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The Impact of Prenatal and Postpartum Vitamin D Supplementation on Cholecalciferol Concentrations in Breast Milk

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Abstract

Vitamin D deficiency is a global health issue which has been shown to impact pregnant and lactating mothers and their infants. Research has shown that maternal oral vitamin D supplementation can serve as a nutritional intervention to increase the vitamin D levels in mothers and infants. While previous studies show the impacts of vitamin D supplementation during pregnancy or lactation on breast milk vitamin D, there is limited research on supplementation during both time periods. Our goal was to examine the impact of maternal oral vitamin D supplementation during pregnancy and continued from pregnancy throughout lactation on maternal breast milk cholecalciferol. This thesis is a sub-study of the Maternal Vitamin D for Infant Growth (MDIG) trial conducted in Dhaka, Bangladesh where vitamin D deficiency is prevalent. The parent trial randomized 1124 pregnant women into five treatment groups (placebo (n=223), prenatal 4200IU/week (n=229), prenatal 16000IU/week (n=224), prenatal 28000IU/week (n=221), and prenatal and postpartum 28000IU/week (n=227). Our study included a sample of 71 pregnant women from three original treatment groups: placebo (n=24), prenatal 28000IU/week (n=23), and prenatal and postpartum 28000IU/week (n=24). The average maternal age was 23.9 ± 4.8 years old and average gestational age of infants at birth was 39.0± 1.4 weeks. For aim 1, we measured the concentration of cholecalciferol in the maternal breast milk samples at 6 months. The mean cholecalciferol concentrations were 14.0 nmol/L for the entire analytical sample, 16.8 nmol/L for the placebo group, 10.9 nmol/L for the pregnancy supplementation group, and 14.1 nmol/L for the prenatal and postpartum supplementation group. An ANOVA test determined no difference between groups (p=0.630). For aim 2, we examined the relationships between maternal serum 25(OH)D levels with maternal breast milk cholecalciferol and maternal breast milk cholecalciferol with infant serum 25(OH)D. Twenty-

eight infants and 25 mothers had serum 25(OH)D data available for analysis. Linear regression models were adjusted for infant sex, birth mode, parity, maternal age and gestational age. Maternal serum 25(OH)D was not significantly associated with breast milk cholecalciferol concentration (unadjusted model: p-value =0.06; adjusted model: p-value = 0.07). Breast milk cholecalciferol was positively associated with infant serum 25(OH)D in both the unadjusted (p-value = 0.02) and adjusted (p-value = 0.03) models. In conclusion, we found that breast milk cholecalciferol did not increase with maternal vitamin D supplementation. However, evidence that infant serum 25(OH)D levels are associated with maternal milk cholecalciferol levels shows the importance of vitamin D in milk. Further studies should continue to examine the relationships between serum vitamin D levels with milk cholecalciferol, as well as the continuation of prenatal vitamins through lactation.

Table of Contents

List of Figures

List of Tables

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Chapter 1: Introduction and Literature Review

Introduction to Vitamin D

Calciferol, more commonly known as vitamin D, is a fat-soluble vitamin found in a few foods in the diet, synthesized from sunlight and available in over-the-counter supplements [1]. It plays a key role in metabolism, gene regulation, calcium absorption, inflammation, and bone health [1,2]. Vitamin D levels in infants depend on their mothers' vitamin D status throughout pregnancy and lactation while also playing a vital role in fetal development [3].

Roles of Vitamin D

Vitamin D is a secosteriod hormone that is required for bone mineralization, calcium absorption, and overall bone health [4]. Bone mineral density (BMD) is correlated with the amount of bone mineralization. Osteoporosis can be considered a condition of inflammation by which pro-inflammatory cytokines are related to bone health. However, fracture risk may be reduced by the ability of vitamin D to rebalance the cytokines and regulate bone metabolism. Additionally, studies show that the likelihood of a child developing rickets disease is increased in breast-fed infants [5]. In all children, vitamin D deficiency can cause bone deformities and growth issues that result in rickets.

In addition to bone health, vitamin D modulates the expression of over 900 genes by interacting with DNA in a cell’s nucleus [2]. Vitamin D can modify gene expression in tumor growth, immune responses, muscle function, and other extra-skeletal effects. Vitamin D binds to specialized receptors called vitamin D receptors (VDRs) which are found in several tissues throughout the body. VDRs are transcription factors that become activated by the binding of vitamin D ultimately resulting in changes in gene expression. Muscle function relies on

expression of VDR to retain strength and muscle mass especially during aging diseases [6]. Additionally, production of T cells in the immune system requires vitamin D, so deficiency can be linked to autoimmune diseases. In general, vitamin D is vital for proper functioning of the body.

Vitamin D Metabolism

There are three major groups of vitamin D molecules: calciferol (including cholecalciferol (vitamin D_3) and ergocalciferol (vitamin D_2)), 25-hydroxyvitamin D (25-(OH)D), and 1,25-dihydroxyvitamin D (1,25 – (OH)D) [7]. Vitamin D_2 and vitamin D_3 differ chemically by different side chains, but both are metabolized into hydroxylate forms in the body [8]. While each of these forms is derived differently, the body needs adequate levels of calciferol, 25(OH)D, and 1,25-(OH)D to support proper immune function, bone health, gene expression and more.

Both cholecalciferol and ergocalciferol are biologically inactive compounds [9]. Therefore, the body must activate them into the biologically active form, calcitriol. Each compound requires hydroxylation both renally and hepatically. Upon reaching the bloodstream, a protein known as vitamin D binding protein (VDBP) binds to them for transport to the hepatocyte cells of the liver. Inside the hepatocytes, the first hydroxylation is catalyzed by cholecalciferol-25-hydroxylase producing calcidiol or 25-hydroxycholecalciferol (25(OH)D). Next, another VDBP transfers the 25(OH)D to the kidneys through the bloodstream. There, the second hydroxylation reaction occurs when 1-α-hydroxylase converts the 25(OH)D into calcitriol or 1,25-hydroxycholecalciferol, the active form of vitamin D which the body can use. In addition to the kidney, T-cells also contain the 1-α-hydroxylase required to convert 25(OH)D into calcitriol [10] as well as the cells of the placenta [11].

Vitamin D3 - Cholecalciferol

Vitamin D3 can be obtained by humans from skin production after ultraviolet B ray (UV-B) absorption of sunlight and through diet [8].

Vitamin D2 – Ergocalciferol

Vitamin D2 is found naturally in very few food sources, but wild mushrooms are a significant source. Additionally, milk from dairy cows contains a low but significant amount of ergocalciferol which is attributed to the hay and grass in their diet [12]. While some foods are fortified with ergocalciferol, most fortification in the US is done with vitamin D3 (e.g. milk and other dairy products) [13].

25-Hydroxylates

While there are both 25(OH)D3 and 25(OH)D2 metabolites, they are together referred to as simply 25(OH)D. The 25(OH)D levels reflect the vitamin D produced endogenously in the skin as well as vitamin D obtained through supplementation and diet [13]. 25(OH)D is a stable metabolite with a half-life of 2-3 weeks [14]. Its stability can be explained by its strong affinity (dissociation constant of 10^{-8} mol) for the VDBP. Thus, serum 25(OH)D is used to determine a human's overall vitamin D status.

Sources of Vitamin D

Diet

Cholecalciferol

As previously stated, sources of cholecalciferol are found in animal sources dietarily – particularly fish and dairy products. A standard 3-ounce portion of fish contains different amounts of cholecalciferol based on the fish: freshwater rainbow trout (645 IU), Tilapia (127 IU), Flounder (118 IU), Herring (182 IU). By fortification, dairy products in the US also contain cholecalciferol [15]. For example, 1 cup of 1% low fat milk contains 117 IU and 8 ounces of plain nonfat yogurt contains 116 IU.

Ergocalciferol

Mushrooms are known to contain approximately 136 IU per 1 cup serving naturally [16]. While less common, some foods are fortified with ergocalciferol. Soymilk, for example, contains about 48 IU of ergocalciferol per 100 mL [17].

Supplementation

Both ergocalciferol and cholecalciferol are available as supplements. A meta-analysis found that compared to ergocalciferol, supplements containing cholecalciferol increased serum 25(OH)D levels significantly higher (p-value = 0.001), making it the more desirable supplement [18]. According to Harvard, approximately 400 IU of vitamin D is found in daily multivitamin supplements [17]. Daily prenatal vitamins for pregnant women typically contain 400 – 600 IU of vitamin D [19]. Therefore, both pregnant and nonpregnant women are likely to obtain similar amounts of daily vitamin D from supplementation [20].

