The determinants of bone health in infancy including vitamin D status at birth and postnatal vitamin D supplementation

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December 2021

A thesis submitted to McGill University in partial fulfilment of the requirements of the degree of Doctor of Philosophy

Abstract

Infancy is a period of rapid bone mineral accretion which may be compromised when vitamin D needs are not met. In Canada, a significant proportion of infants (24-36%) are born with serum 25-hydroxyvitamin D (25(OH)D) <50 nmol/L. For infants, an Adequate Intake (AI) of vitamin D of 400 IU/d was set in accordance with serum 25(OH)D in the range of 40 to 50 nmol/L and for bone health. Whether the AI is enough to meet the needs of infants born with 25(OH)D <50 nmol/L is unclear. The objectives of this thesis were to: 1) provide reference data for bone mineral accretion in infancy and test sex differences; 2) determine whether neonatal bone mass in healthy term, breastfed infants differs according to maternal vitamin D status and gestational weight gain (GWG) and 3) test the impact of 400 vs. 1000 IU/d of vitamin D supplementation on bone mineral content (BMC) and bone mineral density (BMD) from 1 to 12 months of age in healthy term-born infants with 25(OH)D<50 nmol/L.

Study 1 was a secondary analysis of data from a double-blinded randomized trial of healthy term singleton infants with sufficient (25(OH)D ≥50 nmol/L) vitamin D status at 1 month of age. Measurements included: plasma 25(OH)D concentrations, and whole body (WB) BMC, lumbar spine (LS) BMC, and LS BMD (dual-energy x-ray absorptiometry (DXA)) at 1, 3, 6, 9, and 12 months of age. Studies 2 and 3 were completed using data from a double-blinded randomized controlled trial on healthy term singleton infants. Obstetric data were collected and infant serum 25(OH)D concentrations measured within 24-36 h after birth. At 1 month of age, infants with birth serum 25(OH)D: 1) <50 nmol/L were randomized to receive either 400 or 1000 IU/d of vitamin D_3 from 1 to 12 months; and 2) ≥50 nmol/L were allocated to a non-randomized reference group and received 400 IU/d. Infants were followed at 1, 3, 6, and 12 months of age. At the 1 month visit, maternal serum 25(OH)D concentration and anthropometry were measured. At each visit,

infant WB BMC, LS BMC, and LS BMD (DXA), biomarkers of bone metabolism, vitamin D metabolites, and growth were measured.

Study 1 (n=63) showed that WB BMC, LS BMC, and LS BMD increased by 143.2%, 116.8%, and 31.1%, respectively, across infancy. WB BMC was higher (≥4.2%; $p<0.05$) in males than females across the study, but not after adjusting for weight or length. LS BMC and LS BMD did not vary by sex and combined male-female LS BMD growth charts were generated. In study 2, infants (n=142) of mothers with excessive GWG had greater ($p<0.05$) WB BMC (101.40±12.47 g), WB BMC/kg (25.27±3.29 g/kg), LS BMC (2.34±0.38 g), and LS BMD (0.239±0.048 g/cm^2) than those with inadequate GWG (WB BMC: 89.41±13.62 g; WB BMC/kg: 23.47±1.92 g/kg; LS BMC: 1.99±0.34 g; LS BMD: 0.206±0.032 g/cm^2). Additionally, infants of mothers with excessive GWG had greater ($p<0.05$) WB BMC/kg, LS BMC, and LS BMD than those with adequate GWG (WB BMC/kg: 23.93±2.41 g/kg; LS BMC: 2.11±0.40 g; and LS BMD: 0.217±0.036 g/cm^2). Study 3 (n=139) showed that WB BMC, LS BMC, and LS BMD and other biomarkers of bone metabolism are not compromised in neonates born with serum 25(OH)D <50 nmol/L compared to the reference group. In addition, bone mass and biomarkers of bone metabolism were not different between trial groups.

Overall, these results demonstrate that infancy is a period of rapid skeletal mineralization with minimal sex differences. Maternal GWG relates to neonatal bone mass, and this is partly ascribed to infant size. Correction of vitamin D status at birth was achieved with 400 IU/d of supplemental vitamin D within 3 months postnatally, as such a higher dose of 1000 IU/d did not confer additional skeletal advantages in infancy. This reinforces the AI of 400 IU/d of vitamin D supplementation currently endorsed by public health policies in North America.

Résumé

La petite enfance est une période d'accrétion minérale osseuse rapide qui peut être compromise lorsque les besoins en vitamine D ne sont pas satisfaits. Au Canada, une bonne proportion de nourrissons (24 à 36%) naissent avec une concentration sérique de 25-hydroxyvitamine D (25(OH)D) <50 nmol/L. Pour les nourrissons, un Apport Suffisant (AS) en vitamine D de 400 UI/j a été fixé en fonction de la concentration sérique de 25(OH)D dans l'interval de 40 à 50 nmol/L ainsi qu'en fonction de la santé osseuse. Si l'AS répond aux besoins des nourrissons nés avec 25(OH)D <50 nmol/L est toujours inconnu. Les objectifs de cette thèse étaient de : 1) fournir des données de référence pour l'accrétion minérale osseuse dans la petite enfance et tester les différences entre les sexes ; 2) déterminer si la masse osseuse néonatale chez les nourrissons allaités au sein, nés à terme, et en santé diffère selon le statut maternel en vitamine D et le gain de poids gestationnel (GPG) et 3) tester et comparer l'impact de 400 UI/j de supplémentation en vitamine D contre 1000 UI/j sur le contenu minéral osseux (CMO) et la densité minérale osseuse (DMO) de 1 à 12 mois chez les nourrissons nés à terme et en santé et avec 25(OH)D <50 nmol/L.

L'étude 1 était une analyse secondaire des données d'une étude randomisée en double aveugle portant sur des nourrissons uniques nés à terme, en santé et ayant un statut en vitamine D suffisant (25(OH)D ≥50 nmol/L) à l'âge de 1 mois. Les mesures comprenaient: concentrations plasmatiques de 25(OH)D et le CMO du corps entier (CE) et du rachis lombaire (RL) ainsi que la DMO du RL (absorptiométrie à rayons X à double énergie (DXA)) à 1, 3, 6, 9, et 12 mois. Les études 2 et 3 ont été réalisées à l'aide des données d'une étude contrôlée randomisée en double aveugle sur des nourrissons uniques nés à terme et en santé. Les données obstétricales ont été recueillies et les concentrations sériques de 25(OH)D des nourrissons mesurées 24 à 36 h après la naissance. À l'âge

de 1 mois, les nourrissons ayant un niveau sérique de 25(OH)D à la naissance: 1) <50 nmol/L ont été randomisés pour recevoir 400 ou 1000 UI/j de vitamine D_3 de 1 à 12 mois; et 2) ≥50 nmol/L ont été attribués à un groupe de référence et ont reçu 400 UI/j. Les nourrissons ont été suivis à 1, 3, 6 et 12 mois. Lors de la visite à 1 mois, la concentration sérique maternelle de 25(OH)D et les valeurs anthropométriques ont été mesurées. À chaque visite, le CMO (CE et RL), la DMO (RL) (DXA), les marqueurs biologiques du métabolisme osseux, les métabolites de la vitamine D et la croissance ont été mesurés.

