breaths per minute) of infants. Sick children were referred to the local health post or hospital for treatment.

Infant gross motor development

Attainment of gross motor milestones by infants was assessed by CRWs weekly (See Appendix 2 for testing procedures and criteria). The first four milestones (Table 3.3) were taken from the Denver Developmental Scale screening test II [102] The remaining milestones were adopted from the World Health Organization (WHO) multicenter growth reference study [63].

Table 3.3 Gross motor developmental milestones

No.	Motor milestone	No.	Motor milestone
1.	Raising head when on front	5.	Sitting without support
2.	Bearing weight on legs	6.	Hands and knees crawling
3.	Sitting supported by arm	7.	Standing with support
4.	Turning and rolling over	8.	Standing unsupported
		9.	Walking supported
		10.	Walking unsupported

Determination of skin pigmentation

Skin reflectance (pigmentation) was measured at three different locations (middle of the upper inner arm, lower outer hand, and in the middle of the forehead) using a portable ColorTec PCM^{+TM} color meter **(**ColorTec Associates, Inc., Clinotn, NJ)**.** The colorimeter quantifies skin pigmentation using the International Commission on Illumination (CIE) LAB color space. The LAB color space describes all perceivable colors in three dimensions (axes) L* (black to white), a* (red to green) and b* (yellow to blue). L* indicates skin lightness and values can range from 0 to 100 with zero indicating perfect black and 100 indicating perfect white [103]. A person with a lighter skin color will hence have a higher L* value compared to a person with a darker skin tone. L* values have been shown to be highly correlated with skin melanin content and has been used to relate skin pigmentation with 25(OH)D concentrations [42,60]. L* values were used as an indicator of skin color in this study as well.

Ultraviolet (UVB) exposure

Maternal personal UVB exposure was measured using personal electronic dosimeters. The dosimeters measure erythemally-weighted UV irradiance at 8-second intervals. The electronic dosimeters were placed on each participant's wrist (like a watch), and they were instructed not to take them off for five days. Clothing worn during this time was assessed using questionnaires on the fifth day to estimate the portion of body surface area exposed to UVB. The daily UVB exposure data recorded on the dosimeters was then converted into standard erythemal dose (SED). Average usual SED exposure was calculated as mean of the five daily values. One standard erythema dose (SED) is equivalent to an erythemal effective radiant exposure of 100 Jm^2

Dietary intake assessment

At 3 months follow up a one day weighed food record (WFR) was taken to assess dietary intake of all participants. A second nonconsecutive WFR was taken from 40 randomly selected participants to allow estimation of the prevalence of inadequate intake (population percentage at risk). Weekdays and weekends were represented in order to account for any day-of-the-week effects. A diet worker stayed in a participant's home from 6 am to 7 pm and measured and recorded every food and beverage consumed during the day using a digital balance (Model CS 2000, Ohaus Corporation, USA). The Ethiopian Food Composition Table Part III[104] and Part IV[105] were used to calculate nutrient intakes using Food processor (Version 8.1, ESHA Research Inc, Salem, OR).

Questionnaires

Trained research assistants administered questionnaires to mothers in the local language at 3, 6 and 12 months. The questionnaires were used to assess demographics, socioeconomic status, water and sanitation practices, infant and maternal sun exposure behavior, dress habits, breastfeeding practices, complementary feeding practices, diet diversity and household food security. Food security was assessed using the Household food insecurity access scale (HFIAS) [106]. Dietary Diversity was assessed using the FAO individual food security assessment tool [107]. Percent Body Surface Area Exposed (BSAE) was calculated from clothing data collected using a modified version of the rule of nines used for burn assessment [108].

Laboratory Analysis

Maternal 25(OH)D concentrations were measured in samples obtained at baseline, 6 and 12 months. Bone turnover markers were measured in baseline and end line samples. Urinary calcium, phosphorus, and creatinine values were measured in samples obtained at 3, 6 and 12

months. Plasma albumin concentration was determined for samples collected at all four data collection time points. Serum 25(OH)D, bone-specific alkaline phosphatase (BAP) were determined from end-line infant serum samples.

25-hydroxi vitamin D (25(OH)D)

Total plasma 25(OH)D concentration were determined using new VDSP (Vitamin D Standardization Program) certified Immunodiagnostic Systems (IDS) 25-Hydroxy Vitamin D^S ELISA Kits (Immunodiagnostic Systems (IDS) Inc. Gaithersburg, MD) . The method is an indirect detection method that uses a competitive ELISA technique. Samples, calibrators and quality controls (QC) were first diluted with a biotin labeled 25(OH)D. The diluted samples were then pipetted into a 96- well plate coated with a highly specific sheep 25(OH)D. The plate was then incubated and washed. Enzyme labeled avidin was added to selectively bind biotin. Color was developed by adding a chromogenic substrate (TMB). The reaction was stopped, and absorbance was read at 450 nm (reference 650 nm) using a plate reader (Synergy HT, BioTek Instruments Inc., Winooski, VT). Color intensity was inversely proportional to 25(OH)D concentrations. A 4 parametric logistic (4PL) curve was used to calculate results.

To maintain maximum assay performance our lab participated in the Centers for Disease Control and Prevention (CDC) Vitamin D Standardization Certification Program (VDSP)[109]. As part of the standardization program, seven external quality control materials were included along with samples in each ELISA plate. Four of these control materials were obtained from the National Institute of Standards and Technology (NIST) standard reference material 972a, and three serum samples were provided by the VDSCP program. No significant bias (average deviation from a true value) was noted for the seven quality control samples across all plates. The individual sample bias ranged from -8.9% to 15.0%. The imprecision (CV) from the replicate measurements was within suggested criteria of $\leq$10% across all seven samples (range 2.4-7.7%).

Parathyroid hormone (PTH)

Maternal parathyroid hormone (PTH) concentrations were measured in plasma using Immutopics ELISA kits (Immutopics Inc, San Clemente, CA). The kit measures activity of intact PTH (PTH 1-84). Standards, controls and samples were pipetted into Streptavidin coated 96 well plates. A PTH antibody solution was then added to the wells and incubated. After washing, a substrate solution was added and plates were incubated. The reaction was then stopped, and absorbance was read at 450 nm using plate reader (Synergy HT, BioTek Instruments Inc., Winooski, VT).

Bone-specific alkaline phosphatase (BAP)

Maternal plasma and infant serum bone-specific alkaline phosphatase (BAP), a marker of bone formation, was measured using an immunoenzymetric assay (Immunodiagnostic Systems (IDS) Inc. Gaithersburg, MD). Samples, standards, and controls were pipetted in a streptavidin coated 96-well plate and reacted with a solution containing a biotin labeled, BAP-specific monoclonal antibody. During incubation, an antibody/BAP complex was formed. Plates were then washed to remove unbound BAP and incubated with an enzyme substrate. The amount of substrate was determined by measuring absorbance at 405 nm in a 96-well plate reader (Synergy HT, BioTek Instruments Inc., Winooski, VT).

Osteocalcin (OC)

Maternal plasma osteocalcin (OC) activity was measured using ELISA kits (Immunodiagnostic Systems (IDS) Inc. Gaithersburg, MD). The assay uses two monoclonal antibodies against human osteocalcin. Samples, standards, and controls were pipetted into a streptavidin coated 96-well plate. A mixture of biotinylated and peroxidase conjugated antibody was then added to the micro wells. Following incubation, the wells were washed, and a

chromogenic substrate was added. Finally, the reaction was stopped with sulfuric acid and absorbance was measured at 405 nm with 650 nm as a reference in a 96-well plate reader (Synergy HT, BioTek Instruments Inc., Winooski, VT).

Cross-linked telopeptide type 1 collagen (CTX)

Maternal plasma C-terminal telopeptides of type 1 collagen (CTX) were determined using ELISA kits (Immunodiagnostic Systems (IDS) Inc. Gaithersburg, MD). The assay uses two monoclonal antibodies against an amino acid sequence where the aspartic acid residue is β-isomerized. To be detected by the ELISA system, the two chains of the amino acids must be cross-linked. Samples, standards, and controls were pipetted in a streptavidin coated 96-well plate followed by addition of a mixture of biotinylated and peroxidase conjugated antibody. A complex of antigens and the two antibodies was formed which bound to the plates. Following incubation, the wells were washed, and a chromogenic substrate was added. The reaction was stopped with sulfuric acid and absorbance was measured at 405 nm with 650 nm as a reference in a 96-well plate reader (Synergy HT, BioTek Instruments Inc., Winooski, VT).

Urinary Calcium, Phosphorous and Creatinine

Maternal urinary creatinine, calcium and phosphorous were analyzed using a clinical chemistry analyzer (Carolina Liquid Chemistries Corp., Bera, California). The urinary creatinine assay uses a series of coupled enzymatic reactions. Creatinine is converted into creatine by the action of creatininase, which is then converted to sarcosine. Sarcosine is then oxidized to produce hydrogen peroxide. In the presence of peroxidase, hydrogen peroxide is quantified at 546 nm by the formation of a colored dye.

The calcium method measures a colorimetric reaction in which calcium forms a complex with arsenazo III which produces a color. Absorbance is then read at 660 nm. The color intensity

is directly proportional to calcium concentration. To determine urinary phosphorus, it was reacted with ammonium molybdate and sulfuric acid forming a phosphomolybdate complex. Change in absorbance was measured at 340 nm.

Serum Albumin and Cholesterol

Serum albumin was determined using the dye-binding property of albumin. Albumin reacts with bromocresol green (BCG) to form a green color. The intensity of the color is proportional to the quantity of albumin.The color change is measured at 600 nm. Serum cholesterol was determined enzymatically. Cholesterol esters were initially hydrolized to free cholesterol and fatty acids. Cholesterol was then oxidized into cholesterol-4-en-3-one and hydrogen peroxide. Peroxidase catalyzes the oxidative coupling of 4-aminoantipyrine with 3,4-dichlor-2-hydroxybenzenesulfonic acid (DHBS) to form a dye which is read at 505 nm.

Statistical Analysis

Data were analyzed using SAS (Version 9.4, SAS Institute Inc., 2013) and IBM SPSS (version 23, 2016, IBM Corp.) Normality was tested using the one-sample Kolmogorov-Smirnov test. Variables that were skewed were transformed using log or square root transformation before analysis. Differences in 25(OH)D and bone markers between groups were tested using repeated measures ANOVA. An independent-sample *t*- test was used to test cross-sectional mean differences. The Mann-Whitney U test was used for non-parametric data (dietary data). Chi-square test was used to test proportion differences. Binomial regression analysis was performed to test differences in child illness incidences. Differences in the attainment of motor milestones between the two groups were tested using COX hazard ratios (Survival analysis). Statistical significance was set at $P< 0.05$.

Ethical Considerations

Ethical clearance for the study was obtained from the Oklahoma State University Institutional Review Board, the Hawassa University Institutional Review Board and the Ethiopian National Health Research Ethics Review Committee. Final approval for implementation was provided by the Food, Medicine and Health Care Administration and Control Authority of Ethiopia (FMHACA).

Safety Assessment and Monitoring

There were no anticipated serious adverse events (SAE) associated with this intervention in which the vitamin D intake on a daily basis was half of the upper limit (RDA Reference) and much lower than studies that used doses up to five times higher (10,000 IU/day) and reported no adverse events. However to ensure the safety of study participants and because the study was considered as a drug trial for regulatory purposes, morbidity data and information on adverse events were collected weekly. The trial was also be overseen by a Data Safety Monitoring Board (DSMB). The DSMB had the responsibility to ensure the safety of study participants and the validity and integrity of data collected. The DSMB received quarterly reports and reviewed relevant data on serious adverse events, morbidity, study outcomes, enrollment and participant retention. No serious adverse events that can be attributed to the intervention occurred

A written report was provided to the DSMB and FMHAC immediately after a serious adverse events (mortality or hospitalization) regardless of causality. The report contained a description of the event, date and time of onset, clinical history, associated signs and symptoms, temporal association with the study, medical management, severity, and causal relationship with the study intervention.

CHAPTER IV

VITAMIN D SUPPLEMENTATION OF ETHIOPIAN LACTATING MOTHERS IMPROVES MATERNAL PLASMA 25(OH)D BUT NOT INFANT 25(OH)D: A RANDOMIZED, DOUBLE- BLINDED, CONTROLLED TRIAL

Abstract

Background: Vitamin D is crucial for calcium and phosphorous homeostasis and bone health. Vitamin D insufficiency is prevalent in populations residing close to the equator despite abundant ultraviolet light (UVB). Response to vitamin D supplementation among lactating women and effect on infants in such settings are unknown.

Objective: To evaluate the effects of vitamin D supplementation provided to lactating women for 12 months on maternal and infant 25(OH)D and other markers of vitamin D status.

Methods: We conducted a double-blind, randomized controlled trial in Sidama Zone (7^{o}N, 38^{o}E), Southern Ethiopia. Lactating women (n=126) enrolled within two weeks postpartum were randomized to receive weekly vitamin D_3 (15,000 IU/week) or placebo. The primary outcome was change in plasma 25(OH)D. Plasma 25(OH)D concentration was determined using the new VDSP (Vitamin D Standardization Program)

certified Immunodiagnostic Systems (IDS) 25-hydroxy vitamin D^S manual ELISA Kits. Data on demographic characteristics, dress habits and sun-seeking behavior were collected using questionnaires. Skin reflectance was measured using a portable color meter. UVB exposure was quantified using portable electronic dosimeters.

Results: Median (IQR) 25(OH)D concentrations were not significantly different between vitamin D and placebo groups at baseline [45 (39, 56) vs 47 (37, 57) nmol/L, p=0.697]. Medain maternal 25(OH)D was significantly higher in the vitamin D group at one year [109 (93, 121) vs 62 (50, 81) nmol/L, $p < 0.0001$]. All women in the vitamin D group were vitamin D sufficient (> 50 nmol/L) by the end of the study. Furthermore, 95% had attained 25(OH)D concentrations > 75 nmol/L compared to 39% in the placebo group. Infant 25(OH)D concentrations at one year were not significantly different between groups (P = 0.974).

Conclusions: Weekly Vitamin D_3 (15,000 IU/week) supplementation safely eliminated vitamin D insufficiency in lactating women. The intervention did not produce a significant difference in infant 25(OH)D at 12 months. Trial registered at clinicaltrials.gov (NCT02210884).