Recommended Levels

Recommended Dietary Allowance

The Recommended Dietary Allowances (RDA) are the average daily intake (through diet or supplements) of a given nutrient [13]. The RDA for vitamin D assumes minimal sun exposure while aiming to ensure enough vitamin D to maintain normal calcium metabolism and healthy bones [21]. For adults (male and female) ages 18-70 the recommended vitamin D intake level is 600 IU or 15 mcg per day [13]. For pregnant and lactating women ages 14-50 the recommended vitamin D level is also 600 IU or 15 mcg per day. There is currently no RDA for infants, but the adequate intake recommendation for children birth-12 months is 400IU or 10 mcg per day. The tolerable upper intake level for children and adults is 4000 IU/day [21].

Biomarker Cut Points

Vitamin D is metabolized in the liver to a form called 25-hydroxyvitamin D, which is measured to determine a person's overall status: toxic, sufficient, insufficient, or deficient. Biomarker cut points measure the biochemical vitamin D status of a person from all sources including skin and diet.

The official guidelines for vitamin deficiency are provided by the Institute of Medicine (IOM) [22]. For most people, serum 25(OH)D levels less than 30nmol/L put people at risk for vitamin D deficiency, with a level between 30 and 50 nmol/L some people are at risk for inadequacy, and 50 nmol/L is considered sufficient.

The Endocrine Society published cut points that are higher than IOM [23]. According to their literature, toxic levels include all 25-hydroxyvitamin D levels greater than 374 nmol/L and person is considered sufficient vitamin D at just above 75 nmol/L. Thus, insufficiency is less

than 75nmol/L, but greater than 50nmol/L, the currently the accepted cutoff value for deficiency accepted by most clinicians. However, the scientific values from Institutes of Medicine and National Institute of Health (NIH) Office of Dietary Supplements have been set as less than 30nmol/L is deficiency and less than 50nmol/L is insufficiency [1,24]. Despite some physicians, nurses, and other health professionals considering 50 nmol/L as the deficiency cutoff value, scientists and researchers use the NIH value of 30 nmol/L.

Vitamin D Deficiency and Maternal Oral Supplementation

Vitamin D deficiency is a global health issue that particularly impacts pregnant and lactating women in low resource settings. According to Cui et al., 2023, people living in lower-middle-income countries were shown to have a 56% higher prevalence for serum 25(OH)D < 50 nmol/L and 26.7% higher prevalence for serum 25(OH)D < 30 nmol/L globally compared to upper-middle-income countries [25]. As previously studied, maternal health is closely linked to fetal growth and health. Vitamin D deficiency shows links to immunity, glucose metabolism, and dyslipidemia, high levels of blood cholesterol, triglyceride levels and low-density lipoproteins [26].

Previous research shows that maternal vitamin D supplementation increases the vitamin D status of infants. Dawodu & Tsang state that vitamin D status in breastfeeding infants is dependent on the vitamin D intake from milk [27]. Hollis & Wagner published their work from a randomized control trial of lactating women and their infants [28] Women were enrolled at 1 month postpartum and divided into two groups: one group received 1600 IU/day ergocalciferol and the other group received 3600 IU/day ergocalciferol for 3 months. All women also received a 400 IU/day cholecalciferol prenatal vitamin. The infant mean serum 25(OH)D significantly increased from 7.9 ± 1.1 at baseline to 27.8 ± 3.9 ng/mL in the group receiving 2000 IU/day

(1600 IU/day ergocalciferol plus 400 IU/day cholecalciferol) and from 13.4 ± 3.3 to 30.8 ± 5.0 ng/mL in the group receiving 4000 IU/day (3600 IU/day ergocalciferol plus 400 IU/day cholecalciferol).

However, there is limited data about how prenatal maternal vitamin D supplementation affects breastmilk vitamin D and whether prenatal or postpartum maternal supplementation is more beneficial. Previous studies have looked at either prenatal or postpartum vitamin D supplementation on cholecalciferol concentrations, but not both [5,29–31].

Maternal – Infant Vitamin D Supplementation

It is commonly expected that pregnant and/or lactating women regularly consuming prenatal vitamins cannot acquire a deficiency in vitamin D. However, deficiency has been observed in populations consuming supplements. The CDC currently recommends 400IU of vitamin D per day for breastfed infants less than one year of age [32], but a study done in the United States found that 73% of mothers and 80% of their infants were vitamin D deficient at the time of birth despite following the CDC intake recommendations [33]. Takeuchi et al proposes that infant vitamin D supplementation is essential directly or indirectly through the mother [30]. A newer strategy that some research has investigated for increasing infant vitamin D is maternal oral supplementation during lactation [5]. Maternal oral vitamin D supplementation has been previously studied in pregnant women and women during lactation.

Pregnancy Trials

Many researchers have studied the impacts of oral maternal vitamin D supplementation during pregnancy on vitamin D during lactation. The goal of the Wall et al study was to analyze the impacts of oral vitamin D3 supplementation during pregnancy on breast milk vitamin D

activity (total concentration of vitamin D_2, vitamin D_3, 25(OH)D_2, and 25(OH)D_3 per day) during the first 20 months of lactation [31]. Women were enrolled at 27 weeks' gestation and divided into one of three groups: 1000 IU/day supplement, 2000 IU/day supplement, or placebo. At 2 weeks and 2 months after birth, milk samples were collected and analyzed for concentration of vitamin D. It was concluded that the women supplemented with 2000 IU/day had a significantly higher vitamin D activity during the winter months compared to the 1000IU/day group, 92 IU/L compared with 51 IU/L, respectively. While not significant, both supplementation groups had higher vitamin D activity than the placebo. Therefore, vitamin D supplementation during pregnancy alone contributed to higher levels of vitamin D activity in breast milk.

Kunz et al found vitamin D levels in milk to decrease and 25(OH)D levels to increase throughout lactation with a low dose daily maternal supplement of 400 IU/day throughout pregnancy [34]. Therefore, studies show a correlation between oral maternal supplementation during pregnancy and vitamin D concentration in breast milk.

Lactation Trials

In addition to during pregnancy, the impacts of vitamin D supplementation have also been studied in maternal breast milk samples and blood serum throughout lactation. In Japan, Takeuchi et al [30] conducted a trial comparing the serum plasma and breast milk vitamin D concentrations of lactating women with or without additional supplementation [30]. The trial lasted for 4 weeks with one group receiving 1200IU/day of ergocalciferol (vitamin D2) and the other serving as a control not being supplemented. The researchers concluded that both vitamin D2 and vitamin D3 concentrations in serum plasma and milk samples were positively correlated.

This provides evidence that trials measuring plasma vitamin D levels can be used to predict milk vitamin D levels.

There are many studies that have found a positive correlation between oral vitamin D supplementation during maternal lactation and vitamin D concentrations in serum. In New Zealand, a trial was conducted to measure the total serum 25(OH)D concentration after 16 weeks of maternal oral supplementation [5]. 90 mother/infant in pairs were randomly assigned to three groups: placebo, 50,000 IU/month, 100,000 IU/month starting 4 weeks after giving birth. While all three groups did see an increase in serum 25(OH)D at the study's conclusion, the concentration was much higher in the supplementation groups relative to the control.

Hypovitaminosis D in Bangladesh

In many parts of the world, insufficient vitamin D levels (hypovitaminosis D) encompass a large portion of the population. Bangladesh, a small South-Asian country, is the 8th most densely populated country [35]. Due to its location near the equator in a tropical to sub-tropical zone of climates, plenty of sunlight reaches the population year-round, including UV-B rays needed for vitamin D production. This adequacy in sunshine led to researchers to assume vitamin D sufficiency in this population of people. Unfortunately, the analysis conducted by Islam et al found that 27.2-100% of Bangladeshi people are deficient across population group. The high prevalence of hypovitaminosis D has led to more than 8% of children in some parts of Bangladesh having clinically affected rickets, more than 10 million people having diabetes potentially linked to vitamin D deficiency, and an increased likelihood of osteoporosis and bone fractures in the elderly.

Risks for vitamin D deficiency fluctuate throughout the groups within the population. While all groups in Bangladesh are at a high risk, women of childbearing age maintain the

highest risk for hypovitaminosis D for two reasons. First, vitamin D risk is high because of low dietary consumption of vitamin D [36]. While the Bangladesh diet does consist of vitamin D rich foods such as fish and shellfish, women do not eat enough to obtain proper nutrient levels. In low-income households, food insecurity leads to low caloric intake for all family members, especially women. There are patriarchal norms in their culture that result in women sacrificing food to provide for the men of the household. Once a woman is of reproductive age, her body is less demanding, so she receives less food at meals and prepares for marriage and motherhood. Second, cultural dress prevents adequate sun exposure. One study found that about 72% of women wearing the Hijab were deficient in vitamin D due to lack of adequate sun exposure [37]. Using a low cut-off value of S-25OHD<37.5 nmol/L, 38% of low-income women were deficient in vitamin D and 50% of women in high-income regions were deficient [35] showing that all women are impacted.