L' étude 1 (n=63) a montré que le CMO du CE, le CMO du RL, et la DMO du RL augmentaient respectivement de 143,2%, 116,8% et 31,1% pendant la petite enfance. Le CMO du CE était plus élevé (≥ 4,2%; $p < 0,05$) chez les hommes que chez les femmes dans l'ensemble de l'étude, mais pas après ajustement pour le poids ou la longueur. Le CMO du RL et la DMO du RL ne variaient pas selon le sexe et des courbes de croissance combinées mâle-femelle de la DMO du RL ont été générées. Dans l'étude 2, les nourrissons (n=142) de mères avec un GPG excessif avaient un plus grand ($p<0,05$) CMO du CE (101,40 ± 12,47 g), CMO du CE/kg (25,27±3,29 g/kg), CMO du RL (2,34±0,38 g) et DMO du RL (0,239±0,048 g/cm^2) que ceux avec un GPG inadéquat (CMO CE: 89,41±13,62 g ; CMO du CE/kg: 23,47±1,92 g/kg; CMO du RL: 1,99±0,34 g; DMO du RL: 0,206±0,032 g/cm^2). De plus, les nourrissons de mères avec un GPG excessif avaient un CMO du CE/kg, un CMO du RL, et une DMO du RL plus élevés ($p<0,05$), que ceux avec un GPG adéquat (CMO du CE/kg: 23,93±2,41 g/kg; CMO du RL: 2,11±0,40 g; et DMO du RL: 0,217±0,036 g/cm^2). L'étude 3 (n=139) a montré que le CMO du CE et du RL, la DMO du RL, ainsi que les marqueurs biologiques du métabolisme osseux ne sont pas compromis chez les nouveau-nés avec un taux sérique de 25(OH)D <50 nmol/L par rapport au groupe de référence. De

plus, la masse osseuse et les marqueurs biologiques du métabolisme osseux n'étaient pas différents entre les groupes d'essai.

Dans l'ensemble, ces résultats démontrent que la petite enfance est une période de minéralisation squelettique rapide avec des différences sexuelles minimes. Le GPG maternel est lié à la masse osseuse néonatale, et cela est en partie attribué à la taille du nourrisson. La correction du statut en vitamine D à la naissance a été réussie avec 400 UI/j de supplémentation en vitamine D dans les 3 mois après la naissance, et une dose plus élevée de 1000 UI/j n'a pas conféré d'avantages squelettiques supplémentaires dans la petite enfance. Cela renforce l'AS de 400 UI/j de supplémentation en vitamine D actuellement agréée par les politiques de santé publique en Amérique du Nord.

Statement of support

Study 1 was funded by the Canadian Institutes of Health Research, Nutricia Research Foundation, and the Canada Foundation for Innovation. Studies 2 and 3 were funded by the Canadian Institutes of Health Research and the Canada Foundation for Innovation.

The PhD Candidate received financial support from the Canadian Institutes of Health Research and McGill University (Graduate Excellence Fellowship).

Advancement of scholarly knowledge

This thesis dissertation includes three manuscripts that address research questions relating to vitamin D and bone in infancy. The contribution of each manuscript to the body of knowledge is summarized below.

Study 1 from this dissertation provides reference data for bone mineralization in infancy using previously collected data from a trial conducted at McGill University, Canada (2007-2010). In order to ensure that sample means reported are representative of population means, rigorous statistical methods were used. Reference data for bone mass in infancy are scarce and thus this study may help provide further insight on Dietary Reference Intakes for vitamin D during the first year of life. For infants, the AI of vitamin D is based on 25(OH)D concentrations as a functional biomarker of bone health. The use of liquid chromatography tandem mass spectrometry, the gold standard for assessment of vitamin D metabolites in this study increases our confidence in the values reported. In addition, this study helps improve our understanding of sex differences in bone mass in infancy using longitudinal scan data from 1 to 12 months of age from body sites approved by the International Society for Clinical Densitometry. This study has been accepted for publication in the Journal of Clinical Densitometry.

Studies 2 and 3 are based on data from a vitamin D trial that was conducted at McGill University, Canada (2016-2020). Study 2 is focused on neonatal bone outcomes in relation to two maternal factors: vitamin D status and GWG. To the author's knowledge, the relation of maternal vitamin D status and GWG to neonatal bone outcomes has not been thoroughly explored. The latter is important given that excessive GWG may impact on maternal vitamin D status through vitamin D sequestration. As a greater bone mass was observed in infants born to mothers with excessive GWG compared to inadequate GWG and a greater bone mass relative to body weight

was observed in infants born to mothers with excessive GWG compared to adequate and inadequate GWG; this study provides further data that may be considered in future guidelines on GWG for a healthy pregnancy as well as on factors like pre-gravid body mass index.

Study 3 is based on bone outcomes of the vitamin D trial mentioned above in which a greater dose of vitamin D supplementation (1000 IU/d) was compared to the standard of care (400 IU/d) in infants born serum 25(OH)D concentrations <50 nmol/L. For the ease of wording in this thesis, serum 25(OH)D concentrations <50 nmol/L will be labeled as insufficient although this term was not used by the Institute of Medicine in the Dietary Reference Intakes for vitamin D. The trial compared the effect of the two supplementation doses on growth, bone health and biomarkers of calcium homeostasis and bone metabolism, as well as different vitamin D metabolites. The direct implication of this study is the support of the 400 IU/d supplementation dose of vitamin D in infancy endorsed by different regulatory bodies across the globe from North America to Europe and Australia. This trial contributes to the present body of knowledge by comprehensively addressing a research question relevant to an understudied infant population: those born with serum 25(OH)D <50 nmol/L and using a high-quality double blinded randomized controlled trial design. It also provides valuable data from healthy term appropriate for gestational age infants born vitamin D sufficient including reference data on multiple bone outcomes and biomarkers and vitamin D metabolites that are not commonly available in the literature.

Publications and manuscripts being prepared for submission to peer-reviewed scientific journals

1. **Articles**

- Gharibeh N, Gallo S, Sotunde OF, Vanstone CA, Rodd C, Weiler HA. (2021) Patterns of bone mineral accretion and sex differences in healthy term vitamin D replete and breastfed infants from Montreal, Canada: bone mass reference data. Journal of Clinical Densitometry, S1094-6950(21)00058-5 (Chapter 3).
- Gharibeh N, Razaghi M, Vanstone CA, Wei SQ, McNally D, Rauch F, Jones G, Kaufmann M, Weiler HA. (2021) Maternal vitamin D status and gestational weight gain as correlates of neonatal bone mass in healthy term breastfed young infants from Montreal, Canada. Nutrients, *13*(12), 4189, (Chapter 4).
- Effect of vitamin D supplementation (400 vs 1000 IU/d) on bone health parameters: a double-blinded randomized controlled trial in infants born at elevated risk of insufficient vitamin D status. (Chapter 5).

2. **Abstracts at conferences**

- Gharibeh, N., Gallo, S., Sotunde, O. F., Vanstone, C. A., Rodd, C. J., & Weiler, H. A. (2021). No sex differences in bone mineral accretion from 1 to 12 months of age among a sample of healthy term breastfed infants from Montreal, Canada (2021). Current Developments in Nutrition, 5(2), 749. American Society for Nutrition 2021.
- Impact of 25-hydroxyvitamin D concentration at birth on bone mineral accretion in infants between 0 and 6 months of age. Current Developments in Nutrition, 2(11), 10-11. American Society for Nutrition 2018.

- Rapid rescue of vitamin D levels between 1 and 3 months of age in neonates with low vitamin D levels using a daily supplementation dosage of 1200 IU supports higher bone mineral accretion. FASEB J. 31 (S1), 958. ASPET Annual Meeting at Experimental Biology 2017.
- Rapid rescue of vitamin D levels between 1 and 3 months of age in neonates with low vitamin D levels using a daily supplementation dosage of 1200 IU supports higher bone mineral accretion. 9th Symposium of the Reseau Quebecois en Recherche 2016.

