Effect of rapid correction of vitamin D insufficiency on bone health in breastfed infants born with low vitamin D stores: A randomized, double-blind trial in Montreal, Canada

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ABSTRACT

Many Canadian infants are born with an insufficient vitamin D status. Maintaining a low serum 25-hydroxyvitamin D [25(OH)D] concentration of less than 50 nmol/L during infancy could have negative impacts on bone health. In order to support a sufficient 25(OH)D concentration, Health Canada currently recommends that all breastfed infants receive a vitamin D supplement of 400 international units per day (IU/d). It has been shown that higher doses of vitamin D allow for a more rapid increase in 25(OH)D which could benefit those born with insufficient status. However, the lack of evidence in this population limits the development of new guidelines. Thus, the primary objective of this thesis was to determine whether current Canadian supplementation guidelines of 400 IU/d are adequate for infants born with vitamin D insufficiency, or if a higher dose (1000 IU/d) is associated with increased bone mineralization. The secondary objective was to test whether a higher status target of 25(OH)D ≥75 nmol/L, irrespective of supplementation dosage, is associated with changes in bone outcomes. In this preliminary analysis from an ongoing double-blinded randomized controlled trial (RCT) (clinicaltrials.gov: NCT02563015), healthy term-born breastfed infants (n=56) were recruited from a hospital in the Montreal area. Infant blood was sampled 24-36 hours after birth for measurement of total serum 25(OH)D concentration (LIAISON, DiaSorin Inc.). Infants with insufficient status <50 nmol/L were randomized to receive either 400 IU/d (n=19) or 1000 IU/d (n=22) cholecalciferol. Infants with sufficient status ≥50 nmol/L (n=15) formed a reference group receiving 400 IU/d. At baseline (≤1 mo), and at 3 mo, bone mineral content (BMC) and bone mineral density (BMD) of the whole body and lumbar spine were measured using dual-energy X-ray absorptiometry (DXA). Anthropometry, demographic and dietary questionnaires were collected. Data were analyzed using a mixed-model ANOVA (SAS, version 9.3). Data are mean ± SD, significance of $p<0.05$. All mean bone and anthropometric outcomes in the 400 IU/d and 1000 IU/d groups were well within the range of values (mean ± SD) obtained for the reference group from birth to 3 mo, indicating healthy growth. The study population included slightly more male than female infants (32/56) and infants with light skin pigmentation (41/56). Breastfeeding rates were comparable between groups (p=0.32) with 91% (51/56) of infants

received breast milk up to 3 mo of age, and of those 82% (42/51) were exclusively breastfed. By 3 mo, serum 25(OH)D concentration was 77 ± 23 nmol/L for the 400 IU/d group and 131 ± 39 nmol/L for the 1000 IU/d group ($p<0.0001$). No significant differences in absolute or weight-adjusted BMC of the whole body or lumbar spine, or BMD of the lumbar spine were observed between groups. Percent change of total body less head BMC from baseline was significantly higher in the 1000 IU/d group compared to 400 IU/d (46.2 ± 23.5% vs. 37.3 ± 14.8%, $p=0.02$). Infants in the 1000 IU/d group also had significantly greater length velocity with an average growth rate of 0.5 ± 0.2 cm/mo more than infants receiving 400 IU/d ($p=0.03$). Additionally, reaching a higher status target of ≥75 nmol/L 25(OH)D was not associated with any differences in bone outcomes. The preliminary results of this study suggest that there may be some advantages in terms of bone mineral accretion and length velocity by 3 mo associated with the use of 1000 IU/d cholecalciferol. Study participants will continue in the trial until 1 y with follow-up to 3 y to assess the longer-term impacts of the interventions.

RÉSUMÉ

De nombreux enfants canadiens naissent avec un statut en vitamine D insuffisant. Chez les nourrissons, une concentration sérique de 25-hydroxyvitamine D [25(OH)D] de moins de 50 nmol/L peut avoir un impact néfaste sur la santé des os. Dans le but d'obtenir une concentration sérique de 25(OH)D suffisante, Santé Canada recommande actuellement que tous les nourrissons allaités reçoivent un supplément oral de vitamine D de 400 unités internationales par jour (UI/j). Il a été démontré qu'une dose plus élevée de vitamine D permet une augmentation plus rapide de 25(OH)D. Cela pourrait présenter un avantage considérable pour les nourrissons dont le statut osseux est à risque. Toutefois, le manque de données probantes chez cette population restreint l'élaboration d'une nouvelle thérapie. Ainsi, l'objectif principal de cette thèse était de déterminer si les lignes directrices canadiennes actuelles sur la supplémentation (400 UI/j) sont adéquates pour les nourrissons nés avec une insuffisance de vitamine D ou si une dose plus élevée (1000 UI/j) est associée à une minéralisation osseuse accrue. Puis, indépendamment du dosage de la supplémentation, l'objectif secondaire était d'observer si une cible plus élevée de 25(OH)D (≥75 nmol/L) est associée à des changements positifs au niveau de la minéralisation osseuse. Dans cette analyse préliminaire d'un essai contrôlé randomisé à double insu (ECR) (clinicaltrials.gov: NCT02563015), des nourrissons sains et allaités (n=56) ont été recrutés dans un hôpital de la région de Montréal. Des échantillons sanguins ont été prélevés 24 à 36 heures après la naissance pour en mesurer la concentration sérique de 25(OH)D (LIAISON, DiaSorin Inc.). Les nourrissons ayant une valeur insuffisante (<50 nmol/L) ont été randomisés pour recevoir 400 UI/j (n=19) ou 1000 UI/j (n=22) de cholécalciférol. Les nourrissons ayant un statut suffisant (≥50 nmol/L, n=15) ont formé le groupe de référence recevant 400 UI/j. Au commencement de l'étude (≤1 mo) et à 3 mois, le contenu minéral osseux (CMO) et la densité minérale osseuse (DMO) du corps entier et de la colonne lombaire ont été mesurés à l'aide de l'absorptiométrie biphotonique à rayon X (DXA). Des questionnaires anthropométriques, démographiques et alimentaires ont été recueillis. Les données ont été analysées à l'aide d'une ANOVA à modèle mixte (SAS, version 9.3). Les données sont moyennes ± ÉT, niveau de signification $p<0,05$. Tous les résultats moyens des os

et de l'anthropométrie dans les groupes de 400 UI/j et 1000 UI/j se situaient bien dans la fourchette des valeurs (moyenne ± ÉT) obtenues pour le groupe de référence de la naissance à 3 mois, ce qui indique une croissance saine. L'étude comprenait un plus grand nombre de garçons que de filles (32/56) et de nourrissons avec une pigmentation légère de la peau (41/56). Les taux d'allaitement maternel étaient comparables entre les groupes (p=0,32) et 91% (51/56) des nourrissons ont reçu du lait maternel jusqu'à 3 mois d'âge, puis 82% de ceux-ci (42/51) ont été allaités exclusivement. À 3 mois, la concentration sérique de 25(OH)D était de 77 ± 23 nmol/L pour le groupe 400 UI/j et 131 ± 39 nmol/L pour le groupe 1000 UI/j (p<0,0001). Aucune différence significative n'a été observée entre les groupes dans le CMO absolu ou ajusté au poids du corps entier ou de la colonne lombaire, ni pour la DMO de la colonne lombaire. Le pourcentage de variation du CMO du corps entier moins la tête par rapport au commencement de l'étude était significativement plus élevée dans le groupe de 1000 UI/j comparativement à 400 UI/j (46,2 ± 23,5% contre 37,3 ± 14,8%, p=0,02). Les nourrissons du groupe 1000 IU/j avaient également un accroissement statural significativement plus élevé avec un taux de croissance moyen de 0,5 ± 0,2 cm/mo de plus que les nourrissons recevant 400 UI/j (p=0,03). En outre, atteindre une concentration de 25(OH)D ≥75 nmol/L n'a pas été associée à un changement de densité minérale osseuse. Les résultats préliminaires de cette étude suggèrent qu'une supplémentation de 1000 UI/j de cholécalciférol pourrait avoir des avantages en termes d'augmentation de la densité minérale osseuse et d'accroissement statural à 3 mois. Les participants continueront leurs protocoles respectifs pendant 1 an et seront suivis jusqu'à 3 ans afin d'évaluer les impacts des interventions à plus long terme.

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PREFACE AND CONTRIBUTION OF AUTHORS

L. Glenn is the first author included in this thesis for her contributions to the work included. Her roles included shared responsibility for the recruitment of participants and collection of data at the Lakeshore General Hospital. She also had a significant role in communications with study participants for telephone recruitment and scheduling study visits. During study visits, L. Glenn assisted with anthropometric measurements, data collection, and data entry. She conducted and interpreted statistical analysis, reviewed relevant literature, and wrote the manuscript in this thesis.

C. Vanstone was responsible for coordination of the research study and training of all students in the MECNRU. She developed the documents used in the research study (i.e. surveys, pamphlets, consent forms etc.). During study visits, C. Vanstone took anthropometric and body composition measurements on infants as well as collected blood samples.

F. Rauch assisted in the conception of the project and was L. Glenn's committee member and an editor of this thesis.

H. Weiler was the principal investigator on this project and is the director of the MECNRU. She was responsible for the conception and grant proposal stages of this trial. H. Weiler coordinated all authors involved and supervised all aspects of the study to ensure that it was conducted successfully and in a timely manner. She was also L. Glenn's direct supervisor and the primary editor of this thesis.

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ABBREVIATIONS

$1,25(OH)_2D$	1,25-dihydroxyvitamin D
$24,25(OH)_2D$	24,25-dihydroxyvitamin D
25(OH)D	25-hydroxyvitamin D
AI	Adequate Intake
ALP	alkaline phosphatase
ANOVA	analysis of variance
APrON	Alberta Pregnancy Outcomes and Nutrition
BMC	bone mineral content
BMD	bone mineral density
BMI	body mass index
CHMS	Canadian Health Measures Survey
CLIA	chemiluminescence immunoassay
CPS	Canadian Paediatric Society
CT	calcitonin
CTX	C-terminal telopeptide of type I collagen
CV	coefficient of variation
CYP	cytochrome P450
DBP	vitamin D-binding protein
DEQAS	Vitamin D External Quality Assessment Scheme
DXA	dual-energy X-ray absorptiometry
ELISA	enzyme-linked immunosorbent assay
HPLC	high performance liquid chromatography
IOM	Institute of Medicine
ISCD	International Society for Clinical Densitometry
ITA°	individual typology angle
LC-MS/MS	liquid chromatography tandem–mass spectrometry
LSBA	lumbar spine bone area

LSBMC	lumbar spine bone mineral content
LSBMD	lumbar spine bone mineral density
NIST	National Institute of Standards and Technology
NTX	N-terminal telopeptide of type I collagen
P1NP	procollagen type 1 N-terminal propeptide
pQCT	peripheral quantitative computed tomography
PTH	parathyroid hormone
PTHrP	parathyroid hormone-related peptide
RANKL	receptor activator nuclear factor-kB ligand
RCT	randomized controlled trial
RDA	Recommended Dietary Allowance
REDCap	Research Electronic Data Capture
RMP	reference measurement procedure
SD	standard deviation
SPF	sun protection factor
SRM	standard reference material
TBLH	total body less head
UL	tolerable upper intake level
UVB	ultraviolet beta
VDR	vitamin D receptor
Vitamin D_2	ergocalciferol
Vitamin D_3	cholecalciferol
WBBMC	whole body bone mineral content
WHO	World Health Organization

UNITS AND CONVERSION FACTORS

cm	centimeter
d	day
g	gram
IU	international unit
L	liter
m	meter
mL	milliliter
mmol	millimole
mo	month
ng	nanogram
nmol	nanomole
s	second
wk	week
y	year
µg	microgram
µl	microliter

Conversion factors

1 ng/mL of 25(OH)D_3 = 2.50 nmol/L

µg of vitamin D = 40 IU

1 LITERATURE REVIEW

1.1 INTRODUCTION

It is well established that breastfed infants require a vitamin D supplement throughout infancy to prevent deficiency and avoid adverse bone health outcomes. Vitamin D was first identified as a component of cod liver oil in the early 1900s and valued for its ability to cure the childhood bone disease, rickets (1). Following this discovery, significant public health efforts took place to increase the amount of vitamin D in the food supply. Most notable was the mandatory fortification of cow's milk in 1975 in Canada along with the recommendation that infants require 100 international units (IU) of vitamin D per day (2, 3). This recommendation has increased over the years, most recently in 2011 to a level of 400 IU/d as recommended by the Institute of Medicine (IOM) (4). Similarly, recommendations exist for pregnant and lactating women to consume at least 600 IU/d of vitamin D to support fetal and neonatal vitamin D stores (5).

Despite these efforts, low vitamin D status is widespread and remains a public health issue today in both developed and developing countries. A recent study in Quebec City indicates that nearly 1 in 4 infants are born with insufficient vitamin D status based on the status biomarker 25-hydroxyvitamin D [25(OH)D] being below 50 nmol/L (6). This inadequacy is also observed in Canadian children and adults at similar rates (7, 8). There is some evidence that even moderate insufficiency of vitamin D status may have negative consequences on skeletal development in children. Vitamin D insufficiency has been linked to compromised skeletal mineralization (9, 10), increased susceptibility to fractures (11-13), and a reduced ability to achieve genetically programmed peak bone mass (14-16).

The current standard of care is to provide a supplement of 400 IU/d to all breastfed infants to support a serum 25(OH)D concentration of 50 nmol/L (4, 17). Doses exceeding 400 IU/d allow for a more rapid increase of vitamin D status (18-20) which may be beneficial in those infants born with insufficient stores. There is some evidence suggesting improvements to bone outcomes with use of supplements >400 IU/d in infants (20, 21), however there are no studies

specific to infants born with insufficient vitamin D status. Further research in this area is needed to confirm whether the current recommendations are sufficient or if higher doses are beneficial to term-born infants with a vitamin D insufficiency.

1.2 VITAMIN D

1.2.1 Metabolism and forms

1.2.1.1 Forms

Vitamin D is a term that comprises several forms of the fat-soluble compound, which has a four-ringed cholesterol backbone structure (22). *Cholecalciferol* (vitamin D_3) is formed in humans endogenously when ultraviolet beta (UVB) radiation from sunlight is absorbed by 7-dehydrocholesterol found in the epidermis (1). Previtamin D_3 is formed and is subsequently isomerized to cholecalciferol, a stable form of vitamin D_3; a process which takes 2-3 days to complete (23). Cholecalciferol is also found naturally in animal products such as fatty fish and eggs, in fortified foods such as milk, and in supplements (1). *Ergocalciferol* (vitamin D_2) is formed by fungi and yeasts exposed to irradiation which can happen naturally in plankton, or artificially as is done with the mold ergot to produce vitamin D_2 for use in supplements (24). Vitamin D is measured in IU, or in micrograms (µg); 1 µg=40 IU.

1.2.1.2 Metabolic activation

Exogenous vitamin D, whether in the form of cholecalciferol or ergocalciferol, is incorporated into micelles during digestion, absorbed by enterocytes and then packaged into chylomicrons and released into circulation. The efficiency of enteric absorption is reportedly around 50% (25). Endogenously produced vitamin D is bound to vitamin D-binding protein (DBP) in the dermal capillary bed and enters into circulation (25). Both exogenous and endogenous vitamin D are transported to the liver to form biologically active vitamin D. Activation requires several hydroxylation steps, all of which are carried out by cytochrome P450 (CYP) mixed-function oxidases (26).

In the liver, both exogenous and endogenously produced vitamin D are converted by 25-hydroxylase (or CYP2R1) to 25(OH)D also known as *calcidiol*. Calcidiol is the main circulating form of vitamin D and it has a half-life of 2-3 weeks, as compared to 24 hours for cholecalciferol (22). Next, 25(OH)D is further converted by 25-hydroxyvitamin D-1-alpha-hydroxylase (or CYP27B1) into 1,25-dihydroxyvitamin D [1,25$(OH)_2$D], the biologically active hormonal form, also known as *calcitriol* (27). This occurs primarily in the kidney, but also in bone cells, keratinocytes, liver and the placenta (25). The half-life of calcitriol is approximately 4-6 hours. Calcitriol binds to vitamin D receptors (VDRs) in target tissues allowing it to take biological action (22).

All major vitamin D_3 intermediate metabolites can undergo epimerization at the C3 position (25, 28). Epimers are structurally identical to their 25(OH)D or 1, 25$(OH)_2$D counterparts, with the exception of a stereochemical (β to α orientation) difference at the third carbon (26, 29).The physiological importance of these epimers is still unknown. However, there is clinical interest in 3-epi-25(OH)D because when using mass spectrometry 3-epi-25(OH)D will be included in the total circulating 25(OH)D, unless special procedures are used to extract it (26), see **section 1.2.3.2** for further details. In infants, 3-epi-25(OH)D is present in concentrations ranging from 0 to 61% with a mean concentration representing 21% of total circulating serum 25(OH)D (28). A recent longitudinal Canadian study, the Alberta Pregnancy Outcomes and Nutrition (APrON), found that 3-epi-25(OH)D comprised 7.8% of 25(OH)D in infant cord blood samples (n=92) (30). The authors observed a significant correlation between the presence of 3-epi-25(OH)D in infant cord blood and the use of vitamin D supplements by the mother during pregnancy. They also noted that higher maternal supplemental doses (>600 IU/d) were associated with higher 3-epi-25(OH)D concentrations, suggesting a dose-response relationship (30). Gallo et al. (2013) demonstrated that 3-epi-25(OH)D follows a dose-response relationship in infants receiving supplemental intake as well, as assessed at 3 mo (18). Compared to infants, children and adults have lower 3-epi-25(OH)D concentrations of 6% of total 25(OH)D (26, 28).

1.2.1.3 Catabolism

In order to prevent vitamin D toxicity, calcitriol and calcidiol are catabolized by CYP24 to produce biologically inactive excretory forms (25). The degradation is tightly regulated by calcitriol itself which enhances the activity of CYP24 in a negative feedback loop (31). The final end product is either calcitroic acid or a 26,23-lactone derivative; both of which are excreted in the feces via bile (25, 32). The intermediates of this degradation are generally of low or negligible activity (32). However, there is controversy surrounding the intermediary metabolite 24,25-dihydroxyvitamin D [24,25$(OH)_2$D]. Limited evidence from animal studies show that 24,25$(OH)_2$D may have a direct effect on bone metabolism acting in concert with calcitriol, however additional studies are needed to confirm this role (23, 32, 33). In infants, the quantity of 24,25$(OH)_2$D in the blood is associated with dietary intake of vitamin D and total 25(OH)D concentration (18).

1.2.2 Physiological functions

1.2.2.1 Calcium homeostasis

The primary role of 1,25$(OH)_2$D is in calcium homeostasis (**Figure 1.1**). The level of blood calcium is very tightly regulated through homeostatic mechanisms. Total serum calcium includes calcium that is bound to anions or albumin (about 55%) and the remaining 45% circulates as physiologically active ionized calcium (34). If ionized calcium drops, parathyroid hormone (PTH) acts along with 1,25$(OH)_2$D to restore ionized calcium to its normal level. Calcitriol has a direct effect to suppress PTH release from the parathyroid gland for negative feedback once calcium homeostasis has been restored (22). This process occurs in three organ systems:

1) Kidney: Low blood calcium stimulates the release of PTH, which has two important actions on the kidney. Firstly, PTH activates the 1-hydroxylase enzyme allowing 1,25$(OH)_2$D to be formed. Secondly, PTH works with 1,25$(OH)_2$D to enhance calcium reabsorption in the distal renal tubule (24).
2) Bone: PTH and 1,25$(OH)_2$D stimulate osteoblasts to produce receptor activator nuclear factor-kB ligand (RANKL). In turn, RANKL stimulates osteoclasts to mobilize calcium from

bone and release it into circulation (24). This process effectively and rapidly corrects blood calcium concentration (35). However, chronic calcium or vitamin D deficiency can lead to poorly mineralized bone and conditions such as rickets in children (24, 36), discussed in greater detail in **section 1.2.5**. By regulating calcium balance, vitamin D and its metabolites play an indirect, but vital role in bone formation and mineralization (37).

3) Intestine: $1,25(OH)_2D$ functions to increase intestinal calcium absorption. To a lesser extent it also stimulates intestinal phosphate absorption, another important mineral in bone mineralization (24). $1,25(OH)_2D$ acts via the VDR to increase the capacity of calcium transport. Elucidated mechanisms include transcellular intestinal absorption (including facilitated diffusion or vesicular transport) or by paracellular movement through tight junctions (38). In the facilitated diffusion model, calcium bound to calcium binding protein (calbindin D) is transported from the enterocyte cytoplasm through the vitamin D-dependent calcium channel (TRPV6) and released into the blood (38). The vesicular transport model involves movement of calcium across enterocytes in lysosomes that may be vitamin D regulated (38). Lastly, paracellular movement of calcium between enterocyte tight junctions may or may not be influenced by vitamin D (38).