Vitamin D Supplementation and Breast Milk: Current Thesis

As reviewed above, there is limited data about the impact of maternal vitamin D supplementation during pregnancy and lactation on the concentration of vitamin D in breast milk samples. Previous studies focused on supplementation during pregnancy or lactation independently. This thesis examined vitamin D supplementation during pregnancy and/or lactation within the context of a maternal supplementation trial that was previously conducted in Bangladesh. We expect that this research may contribute to the field by providing data to use as reference for maternal vitamin D supplementation. This current project involves developing a method to extract and measure the cholecalciferol concentration in human breast milk samples from the parent trial. A method will be developed for cholecalciferol analysis. In general, human

milk is low in vitamin D activity [31], so it is important to measure cholecalciferol, the most concentrated form.

Aims and Hypotheses

Aim 1: Examine the effect of oral maternal vitamin D supplementation (placebo, prenatal 28,000 IU/week, and prenatal and postpartum 28,000 IU/week) concentrations of cholecalciferol in maternal breast milk samples at 6 months of infant age.

Hypothesis: We expected that milk samples from mothers receiving 28,000 IU/week vitamin D supplements in pregnancy would have a higher concentration of vitamin D than the placebo. Also, we hypothesized that samples from mothers receiving 28,000 IU/week during and after pregnancy would have a higher concentration than the placebo group and mothers receiving 28,000 IU/week only during pregnancy.

Aim 2: Examine the association between cholecalciferol concentrations in maternal breast milk and infant and maternal serum 25(OH)D levels at 6 months.

Hypothesis: Serum vitamin D levels in the mothers and infants would be directly related with the cholecalciferol levels in each participant at 6 months.

Chapter 2: Materials and Methods

Study Design

This thesis was a sub-study of the Maternal Vitamin D for Infant Growth (MDIG) trial in Dhaka, Bangladesh where vitamin D deficiency is common. The parent trial, funded by the Bill and Melinda Gates Foundation, was a randomized, double-blind placebo-controlled trial aimed to assess how weekly prenatal and postnatal maternal vitamin D supplementation impacted the length-for-age z score for infants at one year of age (n=1300) [38,39]. The data and specimen collection of the parent trial included anthropometric data, serum, and breast milk. As the breast milk was not analyzed as part of the parent trial, my honors thesis aimed to measure the impact of oral vitamin D supplementation during pregnancy and lactation on breast milk cholecalciferol concentrations.

Population

Women who were recruited for this study were patients receiving prenatal care at the Maternal and Child Health Training Institute in Dhaka, Bangladesh. This government run facility provides medical care and treatment to children and pregnant women from Dhaka and its surrounding areas. Study inclusion criteria included being at least 18 years old, currently 17-24 weeks gestation (based on maternal report of last menstrual period and an ultrasound), and a singleton pregnancy. Pregnant women were excluded from the study for the following reasons: self-reported history of a medical condition, self-reported intake of medications that may cause a sensitivity to vitamin D, altered metabolism of vitamin D, hypercalcemia; considered a high-risk pregnancy based on point-of-care testing, maternal report or past ultrasound findings; reluctance to stop taking non-study vitamin D, calcium or multivitamin supplements; currently prescribed

vitamin D supplements from a physician; and previous enrollment in the MDIG study with a prior pregnancy. Before study enrollment, eligible women completed a screening process and were provided a detailed description of the trial before written informed consent was sought. Additional details on trial methods are published [38,39].

Ethical Statement

The protocol for the MDIG trial was approved by the research ethics committees at the International Center for Diarrheal Disease Research, Bangladesh (icddr,b) and the Hospital for Sick Children in Toronto. This current thesis was considered human subjects exempt due to the use of de-identified data and samples.

Treatment Groups and Randomization

In the parent trial, 1300 women were enrolled in the study, but due to not meeting all inclusion criteria such as gestational age and geographic location, 176 were dropped out of the study. Thus, 1124 pregnant women were randomized into one of five treatment groups [39]. The first three groups received pregnancy vitamin D only in doses of 4200IU/week (n=229), 16000IU/week (n=224), and 28000IU/week (n=221); these groups received placebo during postpartum. The fourth group received pregnancy and postpartum supplementation of 28000IU/week (n=227). The fifth group served as the placebo group and received a placebo tablet during pregnancy and postpartum (n=223).

In this thesis, three of the original treatment groups were used for breast milk cholecalciferol measurements (n=671): placebo, pregnancy 28,000IU/week supplementation, and pregnancy and postpartum 28,000IU/week supplementation. After importing the data into the software, participants who did not provide milk samples at 6 months of infant age were dropped

(n=102; placebo: n=37, pregnancy: n=36, postpartum: n =29). Then, the “sample” command in Stata was used to randomly sample exactly 24 participants within each group to ensure a power of 90% assuming a minimum difference of 15 nmol/L between the placebo and supplementation groups (Wheeler et al., 2016).

The pre-analytical sample contained n=72 mothers with 24 in each of the three treatment groups. One sample was dropped because it was an outlier in the data set at 3 standard deviations away from the mean. This outlier can be seen in the kernel density plot in Figure 1. This participant was part of the pregnancy group, 28000:0 IU/week, so the analytical sample from this group was n=23, and the total analytical sample was 71.

Batches

After the selection of participants by treatment group, the samples were divided into four batches for vitamin D extraction based upon location in the freezer for easy access. Each batch consisted of a blank, internal standard, quality control, and a duplicate sample. The blank tubes contained deionized water, the surrogate (deuterated 25(OH)D). We used a quality control breast milk sample for QC with each batch. Additionally, the duplicate samples were rotated from the beginning of the batch to the end of the batch. The duplicate samples in batches 1 and 3 occurred at the beginning of the batch and the duplicate samples in batches 2 and 4 occurred at the end of the batches.

Study Visits

Study visits were either conducted at the Maternal and Child Health Training Institute in Dhaka or as home visits. Data was collected weekly from enrollment (second trimester) until delivery for the prenatal intervention and weekly from delivery until 6 months postpartum for the

postnatal intervention. At enrollment, sociodemographic data was collected based on maternal self-report in a questionnaire form.

Maternal Blood Specimen Collection

Maternal venous blood was collected by trained phlebotomists during study visits at baseline, delivery, 3 months postpartum, and 6 months postpartum. Immediately following collection, the serum collection tubes were inverted. Then, after resting for 30 minutes, the samples were centrifuged at a low speed for 15 minutes and then the serum was transferred into 0.25ml aliquot tubes. These tubes were kept in a portable freezer (<-70 °C) until being placed in a -80 °C freezer or liquid nitrogen canister at the end of the day.

Breast Milk Specimen Collection

At 3 and 6 months of infant age, hand-expressed midfeed breast milk spot samples were collected and stored at −80 °C. Moms were instructed to breast feed from the left breast for about 2 minutes before removing the child from her breast. Then, she was asked to hand express 6-8mL of milk into a collection tube. After collection, the study staff inverted the container 5 times to mix the milk. Then, a micropipette was used to transfer 1.5 mL into two microfuge tubes for storage. While there were milk samples from both 3 and 6 months of infant age, for this thesis I analyzed the breast milk samples collected at 6 months of infant age.

Exposures

During weekly study visits, participants were given small oral vitamin D tablets (10mm in diameter) and watched by study personnel to ensure ingestion. Weekly supplementation began at enrollment during the second trimester for all experimental groups throughout pregnancy. For

the prenatal groups, vitamin D supplementation ended at delivery and the participants were switched to the placebo until 6 months of infant age, but the postpartum group continued supplementation until 6 months of infant age [39]. The oral supplementation served as the exposure for Aim 1 of this thesis. During this project, the amount of vitamin D present in breast milk samples at 6 months of infant age was measured. These vitamin D levels were the exposure for that part of Aim 2 examining the relationship between vitamin D in milk and serum 25(OH)D in infant serum.

Outcomes

The measured outcome for Aim 1 in this thesis was the cholecalciferol concentrations in breast milk at 6 months of infant age. We attempted to measure other forms of vitamin D metabolites, but they were below the limit of detection of the mass spectrometry used for analysis. The measured outcome part of Aim 2 the measured breast milk vitamin D levels (with maternal serum 25(OH)D as the exposure).

Vitamin D Extraction for Milk Method

This method was adapted from previous extraction methods [40,41] and completed as part of this thesis.

Preparation

All 6-month postpartum milk samples were kept in an -80ºC freezer until needed. Upon thawing, milk samples were inverted to homogenize prior to use.