Contribution of authors

Manuscript 1: Dr. Hope A. Weiler and Dr. Celia J. Rodd designed, conducted, and supervised the original trial as well as the secondary analysis of data. Catherine A. Vanstone and Dr. Sina Gallo conducted the study visits and collected the data. Dr. Olusola F. Sotunde helped guide analyses and sample estimates. Nathalie Gharibeh analyzed and interpreted the data, wrote, and revised the manuscript. All authors approved the manuscript for publication.

Manuscript 2: Nathalie Gharibeh, Maryam Razaghi, Catherine A. Vanstone, and Dr. Hope A. Weiler contributed to the data collection. Nathalie Gharibeh analyzed and interpreted the data, and elaborated and revised the manuscript. Dr. Shu Qin Wei, Dr. Dayre MacNally, and Dr. Frank Rauch were the steering committee in terms of recruitment and served as data safety monitors. Dr. Glenville Jones and Dr. Martin Kaufmann were the expert analytical chemists collaborators. Dr. Hope A. Weiler, Dr. Shu Qin Wei, Dr. Dayre MacNally, Dr. Frank Rauch, and Dr. Glenville Jones were involved in the design. Dr. Hope A. Weiler was involved in the conduct, guidance, and supervision of the study, staff, graduate trainees, and analyses. All authors approved the manuscript for publication.

Manuscript 3: Nathalie Gharibeh, Maryam Razaghi, Catherine A. Vanstone, Dr. Olusola F. Sotunde, Laura Glenn, Kristina Mullahoo, Zahra Farahnak and Dr. Hope A. Weiler contributed to the data collection. Nathalie Gharibeh analyzed and interpreted the data, and elaborated and revised the manuscript. Dr. Shu Qin Wei, Dr. Dayre MacNally, and Dr. Frank Rauch were the steering committee in terms of recruitment and served as data safety monitors. Dr. Glenville Jones and Dr. Martin Kaufmann were the expert analytical chemists collaborators. Dr. Hope A. Weiler, Dr. Shu Qin Wei, Dr. Dayre MacNally, Dr. Frank Rauch, and Dr. Glenville Jones were involved in the design. Dr. Ali Khamessan designed the product, performed the external testing, and

randomization scheme. Dr. Hope A. Weiler was involved in the conduct, guidance, and supervision of the study, staff, graduate trainees, and analyses. All authors approved the manuscript for publication.

Acknowledgements

This research work would not have been possible without the contribution and support of many. Foremost, this work could not have been possible without the support and guidance of my supervisor Dr. Hope Weiler. I was very lucky to have had the opportunity to learn from her and work under her supervision. Her work ethics, outstanding expertise, great leadership skills as well as kind support have been an inspiration to me throughout my PhD journey and have made this journey a very memorable one. For all that, I thank her dearly.

I would also like to thank Dr. Sarah Kimmins, my co-supervisor for her support and guidance throughout this journey and for inspiring me to make my presentations more attractive. To Dr. Stephanie Chevalier, thank you for being a wonderful graduate program director and committee member, for offering to help and provide guidance on several occasions and for involving me in different departmental activities. I would also like to acknowledge another committee member, Dr. Grace Marquis, for her contribution and advice throughout.

To all the co-applicants on the vitamin D trial, thank you for your contribution to the study. To Dr. Celia Rodd, thank you for your guidance on the statistical methods for the generation of the growth charts. Dr. Cue thank you for all your help and guidance in statistics and for your prompt replies even on weekends.

To the study team members including Maryam Razaghi, Catherine A. Vanstone, Olusola F. Sotunde, Sherry Agellon, Paula Lavery, Dr. Sina Gallo and all the students who helped with the different research projects; thank you for your contribution to these projects. Maryam, thank you for being a wonderful friend and colleague and for all your help and support. You have made this journey more fun. Catherine, thank you for all of your hard work during study visits and for your advice when needed.

To all the families who participated in this study, this research would not have been possible if it were not for you. Thank you for the time you invested in this.

A big thank you to all my friends who were supportive throughout this journey especially Wissam and Carla. Thank you to family Khoury for making me feel home away from home, for their endless love and support throughout the past few years.

Last but not least, I am forever grateful to my sister, my brother-in-law and parents for always believing in me, for their encouraging words and support all the way across the globe. I dedicate this PhD to the four of you.

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$1,25(OH)_2D$	1,25-dihydroxyvitamin D
$24,25(OH)_2D_3$	24,25-dihydroxyitamin D_3
25(OH)D	25-hydroxyvitamin D
7-DHC	7-dehydrocholesterol
AI	Adequate Intake
AGA	Appropriate weight for gestational age
BAZ	BMI-for-age z-score
BMI	Body mass index
BMC/kg	Bone mineral content per kilogram body weight
BMC	Bone mineral content
BMD	Bone mineral density
CAD	Canadian dollars
CPS	Canadian Paediatric Society
CTX-I	Carboxy-terminal crosslinked telopeptide of type 1 collagen
CLIA	Chemiluminescent immunoassay
CYP	Cytochrome P450 oxidase
DBP	Vitamin D binding protein
DRIs	Dietary Reference Intakes
DXA	Dual-energy x-ray absorptiometry
FGF23	Fibroblast growth factor 23
F	Fitzpatrick skin type scale

GWG	Gestational weight gain
HC	Head circumference
HCAZ	Head-circumference-for-age z-score
ITA°	Individual typology angle
IOM	Institute of Medicine
IGF-I	Insulin-like growth factor I
ISCD	International Society for Clinical Densitometry
IU	International units
iCa	Ionized calcium
LAZ	Length-for-age z-score
LGA	Large for gestational age
LC-MS/MS	Liquid chromatography tandem mass spectrometry
ln	Length
LS	Lumbar spine
NIST	National Institute for Standards and Technology
pQCT	Peripheral quantitative computed tomography
P1NP	Procollagen type 1 N-terminal propeptide
RANKL	Receptor activator of nuclear factor kappa-B ligand
SGA	Small for gestational age
UL	Tolerable upper intake level
UVB	Ultraviolet B
DBP	Vitamin D binding protein
DEQAS	Vitamin D External Quality Assessment Scheme

VDR	Vitamin D receptor
VDREs	Vitamin D response elements
VDSCP	Vitamin D Standardization Certification Program
Weight-for-age z-score	WAZ
Wt	Weight
WB	Whole body
WBLH	Whole body less head

Chapter 1: Introduction

1.1 Background and thesis rationale

Infancy is a period of rapid bone mineral accretion during which bone mass increases by 300% (1). Vitamin D is known for its classic function in bone in addition to the pleiotropic functions in other tissues like the immune system. While vitamin D can be produced endogenously in humans and is obtained from exogenous dietary and supplemental sources; infants rely mainly on vitamin D supplements to meet their vitamin D needs. Direct exposure to the sun is not recommended during the first six months of life. Breast milk, the ideal food for infants, does not provide vitamin D in amounts that meet the needs of infants for growth, and solid foods are not consumed in amounts to help meet the recommendations (2).