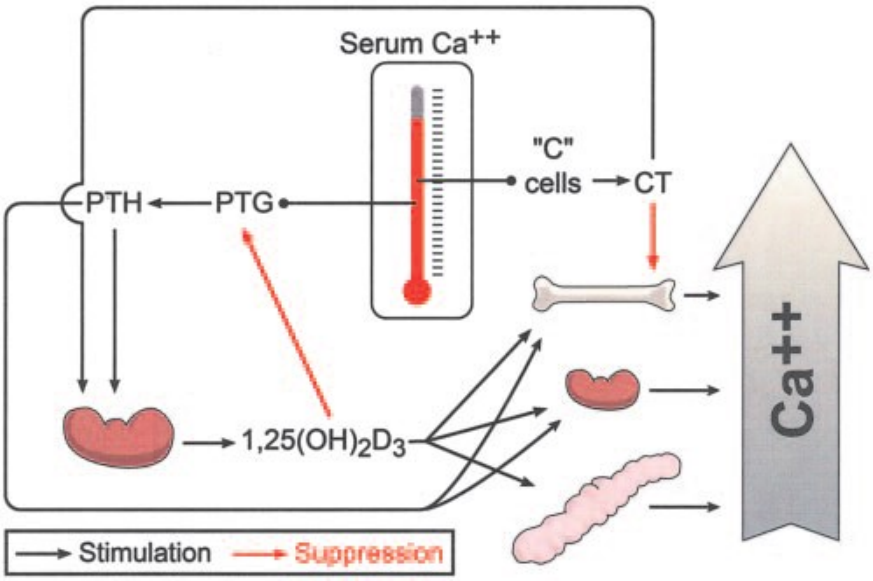

Figure 1.1. Calcium homeostasis

Low serum ionized calcium concentration signals a release of parathyroid hormone (PTH) from the parathyroid gland (PTG). PTH acts in tandem with calcitriol on bone, the kidneys, and intestine to increase serum calcium. In the situation of high serum calcium, the C cells of the thyroid gland secrete calcitonin (CT) to reduce serum calcium by acting on bone to inhibit osteoclasts.

Diagram reproduced with permission from DeLuca, HF, 2004 (24).

1.2.2.2 Vitamin D and bone cells

Besides its important role in calcium homeostasis, $1,25(OH)_2D$ may also act directly on bone cells via genomic and non-genomic actions, the mechanisms of which are not well understood. The expression of genes in osteoblasts, osteoclasts and chondrocytes seems to be modulated by $1,25(OH)_2D$, which will affect cellular growth and differentiation (39). The genomic action of $1,25(OH)_2D$ has been shown to occur via the VDR binding to vitamin D response element (VDRE); a DNA sequence in the genes it regulates (26). The non-genomic actions of $1,25(OH)_2D$ on bone cells are rapid and seem to occur directly in the cell membrane, independent of VDR (40). This area of research remains to be explored but $1,25(OH)_2D$ has many different potential mechanisms of action involved in bone development.

1.2.2.3 Other functions

While the discussion of non-skeletal functions of vitamin D is outside the scope of this literature review, it is interesting to note that this is an active area of research pointing to many roles in the body in addition to calcium homeostasis and bone health (7). Vitamin D receptors have been discovered in all organs (ex: brain, heart, pancreas, intestine, immune cells) suggesting biological activity. Evidence shows that maintaining sufficient vitamin D status may be important for healthy lean tissue development, immune response, and the prevention of chronic diseases (41).

1.2.3 Measurement of vitamin D status

1.2.3.1 Forms of vitamin D

Serum 25(OH)D is generally considered the best measure of vitamin D status and is the clinical standard. It is the major circulating form of vitamin D and reflects both endogenous and exogenous sources (24, 42). Although $1,25(OH)_2D$ is the active form of the vitamin, in comparison it is not a good indicator of vitamin D status. It has a very short half-life and has been shown to remain normal or even elevated in cases of vitamin D deficiency (1). Although the biological role of 3-epi-25(OH)D is unclear, it should be reported separately from estimates of total vitamin D status. Otherwise it may inflate the values and mask insufficiencies in vitamin D (43).

1.2.3.2 Assays

Due to increasing awareness of vitamin D insufficiency, there has been a large rise in vitamin D testing worldwide. A variety of assays are available including immunoassays, high performance liquid chromatography (HPLC), and liquid chromatography–tandem mass spectrometry (LC-MS/MS) (44). The lack of a standardized assay of vitamin D is a research limitation, making it difficult to pool results from different studies in order to determine clinical cut-points (45). However, efforts are being made to standardize methods amongst laboratories (42, 45). Reference measurement procedures (RMPs) and standard reference materials (SRMs) have been developed to set a gold standard for bringing together the results of different assays and methods. RMPs and SRMs have been designed by the National Institute of Standards and Technology (NIST) and have improved standardization for assay calibration. The Vitamin D External Quality Assessment Scheme (DEQAS) is a program that monitors laboratory proficiency in testing 25(OH)D. They also provide advice and samples to participants and manufacturers wishing to introduce or develop new methods and troubleshoot existing methods (46).

LC-MS/MS is generally considered the most accurate method of measuring 25(OH)D (7, 44, 45). In addition to precision, another advantage of this assay is that it provides separate values for $25(OH)D_2$ and $25(OH)D_3$ isomers. The LC-MS/MS assay can also provide a value for metabolites 3-epi-25(OH)D and $24,25(OH)_2D$ which may otherwise interfere in immunoassays (18). Many institutions use radioimmunoassay and automated immunoassay systems for their lower cost (7, 44, 45). Immunoassays have the advantage of automation and can process more samples at one time than the LC-MS/MS method (43). The disadvantage is that immunoassays have variable precision and are sometimes inaccurate. This could be due to the method of standardisation, instrument maintenance and water quality (43). These systems measure total 25(OH)D but are known to underestimate $25(OH)D_2$ (43). Although the LIAISON (DiaSorin Inc.) chemiluminescence immunoassay (CLIA) has shown a substantial negative measurement bias compared with LC-MS/MS-based assays (10.6 nmol/L) (43), it is still generally regarded to have superior agreement with LC-MS/MS when compared against other immunoassays (concordance correlation coefficient=0.95) (44).

1.2.3.3 Site of blood collection

Venous blood is the typical sample collection for measuring blood biomarkers in adults. However, for infants and young children 25(OH)D is typically tested in serum from a capillary blood sample by lancing the heel or fingertip. This method is less invasive, more cost-effective, and requires only a small blood volume (47). In certain studies (6, 48, 49), umbilical cord blood (venous) is used to measure vitamin D status non-invasively. No studies have compared umbilical cord blood with infant capillary samples *per se*. However, a recent study from Montreal, QC compares venous and capillary measurements in young children (1 to 5 y) and demonstrates that the two are highly correlated over a wide range of values (50). Until additional studies confirm these findings the methods should not be used interchangeably (47).

1.2.4 Sources of vitamin D and their limitations

There are three ways to obtain vitamin D: endogenous synthesis, food sources, and supplements. Each of these makes a unique contribution to vitamin D status that varies across the lifespan.

1.2.4.1 Endogenous production

Most people worldwide depend on sun exposure to meet their vitamin D needs (42, 51). The ability to endogenously synthesize vitamin D is very efficient (22). In fact, it is estimated that just 10 to 15 minutes of sun exposure on unprotected skin during peak daylight hours (10 am to 3 pm) provides adequate vitamin D (equivalent of 3000 IU vitamin D_3) in light-skinned individuals (52). Additionally, prolonged sun exposure will not cause an overproduction of vitamin D as the skin will only convert from previtamin D_3 what is needed and the remainder will be converted to inactive metabolites (42).

Despite the advantages of endogenous synthesis, many factors can negatively influence cutaneous production of vitamin D. The first of these is skin pigmentation. Individuals with darker skin have higher amounts of melanin, which absorbs UVB and prevents it from eliciting vitamin D synthesis. As a result, these individuals require much longer sun exposure to produce adequate vitamin D and tend to be at higher risk of deficiency (1, 7). Secondly, physical or chemical barriers to sunlight prevent vitamin D synthesis. Wearing clothing that covers the skin,

spending many hours indoors, and use of sunscreens containing sun protection factor (SPF) of 8 or greater prevent cutaneous synthesis almost entirely (1, 17). Lastly, environmental factors including time of day, season, latitude, and air pollution also have an important effect. Canada is located north of the 42nd parallel and as a result endogenous synthesis of vitamin D is prevented between the months of October and April (7). Because endogenous synthesis of vitamin D is highly variable and dependent on many factors, and due to concerns of skin cancer, the IOM recommendations for vitamin D intake have been set assuming minimal or no exposure to sun (4). Additionally, Health Canada recommends keeping infants under one year of age out of direct sunlight (53). This means that Canadian infants under 1 y are not anticipated to acquire vitamin D endogenously and are thus fully dependent on stores developed *in utero*, dietary sources and supplements.

1.2.4.2 Maternal-fetal transfer in utero

A source of vitamin D that is specific to infants is *in utero* transfer. Prenatally, fetal vitamin D status is dependent on placental transfer of 25(OH)D from the mother. The exact mechanism by which transfer occurs is not known, however there is a strong correlation between maternal and infant cord blood concentration of 25(OH)D, suggesting passive transfer (49, 54). Typically, a term-born infant's serum 25(OH)D concentration is 75% that of the mother's. It is generally believed that fetal demands are small and are not thought to deplete the mother's vitamin D stores (7, 54). A recent cohort study by Wegienka et al. (2016) does suggest, however, that a threshold may exist below which the mother will limit her contribution of 25(OH)D to the fetus. The authors observe that the correlation between prenatal maternal vitamin D status and cord blood is reduced when maternal vitamin D is less than 50 nmol/L (r=0.29) and is even weaker when maternal vitamin D is less than 37.5 nmol/L (r=0.16) (49). Regardless, it is well understood that if an infant is born with low vitamin D status it is primarily due to an underlying low vitamin D status of the mother. After birth, once placental transfer has ceased, infant vitamin D reserves will deplete quickly unless adequate supplementation is given (55).

1.2.4.3 Dietary sources

Vitamin D in either form (D_2 or D_3) is found naturally in very few foods (egg yolk, fatty fish, beef and mushrooms) (22). Commonly available food sources with the highest content of vitamin D are salmon and cod liver oil (**Table 1.1**), however these foods are not eaten regularly by most people (56) and not usually by infants. Cod liver oil was widely used in the prevention of rickets for infants in the 1900s but gradually lost its popularity due to concerns with vitamin A toxicity and was replaced with more refined forms of vitamin D. The combination of limited dietary sources and low sun exposure in Canada has led to the mandatory fortification with vitamin D of fluid milk, margarine and infant formula (7). Fluid milk or alternative beverages are the most commonly consumed dietary source and contain approximately 100 IU vitamin D per 250 mL (56). Optionally fortified foods include rice/soy/almond beverages and orange juice (7) as well as yogurt when made with vitamin D fortified milk (57). Fortified foods have demonstrated equal efficacy to supplements in their ability to raise 25(OH)D in older children and adults (58, 59); however these dietary sources are not applicable to the diet of newborn infants.

It is unanimously recommended in Canada that infants be exclusively breastfed for the first 6 months, with continued breastfeeding until 2 years of age or longer along with appropriate complimentary foods (53). Although breast milk is the best food for an infant, it is naturally quite low in vitamin D. If a breastfeeding mother takes a supplement of 400 IU/d, her milk's vitamin D content will range from <25 to 80 IU/L (17, 60). The vitamin D content of breast milk is highly dependent on maternal vitamin D status and reflects significant seasonal variability (60). For these reasons, in the absence of adequate supplementation breastfed infants will likely deplete their vitamin D stores, especially in the case where mother's stores are low to begin with (17, 53). Commercially available infant formulas are fortified with vitamin D. Consuming approximately 1 L of infant formula will provide an infant with 400 IU/d of vitamin D (7). However most infants do not consume this quantity until after the first month of life, or later if partially breastfed (17). It is clear that there are many barriers for infants to meet their dietary requirements and that supplementation is necessary, particularly for breastfed infants.

Table 1.1. Selected common food sources of vitamin D in Canada

Food	Serving size	Vitamin D content (IU)
Milk and alternatives		
Cow's milk (whole, 2%, 1%, skim, chocolate)	250 mL (1 cup)	103-105
Soy beverage, fortified with vitamin D	250 mL (1 cup)	86
Yogurt, made with vitamin D fortified milk	175 g (3/4 cup)	58-71
Rice, almond or oat beverage, fortified with vitamin D	250 mL (1 cup)	88-90
Meat and alternatives		
Egg, yolk, cooked	1 large	29-44
Pork, various cuts, cooked	75 g (2 ½ oz)	6-60
Salmon, canned or fresh, cooked	75 g (2 ½ oz)	181-699
Mackerel, fresh, cooked	75 g (2 ½ oz)	342
Halibut, cooked	75 g (2 ½ oz)	144
Tuna, albacore, cooked	75 g (2 ½ oz)	82-105
Vegetables and fruit		
Orange juice, fortified with vitamin D	250 mL (1 cup)	100
Fats and Oils		
Cod liver oil	5 mL (1 tsp)	427
Margarine	5 mL (1 tsp)	25
Other		
Infant formula	250 mL (1 cup)	100[1]
Breast milk	250 mL (1 cup)	8

Data obtained from the Canadian Nutrient File, 2015 (61) and [1]Canadian Food Inspection Agency, 2017 (62).

1.2.4.4 Supplements

Vitamin D supplements are effective for the prevention or treatment of vitamin D deficiency. Health Canada recommends that breastfed infants and adults over the age of 50 y take a vitamin D supplement of 400 IU/d (5). Children, adults and pregnant or breastfeeding women may be recommended to take a vitamin D supplement if they are unable to meet their requirements through diet alone (5). Much debate exists over current recommendations, which will be discussed further in **section 1.2.7**.

Vitamin D supplements are widely available in Canada and are regulated as a natural health product. In Quebec, vitamin D supplements can be covered under the government health insurance plan (Régie de l'assurance maladie du Québec) if prescribed by a physician. If purchased over-the-counter the price of infant vitamin D supplements is approximately $10-15 Canadian dollars for a 90-day supply. Vitamin D content varies by brand ranging from 400 IU to 1000 IU per unit (63). Infants receive vitamin D in liquid, oil-drop or strip forms whereas children and adults have the options of oils and drops, capsules, chewables, powders, strips or tablets (64). Supplements are available in two forms, vitamin D_2 or D_3, with the latter being used more commonly in Canada. In adults, vitamin D_3 is approximately 1.7 to 3 times more effective at raising circulating 25(OH)D concentrations than vitamin D_2, and has a longer half-life making it more likely to maintain vitamin D status even if taken irregularly (7). In infants (<4 mo), it has been shown that either isoform is equally effective at raising plasma 25(OH)D concentration (65). Vitamin D for older children and adults is also available in combination with calcium carbonate, or in multivitamins, including prenatal multivitamins (contains 200-600 IU of vitamin D per dose).

A large number of factors complicates the relationship between vitamin D supplementation and the resulting circulating 25(OH)D concentration. When supplemented with a given dose, the rise in 25(OH)D differs significantly between individuals (66). Both individual and environmental factors have been identified as modulating the individual response to vitamin D supplementation. Major individual factors include baseline 25(OH)D concentration, body mass index (BMI) and body fat, age, ethnicity, dietary calcium and fat intake, genetics, diseases and medications. Environmental factors include season (significant

over and above the effect of baseline 25(OH)D) and dosing regimen (including isoform of vitamin D, dose, route, frequency and duration) (66, 67). All of the above factors have been identified in adults with limited research in infants and children. Nonetheless, it is important to account for these factors when examining the effects of vitamin D supplements in any age group.

1.2.5 Skeletal consequences of vitamin D deficiency and insufficiency

There are many known and suspected consequences of both deficient and insufficient vitamin D status on skeletal development and maintenance throughout the life stages.

1.2.5.1 Rickets

Nutritional rickets was an epidemic that began during the Industrial Revolution when people began living in cities and spending less time exposed to direct sunlight. A high proportion of infants were affected with reports ranging from 50-95% in some U.S. cities. It was greatly reduced with the discovery and widespread use of vitamin D (in the form of cod liver oil) in the early 1930s (68).

Nutritional rickets can be caused by a severe deficiency of vitamin D. In a vitamin D-deficient state, the small intestine has a limited ability to absorb calcium and phosphorus (1). It can only absorb approximately 10-15% of dietary calcium, as compared to 30-40% when status is sufficient as studied in adults (69). The parathyroid gland is stimulated to secrete PTH, which works to maintain calcium homeostasis as the priority. As a consequence, less calcium is available for skeletal mineralization during bone development (70, 71). This compromised mineralization occurs mainly at the growth plates. In a healthy state, chondrocytes - which are cartilage forming cells, proliferate and enlarge at the growth plate forming a columnar structure that becomes mineralized to form bone (55). In rickets the cartilage accumulates, chondrocyte formation is disorganized and mineralization is delayed or prevented (39). This leads to thickening and widening at the growth plate, as well as slowed longitudinal bone growth and skeletal deformities (39). These changes may be seen as an adaptation to compensate for weakened bones by forming wider bones (55, 72). Regardless, the strength of the bone is

compromised and typically appears as bowing of the legs in rachitic children who have begun to stand (42, 55). Nutritional rickets usually occurs between 3 mo and 3 y of age (55).

Rickets is detected with X-rays and is best visualized at the growth plate of rapidly growing long bones. In the forearm, the distal radius and ulna are the typical sites of detection and in the legs, the femoral and tibial metaphyses near the knees (55). Even when typical skeletal deformities are not evident, rickets can present as hypocalcemic seizures (about 20% of cases in Canada) (73), respiratory distress syndrome, or hypotonic musculature, and is later diagnosed based on clinical biochemistry and radiological investigations (7, 55). For infants and children, the signs and symptoms of rickets generally appear when serum 25(OH)D is <30 nmol/L (3, 51). Other abnormal biochemical findings exist including elevated serum alkaline phosphatase (ALP), which is involved in the mineralization of bone. The serum concentration of PTH is also typically elevated, indicative of a vitamin D deficiency. Other biochemistry sometimes affected includes low serum phosphorus, 25(OH)D and serum calcium (55). The treatment of vitamin D deficiency rickets for infants is supplemental vitamin D at a dose of 2000 IU/d or 50,000 IU weekly for 6 weeks, followed by a maintenance dose of 400-1000 IU/d thereafter (31). Skeletal deformities are reversible if treated correctly and rapidly (55).

Some experts have described a recent resurgence of rickets in developed countries (41), while others are skeptical and believe this apparent rise may merely be due to changes in population demographics and reporting of medical diagnoses (12). Nevertheless, it remains a persistent public health issue in Canada and abroad. According to Canadian data collected from 2002 to 2004, 104 cases of vitamin D-deficient rickets were confirmed, representing an annual incidence rate of 2.9 cases per 100,000 (73). In each case, vitamin D supplementation was not adhered to. This number is considerably high given that supplemental vitamin D has been proven to prevent the disease. Some experts believe that cases of severe vitamin D deficiency represent only the tip of the iceberg to a much greater problem; vitamin D insufficiency. Although not as dramatic as rickets, it is widespread amongst Canadian infants and children and may carry very real health consequences (41).

1.2.5.2 Vitamin D insufficiency

In recent years, researchers have expressed concerns over the potential skeletal consequences of vitamin D insufficiency. Vitamin D insufficiency refers to a vitamin D status that is suboptimal for health, but not causing overt rickets (41). This condition is sometimes referred to in the literature as "vitamin D inadequacy", or "sub-clinical/asymptomatic vitamin D deficiency". Health Canada and the IOM define vitamin D deficiency as serum 25(OH)D below 30 nmol/L and vitamin D insufficiency as serum 25(OH)D 30-50 nmol/L (3). Vitamin D insufficiency has been linked to suboptimal skeletal mineralization in infants, preventing children from attaining their genetically programmed peak bone mass, and increased risk of fractures in children (42). Further evidence is needed to substantiate these claims, particularly studies in infants and young children.

1.2.5.2.1 Skeletal mineralization

Various studies have investigated whether bone mineralization suffers in infants with insufficient vitamin D status and so far, the evidence is divided. A cross-sectional study in Winnipeg, Canada by Weiler and colleagues (2005) sought to determine if there is a relationship between vitamin D status and bone mineral content (BMC) of infants. Healthy term-born infants (n=50) of appropriate birth weight were recruited from a local hospital. Vitamin D status was assessed via samples of cord blood analysed for 25(OH)D. Dual-energy X-ray absorptiometry (DXA) was used to measure whole body bone mineral content (WBBMC), lumbar spine bone mineral content (LSBMC) and whole femur. Results indicate that 18 infants (36%) were deficient in vitamin D with a cord blood 25(OH)D concentration <27.5 nmol/L used as the cut-point at the time. Infants deficient in vitamin D were overall larger (weight, length and head circumference) when compared to those with sufficient vitamin D status. Despite their larger body size, they had lower WBBMC and femur BMC relative to weight. It is hypothesized that the low vitamin D status observed amongst these infants may have resulted in inadequate mineralization of bone relative to body size. The authors suggest that this lower BMC may have negative implications for bone health later in life and propose that further research is warranted toward correction of insufficient vitamin D status in infancy (10).

Gordon et al. (2008) studied a cross-sectional sample of healthy infants and toddlers (n=380) in Boston, MA (9). They determined that the prevalence of vitamin D insufficiency (<50 nmol/L) was 12% within the sample. Of those vitamin D insufficient participants, 3 participants (7.5%) showed rachitic changes on radiographs and another 13 (32.5%) had evidence of inadequate mineralization. Inadequate mineralization in this study was assessed visually by radiologists and rated from 0 to 5 (9). The authors interpret the findings to indicate that even subclinical insufficiencies in vitamin D could have deleterious effects on bone (9). However, we must interpret the clinical significance of these findings carefully. The inadequate mineralization seen on radiographs of healthy infants and children is not well understood. While is it possible that this asymptomatic finding could lead to short or long term effects on bone health, it could equally be a transient phase that will resolve when adequate vitamin D is provided (74).