Saponification

A 2.5 mL of a saponification mixture of 25% potassium hydroxide, 1% pyrogallol in 75% ethanol was added to each extraction tube followed by 1 mL of deionized water for blank samples or 1mL of milk samples. Then, samples were incubated in a warm water bath at 55°C with gentle stirring. After the incubation, the samples were cooled back to room temperature.

Lipid Extraction

5mL of 100 µg/mL butylated hydroxytoluene and 50 µL deuterated 25(OH)vitamin D3 (surrogate) were added to each tube and then vortexed for 30 seconds. Then, 2.5 mL of 7% sodium sulfate was added, the samples were vortexed for another 30 seconds followed by 10 minutes of room temperature centrifugation at 1600 rpm. After centrifuging, the entire upper hexane phase was transferred to a clean tube.

Concentration and Reconstitution

After the samples were dried under N_2 gas with a 37°C water bath, they were reconstituted with 1mL of 60% methanol and vortexed to dissolve.

Solid Phase Extraction Purification

Vitamin D was purified from hexane extracts using hydro-lipid balanced solid phase extraction cartridges (Machery Nagel, City, Country; Chroma bond 3cc 60 mg) were added to a vacuum manifold. Each cartridge was washed with 3 mL of hexane, then 3 mL of pure methanol, followed by conditioning with 3 mL of 5% methanol in deionized water. Then, the samples were loaded onto the column. Each extraction tube was washed out with 1 mL 60% methanol and

added to the column to ensure that all sample was properly transferred. A light vacuum of 0.5-3 mmHg was applied to load the samples. Then, each column was washed with 1.5 mL of 40% methanol using a light vacuum of 0.5-3 mmHg to elute polar lipids. Next, the vacuum was increased to approximately 10mmHg to dry the columns. Finally, the vitamin D metabolites were eluted with 0.5mL of pure methanol into 2mL vials twice. After the metabolites were collected, the columns were washed twice by pulling through 3 mL of methanol.

Reconstitution and Analysis

We placed the vitamin D metabolites under N_2 gas with a 37°C water bath until fully dry. 50 µL of 10 nM CUDA in liquid chromatography mass spectrometry grade methanol was used to reconstitute the samples and then they were vortexed to dissolve. The vortexed samples were then transferred into limited volume inserts in labeled amber autosampler vials for analysis by liquid chromatography mass spectrometer.

Method Development

The original intended research aim for this thesis was to develop an Ultra Performance Liquid Chromatography – Photo Diode Array method for the measurement of vitamin D in breast milk. However, the amount of vitamin D in the breast milk samples was lower than expected, and therefore below the limit of the UPLC's detection. As a result of this issue, we used mass spectrometry for measurement of vitamin D in milk instead.

Liquid Chromatography Mass Spectrometry Vitamin D Method

We used liquid chromatography-mass spectrometry (Waters™ Xevo™ TQD with a pentafluorophenyl column) electro-spray ionization to separate and measure 25-

hydroxycholecalciferol [25(OH)D3], and cholecalciferol concentrations chromatographically from the breast milk samples adapted from Albrahani et al., and Yazdanpanah et al., [41,42]. After injecting 5uL of sample, metabolites were separated using a XSelect HSS PFP 2.5 µM 2.1x100 mm particle size column. A 0.6 mL/min flow rate was set consisting of a gradient run using water with 0.1% formic acid, 5mM ammonium formate (Solvent A), and methanol (Solvent B) for 10 minutes (0-8 min 35% B to 95% B, 8-8.5 min 95% B, 8.5-10 min 35% B). The electrospray ionization operated with the capillary set at 1.8 kV and in positive ion mode. The desolvation temperature was set at 500 °C and source temperature at 150 °C. Vitamin D standards were directly injected on the mass spectrometer to optimize cone voltage, collision energy, and most prevalent daughter fragments.

A standard curve was created using cholecalciferol, 25(OH)D3 and deuterated 25(OH)D3. The limit of quantification of our standard curve was 0.5 nmol/L for cholecalciferol, but we were able to detect lower than that. There were inconsistent readings for 25(OH)D3, so the limit of quantification of the standard curve was not considered due to limit of detection. The overall coefficient of variation between batches was 0.71. The coefficients of variation for batches 2-4 were 0.72, 0.64, and 1.23 respectively. There was no coefficient of variation for batch 1 due to an error with the lipid extraction in the duplicate sample. We used the deuterated 25(OH)D3 as a surrogate for calculating our extraction efficiency. Using a spectrophotometer, we calculated the UV absorbance of the surrogate at 265 nm. This absorbance was used to calculate dilutions which we ran on the mass spectrometer. However, the concentrations were not quantifiable by the mass spectrometer and thus could not be used as a control.

Measurement of Maternal Serum 25-Hydroxyvitamin D Concentrations

Using a high-performance liquid chromatography-tandem mass spectrometry, 25-hydroxyvitamin D concentrations were measured. The specificity of the machine allowed for separate measurements of 25(OH)D3, 25(OH)D2, and 25(OH) D3 C-3 epimer concentrations. All these measurements took place at the Analytical Facility for Bioactive Molecules at the Hospital for Sick Children in Toronto, Canada as part of the parent trial.

Statistical Analysis

All statistical analysis for this study was conducted in the Stata 18.0 (College-Station, TX) software. We first examined distributions of continuous variables and frequencies of categorical variables. Kernel density plots were created to examine the distribution of the continuous variables, as well as mean, median, skewness and kurtosis. For aim 1, we performed an ANOVA test to test the mean differences of milk cholecalciferol concentrations between the three vitamin D treatment groups. For aim 2 we examined the relationship between maternal serum (exposure) and breast milk (outcome) vitamin D and the relationship between breast milk (exposure) and infant serum (outcome) vitamin D. We performed a linear regression to examine the association between the maternal serum 25-hydroxyvitamin D levels and the breast milk cholecalciferol levels both at 6 months. We performed a second linear regression to examine the association between the breast milk cholecalciferol levels and infant serum 25-hydroxyvitamin D levels both at 6 months. We ran the models unadjusted (only serum and milk values) and adjusted (including maternal age, parity, gestational age, birth mode and infant sex). In sensitivity analysis, we examined the four batches run in the mass spectrometry to look at the overall distribution and the distributions by batch.

Chapter 3: Results

Maternal and Infant Characteristics

As seen in Table 1, the mean maternal age was 23.9 years old with participants ranging in age from 18-38 years old. Thirty-seven percent of women were pregnant with their first child. About 56% of children were born vaginally, leaving the other 44% to have been born by cesarian section. There was an even split of male and female babies with 51.5% male.

Table 1. Maternal and infant characteristics by vitamin D supplementation treatment group for participants in a randomized controlled trial of maternal supplementation in Bangladesh

	All Participants (n = 71)	Placebo (0:0 IU/wk) (n = 24)	Pregnancy 28000:0 IU/wk (n = 23)	Postpartum 28000:28000 IU/wk (n = 24)
Maternal Characteristics				
Maternal Age, years	23.9 ± 4.8	25.8 ± 6.1	22.7 ± 3.6	23.1 ± 3.5
(Primiparous, %)	36.6	12.5	47.8	50.0
Infant Characteristics				
Gestational Age, weeks	39.0 ± 1.4	38.8 ± 1.5	39.0 ± 1.6	39.2 ± 1.2
Infant Sex (% male)	50.7	50.0	52.2	50.0
Birth Mode (% vaginal births)	56.3	54.2	52.2	62.5

Values are mean ± standard deviation or percent

Aim 1

Figure 1 shows the distribution of cholecalciferol concentrations in the breast milk by supplementation group. The mean cholecalciferol concentration of the entire analytical sample was 13.98 nmol/L (SD= 20.97 nmol/L) with a median of 8.13 nmol/L (IQR = 4.22-14.61 nmol/L). The range for all values was 1.33 to 147.31 nmol/L. For the placebo group (0:0 IU/week vitamin D), the mean was 16.84 nmol/L (SD = 31.17 nmol/L) with a median of 6.48 nmol/L (IQR = 4.17-13.50 nmol/L). For the pregnancy group (28000:0 IU/week vitamin D supplement), the mean was 10.9 nmol/L (SD = 11.50 nmol/L) with a median of 7.45 nmol/L (IQR = 4.22-12.05 nmol/L). For the pregnancy and postpartum group (28000:28000 IU/week vitamin D supplement), the mean was 14.1 nmol/L (SD = 14.92 nmol/L) with a median of 9.89 nmol/L (IQR = 3.86-18.48 nmol/L). When tested by ANOVA, mean milk cholecalciferol concentrations did not vary between treatment groups (p=0.630).