Dietary Reference Intakes (DRIs) set by the Institute of Medicine for vitamin D in infancy include an Adequate Intake (AI) of 400 IU/d, tolerable upper intake level of 1000 IU/d during the first six months of life and 1500 IU/d from 7 to 12 months of age (2). Health Canada also endorses the AI of 400 IU/d and recommends that all breastfed infants receive vitamin D supplementation at this amount soon after birth (3). The AI of vitamin D was set according to levels of intake that are consistent with serum 25-hydroxyvitamin D (25(OH)D) concentrations of 40 to 50 nmol/L that are in support of bone health. Serum 25(OH)D is the best indicator of vitamin D status and as per the DRIs serum 25(OH)D concentrations of 50 nmol/L and above are considered sufficient for bone health. Despite public health policies and efforts by regulatory bodies to help infants achieve sufficient vitamin D status, a significant proportion of infants in Canada are vitamin D deficient (25(OH)D <30 nmol/L) or below the cut-point for sufficiency; termed as insufficient (25(OH)D <50 nmol/L) (4-6). Although insufficiency is not a term defined by the Institute of Medicine in classification of vitamin D status, it is used throughout this thesis for ease of wording. In Canada,

we lack national data to inform us about proportions of infants who are vitamin D deficient. However, based on studies from different cities and provinces we know that infants with insufficient vitamin D status range from 24.4% in Quebec City (cord serum 25(OH)D <50 nmol/L) to 28% in Calgary and Edmonton (cord serum 25(OH)D <50 nmol/L) and 36% in Winnipeg (cord serum 25(OH)D <27.5 nmol/L) (4-6).

Vitamin D status has implications for bone health in the short term and long term. Infants born vitamin D deficient present with lower whole body bone mineral content per kilogram body weight (BMC/kg) compared to those born vitamin D sufficient (4-6). In addition, prolonged vitamin D deficiency may lead to vitamin D deficiency rickets, which may result in skeletal deformities and poor skeletal and developmental outcomes in the long run (7). In Canada, annual incidence of vitamin D deficiency rickets for 2002 to 2004 was of 2.9/100 000 with the highest incidence reported to take place between 3 and 18 months of age and in breastfed infants not receiving vitamin D supplementation (7). Therefore, implementing public health policies on vitamin D supplementation is important to help circumvent an easily preventable disease.

To date, there is a lack of reference data on bone mineral accretion in infancy which would be of great value in assisting clinicians in assessment of infantile bone health. In addition, it is not established whether sex differences in bone mass exist since the first year of life. Therefore, a study that may provide reference data on bone mineral accretion in infancy, shed the light on patterns of skeletal mineralization and explore sex differences is needed.

In addition, bone mineral accretion that takes place during the first year of life may be the object of many different factors including the amount of minerals deposited in an infant's skeleton since early infancy and which roots back to the gestational phase. Therefore, a better understanding

of maternal determinants of BMC and bone mineral density (BMD) in the neonatal phase is required given that many of these factors have not been fully explored yet.

Factors that are modifiable such as vitamin D supplementation may also impact mineral accretion throughout infancy. Given the skeletal risks associated with low vitamin D status in infancy, ensuring that sufficient 25(OH)D concentrations are achieved in all infants and especially in those born vitamin D insufficient is important for the development of healthy bones.

1.2 Objectives and hypotheses

The overarching aim of the studies described in this thesis is to provide a better understanding of bone mineralization in infancy and its influencing factors including vitamin D supplementation.

Study 1: (Manuscript 1): The objectives of this report were to provide reference data for bone mineral accretion, investigate patterns of bone mineral accretion and sex differences, and generate age specific WB BMC and LS BMD curves with respective percentiles for the purpose of informing clinical judgement of skeletal growth and mineralization of primarily breastfed, vitamin D sufficient infants.

Hypothesis: BMC is not different between males and females throughout infancy.

Study 2: (Manuscript 2): The objective of this study was to determine whether neonatal bone mass in healthy term, breastfed infants differs according to maternal vitamin D status and GWG.

Hypothesis: Sufficient maternal vitamin D status and healthy gestational weight gain (GWG) are associated with greater BMC/kg compared to insufficient and excessive GWG.

Study 3: (Manuscript 3): The objective was to investigate whether correction of insufficient vitamin D status early in infancy using 1000 IU/d of vitamin D normalizes bone mass across infancy.

Hypothesis: Neonates with insufficient vitamin D status and provided a supplement of 400 IU/d (compared to 1000 IU/d) have lower bone mineral accretion by 3 months without resolution at 12 months of age.

In order to provide a framework for these 3 studies, an overview of the literature on bone mineralization in infancy, vitamin D sources and metabolism in infancy, and its action on bone as well as bone response to different doses of vitamin D supplementation is provided in the next chapter.

Chapter 2: Review of the literature

2.1 Growth in infancy

Infancy (birth to 12 months) is a period of significant growth. According to the World Health Organization standards of growth, infants around the world, if raised in healthy environments, would experience comparable patterns of growth (8). Weight increases remarkably during this period of life; it is doubled by 4-6 months of age and tripled by the end of infancy (9). Body composition is also subject to major changes; total body water percentage is highest at birth (75-85%) and decreases to 61% by the end of infancy (9). Moreover, lean mass and fat percentage increase by 100% and 7 to 12 % by 12 months of age, respectively (10). During infancy, length increases by more than 50% with an average linear growth rate of 2.5 cm/month during the first 6 months of life which then decreases to 1.3 cm/month during the second half of infancy. To accommodate linear growth, tremendous skeletal modelling occurs during the first year of life (11). Overall, by the end of infancy, bone mass triples due to rapid bone mineral accretion required for linear and appositional bone growth (12). Thus, it is of prime importance to provide nutrition consistent with attainment of bone mineral accretion rates given the potential for early life programming of bone health (13). A more in-depth overview of bone tissue composition and its development in the postnatal phases of life is provided in the following section.

2.2 Bone tissue and its development in the postnatal phases

Bone tissue is a major structural connective tissue of the human body (11). Development of bone tissue starts in the embryonic stage along with skeletal patterning. Skeletal mineralization follows and involves a series of enzymatic reactions during which crystals of hydroxyapatite ($(Ca)_{10}(PO_4)_6(OH)_2$) and small amounts of other salts of magnesium, sodium carbonate and citrate are embedded in an extracellular matrix consisting of collagen, proteoglycans, and other non-

collagen proteins (11). Calcium and phosphorous constitute 39.7% and 18.4% of the weight of hydroxyapatite crystals, respectively.

Skeletal mineralization targets mesenchymal connective tissue or cartilage and bone development begins by either one of two processes, respectively: intramembranous ossification or endochondral ossification. Intramembranous ossification involves the mineralization of connective tissue and (11) occurs throughout life in bones of the skull, face, mandible and clavicle. Endochondral ossification involves the use of cartilage as a template on which to lay down mineral matrix and form bones and occurs in bones that involve joints. At the epiphyseal plates of these long bones, cartilage remains allowing bones to grow in length. Chondrocytes proliferate closer to the epiphyseal end of the growth plate and then senesce at its diaphyseal side where invasion by osteoblasts takes place and bone tissue emerges. Ossification of the growth plate continues until puberty. The latter is considered another key period for bone development. The highest bone mineral accretion rate during puberty occurs during Tanner stages III - IV (14, 15) but is comparatively slower than observed in infancy during which a 300% increase in bone mass occurs by the end of the first year of life (12). By age 13-15 and 15-17 years, epiphyseal fusion normally takes place in females and males, respectively, and adult height is attained by 20-21 years of age as the vertebrae complete growth (14, 15).

The process of skeletal mineralization necessitates an adequate supply of energy and nutrients. Nutrients required include certain amino acids such as alanine, arginine, glutamic acid, glycine, and proline (16), in addition to minerals such as calcium, phosphate, magnesium, zinc, copper, manganese, carbonate, and citrate as well as certain vitamins such as vitamin C, D, and K. The roles these nutrients play in bone modelling vary as these nutrients may constitute structural components of bone or may have a function in bone metabolism and/or maintenance of calcium

and phosphate homeostasis (11). Physiological needs, net absorption, balance of calcium and phosphate as well as metabolism are different in infants and children vs. adults (17). For instance, in early infancy, normal ranges of ionized calcium (iCa) are relatively high and they decrease as liver function matures and more calcium becomes bound to albumin (18). In addition, calcium absorption is high especially in breastfed infants given the higher bioavailability (20%) of calcium in breast milk compared to formula milk(19).