Dror et al. (2012) investigated the association of infant WBBMC, assessed by DXA, at 8-21 days after birth with maternal and cord blood 25(OH)D concentration. The study was a cross-sectional design carried out in a multi-ethnic population (n=120) in California. Contrary to previous studies, no association was found between WBBMC and feto-maternal vitamin D status over a range of 25(OH)D concentrations from 13.0–157.5 nmol/L in cord blood (75). This study, however, did not adjust for body weight or size.

1.2.5.2.2 Skeletal fractures

Childhood fractures are common and lower bone density has been shown to be a risk factor. Improvements in bone mineral density (BMD) are thus likely to reduce the risk of childhood fractures, however this remains to be investigated in prospective studies (76).

There is some evidence relating fracture risk to vitamin D status in children. An observational study from Australia collected data from children 2-17 y presenting with acute fractures. Results show that one third of all participants were vitamin D insufficient (<50 nmol/L of 25(OH)D). Of those children identified as having risk factors (dark skin pigmentation, minimal sun exposure, sunscreen use, obesity) the majority (52%) were considered vitamin D insufficient (11). Two cross-sectional studies in the US studied children presenting with upper

limb fractures and their associated vitamin D status. The first study by Ryan et al. found that 10 of 17 (59%) African-American children presenting with forearm fractures had a 25(OH)D concentration <50 nmol/L (77). The second study by James et al. (2013) found that 41% of children who experienced fractures had a serum 25(OH)D concentration of 50-75 nmol/L and another 24% of children had a value of <50 nmol/L (78). None of these studies included a control group and thus we cannot conclude if individuals with lower vitamin D values are at an increased risk compared to those with adequate stores. A follow-up case-control study by Ryan et al. in 2012 demonstrated that children with a serum 25(OH)D concentration <50 nmol/L are at a 3.5 times higher risk of experiencing a forearm fracture compared to a control group (13). However, the control group used in this study may not be representative of the general population as it included many children with asthma and individuals that proactively joined the study. Other similarly designed case-control studies do not agree with these results, finding no significant differences in fracture incidence according to vitamin D status (12). Similarly, a study of healthy infants (n=38) in Texas found no differences in bone mineralization amongst those born with low vs. healthy vitamin D status (79). The authors report that about 60% of infants are born with cord blood 25(OH)D <50 nmol/L. Despite this, BMC and BMD at birth was normal as measured by DXA and did not differ significantly based on 25(OH)D concentration. After 3 months of supplementation with 400 IU/d of vitamin D, all infants had a 25(OH)D concentration above 50 nmol/L and changes to bone mineralization were not significantly different between groups (79). The sample size of this study is small and due to the high proportion of Hispanic participants and the sunny climate, the data are not representative of Canadian infants. This same finding is supported by previous research by Park et al. (1998) on Korean infants who found no association between 25(OH)D concentration and lumbar spine BMC assessed by DXA, even though 44% of the infants had values <28 nmol/L (80). Clearly, there is a paucity of evidence relating vitamin D insufficiency and fracture risk, and well-designed trials are needed.

1.2.5.2.3 Peak bone mass

In older children there is substantial evidence that poor vitamin D status *in utero* and at birth, can reduce the ability to achieve peak bone mass. In 2006, Javaid et al. published a mother/offspring cohort identifying a significant link between suboptimal maternal vitamin D

status (at 34 wk gestation) and whole body and lumbar spine BMC and BMD of children at 9 y (15). Similar results were found in a prospective birth cohort at 20 y. Findings indicate that those born to mothers with a vitamin D deficiency (<50 nmol/L 25(OH)D) have lower WBBMC and BMD and thus achieve lower peak bone mass (16). These findings have been reproduced subsequently by other researchers (14). This suboptimal bone growth during childhood and adolescence is as important as bone loss later in life in the development of osteoporosis. A 10% improvement in peak bone mass has been estimated to delay the occurrence of osteoporosis by 13 y and reduce the risk of hip fracture by about 50% (76).

1.2.6 Risk factors for insufficient vitamin D status

Identified risk factors for insufficient vitamin D status amongst Canadian children and adults are listed in **Table 1.2**. Factors that are relevant to healthy, term-born infants include being breastfed, inadequate supplementation, being born to a mother with insufficient vitamin D status, and inadequate sun exposure (2, 9). As previously mentioned, sun exposure is not recommended for infants, and exclusive breastfeeding is strongly recommended. This leaves vitamin D supplementation as the main modifiable risk factor once the infant is born.

Table 1.2. Risk factors for insufficient vitamin D status

Risk factor	Explanation
Infants	
Inadequate dietary intake - Exclusive breastfeeding - Inadequate use of supplements	Breast milk is naturally low in vitamin D (17, 60), exclusively breastfed infants require a vitamin D supplement of 400 IU/d (5, 17).
Insufficient vitamin D *in utero*	Prenatally, fetal vitamin D status is dependent on placental transfer of 25(OH)D from the mother (49, 54).
Inadequate sun exposure - Season, latitude, angle of sun (81) - Air pollution (82) - Time of day (81) - Dark skin pigmentation (81) - Covering skin / use of sunscreen (83) - Indoor lifestyle (84)	All these factors reduce the amount of vitamin D being synthesized endogenously in the skin.
Mothers	
Inadequate dietary intake - Avoidance of dairy / lactose intolerance - Not consuming foods fortified with vitamin D - Inadequate use of supplements	Natural dietary sources of vitamin D are limited and only a small number of foods are fortified with vitamin D (85).
Obesity	Vitamin D is sequestered in body fat, especially in those with BMI >30 (86).
Pregnancy	Vitamin D requirements are elevated during pregnancy to ensure health to the fetus and mother (87, 88).
Age	The skin's ability to synthesize vitamin D decreases with aging as does renal conversion (89). Elderly are often housebound and may also have less consumption of milk due to lactose intolerance (2).
Comorbid conditions	Malabsorption syndromes, including Crohn's disease, colitis, cystic fibrosis, and liver disease (90).
Drug interactions	Certain medications impair vitamin D absorption, metabolism and clearance (2).
Inadequate sun exposure - Season, latitude, angle of sun (81) - Air pollution (82) - Time of day (81) - Dark skin pigmentation (81) - Covering skin / use of sunscreen (83) - Indoor lifestyle (84)	All these factors reduce the amount of vitamin D being synthesized endogenously in the skin.

1.2.7 The current state of vitamin D in Canada

Vitamin D insufficiency is a largely unrecognized problem in many countries, across different climates, and affecting all age groups (42). Absent of the obvious skeletal deformities associated with rickets, it can be difficult to detect. Additionally, since testing of vitamin D is not done routinely, it often goes unnoticed.

1.2.7.1 Women of child-bearing age

1.2.7.1.1 Vitamin D status

Vitamin D insufficiency is relatively common in Canada. According to cycle 2 (2009-2011) of the Canadian Health Measures Survey (CHMS), approximately 40% of Canadians ages 20-39 y fall below the cut-off of 50 nmol/L 25(OH)D (8). The average serum 25(OH)D concentration for women in this age group was 66 nmol/L (8). This value appears to be representative of the Quebec population as well; a study of 741 women of child-bearing age (mainly white and of French descent) found that average plasma 25(OH)D concentration was 64.9 nmol/L (91). Certain populations within Canada have a higher prevalence of vitamin D insufficiency including those of aboriginal ancestry (2, 92) and non-white ethnicity (32, 93, 94). Seasonal variability is also a factor effecting Canadians. A subset (n=188) of the Canadian Multicentre Osteoporosis Study cohort participated in a vitamin D study in which 25(OH)D was measured every 3 months over the course of one year (95). Participants were ≥25 y and mean 25(OH)D was 52.9 ± 17.2 nmol/L in late fall/early winter and 71.6 ± 23.6 nmol/L in summer. Results indicate that 34% of participants had a 25(OH)D concentration less than 40 nmol/L; 115 (61%) had a concentration less than 50 nmol/L; and 182 (97%) had a concentration less than 80 nmol/L for at least one point during the year (95). It is likely that these values would underestimate those of other Canadian cites because the population of Calgary is mostly white, and it receives more hours of sunlight than other Canadian cites. For these reasons the seasonal drop-off in vitamin D is likely more severe elsewhere in Canada (95).

1.2.7.1.2 Recommended intake

Health Canada's Recommended Dietary Allowance (RDA) for vitamin D states that adults should consume 600 IU/d (5). This is under the assumption that this amount will allow 97.5% of

the general Canadian population to achieve a serum 25(OH)D concentration of 50 nmol/L or higher. The consensus amongst other experts in the field is that concentrations of 75 nmol/L or greater are considered optimal for adults (96). To obtain this higher status at least 1000 IU/d of vitamin D is required (32). Health Canada also recommends a tolerable upper intake level (UL) for daily intake of 4000 IU/day in adults to avoid adverse health outcomes (5).

Despite these recommendations, average intake from food tends to be less than 400 IU/d according to the IOM (4). Additionally, CHMS data indicate that only 41% of females of all ages use a vitamin D containing supplement and that supplement use was the lowest amongst those ages 12-39 y (8). Even among those taking a supplement containing vitamin D, 25% still had a serum 25(OH)D concentration below 50 nmol/L (8). Particularly during winter, it seems that current recommendations may not be adequate. Research out of Toronto, ON found that 20% of young women consuming 400 IU/d or higher still had concentrations of 25(OH)D below 40 nmol/L during winter. This is comparable to the rate of insufficiency found amongst those who consumed essentially no vitamin D all winter (97). These findings suggest that a higher amount may be needed to support vitamin D status of women of reproductive age throughout winter in Canada.

1.2.7.2 Pregnant and lactating women

1.2.7.2.1 Vitamin D status

Vitamin D status is more often low in pregnant women compared to non-pregnant women (25). The mother's vitamin D supply must support her own physiological needs in addition to those of the growing fetus or breastfeeding newborn. Data from two prospective Canadian cohorts in Halifax and Quebec City indicate that approximately 45% of women had serum 25(OH)D concentrations below 50 nmol/L in early pregnancy. Of these, 8% were deficient at <30 nmol/L (98). Similar results were found in a study of pregnant women 20-25 weeks' gestation carried out in Vancouver and found that 46% of women had insufficient vitamin D status, despite the majority using a prenatal supplement of 400 IU/d (99). A study in Toronto measured women's 25(OH)D concentration during pregnancy and postpartum and found that mean 25(OH)D was 63.7 ± 24.5 nmol/L in pregnancy, 62.6 ± 24.2 nmol/L at 3 mo, and 61.4 ±

26.4 nmol/L at 12 mo postpartum (100). The data from these studies illustrate how prevalent vitamin D insufficiency is amongst women during pregnancy and postpartum.

1.2.7.2.2 Recommended intake

Health Canada's RDA for vitamin D during pregnancy and lactation is the same as for adults; 600 IU/d to support concentrations of 25(OH)D at 50 nmol/L (5). The Canadian Paediatric Society (CPS), however, recommends a higher concentration target of 75 nmol/L during pregnancy (7).

Use of prenatal supplements (containing 400 IU vitamin D) is common, but still not adequate. In a Saskatchewan study, 85% of mothers reported taking a prenatal supplement containing vitamin D but even so 70% of their newborns were born with insufficient or deficient cord blood concentrations for vitamin D (48). In a study of 50 women from mixed ethnic backgrounds in Winnipeg, 78% of the women reported taking vitamin D containing multivitamins during pregnancy and still 46% were deficient in circulating vitamin D (10). Data from Quebec City indicate that 85% of mothers consumed up to 400 IU/d of vitamin D, while only 3% took the recommended >400 IU/d vitamin D during their pregnancy. Despite this, 38% of Quebec City mothers taking a supplement >400 IU/d still had 25(OH)D concentration <50 nmol/L. This could be caused by low adherence, or it could mean that the dose taken was not high enough (101).

Evidently, the recommended dose of 600 IU/d is not adequate to support both the mother and her infant. There has recently been research interest in determining if mothers can be supplemented with a dose of vitamin D that would result in 25(OH)D concentration ≥50 nmol/L in exclusively breastfed infants. Two recent studies have demonstrated that 4000-6400 IU/d is needed to achieve a sufficient concentration of vitamin D in breast milk and appeared to be safe with respect to biochemistry (88, 102, 103). However, studies included only exclusively breastfed infants and did not account for the real-world scenario of mothers introducing fortified infant formula. Until additional studies are carried out to validate safety this high dose is not recommended.

1.2.7.3 Term-born infants

1.2.7.3.1 Vitamin D status

A recent estimate from Quebec City indicates that 25% of neonates are born with a cord blood 25(OH)D concentration below 50 nmol/L (6). Of those born with sufficient vitamin D, about 43% were in the range of ≥50 to <75 nmol/L and the remaining 32% were ≥75 nmol/L. The infants studied were in a control group that were healthy and of normal birth weight. Nearly all mothers (97%) were Caucasian so these data may represent a best-case scenario for vitamin D status in the region owing to higher efficiency of endogenous synthesis. In the longitudinal APrON study, 92 samples of infant cord blood were tested and 28% were <50 nmol/L. All of the mothers in this study reported taking a supplement containing vitamin D during pregnancy (30). In Newfoundland and Labrador, a cross-sectional analysis of 25(OH)D concentration of cord blood was done. The authors found that 35% of samples were below 50 nmol/L (104). In more Northern regions of Canada, or in aboriginal groups the prevalence of vitamin D insufficiency and deficiency is more severe and ascribed to darker melanin content of the skin and geographic location (7). It is clear that vitamin D insufficiency is widespread amongst newborn infants in Canada, despite the use of prenatal supplements.

1.2.7.3.2 Recommended intake

Health Canada, the Endocrine Society and the American Academy of Pediatrics agree that all infants, including those who are exclusively breastfed should receive 400 IU of supplemental vitamin D daily starting from birth (5, 52). This is now the Adequate Intake (AI) for the prevention of rickets and to maintain 25(OH)D at a concentration of 50 nmol/L or higher in most infants. This supplementation should continue until the infant is getting 400 IU of vitamin D daily from other dietary sources such as formula or cow's milk. The CPS adds to their recommendation that requirements for infants of darker-skinned mothers and those living in northern latitudes may be much higher (105). For breastfed infants living in northern communities (north of 55th latitude), and for those living between the 40th and 55th parallel who have additional risk factors they recommend 800 IU of vitamin D daily in winter (October to April) (105). It is unclear whether these recommendations are adequate for those born with

an insufficiency as research is lacking in this population. The UL for vitamin D is set at 1000 IU/day from 0-6 mo, although some evidence indicates that higher doses may be safe (18, 20, 106). A study by Gallo et al. demonstrates that 1200 IU daily appeared safe, but 1600 IU/d brought serum 25(OH)D into a range that may be associated with hypercalcemia (18). A safe upper limit for serum 25(OH)D in infants has been identified in the range of 125-374 nmol/L (106) with the most common definition of 225 nmol/L by the CPS (105).

1.2.7.3.3 Use of supplements

According to Health Canada nearly three quarters of breastfed infants receive vitamin D supplements (53). Research in Quebec suggests that rates of vitamin D supplementation could be improved. Millette et al. performed a retrospective cohort study looking at infants born from 1998-2008 who were eligible to receive free vitamin D supplements under the provincial prescription drug program. Results indicate that <20% of eligible infants received a vitamin D supplement. Furthermore half of these people obtained only one bottle of 50 doses and the median age for getting the first dose was 36 days of age (107). Data from Montreal were more encouraging. In a cross-sectional telephone survey of mothers delivering healthy infants from December 2007 to May 2008, 74% of exclusively breastfeeding mothers reported that they followed the Health Canada recommendations by starting supplementation within the first week and continuing until 6 mo (108). There appears to be a lack of knowledge regarding the need for supplementation when mixed milk feedings are used (108). Demographic factors including socio-economic status, living as a couple, older mothers, and a prescription by a pediatrician appear to significantly increase the likelihood of infants to obtain vitamin D (107).

1.2.8 Challenges in determining vitamin D requirements

There is no consensus in the literature as to how much vitamin D the human body requires firstly to avoid deficiency and secondly for optimal health. There is a very large range between the serum concentration of 25(OH)D needed to prevent rickets in infants (approximately 30 nmol/L) and the amount that poses a risk of hypercalcemia (>225 nmol/L) (18, 105). Within this range it remains to be determined if there are specific thresholds desirable for health. Because impaired calcium levels cause secondary hyperparathyroidism, and increased bone resorption,

an optimal range for vitamin D has been suggested as one that will keep PTH at a minimum, and calcium absorption at a maximum (42). Several studies have concluded that this steady state occurs when 25(OH)D concentration reaches 75 nmol/L or higher in adolescents and adults (1, 7, 42). There is insufficient evidence to determine if this same cut-point applies to infants, especially since PTH is very low in young infants (109). As such, 50 nmol/L has been recommended as the value indicating vitamin D sufficiency in infants. As previously discussed, the imprecision of different vitamin D assays is also a challenge in determining requirements.

1.2.9 Serum response to vitamin D supplementation in infants

To recapitulate, the vitamin D status categories based on serum 25(OH)D concentrations for infants: <30 nmol/L is deficient; ≥30-50 is insufficient; ≥50 nmol/L is recommended; ≥75 nmol/L may confer additional health benefits; and ≥225 nmol/L is considered at risk of toxicity. This is important to keep in mind when looking at how infants respond to vitamin D supplementation of different doses.

Research clearly demonstrates that most infants supplemented with 400 IU/d of vitamin D will be able to achieve serum 25(OH)D of 50 nmol/L or higher by 3 mo of age. Despite continued supplementation, maintaining this concentration at 6 mo of age appears more difficult and may require higher doses (18, 19). Additionally, reaching the potentially desirable concentration of 75 nmol/L or higher appears to require higher dose supplements (18). The following studies illustrate these points. In a 2013 Montreal study by Gallo et al., healthy infants (n=132) were provided with vitamin D supplements in a dose-response trial (18). Vitamin D measurements at birth were not taken, but a baseline measurement was done at 1 mo of age. Infants were randomly assigned to receive either 400, 800, 1200, or 1600 IU/d of oral cholecalciferol for 11 mo. For all doses, 97% of the study population achieved a serum 25(OH)D concentration of 50 nmol/L at 3 mo and 98% maintained this concentration by 6 and 12 mo. These data however do not represent the most at-risk population. The sample population consistent largely of white, well-educated, high-income mothers, and infant's mean baseline vitamin D was 59 nmol/L (95% CI, 55-63). Only the highest dose of 1600 IU/d was able to achieve 25(OH)D ≥75 nmol/L in 97.5% of the population at 3 mo, however it was discontinued due to concerns of risk of hypercalcemia. The second highest dose, 1200 IU/d, came close to

being adequate as it allowed 92% of the study population to achieve a concentration ≥75 nmol/L at 3 mo. In all groups, 25(OH)D concentrations peaked at 3 mo and declined during the remaining study period while still maintaining concentrations of 50 nmol/L but not 75 nmol/L. The authors hypothesize that this 3 month drop-off reflects the change in relative intake (per kg body weight) over time (18). Intake by body weight has been shown to be an important predictor of vitamin D sufficiency in children (110), but studies in infants are lacking and dosing by body weight is not practical given the rapidly changing body weight of infants. Another possible explanation for this characteristic drop in vitamin D status is that vitamin D is more highly utilized for calcium absorption at 3 mo (79). This could imply that recommendations, although sufficient for 0-3 mo of age, are not sufficient for the rest of the first year of life. A New Zealand study (19) had similar findings. This study had two arms: prenatal maternal supplementation followed by infant supplementation. Mother/infant pairs (n=260) were provided with either placebo/placebo, 1000/400 IU/d or 2000/800 IU/d of vitamin D for mothers and infants respectively. They found that at age 2 mo, serum 25(OH)D concentrations ≥50 nmol/L were achieved in 82% of infants in the 400 IU/d group and 92% in the 800 IU/d group. When establishing an RDA, the Health Canada criteria is that a given intake should support healthy status in 97.5% of the population, so both 400 IU and 800 IU fall short of that. Interestingly, the authors also observed the same drop-off in serum vitamin D occurring between 2 and 4 mo. By 6 mo of age, only the highest dose group (800 IU/d) was able to sustain 25(OH)D ≥50 nmol/L in significantly more infants than placebo (89% vs. 74%) (19).