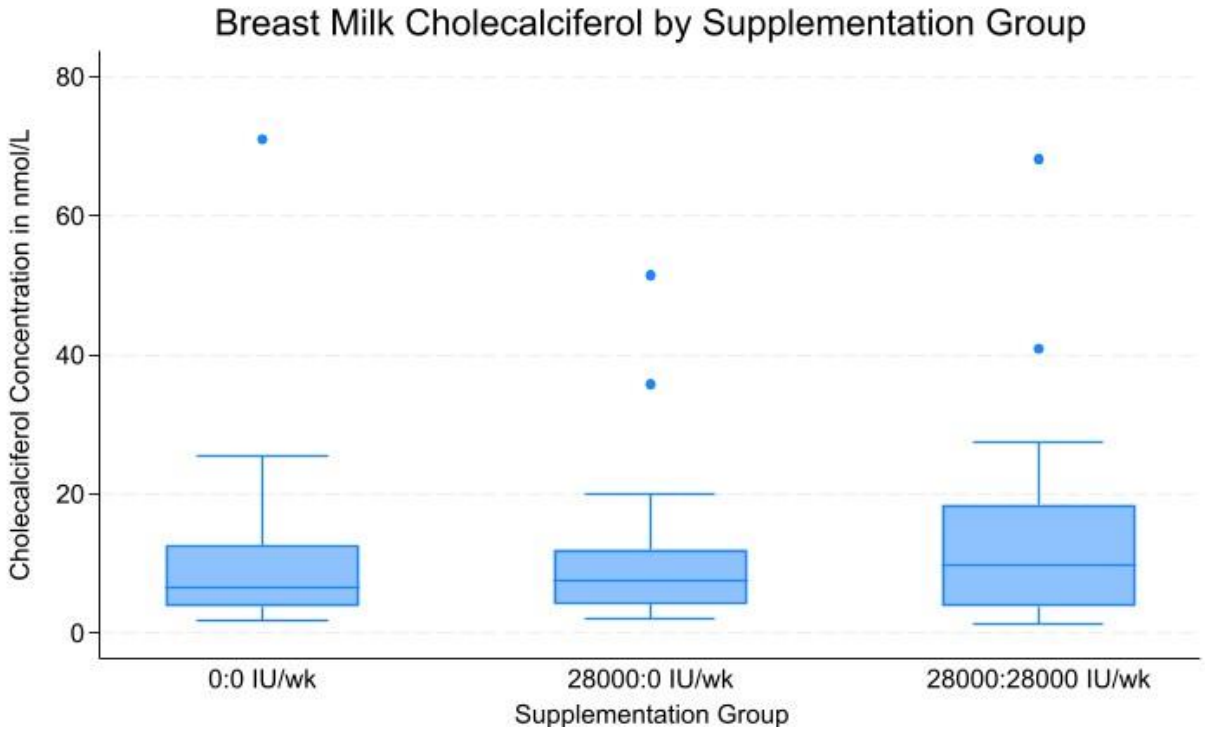

Figure 1. Cholecalciferol concentration (in nmol/L) in breast milk by supplementation group (n=71). Boxes represent the median and interquartile range, and whiskers represent the 95th and 5th percentiles. All samples outside the 95th percentile are represented as dots. One sample at 147.3 nmol/L (0:0 IU/wk group) was dropped from the figure to improve visualization of the center of the distributions.

Aim 2

28 infants and 35 mothers had serum 25(OH)D data available for analysis in aim 2. Figure 2 shows the kernel density estimate of maternal serum 25(OH)D at 6 months with a mean of 65.0 nmol/L. Figure 3 shows the kernel density estimate for the infant serum 25(OH)D at 6 months of age. It has a normal distribution with a mean of 65.2 nmol/L. Figure 4 shows the locally Weighted Scatterplot Smoothing (Lowess) plot of maternal 25(OH)D (nmol/L) and breast milk cholecalciferol concentration (nmol/L) at 6 months. It shows this relationship as linear. Figure 5 shows the Lowess plot of maternal breast milk cholecalciferol concentration (nmol/L) and infant serum 25(OH)D at 6 months. The results of two linear regressions can be seen in Table 2. The adjusted models were adjusted for the babies' gestational age at birth, infant sex, birth mode, parity and maternal age. The maternal regression (maternal serum 25(OH)D with breast milk cholecalciferol concentration) was not statistically significant in either the unadjusted or adjusted models with p-values of 0.06 and 0.07, respectively. The infant regression (breast milk cholecalciferol concentration with infant serum 25(OH)D at 6 months), however, was statistically significant in both the unadjusted and adjusted models with p-values of 0.02 and 0.03, respectively.