Introduction

Vitamin D is produced in the skin from 7-dehydrocholesterol by the action of ultraviolet light (UVB) and can be obtained from the diet[1-3]. Its main function is regulation of calcium and phosphorous homeostasis thus influencing mineralization of bone[4]. Possible non-calcemic function of vitamin D includes regulation of immune response and cell proliferation[4-6]. Vitamin D deficiency in adults leads to osteomalacia and is also associated with muscle weakness and osteoporosis[2]. In children, deficiency results in rickets and is linked to increased risk of respiratory infections and poor growth [7,8]. Lactating women and infants are at an increased risk of vitamin D deficiency due to increased physiological needs [9,10]. With a high rate of skeletal growth, infants meet their vitamin D needs mainly through breastmilk unless they are supplemented with vitamin D [9]. However, breast milk has been shown to be low in vitamin D, which increases the risk of deficiency[11]. Maternal vitamin D deficiency and exclusive breastfeeding are commonly seen in infants with rickets [4].

Regions located between 37^{O} N and 37^{O}S have adequate sunlight to maintain optimal concentrations of 25(OH)D throughout the year[12,13]. In some of these regions foods are not commonly fortified with vitamin D, so synthesis in the skin is a primary source. Despite adequate ambient UVB hypovitaminosis D has been reported in India[14,15], Iran[16] and Turkey[17] among pregnant women and newborns and in the United Arab Emirates (UAE)[18] among exclusively breastfed infants and lactating mothers. Vitamin D deficiency also has been reported in other target groups residing in countries with abundant sunshine[19,20]. Latitude, season, a conservative dress style limiting the amount of body surface area exposed to the sun, sunscreen use, and air pollution[21] are some factors that affect the amount of UV light that reaches the surface of the skin hence reducing synthesis [2,22]. Individuals with darker skin are at an increased risk of vitamin D deficiency because they have a higher level of melanin that acts as a UV filter[22]. The degree to

which these factors modulate response to vitamin D supplementation in such settings is not known.

Ethiopia is located near the equator and has abundant UVB throughout the year, which is the main source of vitamin D. A conservative dress style and cultural practices such as extended confinement after delivery limit sun exposure. Vitamin D insufficiency is prevalent in pregnant and non-pregnant women[23,24] and adolescents[25]. Exclusive breastfeeding for the first months is widely practiced. Prenatal supplements mainly contain iron and are not widely consumed[26]. Infants are not supplemented with vitamin D. Determination of response to vitamin D supplementation can improve our understanding of vitamin D nutrition in similar settings. Thus, the aim of this study was to evaluate changes in maternal and infant vitamin D status in response to a weekly vitamin D supplement (15,000 IU) vs. placebo administered to lactating women.

Methods

Study setting

The study was conducted from October 2014 to January 2016 in four rural communities (Alamura, Finchawa, Chefe Koti Jebesa, and Tullo) located near Hawassa town (7^{o}N, 38^{o}E), Southern Ethiopia. Subsistence farming is the main source of income for the study population. Maize (Zea maysL.) and enset (Enset ventricosum) are the main staple foods [27]. A preliminary analysis of vitamin D status of women in the study area showed that 84% of the participants had 25(OH)D concentrations below 50 nmol/L [24]. Exclusive breastfeeding for the first six months of life is widely practiced (58%), and breastfeeding continues well after the first year with 91% of children aged 12-17 months currently breastfeeding in 2016 [26]. Foods are not fortified with vitamin D in the country. Ethiopia has UVB exposure adequate to produce sufficient vitamin D all year round.

Participants

Lactating women and infant dyads were enrolled in the study if they were apparently healthy, delivered within two weeks and were currently breastfeeding and planned to continue for 12 months. Screened mothers were excluded if they had any self-reported chronic or acute disease condition (liver and kidney disease, diabetes, tuberculosis, or HIV), had twin births or were not permanent residents of the area.

Study Design

The study was a parallel group, double-blind, randomized, controlled vitamin D supplementation trial. Lactating women enrolled in the trial were randomized to one of the study arms and received 15,000 IU vitamin D_3 or placebo capsules weekly for 12 months.

Intervention

Vitamin D and placebo (composed of plant fiber) tablets were formulated by Pure Encapsulations (Pure Encapsulations Inc., Sudbury,MA) and were identical in appearance. Community Research Workers (CRW) made weekly home visits and gave the intervention to the lactating women. To guarantee compliance mothers took the capsules while the CRW observed.

Simple randomization was used to randomly assign the study participants to one of the two intervention groups. The randomization sequence was generated using the website Randomization.com (http://www.randomization.com/) [28]. The sequences with the corresponding codes were sealed in opaque envelopes that were sequentially numbered on the outside. When a mother met the screening criteria and gave informed consent, she was given an envelope that contained an allocation sequence. Envelopes were allocated to participants sequentially following the number on the outside of the envelopes. After assignment, the researcher (MG) opened the sealed envelope and recorded the mother's intervention code in a separate master sheet.

Ethical clearance for the study was obtained from the Oklahoma State University Institutional Review Board, the Hawassa University Institutional Review Board, the Ethiopian National Health Research Ethics Review Committee and the Food, Medicine and Health Care Administration and Control Authority of Ethiopia (FMHACA). The trial was registered at clinicaltrials.gov (Registration number: NCT02210884). Study participants provided an informed written consent before enrollment.

Outcome assessment

Laboratory analysis

Maternal venous blood samples were collected at baseline, 6 and 12 months using lithium heparin coated tubes (Sarstedt, Inc., Newton, NC.). Infant venous blood samples were collected at end-line. All blood samples were non-fasting. Blood samples were centrifuged at 2000 x *g* for 10 minutes at room temperature to separate plasma from red blood cells. Maternal spot urine samples were collected at 3, 6, and 12 months. All samples were stored at -15 to -20 ^{O}C at Hawassa University before shipment (in dry ice) to Oklahoma State University for further analysis.

Total plasma 25(OH)D concentration was determined using VDSP (Vitamin D Standardization Program) certified Immunodiagnostic Systems (IDS) 25-Hydroxy Vitamin D^{S} EIA manual ELISA Kits. To maintain maximum assay performance our lab participated in the CDC Vitamin D Standardization Certification Program (VDSCP)[29]. As part of the standardization program, seven external quality control materials were run along with samples in each ELISA plate. These control materials were obtained from the National Institute of Standards and Technology (NIST), and the VDSP program. No significant bias (average deviation from a true value) was noted for quality control samples across all plates. The individual sample bias ranged from -8.9% to 15.0%. The imprecision (CV) from the replicate measurements was within suggested criteria of $\leq$10% across all seven samples (range 2.4-7.7%).

Maternal parathyroid hormone (PTH) concentrations were measured in plasma using Immutopics ELISA kits (Immutopics Inc, San Clemente, CA). Urinary creatinine, calcium, phosphorous and serum albumin and cholesterol were analyzed using a clinical chemistry analyzer (Carolina Liquid Chemistries Corp., Bera, CA).

Determination of Skin pigmentation

Skin reflectance (pigmentation) was measured at three different locations (middle of the upper inner arm, outer hand, and in the middle of the forehead) using a portable ColorTec PCM^{+TM} color meter **(**ColorTec Associates, Inc., Clinton, NJ)**.** Measurements were expressed using the International Commission on Illumination (CIE) LAB color space (L* (black to white), a* (red to green) and b* (yellow to blue). L* indicates skin lightness and values can range from 0 to 100. Zero indicated perfect black and 100 indicated perfect white[30]. L* values were used as a marker for skin pigmentation in this study.

Ultraviolet (UVB) Exposure

Individual maternal personal UVB exposure was measured using personal electronic dosimeters after end-line assessment. The dosimeters measure UVB irradiance at 8-second intervals, which is converted to erythemally-weighted standard erythema dose (SED). One SED is equivalent to an erythemal effective radiant exposure of 100 J/m^2. SED provides a measure of UVB exposure independent of skin type. The electronic dosimeters were placed on participant's outer wrist (like a watch) for five days. Clothing worn during this time was assessed using questionnaires to estimate the portion of body surface area exposed to UVB. Usual SED exposure was calculated by taking the average of daily SED values. SED values were adjusted for body surface area exposed BSAE.

Anthropometric assessment and Questionnaires

Maternal height and weight and, infant weight and length were measured using standardized techniques [31] and calibrated equipment. Trained research assistants administered questionnaires to mothers in the local language. The questionnaires were used to assess demographics, socioeconomic status, and infant and maternal sun exposure behavior and dress habits. Percent BSAE was calculated from clothing data collected using a modified version of the rule of nines used for burn assessment [32].

Sample size

The primary endpoint of this study was to assess mean differences in 25(OH)D concentrations between the two study groups (placebo and Vitamin D). Hence sample size was estimated for a repeated measure, between factors ANOVA test using the software G*Power version 3.0.10 (Franz Faul, Kiel University, Kiel, Germany). A sample size of 108 was calculated to be adequate using a small effect size of 0.25, a 0.05 level of significance and 90% power. After a 15% adjustment for a possible loss to follow-up, the final sample size for the study was 126 (63 in each group).

Statistical analysis

Data collected were analyzed using SAS (Version 9.4, SAS Institute Inc., 2013) and IBM SPSS (version 23, 2016, IBM Corp.) Normality was tested using the one-sample Kolmogorov-Smirnov test. Variables that were not normally distributed were transformed using log (25(OH)D) or square root (PTH) transformation before analysis. Predictors of 25(OH)D at baseline were determined using linear regression. Differences in 25(OH)D and other biochemical outcomes between groups were tested using repeated measures ANOVA. An independent-sample *t*- test was used to test cross-sectional mean differences. Chi-square test and Fisher's exact tests were used to test proportion differences. Statistical significance was set at $P< 0.05$.

Results

A total of 152 mother-infant pairs were assessed for eligibility. Of those screened the eligible 126 women were randomized into either the vitamin D (n=63) or placebo (n=63) groups. Of those randomized 11 were lost to follow-up. Reasons for discontinuation were moving away (n=6), infant death (n=2), pregnancy (n=1) and withdrawal of consent (n=2) (**Figure 3.1**). Eleven of women in the Vitamin D and 9% in the placebo group were lost to follow-up. Total compliance (proportion of capsules consumed to distributed) was 99%. The vitamin D group had 99.2% compliance, and the placebo group had 99% compliance. Eighty-one percent of the mothers were 100% compliant. More than half 60% of the mothers reported that they avoided going outside the home for 4 weeks after delivery. However, 97% of mothers reported that they expose newborns to the sun during the first 12 weeks after birth.

Maternal and infant characteristics were similar between groups at baseline (**Table 4.1**). Maternal 25(OH)D concentrations were not significantly different between the vitamin D (44.9 nmol/L) and placebo (46.9 nmol/L) group ($P = 0.697$). The majority of mothers in both groups were vitamin D insufficient using the Institute of Medicine (IOM) cutoffs[33] (58% in placebo group and 59 % in Vitamin D group). Age ($P< 0.001$) and weight ($P= 0.02$) were significant predictors of maternal 25(OH) D concentrations at baseline (**Table 4.2**). Percent BSAE exposed did not significantly predict 25(OH)D concentrations ($P = 0.625$, $R^2 = 14.2\%$). Skin lightness was positively correlated with 25(OH)D ($r = 0.176$, $P = 0.06$,). However, it did not predict 25(OH)D in a multivariate regression model ($P = 0.151$, $R^2 = 14.2\%$).

Changes in maternal 25(OH)D concentrations over time are shown in **Figure 4.1**. Supplementation with Vitamin D_3 had a substantial significant effect on maternal vitamin D status at six months and end-line. At six months, median (IQR) maternal 25(OH) D concentrations were 121 (91, 147) in the vitamin D group compared to 65 (57,79) in the placebo group ($P < 0.001$).The 25(OH)D concentrations declined at end-line in the vitamin D group

compared to the values at six month 109 (93, 121) (p= 0.003). However, values were still significantly higher compared to the placebo group (63 (49, 81), P < 0.001). In the placebo group 25(OH)D concentrations increased significantly at six months compared to baseline (P < 0.001) which was expected since baseline assessment was carried out towards the end of the rainy season where 25(OH)D levels will be at their lowest. Furthermore maternal activity outside the home is likely to be limited towards the end of pregnancy. No change was seen from 6 months to end-line. All lactating women in the Vitamin D group were vitamin D sufficient (> 50 nmol/L) at six months and 12 months (**Table 4.3)**. In contrast, 15.3% and 23.8 % of mothers in the placebo group were insufficient at 6 and 12 months respectively. Controlling for baseline 25(OH)D, maternal weight (p= 0.227), maternal age (p= 0.490), skin lightness (P=0.412), and PTH (P=0.361) did not modify the effect of vitamin D supplementation on end- line 25(OH)D. Infant 25(OH)D concentrations at end-line ranged from 44.3 to 170 nmol/L in the placebo group and 60.2 to 174 nmol/L in the Vitamin D group. There was no statistically significant difference in infant 25(OH)D by treatment group (p = 0.974).

Serum PTH increased at end-line compared to baseline in both groups (**Table 4.4**). PTH concentrations were negatively correlated with 25(OH)D concentrations (**Figure 4.2**).Median (IQR) urinary calcium to creatinine ratio remained similar between the two groups over time. Furthermore, there was no significant difference in the proportion of mother with ratios > 0.7 and > 0.14 in either group. In the vitamin D group urinary phosphorous significantly increased over time (P= 0.013). Unadjusted UVB and adjusted UVB exposure (adjusted for body surface area exposed) measured as standard erythema dose (SED) did not significantly differ between the two groups (P = 0.466) (**Table 4.5).**

Discussion

This study aimed to examine the effect of long-term maternal vitamin D_3 supplementation (15,000 IU/week) during lactation on maternal and infant 25(OH)D concentrations. Supplementation significantly raised maternal plasma 25(OH)D concentrations at 6 and 12 months (end-line). However, we did not see significant differences in infant 25(OH)D concentration between the vitamin D and placebo groups at end-line. All the women and infants in the vitamin D group were vitamin D sufficient using the IOM cut-off for sufficiency (50 nmol/L)[33]. This study provides data on response to vitamin D supplementation relevant for setting with abundant UVB exposure, variable sun exposure, and darker skin pigmentation.