It is not well understood whether infants born with low vitamin D stores are able to achieve concentrations of 50 nmol/L given 400 IU/d of vitamin D or if higher doses are required; evidence on this is very limited. In the New Zealand study (19), 80% of infants in the placebo group were born with cord blood below 50 nmol/L (median 32 nmol/L). Without supplementation, 50% of this group achieved a sufficient status (≥50 nmol/L) by 2 mo, and 74% by age 6 mo. In comparison, the two supplemented groups fared better: 400 IU group (82% at 2 mo, 82% at 6 mo) and the 800 IU group (92% at 2 mo, 89% at 6 mo). Although it is impossible to separate out the effects of prenatal supplementation, it is clear that supplemental vitamin D will bring infants up to a level of sufficiency more rapidly compared to placebo, and that a dose-

response relationship exists. The authors did not analyze the results by cord blood 25(OH)D concentration irrespective of supplementation which would have provided more insight on the relationship between baseline status and the storage pools in response to supplementation. More studies on this are needed. In a small non-randomized study from Texas, 60% of Caucasian and Hispanic infants (n=38) had cord 25(OH)D concentrations below 50 nmol/L at birth. Following supplementation with 400 IU/d of vitamin D, serum values were above 50 nmol/L in all infants regardless of 25(OH)D at birth. This would indicate that those with the lowest vitamin D status were able to catch up when supplemented. There are several limitations of this study such as intake of formula not being controlled for, the short study duration of 3 mo and small sample size (79). Nonetheless, this study adds to the growing data that suggests 400 IU is appropriate for infants born with either low or healthy status. Whether it is enough to support optimal growth and quality of growth is not clear. A Polish study by Czech-Kowalska et al. (2012), tested the serum response to vitamin D supplements in deficient infants (<27.5 nmol/L) and those with vitamin D insufficiency (27.5-50 nmol/L). After 10 weeks of supplementation with 550 IU/d, initially vitamin D-deficient infants increased from median 21 nmol/L to 136 nmol/L 25(OH)D as measured using CLIA (LIAISON, DiaSorin Inc.). As expected, the initially deficient group revealed significantly higher percentage changes for 25(OH)D (745% vs. 167%, $p<0.0001$) compared to the insufficient group (111). This gap levels off over time, once infants are approximately 6 mo (19).

In summary, it appears that supplemental doses of 400 IU/d are adequate to maintain serum 25(OH)D concentration at 50 nmol/L in most infants, throughout the first 3 mo but not 6 mo of life. Those born to vitamin D-deficient mothers, mothers with darker skin pigmentation and those living in northern climates may be at greater risk of vitamin D insufficiency. Higher doses may be required to support infant 25(OH)D concentration at 50 nmol/L year-round. In order to support serum 25(OH)D concentration at 75 nmol/L in 97.5% of infants throughout the first 6 months of life doses exceeding 400 IU/d appear necessary.

1.3 BONE

1.3.1 Structural and cellular components

Bone is a dynamic organ that serves both the structural functions of supporting the body and protecting organs and the metabolic functions of storing and mobilizing minerals. Bone tissue is a matrix composed of an organic component that is mostly collagen and an inorganic component of bone mineral made mostly of hydroxyapatite or other salts of calcium and phosphate (112). Collagen provides the skeleton with flexibility, and hydroxyapatite and other minerals give it its' strength (113).

1.3.1.1 Skeletal components

Within the skeleton, there exists two main types of bone tissue: cortical bone and trabecular (or cancellous) bone. *Cortical bone* forms the rigid outer part of all bones and is dense and compact. It is found in many bones including: the outer ends of long bones and the diaphyses, and the exterior of the spinal vertebrae, flat bones, and the pelvic bones. It is estimated that 80% of the adult skeleton by weight is cortical bone. Its major function is to provide strength, but it can be involved in metabolism especially in mineral deficits. *Trabecular bone* is part of the interior of bones. It is porous, appears sponge-like, and constitutes the remaining 20% of skeletal mass in adults. Trabecular bone is metabolically active and releases mineral in situations of deficit. It also contributes to mechanical strength, especially in the spine (112).

1.3.1.2 Cell types

- Mesenchymal stem cells: can differentiate into a variety of cell types including chondrocytes and osteoblasts (114).
- Chondrocytes: cartilage-producing cells.
- Osteoblasts: bone-forming cells that form osteoid protein on the surface of a bone, which then becomes mineralized. Osteoid is mainly made of type 1 collagen. (113)

- Osteocytes: formed from osteoblasts that have become trapped and surrounded by the bone matrix they themselves produced, and became mineralized. Osteocytes have processes that connect to other osteocytes and osteoblasts for communication (113).
- Osteoclasts: responsible for bone breakdown or resorption. They secrete acid and enzymes against the surface of bone which releases minerals when stimulated (113). Osteoclasts are derived from hematopoietic stem cells located in the bone marrow (115).

1.3.2 Bone formation

1.3.2.1 In utero

Flat bones are formed by *intramembranous ossification*; a process in which the initial bone pattern is laid out by osteoblasts directly into mesenchymal connective tissue producing osteoid and rapid growth of woven bone occurs (114). Ossification with more resilient lamellar bone (cortical and trabecular) begins around week 6 of gestation and progresses sequentially in different bones into early infancy (113). A different method, *endochondral ossification*, is responsible for the formation of the long bones. This process involves chondrocytes forming a cartilage scaffold which is later replaced by bone (114). Clearly, bone formation is a complex process involving the coordinated action of osteoblast differentiation, matrix production, mineralization, and vasculogenesis. To accomplish this, the fetus requires a large amount of building blocks including proteins and minerals from the placenta to form bone. The primary hormone involved with *in utero* bone development is parathyroid hormone related peptide (PTHrP). Vitamin D and PTH are present only in low levels in fetal serum; bone formation seems primary dependent on high levels of calcium being available (113). In fact, studies suggest that *in utero* skeletal development occurs independently from vitamin D status of the mother except in cases of severe deficiency which can result in congenital rickets (54, 75).

1.3.2.2 Infancy

After birth, the skeleton continues its rapid growth rate with WBBMC more than tripling in the first year of life (116, 117). This requires significant mineral input. Now that placental transfer has ceased, the newborn must obtain minerals through intestinal absorption. Also, PTH

and 1,25$(OH)_2$D become important factors in normal bone development (113). Despite this rapid growth, a drop in DXA-derived BMD is frequently observed in healthy infants, which reflects a redistribution of bone tissue as the bone dimensions widen and not a loss of bone mineral *per se* (116, 118).

1.3.2.3 Peak Bone Mass

According to Weaver et al. (2016), peak bone mass is "the amount of bone gained by the time a stable skeletal state has been attained during young adulthood." Skeletal growth occurs with the coordination of new bone formation and resorption to allow bones to expand and lengthen into their adult form. This process occurs until epiphyseal fusion, by the end of the second decade of life (119). Many factors influence peak bone mass including genetics, sex, nutrition, physical activity, body weight and hormones (120). Nutrition is one of the modifiable factors and therefore an area of active research. There is evidence from longitudinal studies that vitamin D status during childhood (119) and as early as during the intrauterine period (16) may program for higher peak bone mass.

The magnitude of the variance of bone mass and size by age is large between individuals. For example, individuals in the 95th and 5th percentile will differ in bone size by about 50%. This difference may be an important determinant of fracture risk later in life, and illustrates why attaining genetically programmed peak bone mass can be protective against fractures later in life (113).

1.3.3 Bone modeling and remodeling

The interplay between bone formation and bone resorption results in mineral turnover, growth, and maintenance of the skeleton. *In utero* and during early childhood, bones are sculpted by the removal of bone by osteoclasts and the deposition of bone at a different site by osteoblasts. This process is called bone *modeling*, and is regulated by the action of osteocytes. In periods of growth, formation exceeds resorption resulting in increasing BMC. Bone regeneration continues periodically in adult bone and is called *remodeling*. The adult skeleton regenerates completely approximately every ten years (112). The role of bone remodeling is not very clear but most likely is to remove dead osteocytes, maintain oxygen and nutrient

supply, and repair damaged tissue. Bone remodeling occurs in three phases: resorption, reversal and formation. Each phase occurs sequentially over a specific period. In adults, bone remodeling should maintain equilibrium between resorption and formation to maintain BMC. Whereas in infants, bone formation dominates in a healthy state.

1.3.4 Factors influencing bone development and strength

Bone growth is a complex process that is affected by both genetic and environmental factors. These include, but are not limited to: genetic programming, weight, ethnicity, sex, hormones, fetal and infant movement, nutrition, toxins and drugs, and bone disorders (113).

1.3.4.1 Ethnic differences

Ethnic differences in bone and mineral metabolism have been identified in children, adolescents and adults thus far. Among adults in the United States, African-American, Asian, and Hispanic populations have a lower prevalence of hip fractures and osteoporosis compared to white populations (113). They also tend to have greater peak bone mass, greater muscle mass, lower bone turnover rates, and have increased body weight that contributes to bone loading. All of this is true, even despite African-Americans having lower circulating concentrations of 25(OH)D compared to white Americans (25). Similarly, studies in children have shown that fracture rates in black children from South Africa are less than half of those found in white children of the same age (113). Data from Asian and Hispanic children indicate that their bone mass is similar to that of Caucasians, but lower than African-American children (113). Bone length and bone size account for much of the observed differences in BMD between ethnicities, meaning that African-Americans tend to have longer limbs with a larger cross-sectional bone area – resulting in greater bone strength (113, 121). It is important to mention that the differences observed may be related to environmental factors, and not solely ethnicity. For example, one study compared white and black children from the US and South Africa, and found that South African children have significantly higher WBBMC compared with US children in each ethnic group (113). These differences between the same ethnic groups provide evidence that geography and environment may have a significant impact on bone and that studies comparing ethnicities cannot be generalized outside of the region.

Most studies in infants did not find a difference in BMC, BMD or body size by ethnicity. It is however possible that study sample sizes were too small or that the duration of studies was not long enough for differences to become apparent – more research is needed. In a study by Abrams et al., 38 infants (19 Hispanic and 19 Caucasian) were supplemented with 400 IU/d of vitamin D and whole body BMC and BMD were measured using DXA. Hispanic infants had greater WBBMC and BMD at 3 mo but not at 1 wk of age. Thus the change in BMC was significantly greater in Hispanic infants than Caucasians (51.0 ± 11.3 g vs. 41.2 ± 10.1 g, p=0.006). For BMD, changes were greater for Hispanics than Caucasians (0.019 ± 0.012 g/cm^2 vs. 0.010 ± 0.012 g/cm^2, p=0.017). When body weight at 3 mo was included as a covariate in this analysis, changes in BMC remained significant (p=0.03) but changes in BMD were not (p=0.06). Overall, there was no relationship between changes in 25(OH)D and BMC (79). An infant study by Weiler and colleagues compared bone mass of First Nations, white and Asian infants using DXA. After controlling for body size and vitamin D status, the Asian infants had 29% lower lumbar spine BMC compared with white infants, with First Nations being intermediate. No significant differences between groups were found for whole body or femur BMC (122).

1.3.4.2 Sex differences

Sex differences in bone length, width, or mass emerge mainly during puberty. Before puberty, some studies have shown that male bones may have larger width than female bones, with this difference beginning *in utero* (113). Females tend to have lower WBBMC as newborns and until 1 y as well as lower spine BMC, but not BMD, until 36 mo of age. It makes sense that BMD shows no sex differences due to a smaller bone area in females compared to males (123).

1.3.4.3 Nutrition

There are several nutritional factors that may contribute to bone mineral accretion in infants including maternal nutrition during pregnancy and breastfeeding, use of supplements, type of infant feeding, calcium and phosphorus content of infant formula, and introduction of weaning foods (124).

Studies have found that infants fed formula tend to have higher total body BMC accretion compared to exclusively breastfed infants (125). Some key factors that may be responsible for this are the higher calcium, vitamin D, and phosphorus content of formulas (124). However, longer-term research has shown that this initial lower bone accretion in breastfed infants is temporary and that catch-up occurs later in childhood (113, 126). Infant growth curves are based on the human milk-fed infant, the gold standard for defining normal growth (127).

As previously discussed, vitamin D has a vital role in bone health via calcium homeostasis. It is well recognized that maternal 25(OH)D blood concentrations exhibit seasonal variability. As a consequence, newborn BMC follows a similar pattern being lowest in winter in four-season climates (116, 128). Circulating $1,25(OH)_2D$ may or may not act directly on bone. Animal studies exist in support of either hypothesis. VDR knockout mice experienced secondary hyperparathyroidism, hypocalcemia and rickets. However, when fed high amounts of calcium and phosphorus, ionized calcium and PTH concentrations are normalized and rickets is prevented. This suggests that vitamin D may be involved solely in the absorption of calcium and phosphorus. Interestingly, when these mice are fed the rescue diet, not all changes in osteoblast number, mineral apposition rate, and bone volume are rescued, so there may also be direct skeletal effects of $1,25(OH)_2D$ as well (113). Other research indicates that $1,25(OH)_2D$ binds to VDRs on osteoblasts and osteoprogenitor cells to alter the expression of osteoblast genes (25).

It is hypothesized that in early life calcium absorption is independent of vitamin D, but that by 3 mo of age vitamin D begins to play an important role in calcium absorption. Vitamin D may be of particular importance to breastfed infants who are consuming less, albeit more bioavailable calcium, than formula-fed infants (79). Studies on the effects of calcium supplementation and bone outcomes are limited. A 7-year randomized controlled trial (RCT) involved supplementing children with 670 mg/d calcium on top on their current intakes. By adolescence, it was observed that calcium supplementation was associated with greater increases of BMD in the forearm and whole body BMD compared to placebo. These differences however disappeared once supplementation was stopped (113). Calcium balance studies in children (ages 12-15 y) suggest that there is a threshold of calcium (1300 mg, the RDA for this

age group) below which skeletal uptake of calcium is limited by intake, and above which skeletal accumulation remains constant (113, 129). However calcium balance studies represent a short term response and do not necessarily reflect impact on bone in the long-term (113). Overall the results from meta-analyses suggest that there is either no effect, or only a small short-term effect of calcium supplementation on bone mineral accrual and that the effect does not persist once supplementation is ceased (113).

1.3.5 Dual-energy X-ray absorptiometry (DXA)

The main bone outcomes of consequence are fractures. Although this is the ultimate endpoint to determining if a skeleton performed under the forces placed upon it, it is not a feasible outcome for most prospective studies. As such, surrogate measures of bone strength are used including BMD and BMC which are direct indicators of fracture risk (12, 25, 130) and can be measured using DXA. A DXA scanner includes: a scanning X-ray source, an X-ray detector that records absorption data at two different photon energies, and an interface with a computer system for processing and analysis of the absorption data (131). The two X-ray photon energies (70 and 140 kilovolts) are emitted and absorbed differently by various tissue parts, which allows for detection of body composition (132). For Hologic DXA scanners, the BMC (g) and bone area (cm^2) are estimated. To calculate areal BMD (g/cm^2), BMC is divided by the bone area (133).

1.3.5.1 Anatomical sites of measurement

DXA measurements are performed on specific anatomical sites. In adults this is commonly the spine and hip (113). For infants there are no data to determine which skeletal sites are optimal for fracture risk or disease identification (127). According to the 2013 guidelines from the International Society for Clinical Densitometry (ISCD) the lumbar spine is the most commonly used regional scan site in infants and young children and has been successfully obtained even in small infants (127). Precision estimates with the Hologic device for BMC and BMD were 2.2% and 1.8% respectively and improved as children got older. The spine region is a feasible measurement site in infants in terms of positioning without the use of any sedatives or restraints (127). The drawbacks to using the lumbar spine are that most of the skeletal growth

in infants and children occurs in the extremities and fractures of the spine are extremely rare, making its clinical significance undefined. At birth, BMC of the lumbar spine (L1-L4) region is approximately 2 g and increases to about 10 g by 36 mo. Gallo et al. found that LSBMC increased by 102% (doubled) between 1 and 12 mo of age (18). LSBMC represents less than 3% of the total body BMC in infants <1 y. Even a 20% change in spine BMC represents a very small absolute quantity of bone mineral that may or may not be clinically important (127). Spine BMC is related to weight, height, BMI and body surface area ($r>0.6$, $p<0.0001$) in healthy infants less than 1 y of age (127).

The other scan site commonly used for infants is the whole body scan. This scan provides information on BMC, bone area, and lean and fat mass. Changes in WBBMC occur rapidly in infancy and thus this region is appropriate for use in growth studies where changes can be observed even over a relatively short period of time (134). WBBMC is largely dependent on infant body size and therefore it is important to view measurements keeping in mind length (134). The head region may be excluded from the whole body scan because it contributes largely to WBBMC at young ages (nearly 50% of BMC at birth) and may reduce the sensitivity of detecting changes in BMC occurring in the sub-cranial skeleton. Using total body less head (TBLH) avoids this issue (127).

Regional measurement of the forearm and femur are also possible because of the short scan time required (<30 s) and possibility of holding the infant without interfering with the scan. Although interesting for research, the clinical utility of both these regions is presently limited due to a lack of precision estimates and reference data in infants. Femur scans are not currently recommended for clinical use in children <5 y of age due to difficulty analysing these scans (134).

1.3.5.2 Reference data

Kalkwarf et al., in the 2013 ISCD Pediatric Official Positions, stress the importance of using reference data generated with the same DXA platform and software version for proper interpretation of results. Ideally, BMC and BMD should be expressed as z-scores (or number of standard deviations above or below the mean). Reference data in infants for DXA should be

age, sex, and race-specific (127). Unfortunately, there is a lack of national reference data for infants and children from 0 to 5 y. Only normative data exist and most studies have been based on convenience samples and have been composed of predominantly white children. The largest studies are those of Gallo et al. (18) and Kalkwarf et al. (123) which provide data on LSBMC and LSBMD in infants as well as WBBMC for Gallo et al. For Hologic DXA devices, reference data are only available for children ages 3 and up for the whole body BMC and lumbar spine BMD (127).

1.3.5.3 Advantages of DXA

DXA is the preferred method for assessment of bone health in infants (134). The instrument is accepted as safe and accurate for use in both infants and children, meaning that comparable measurements can be taken throughout growth. The advantages of this technology include the low ionizing radiation exposure, excellent precision, low cost, and short scan time of 3 min or less depending on scan type (113). The infant whole body scan was developed as a research application for children ages 0-2 y and is optimized to detect bone of very low density. Precision, expressed as the coefficient of variation (CV) of BMC and BMD measured with the infant whole body software were 1.9% and 1.7% respectively, from duplicate scans of 40 piglets between 0.6 and 21.1 kg using a fan-beam DXA device (Hologic QDR 4500A). Precision with infants is unknown, but with repositioning, is likely to be greater (127).

1.3.5.4 Limitations of DXA

Certain limitations exist with DXA. Firstly, the fan-beam device has only been validated for measuring BMC in small animals (compared to chemical carcass analysis) (127). Secondly, there are technical challenges with infants and young children to remain still for the duration of the scan. Movement in the scanned region results in unpredictable motion-related artifacts that can greatly affect results. This can be overcome by having a skilled technician who is able to calm and swaddle infants and children prior to the scan and if multiple attempts are made (127). Thirdly, the images generated are two-dimensional projections of a three-dimensional bone structure; so only areal BMD, and not volumetric BMD is measured. Thus the results are dependent on projected bone area which can be a particular issue in growth states or due to positioning (113). Another issue arising from this two-dimensional image is the inability to

distinguish cortical from trabecular bone. It has been hypothesized that in low vitamin D states cortical bone suffers more than trabecular and that the two should be analyzed separately to observe the true relationship with vitamin D status (84). Although the whole body scan may be of limited clinical utility for BMD due to the inability to standardize positioning in infants, it is recommended as an assessment of therapy that may affect BMC (127). In terms of clinical utility of DXA, it is difficult to assess the bone strength of a child relative to the types of strain that will be placed upon them in their life. Whether or not bone accretion during infancy and early childhood are related to bone health outcomes at older ages is also unknown (127).

1.3.6 Biomarkers of bone mineral metabolism

While DXA offers important information on BMC and BMD, it provides a static measurement and does not reflect the live changes happening in bone. Assays are available to measure biomarkers of bone formation and resorption which reflect the dynamic aspect of bone (135). Bone formation involves the production of collagen by-products and other proteins, as they form osteoid. These can be measured in the serum and indicate the rate of formation. Some examples include ALP, amino terminal propeptide of type 1 procollagen (P1NP) and osteocalcin (136). Bone resorption results in mineral and osteoid (mainly collagen) being released and broken down into peptides. Some biomarkers of bone resorption include urinary N-terminal telopeptide of type I collagen (NTX) and serum C-terminal telopeptide of type I collagen (CTX).

Infants and children have dramatically elevated bone biomarker concentrations compared to adults because they are experiencing rapid growth and bone modelling. Bone biomarkers in infants and children reflect physiological and pathological factors. Age, sex, growth velocity and nutritional status are physiological factors. Gestation, nutritional deficiencies (including vitamin D) or bone diseases are pathological factors (135).

P1NP is produced in the conversion of procollagen to collagen during bone formation. The serum concentration of P1NP is directly representative of the amount of newly formed collagen. It is thus a reliable and routinely used biomarker of bone formation. It is considered reliable because is it stable, reproducible and has not been found to be modified by circadian

rhythm (137). Levels of P1NP are highest during the first year of life, with a second peak during puberty due to rapid bone growth. P1NP is not significantly correlated with actual height, weight, or BMI at any age (137). Osteocalcin is a bone protein produced by osteoblasts that is used as a biomarker for bone formation (136). $1,25(OH)_2D$ appears to modulate osteocalcin through a VDR binding to a VDRE in the osteocalcin gene (138). Although the function of osteocalcin in bone metabolism is debated (138), it warrants attention as a biomarker because of its regulation by $1,25(OH)_2D$. A postnatal peak in osteocalcin has not been observed, but levels are elevated in childhood compared to adult levels (137). Circadian variations exist for osteocalcin with highest concentrations in the morning (137). CTX is a cross-linked telopeptide that is a product of mature collagen degradation (111). It is a specific and sensitive biomarker of bone resorption. Reference data indicate that CTX is highest in neonates and decreases significantly after 1 year of age. There is also a peak during pubertal growth later on (135).