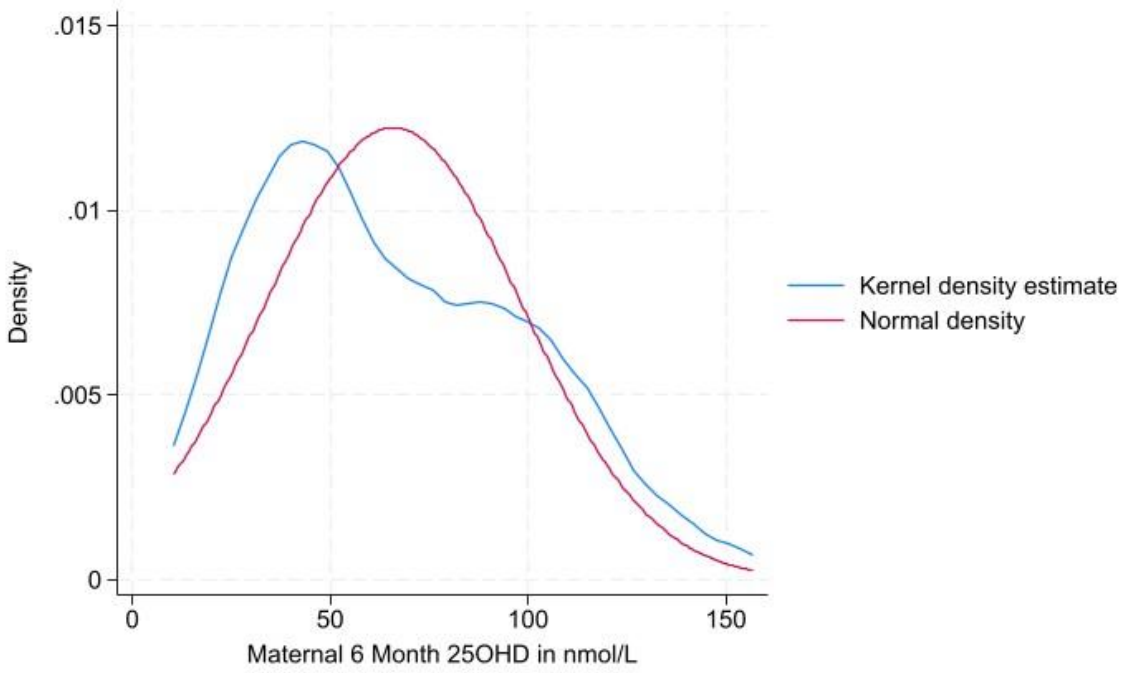

Figure 2. The kernel density plot of maternal serum 25(OH)D at 6 months

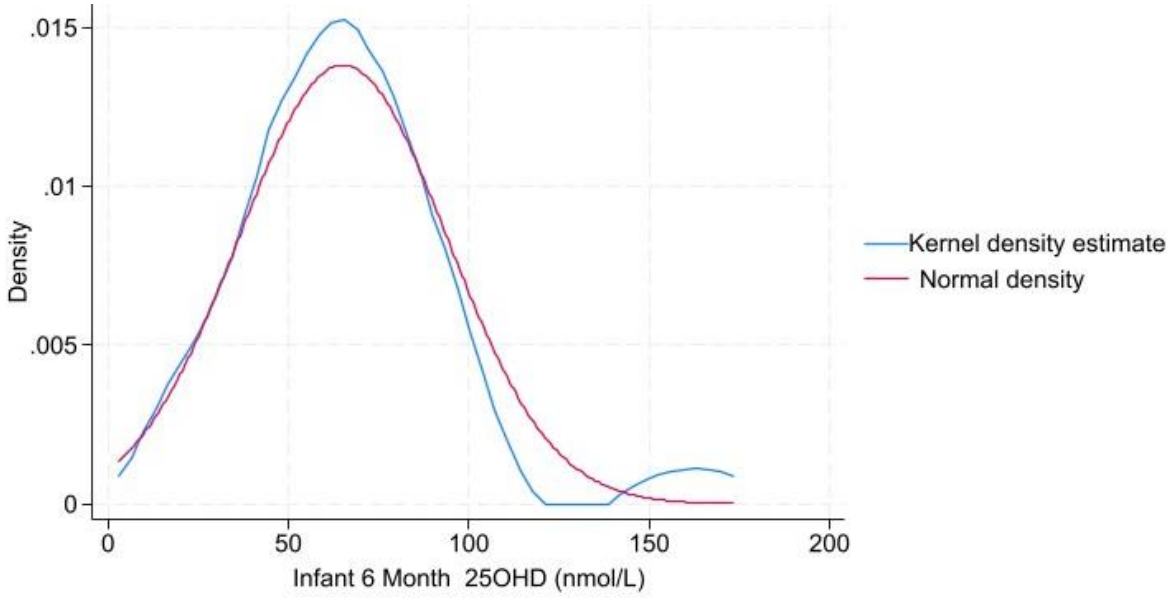

Figure 3. The kernel density plot of infant serum 25(OH)D at 6 months

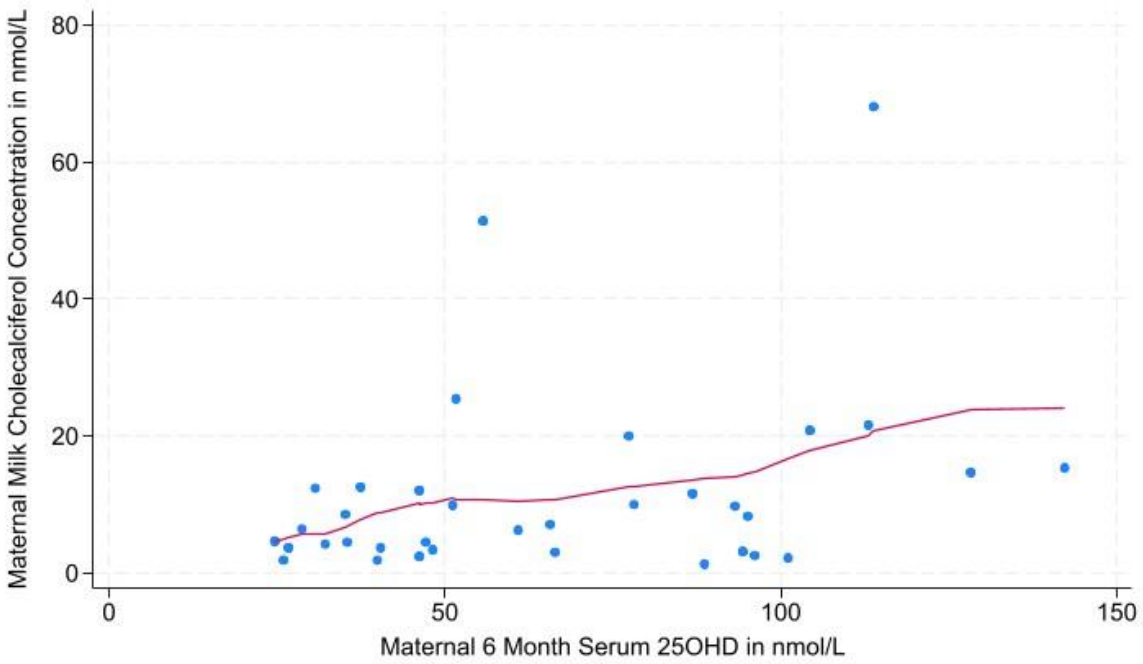

Figure 4. Locally Weighted Scatterplot Smoothing (Lowess) plot of maternal 25(OH)D (nmol/L) and breast milk cholecalciferol concentration (nmol/L) at 6 months

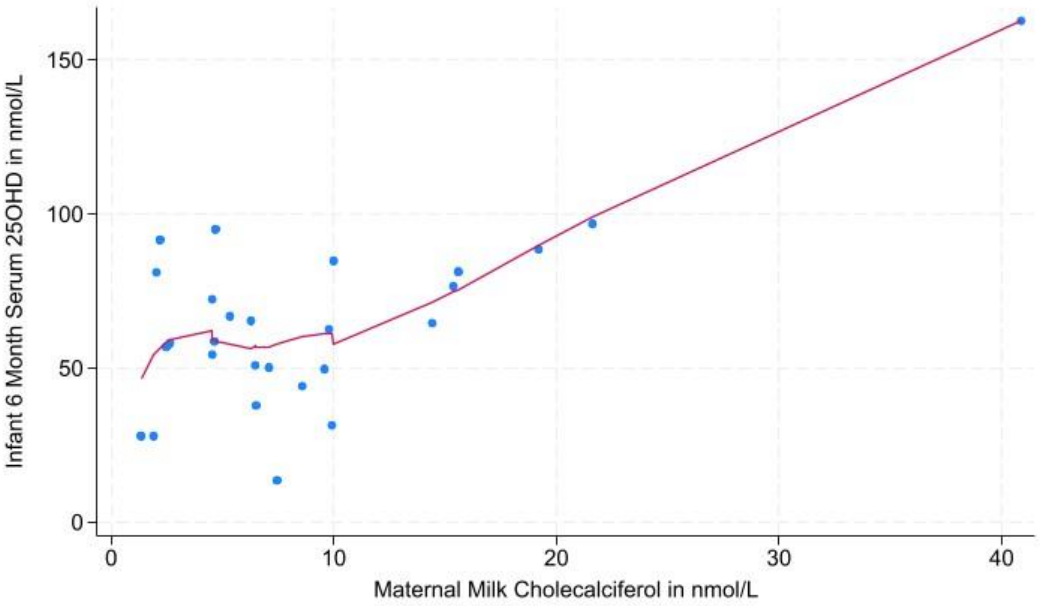

Figure 5. Locally Weighted Scatterplot Smoothing (Lowess) plot of maternal breast milk cholecalciferol concentration (nmol/L) and infant serum 25(OH)D at 6 months. One sample of 162.7 nmol/L (infant serum) was dropped from the figure to improve visualization of the center of the distributions.

Table 2. Linear regression models for maternal 25(OH)D and breast milk cholecalciferol concentration with maternal breast milk cholecalciferol concentration with infant serum 25(OH)D at 6 months

	Unadjusted		Adjusted*	
	Mean	95% CI	Mean	95% CI
Maternal (n=35)				
25(OH)D, per 10 nmol/L [outcome cholecalciferol, nmol/L]	1.35	-0.063, 2.77	1.47	-0.129, 3.07
Infant (n=28)				
Cholecalciferol, nmol/L [25(OH)D, nmol/L]	0.921	0.167, 1.68	0.952	0.119, 1.78

*Adjusted for gestational age, infant sex, birth mode, parity, and maternal age

Chapter 4: Discussion

In this study measuring the impact of prenatal and postpartum vitamin D supplementation on cholecalciferol concentrations in breast milk at 6 months, we examined the effect of oral maternal vitamin D supplementation on the concentrations of cholecalciferol in breast milk at 6 months as well as the associations between cholecalciferol concentrations in maternal breast milk with infant and maternal serum 25(OH)D levels at 6 months. We found that mean milk cholecalciferol concentrations did not vary between supplementation groups and were not related to maternal serum 25(OH)D. However, we did find that breast milk cholecalciferol concentrations were related to infant serum 25(OH)D.

Aim 1

For the first aim of this thesis, we examined the effect of oral maternal vitamin D supplementation (placebo, prenatal 28,000 IU/week, and prenatal and postpartum 28,000 IU/week) concentrations of cholecalciferol in maternal breast milk samples at 6 months of age. We found no statistically significant differences between the mean concentration of cholecalciferol in breast milk by supplementation group. The placebo group had the highest mean cholecalciferol concentration (16.8 nmol/L), followed by the pregnancy and postpartum group having a mean cholecalciferol concentration of 14.1 nmol/L. The pregnancy supplementation group had the smallest cholecalciferol concentration of 10.9 nmol/L. These results did not support our hypothesis for this aim as we predicted that the amount of breast milk cholecalciferol would increase with increased supplementation.

We were unable to find any randomized controlled trials that measured cholecalciferol in breast milk. However, several studies looked at the total vitamin D concentration in breast milk.

One longitudinal study in Finland explored the potential benefits of oral maternal vitamin D supplementation for mothers living in country with four distinct seasons [29]. One group of 45 mothers who delivered in the winter were divided into three groups (2000IU/day cholecalciferol supplement, 1000IU/day cholecalciferol supplement, or placebo). A second group of 40 mothers (20 delivered in the winter and 20 delivered in the summer) were divided into two groups: 1000IU/day ergocalciferol and placebo. Samples from group 1 were collected at 8 and 20 weeks postpartum and samples from group 2 were collected at 8 and 15 weeks postpartum. The researchers found that while maternal supplementation increased milk 25(OH)D levels slightly, it did not influence overall milk vitamin D levels. This is consistent with our findings in general because cholecalciferol is the most prominent form of vitamin D in breast milk. Since the breast milk total vitamin D levels did not increase which could be attributed to a lack of cholecalciferol increase with supplementation amount. However, the timing of supplementation was not stated in Finland study, making it difficult to compare to our work.