The present findings build on work done by Saadi et al. among women in the United Arab Emirates. Saddi and colleagues supplemented 90 lactating women from 1-month post-partum to 3 months with 2000 IU vitamin D_2/ week or 60,000 IU vitamin D_2/ month. Infants received 400 IU vitamin D_2/week [18,34]. Baseline mean 25(OH)D concentrations were 27 nmol/L (weekly group) and 23 nmol/L (monthly) compared to 48 nmol/L in both groups in our study[34]. At 3 months maternal 25(OH)D concentrations changed (from baseline) from 27 to 42 nmol/L in the weekly group and 23 to 28 nmol/L in the monthly group[34]. Infant 25(OH)D was raised 14 to 50 nmol/L in the weekly group and 14 to 45 nmol/L in the monthly group[18]. Mean increment in maternal 25(OH) was lower compared to our study. The change we saw in maternal 25(OH)D is closer to the response to supplementation reported by Hollis[35,36], and Basile[37] for lactating women in South Carolina. In their most recent, study Hollis et al.[38] evaluated response to two doses of vitamin D_3: 400 IU/day, and 6400 IU/day in lactating women (for 6 months). Infants in the 400 IU group were supplemented with 400 IU vitamin D_3/day. Maternal mean 25(OH)D concentrations at visit 1 and end-line were 89 and 79 nmol/L in the 400 IU group and 99 and 151 nmol/L in the 6400 IU group (an increase of -10 and 52 nmol/L respectively).

Based on equations derived by Heaney et al.[39] we can expect that serum 25(OH)D concentrations will increase by 0.7 – 1.0 nmol/L per 1 µg (40 IU) daily intake of vitamin D.

Accordingly, for the dose we provided, an increase in the 37 to 64 nmol/L range is reasonable. The increase we saw in the vitamin D group (60-75 nmol/L) in our study corresponds to the widely used Heaney et al[39], adult estimates, especially when we take into account the increase in the placebo group (19-22 nmol/L). Our changes were similar to changes seen by Hollis[38] for a 6400 IU vitamin D_3/day dose and 5000 IU vitamin D_3/day for women in the US[34]. However, direct comparisons between our study and these two studies may not be appropriate due to differences in availability of ambient UVB, dress habits and differences in dietary vitamin D intakes.

Interestingly contrary to what has been reported by other studies[38,40,41] maternal supplementation did not result in a significantly higher infant 25(OH)D compared to placebo. At end-line 98% of infants in the placebo group were vitamin D sufficient. Several factors might explain our results. The duration of intervention in other studies ranged from 28 days to 6 months. Our intervention was provided for 12 months, and infant 25(OH)D was assessed at 12 months. It is possible that we might have been able to detect a difference if outcome was assessed earlier in the study, since infant sun exposure increases with age. Our findings also suggest that sun exposure in Ethiopian infants maybe higher compared to other countries.

This study is the first to characterize biochemical response to vitamin D_3 supplementation (15,000 IU/week) among lactating women and their infants living close to the equator. However, this study had several limitations. We measured infant 25(OH)D concentrations only at end-line consequently we cannot make comparisons with baseline 25(OH)D. Furthermore, the inclusion of only one assessment time point prevents us from evaluating change over time. We also did not quantify UVB exposure among infants. Future studies should quantify vitamin D synthesis through this exposure. Moreover, breastmilk vitamin D concentrations should also be measured to determine how much benefit is transferred to infants. The World Health Organization (WHO) sets nutrient intake recommendations for the global population. Its current RDA for vitamin D is 200 IU per day. These recommendations have not been updated since 2004 and the WHO has stated

that more research is needed to update recommendations, especially with regards to the role of vitamin D in infant respiratory health and benefit during pregnancy[42]. Thus future studies should assess effect of supplementation on maternal and infant health outcomes in UVB abundant locations.

Conclusion

In conclusion, in a population residing closer to the equator with darker skin pigmentation, long-term oral maternal vitamin D supplementation significantly and safely improved maternal vitamin D status. The intervention did not produce significantly different improvements in infant vitamin D status.

Acknowledgement

We would like to thank the mothers and infants who participated in the study. We are also grateful for the cooperation we received from the local community leaders and health care providers.

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Table 4.1 Baseline Characteristics of lactating women and infants, Southern Ethiopia 2014

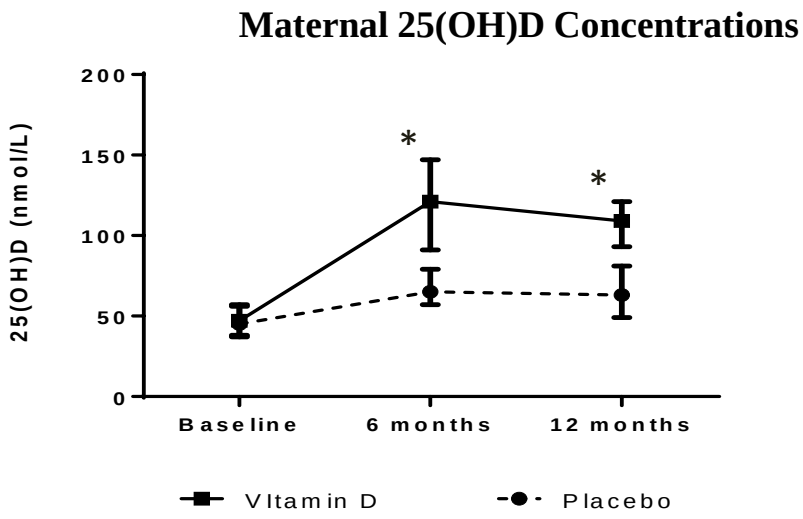
	Placebo (n= 59)	**Vitamin D (n= 56)**	**P-value**
Maternal Age (years)[1]	25 (20,30)	26 (20, 30)	0.469
Weight (kg)[2]	54.2 ± 7.4	53.3 ± 7.3	0.734
Height (m)	1.59 ± 0.06	1.57 ± 0.06	0.422
Infant weight (kg)	3.3 ± 0.5	3.4 ± 0.5	0.880
Infant sex ratio (M:F)	1.1	1.3	0.680
Skin lightness (*L)	34.1 ± 3.5	34.0 ± 3.2	0.647
Body surface area exposed (BSAE)	0.23 (0.16, 0.28)	0.24 (0.20, 0.29)	0.802
Maternal 25(OH)D nmol/L	44.9 (38.5,55.6)	46.9 (37.2,57.1)	0.697
PTH (pg/mL)[1]	54.7 (35.0, 86.3)	50.7 (35.0, 76.4)	0.381
Number of pregnancies [3]			
Primiparous	23.0 (39.0)	22.0 (39.3)	
Multiparous	36.0 (61.0)	34.0 (60.7)	0.937
Maternal education			
No formal education	23.0 (39.0)	16.0 (28.6)	
Primary education	30.0 (50.8)	31.0 (55.4)	0.407
Secondary education or higher	6.0 (10.2)	9.0 (16.0)	
Maternal occupation			
Housewife	51 (86.4)	47 (83.9)	
Employed outside the house	1 (1.7)	3 (5.3)	0.683
Petty trading	7 (11.9)	6 (10.7)	

[1] Median (25th percentile, 75th percentile) (all such values) [2] Mean ± SD (all such values)[3] n(%) (all such values). PTH: Parathyroid hormone.

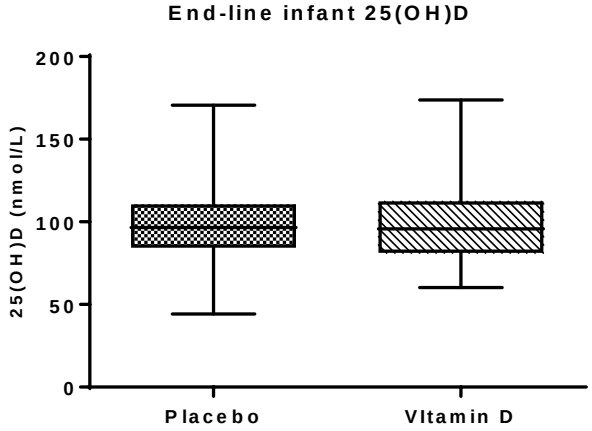
Table 4.2. Predictors of maternal 25(OH)D concentrations at baseline (n= 115), Southern Ethiopia 2014

	β	95 % CI for β		P-value
		Lower	Upper	
Maternal age	-0.741	-1.149	-0.334	0.001
Maternal weight	-0.386	-0.713	-0.059	0.021
Skin Lightness (L*)	0.539	-0.199	1.277	0.151
BSAE	-10.36	-52.4	- 31.6	0.625
Adj. R^2		0.142		

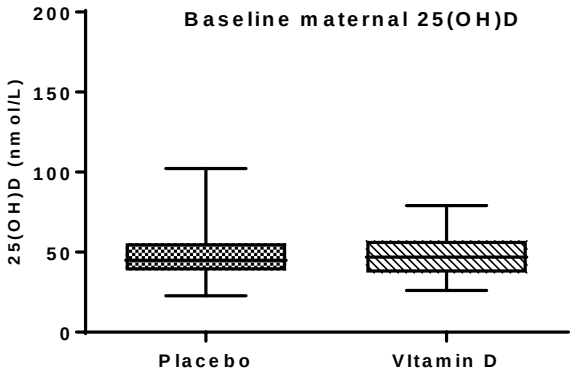
A)

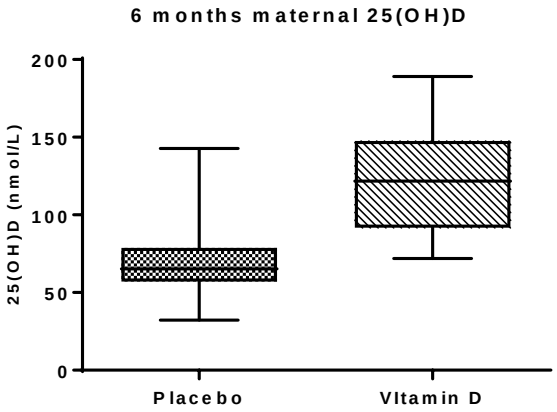

Maternal 25(OH)D Concentrations
25(OH)D (nmol/L)
200
150
100
50
0
*
*
Baseline
6 months
12 months
VItamin D
Placebo

B)


End-line infant 25(OH)D
25(OH)D (nmol/L)
200
150
100
50
0
Placebo
VItamin D

C)





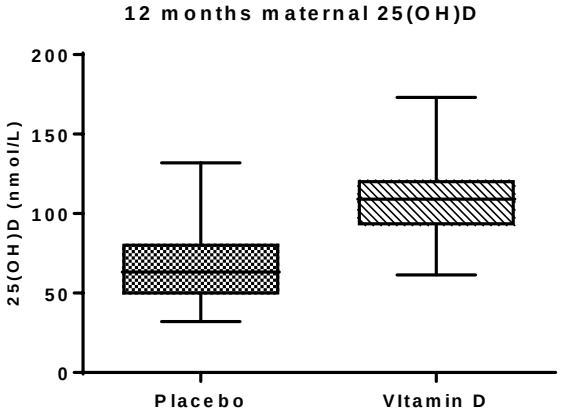


Figure 4.1. A) Significant group and time differences were observed (P< 0.001). Error bars indicate 25th and 75th percentiles. B) & C) Box-and-whiskers plots. Vertical line inside boxes indicates the median. Ends of boxes indicate the first and third quarters. Whiskers show highest and lowest observations. C) No significant differences seen between groups (P = 0.975). Southern Ethiopia 2014 -2015.

Table 4.3. Maternal and infant plasma 25(OH)D categorical distributions by supplementation group, Southern Ethiopia 2014 - 2015

	Placebo	**Vitamin D**	***p* value**
Materanl 25(OH)D concentrations baseline [1]			0.738
< 30 nmol/L	2 (3.4)	3 (5.4)	
30 – 49 nmol/L	32 (54.2)	30 (53.6)	
50-75 nmol/L	20 (33.9)	21 (37.5)	
> 75 nmol/L	5 (8.5)	2 (3.6)	
Maternal 25(OH)D concentrations 6 months			< 0.001
< 30 nmol/L	0	0	
30 – 49 nmol/L	9 (15.3)	0	
50-75 nmol/L	31 (52.3)	2 (3.6)	
> 75 nmol/L	19 (32.2)	54 (96.4)	
Maternal 25(OH)D concentrations 12 months			< 0.001
< 30 nmol/L	0	0	
30 – 49 nmol/L	14 (23.7)	0	
50-75 nmol/L	22 (37)	3 (5.4)	
> 75 nmol/L	23 (39)	53 (94.6)	
Infant 25(OH)D concentrations infants			0.933
< 30 nmol/L	0	0	
30 – 49 nmol/L	1 (1.7)	0	
50-75 nmol/L	5 (8.5)	4 (7.1)	
> 75 nmol/L	53 (89.8)	52 (92.8)	

[1] n(%) (all such values), Chi- square test or Fischer's exact test

Table 4.4. Calcium and vitamin D status markers by supplementation group, Southern Ethiopia 2014 - 2015

	n	Placebo	n	Vitamin D	*p* value		
					Group	time	Group by time
Urinary Ca/Cr ratio[1]					0.586	0.756	0.322
3 months	58	0.003 (0.001, 0.008)	54	0.008 (0.002, 0.024)			
6 months	58	0.006 (0.002, 0.021)	55	0.007 (0.002, 0.022)			
12 months	59	0.011 (0.007, 0.027)	56	0.014 (0.007, 0.032)			
Urinary calcium (mg/dL)					0.973	0.016	0.235
3 months	58	0.2 (0.1, 0.6)	55	0.5 (0.1, 1.0)			
6 months	59	0.5 (0.2, 1.2)	55	0.5 (0.2, 1.2)			
12 months	59	0.8 (0.4, 2)	56	1.0 (0.6,2.0)			
Urinary Creatinine (mg/dL)					0.571	0.001	0.684
3 months	58	62.7 (43.9, 92.5)	54	58.2 (39.1, 77.3)			
6 months	58	74.2 (43.8, 103)	55	68.5 (51.6, 98.8)			
12 months	59	70.8 (46.4, 114.8)	56	62.4 (53.0, 101.0)			
Urinary phosphorus (mg/dL)					0.801	0.001	0.013
3 months	58	16.2 (3.4, 24.1)	55	9.8 (4.3, 24)			
6 months	59	19.5 (6.6, 26.6)	54	16.5 (7.2, 24.9)			
12 months	59	15.2 (9.4, 36.4)	56	29.2 (13, 44.3)			
Albumin (g/dL)					0.060	0.000	0.925
Baseline	59	3.3 (2.7,3.6)	56	3.3 (2.9, 3.7)			
3 months	59	3.9 (3.4,4.4)	56	4.1 (3.5, 4.6)			
6 months	49	3.7 (3.3,3.9)	48	3.8 (3.3, 4.1)			
12 months	58	3.8 (3.4, 4.3)	56	3.9 (3.5, 4.2)			
PTH (pg/mL)					0.463	0.014	0.331
Baseline	59	54.5 (34.7, 85.1)	56	50.7 (35.0, 76.4)			
12 months	59	57.9 (45.3, 84.4)	56	61.2 (42.9, 82.6)			