The clinical utility of bone mineral metabolism biomarkers in infants and children is hindered because of high assay variability and lack of reference data (135). They are however very useful in clinical trials for understanding the underlying mechanisms of action in response to a treatment (139). Markers can be measured in both serum and urine. Serum assays are the preferred method because they have coefficients of variation about half that of urinary assays (137). In serum the standard laboratory quantification method is enzyme linked immunosorbent assay (ELISA) and kits are available for each specific biomarker being measured (135). Biological variability has an effect on bone metabolism and consequently on biomarkers of bone formation and resorption. Sources of biological variation have not been studied in infants. In children and adults, factors known to effect biomarker concentrations include diurnal variation, age, sex, body size and recent food consumption (135, 136, 140).

1.4 Review of the literature on vitamin D supplementation and bone health in infants

Unfortunately, RCT data seeking to identify the best vitamin D supplementation regimen for bone health in infants are sparse and unconvincing. Most studies have found no significant differences to bone mineral accretion, but it is imperative to mention that study populations have been made up mostly of infants or older children who had a sufficient vitamin D status

(>50 nmol/L 25(OH)D) to begin with. It is likely that vitamin D supplements are of the biggest benefit to deficient infants and RCTs in this population are urgently needed before benefits on bone mineralization can be ruled out. A summary of relevant studies is presented in **Table 1.3**.

A Swiss retrospective cohort study carried out by Zamora et al. (1999), sought to determine whether vitamin D supplementation (400 IU/d) of breastfed infants during the first year of life is associated with greater BMC and BMD in later childhood. One hundred and six healthy prepubertal Caucasian girls (ages 7-9 y) participated and a questionnaire was sent to the child's family and pediatrician to classify them as supplemented or unsupplemented during the first year of life. Vitamin D supplementation in infancy was associated with increased DXA-obtained BMD at specific skeletal sites (radial metaphysis, femoral neck, and femoral trochanter) (141). Because this study is observational, no causal relationship can be drawn. Additionally, serum measures of vitamin D were not taken so sufficiency/deficiency cannot be assessed. However, it is likely that some infants in the study were vitamin D-deficient at birth given Geneva's northern latitude and thus long winter periods of minimal UVB exposure. Lastly, comparing against placebo is not clinically relevant in Canada's current situation where the benefits of supplementation with 400 IU/d are already well recognized.

Czech-Kowalska et al. (2012), tested the biochemical response to supplements in vitamin D-deficient infants (<27.5 nmol/L) and in those with vitamin D insufficiency (27.5-50 nmol/L). After 10 weeks of supplementation with 550 IU/d, they found that median 25(OH)D concentrations in the two groups were 155 nmol/L and 105 nmol/L respectively. The initially-deficient group revealed significantly higher percentage changes for 25(OH)D (745% vs. 167%, $p<0.0001$) compared to the insufficient group (111). Although the authors did not perform radiographic tests, they did measure markers of bone mineral metabolism including ALP, PTH, osteocalcin, P1NP and CTX. Results indicate that vitamin D supplementation had little to no impact on biomarkers of bone mineral metabolism in term infants in the first few months of life, with the exception of osteocalcin (113% vs. 40%, $p<0.05$) which increased significantly with supplementation and may be representative of osteoblastic (bone building) activity.

Holmlund-Suila et al. (2012), carried out a double-blind randomized dose-response trial in Finland on healthy newborn infants with median baseline cord blood 25(OH)D of 53 nmol/L; 113 infants were randomized to receive vitamin D in doses of 400, 1200, or 1600 IU/d from age 2 wk to 3 mo. At 3 mo, the mean 25(OH)D in each group was 88, 124, and 153 nmol/L ($p<0.001$) per respective dosage. Peripheral quantitative computed tomography (pQCT) was used to assess bone outcomes and showed a trend toward larger tibial total bone cross-sectional area and cortical bone area as dosages increased (20). The main limitations of this study are that pQCT is not ideal for use in infants and the authors report a significant amount of motion artifacts in their scans. Additionally, 10 weeks of treatment is a short period for changes in bone architecture and mineral accrual. These results would need to be validated using a longer trial with DXA technology for bone mineral accretion.

A dose-response study by Gallo et al. (2013) was similar in design but used superior methods for blood sampling and bone densitometry. Infants were randomized to vitamin D treatment groups (400 IU, 800 IU, 1200 IU, or 1600 IU) and bone outcomes (BMC and BMD of whole body, lumbar spine L1-L4 and femur) were measured. No significant differences were found between treatment groups (18). However, when sub-analyses were performed to separate groups by baseline 25(OH)D concentration interesting results were revealed. It was found that amongst those with baseline 25(OH)D <50 nmol/L (n=18), those who achieved a 25(OH)D concentration of ≥100 nmol/L by 3 mo had higher LSBMC compared to those who achieved a 25(OH)D concentration of 50-99 nmol/L. Of this second group, 8 participants even experienced a slight decrease in LSBMC ($p=0.132$) (21). This study also demonstrated a clear dose-response relationship between vitamin D supplements and serum 25(OHD) (18). This would suggest that infants with deficient baseline vitamin D status that received the low dose supplements (400 IU) might have experienced lower LSBMC compared to those on higher doses.

Table 1.3. Vitamin D supplementation studies relating to bone health in infants

Reference	Design	Duration	Population, N	Baseline 25(OH)D (nmol/L)	Vitamin D measures	Vitamin D dose (IU/d)	Bone Measures	Conclusions
Zamora et al., 1999	Retrospective cohort (Geneva)	N/A	Healthy prepubertal Caucasian girls, n=106	N/A	N/A	400	Radius, femur, lumbar spine L2-L4 (DXA, Hologic QDR-2000)	Vitamin D associated with increased BMD compared to placebo at radius and femur but not lumbar spine.
Czech-Kowalska et al., 2012	Intervention study (Warsaw)	10 wk	Healthy, term-born infants, deficiency/ insufficiency, n=19/11	<27.5 and 27.5-50	Serum 25(OH)D, $1,25(OH)_2D$ (CLIA)	550	Serum bone biomarkers (ALP, PTH, OC, P1NP, CTX, NT-proCNP)	Vitamin D-deficient infants at baseline, compared to the insufficient group, revealed significantly higher percentage changes for 25(OH)D, $1,25(OH)_2D$ and OC.
Holmlund-Suila et al., 2012	Double-blind randomized dose-response trial (Helsinki)	3 mo	Healthy, term-born infants, n=113	Median 53	Cord blood, and serum 25(OH)D (CLIA)	400, 1200, 1600	Left tibia (pQCT, Stratec XCT-2000)	Larger tibial total bone cross-sectional area and cortical bone area with higher doses of vitamin D.
Gallo et al., 2013	Double-blind randomized trial, dose-response (Montreal)	11 mo	Healthy 1-mo old breastfed infants, n=132	Mean 59	Plasma 25(OH)D (LC-MS/ MS)	400, 800, 1200, 1600	Whole body, lumbar spine L1-L4, femur (DXA, Hologic 4500A Discovery)	Dosages of vitamin D >400 IU/d provided no additional benefits for bone mineral accretion.

DXA dual-energy X-ray absorptiometry; CLIA chemiluminescence immunoassay; ALP alkaline phosphatase; PTH parathyroid hormone; OC osteocalcin; P1NP N-terminal propeptide of type 1 procollagen; CTX C-telopeptide; NT-proCNP amino-terminal propeptide of C-type natriuretic peptide; pQCT peripheral quantitative computed tomography; LC-MS/MS liquid chromatography tandem-mass spectroscopy

1.5 RATIONALE AND OBJECTIVES

Upon reviewing the literature, it is evident that research investigating the effects of rapid correction of vitamin D status on bone in infants born with 25(OH)D concentrations <50 nmol/L is very limited. RCTs to date have been carried out in populations of healthy infants with mostly sufficient vitamin D status. Results from studies are mixed, but some evidence suggests that vitamin D supplements above 400 IU/d may improve bone mineral accretion and BMD, particularly in infants with insufficient vitamin D status. There is a need for studies focusing on at-risk infants to provide evidence regarding their vitamin D requirements and whether or not current supplementation practices are adequate or if higher doses offer improved bone health outcomes.

Primary objective: To test whether rapid correction of vitamin D status early in the neonatal period significantly improves surrogate measures of bone health by 3 mo of age using an RCT in otherwise healthy term-born infants with serum 25(OH)D below 50 nmol/L. It is hypothesized that infants supplemented with a recovery dose (1000 IU/d) of vitamin D will have a greater change in BMC and BMD at the lumbar spine and/or greater change in whole body BMC at 3 months compared to those given 400 IU/d.

Secondary objective: To determine if a serum concentration of 25(OH)D of 75 nmol/L or greater at 3 mo is associated with greater change in BMC and BMD at the lumbar spine and/or greater change in whole body BMC, irrespective of vitamin D dosage. It is hypothesized that infants achieving a serum concentration of 25(OH)D of ≥75 nmol/L at 3 mo will show greater improvements in these bone measures.

2 MANUSCRIPT

Effect of rapid correction of vitamin D insufficiency on bone health in breastfed infants born with low vitamin D stores: A randomized, double-blind trial in Montreal, Canada

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2.1 ABSTRACT

Evidence is lacking regarding the vitamin D requirements of breastfed infants born with 25-hydroxyvitamin D [25(OH)D] concentration <50 nmol/L. The objective was to determine whether current Canadian supplementation guidelines (400 IU/d) are adequate for this population, or if a higher dose (1000 IU/d) confers benefits to bone health. In this preliminary trial analysis, healthy term-born breastfed infants were recruited from a hospital in Montreal, QC. Infant serum 25(OH)D was sampled at birth (LIAISON, DiaSorin Inc.). Infants with 25(OH)D <50 nmol/L were randomized to receive 400 IU/d (n=19) or 1000 IU/d (n=22) of cholecalciferol. Those with ≥50 nmol/L (n=15) formed a reference group receiving 400 IU/d. At baseline (≤1 mo), and at 3 mo, bone mineral content (BMC) and bone mineral density (BMD) of the whole body and lumbar spine were measured using dual-energy X-ray absorptiometry (DXA), as well as anthropometry. Analysis was done using a mixed-model ANOVA. The trial population was 57% male, 73% light skinned and 91% breastfed until 3 mo. By 3 mo, 25(OH)D concentration was 77 ± 23 nmol/L (400 IU/d group) and 131 ± 39 nmol/L (1000 IU/d group) ($p<0.0001$). Bone and anthropometric outcomes indicated healthy growth in all infants. No significant differences in absolute or weight-adjusted BMC or BMD of the whole body and lumbar spine were observed between groups. Percent change of total body less head BMC from baseline to 3 mo was significantly higher with 1000 IU/d (46.2 ± 23.5% vs. 37.3 ± 14.8%, $p=0.02$). The 1000 IU/d group also had a 0.5 ± 0.2 cm/mo greater length velocity vs. 400 IU/d ($p=0.03$). The results suggest that infants with vitamin D insufficiency may experience improvements in BMC accretion and length velocity by 3 mo with the use of 1000 IU/d cholecalciferol. Study participants will continue in the trial to 3 y to assess the longer-term impacts of the intervention.

2.2 INTRODUCTION

It is well established that breastfed infants require a vitamin D supplement throughout infancy to prevent deficiency and avoid adverse bone health outcomes. The current standard of care is to provide a supplement of 400 international units (IU) per day of vitamin D to all breastfed infants to support a sufficient status of 50 nmol/L circulating 25(OH)D as recommended by the Institute of Medicine (IOM) (4). Similarly, recommendations exist for pregnant and lactating women to consume at least 600 IU/d of vitamin D to support fetal and neonatal vitamin D stores (5). Despite this, low vitamin D status is widespread and remains a health problem in Canada. A recent cohort study from Quebec City estimates that 1 in 4 infants are born with insufficient vitamin D status below <50 nmol/L of 25(OH)D (6).

Doses exceeding 400 IU/d allow for a rapid increase of vitamin D status (18-20), effectively reducing the amount of time spent in a state of insufficiency. This recovery dose may be particularly beneficial for those infants born with low stores. There is some evidence suggesting that a recovery dose of vitamin D exceeding 400 IU/d may lead to improvements in bone mineral accrual and bone mineral density in infants with low status (142). Further research in this area is needed to confirm whether the current recommendations are sufficient or if higher doses are beneficial to infants born with a vitamin D insufficiency.

The primary objective of this study was to test whether a recovery dose (1000 IU/d) of vitamin D vs. a standard dose (400 IU/d) significantly improves surrogate markers of bone health as measured by DXA by 3 mo of age in otherwise healthy term-born exclusively-breastfed infants with serum 25(OH)D below 50 nmol/L. The secondary objective was to determine if a serum 25(OH)D concentration of 75 nmol/L or greater at 3 mo is associated with improved surrogate markers of bone health as measured by DXA, irrespective of the vitamin D supplement dosage given.

2.3 SUBJECTS AND METHODS

Overview

The preliminary data from baseline to 3 mo of age used in this report are from a pilot phase of a randomized double-blind parallel-group trial that is currently ongoing. Recruitment began in March 2016; the entire study will have follow up until children reach 3 y. Each infant participating in the trial was assigned an individual study code that was recoded for this thesis. As such, the full trial will continue as double-blinded.

Infant serum 25(OH)D was screened 24-36 h after birth. Infants with insufficient vitamin D status (<50 nmol/L) were randomized to either the standard of care 400 IU/d vitamin D_3 or a recovery dose of 1000 IU/d vitamin D_3. Randomization was done in blocks by Randomize.net website enabling randomization according to 2x2 block sizes and stratification by sex and skin pigmentation. A group of healthy infants with a sufficient initial vitamin D status (≥50 nmol/L) was given 400 IU/d vitamin D to serve as a local reference group for bone outcomes. Treatment began within the first month of life and will continue until 1 y. For the secondary objective, infants were grouped by those who achieved a status ≥75 nmol/L 25(OH)D by 3 mo, and those who remained <75 nmol/L; 75 nmol/L was chosen as a target because this an optimal 25(OH)D concentration in adolescents and adults (1, 7, 42), but has yet to be shown in infants.

Supplements were formulated by Euro-Pharm International Canada Inc. and administered by parents in 1-mL/d volume using a standardized dropper; both doses had similar taste, smell and appearance. Bottles contained 50-mL volume and were coded with a unique randomization number for each participant. Parents and researchers were blinded to treatment dosage. Visits were scheduled with participants at the research facility at baseline (≤1 mo) and 3 mo. At each visit anthropometry, body composition, bone densitometry, biochemistry, and skin pigmentation were assessed. Questionnaires regarding demographic information, feeding practices and sun exposure were also administered. By 3 mo, infants are still predominantly breastfed, other sources of vitamin D (endogenous, dietary) are limited (143), and bone modelling is rapid (118), making it a reasonable age to analyze the preliminary trial data. See **Table 2.1** for summary of study measurements and timing.

Subjects

Newborns (n=56) were recruited from the post-natal unit at the Lakeshore General Hospital in Pointe-Claire, QC. For the randomized controlled trial (RCT), infants had serum 25(OH)D <50 nmol/L while those ≥50 nmol/L formed the reference group. For both the trial and the reference groups, inclusion criteria were: born to mothers with an otherwise healthy pregnancy, free of medications that impact vitamin D metabolism (except vitamin/mineral supplements) or fetal growth, and intent to breastfeed to at least 3 mo. Exclusion criteria were: maternal smoking in pregnancy as it limits growth, diabetes, preeclampsia, celiac disease, inflammatory bowel disease, liver disease and medications that impact vitamin D/mineral metabolism. Additionally, mothers in the reference group could not have a pre-pregnancy body mass index (BMI) exceeding 27.

Bone and body composition

The main outcome of interest, BMC, was assessed using a fan-beam DXA (APEX version 13.3:3, Hologic 4500A Discovery Series, Bedford, MA). Each infant wore a light sleeper with no metal or plastic components and a diaper and was scanned using the infant whole body software. Whole body scans provided whole body bone mineral content (WBBMC, g) and total body less head BMC (TBLH, g) as well as lean mass (g) and fat mass (g). Lumbar spine vertebrae L1-L4 were also scanned to capture lumbar spine bone area (LSBA, cm^2), lumbar spine bone mineral content (LSBMC, g) and lumbar spine areal bone mineral density (LSBMD, g/cm^2) according to the International Society for Clinical Densitometry (ISCD) guidelines (127). Change in BMC between visits (g/mo) was calculated using time as a continuous variable to adjust for age at visit. The instrument was calibrated daily with a phantom spine (Hologic phantom No. 14774). The coefficient of variation (CV) over the study period for the phantom was 0.6% for LSBMC and 0.4% for LSBMD, which indicated satisfactory instrument stability with no sign of drift. An experienced member of the research team (CV) closely scrutinized all the DXA scans for movement artifacts and lumbar spine regions of interest. To reduce movement during the scan, mothers were invited to breastfeed their infants prior to the scan to encourage infants to

sleep. Due to technical problems with the DXA, one infant whole body scan was not obtained at the 3 mo visit.

Biochemistry

At birth, a hospital nurse collected capillary blood samples (0.5 ml) via infant heel lance in a gel-containing microtainer. Screening samples were centrifuged (4000 x g for 20 min at 6°C) and serum transferred to 2-mL Axygen tube at McGill. At study visits, capillary blood samples (1.0 ml yields ~500-600 µl serum) were collected from infant heel or finger. Samples were centrifuged and analyzed immediately, or stored frozen at -80°C until analysis. One 5 ml venous sample was electively taken from parents at baseline for measurement of serum 25(OH)D. Total 25(OH)D was measured by chemiluminescent immunoassay (CLIA) using a dedicated autoanalyzer (LIAISON, DiaSorin Inc.); this assay was also used for safety assessments at 3 mo. The laboratory is certified by the Vitamin D External Quality Assessment Scheme (DEQAS) and participates in the National Institute of Standards and Technology (NIST) quality assurance program. Internal quality control methods included duplicate measures of high and low controls supplied in the manufacturer kits, and a pooled serum sample from healthy adults. The inter-assay CV was 6.7% and the range of intra-assay CVs was 5.2-13.4% with a mean of 7.3%.

Blood-ionized calcium was measured using a portable unit (ABL80 FLEX Radiometer Medical A/S, Denmark) and compared to published standards for safety (144). Urinary calcium, phosphate and creatinine were measured in a urine spot sample collected at each visit during the trial (Beckman Coulter UniCel DxC600 autoanalyzer) (116).

Anthropometry

Weight was measured using an electronic scale with a dynamic weighing program (Mettler-Toledo Inc., Switzerland). Length (0.1 cm) was measured using an infantometer (O'Learly Length Boards, Ellard Instrumentation Ltd., US). Length velocity between visits was calculated by change in length over time (continuous) between visits. Head circumference and mid-upper-arm circumference were measured (0.1 cm) using a non-stretchable tape (Perspective Enterprises, US) to complete the anthropometry panel. Weight-for-age and length-for-age Z-

scores were calculated using World Health Organization (WHO) software (WHO AnthroPlus, Switzerland).

Endogenous vitamin D

At each visit, skin color of the infant was measured by taking the average of three measurements at each site for constitutive (natural) pigmentation at the inner upper arm and facultative pigmentation [ultraviolet beta (UVB) exposure] at the forehead, mid-forearm and lower leg using a spectrophotometer (CM-700d/600d, Konica Minolta, USA). Individual typology angle (ITA°) was calculated with the L* (Luminance) and b* (yellow/blue component) values using published equations (145). Using constitutive pigmentation at baseline, infants were classified into skin types (I-III: light; IV-VI: dark) based on Fitzpatrick descriptions (146, 147). Sun exposure, winter travel and use of sunscreen was also surveyed. Changes to skin pigmentation from baseline to 3 mo were calculated at the mid-forearm to assess if sun exposure caused a change in skin pigmentation in infants (148). Month of birth was defined as within synthesizing (Apr-Oct inclusive) vs. non-synthesizing periods (Nov to Mar inclusive) (149).

Demographic data

Parents completed a survey that included self-identified race and ethnicity, household income, and education using Canadian Census criteria. Mothers were also surveyed at each study visit regarding their use of vitamin and mineral supplements during pregnancy and while breastfeeding, and type of infant feeding. This information was used to assist in interpretation of vitamin D status and bone health assessments.

Adherence

Parents were asked at what frequency they gave the assigned vitamin D supplement to their infant on a 5-point scale with descriptors “every day”, “almost every day”, “2 to 3 times a week”, “once a week”, or “never”. Parents also completed a compliance calendar on which they recorded whether or not the supplement was given to their infant every day. These were checked by study staff at each visit and recorded as a percentage of assigned doses given.

2.4 DATA MANAGEMENT AND SAFETY

Screening data and study visit data were entered electronically into Research Electronic Data Capture (REDCap) which is a secure, online data management system licensed for use in this trial. Data were entered as soon as available, and audited by members of the research team. For safety, a registered nurse monitored all biochemistry data and any abnormal results were communicated to a study physician. A protocol was in place to repeat any abnormal results within 24 h and if still abnormal, the child would move to standard of care (400 IU/d open label) and be followed as intent-to-treat.

2.5 ETHICS

This study was approved by the Research Ethics Committee of the St. Mary's Hospital Centre and valid for the Lakeshore General Hospital. Written informed consent was provided by the parent/guardian prior to the infant's participation in screening and in the study.