Mineralization of bone matrix is also under the control of multiple hormones including parathyroid hormone (PTH), calcitonin, fibroblast growth factor 23 (FGF23), and the active form of vitamin D: calcitriol, which tightly controls phosphorous and calcium homeostasis. A focus on vitamin D, its synthesis and metabolism, recommended levels of intake and mechanism of action on bone follows below as the main topic pertaining to this thesis.

2.3 Vitamin D sources in infancy

Vitamin D is a secosterol molecule also known as calciferol that consists of a cyclopentanoperhydrophenanthrene structure (20). Two main isoforms of vitamin D exist: ergocalciferol (vitamin D_2) and cholecalciferol (vitamin D_3). Difference between the two isoforms is in their structure as vitamin D_2 possesses an additional double bond located between carbons 22 and 23 and an additional methyl group located on carbon 24 in comparison with its mammalian isomer vitamin D_3 (21).

In humans, sources of vitamin D_3 include both endogenous and exogenous sources. Vitamin D_2 can only be obtained exogenously and is produced in mushrooms and yeasts subsequent to exposure to ultraviolet B (UVB) radiation. Vitamin D_3 on the other hand is found naturally in certain foods of animal origin such as cod liver oil (400 IU in 1 teaspoon), salmon, tuna, mackerel

and in higher amounts in wild fish compared to farmed fish (22). Moreover, vitamin D_3 is commonly used to fortify foods in North America including milk, margarine, and orange juice. Although these foods are excellent sources of vitamin D, given that they are consumed in small amounts in infants, they are unlikely to be major contributors to vitamin D intake (23).

Additionally, vitamin D_3 can be synthesized by the mammalian body, including humans, subsequent to UVB radiation exposure (24). However, infants cannot rely on endogenous production given that sun exposure is not recommended prior to 6 months of age (25). Nonetheless, sun exposure may be considered for therapeutic purposes in infancy including the treatment of hyperbilirubinemia (26) and shows promise in support of motor development in infants born to mothers with perinatal depression (27). Although infants may be even better than adults at producing vitamin D due to the relatively more porous layers of the skin and its lower melanin content, other factors such as immaturity of organs involved in vitamin D production (liver and kidneys) are to be considered as well (28).

Therefore, neonatal vitamin D stores depend on three main sources of vitamin D: 1) maternal-fetal transfer of 25-hdroxyvitamin D (25(OH)D) during gestation, 2) maternal-fetal transfer of vitamin D and 25(OH)D via breast milk, and 3) vitamin D from postnatal vitamin D supplements. Serum 25(OH)D is the most abundant vitamin D metabolite in healthy states and is the best biomarker of vitamin D status (2). In order for infants to be born with vitamin D sufficiency, defined by the Institute of Medicine as serum 25(OH)D concentrations of 50 nmol/L and above, the mother's vitamin D status needs to be in the sufficient range (29).

Maternal-fetal transfer of 25(OH)D occurs during gestation via the placenta and endogenous synthesis of vitamin D by the mother supports building of fetal stores (30). This has been supported by evidence generated in rats (31) and sheep (32) and by the fact that cord blood 25(OH)D

concentrations correlate (r=0.57) with maternal serum 25(OH)D concentrations (4). Indeed, cord blood 25(OH)D concentrations have been shown to be equal to or lower than maternal concentrations (up to 20% less) (33, 34).

The placenta is selectively more permeable to 25(OH)D in comparison with calcitriol (1,25-dihydroxyvitamin D ($1,25(OH)_2D$) (35) and placental onset of $1,25(OH)_2D$ production happens relatively early in pregnancy (36). While $1,25(OH)_2D$ is produced by both the fetal and the maternal placental components (36), it is not considered a biomarker of vitamin D status (2). Infant free 25(OH)D and free $1,25(OH)_2D$ concentrations in the fetus nonetheless exceed maternal concentrations and that is due to lower vitamin D binding protein (DBP) and albumin concentrations (35).

After birth and in breastfed infants, maintenance of vitamin D stores depends on infant vitamin D supplements as 1L of breast milk contributes 40 IU of vitamin D (37). Few studies show that maternal supplementation doses as high as 4000 IU/d and 6400 IU/d are required for the breast milk to have a sufficient content of vitamin D and may substitute for the infant's need for direct supplementation (23, 38); evidence in support of this remains scarce and 4000 IU/d is the tolerable upper intake level (UL) for adults. Therefore, direct infant supplementation is preferred as per public health policies.

The IOM's updated Dietary Reference Intakes (2011) defined the Adequate Intake (AI) of vitamin D during infancy as 400 IU/day and the UL as 1000 IU/d during the first 6 months of life and 1500 IU/d from 6-12 months of age (2). As per Health Canada (2015), all exclusively and partially breastfed, healthy, term infants in Canada should be supplemented with 400 IU/day of vitamin D until this amount of vitamin D is secured from other dietary sources (3). The latter is derived from the fact that human milk does not provide vitamin D in amounts consistent with the

AI to support growth and prevent vitamin D deficiency rickets; a condition with peak incidence between 3 and 18 months of age in which the growing bones fail to mineralize well due to severe vitamin D deficiency (7). Accordingly, formulas and other types of milk are fortified with vitamin D (400 IU of vitamin D per 1 L) (23) in order to help infants meet their vitamin D needs. In addition, exclusive breastfeeding is recommended by Health Canada for up to 6 months and to be continued up to 2 years along with alternative feeds (39). This recommendation is also endorsed by the World Health Organization and the Dietary Reference Intakes (2).

Vitamin D supplements for infants marketed in Canada range from 400 IU to 1000 IU per day, are available more commonly in the D_3 isoform and in both water-based and oil-based solutions. National data on the vitamin D status of infants is lacking. According to a study from Quebec City, 1 in 4 infants are vitamin D sufficient. The majority of mothers in Canada in 2011–2012 (89%) breastfed their newborns and 3 out of 4 infants receiving breast milk were supplemented with vitamin D (39). Indeed, infants in the Canadian Paediatric Surveillance study who developed rickets were typically breastfed and not receiving supplements (7).

2.4 Vitamin D production and metabolism

Endogenous production of vitamin D_3 is relevant to maternal-fetal and maternal-infant transfer of vitamin D. It is a thermal process that requires exposure of epidermal cells to UVB radiation (280-320 nm) (40). UVB radiation is one of three different types of ultraviolet radiation emitted by the sun and that reach planet earth (41). During UVB exposure, 7-dehydrocholesterol (7-DHC) which is found in the epidermal layer of the skin and specifically stratum basale and stratum spinosum (42) is cleaved between carbons 9 and 10 and leads to the formation of pre-vitamin D_3 (41). Pre-vitamin D_3 is thermodynamically unstable and rearranges its double bonds over a 3 day

period (43) in a process known as thermo-isomerization and which results in the formation of a thermodynamically stable molecule: vitamin D_3 or cholecalciferol (24). Extended exposure to UVB leads to the transformation of pre-vitamin D_3 to lumisterol and tachysterol and thus prevents vitamin D_3 intoxication due to endogenous synthesis (44).