[1] Median (25th percentile, 75th percentile)

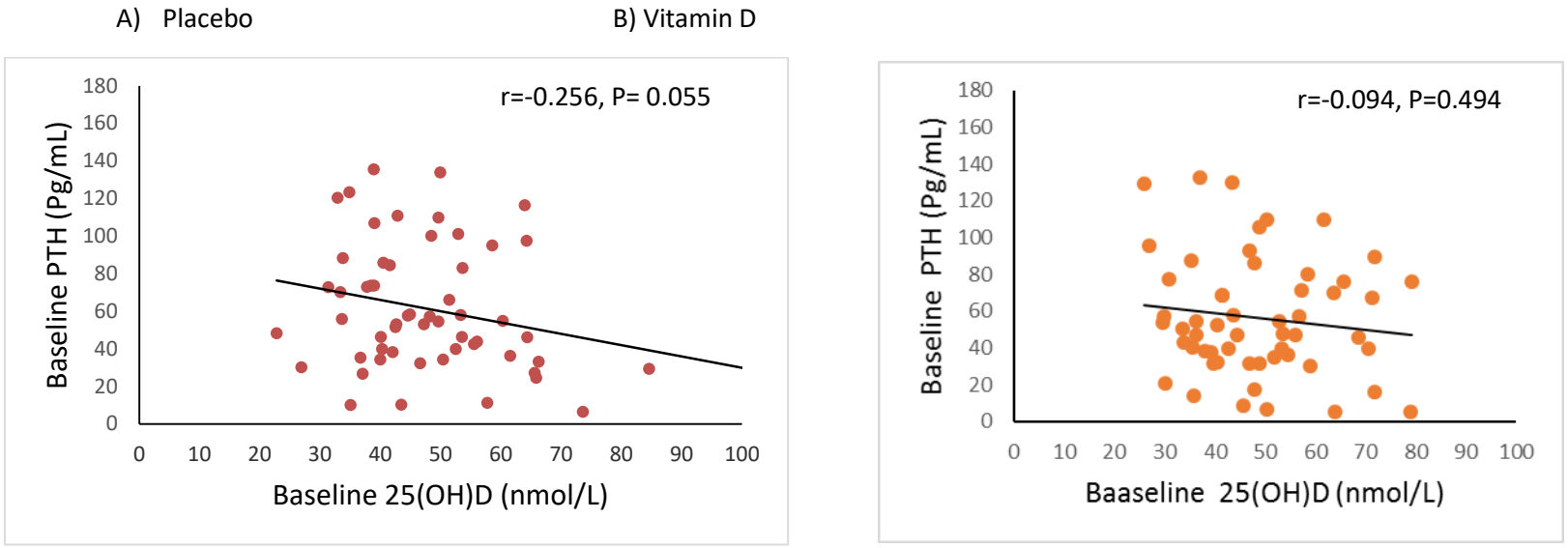
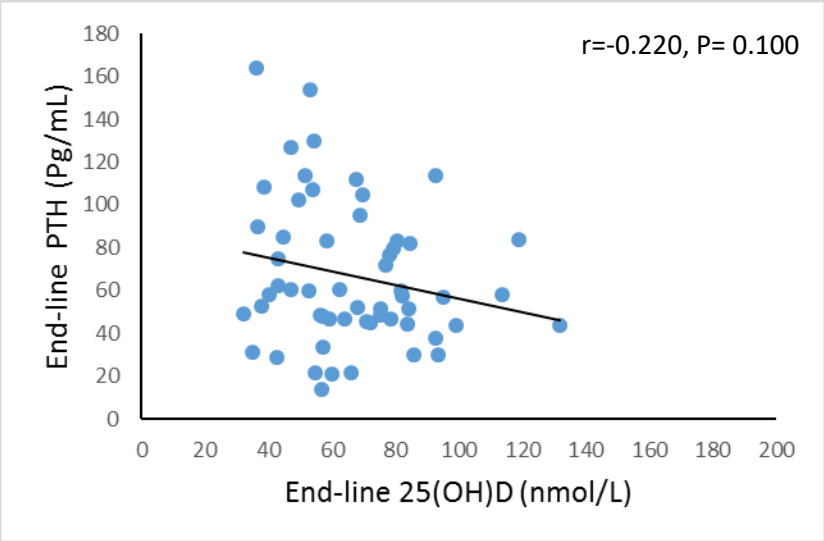


Figure 4.2. Correlation between maternal PTH (pg/mL) and 25(OH)D (nmol/L) at baseline, Southern Ethiopia 2014 - 2015

C) Placebo



D) Vitamin D

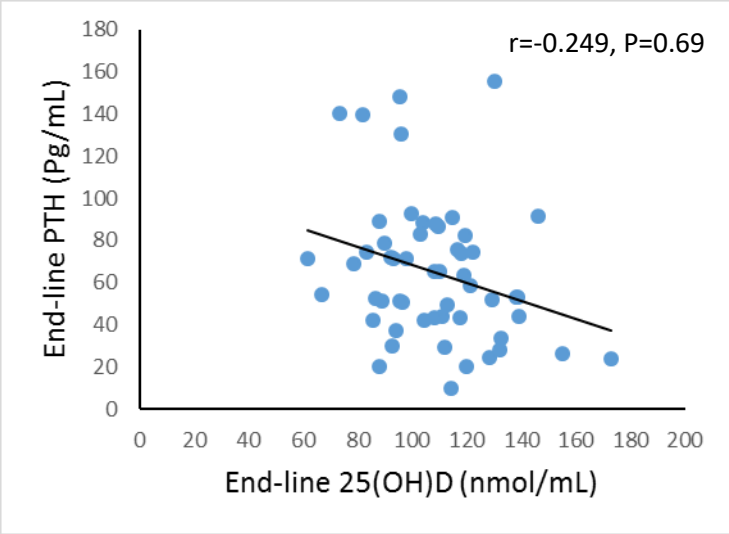


Figure 4.3. Correlation between maternal PTH (pg/mL) and 25(OH)D (nmol/L) at end-line, Southern Ethiopia 2014 - 2015

Table 4.5. Usual UVB exposure (SED) by supplementation group, Southern Ethiopia 2015

	Placebo	**Vitamin D**	***p value***
Usual UVB (SED)[1]	2.0 (1.1, 3.0)	1.7 (0.7. 3.0)	0.466
Adjusted usual UVB (Adj. SED)[2]	0.43 (0.22, 0.73)	0.37 (0.17, 0.77)	0.593

[1] Median (25th percentile, 75th percentile), Mann-Whitney U test. [2] Adjusted for Body Surface Area Exposed (BSAE), SED: Standard erythemal dose

CHAPTER V

EFFECTS OF VITAMIN D SUPPLEMENTATION ON MARKERS OF BONE TURNOVER IN LACTATING WOMEN AND THEIR INFANTS: A 12 MONTHS RANDOMIZED, DOUBLE- BLIND, CONTROLLED TRIAL

Abstract

Background: Vitamin D is crucial for calcium and phosphorous homeostasis and bone health. No study has evaluated whether vitamin D supplementation alters skeletal resorption during lactation, in women with marginal to low dietary calcium intakes.

Objective: To evaluate the effect of a vitamin D_3 vs. placebo supplement provided to lactating women from 2 weeks after delivery for 12 months on maternal and infant markers of bone turnover.

Methods: We conducted a double-blind, randomized controlled trial in Sidama Zone (7^o N, 38^o E), Southern Ethiopia. Lactating women (n=126) enrolled within two weeks postpartum were randomized to receive weekly vitamin D_3 (15,000 IU/week) or placebo for 12 months. Plasma 25(OH)D, bone-specific alkaline phosphatase (BAP), C-terminal telopeptides of type 1 collagen (CTX), Osteocalcin (OC), and parathyroid hormone (PTH) concentrations were measured using ELISA kits. Dietary intake was assessed using weighed food records at three months post-partum. Differences in bone turnover markers were tested using repeated measures ANOVA.

Results: Bone turnover markers and PTH were not significantly different between vitamin D and placebo groups at baseline. At baseline, OC was significantly higher in participants who were vitamin D sufficient (> 50 nmol/L) compared to those participants who were vitamin D insufficient (P = 0.004). There was no significant difference change in bone markers in the vitamin D group compared to placebo. Bone formation markers OC (P = 0.006) and BAP (*p* = 0.018) were significantly higher at end-line in both groups. Infant BAP was numerically higher in the vitamin D group at end-line (*p* =0.128)

Conclusions: Vitamin D supplementation did not significantly influence bone turnover markers in African women with low habitual dietary calcium intake. Trial registered at clinicaltrials.gov (NCT02210884).

Introduction

Vitamin D is essential for calcium homeostasis and bone health. Deficiency is known to cause osteomalacia in adults and rickets in children[2]. Bone is constantly being remodeled and is thus highly metabolically active[89]. Lactation is a highly bone catabolic life stage. Lactating women lose up to 10% of their bone mineral density to provide sufficient calcium in breast milk for the rapidly growing infant[84,110]. A lactating mother provides 200-300 mg of calcium in a day to the infant[84,110,111]. These physiological changes are coupled with changes in bone turnover markers. These markers are of interest since they have a potential to predict osteoporosis and fracture risk[87]. Both markers of bone formation and bone resorption are elevated during lactation indicating high bone metabolic activity[90]. Furthermore, ionized calcium levels are reported to be slightly higher, serum phosphate levels are elevated, and parathyroid hormone (PTH) and calcitriol concentrations are lower[78,111]. Bone resorption during lactation is mainly directed by increased secretion of parathyroid hormone-related protein (PTHrp) during nursing and reduced

estradiol secretion[84]. Several studies have reported that bone loss during lactation is reversed after weaning and has no residual effect on long-term maternal bone health[83,110,112].

However, changes in some biomarkers are not universal to all women and can differ by race and dietary calcium intake and breastfeeding duration. Several studies have reported elevated PTH concentrations, and resistance to PTH stimulated bone resorption in African Americans and South Asians [86,113-115]. In Gambian lactating women with habitually low dietary calcium intake PTH and calcitriol levels were elevated at 1 month post-partum and increased as lactation progressed[86] suggesting atypical physiological responses to lactation.

Ethiopian women commonly breastfeed until a child is two years old. In the most recent demographic and health survey 85% of children age 12-23 months and 76% aged 20-23 months were still breastfeeding[98]. Additionally, the diet is low in calcium with a median (IQR) intake of 479 (220, 680) mg/day reported for pregnant women in the study area[50]. The interval between pregnancies is short, and fertility rate is high. In a study that evaluated response to lactation in Gambian women that had similar characteristics to our study population, lactating women had lower BMD compared to Caucasians at 1-year post-partum[116]. The lower BMD in these Gambian women is contrary to the findings of studies that have reported higher BMD in African Americans despite low 25(OH)D and high PTH concentrations compared to caucasians[113,114].

The Institute of Medicine (IOM) classifies 25(OH)D concentrations < 50 nmol/L as inadequate for bone and overall health [11] 25(OH)D concentrations below 30 nmol/L are associated with rickets and ostomalacia[11]. Studies that have measured vitamin D in Ethiopian women have shown that vitamin D insufficiency and deficiency are prevalent [36,38,117]. Vitamin D status may influence the physiological adaptation to meet calcium needs during lactation. This combined with low dietary calcium intake and long-term exposure to bone loss due to repeated pregnancy can compromise bone health in this population. To our knowledge, no study has

evaluated whether vitamin D supplementation alters bone resorption during lactation, in women with marginal to low dietary calcium intakes. Thus the aim of this study is to assess the effect of vitamin D supplementation on markers of bone turnover in a population that has low calcium intake and prolonged lactation.

Methods

Participants & settings

Lactating women and their infants residing in Alamura, Finchawa, Chefe Koti Jebesa, and Tullo, located near Hawassa town (7^{o}N, 38^{o}E), Southern Ethiopia were enrolled in the study from October 2014 to January 2016. The women were eligible for enrollment if they delivered in the past two weeks, were breastfeeding at the time of enrollment and were apparently healthy. The study area is characterized by a reliance on subsistence farming as a main source of income. Maize (Zea maysL.) and enset (Enset ventricosum) are staple foods.

Study Design and intervention

The study was a parallel group, double-blind, randomized, controlled vitamin D supplementation trial. Lactating women enrolled within two weeks after delivery were randomized to one of the study groups and received either 15,000 IU vitamin D_3 or Placebo capsules weekly for 12 months. Ethical clearance for the study was obtained from the Oklahoma State University Institutional Review Board, the Hawassa University Institutional Review Board, and the Ethiopian National Health Research Ethics Review Committee and the Food, Medicine and Health Care Administration and Control Authority of Ethiopia (FMHACA). The trial was registered at clinicaltrials.gov (Registration number: NCT02210884).

Outcome measures

Laboratory methods

Non-fasting venous blood samples were collected using Lithium Heparin coated tubes (Sarstedt, Inc., Newton, NC.). Infant blood samples were collected only at end-line (12 months). Blood samples were centrifuged at 2000 *x g* for 10 minutes at room temperature to separate plasma from red blood cells. Samples were stored at -15 to -20 ^{O}C at Hawassa University before shipment (in dry ice) to Oklahoma State University for further analysis. Maternal bone turnover markers were analyzed at baseline and end-line.