2.6 SAMPLE SIZE

For this preliminary analysis, the treatment groups had a sample size of 19 and 22 for the routine care (400 IU/d) and recovery dose (1000 IU/d) respectively; plus, the reference group (n=15). A target sample size of 28 infants in each group was estimated to provide 80% power to detect a 14.6% difference in change of lumbar spine BMC by 3 mo of age at $p<0.05$. This estimate was calculated from unpublished data by Gallo et al. in a dose-response trial of a comparable population of infants with 25(OH)D concentration <50 nmol/L at birth from Montreal, QC (18).

2.7 STATISTICAL ANALYSIS

Data analyses were conducted using SAS (version 9.3 SAS Institute Inc., Cary, NC, USA) and intent-to-treat analyses. Screening and baseline characteristics were tested for differences between trial groups using Student's t-test for normally distributed continuous variables, Wilcoxon two-sample nonparametric test for non-normal and categorical variables and Chi-

squared test for proportions. Group differences at 3 mo including infant feeding type (exclusively breastfed, partially breastfed, or exclusively formula fed); supplement adherence (5-point scale); and sun exposure (yes or no: infant typically exposed to direct sunlight, without sunscreen, during the synthesizing period or during travel) were tested using Chi-squared test for proportions.

Repeated measures mixed-model analysis of variance (ANOVA) was used to test for bone outcomes between treatment groups from baseline to 3 mo. Bone outcomes analyzed include WBBMC (g), TBLH (g), LSBA (cm^2), LSBMC (g), and LSBMD (g/cm_2) and body weight-adjusted versions of these variables at 3 mo. Percent change in bone outcomes and mineral accretion over time (g/mo) were also tested using mixed-model ANOVA. The model accounted for fixed effects including sex, treatment, and skin pigmentation. In addition to the fixed effects listed, the model also accounted for random effects of season of birth, exact age at visits, gestational age and BMI. These covariates were chosen based on the current literature and biological plausibility. Shapiro-Wilk test for normality of residuals was used along with Bonferroni correction for multiple comparisons.

For the secondary outcomes, the proportion of infants in each trial group (400 IU/d and 1000 IU/d) reaching the threshold of ≥75 nmol/L 25(OH)D was tested using Chi-squared test. Relationships between infant 25(OH)D concentration and bone outcomes were tested using Pearson's correlation. Additionally, bone outcomes between trial infants who achieved a 25(OH)D concentration of ≥75 nmol/L, and those who did not, were tested from baseline to 3 mo with repeated measures mixed-model ANOVA. The same fixed and random effects as listed for the primary outcome were used. Statistical significance for all tests was set at $p<0.05$ after adjustment for multiple comparisons where applicable. Data are mean ± SD unless otherwise specified.

2.8 RESULTS

Study population

Of 555 infants screened, 69 were enrolled from March 2016 to May 2017 inclusive. The remaining families declined to participate (n=364), did not meet inclusion criteria (n=92), or had insufficient blood sample for testing (n=30). Of the 69 infants who were enrolled, 56 infants had complete biochemistry for serum 25(OH)D results and DXA scans at the 3 mo time point and were thus included in the analysis (**Figure 2.1**). This sample was analyzed as a pilot phase only and the full trial is ongoing. All maternal and infant screening and baseline characteristics were similar between groups (**Table 2.2** and **Table 2.3**). The study population included slightly more male than female infants (32/56) and infants with light skin pigmentation (41/56). Breastfeeding rates were comparable between groups (p=0.32). Overall, 91% (51/56) of infants received breast milk up to 3 mo of age, and of those 82% (42/51) were exclusively breastfed. There was no significant difference in supplement adherence between treatment groups (p=0.76) with "almost every day" being the most frequently reported response, which corresponds with a recorded 90% and 88% mean adherence in the 400 IU/d and 1000 IU/d treatment groups respectively. From baseline to 3 mo, there were no significant differences in reported infant sun exposure between groups (p=0.68); 21% (4/19) of infants in the 400 IU/d group and 14% (3/22) of infants in the 1000 IU/d group were exposed to direct sunlight during the months when endogenous synthesis is possible. However, sun exposure was not associated with a change in facultative skin pigmentation as measured at the mid-forearm (p=0.92).

An equal number of infants (n=2) were lost to follow up in each treatment group. Infants who dropped out before the 3 mo visit had significantly higher mean 25(OH)D concentration at birth ($p<0.01$) compared to those who completed the 3 mo visit and all were born during the vitamin D synthesizing period (100%). There were no other statistically significant differences at baseline between completers and non-completers (**Table 5.1**).

Primary outcome

By 3 mo of age, serum 25(OH)D concentration was 77 ± 23 nmol/L for the 400 IU/d group and 131 ± 39 nmol/L for the 1000 IU/d group (p<0.0001) (**Figure 2.2**). In the 400 IU group, 94% of infants achieved a sufficient 25(OH)D concentration of ≥50 nmol/L compared to 100% of infants in the 1000 IU group. Ionized calcium, urinary calcium creatinine ratio and urinary phosphorus creatinine ratio (**Table 2.4**) did not differ between groups and were all within the normal range for age.

In our ANOVA analyses, there were no significant differences between groups in absolute or weight-adjusted WBBMC, TBLH, LSBMC or LSBMD by 3 mo. There was a statistically significant difference in percent change in TBLH between groups with 37.3 ± 14.8% change for the 400 IU/d group and 46.2 ± 23.5% change for the 1000 IU/d group from baseline (≤1 mo) to 3 mo (p=0.02) (**Figure 2.3**). This can also be presented as grams per month accretion of TBLH: 7.82 ± 3.26 g/mo and 9.09 ± 3.98 g/mo respectively (p<0.05). Length velocity between groups was significant; infants in the 1000 IU/d group had significantly greater length velocity with an average growth rate of 0.5 ± 0.2 cm/mo more than infants receiving 400 IU/d (p=0.03) (**Figure 2.4**). When adjusting for adherence, these relationships remained significant. There were no other statistically significant differences between treatment groups on weight or bone outcomes at the lumbar spine, or whole body (**Table 2.5**).

Sex differences were apparent only in bone outcomes of the lumbar spine (L1-L4). Male infants had larger LSBA 0.67 ± 0.26 cm^2 (p=0.01), greater percentage change in LSBMC 15.1 ± 5.7% (p=0.01) and greater monthly change in LSBMC 0.13 ± 0.05 g/mo (p<0.01) compared to female infants. Absolute LSBMC, weight-adjusted LSBMC, LSBMD and percentage change in LSBMD did not differ significantly based on sex. Skin pigmentation was associated only with change in length from baseline to 3 mo, with darker skinned infants growing 1.6 ± 0.7 cm more than lighter skinned infants (p=0.03). All mean bone and anthropometric outcomes in the 400 IU/d and 1000 IU/d groups were well within the range of values (mean ± SD) obtained for the reference group. This indicates that infants in both treatment groups grew and gained bone mineral in an age-appropriate manner from baseline to 3 mo.

Secondary outcome

By 3 mo, 7 (39%) infants in the 400 IU/d group and 21 (95%) infants in the 1000 IU/d group achieved a 25(OH)D concentration of ≥75 nmol/L (p=0.0002). Overall amongst infants in the trial, serum 25(OH)D concentration at 3 mo was inversely correlated with absolute WBBMC (r=-0.33, p=0.04), absolute TBLH (r=-0.35, p=0.03) and percentage change in LSBMD (r=-0.40, p=0.01). When adjusting for covariates in ANOVA analyses, these associations disappeared. Amongst infants in the trial (n=41) there were no significant differences in WBBMC, TBLH, LSBMC or LSBMD between those above or below 75 nmol/L at 3 mo. Additional outcome variables including accretion over time in all four variables, and weight-adjusted versions were also analyzed; none were found to be significantly different between groups.

2.9 TABLES

Table 2.1. Summary of study measurements and timing

Measurement	Screening	Baseline	3 mo
Biochemistry			
Total 25(OH)D by LIAISON	x		x
Vitamin D metabolites by LC-MS/MS[a] 25(OH)D 3-epi-25(OH)D $24,25(OH)_2D$ $1,25(OH)_2D$		x	x
Parathyroid hormone[a]		x	x
Ionized calcium		x	x
Biomarkers of bone mineral metabolism[a]		x	x
Urinary Ca:Cr and P:Cr		x	x
Anthropometry			
Weight	x (chart)	x	x
Length		x	x
Head circumference	x (chart)	x	x
Mid-upper arm circumference		x	x
Body composition and bone by DXA		x	x
Surveyed Information			
Obstetrical record	x (chart)		
Demographic surveys	x		
Adverse events/any illnesses		x	x
Dietary/supplement intake and adherence		x	x
Sun exposure/skin pigmentation		x	x

LC-MS/MS liquid chromatography tandem-mass spectroscopy; Ca:Cr Calcium creatinine ratio; P:Cr Phosphorus creatinine ratio; DXA dual-energy X-ray absorptiometry

[a] Measurement results not yet available.

Table 2.2. Screening and baseline characteristics of participating infants and their mothers

Characteristics	Treatment 400 IU/d (n=19)	Treatment 1000 IU/d (n=22)	p value	Reference 400 IU/d (n=15)
Infants				
Sex				
Male, no. (%)	10 (53%)	13 (59%)	0.76	9 (60%)
Female, no. (%)	9 (47%)	9 (41%)		6 (40%)
Skin pigmentation				
Light, no. (%)	15 (79%)	13 (59%)	0.20	13 (87%)
Dark, no. (%)	4 (21%)	9 (41%)		2 (13%)
Gestational age, wk	39.8 ± 0.9	39.5 ± 0.9	0.20	40.0 ± 0.8
Anthropometrics at birth				
Birth weight, kg	3.446 ± 0.464	3.321 ± 0.446	0.38	3.506 ± 0.303
Weight-for-age z-score,	0.21 ± 1.00	-0.05 ± 1.00	0.40	0.40 ± 0.59
Weight-for-length z-score	-0.93 ± 1.38	-0.92 ± 1.80	0.99	-0.97 ± 1.67
Birth length, cm	51.6 ± 2.1	51.0 ± 2.4	0.35	52.0 ± 2.8
Length-for-age z-score	0.95 ± 1.02	0.64 ± 1.16	0.37	1.28 ± 1.52
Birth during vitamin D synthesizing period[a], no. (%)	9 (47%)	8 (36%)	0.54	10 (67%)
Screening 25(OH)D concentration, nmol/L	28.5 ± 10.0	29.7 ± 12.4	0.74	67.6 ± 14.4
Age at baseline, mean ± SD, d	18 ± 5	20 ± 5	0.43	20 ± 7
Receiving vitamin D supplement at baseline, no. (%)	17 (89%)	21 (95%)	0.59	15 (100%)
Mothers				
Age at delivery, y	32 ± 5	31 ± 6	0.43	32 ± 4
Primiparous, no. (%)	8 (42%)	11 (50%)	0.37	5 (33%)
Household income ≥$70,000 CAD, no. (%)	10 (53%)	10 (45%)	0.35	13 (87%)
Not disclosed, no. (%)	1 (5%)	4 (18%)	0.89	-
Completed university, no. (%)	12 (63%)	18 (82%)	0.44	13 (87%)
Used prenatal multivitamin during pregnancy, no. (%)	17 (89%)	21 (95%)	0.28	14 (93%)

Data are reported as mean ± SD unless otherwise indicated.

[a] Infants born during Apr-Oct when endogenous vitamin D production is possible based on latitude.

Table 2.3. Infant bone and anthropometric outcomes at baseline (≤1 mo)

Variable	Treatment		p value	Reference
	400 IU/d (n=19)	1000 IU/d (n=22)		400 IU/d (n=15)
Dual-energy X-ray absorptiometry				
Whole body bone outcomes				
WBBMC, g	95.67 ± 15.13	93.52 ± 12.20	0.62	92.23 ± 16.20
Weight-adjusted WBBMC, g/kg	24.83 ± 3.81	24.80 ± 2.90	0.98	23.69 ± 2.53
TBLH, g	53.94 ± 8.67	49.91 ± 6.65	0.10	51.31 ± 8.49
Weight-adjusted TBLH, g/kg	14.09 ± 2.81	13.23 ± 1.57	0.25	13.27 ± 1.96
Lumbar spine bone outcomes				
LSBA, cm^2	9.63 ± 0.92	9.34 ± 0.86	0.31	9.64 ± 0.60
LSBMC, g	2.22 ± 0.47	2.23 ± 0.36	0.94	2.34 ± 0.41
Weight-adjusted LSBMC, g/kg	0.58 ± 0.14	0.60 ± 0.14	0.65	0.61 ± 0.10
LSBMD, g/cm^2	0.229 ± 0.040	0.243 ± 0.056	0.37	0.242 ± 0.038
Anthropometrics				
Weight, kg	3.894 ± 0.618	3.789 ± 0.424	0.52	3.893 ± 0.557
Weight-for-age z-score	-0.04 ± 1.04	-0.28 ± 0.62	0.37	-0.09 ± 0.56
Weight-for-length z-score	-0.29 ± 1.08	-0.57 ± 0.79	0.35	-0.70 ± 0.85
Length, cm	52.8 ± 1.8	52.7 ± 2.2	0.89	53.2 ± 2.3
Length-for-age z-score	0.00 ± 0.87	-0.06 ± 0.91	0.83	0.25 ± 0.91
BMI, kg/m^2	13.9 ± 1.5	13.6 ± 0.8	0.44	13.7 ± 1.0
BMI-for-age z-score	-0.06 ± 1.10	-0.35 ± 0.58	0.28	-0.31 ± 0.63

Data are reported as mean ± SD.

Table 2.4. Biochemistry values at 3 mo and normative values used in safety monitoring

Analyte	400 IU/d (n=19)	1000 IU/d (n=22)	p value[a]	Reference (n=15)	Normal range
Serum 25(OH)D (nmol/L) (LIAISON, DiaSorin Inc.)	77.1 ± 23.1	130.9 ± 39.5	<0.0001	83.9 ± 35.2	≥50[b] - <225[c]
Blood iCa (mmol/L)	1.41 ± 0.02	1.41 ± 0.04	0.77	1.43 ± 0.04	1.31 - 1.46[d]
Urinary Ca:Cr[e] (mmol:mmol)	1.1 ± 0.9	1.6 ± 0.9	0.16	1.5 ± 1.2	<2.2[f]
Urinary P:Cr[e] (mmol:mmol)	1.8 ± 1.8	0.6 ± 0.4	0.06	0.9 ± 0.7	Not established[g]

Data are reported as mean ± SD.
[a] Wilcoxon two-sample nonparametric test
[b] Health Canada, 2012 (5)
[c] Godel, C and CPS, 2017 (105)
[d] Gallo et al., 2013 (18) and Gallo et al., 2014 (65)
[e] n=12 in each of the 400 IU and the 1000 IU groups
[f] Based on values currently used at the Montreal Children's, Shriners, CHEO and General Hospitals
[g] Normal range not established for infants 3 mo, however in infants of 1-12 mo, 5th-95th percentile is 1.2-19.0 mmol:mmol (150)

Table 2.5. Bone and anthropometric outcomes at 3 mo

Variable	Treatment 400 IU/d (n=19)	Treatment 1000 IU/d (n=22)	p value	Reference 400 IU/d (n=15)
Dual-energy X-ray absorptiometry				
Whole body bone outcomes[a]				
WBBMC, g	142.62 ± 17.43	141.75 ± 23.13	0.67	142.64 ± 16.88
Weight-adjusted WBBMC, g/kg	22.55 ± 2.57	22.72 ± 2.28	0.95	23.02 ± 1.98
Δ WBBMC per month, g/mo	17.73 ± 4.64	19.31 ± 7.41	0.32	20.24 ± 5.73
% Δ WBBMC, %	48.7 ± 16.8	52.5 ± 23.9	0.40	53.2 ± 23.6
TBLH, g	74.54 ± 13.60	72.72 ± 13.16	0.31	72.39 ± 13.01
Weight-adjusted TBLH, g/kg	11.73 ± 1.69	11.65 ± 1.45	0.39	11.67 ± 1.78
Δ TBLH per month, g/mo	7.82 ± 3.26	9.09 ± 3.98	<0.05	8.45 ± 5.34
% Δ TBLH, %	37.3 ± 14.8	46.2 ± 23.5	0.02	43.5 ± 28.9
Lumbar spine bone outcomes				
LSBA, cm^2	11.99 ± 0.93	12.24 ± 1.04	0.78	12.11 ± 0.60
LSBMC, g	2.62 ± 0.44	2.62 ± 0.49	0.96	2.86 ± 0.47
Weight-adjusted LSBMC, g/kg	0.42 ± 0.07	0.42 ± 0.08	0.58	0.46 ± 0.06
Δ LSBMC per month, g/mo	0.15 ± 0.09	0.16 ± 0.22	0.65	0.20 ± 0.18
% Δ LSBMC, %	19.3 ± 12.8	19.8 ± 26.4	0.75	25.0 ± 26.6
LSBMD, g/cm^2	0.218 ± 0.031	0.214 ± 0.032	0.58	0.236 ± 0.034
% Δ LSBMD, %	-4.1 ± 8.9	-8.9 ± 18.6	0.13	-0.9 ± 19.5
Anthropometrics				
Weight, kg	6.343 ± 0.952	6.262 ± 1.013	0.54	6.191 ± 0.431
Weight-for-age z-score	0.02 ± 1.19	-0.04 ± 1.19	0.58	-0.11 ± 0.61
Weight-for-length z-score	0.17 ± 1.24	-0.23 ± 1.04	0.23	-0.54 ± 0.74
Δ Weight from baseline, kg	2.449 ± 0.610	2.474 ± 0.809	0.23	2.297 ± 0.441
Weight velocity, kg/mo	0.946 ± 0.223	0.994 ± 0.296	0.10	0.915 ± 0.131
Length, cm	61.2 ± 2.9	61.7 ± 3.3	0.87	62.1 ± 2.0
Length-for-age z-score	-0.03 ± 1.40	0.29 ± 1.47	0.73	0.45 ± 0.91
Δ Length from baseline, cm	8.4 ± 2.1	9.0 ± 2.2	0.09	8.9 ± 1.8
Length velocity, cm/mo	3.2 ± 0.7	3.7 ± 0.9	0.03	3.5 ± 0.4
BMI, kg/m^2	16.9 ± 1.7	16.4 ± 1.6	0.24	16.5 ± 1.7
BMI-for-age z-score	0.06 ± 1.15	-0.28 ± 1.07	0.39	-0.49 ± 0.68