Melanin pigmentation is produced by melanocytes found in the stratum basale and is moved to keratinocytes found in the stratum spinosum where some of the 7-DHC molecules are located (45). It is highly effective at absorbing UVB and therefore competes with 7-DHC at UVB absorption and limits the conversion of 7-DHC to pre-vitamin D_3 (46). The latter, in part, explains why people of relatively darker skin are more prone to vitamin D deficiency than people of lighter skin color. In the US (2001-2004), a substantially greater proportion of expecting mothers of Fitzpatrick skin type IV to VI were deficient compared to those of Fitzpatrick skin type I-III (47). This could be explained by the fact that more extended sun exposure and of a larger body surface area is required in individuals with Fitzpatrick skin type V compared to skin types I-III to reach specific serum 25(OH)D concentrations (48). The latter has implications for pregnant women of Fitzpatrick skin type IV to VI and consequently to their fetuses. However, the efficacy of vitamin D supplementation may be greater and potentially safer and more consistent than sun exposure at improving serum 25(OH)D concentrations in pregnant women (49).

Other factors that may influence endogenous production of vitamin D include latitude, altitude, season of the year, clothing coverage, use of sun screen creams and time of the day (46). The solar zenith angle which is the angle formed by the sun's light and the vertical is larger at higher latitudes, during winter season and daily prior to 9 am or after 3 pm (50). A larger zenith angle leads to a more pronounced absorption of UVB radiation by the ozone layer, and thus a smaller amount of radiation reaching Earth at higher latitudes, and a less efficient dermal vitamin D_3

production. This explains why vitamin D is not readily synthesized year-round in the northern and southern hemispheres.

In Canada, the time of year that UVB is sufficiently strong to elicit endogenous synthesis of vitamin D extends from the beginning of April until the end of October (51); this is often termed the vitamin D synthesizing period. In addition, at any given latitude, altitude is also a factor with lower altitude hindering vitamin D3 production as a consequence of the longer path length that the sun's light needs to travel in order to reach the surface of the Earth and dermis (46). Another factor that may impact vitamin D bioavailability is obesity. In obese individuals, the process of vitamin D_3 production in the skin may be impaired while its release into the circulation may be affected. Subcutaneous fat stores vitamin D_3 and thus in an obese individual where more fat is available, more of the synthesized vitamin D_3 is sequestered by subcutaneous fat (52). According to a study by Wortsman et al. (52), individuals with BMI $\geq$30 kg/m^2 have a lower bioavailability (within 24 hours) compared to those with BMI $\leq$25 kg/m^2 subsequent to ultraviolet exposure or oral dose of vitamin D.

Once synthesized in the epidermal layer of the skin, vitamin D_3 enters the dermal capillary blood system and given its hydrophobic properties is transported to the liver bound with high affinity to the DBP (53). Other non-predominant forms in which vitamin D circulates in the blood include bound to albumin but with weaker affinity compared to DBP ($x10^{-3}$) (54) and free. By binding to the product of the thermo-isomerization reaction, vitamin D_3 production from pre-vitamin D_3 is reinforced. Ingested vitamin D_2 and D_3 are transported to the liver in chylomicrons after being incorporated into micelles and being absorbed by enterocytes (21). A sequence of hydroxylation reactions: 25-hydroxylation, 1α-hydroxylation, and 24-hydroxylation follows; all

performed by cytochrome P450 oxidases (CYPs) located either in the endoplasmic reticulum (CYP2R1) or in the mitochondria (CYP27A1, CYP27B1, and CYP24A1) (21).

Once in the liver, vitamin D is hydroxylated using alpha-hydroxylases to calcidiol or 25(OH)D. Main alpha-hydroxylases involved in this reaction include CYP27A1 and CYP2R1 with the first acting exclusively on vitamin D_3 and the latter on both vitamin D_2 and vitamin D_3. Other enzymes may also possess alpha-hydroxylase activity and may contribute to the total body 25(OH)D pool; nonetheless CYP2R1 has been shown to be the major contributor (21). A major contributor to $25(OH)D_3$ concentrations in infancy is the C-3α epimer of 25(OH)D or 3-epi-$25(OH)D_3$ which is present in higher amounts in early life (up to 60% of total $25(OH)D_3$ vs. adulthood (17%) (55). Nonetheless the function and mechanisms of production of this molecule remain poorly understood (55) and its concentrations are not included in the interpretation of vitamin D status (2). So far, it has been established (56) that isomerization takes place outside the kidney and that 3-epi-$25(OH)D_3$ may be less efficient than its isomer at exerting physiological functions. It also shows a dose response to vitamin D supplementation (1) in infants, positively associates with $25(OH)D_3$ and its concentrations are highest during the first 3 months of life (57). The latter could be due to a higher relative intake of vitamin D per kg body weight during the first 3 months of life. Evidence from weanling and adult rats also indicates that 3-epi-$25(OH)D_3$ may support bone health (55, 58).

Subsequently within the endocrine system, 1α-hydroxylation takes place in the kidneys and 25(OH)D is hydroxylated to calcitriol by CYP27B1 exclusively (21). CYP27B1 is found to be expressed in a variety of other cells and tissues including proximal renal tubules (59), where megalin, a low density lipoprotein receptor frees 25(OH)D from DBP allowing its conversion to $1,25(OH)_2D$ (60). CYP27B1 is also found in epithelial cells, immune cells, chondrocytes,

osteoblasts and endocrine glands; nonetheless the kidneys remain the main contributor to the circulating concentrations of $1,25(OH)_2D$. Evidence from bovine models points at a suppression of PTH by 3-epi-1, $25(OH)_2D_3$ with potency similar to $1,25(OH)_2D_3$ (61).

In addition to its endocrine action, $1,25(OH)_2D$ has been shown to exert autocrine and paracrine actions in different cell types. For instance, $1,25(OH)_2D$ promotes keratinocyte differentiation through an autocrine action. Its autocrine and paracrine actions have also been described in the immune system in which it is implicated in signal transduction and cell division (62, 63). In bone, autocrine and paracrine actions of vitamin D have been shown to exist wherein $1,25(OH)_2D$ regulates the activity of osteoblasts and is in this case regulated by non-classical regulators like interleukin 1. Nonetheless, in these cells, unlike in the kidney, when concentrations of 25(OH)D drop below 15 nmol/L, CYP27B1 expression may not be increased which leads to reduced amounts of $1,25(OH)_2D$ in the tissues (63).

Tight regulation of renal 1α-hydroxylase is exerted by multiple hormones: PTH, FGF23, insulin-like growth factor I (IGF-I) and $1,25(OH)_2D$ itself. PTH and IGF-I stimulate whereas FGF23 and $1,25(OH)_2D$ inhibit CYP27B1 (21). Disposal of excess 25(OH)D and $1,25(OH)_2D$ is catalyzed by CYP24A1 which adds a hydroxyl group at carbon 24 of either calcidiol or calcitriol. Calcitriol is a preferred substrate of hydroxylation of CYP24A1 in comparison with calcidiol and the product of hydroxylation is the inactive molecule of calcitroic acid. CYP241 is found in all target tissues and is up-regulated by high concentrations of $1,25(OH)_2D$ (21). The half-life of all of these metabolites is affected by DBP and varies according to binding affinities; highest affinity to 25(OH)D and 24,25-dihydroxyvitamin D ($24,25(OH)_2D$) followed by $1,25(OH)_2D$ and vitamin D_2 or D_3, respectively (53). Affinity of DBP to the C-3 epimers is lower (36-46%) (64).