Maternal and infant plasma bone-specific alkaline phosphatase (BAP), a marker of bone formation, was measured using EIA immunoenzymetric assay (Immunodiagnostic Systems (IDS) Inc. Gaithersburg, MD). Maternal plasma osteocalcin (OC) activity, a marker for formation, was measured using ELISA kits (Immunodiagnostic Systems (IDS) Inc. Gaithersburg, MD). The bone resorption marker cross-linked telopeptide type 1 collagen (CTX) was determined in maternal plasma using ELISA kits (Immunodiagnostic Systems (IDS) Inc. Gaithersburg, MD). Maternal intact parathyroid hormone (PTH) concentrations were measured in plasma using Immutopics ELISA kits (Immutopics Inc, San Clemente, CA). The kit measures activity of intact PTH (PTH 1-84). Total plasma 25(OH)D concentration was determined using the new VDSP (Vitamin D Standardization Program) certified Immunodiagnostic Systems (IDS) 25-Hydroxy Vitamin D^{S} EIA manual ELISA Kits.

Assessment of rickets

At end-line, a pediatrician screened the infants for clinical signs of rickets. Signs that were examined were delayed fontanel closure, wrist widening, rachitic rosary, double malleoli, bow legs, ping-pong sensation on the skull (craniotabes), and delayed teeth eruption or dental

abnormalities. The pediatrician made a clinical diagnosis based on the presence of a combination of two or more symptoms.

Dietary intake assessment

At 3 months follow up a one day weighed food record was taken to assess dietary intake of all participants. Weekdays and weekends were represented in order to account for any day-of-the-week effects. A diet worker stayed in a participant's home from 6 am to 7 pm and measured and recorded every food and beverage consumed during the day using a digital weighing scale (Model CS 2000, Ohaus Corporation, USA). The Ethiopian Food Composition Table Part III and Part IV were used to calculate nutrient intakes using Food processor (Version 8.1, ESHA Research Inc, Salem, OR).

Anthropometric assessment and Questionnaires

Maternal height and weight, infant weight and length were measured using standardized techniques [100] and calibrated equipment. Trained research assistants administered questionnaires to mothers in the local language. The questionnaires were used to assess demographics, socioeconomic status.

Statistical analysis

A sample size of 108 was calculated to be adequate to test mean differences in outcome variables, using a small effect size of 0.25, a 0.05 level of significance and 90% power. After a 15% adjustment for a possible loss to follow-up, the final sample size for the study was 126 (63 in each group). Normality of continuous variables was tested using the one-sample Kolmogorov-Smirnov test. Data was presented as mean (SD) and median (IQR). Variables that are not normally distributed were transformed using log or square root transformation. Baseline predictors of bone markers were tested using linear regression. Differences in bone turnover

markers were tested using repeated measures ANOVA. An independent-sample *t*- test was used to test cross-sectional mean differences. Mann-Whitney U test was used for non-parametric data (Dietary data). Statistical significance was set at P< 0.05. Data were analyzed using SAS (Version 9.4, SAS Institute Inc., 2013) and IBM SPSS (version 23, 2016, IBM Corp.)

Results

Baseline characteristics of participants

Participant flow through the trial has been reported elsewhere (**Figure 3.1**). Maternal haracteristics and bone markers at baseline were not significantly different between the vitamin D and placebo groups (**Table 5.1**). Mean maternal age at enrollment was 26 years and number of days postpartum ranged from 1 to 15 days. A majority of the lactating women initiated breastfeeding (94 %) immediately after birth. All women (100%) were still breastfeeding at end-line and 90% were breastfeeding up to 7-9 times in a day. At baseline median OC concentrations were significantly higher in women who were vitamin D sufficient (> 50 nmol /L) compared to those who were insufficient (< 50 nmol/L) (P = 0.004) (**Table 5.2**). BAP concentrations were higher in vitamin D sufficient women and PTH was lower in Vitamin D deficient women (**Table 5.3**). A unit increase in 25(OH)D was associated with a 0.004 increase in OC in a model that included age and days post-partum (P = 0.01, Adj. R^2 = 0.221). 25(OH)D was significantly negatively correlated with the ratio of CTX to OC (CTX:OC) (r= -0.197, P = 0.037). BAP positively correlated with 25(OH)D (r=0.19, P= 0.044) and negatively with age (r = -0.248, P = 0.008). Median (IQR) dietary intake at 3 months post-partum was 624 (389, 884) mg/day and calcium intake was not statistically different between the two groups (P = 0.52) (**Table 5.4**).

Effect of vitamin D supplementation

Bone formation and bone resorption markers were positively correlated at baseline and end-line. PTH was negatively correlated with both formation and resorption markers. Infant end-line BAP was positively correlated with maternal end-line BAP and OC **(Table 5).**

There was no significant difference in maternal BAP, OC, CTX, and PTH by supplementation group. Vitamin D supplementation did not significantly affect maternal and infant bone turnover markers. Maternal bone formation markers OC and BAP were significantly higher at end-line compared to baseline in both groups ($P = 0.006$ and $P = 0.018$ respectively). PTH was also significantly higher at end-line compared to baseline in mothers. However, this relationship disappeared when controlling for maternal age. Percent change in 25(OH)D did not predict percent change in maternal BAP, OC, CTX and PTH. Infant BAP was numericaly higher in the Vitamin D group compared to placebo ($P = 0.128$). Infant BAP was significantly correlated with age ($r = 0.298$, $P < 0.001$) but was not significantly correlated with height velocity ($r = -0.046$). Infants who had clinical signs of rickets had a higher mean BAP concentration compared with those with no signs of rickets [51.6 (20.5) vs. 46.8 (20.9)].

Discussion

Among lactating women with relatively low dietary calcium intake, maternal vitamin D_3 supplementation (15,000 IU/week) for 12 months did not significantly influence bone turnover markers OC, BAP, CTX, and PTH. Bone formation markers were significantly higher at end-line in both the vitamin D and placebo groups. Supplementation did not result in PTH suppression in the study participants. Infant BAP concentrations were slightly higher in the vitamin D group. This study provides information on the effect of vitamin D supplementation on markers of bone turnover in lactating East African women, with limited dietary calcium intake for whom no data exist.

Similar to our findings, vitamin D supplementation did not affect bone turnover markers in studies done among Irish adult men and women, [23] Austrian hypertensive patients [24] and healthy Irish young and elderly adults [25]. This finding support existing evidence that maternal physiological adaptations during pregnancy and lactation enable mothers to meet needs despite marginal intakes of calcium and vitamin D intakes [4,26]. Another factor that might explain the lack of benefit is that baseline 25(OH)D concentrations were closer to sufficiency (46 nmol/L) in our study, and very few participants had values < 30 nmol/L. More benefit might have been observed if more participants had baseline 25(OH)D concentrations below 25 nmol/L which have been linked with bone disease[18].

Our findings build on work carried out by Prentice[13] and Sawo[27] that characterized longitudinal changes in biochemical markers of bone metabolism and bone mineral content in African lactating women, with low habitual dietary calcium intake. Prentice et al[13]. supplemented 60 Gambian lactating women with calcium (714 mg/day) for 6 months post-partum and assessed changes in bone metabolism markers at baseline, 13 weeks and 6 months after supplementation stopped (at 52 weeks). Similar to our findings PTH levels increased during lactation compared to baseline. Osteocalcin and BAP levels also increased during lactation in both groups, although the

increase was slight for BAP. In 2015, Madar et al.[28] supplemented 251 immigrants (from Sub-Saharan Africa, South Asia, Middle East and North Africa) in Norway with either 400 IU, 1000 IU vitamin D_3/day or placebo for 16 weeks. Supplementation did not affect bone markers including CTX despite an increase in 25(OH)D. Supplementation however suppressed PTH levels. In our study supplementation did not suppress PTH levels. Furthermore, the increase in PTH did not result in a significant corresponding increase in CTX. The elevated PTH concentrations in our study population is similar to what has been reported for lactating African American women[29] and African-Americans in general[11,30,31].

We saw a positive correlation between bone formation and bone resorption markers which supports the existing evidence that bone formation and bone resorption are coupled. Hence we would expect to see increased levels of markers of formation and resorption. Similar to what has been reported in other studies [32], study children with clinical signs of rickets had higher BAP concentrations which is expected for recovery.

This RCT had several limitations. Detection of changes in bone turnover markers was not the primary outcome of this study. Consequently, we had limited power to detect changes in infant bone markers in response to supplementation. We also did not measure BMD and BMC that are better markers of bone health. CTX levels have been shown to be influenced by diurnal variations[6]. Due to logistical difficulties, our blood samples were not all drawn at the same time, and we did not adjust for time of collection during analysis. Thus our results should be interpreted with caution. Finally, due to ethical reasons, we were not able to collect fasting blood samples.

Conclusion

In conclusion, in this RCT vitamin D supplementation during lactation did not impact bone markers. Further studies should measure bone markers in non-pregnant and non- lactating women to characterize bone turnover in other life stages. Moreover, measurement of bone markers will enable the establishment of population-specific reference ranges.

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Table 5.1. Baseline Characteristics of lactating women, Southern Ethiopia 2014

	Control group (n= 59)	**Vitamin D group (n= 56)**	***P* value**
Days postpartum [1]	9 ± 3.8	10.1 ± 4.4	0.281
BMI (kg/m^2)	21.2 ± 2.2	21.4 ± 2.5	0.827
Frame size[2]			0.636
Small	10 (16.9)	9 (16.1)	
Medium	37 (62.7)	39 (69.9)	
Large	12 (20.3)	8 (14.3)	
Albumin (g/dL)	3.2 ± 0.6	3.2 ± 0.7	0.338
Intact PTH (Pg/mL)[3]	54.7 (35.0, 86.3)	50.7 (35.0, 76.4)	0.381
BAP (µg/L)	8.0(7.1, 10.7)	9.1 (7.1, 10.5)	0.370
Osteocalcin (ng/mL)	10.5 (7.2,14.8)	9.8 (7.9, 15.9)	0.509
CTX (ng/mL)	0.4 (0.3, 0.5)	0. (0.2, 0.6)	0.464
Median interpregnancy Interval	36 (21.0, 45)	36 (24,60)	0.233
Less than a year	7 (11.7)	5 (8.9)	
1 to 2 years	10 (16.7)	6 (10.7)	
2 to 5 years	15 (25.0)	19 (33.9)	
> 5 Years	4 (6.7)	4 (7.1)	

[1] Mean ± SD (all such values) [2] n(%) (all such values) [3] Median (25th percentile, 75th percentile) (all such values). Frame size= Height (cm)/ Wrist circumference (CM) (Small frame ≥ 11, Medium frame= 10.1-11 and Large frame ≤ 10.1). PTH: Parathyroid hormone, BAP: Bone specific Alkaline phosphatase, CTX: Cross-linked telopeptide type 1 collagen.

Table 5.2. Bone turnover markers at baseline by vitamin D status in lactating women, Southern Ethiopia 2014

	Vitamin D insufficient (25(OH)D < 50 nmol/L) (n= 69)	**Vitamin D sufficient (25(OH)D ≥ 50 nmol/L) (n= 46)**	*p value*
PTH (Pg/mL)[1]	62.1 (38.0, 85.9)	53.6 (32.3, 76.3)	0.101
BAP (µg/L)	8.9 (7.2, 10.0)	9.7 (6.7, 12.3)	0.395
Osteocalcin (OC) (ng/mL)	10.1 (6.7, 12.5)	14.6 (8.6, 19.7)	0.004
CTX (ng/mL)	0.4 (0.3, 0.5)	0.4 (0.3, 0.5)	0.376

[1] Median (25^{th} percentile, 75^{th} percentile) (all such values) . PTH: Parathyroid hormone, BAP: Bone Specific Alkaline Phosphatase and v CTX: C-terminal telopeptides of type 1 collagen

Table 5.3. Simple and multiple regression analysis for predictors of OC and CTX in lactating women at baseline, Southern Ethiopia 2014

				95 % CI for β	
	β	SE	*P*	Lower	Upper
OC Model[1]					
25(OH)D	0.004	0.002	0.011	0.001	0.008
Maternal age	-0.342	0.233	0.145	-0.804	0.12
Days postpartum	0.027	0.006	< 0.001	0.016	0.038
Adj. R2			22.1		
CTX Model[2]					
Days postpartum	0.016	0.004	< 0.001	0.008	0.025
Adj. R2			11.5		

[1] Predictors of OC in a Multiple regression analysis [2] Predictor of CTX in a simple regression analysis

Table 5.4. Maternal dietary calcium and phytate intake at 3 months postpartum, Southern Ethiopia 2015

	Control group (n= 59)	Vitamin D group (n= 56)	*p value*
Dietary Calcium (mg/day)[1]	594 (385, 879)	663 (472, 894)	0.52
Phytate: Calcium molar ratio[1]	0.20 (0.14, 0.30)	0.16 (0.10, 0.25)	0.077

Table 5.5 Correlation between bone turnover markers and 25(OH)D at baseline and end-line in lacataing women and infants, Southern Ethiopia 2014-2015

	Baseline					End-line				
	25(OH)D	**OC**	**BAP**	**CTX**		**25(OH)D**	**OC**	**BAP**	**CTX**	**PTH**
25(OH)D	1				25(OH)D	1				
OC	0.238[b]	1			OC	-0.035	1			
BAP	0.190[a]	0.481[c]	1		BAP	-0.205[a]	0.477[c]	1		
CTX	0.069	0.411[c]	0.223[a]	1	CTX	0.017	0.177	0.384[a]	1	
PTH	-0.195[a]	-0.021	-0.041	-0.040	PTH	-0.195a	0.249[b]	0.365[c]	0.367[c]	1
					Infant BAP	-0.088	0.265[b]	0.219[a]	0.108	0.034

Values in table are Pearson correlation coefficients (r). OC: Osteocalcin, BAP: Bone Specific Alkaline Phosphatase, CTX: C-terminal telopeptides of type 1 collagen,PTH: Parathyroid hormone. [a] $P < 0.05$, [b] $P < 0.01$, [c] $P < 0.001$.