Data are reported as mean ± SD.
[a] n=18 in the 400 IU/d group

2.10 FIGURES

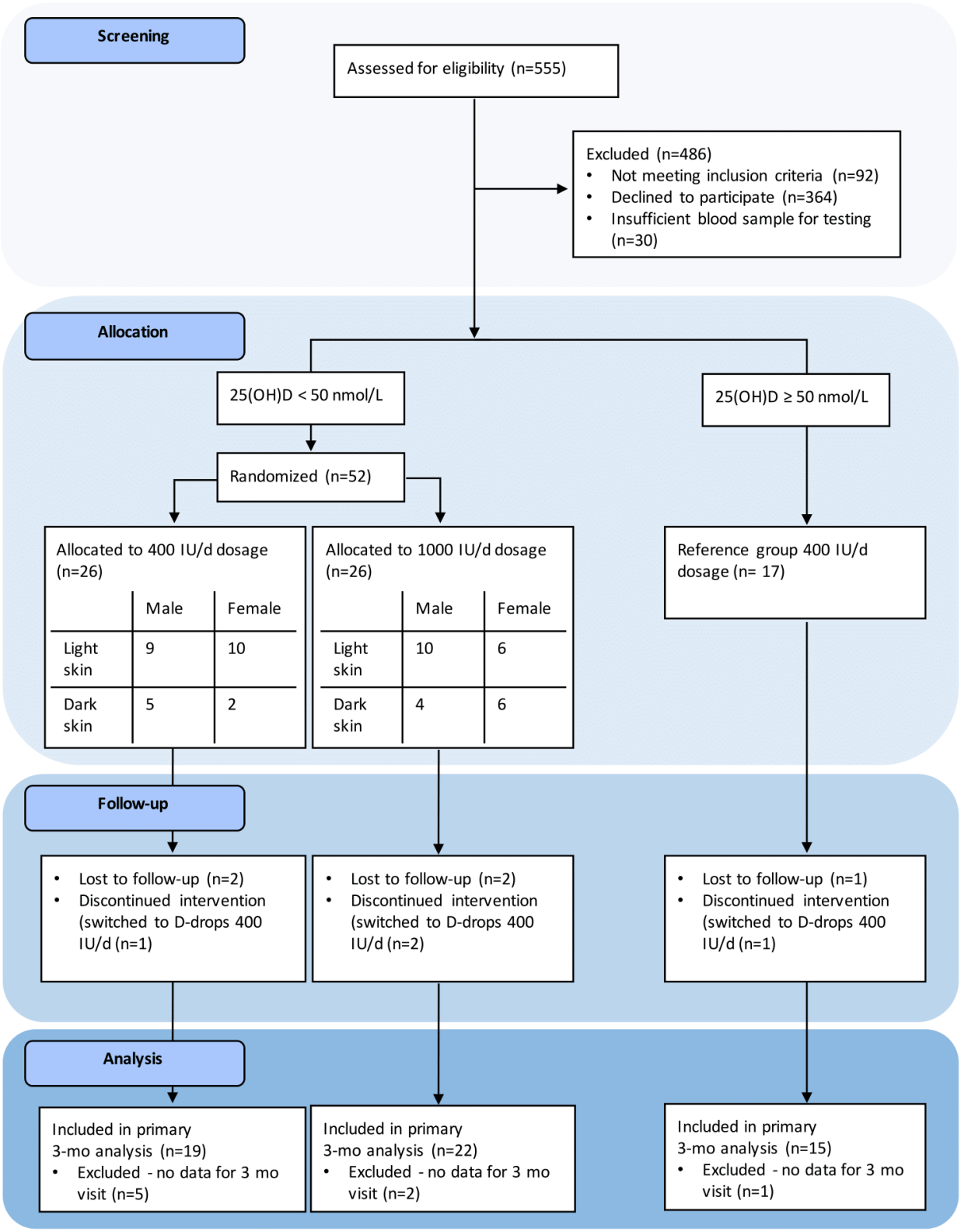

Figure 2.1. CONSORT diagram

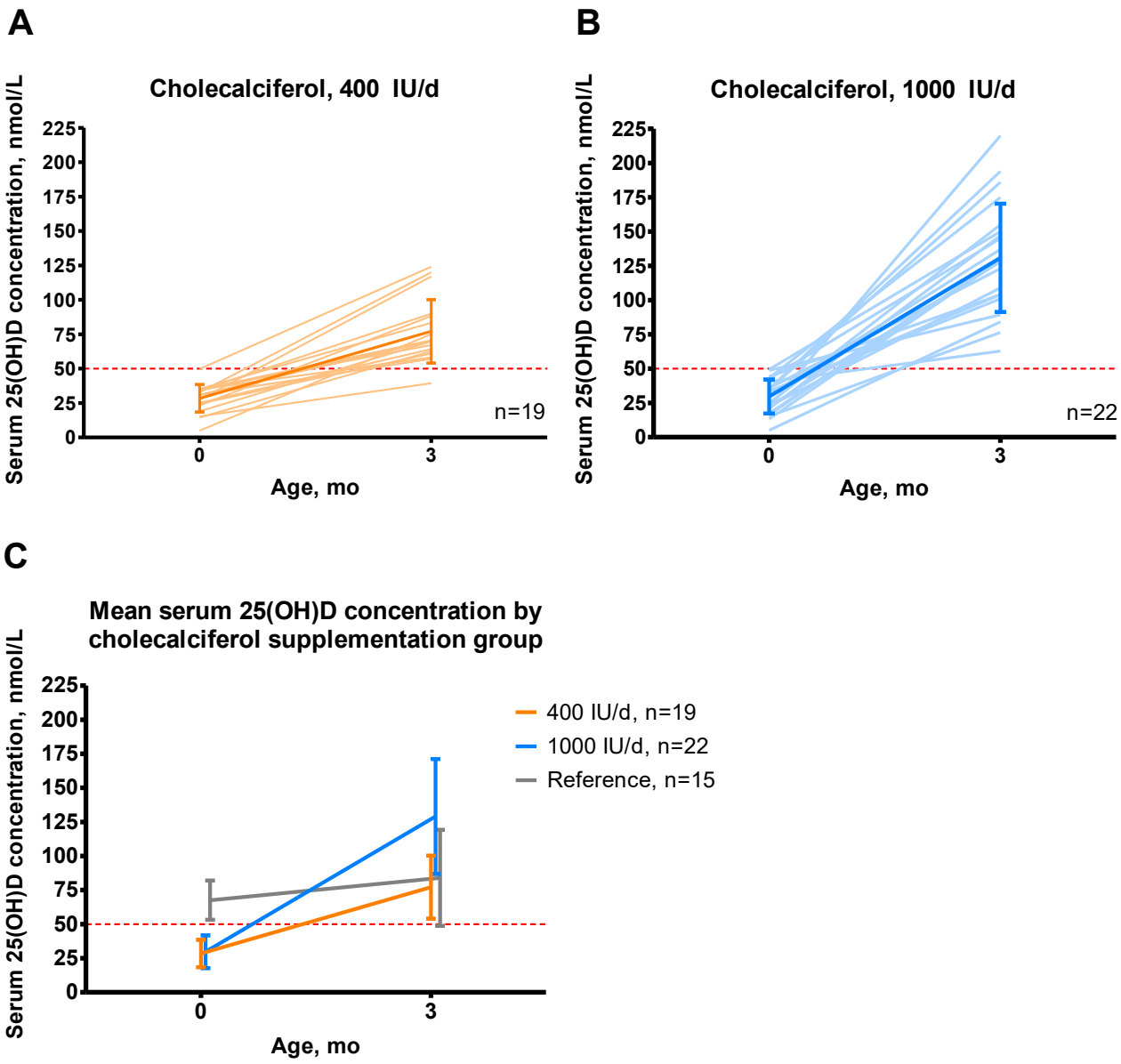

Figure 2.2. Serum 25(OH)D concentrations at screening (0 mo) and follow-up (3 mo) in trial infants who received vitamin D_3 supplementation dosage of 400 IU/d (A) or 1000 IU/d (B), and compared to a reference group receiving 400 IU/d (C) using CLIA

Data for each participant are shown as a spaghetti plot underlying the bold group means; error bars indicate SD.
CLIA chemiluminescence immunoassay

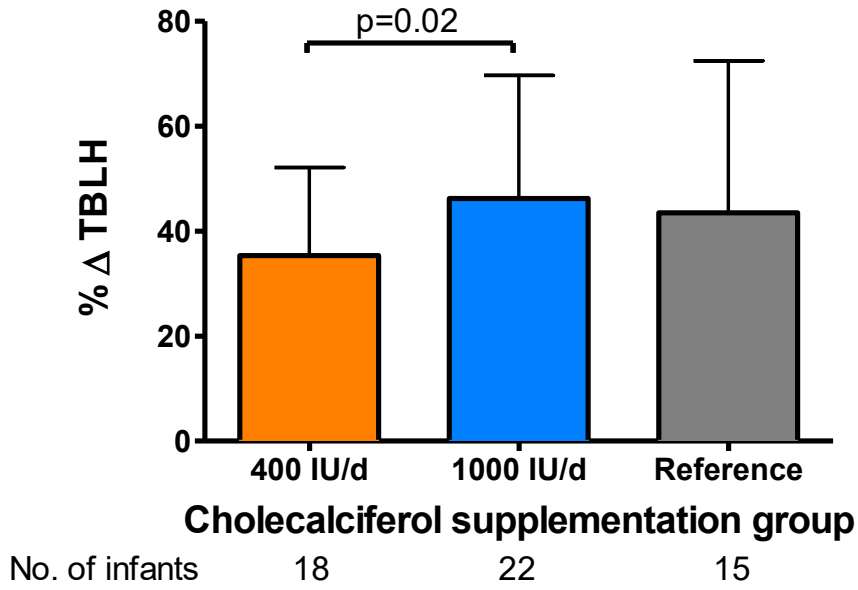

Figure 2.3. Percent change in TBLH bone mineral content from baseline (≤1 mo) to follow-up (3 mo) in trial infants who received vitamin D_3 supplementation dosage of 400 IU/d or 1000 IU/d, and compared to a reference group receiving 400 IU/d using DXA

Percent change TBLH was calculated using actual time elapsed between study visits.
TBLH total body less head; DXA dual-energy X-ray absorptiometry

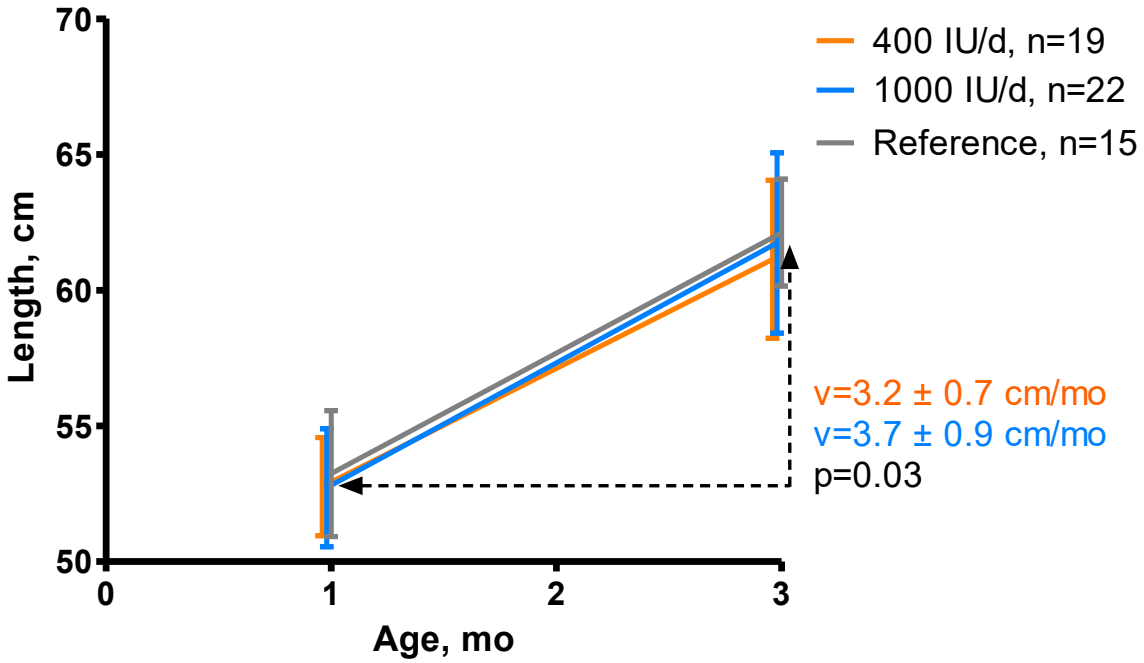

Figure 2.4. Infant length velocity (cm/mo) from baseline (≤1mo) to follow-up (3 mo) in trial infants who received vitamin D_3 supplementation dosage of 400 IU/d or 1000 IU/d, and compared to a reference group receiving 400 IU/d using an infantometer

Length velocity (v) was calculated using actual age (continuous) at baseline and follow-up.

2.11 DISCUSSION

This preliminary analysis set out to determine whether bone outcomes at 3 mo, as assessed by DXA, differed based on the dosage of vitamin D supplementation given to infants born with vitamin D insufficiency. Overall, both groups had normal BMC at baseline and increased BMC over the 3 mo study period at a rate comparable to the reference group. A significant difference in the percent change, and accretion per month of TBLH was found whereby infants in the 1000 IU/d group gained bone mineral faster than the 400 IU/d group over the study period, despite having comparable BMC in this region at baseline. Infants in the 1000 IU/d group also grew in length significantly more rapidly than those in the 400 IU/d group, which is consistent with greater bone mineral accretion. There were no significant differences in any other anthropometric or bone outcomes of the whole body or lumbar spine.

It is not surprising that no differences in the lumbar spine were detected by 3 mo between trial groups as this area is small and growth is much slower than in the long bones (127). Thus, any treatment effects at the lumbar spine are expected to take longer to present, as shown in longer-term trials and prospective studies (15, 16, 142). Percent change in LSBMD was negative in both treatment groups, which is commonly observed during early infancy and indicates bones are becoming larger before they become denser (116, 118). The total spine, together with the skull (included in WBBMC) make up a large portion of the axial skeleton. Since a significant change in bone mineral accretion was only detected in TBLH, but not WBBMC or LSBMC, this suggests that the change took place mainly in the appendicular skeleton. Combined with the information that change in length was also more rapid, these findings imply that infants in the 1000 IU/d group gained bone mineral more rapidly in the long bones compared to those in the 400 IU/d group. It is important to mention that although not statistically significant ($p=0.10$), the 1000 IU/d group did begin the trial with slightly lower TBLH. Therefore, it is possible that this group experienced catch-up growth rather than an effect due to the treatment. These results are interesting and further investigation is warranted to confirm the effects of supplementation.

There are limited studies against which we can compare our findings, and unlike in our trial most have been carried out in infants with sufficient vitamin D status. Gallo et al. measured whole body, lumbar spine L1-L4, and femur using DXA (Hologic 4500A Discovery) in their vitamin D dose-response study. Infants (1 mo) were randomized to a supplementation treatment (400, 800, 1200, and 1600 IU/d) and bone outcomes were assessed at 12 mo; no significant differences in bone outcomes were found (18). In a study of similar design, Holmlund-Suila et al. found a trend towards larger tibial total bone cross-sectional area and cortical bone area associated with vitamin supplementation exceeding 400 IU/d (1200 and 1600 IU/d). These outcomes were assessed by peripheral quantitative computed tomography (pQCT) at 3 mo, but results were not statistically significant (20). Contrary to both these trials, our study is the first to find a significant difference in TBLH and length velocity associated with vitamin D supplementation of 1000 IU/d vs. 400 IU/d. Our findings also potentially challenge previous studies which have reported that vitamin D intakes between 340 and 600 IU/d have the maximum effect on linear growth (151). In future studies, the measurement of blood biomarkers of bone formation and resorption could help to provide insight into the dynamic changes occurring in bone. For example, procollagen type 1 N-terminal propeptide (P1NP) and osteocalcin are used as markers of bone formation (111, 136) and could indicate changes occurring during endochondral ossification of the long bones (114). Biomarkers respond rapidly to changes occurring in bone compared to DXA, making it a complimentary measurement for a study of short duration.

Serum total 25(OH)D concentration is a reflection of both exogenous and endogenous vitamin D sources. In our study, endogenous synthesis was unlikely to contribute to 25(OH)D concentration as few infants were reported to have sun exposure at 3 mo, and this sun exposure was not correlated to a change in 25(OH)D concentration, nor a change in facultative skin pigmentation at the mid forearm. Exogenous vitamin D was provided via supplements, with very little estimated from other sources such as infant formula and breast milk. The majority (82%) of infants were exclusively breastfed and had an equivalent status to those receiving formula. A vitamin D dosage of 400 IU/d was effective in achieving and maintaining a serum concentration of 25(OH)D above the recommended 50 nmol/L (4) in the majority (94%)

of infants, all of whom were born with an insufficient vitamin D status. This had not previously been demonstrated as trials to date have mainly involved infants with sufficient vitamin D status (18, 19, 79). As expected, the recovery dose of 1000 IU/d increased 25(OH)D to a much higher concentration (mean 131 nmol/L) by 3 mo and 100% achieved a sufficient status. Previous studies show similar findings where vitamin D supplements exceeding 400 IU/d resulted in mean 25(OH)D concentrations over 100 nmol/L (18, 19, 111). No infants in our study exceeded the suggested safe concentration of 225 nmol/L 25(OH)D (105) and no infants were identified as being at risk of hypercalcemia based on normative values for ionized calcium and urinalysis results.

The secondary aim of this thesis was to relate serum 25(OH)D concentration to bone mineralization measured with DXA. There were no significant differences in absolute values or changes in BMC or BMD between those above or below 75 nmol/L, indicating that a serum 25(OH)D target of 75 nmol/L was not able to predict differences in bone outcomes. Not surprisingly, the 1000 IU/d dosage was significantly more effective than 400 IU/d at raising serum 25(OH)D ≥75 nmol/L, and did so in 95% of infants. The response to supplementation in our study was stronger than what was observed in the Gallo et al. dose-response study where doses of 800 IU/d and 1200 IU/d resulted in 81% and 92% of infants reaching ≥75 nmol/L 25(OH)D respectively (18). This is perhaps because the infants in our study began with an insufficient status; an inverse relationship between initial status and response to supplementation has been observed in previous studies in infants and adults (67, 111, 152, 153). It is hypothesized that hepatic 25-hydroxylation (CYP2R1) is suppressed once a sufficient vitamin D status is reached (154, 155).

Previous supplementation studies have observed that 25(OH)D concentration is typically at its maximum at 3 mo of age and decreases thereafter (18, 19). This peak may represent a moment when vitamin D intake relative to body size is high (18). This could explain why we found a significant inverse correlation between serum 25(OH)D concentration and absolute WBBMC, TBLH and percentage change in LSBMD – but not when adjusting for body size. Data on vitamin D metabolites were not available for analysis in this thesis. Analysis by liquid chromatography tandem-mass spectrometry (LC-MS/MS) is suggested for future studies to

quantify 3-epi-25(OH)D, 24,25-dihydroxyvitamin D, 1,25$(OH)_2$D, ergocalciferol and cholecalciferol separately. These could offer a better understanding of the metabolism of vitamin D in infancy and in relation to bone outcomes.

We explored several demographic infant and maternal characteristics in our analyses but found that only infant sex and skin pigmentation had any significant effects on bone outcomes. In agreement with existing research (113, 123), we found that females had lower LSBA and a smaller increase in LSBMC compared to male infants, but that LSBMD was not significantly different. Skin pigmentation was associated with change in length from baseline to 3 mo, with darker skinned infants growing 1.6 ± 0.7 cm more than lighter skinned infants (p=0.03). Darker skinned infants were significantly shorter at baseline (p=0.03) but had similar vitamin D status at birth. There is very little research regarding ethnicity or skin pigmentation in relation to bone development in infants, however a cross-sectional in children (age 9 y) found that black children showed more rapid growth in the appendicular skeleton (limbs), whereas white children had more growth in the axial skeleton (trunk) (121). Future studies examining such differences should include examination of limb lengths, or regional DXA scans to validate these findings.

Our study has many strengths including the rigorous double-blind randomized design, control of multiple possible confounders, multicultural population, and the use of DXA, which is accurate even in very small subjects. This is one of the first trials focusing on infants born with an insufficient vitamin D status and it contributes important data on the vitamin D status and DXA bone data in Canadian infants. One of our study's limitations is that the majority of mothers were university educated women with an annual household income ≥70,000 Canadian dollars, placing them above the median for the country (156, 157) which could influence the generalizability of results. A second limitation is that the statistical power of the study was reduced by the small sample size, but our results do support the need for the larger population study.

In summary, the preliminary results of this study found no significant differences in absolute BMC or BMD associated with the use of a higher dose (1000 IU/d) compared to a

standard dose (400 IU/d) of vitamin D in infants. The findings do suggest some growth advantages in terms of bone mineral accretion and length velocity by 3 mo associated with higher dose supplements. The relationship between vitamin D status and bone outcomes remains unclear and at this time there is no evidence to suggest that a 25(OH)D concentration target of 75 nmol/L is useful in infants. Study participants will continue in the trial until 1 y with follow-up to 3 y to assess the longer-term impacts of the intervention, in a larger sample size.

3 GENERAL DISCUSSION

3.1 FINDINGS

This thesis was designed to investigate whether rapid correction of vitamin D status early in the neonatal period significantly improved surrogate markers of bone health by 3 mo of age using a pilot phase of a randomized controlled trial in otherwise healthy term-born infants with 25(OH)D concentration below 50 nmol/L. It was important to focus on newborns with insufficient vitamin D status as they are more at risk and previous studies have mainly addressed supplementation in infants with already-sufficient status. It was hypothesized that infants supplemented with 1000 IU/d of vitamin D would have a greater change in LSBMD and LSBMC and greater change in WBBMC and TBLH at 3 mo compared to those given 400 IU/d. The main findings were a significantly higher percent change and monthly accretion in TBLH and in length velocity amongst infants in the 1000 IU/d compared to the 400 IU/d group. These results suggest that infants in the 1000 IU/d group may be growing more rapidly than those in the 400 IU/d group, particularly in the long bones. It is possible that the slight, although not statistically significant (p=0.10), difference in baseline TBLH between groups could be contributing to the change in TBLH by 3 mo. That is to say that the 1000 IU/d group may have experienced catch-up growth rather than a true treatment effect. This will become clear with a larger sample size and over the duration of the full trial. There were no significant differences in the lumbar spine bone outcomes, likely due to the slower growth in this region compared to the long bones (127). The secondary objective was to determine if infants reaching a 25(OH)D concentration of 75 nmol/L or greater by 3 mo have improved bone outcomes compared to those below this target, irrespective of vitamin D intake. There were no significant differences found between groups, suggesting that a serum target of 75 nmol/L was not able to predict differences in bone outcomes by 3 mo.

The prevalence of 25(OH)D insufficiency at birth in this preliminary study population was 66% (n=322/488 sampled in hospital) which is significantly higher than previous estimates in the Canadian population ranging from 25-35% of infants (6, 30, 104). Perhaps this is related to

the relatively high proportion of infants born to non-white parents in our screening population (45%, 219/488), who may be at increased risk of vitamin D insufficiency due to limited endogenous vitamin D synthesis. In comparison, other studies of Canadian infants had a lower proportion of non-white infants including 35% in an Alberta study (30), 3% in Quebec City (6), and approximately 2% Newfoundland and Labrador (104). Comparison between studies is complicated by the fact that other studies sampled cord blood for testing (6, 30, 104) whereas we used capillary blood. To date, no studies have compared umbilical vein blood with infant capillary samples *per se*, although we do know that venous blood and capillary blood are highly correlated, with capillary blood estimates tending to be the higher of the two (47).