Another observational study found that total milk vitamin D was associated with maternal vitamin D intake ($p = 0.005$) [42]. They also found that maternal milk cholecalciferol and vitamin D intake were correlated ($p=0.03$) in an unadjusted model. This pattern is different than that of our results which showed no difference between cholecalciferol levels in milk even with increased or prolonged oral supplementation.

Compared to our mean findings of cholecalciferol in breast milk at 6 months (placebo: 16.8 nmol/L, pregnancy 28,000 IU/week supplementation: 10.9 nmol/L, and pregnancy and postpartum: 28,000 IU/week supplementation: 14.1 nmol/L), Hollis and Wagner recorded lower amounts of vitamin D in breast milk after their 4-month trial, but did find that breast milk vitamin D increased with oral supplementation [28]. After 4 months of 2000IU/day lactation

supplement, they recorded a total breast milk vitamin D concentration of 69.7 IU/L (2.28 nmol/L), a significant increase forms the 35.5 IU/L (1.16 nmol/L) concentration at baseline. After 4 months of a 4000IU/day lactation supplementation, total vitamin D concentrations in milk increased significantly from 40.4 IU/L (1.32 nmol/L) at baseline to 134.6 IU/L (4.40 nmol/L). Contrarily, Greer et al reported antirachitic activity of 6700 IU/day (218.9 nmol/L) of ergocalciferol in human milk from a mother receiving a daily supplement of 100,00 IU/day during pregnancy to maintain serum calcium concentration for thyroid-parathyroidectomy [43] . This data supports the validity of our 147.31 nmol/L cholecalciferol concentration measured from one participant. Finally, in a paper by Ala-Houhala et al., the range of reported hindmilk total vitamin D levels from the participants receiving 0-2000IU/day at 8 weeks was 286-403 pmol/L (0.286-0.403 nmol/L) and at 15 weeks was 359-390 pmol/L (0.359-0.390 nmol/L) [29]. In general, our cholecalciferol concentration measurements were higher than studies measuring total vitamin D concentrations in breast milk.

Aim 2

For the second aim, we examined associations between breast milk cholecalciferol and serum 25-hydroxyvitamin D at 6 months. First, we looked at the relationship between maternal serum 25-hydroxyvitamin D levels and the breast milk cholecalciferol concentration. Second, we examined the relationship between breast milk cholecalciferol concentrations and infant serum 25-hydroxyvitamin D levels. We hypothesized that the serum levels in mothers and infants would both be correlated with breast milk cholecalciferol. However, our results showed no statistically significant association between maternal serum 25(OH)D with breast milk cholecalciferol concentration in an unadjusted model. These results remained nonsignificant after adjusting for gestational age at birth, infant sex, birth mode, parity and maternal age. On the

contrary, both the unadjusted and adjusted models for the association between breast milk cholecalciferol concentration and infant serum 25(OH)D at 6 months were statistically significant. Therefore demonstrating the importance of milk cholecalciferol levels and infant vitamin D.

Like our results, in the Ala-Houhala et al study previously mentioned, the researchers also found no significant correlation between 25(OH)D levels in maternal serum and breast milk [29].

While Hollis et al., did not measure the vitamin D in milk, they did look at the impact of maternal supplementation on infant serum levels [44]. Interestingly, the results showed that there was no difference in infant serum 25(OH)D between infants receiving a 400IU/day cholecalciferol supplement and mothers receiving a 6400IU/day supplement. Therefore, demonstrating maternal supplementation at high enough doses can increase sufficiency in breastfed infants. Hollis and Wagner found that maternal supplementation of 2000IU/day increase infant serum 25(OH)D levels significantly from 7.9 ± 1.1 to 27.8 ± 3.9 ng/mL ($P < 0.02$) and daily maternal supplementation of 4000IU/day increased infant serum 25(OH)D levels from 13.4 ± 3.3 to 30.8 ± 5.0 ng/mL ($P < 0.01$) [28].

Dawodu & Tsang reported that the vitamin D status of infants that are breastfed reflects the vitamin D concentrations in maternal breastmilk [27]. After three months of oral vitamin D supplementation, infants of mothers receiving 4000IU/day had significantly higher serum 25(OH)D-2 (30.0 ± 3.5 nmol/L) than infants of mother receiving 2000IU/day (15.0 ± 2.7 nmol/L). These studies suggest a correlation between maternal supplementation and infant serum like that seen in our study between breast milk cholecalciferol concentrations and infant serum 25(OH)D. A Danish study found no correlation between infant plasma 25(OH)D concentrations

and vitamin D concentrations in milk samples [45]. However, the authors contributed that to the mother's likely following the recommendation to supplement their infants with 400IU/day of vitamin D.

Another study, published in 1985, found no correlation between breast milk vitamin D and infant serum 25(OH)D from a baseline measurement used to compare differences between two races [42]. They suggested that even in exclusively breast-fed infants that infant sun exposure has a greater impact on vitamin D status than maternal dietary vitamin D. It is important to note that this study was conducted before public health recommendations to keep infants out of the sun to prevent sunburn and reduce risk for cancer later in life. Unlike the Specker study, our study did find a significant correlation between breast milk vitamin D and infant serum 25(OH)D. While we only measured cholecalciferol, as the most concentrated form of vitamin D in breast milk, it should play a huge role in the overall vitamin D levels.

Limitations

This study had several limitations particularly involving measuring cholecalciferol in breast milk. The original plan for this thesis was to develop a vitamin D method for the UPLC-PDA, but after determining the limit of detection was not low enough, we switched to using a mass spectrometer. While we were able to measure vitamin D on the mass spectrometer, there were still several challenges. First, of all we had issues with the quality control resulting in an overall coefficient of variation between batches being 0.71. Additionally, the surrogate was inconsistent throughout the samples in the study making it difficult to validate our methods. Next, for our study, we had small volumes of breast milk, so we were only able to analyze a maximum volume of 1 mL of milk from each participant. Several studies used a higher volume of milk [42,46–48] which could have increased our ability to detect vitamin D.

As a comparison, one study measured total milk vitamin D (ergocalciferol, cholecalciferol, 25(OH)D2 and 25(OH)D3) from a total of 9 milliliters of breast milk from each participant [42]. However, the milk was assayed by ligand-binding assay with a less than 10% variation in the intra-assay and interassay coefficients for the milk vitamin D metabolites. Other methods used to detect vitamin D in milk included the use of positive ion Atmospheric Pressure Chemical Ionization (APCI) mode on the mass spectrometer [48] and derivatization [49] . Finally, vitamin D detection methods beyond LC/MS and UPLC-PDA should be considered for future studies.

Another important limitation of this study was the collection of breast milk samples in the parent trial. The primary focus of the parent trial was not about breast milk, so we only had spot samples from the trial. There is also limited published information about the way in which the milk was collected from the breast-feeding mothers.

Finally, for the second aim of this thesis, only 28 and 35 out of the 71 selected participants had blood samples that could be used for analysis. We should have considered data availability when selecting participants at the beginning of the study and aligned our milk samples with availability of serum in both mother and infant.

Strengths

Our study was the first study, to our knowledge, to examine the breast milk cholecalciferol levels for women continuing oral vitamin D supplementation from pregnancy through lactation. Previous studies have examined the effects of vitamin D supplementation on breast milk cholecalciferol concentrations either prenatally or postpartum, but none have studied milk cholecalciferol levels from women who were supplemented during both time periods [5,29–

31]. Another strength of this thesis was the design. The parent trial was a randomized control trial, and this sub-study was also designed with randomized groups making all results applicable.

Summary and Future Directions

In conclusion, we found that breast milk cholecalciferol did not increase with supplementation, nor did we see any benefit in continuing prenatal supplementation through lactation. However, infant serum 25(OH)D levels were related to maternal milk cholecalciferol levels. Other studies should continue to examine the possibility that maternal supplementation may increase milk concentrations and in turn could be used to mitigate infant deficiency. Further studies should examine the relationships between maternal and infant serum vitamin D levels with milk cholecalciferol on a broader scale, as well as continue to study the continuation supplement with vitamin D throughout lactation.

Chapter 5: Appendix

Analytical Sample Distribution

The kernel density of both the preanalytical and analytical samples are rightly skewed as seen in Figure 6 and 7, respectively. Figure 8 displays the kernel density plots for each of the four batches of samples. Figure 8A shows the kernel density estimate of batch 1 with a mean of 18.0 nmol/L. Figure 8B shows the kernel density estimate of batch 2 with a mean of 11.6 nmol/L. Figure 8C shows the kernel density estimate of batch 3 with a mean of 19.5 nmol/L. Figure 8D shows the kernel density estimate of batch 4 with a mean of 6.9 nmol/L.