Table 5.6. Bone turnover markers at baseline and end-line by supplementation group among lactating women and infants, Southern Ethiopia, 2014 - 2015

	Placebo	Vitamin D	P-value		
			Group	time	Group x time
BAP (µg/L)[1]					
Baseline	9.1 (7.1,10.7)	9.4 (7.1, 10.5)	0.471	0.018	0.216
12 months	25.7 (16.4, 32.2)	23.6 (14.9, 29.0)			
Osteocalcin (ng/mL)					
Baseline	11.3 (7.2, 14.8)	12.6 (7.9, 15.9)	0.297	0.006	0.934
12 months	20.4 (14.0, 27.2)	21.7 (14.8, 28.3)			
CTX (ng/mL)					
Baseline	0.42 (0.28, 0.52)	0.40 (0.24, 0.57)	0.999	0.127	0.593
12 months	0.39(0.21, 0.59)	0.41 (0.21, 0.50)			
PTH (pg/mL)[2]					
Baseline	54.5 (34.7, 85.1)	50.7 (35.0, 76.4)	0.463	0.238	0.398
12 months	57.9 (45.3, 84.4)	61.2 (42.9, 82.6)			
Infant BAP (ng/mL)[3]					
Baseline	--	--			
12 months	43.5 (26.8, 55.8)	49.5 (32.6, 67.1)	0.128	--	--

[1] Median (25th percentile, 75th percentile) (all such values),[2] Controlled for maternal age

CHAPTER VI

EFFECTS OF MATERNAL VITAMIN D SUPPLEMENTATION DURING LACTATION ON INFANT GROWTH, GROSS MOTOR DEVELOPMENT AND MORBIDITY: RESULTS FROM A RANDOMIZED, DOUBLE-BLIND, CONTROLLED TRIAL

Abstract

Background: Vitamin D is a potent modulator of the immune system. Furthermore, it plays an important role in bone mineral mineralization and skeletal growth. Studies that explore the role of vitamin D in the alleviation of infectious disease and promotion of growth are needed.

Objective: To evaluate the effect of vitamin D supplementation provided to lactating women for 12 months on infant growth, attainment of gross developmental milestones and incidence of common childhood illnesses.

Methods: We conducted a double-blind, randomized controlled trial in Sidama Zone (7^{o} N, 38^{o} E), Southern Ethiopia. Lactating women (n=126) enrolled within two after delivery were randomized to receive weekly vitamin D_3 (15,000 IU/week) or placebo for 12 months. Infant length, weight, and mid upper arm circumference (MUAC) were measured at baseline, 3, 6, and 12 months. Attainment of ten gross motor milestones and incidence of cough, diarrhea, vomiting and runny nose were assessed weekly.

Differences in anthropometric status were tested using repeated measures ANOVA. Negative binomial regression analysis was performed to test differences in child illness incidences. Differences in the attainment of motor milestones between the two groups were tested using COX hazard ratios.

Results: Infant and maternal anthropometric indices were not significantly different between vitamin D and placebo groups at baseline. There was no significant difference in change in height-for-age Z scores (P=0.5), weight-for-age z scores (P=0.267) or weight-for-height z scores (P = 0.299) in the vitamin D group compared to placebo. There was also no significant difference in the attainment of gross motor milestones and incidence of common childhood illnesses.

Conclusions: Vitamin D supplementation did not significantly affect growth, gross motor development or incidence of common childhood illnesses. Trial registered at clinicaltrials.gov (NCT02210884).

Introduction

Vitamin D has been shown to modulate innate and adaptive immune systems. It plays a role in the innate immune system through enhancing chemotaxis and the phagocytic capabilities of macrophages[1]. It also promotes the production of antibacterial peptides such as cathelicidins and defecins that destroy microbial agents such as Mycobacterium tuberculosis and other infectious agents[2,3]. Moreover, vitamin D down regulates inflammatory cytokines such as tumor necrosis factor (TNFα), interleukin 2 (IL- 2) and IL-12. Vitamin D also promotes T lymphocyte function and balance. This alteration in cytokine response and enhancement of innate immunity can explain associations between vitamin D and infectious disease[4,5]. Low plasma levels of

25(OH)D have been associated with increased infectious disease, including respiratory tract infections[6,7].

Vitamin D regulates bone mineral metabolism and skeletal development. Adequate vitamin D is required for bone mineral accretion and skeletal development[6]. Vitamin D deficiency in children has been linked to linear growth retardation in the presence of rickets[6,8]. Children with vitamin D-dependent rickets present with secondary hyperparathyroidism and demineralization of the growing skeleton, as well as impairment of bone elongation[9]. Studies have shown treatment with vitamin D leads to resolution of hyperparathyroidism and catch up growth[10]. The relationship between child linear growth and vitamin D have not been explored widely. In a study in Indian, weekly vitamin D supplementation (1400 IU/week) of low birth weight infants reduced the risk of stunting[11] In another study in Bangladesh maternal vitamin D supplementation (35, 000 IU/week) starting the third trimester of pregnancy trough delivery resulted in higher length-for-age z score at year one[12].

In sub-Saharan Africa, stunting occurs in more than a third of children and accounts for 21% of disability- adjusted life years[13]. In Ethiopia despite a substantial decline in the past decade, 38% of children under five years of age are stunted[14]. Acute respiratory infection, fever, and diarrhea are important contributing causes of childhood morbidity and mortality. Infant mortality was 48 deaths per 1000 live births in the Demographic and Health Survey conducted in 2015. Neonatal mortality rate is 29 deaths per 1000 live births[14]. The role of vitamin D in immune function and bone health make it a viable option as a new intervention that can be used to improve maternal and infant health outcomes in low-income settings. To our knowledge no study has explored the role of vitamin D in relation to infant growth and morbidity in Ethiopia which has high infectious disease burden. Thus the aim of this study was to evaluate the effect of a vitamin D supplementation on infant growth, attainment of gross developmental milestones and incidence of common childhood illnesses.

Methods

Trial design

The study was a parallel group, double-blind, randomized, controlled vitamin D supplementation trial conducted from October 2014 to January 2016. Lactating women enrolled in the trial within two weeks after delivery were randomized to one of the study groups and received 15,000 IU vitamin D_3 or placebo capsules weekly for 12 months. Ethical clearance for the study was obtained from the Oklahoma State University Institutional Review Board, the Hawassa University Institutional Review Board and the Ethiopian National Health Research Ethics Review Committee and the Food, Medicine and Health Care Administration and Control Authority of Ethiopia (FMHACA). The trial was registered at clinicaltrials.gov (Registration number: NCT02210884).

Participants

The study was conducted in four subsistance farming communities (Alamura, Finchawa, Chefe Koti Jebesa, and Tullo) in Southern Ethiopia (7^oN, 38^oE). Lactating women and infant dyads were enrolled in the study if: they were apparently healthy, delivered within two weeks, were currently breastfeeding, and planned to continue breastfeeding for 12 months.

Outcome assessments

Anthropometric measurements

Maternal height, weight, mid-upper arm circumference (MUAC), and wrist circumference were measured at baseline, 3 and 6 and 12 months. Infant weight and MUAC were also measured at these time points. Infant recumbent length was measured at 3, 6 and 12 months. Measurements were taken using standardized techniques and calibrated equipment. Height and length were measured to the nearest 0.1 cm using the Shorr measuring board (Shorr Productions, Olney, MD, USA). Weight was measured to the nearest 0.1kg using a digital scale (SECA

Uniscale, UNICEF, Copenhagen). At baseline, infant weight was measured to the nearest 0.01 kg using a portable baby scale (SECA354, UNICEF, Copenhagen) and was measured with the SECA Uniscale weighing scale on subsequent visits. A non- stretchable Teflon measuring tape was used to measure MUAC and wrist circumference. Infant anthropometric indices; length-for-age (LAZ), weight-for- age (WAZ), weight-for-height (WHZ), and BMI-for-age (BMIZ) were calculated from the measurements using the WHO Anthro software (Version 3.2.2.). Stunting (short for age) was defied as LAZ < -2. Underweight (thin for age) was defined as WAZ < -2. Wasting was defined as WLZ < -2.

Infant Morbidity

Trained community research workers (CRW) assessed the incidence of common childhood illnesses weekly during home visits. Illnesses were defined based on the WHO Integrated Management of Childhood Illness (IMNCI) guidelines[15]. CRWs asked the mother the number of times the infant had vomited, coughed, and had diarrhea in the past seven days. Vomiting was defined as the forceful evacuation of stomach contents. Reflux, which is common in breastfeeding infants was not counted as vomiting. Diarrhea was defined as three or more loose stools in a day. The CRWs also observed if the child had a runny nose at the time of the visit. Sick children were referred to the local health post or hospital for treatment. Longitudinal prevalence was calculated using the number of days an infant had the outcome (illness) as a numerator and the total number of days the outcome was assessed (visit) as a denominator.

Infant Gross Motor Development

Attainment of gross motor milestones by infants was assessed by CRWs weekly (See Appendix 2 for testing procedures and criteria). Four milestones (1. Raising head when on front, 2. Bearing weight on legs, 3. Sitting supported by arm, 4. Turning and rolling over) were used from the Denver Developmental Scale screening test II [16] All the gross motor milestones used in the World Health Organization (WHO) multicenter growth reference study[17] were also used

together with the Denver II milestones. The WHO gross motor milestones were sitting without support, hands and knees crawling, standing with support, standing unsupported, walking supported and walking unsupported.

Statistical methods

Data were analyzed using SAS (Version 9.4, SAS Institute Inc., 2013) and IBM SPSS (version 23, 2016, IBM Corp.) Normality was tested using the one-sample Kolmogorov-Smirnov test. Variables that were skewed were transformed using log transformation. Differences in anthropometric indices were tested using repeated measures ANOVA. An independent-sample *t*-test was used to test cross-sectional mean differences. Chi-square test was used to test proportion differences. Negative binomial regression analysis was performed to test differences in child illness incidences. Differences in the attainment of motor milestones between the two groups were tested using COX hazard ratios (Survival analysis). Statistical significance was set at $P< 0.05$.

Results

A total of 152 mother-infant pairs were assessed for eligibility. In total, 126 mothers were randomized into intervention (n=63) and control (n=63) groups **(Figure 3.1)**. Baseline characteristics of the study groups were similar (**Table 6.1)**. Compliance and loss to follow-up were similar between the placebo and Vitamin D groups.

We found no statistical difference in attainment of all 10 motor milestones between the placebo and intervention group (**Table 6.2**). In addition, no significant differences were seen in the first incidence or longitudinal prevalence of common childhood illnesses (**Table 6.3**).

There were no statistically significant differences in the change in LAZ, WAZ, WHZ, MUACZ and BMI-for-age over time between study groups (**Table 6.4**). At 3, 6, and 12 months fewer children tended to be stunted and underweight in the vitamin D group compared to placebo group (**Table 6.5**). Wasting tended to be higer in the treatment group at 3 months.

Discussion

This study investigated the effects of vitamin D supplementation to lactating mothers on growth and morbidity of their infants in a setting with high prevalence of growth faltering and heavy burden of infectious. The intervention resulted in significant improvements in maternal 25(OH)D concentrations. However, we found no significant effect of the intervention on infant growth, gross motor development, or morbidity.

Vitamin D is important for fetal growth and bone development[2]. Several studies have shown that improved vitamin D status during pregnancy can result in improved birth outcomes[18]. In this study maternal 25(OH)D concentrations at baseline were not related to infant weight. This finding is in agreement with a study conducted in a similar setting among Gambian [19] women with comparable maternal 25(OH)D with women in our study. Maternal 25(OH)D concentrations were measured at 20 weeks and 36 weeks during pregnancy and all women were vitamin D sufficient (> 50 nmol/L). Vitamin D status was not related to birth weight and growth, and did not influence infant weight, length, head circumference and bone mineral accretion (BMC and BMD) at any time during 52 weeks of postpartum follow-up. Furthermore, in a Dutch multi-ethnic cohort, vitamin D deficiency (< 30 nmol/L) and not insufficiency and adequacy were significantly associated with lower birth weight and a higher risk of SGA[20].

The lack of an overall intervention effect on infant growth and motor development is consistent with previous studies. In a cross-sectional analysis of the association between vitamin

D status and gross motor performance in 912 Indian children aged 5 years, vitamin D status was not associated with motor performance[21]. In contrast, among low birth weight infants supplemented with vitamin D (1400 IU/week) from 1 week to 6 months of age the intervention resulted in increased length and weight at 6 months[11]. However, when these children were followed at age 3 to 6 years, children in the vitamin D group had lower BMI_for_age z scores, thigh circumference and arm muscle area and borderline lower mid-upper arm circumference showing that effects seen earlier were not preserved in the long-term [22].

However, several studies have shown a negative relationship between vitamin D deficiency and infant growth. A large US multi-center cohort study (n= 2473) tested the association between maternal 25(OH)D at 26 weeks of gestation and infant length-for-age z score (LAZ) at birth, 4 , 8 and 12 months. In this study, infants whose mothers were not deficient had significantly higher LAZ across the first year of life compared to infants whose mothers were vitamin D deficient (< 30 nmol/L)[23]. Furthermore, in Mongolian school children (n=113) with low baseline mean 25(OH)D (17 nmol/L), 6 month supplementation with 800 IU/day of vitamin D resulted in a statistically significant greater increase in height compared to children who received placebo[24]. The lack of effect of the intervention on growth in this study might be due to the fact that baseline 25(OH)D status (an indicator of maternal status during pregnancy) was much higher (45 nmol/L and 46 nmol/L in the placebo and vitamin D groups) compared to these studies that reported positive relationships with vitamin D status and growth.

We hypothesized that improved vitamin D status would modulate infant immune response to infectious challenges and downregulate inflammation, which would subsequently result in reduced morbidity. However, we were unable to detect an effect on morbidity. In a study that supplemented low birth weight infants with vitamin D (1400 IU/week) or placebo for 6 months [25], the authors found no significant differences in C-reactive protein (CRP), tumor

necrosis factor-a (TNFa), interferon-g (INFg), interleukin (IL)-10 and IL-13. These findings suggest that vitamin D supplementation might not have influenced inflammation in our study. Continuing with what has been reported for growth, beneficial effects of vitamin D in relation to morbidity have been reported in vitamin D deficient populations. In Mongolian school age children who received vitamin D fortified milk (300 IU) or unfortified milk for 7 weeks, compared with control, children receiving vitamin D fortified milk had lower incidence of acute respiratory infections[26]. Baseline 25(OH)D ranged between 12.5 and 25 nmol/L in the children. In a cross-sectional analysis newborns with acute lower respiratory tract infections had lower mean 25(OH)D concentrations (23 nmol/L) compared to healthy controls and more had values less than 25 nmol/L[27].