A decrease in serum 25(OH)D concentration below 20 nmol/L is associated with a decrease in serum 24,25$(OH)_2$D and 1,25$(OH)_2$D as 25(OH)D is their substrate (65). It is also associated in older children and adults with higher PTH concentrations (>60 pg/mL) which if sustained negatively impacts bone by stimulating bone resorption (66). When 25(OH)D concentrations decrease to less than 40 nmol/L (67), 1,25$(OH)_2$D concentration starts to increase, but declines eventually when 25(OH)D drops below 20 nmol/L. The former could be explained by activation of 1 α -hydroxylase by PTH (67). However, in infants, it is not clear if and at what cut-point for 25(OH)D leads to increased PTH concentrations. PTH concentrations are usually low especially during the first couple of months of life in breastfed infants receiving vitamin D supplements (18). In addition, the negative relation between iCa and PTH starts to become evident only after the first month of life (68).

In summary, vitamin D can be obtained from endogenous and exogenous sources and are metabolized the same way once converted to 25(OH)D. A better understanding of vitamin D metabolism has been possible given the development of assays that assess vitamin D status and measure the different vitamin D metabolites.

2.5 Vitamin D assays

A wide variety of assays are currently in use for vitamin D measurement including competitive protein-binding assays, enzyme-immunoassays, chemiluminescent immunoassay, radio-immunoassays, high-pressure liquid chromatography and mass spectrometry (69) with the gold standard being liquid chromatography tandem mass spectrometry (LC-MS/MS). Efforts have been drawn lately in order to address between-assay variability and within-assay variation and to standardize 25(OH)D measurement. The Vitamin D Standardization Certification Program

(VDSCP), is a collaborative approach launched in 2010 by the Office of Dietary Supplements of the National Institutes of Health, the Centers for Disease Control and Prevention, the National Institute for Standards and Technology (NIST), the Vitamin D External Quality Assessment Scheme (DEQAS), the International Federation of Clinical Chemistry and Laboratory Medicine, the College of American Pathologists, and the American Association for Clinical Chemistry. The VDSCP aims to promote standardized vitamin D measurements using the "gold standard" reference measurement procedures developed at the NIST and Ghent University and using NIST Standard Reference Materials. The VDSCP reference measurement system also requires the participation in accuracy-based performance testing or External Quality Assessment schemes provided by the College of American Pathologists and DEQAS. Laboratories performing up to the targets set by the DEQAS advisory panel are provided a certificate of proficiency (accuracy <5% and precision <10%). It also specifies the need to measure total 25(OH)D defined as the summation of $25(OH)D_2$ and $25(OH)D_3$ (70).

LC-MS/MS is considered to be the gold standard for measurement of 25(OH)D (71, 72) given its high sensitivity, specificity, and reproducibility at measuring total serum 25(OH)D concentrations in addition to its ability to detect and to measure multiple vitamin D metabolites simultaneously and at high specificity each. Nonetheless LC-MS/MS measurements are costly, require well trained and experienced personnel and quality control and equipment calibration in order to obtain valid results (73). They also have a limited sensitivity to $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$ given their low concentrations (picomolar). Measurements of these metabolites is performed using a separate assay that requires a higher volume and derivatization of these metabolites is needed to increase sensitivity (74). Separation of 3-epi-$25(OH)D_3$ from measurement of metabolites including $1,25(OH)_2D$ and $24,25(OH)_2D$ have not been standardized

as of yet. Despite the advantages of LC-MS/MS that outweigh its limitations, many laboratories rely on immunoassays for various reasons. The latter are less expensive and are time effective if they are automated which is desirable in a clinical setting. However, these assays demonstrate a high cross reactivity to other metabolites (24,25$(OH)_2$D, cholecalciferol), and therefore are not as accurate at measuring total 25(OH)D (71) and can have a lower precision (73).

The majority if not all of these assays are capable of measuring total 25(OH)D concentrations. Total 25(OH)D is the most commonly measured vitamin D metabolite as it reflects vitamin status and is available among immunoassays as well as analytical chemistry assays. Cut-points for vitamin D status as defined by different societies and regulatory bodies are summarized in the following section.

2.6 Vitamin D status in infancy

Serum 25(OH)D is considered to be the best indicator of vitamin D status as it is the most concentrated and most stable vitamin D metabolite with a half-life of 3 weeks in comparison with a range of four to 6 hours in the case of 1,25$(OH)_2$D. In addition, vitamin D toxicity is mainly related to an excess of 25(OH)D rather than an excess of 1,25$(OH)_2$D (75). Physiological levels of vitamin D required for healthy skeletal growth and development during infancy are not clearly defined.

In its 2011 recommendations, the IOM defined four categories of vitamin D status based on serum 25-hydroxyvitamin D (25(OH)D): (i) risk of deficiency: <30 nmol/L, (ii) risk of inadequacy: 30-49 nmol/L, (iii) sufficiency: 50-125 nmol/L, and (iv) level above which there may be reason for concern >125 nmol/L (7). In infants, 40 to 50 nmol/L concentrations were considered to be

desirable. The term vitamin D insufficiency is commonly used in the literature and refers to serum 25(OH)D <50 nmol/L.

The vitamin D status cut-point values and categories suggested by the IOM are supported and used by Health Canada; the 30-49 nmol/L category is not listed per se (76) and the median of 40 nmol/L is used to define adequacy. Similarly, the Canadian Paediatric Society (9) (CPS) defined vitamin D deficiency (2007) as serum 25(OH)D <25 nmol/L and vitamin D insufficiency as 25(OH)D concentrations that extend up to 75 nmol/L. Interestingly, higher cut-points of sufficiency (>75 nmol/L) have been suggested by the CPS (23) and the Endocrine Society (77) for skeletal health **(Table 2.1).** Cut-points vary across nutrition advisory committees/ societies. For instance, in Europe, the Scientific Committee on Food recommends a range of 25 to 100 nmol/L for 25(OH)D in the general population. In the UK, the Scientific Advisory Committee on Nutrition in the UK supports concentrations greater than 20 to 30 nmol/L for extra-skeletal health (78). Nonetheless, there is no evidence for such recommendations in healthy term born infants based on findings from the Gallo et al. (2013) study (1).

In summary, serum 25(OH)D concentrations in support of skeletal health in infancy are based on limited evidence and whether the cut-point for sufficiency may vary throughout infancy as growth decelerates and dietary transitioning takes place remains to be explored. A review of studies reporting on the response of serum 25(OH)D concentrations to different doses of vitamin D supplementation in infants follows.

Table 2.1. Serum 25(OH)D concentrations cut-offs in infancy.

Concentrations (nmol/L)	Status
Institute of Medicine[2]	
<30	Risk of deficiency
30-50	Risk for inadequacy in some but not all; population target is median concentration 40 nmol/L or above
40-50	Desired target for infants corresponding to the Adequate Intake
≥50	Sufficient for most people for bone health
>75	Not consistently associated with health benefits
>125	Potential reason for concern
Canadian Paediatric Society[18]	
<25	Deficient
25-75	Insufficient
75-225	Optimal
>225	Pharmacological (potential adverse effects)
>500	Potentially toxic
Endocrine Society[77]	
<50	Deficiency
50-74.9	Insufficiency
75-250	Sufficiency

2.7 Higher vitamin D supplementation doses and safety

The AI in infancy was set according to levels of intake that are consistent with serum 25(OH)D concentrations in the range of 40 to 50 nmol/L and that are in support of bone health (25(OH)D >50 nmol/L). Nonetheless given that not enough data are available to generate Recommended Dietary Allowance values, and that most studies are conducted in infants with sufficient vitamin D status, additional data on higher levels of supplementation for infants with insufficient vitamin D status is needed. Evidence of the impact of higher doses of vitamin D supplementation than the standard of care (400 IU/d) on the vitamin D status of infants born with low vitamin D concentrations is scarce and unclear. In a meta-analysis by McNally et al. (79), it was shown that relatively higher doses of vitamin D supplementation (1000-4000 IU/d) than the UL are required to raise serum 25(OH)D concentrations of children with vitamin D deficiency. Other studies on infants with vitamin D deficiency (80, 81) showed that 400 IU/d of vitamin D is successful at correcting the deficiency after 3 months and at supporting 25(OH)D concentrations >50 nmol/L.