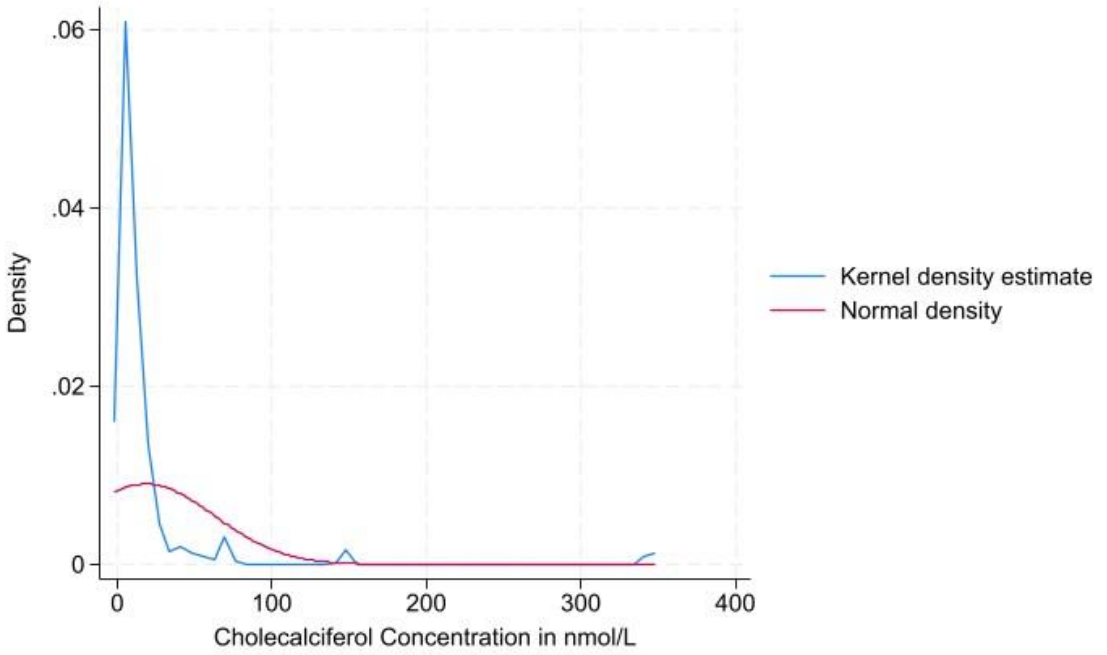

Figure 6. The kernel density plot of cholecalciferol (n=72)

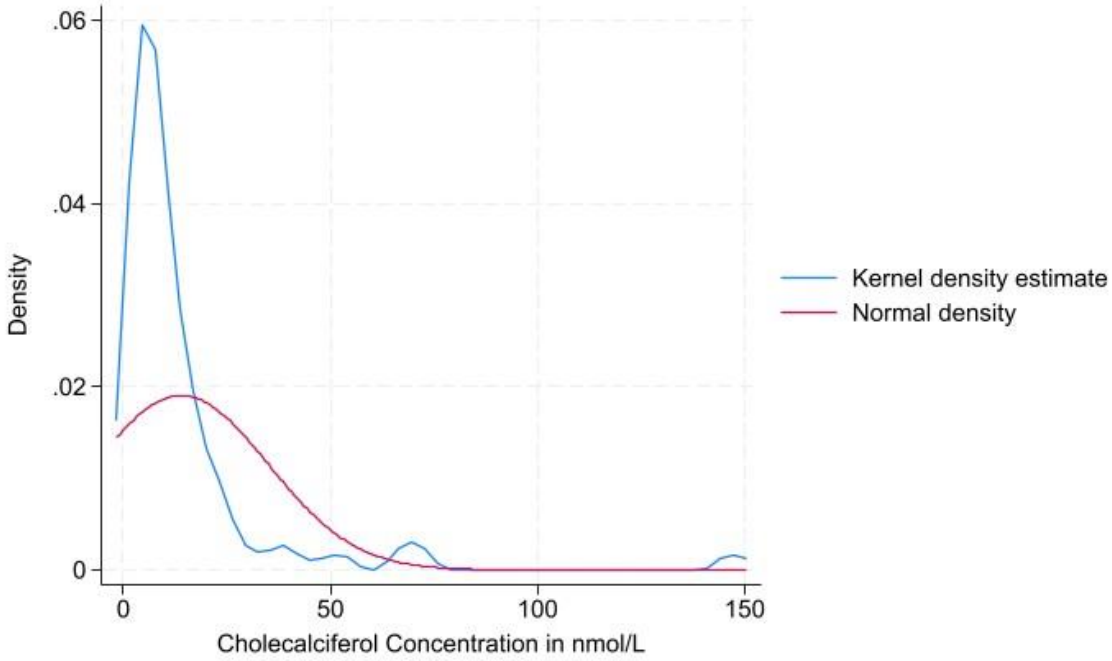

Figure 7. The kernel density plot of cholecalciferol (n=71)

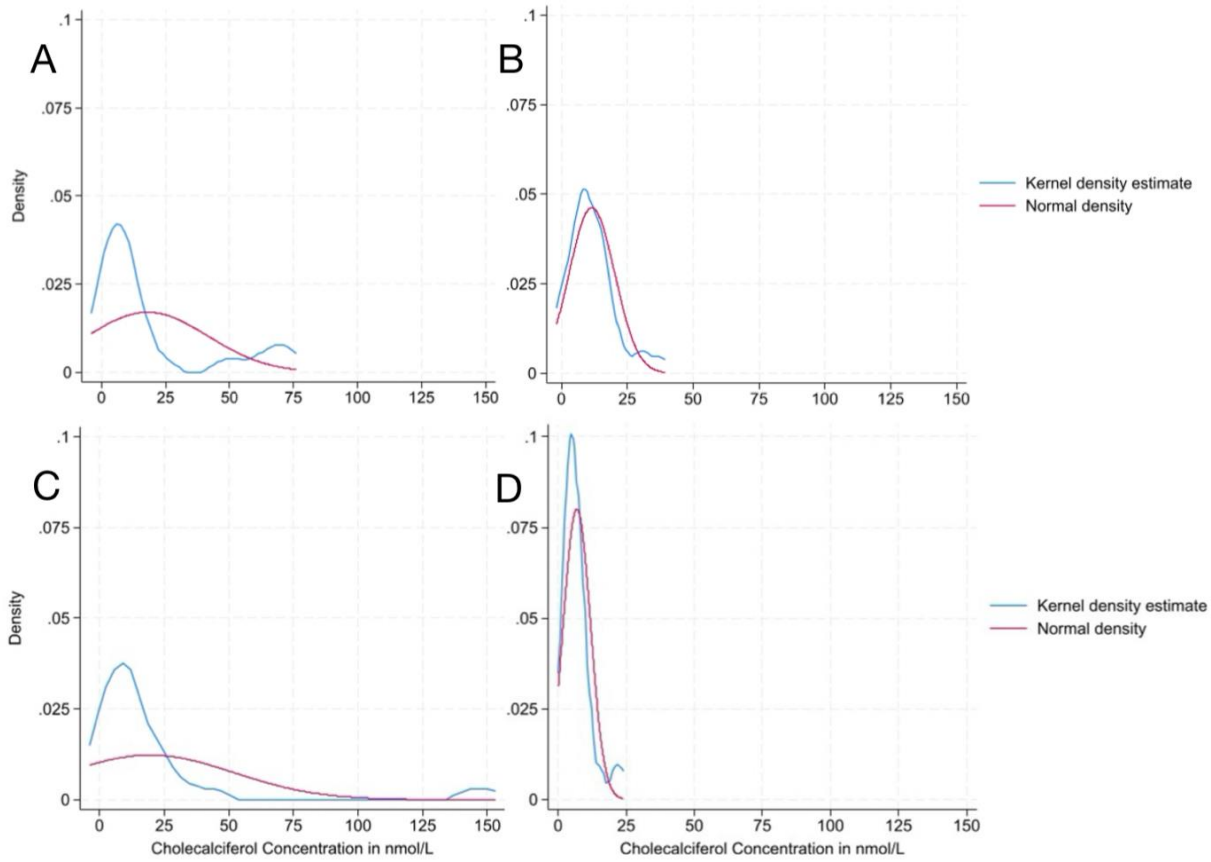

Figure 8. The kernel density plot of cholecalciferol for each of the four batches of samples run (Batch 1 (n=16): A, Batch 2 (n=18): B, Batch 3 (n=19): C, Batch 4 (n=19): D).

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