This study provides additional data on the lack of relationship between vitamin D status and infant growth, gross motor development and morbidity. The main limitation of this study is its small sample size. Growth and morbidity were not primary outcomes hence we had low power to detect the effect of vitamin D on the infant functional outcomes discussed in this paper. Furthermore, our morbidity assessment relied on recalled infant events. For ethical purposes, we were required to cover health costs of the study participants. This combined with close follow-up of subjects may have led to reduced morbidity incidences compared to the general population. We also did not measure markers of inflammation in the study participants.

Conclusion

In conclusion, in this study, vitamin D supplementation of lactating mother starting from 2 weeks post-partum to 12 months had no effects on infant growth and gross motor development in a population where growth is influenced by multiple factors. The intervention did not also improve incidence of common childhood illnesses.

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Table 6.1. Infant characteristics at baseline, Southern Ethiopia 2014

	Control (n=59)	**Vitamin D group (n=56)**	**P-value**
Infant Age (age)[1]	9.3 (3.8)	10.1 (4.4)	0.281
Infant Weight (kg)	3.3 0(0.5)	3.4 (0.5)	0.889
Infant MUAC (cm)	10.5 (0.8)	10.6 (0.8)	0.413
Weight-for-age Z score	-0.4 (1.0)	-0.4 (0.9)	0.834
Maternal weight (kg)	54.2 (7.4)	53.8 (7.3)	0.804
Maternal Height (m)	1.6 (0.06)	1.6 (0.06)	0.373
Maternal MUAC (cm)	25.3 (2.3)	25.2 (2.50	0.825
BMI (kg/m^2)	21.2 (2.2)	21.3 (2.5)	0.697

[1]Mean(SD). MUAC: Mid upper arm circumference. BMI: Body Mass Index

Table 6.2. Infant mean motor milestones attainment by supplementation group, Southern Ethiopia 2015

Gross motor milestones	**Control**	**Treatment**	**Exp(B) (95% CI)[1]**
Raise head when lying on front[1]	82.0 (19.4)	85.2 (22.8)	0.82 (0.6,1.2)
Bears weight on leg	98.1(23.8)	103.6 (22.9)	0.83 (0.6, 1.2)
Chest up arm support	135.1 (31.6)	145.1 (40.0)	0.76 (0.5, 1.1)
Turning and rolling over	144.4 (29.7)	144.7 (29.6)	0.98 (0.7, 1.4)
Sitting without support	154.4(24.7)	151.9 (19.5)	1.15 (0.8, 1.7)
Hands and knees crawling	248.3 (35.5)	244.7 (26.6)	1.17 (0.8, 1.7)
Standing with assistance	266.4 (35.6)	261.96 (31.97)	1.18 (0.8, 1.7)
Walking with assistance	30.2.1 (30.36)	303.8 (31.1)	0.90 (0.6, 1.3)
Standing alone	325.3 (37.1)	331.3 (41.4)	0.83 (0.5, 1.3)
Walking alone	346.8 (43.4)	357.7 (45.5)	0.96 (0.6, 1.5)

[2] Mean(SD) values in table show mean days of attainment, [1] Cox Hazard ratio using the control group as reference (1)

Table 6.3. Infant morbidity outcomes by supplementation group, Southern Ethiopia 2015

Morbidity outcomes	**Placebo**	**Vitamin D**	**P-value**
Incidence of childhood illnesses[1]			
Cough	37 (61.6)	35 (62.50)	0.981
Runny nose	50 (83.3)	47 (83.9)	0.904
Vomiting	34 (56.67)	26 (46.43)	0.230
Diarrhea	41 (68.3)	36 (64.3)	0.553
Longitudinal prevalence of childhood illnesses[2]			
Cough	0.03	0.03	0.911
Runny nose	11.86	11.84	0.605
Vomiting	0.02	0.02	0.53
Diarrhea	0.03	0.02	0.244

[1] n(%) all such calues. Incidence represents first incence of event. [2] Mean(SD) Longitudinal prevalence = number of days the infant had illness/ total number of days visit

Table 6.4. Infant mean anthropometric Z scores by supplementation group, Southern Ethiopia 2015

Anthropometric Indices	Placebo	Vitamin D	P-value
Height-for-age[1]			0.5
3 months	-0.5 (1.2)	-0.1 (1.2)	
6 months	-0.9 (1.3)	-0.7 (0.8)	
12 months	-1.2 (1.2)	-1.3 (0.9)	
Weight-for-age			0.267
Baseline	-0.4 (0.9)	-0.4 (0.9)	
3 months	-0.2 (1.2)	0.1 (1.0)	
6 months	-0.3 (1.3)	-0.01 (1.0)	
12 months	-0.5 (1.1)	-0.4 (0.9)	
Weigh-for-Height			0.299
3 months	0.3 (1.1)	0.3 (1.5)	
6 months	0.4 (1.1)	0.6 (1.0)	
12 months	0.1 (0.9)	0.3 (1.0)	
BMI-for-Age			0.179
3 months	0.1 (1.1)	0.2 (1.4)	
6 months	0.2 (1.1)	0.6 (1.0)	
12 months	0.2 (0.9)	0.5 (1.0)	
MUAC-for-age			0.320
3 months	-0.01 (1.1)	0.1 (0.9)	
6 months	-0.1 (1.7)	0.1 (0.9)	
12 months	-0.2 (1.1)	0.04 (0.9)	

[1] Mean (SD) all such values

Table 6.5. Incidence of growth faltering by supplementation group, Southern Ethiopia 2015

Anthropometric Indices	**Placebo**	**Vitamin D**	**P-value**
Stunting			
3 months	6 (5.2)	1 (0.9)	0.065
6 months	13 (11.3)	7 (6.1)	0.178[a]
12 months	15 (13.2)	10 (8.7)	0.325[a]
Underweight			
3 months	3 (2.6)	2 (1.7)	0.525
6 months	7 (6.1)	2 (1.7)	0.094
12 months	6 (5.2)	2 (1.7)	0.153
Wasting			
3 months	1 (0.9)	5 (4.3)	0.092
6 months	2 (1.7)	0	0.216
12 months	1 (0.9)	0	0.513

[a] Chi-square test. All other P-values are for Fisher's exact test.

CHAPTER VII

SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

Summary of finding

The purpose of this study was to determine the effects of a weekly vitamin D supplement (15,000IU) vs. placebo administered to lactating women (for 12 months starting from 2 weeks after birth) on maternal and infant markers of vitamin D status. Secondary outcome measures considered include maternal markers of bone turnover, infant growth, infant gross motor developmental milestones and incidence of common childhood illnesses.

Null hypothesis for primary objectives

.***HO_1***: There is no significant differences in maternal 25(OH)D concentration between the treatment and control groups.

- HO1 was rejected since there was a significant difference in maternal 25(OH)D concentrations between the placebo and control group

HO_2: There is no significant differences in infant 25(OH)D concentration between the treatment and control groups.

- We failed to reject this hypothesis since there was no significant differences in infant 25(OH)D concentrations between groups.

HO_3: Skin reflectance, UVB exposure and dress habits will not significantly predict 25(OH)D concentrations.

- We failed to reject this hypothesis since skin reflectance, UVB exposure and percent body surface area exposed were not significantly associated.

Null Hypothesis for secondary objectives

HO_1: There is no significant difference in parathyroid hormone (PTH) concentrations between the treatment and control groups.

- We failed to reject this hypothesis as PTH was not significantly different between the v vitamin D and placebo group.

HO_2: There is no significant difference in maternal and infant bone specific alkaline phosphatase (BAP) concentrations between the treatment and control groups.

- We failed to reject this hypothesis as BAP was not significantly different between the vitamin D and placebo group.

HO_3: There is no significant difference in osteocalcin (OC) concentrations between the treatment and control groups.

- We failed to reject this hypothesis as OC was not significantly different between the vitamin D and placebo group.

***HO*$_4$**: There is no significant difference in C-terminal telopeptides of type 1 collagen (CTX) concentrations between the treatment and control groups.

- We failed to reject this hypothesis as CTX was not significantly different between the vitamin D and placebo group.

***HO*$_6$**: There is no significant difference in the date of attainment of gross motor milestones between infants of mothers in the treatment and control group.

- We failed to reject this hypothesis as date of attainment was not significantly different between the vitamin D and placebo group.

***HO*$_7$**: There is no significant difference in the infant growth between infants by treatment group.

- We failed to reject this hypothesis as infant growth was not significantly different between the vitamin D and placebo group.

***HO*$_7$**: There is no significant difference in the incidence of common childhood illnesses between infants of mothers in the treatment and control group.

- We failed to reject this hypothesis as incidence of illness was not significantly different between the vitamin D and placebo group.

Conclusions and recomedations for future research

Weekly Vitamin D3 (15,000 IU/week) supplementation of lactating women starting 2 weeks post-partum and extending to 12 months significantly improved maternal 25(OH)D. However, the intervention did not result in a significant difference in infant 25(OH)D, maternal and infant markers of bone turnover, infant growth, gross motor development or infant

morbidity. Further research is needed to confirm if the results of this study can be replicated in similar settings. Future studies should also quantify vitamin D synthesis through UVB exposure for 117 infants. Large randomized controlled trials that powered to detect functional outcomes are needed to establish the benefit of vitamin D supplementation on functional outcomes related with bone health, infant growth and morbidity

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APPENDICES

Appendix 1: Percent body surface area exposed sunlight calculation

			Head		Top			Bottom	
	Rule of nines[108]	Adapted value	No Head scarf	No neck scarf	No sleeve	Short sleeve	Long sleeve	Long bottom	Short bottom
Anterior head	3.5	3.5	7 (0.07)	2 (0.02)					
Posterior head	3.5	3.5							
Anterior neck	1	0.75							
Posterior neck	1	1.25							
Anterior upper arm (Both)	4	4			20 (0.20)	12 (0.12)	6 (0.06)		
Posterior upper arm (Both)	4	4							
Anterior lower arm (Both)	2.5	3							
Posterior lower arm (Both)	2.5	3							
Anterior hand (Both)	2.5	3							
Posterior hand (Both)	2.5	3							
Anterior upper leg (Both)	9.5	9.5							
Posterior upper leg (Both)	9.5	9.5							
Anterior lower leg (Both)	7	5						2 (0.02)	12 (0.12)
Posterior lower leg (Both)	7	5							
Foot	3	2							

Appendix 2: Gross motor milestone testing procedure and performance criteria

No.	Gross motor milestone	Testing procedure	Performance criteria
1.	Raise head when lying on front 	Place the child on his/her stomach on a flat surface.	Child lifts head so that face makes an approximate 45 degree angle with the surface for 10 seconds.
2.	Bears weight on legs	Hold the child in standing position, his/her legs and feet on a flat surface. Then slowly loosen your hand support.	Child is able to support weight on legs and feet when support is lessened for 5 seconds.
3.	Chest up arm support (sitting supported by arm) 	Place the child on his/her stomach on a flat surface	Child lifts head and chest off the surface using the support of outstretched arms. Child is looking straight ahead or up.
4.	Turning and rolling over 	Place the child on his/her stomach/back.	The child rolls from back to stomach or from stomach to back.

5.	Sitting without support 	Facing the child and smiling, places the child in a sitting position. Than give the child an object to handle with both hands.	Child sits up straight with the head erect for at least 10 seconds. Child does not use arms or hands to balance body or support position.
6.	Hands-and-knees crawling 	Places the child in the prone position with the abdomen above the supporting surface. CRW places herself in front of the child. If the child does not crawl spontaneously, show the child an object that attracts the child's visual attention.	Child alternately moves forward or backward on hands and knees. The stomach does not touch the supporting surface. There are continuous and consecutive movements, at least three in a row
7.	Standing with assistance 	Place the child in a standing position by holding onto a stable object (e.g., furniture)	Child stands in upright position on both feet, holding onto a stable object with both hands without leaning on it. The body does not touch the stable object, and the legs support most of the body weight. Child stands with assistance for at least 10 seconds

8.	Walking with assistance 	Place the child in a standing position by holding onto a stable object (e.g., furniture). If the child does not move spontaneously, show the child an object that attracts the child's visual attention.	Child is in upright position with the back straight. Child makes sideways or forward steps by holding onto a stable object with one or both hands. One leg moves forward while the other supports part of the body weight. Child takes at least five steps.
9.	Standing alone 	Place the child with both feet flat on the floor and supports the child to an erect position. Then withdraw the support gradually and temporarily.	Child stands in upright position on both feet (not on the toes) with the back straight. The legs support 100% of the child's weight. There is no contact with a person or object. Child stands alone for at least 10 seconds.
10	Walking alone	Place the child in an erect position out of the reach of any supporting object.	Child takes at least five steps independently in upright position with the back straight.

		Stand one or two steps in front of the child and call the child to move towards you.	One leg moves forward while the other supports most of the body weight. There is no contact with a person or object.

- Date of attainment will be recorded in consultation with the mother.

-The CRW must see when the child performs the milestones.

- If the child does not perform one milestone for 3 consecutive visits (recorded 2) inform the researcher.