Our study has shown that a dose of 400 IU/d vitamin D was effective in achieving and maintaining a serum 25(OH)D concentration of ≥50 nmol/L in 94% of trial infants by 3 mo and 87% of the reference group. Currently 400 IU/d is the Adequate Intake (AI) set by the IOM and Health Canada as a level at which rickets is prevented and 25(OH)D concentrations are ≥50 nmol/L (5, 52). When establishing a Recommended Dietary Allowance (RDA), the criteria is that a given intake should support healthy status in 97.5% of the population. There is currently a lack of data to establish an RDA in infants. Based on our preliminary findings, and previous research, it seems as though 400 IU/d supports a sufficient vitamin D status in 82-97% of a population including those born with sufficient and insufficient status (18, 19). In our study 1000 IU/d resulted in sufficient vitamin D status in 100% of the population and similarly Gallo et al. found that 1200 IU/d gave 97% of the population a 25(OH)D concentration of ≥50 nmol/L (18). Furthermore, existing studies suggest that infant status is expected to decline after 3 mo (18, 19), making it an even greater challenge for those on the 400 IU dosage to achieve and maintain serum 25(OH)D concentration of 50 nmol/L as they develop. Based on this information, if an RDA were to be established, 1000 IU/d might be more appropriate than 400 IU/d to meet the needs of 97.5% of Canadian infants. No infants in our study exceeded the suggested safe concentration of 225 nmol/L 25(OH)D (105) and no infants were identified as being at risk of hypercalcemia based on normative values for ionized calcium and urinalysis results (**Table 2.4**). For this reason, there are no suggested changes to the study protocol regarding safety at this time.

In the assessment of bone outcomes, the finding that TBLH and length increased more rapidly in the 1000 IU/d group suggests that this group may have experienced a greater accretion of bone mineral in the long bones. Femoral growth is particularly susceptible to environmental and physiological conditions with changes being detectable as early as *in utero* (121, 158). Additionally, the hip is a common fracture site in osteoporosis and the incidence of hip fracture has been linked to slow rate of childhood growth (height and weight) in a large longitudinal study (159). For these reasons, the femur is an area that warrants further investigation when studying the potential effects of vitamin D on linear velocity and BMC. However, software for the measurement of infant femur does not exist. Previous studies on vitamin D supplementation in infants have included an assessment of femur growth using software for a subregion of the hip; however, it has not been validated for use in infants. Nonetheless, Gallo et al., found no significant differences in BMC of the whole femur based on vitamin D supplementation dosage in their dose-response study (18). In the retrospective study by Zamora et al., DXA (Hologic, Inc., QDR-2000) was used to assess the femoral neck, femoral trochanter, and femoral mid-diaphysis in girls (7–9 y) that were either supplemented or supplemented with vitamin D during the first 6 mo of life. They found that the supplemented group had significantly higher BMC and BMD at the level of femoral neck and femoral trochanter (141). To further explore measurements of femoral BMC in our study, we performed a sub-analysis on infant scans where an unobstructed view of the left femur was available at baseline and 3 mo. The whole femur was manually traced from the whole body scan and analyzed for BMC (**Figure 5.1**). In the 400 IU/d group, 13 pairs of infant scans were suitable for analysis and in the 1000 IU/d group, 19 pairs of scans. When analyzed using ANOVA with repeated measures adjusting for sex, skin pigmentation and weight, we found no significant difference in femur area, femur BMC, or femur BMD between the two trial groups (**Table 5.2**). It should be noted that the whole body scan is not designed for the extraction of the femur as a sub-region, and to improve resolution future studies should include either a femoral regional scan (134) or measurement of limb length. Thus, forearm measurements are recommended at the 2 and 3 y time-point assessments.

Several covariates were explored in our primary models including sex, skin pigmentation, body size, season of birth, nutrition, and mother's demographic information. Existing research on sex differences in infants has found that females may have lower whole body and spine BMC, but that spine BMD is the same due to smaller bone area in females compared to males (113, 123). Our results are in agreement with male infants having significantly higher LSBA (0.67 ± 0.26 cm^2, p=0.01) and greater percentage change in LSBMC (15.1 ± 5.7%, p=0.01) as well as change in LSBMC per mo (0.13 ± 0.05 g/mo, p<0.01), but not LSBMD compared to females. These differences are expected to become more pronounced with age (113).

There is limited previous research in infants examining the relationship between bone mineral accretion and ethnicity however some evidence finds that those of African-American descent experience fewer fractures and have denser bones than those who are Caucasian, Asian, and Hispanic (25, 113). In infants, one study showed that Hispanic infants had higher change in WBBMC compared to Caucasian infants (79). Differences in bone morphology by ethnicities are attributed to genetics, environmental factors (geographic, socioeconomic, lifestyle) and perhaps differences in endogenous vitamin D synthesis (113). It was not possible to classify infants based on parent's self-identified ethnicity because of the small sample size and many being of mixed ancestry. Instead, ITA° was measured by spectrophotometer and used to objectively classify infants by skin type (I-III: light; IV-VI: dark) based on Fitzpatrick descriptions (146, 147) to be included as a fixed effect in our models. It was found that infants with darker skin pigmentation grew significantly more in length from baseline to 3 mo (1.6 ± 0.7 cm, p=0.03). Darker skinned infants were shorter at baseline (p=0.03) but had comparable vitamin D status. There were no significant differences in bone outcomes between skin pigmentation groups by 3 mo; any differences may take longer to become evident. Change in skin pigmentation from baseline to 3 mo was calculated at the mid forearm to assess sun exposure. Only 4 of 41 infants got darker while the remainder stayed the same, or became lighter. Some of these changes could be attributed to flushing of skin, or lower subcutaneous fat in very young infants. As expected at this age, reported sun exposure was minimal as recommended by Health Canada (5) and any changes to facultative skin pigmentation were not

correlated with reported sun exposure. The fact that infants receive minimal sun exposure reinforces the importance of vitamin D supplements to meet their requirements.

It is well understood that WBBMC is largely dependent on infant body size (134). In line with this, we found that WBBMC was highly correlated with weight and length and considered this in our models. What is less well understood is the relationship between vitamin D status, body size, and BMC in infants. A Canadian study by Weiler et al. found that sufficient (≥27.5 nmol/L) vs. deficient (<27.5 nmol/L) 25(OH)D concentration at birth was associated with higher weight-adjusted BMC (10). Making a comparison is difficult as our study used different sufficiency cutoffs, but no association was found between 25(OH)D concentration and weight-adjusted BMC at baseline or 3 mo.

The seasonal variation in 25(OH)D concentration is well documented and is known to relate to infant vitamin D status at birth secondary to maternal status (95). In our study, 25(OH)D concentration was significantly higher for infants born from Apr-Oct inclusive vs. those born during Nov-Mar (33.5 ± 8.3 nmol/L vs. 26.0 ± 12.1 nmol/L, $p<0.05$) by Student's t-test. In addition to vitamin D status, a Canadian study also found seasonal differences in bone health whereby LSBMC was lower in infants born in the fall compared to spring (116). The effect of season on WBBMC are less clear with some studies showing higher bone mineral mass in the winter and others in the summer (160). When testing for this in our data by Student's t-test, the only variable which showed a significant difference by season was percent change in LSBMD, with infants born Apr-Oct having 11 ± 14% higher change in LSBMD ($p=0.02$) by 3 mo compared to those born Nov-Mar. The clinical significance of this change is not defined (127) and differences between season of birth are expected to disappear by about 6 mo of age (116).

Nutritional factors were not included in the ANOVA models in this thesis; however, they were explored separately to rule out possible effects. Nutritional factors that may affect bone mineral accretion in early infancy include: maternal nutrition during pregnancy and breastfeeding, use of supplements, and type of infant feeding (124). Maternal transfer of 25(OH)D *in utero* to the fetus is highly effective and at birth an infant's serum 25(OH)D concentration is highly correlated to the mother's (49, 54). Maternal use of prenatal

supplements was thus explored and no correlation was found between frequency of use of prenatal multivitamins or vitamin D supplements with infant 25(OH)D concentration at birth or with infant bone outcomes at baseline or 3 mo. Within the context of this thesis, maternal diet during lactation was not analyzed as it was not expected to significantly alter the already low vitamin D content of breast milk (17). The type of infant feeding (breast milk or infant formula) is more relevant and previous research has shown that infants fed formula tend to have higher WBBMC accretion by 6 mo compared to exclusively breastfed infants possibly due to the higher vitamin D or calcium content in formula (125). Our study did not find any significant correlations with bone outcomes and type of infant feeding, however all infants were breastfed at baseline and the majority of infants (82%) were exclusively breastfed until 3 mo with the remainder receiving some infant formula but no solid foods at this age. Adherence to trial supplements was reported at 90% and 88% in the 400 IU/d and 1000 IU/d treatment groups respectively, which is acceptable. We adjusted for adherence in our models but found that it did not significantly alter the results. In the ongoing trial, additional nutritional indices will be analyzed including maternal diet during pregnancy, infant breast milk feeding volume (by test weighing), and infant diet records. This information will be useful in order to tease out the effect of the supplement versus dietary sources. Lastly, in our analyses mother's demographic information was explored including education level, income, parity, and age and none were found to have a significant effect on infant vitamin D status or bone outcomes.

3.2 STRENGTHS AND LIMITATIONS

The present study included a randomized double-blind design that limits bias and multiple confounding variables and covariates were controlled for in the analyses. The methods used to quantify our variables were consistent from baseline to 3 mo, each of which will be discussed in further detail. We estimated vitamin D intake from the supplements provided while accounting for all other sources of vitamin D including endogenous synthesis, type of infant feeding. Factors potentially effecting individual responsiveness to supplements were also taken into account including body size, skin pigmentation, age, any illnesses or medications (none present), 25(OH)D concentration and season (66, 67).

We measured serum 25(OH)D concentration using LIAISON CLIA for purposes of classification into the trial or reference group, and also to identify infants with total 25(OH)D concentration ≥75 nmol/L. LIAISON results were available quickly, which was necessary for timely enrolment of participants in the trial. Unfortunately, this method can have variable accuracy possibly due to the method of standardisation, instrument maintenance and water quality (43). LIAISON is known to have a negative measurement bias compared with LC-MS/MS based assays, but is still generally considered to have superior agreement with LC-MS/MS compared to other immunoassays (44). Since LC-MS/MS will be used in the full trial, preliminary results (n=16) of serum total 25(OH)D at baseline and 3 mo were available and were highly correlated with LIAISON ($r=0.94$, $p<0.0001$) (**Figure 5.2**). One of the major limitation of LIAISON is that it cannot distinguish between 25(OH)D and 3-epi-25(OH)D which it likely to impact our assessment of vitamin D status, especially since 3-epi-25(OH)D is present in high quantities in infants (26, 28). Specifically, 3-epi-25(OH)D may serve to mask insufficiencies in vitamin D status as was explored in the APrON study. For example, when 3-epi-25(OH)D was excluded from their vitamin D estimations 80% of cord blood samples had concentration <75 nmol/L; however, with 3-epi-25(OH)D included, the estimate was 73% (30). This method will also allow us to quantify 24,25-dihydroxyvitamin D which has suspected roles in bone mineral metabolism (23, 32, 33) and $1,25(OH)_2D$ the biologically active form (27). Our secondary objective was designed around the premise that 75 nmol/L 25(OH)D keeps PTH at a minimum in adolescents and adults (1, 7, 42), and is therefore beneficial to maximize calcium absorption (42). Our findings are inconclusive regarding whether this same target is useful in infants; however, the measurement of PTH by immunoassay could provide further insight.

Regarding the measurement of bone outcomes, DXA is considered the gold standard for infant BMC measurement because of its low ionizing radiation exposure, excellent precision, and short scan time (113). Once infants were sleeping, the use of this instrument was very successful in our study for obtaining quality scans free of movement artifacts with only 1 missing due to technical difficulties of the instrumentation. Despite all the advantages of this instrument, one limitation is that it is not designed to capture morphological changes in bone. Although none of the infants in this study are expected to experience rachitic structural

changes, our study is not set up to detect these. In future studies, an infant forearm or femur scan could be included in order to isolate changes happening in these areas particularly prone to rachitic changes (55). It would also be interesting to have results for bone biomarkers from infant blood to get an idea of the dynamic changes happening in the bone, which could help better explain our results. Testing of biomarkers will be included in the full trial.

Another limitation of this thesis is that the sample size was smaller than our target. The original sample size was estimated based on a small sub-population of 18 participants from Gallo et al. with very few of darker skin pigmentation (18). Now that the results of this thesis have shown some significance, a new and more accurate estimated sample size was calculated for future analyses based on the 41 participants in our trial groups. Given that the main outcome of interest was bone, the results for percentage change TBLH were used. An estimated sample of 73 participants per treatment group is needed (total 219 for the 2 treatment groups + reference). The full trial is aiming for a sample size of 74 infants per group, so this is feasible.

The study is novel because it is the first to look at bone outcomes specifically in infants born with a vitamin D insufficiency in an RCT format. It is also the first study to have found a statistically significant difference in TBLH for those receiving a recovery dose of vitamin D. Additionally, this study begins to contribute much-needed reference data regarding 25(OH)D concentration and bone measurements of Canadian infants from birth to 3 mo. The clinical relevance of our preliminary findings is unclear as the intention was to guide the full trial rather than draw conclusions. The 1000 IU dosage of vitamin D raised serum 25(OH)D concentrations above 50 nmol/L in a higher proportion of infants (100%) compared to 400 IU (94%) by 3 mo. The 1000 IU dosage also showed potential benefits in terms of length velocity and change in TBLH. Despite this, the longer-term impacts on bone and other health outcomes need to be examined before a recommendation can be made.

3.3 CONCLUSION

The preliminary results of this study validate that vitamin D doses of 1000 IU/d established healthy serum 25(OH)D concentration in a higher proportion of infants born with insufficient

vitamin D status compared to the standard care dosage (400 IU/d) by 3 mo. Despite this, there were no significant differences in BMC of the whole body or BMC and BMD of the lumbar spine with the use of the higher vitamin D dosage. Results do suggest that there may be some advantages to bone mineral accretion of TBLH and length velocity associated with the use of a 1000 IU/d cholecalciferol compared to a standard dose (400 IU/d) by 3 mo of age. The association between vitamin D status and bone outcomes remains unclear, and at this time there is no evidence to suggest that a 25(OH)D concentration target of 75 nmol/L is useful in infants. Further research in this area is needed to confirm whether the current recommendations are sufficient or if higher doses are beneficial to term-born infants with a vitamin D insufficiency. The results of this thesis can help begin to address the gap in the literature regarding the use of vitamin D supplements in infants born with an insufficiency and the effects on bone. This study will continue with follow-up until 3 y and is expected to have a sample size of 74 participants per group. The results of the trial in combination with additional scientific research will provide a greater understanding of the influence of vitamin D on infant bone development and other health outcomes. This information can be used to make clinical recommendations regarding the appropriate dosage of vitamin D supplements for infants born with an insufficiency.

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5 APPENDIX

Table 5.1. Screening and baseline characteristics of completers vs. non-completers

Characteristics	Completed 3 mo visit (n=41)	Drop out before 3 mo visit (n=4)	p value
Infants			
Treatment			
400 IU/d	19 (46%)	2 (50%)	1.00[b]
1000 IU/d	22 (54%)	2 (50%)	
Sex			
Male	23 (56%)	3 (75%)	0.63[b]
Female	18 (44%)	1 (25%)	
Skin pigmentation			
Light	28 (68%)	2 (50%)	0.59[b]
Dark	13 (32%)	2 (50%)	
Gestational age, mean ± SD, wk	39.6 ± 0.9	39.4 ± 0.6	0.57[c]
Age at baseline, mean ± SD, d	19 ± 5	19 ± 7	0.92[c]
Birth during vitamin D synthesizing period[a]	17 (41%)	4 (100%)	0.04[b]
Screening 25(OH)D concentration, mean ± SD, nmol/L	29.1 ± 11.2	45.1 ± 2.2	<0.01[c]
Mothers			
Age at delivery, mean ± SD, y	31 (30-33)	33 (29-37)	0.53[b]
Primiparous	19 (46%)	0 (0%)	0.13[b]
Household income ≥$70,000 CAD	25 (61%)	2 (50%)	1.00[b]
Not disclosed	0 (0%)	0 (0%)	-
Completed university	30 (73%)	2 (50%)	0.57[b]
Use of prenatal multivitamin during pregnancy	38 (93%)	4 (100%)	1.00[b]

Data are reported as No. (%) unless otherwise indicated.
[a]Infants born during Apr-Oct when endogenous vitamin D production is possible based on latitude.
[b]Fisher's exact test
[c]Student's t-test

Table 5.2. Whole femur bone outcomes at 3 mo using dual-energy X-ray absorptiometry

	Treatment		p value	Reference
Variable	**400 IU/d (n=13)**	**1000 IU/d (n=19)**		**400 IU/d (n=11)**
Whole left femur bone outcomes[b]				
Femur area, cm^2	23.19 ± 2.17	23.43 ± 2.96	0.74	23.75 ± 1.88
Femur BMC, g	4.74 ± 0.89	4.44 ± 0.63	0.09	4.75 ± 0.54
Weight-adjusted Femur BMC, g/kg	0.74 ± 0.16	0.71 ± 0.13	0.27	0.76 ± 0.06
Δ Femur BMC per month, g/mo	0.40 ± 0.25	0.45 ± 0.27	0.81	0.43 ± 0.35
% Δ Femur BMC, %	28.2 ± 17.9	36.4 ± 21.4	0.30	30.2 ± 25.5
Femur BMD, g/cm^2	0.203 ± 0.023	0.189 ± 0.018	0.07	0.199 ± 0.011
% Δ Femur BMD, %	-10.6 ± 16.9	-7.4 ± 16.4	0.66	-11.8 ± 10.3

Data are reported as mean ± SD.

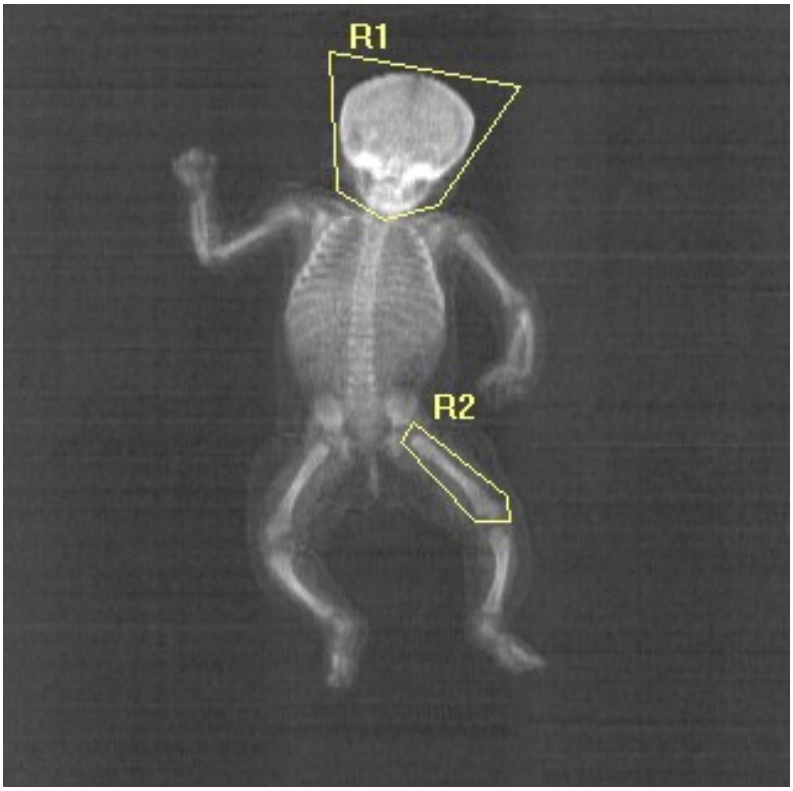

Figure 5.1. Sample whole body DXA analysis with head (R1) and whole femur (R2) sub-regions isolated on a 3 mo infant

Image with permission.
DXA dual-energy X-ray absorptiometry

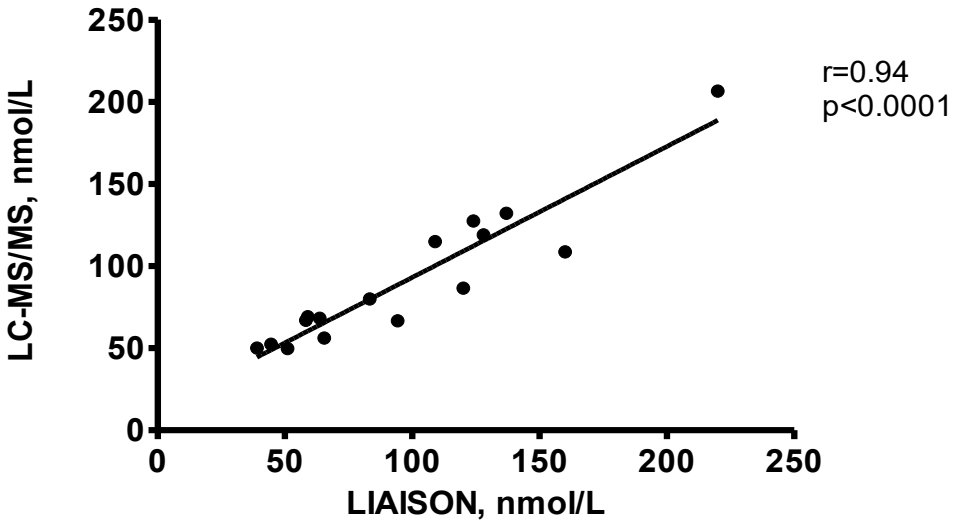

Figure 5.2. Total serum 25(OH)D concentration sampled from trial infants at baseline (≤1 mo) and follow-up (3 mo) measured by LIAISON CLIA vs. LC-MS/MS

CLIA chemiluminescence immunoassay; LC-MS/MS liquid chromatography tandem–mass spectrometry