Appendix 3: Infant illness referral and reporting chart

Signs and Symptoms	Where to refer	When to notifying investigators
Urgent referral signs and symptoms		
Danger signs	If the child has one of these danger signs Urgently refer to Bushulo, Health Center, Referral or Adare Hospital	Immediately
1. Is not able to eat or breastfeed 2. Vomits everything 3. Has had convulsions or has current convulsions 4. Is Lethargic or unconscious		
Other signs		
5. Cough with: fast breathing (>50 breaths per min) 6. Long lasting fever (7 days) 7. Diarrhea with: Sleepy or unconscious, Sunken Eyes, and Blood in stool 8. Ear problem with swelling around the ears		
Referral : Other signs and symptoms		
1. Cough	Refer to Health Center or Hospital	Weekly (through weekly report form or thorough phone calls)
2. Diarrhea (Irritable or restless, Drinks eagerly)		
3. Fever		
4. Ear infection : Liquid in ear for 2 weeks		
5. Eye infection		
6. Generalized rash		

Appendix 4: Questinnaires

Effects of maternal vitamin D supplementation on markers of vitamin D Status and related infant and maternal health outcomes in Southern Ethiopia

Baseline maternal questionnaire

Maternal code: _____

Interviewer _____ Date of data Collection ___/____/__
Child birth date___/____/___ Child sex_____ Village _____

Part I. Demographic and socioeconomic characteristics

No.	Questions	Responses
1.	How old are you? (Interviewer: How certain was the mother about age 1. Certain 2. Fairly certain 3. Uncertain)	_____ Years
2.	What is your current marital status?	1. Married 2. Single 3. Divorced 4. Widowed Others (specify) _____
3.	What is your religion?	1 Orthodox Christian 2. Muslim 3. Protestant 4. Catholic Others (specify) _____
4.	How many people reside in your home?	_____ Household members
5.	What is the highest grade/number of years of school you completed?	_____ Years 99. Non-formal education
6.	What is the highest grade/number of years of school your husband/partner has completed?	_____ Years 99. Non-formal education
7.	What is your occupation?	1. Housewife 2. Daily-laborer 3. Employed government 4. Employed non-government 5. Petty trade Others (specify) _____
8.	What is the occupation of the father?	1. Farmer 2. Employed government 3. Employed non-government 4. Daily-laborer

		5. Self-employed non farming Others (specify)_____

9.	Do you have any of the following things in your house that are functioning?	1. Electricity 2. Radio/ tape player 3. Television 4. Bicycle 5. Motor bike 6. Car 7. Mobile telephone 8. Non-mobile telephone 9. A savings account
10.	How many rooms do you have in your house?	_____ Rooms
11.	Does your house have a separate kitchen?	0. No 1. Yes
12.	What material is the roof of your house made of?	1. Thatched roof 2. Plastic sheets 3. Corrugated iron 4. Cement/ concrete Others (specify)_____
13.	What material is your floor made of?	1. Natural floor (earth) 2. Cow dung 3. Wood 4. Cement Others (specify)_____
14.	What material is the inside wall of your house?	1. Wood with mud 2. Stone with mud 3. Cement/ Cement blocks 4. Brick Others (specify)_____
15.	What is the main source of drinking water for members of your household?	1. River/ Lake 2. Spring 3. Well 4. Public/ shared tap 5. Private tap Others (specify) _____
16.	How long does it take you to go to your water source, get water and come back home?	_____Minutes

17.	What kind of toilet facility does your household use? (If the respondent does not understand ask to observe the toilet and circle the appropriate answer.)	1. No facility/Bush/field 2. Pit latrine without slab 3. Pit latrine with slab cover 4. Ventilated improved pit latrine 5. Flush toilet Others (specify) _____
18.	Is the toilet facility shared with other households?	0. No 1. Yes
19.	Does your household own agricultural land?	0. No 1. Yes
20.	How many units (local) of land does your household own?	_____ hectare or_____ timad
21.	Which crops do you grow? (Read the list)	Yes No 1.Coffee 1 0 2.Chat 1 0 3.Maize 1 0 4.Enset 1 0 5.Barley 1 0 6.Sugar cane 1 0 Others (specify)_____
22.	Does the household own any of the listed animals? (Read the list)	No Yes Number Cows 0 1 _____ Oxen 0 1 _____ Horse/ Donkey 0 1 _____ Sheep 0 1 _____ Goat 0 1 _____ Chicken 0 1 _____

Part II Maternal care and dietary practice

23.	How many children did you give birth to?	_____ Number
24.	How many children are living ?	_____
25.	Did you receive antenatal care at a health facility during you last pregnancy?	0. No (Skip to 29) 1. Yes
26.	If yes whom do you usually see? (More than one answer possible)	1. Doctor 2. Nurse/midwife 3. Health extension worker Others (specify)_____
27.	Where did you receive antenatal care?	1. Government hospital 2. Public hospital 3. Public health center 4. Health post

		Others (specify)_____
28.	How many months pregnant were you when you first received antenatal care?	_____ Months
29.	How many times did you receive antenatal care during your last pregnancy?	_____
30.	Where did you give birth to your last baby (NAME)?	1. Home 2. Public hospital or health center 3. Private hospital or health center Others (specify)_____
31.	If NAME was born at home, why did you not deliver in a health facility?	1. Cost 2. Facility not open 3. Facility too far 4. Family did not allow 5. Afraid to go to a health facility Others (specify)_____
32.	Were any of your children delivered by caesarean? (Did they cut your belly to take the baby out?)	0. No (Skip to 33) 1. Yes
33.	Was your last baby (NAME) delivered by caesarean	0. No 1. Yes
34.	Did you take any supplements (Iron/folic acid iodine, or other) when you were pregnant with your last child?	Iron/folic acid: No 0, Yes 1 Iodine: No 0, Yes 1 Other (specify) _____
35.	Did you take cod liver oil during pregnancy?	0. No 1. Yes
36.	Did you take cod liver oil since giving birth?	0. No 1. Yes

Part IV Household hunger index

37.	In the past month was there ever no food to eat in your house because of lack of resources to get food?	0. No (Skip to 38) 1. Yes
38.	How often did this happen in the past month?	1. Rarely (1-2 times) 2. Sometimes (3-10 times) 3. Often (more than 10 times)
39.	In the past month did you or any household member go to sleep at night hungry because there was not enough food?	0. No (Skip to 40) 1. Yes
40.	How often did this happen in the past month?	1. Rarely (1-2 times) 2. Sometimes (3-10 times) 3. Often (more than 10 times)

41.	In the past month did you or any household member go a whole day and night without eating anything at all because there was not enough food?	0. No (Skip to 42) 1. Yes
42.	How often did this happen in the past month?	1. Rarely (1-2 times) 2. Sometimes (3-10 times) 3. Often (more than 10 times)
43.	How many times did you eat yesterday? (Coffee not counted)	1. Once 2. Twice 3. Three times 4. More than three times

Part IV Child feeding practices

44.	Are you breastfeeding your last child (NAME) ?	0. No (Skip to 45) 1. Yes
45.	How long after birth did you start breastfeeding NAME ?	0. Immediately _____Hours _____ Days
46.	After delivery was NAME given anything to drink other than breast milk?	0. No (Skip to 47) 1. Yes
47.	What was NMAE given to drink?	1. Milk other than breast milk 2. Plain water 3. Sugar with water 4. Fenugreek 5. Cod liver oil Other (specify)_____
48.	Was the child given any supplements after birth?	No Yes Vitamin A 0 1 Iodine 0 1 Other _____
49.	For how long do you intend to exclusively breastfeed NAME?	_____Months
50.	For how long do you intend to breastfeed NAME?	_____Months
51.	Do you own a baby bottle?	0. No 1. Yes
52.	Do you bottle feed NAME?	0. No (Give positive reinforcement) 1. Yes

Part IV Sun exposure behavior

53.	Did you expose your previous child to the sun?	0. No 1. Yes 2. First baby
54.	If yes how long after birth did you start exposing your baby to sunlight?	_____days _____ Weeks
55.	Do you plan on exposing your last baby NAME to the sun?	_____days _____ Weeks
56.	Do you usually stay outside in the sun without an umbrella?	0. No 1. Yes
57.	If yes how often do you stay outside?	0. Once every two weeks 1. Once per week 2. Twice per week 3. Every other day 4. Daily Other (specify)_____
58.	On a typical day for how long do you stay outside?	_____Minutes _____ Hours
59.	How would you characterize your typical dress style when you stay outside? (Interviewer: Show the respondent the clothing picture chart and record the appropriate letter)	Head wear_____ Blouse_____ Skirt _____
59.	How would you characterize your typical dress style when you are at home? (Interviewer: use picture chart)	Head wear_____ Blouse_____ Skirt _____
60.	How often do you wear sleeveless clothes?	0. Never 1. Once every two weeks 2. Once per week 3. Twice per week 4. Every other day 5. Daily Other (specify)_____
61.	How often do you wear short sleeve clothes?	0. Never 1. Once every two weeks 2. Once per week 3. Twice per week 4. Every other day 5. Daily Other (specify)_____

62.	How often do you wear long sleeve clothes?	0. Never 1. Once every two weeks 2. Once per week 3. Twice per week 4. Every other day 5. Daily Other (specify)_____
63.	Do you avoid staying out in the sun?	0. No 1. Yes
64.	If yes why do you avoid sunlight?	_____ _____
65.	Please describe all the outdoor activities you performed yesterday, including walking outside, from early in the morning until sundown? (Interviewer: Start with the first outdoor activity and list all the activities performed in the day. Use the clothing style picture chart to record the type of cloth worn during each activity. Probe for activities.)	

Time of day	Outdoor activity	Time spent on activity	Clothing worn during activity

Thank you for your time!!!

Name of mother_____ Name of child_____

✂

Maternal code: _____

Effects of maternal vitamin D supplementation on markers of vitamin D Status and related infant and maternal health outcomes in Southern Ethiopia

Follow up maternal questionnaire

Interviewer _____ Date of data Collection ___/____/__Child sex_____
Village _____

Part I Dietary practices and household hunger index

1.	Did you take any supplements in the past three months?		No	Yes
		Iron	0	1
		Cod liver oil	0	1
		Other (specify)	_____	
2.	Did your last child (NAME) recently take any supplements?		No	Yes
		Vitamin A	0	1
		Cod liver oil	0	1
		Other (specify)	_____	
3.	Are you breastfeeding your last child NAME?	0. No (Skip to 7) 1. Yes		
4.	After delivery was NAME given anything to drink other than breast milk? (***Asked only at 3 months)***	0. No (Skip to 6) 1. Yes		
5.	If yes what was NAME given to drink?	_____		
6.	For how long do you intend to exclusively breastfeed NAME? ***(Asked only at 3 months)***	_____Months		
7.	Do you bottle feed NAME?	0. No (Give positive reinforcement) 1. Yes		
8.	Have you started giving NAME any semi-solid or solid foods?	0. No (Skip to 11) 1. Yes		
9.	If yes how many times was NAME given semi-solid or solid foods yesterday?	_____		
10	Did NAME eat any of the foods/food groups listed yesterday?		No	Yes
		Grains, roots and tubers	0	1
		Legumes and nuts	0	1
		Dairy products	0	1
		Meat, fish, poultry	0	1
		Eggs	0	1
		Vitamin A rich fruits and vegetables	0	1
		Cod liver oil	0	1

11.	Have you ever seen a child with rickets?	0. No (Skip to 13) 1. Yes
11.	In the past month was there ever no food to eat in your house because of lack of resources to get food?	0. No (Skip to 13) 1. Yes
12.	How often did this happen in the past month?	4. Rarely (1-2 times) 5. Sometimes (3-10 times) 6. Often (more than 10 times)
13.	In the past month did you or any household member go to sleep at night hungry because there was not enough food?	0. No (Skip to 15) 1. Yes
14.	How often did this happen in the past month?	4. Rarely (1-2 times) 5. Sometimes (3-10 times) 6. Often (more than 10 times)
15.	In the past month did you or any household member go a whole day and night without eating anything at all because there was not enough food?	0. No (Skip to 17) 1. Yes
16.	How often did this happen in the past month?	4. Rarely (1-2 times) 5. Sometimes (3-10 times) 6. Often (more than 10 times)
17.	How many times did you eat yesterday? (Coffee not counted)	5. Once 6. Twice 7. Three times 8. More than three times

Part II Sun exposure behavior

18.	After giving birth to NAME did you stay inside your home for some time? (***Asked only at 3 months)***	0. No (Skip to 20) 1. Yes
19	If yes for how long did you stay inside your house? (***Asked only at 3 months)***	_____ weeks
20	Did you expose NAME to the sun? (***Asked only at 3 months)***	0. No (Skip to 23) 1. Yes
21.	If yes how long after birth did you start exposing NAME to sunlight? (***Asked only at 3 months)***	_____days _____ Weeks

22.	Do you put oil/butter on NAME skin when exposing to the sun? (***Asked only at 3 months)***	0. No 1. Yes
23.	Do you usually stay outside in the sun without an umbrella?	0. No (Skip to 26) 1. Yes
24.	If yes how often do you stay outside?	5. Once every two weeks 6. Once per week 7. Twice per week 8. Every other day 9. Daily Other (specify)_____
25.	On a typical day for how long do you stay outside in the sun?	_____Minutes _____ Hours
26.	How would you characterize your typical dress style when you stay work outside? (Interviewer: Show the respondent the clothing picture chart and record the appropriate letter)	_____ _____ _____
27.	How would you characterize your typical dress style when you are at home? (Interviewer: use picture chart)	_____ _____ _____
28.	How often do you wear sleeveless clothes?	1. Once every two weeks 2. Once per week 3. Twice per week 4. Every other day 5. Daily Other (specify)_____
29.	How often do you wear short sleeve clothes?	1. Once every two weeks 2. Once per week 3. Twice per week 4. Every other day 5. Daily Other (specify)_____
30.	How often do you wear long sleeve clothes?	1. Once every two weeks 2. Once per week 3. Twice per week 4. Every other day 5. Daily Other (specify)_____

31.	Do you avoid staying out in the sun?	0. No 1. Yes
32.	If yes why do you avoid sunlight?	_____ _____
33.	Please describe all the outdoor activities you performed yesterday, including walking outside, from early in the morning until sundown? (Interviewer: Start with the first outdoor activity and list all the activities performed in the day. Use the clothing style picture chart to record the type of cloth worn during each activity. Probe for activities.)	

Time of day	Outdoor activity	Time spent on activity	Clothing worn during activity

Clothing style picture chart

A 	B 	C 	D 
E 	F 	G 	H 
L 	J 	K 	

VITA

Meron Girma Wondimagegnhu

Candidate for the Degree of

Doctor of Philosophy

Dissertation: EFFECTS OF MATERNAL VITAMIN D SUPPLEMENTATION ON MARKERS OF VITAMIN D STATUS AND RELATED INFANT AND MATERNAL HEALTH OUTCOMES IN SOUTHERN ETHIOPIA

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- Graduate Research Assistant & Graduate Teaching assistant: Department of Nutritional Sciences, Oklahoma State University, August, 2011-Present

- Lecturer: School of Nutrition and Food science, Hawassa University, Ethiopia, January, 2010 - August, 2011

- Research Assistant: Maternal and Child Nutrition Research Project, Hawassa University, Ethiopia, October, 2005 - December, 2009

Professional Memberships:

- American Society of Nutrition (2012 to date)
- Food and Nutrition society of Ethiopia (2008 to date)