Safety of higher doses of vitamin D supplementation remains unclear. While a systematic review by Mimouni et al.(82) showed that larger amounts of daily vitamin D beyond the recommended 400 IU daily dose do not result in better health outcomes, they noted such intakes are potentially associated with adverse effects. A study by Vervel et al. (83) showed that a vitamin D supplementation dose of 1000 IU/d during the first 3 months of life is safe and may be advantageous in improving vitamin D status especially in infants at risk of vitamin D deficiency. Safety of doses as high as 1600 IU/d were supported by a study by Holmlund-Suila et al. (2012) (84) in which 1600 IU daily supplementation dose over a period of 3 months was successful at maintaining serum 25(OH)D in all infants above the CPS cut-off for skeletal health (75 nmol/L). Similarly, in a study by Gallo et al. (2013) only a higher dose of 1600 IU/d was able to achieve

serum 25(OH)D concentrations that align with the 75 nmol/L cut-point in 97.5% of infants at 3 months of age while all the other lower doses (400, 800, 1200 IU/d) were able to achieve concentrations of 50 nmol/L at 3 up to 12 months.

Defining doses of supplementation that may help achieve sufficient serum 25(OH)D concentrations in infancy are important to help support bone health during this period of rapid skeletal mineralization. The role of vitamin D in bone is summarized in the following section.

2.8 Physiological actions of vitamin D in relation to bone

a) Calcium homeostasis

The active form of vitamin D ($1,25(OH)_2D$) is a calciotropic hormone that maintains blood calcium homeostasis (2). Other key hormones, organs and tissues involved in calcium homeostasis include PTH and calcitonin from the parathyroid gland, along with the actions of PTH and calcitriol in the intestine, the kidneys and bone (2). In general, in the case of low iCa, the parathyroid gland releases PTH which stimulates the production of $1,25(OH)_2D$ and calcium reabsorption from the renal tubules while limiting phosphate reabsorption. $1,25(OH)_2D$ then acts on the small intestine and kidneys, respectively (2).

At the level of the small intestine, $1,25(OH)_2D$ increases calcium and phosphate absorption (2) but this process takes time. Both the active or transcellular and the inactive or paracellular modes of calcium transport are affected by $1,25(OH)_2D$ (88). Active calcium transport is mainly stimulated in response to $1,25(OH)_2D$, in the proximal intestine but has also been shown to occur in the distal intestine including the ileum and colon (88). Transcellular calcium transport occurs in a three-step process all under the influence of $1,25(OH)_2D$ (88). The first step involves calcium entry into the enterocyte which is stimulated by $1,25(OH)_2D$ via activation of Transient Receptor

Potential Cation Channel Subfamily V Member 6, an epithelial calcium selective channel which is a relatively quick process (88). The second step of the process involves movement of calcium in the enterocytes which is modulated by the calcium binding protein calbindin-D9k (85). Calbindin-D synthesis is increased by $1,25(OH)_2D$ but this process takes time: ~8 hours for peak gene expression to occur (86). The third step of the process involves calcium extrusion from the enterocytes at the basolateral membrane via the intestinal plasma pump: plasma membrane Ca^{2+} ATPase 1b. This calcium pump synthesis has been shown to be stimulated by $1,25(OH)_2D$. The paracellular calcium transport pathway involves tight junctions located within the intercellular spaces and occurs throughout the intestine. Evidence is not clear on how vitamin D regulates the paracellular transport of calcium. Nonetheless studies have shown that two of the most abundant ileal tight junctions known as claudin-2 and 12 may serve as para-cellular calcium channels in the epithelial lining of the intestine and that they are possibly regulated by $1,25(OH)_2D$. The latter may elucidate the mechanism by which vitamin D influences paracellular calcium absorption (87). In infants, calcium absorption is high (30 to 60%) in order to help support skeletal growth and development (88) compared to 25-40% in older children and 15-20% in adults (89) and calcium is also more bioavailable in breast milk compared to formula milk (88).

At the level of the kidney, $1,25(OH)_2D$ enhances calcium reabsorption in the distal tubule luminal membrane, a relatively faster process compared to the response in the intestines. Once calcium enters the renal distal tubule cells, it is sequestered by calbindin-D28k, a vitamin D-dependent calcium binding protein. Binding of calcium to calbindin-D28k reduces concentrations of free cytosolic calcium and therefore promotes calcium diffusion into the cell. Free calcium is then pumped out of the distal tubule cells and into the plasma by ATP-dependent plasma

membrane calcium pumps (85). Nonetheless, it is important to note that an infant's kidneys are yet not fully matured before 6 months of age (90).

Circulating $1,25(OH)_2D$ also acts on bone and stimulates calcium release from a rapidly exchangeable calcium pool to restore normal iCa concentrations in the blood (91, 92). This process helps re-establish normal blood calcium levels quickly. It also leads to remodeling of bone by promoting the production of receptor activator of nuclear factor kappa-B ligand (RANKL) by osteoblasts which activate osteoclasts to resorb bone in order to raise serum calcium, although in a much slower way and in lower amounts (93, 94). Calcitriol is also produced in the growth plate (95) where it was shown to be involved in endochondral ossification. Aside from its classical function in calcium homeostasis, vitamin D also exerts molecular actions on bone.

b) Molecular action of vitamin D on bone

Vitamin D is well known as a nuclear hormone involved in bone growth and remodeling. The genomic mechanism of action of vitamin D on bone involves binding of the active form of vitamin D, $1,25(OH)_2D$, to a high affinity nuclear receptor, the vitamin D receptor (VDR) which belongs to the family of steroid hormone receptors (21, 96) and which is ubiquitously expressed in at least 38 human cells including chondrocytes, osteoblasts, osteocytes, and osteoclasts (97). The ubiquitous expression of VDR renders $1,25(OH)_2D$ a pleiotropic molecule that acts on a variety of cells and tissues (98).

VDR is mainly located in the cytoplasm and shifts to the nucleus upon binding of its ligand, the active form of vitamin D ($1,25(OH)_2D$ or calcitriol) (99). Once calcitriol binds to VDR, the latter heterodimerizes with other nuclear hormone receptors especially retinoid X receptor and translocates to the nucleus (99). The complex then binds to DNA sequences called vitamin D

response elements (VDREs) in the genes which it regulates and results in a gene-specific recruitment of coregulatory complexes (100). Coregulatory complexes include a VDR interacting component and other subunits with enzymatic actions and which are involved in epigenetic regulation mechanisms including methyltransferase, demethyltransferase, acetyltransferase and acetyldetransferase actions and ATPase (100). These enzymatic actions will result changes in gene expression; either activation (+VDRE) or repression of transcription (-VDRE) (101). For instance, upregulation of VDR expression by $1,25(OH)_2D$ leads to transcription of the gene for the enzyme CYP24A1 and increases conversion of $1,25(OH)_2D$ into inactive metabolites while limiting transcription of the gene CYP27B1 which converts 25(OH)D into $1,25(OH)_2D$ (102). VDR is a ligand-activated transcription factor that regulates the expression of genes that mediate its activity (11). VDR expression is upregulated by $1,25(OH)_2D$ (100) while it is downregulated by 25(OH)D (68). The ability of ligand-activated VDR to modulate mRNA production and as such protein synthesis, illustrates the molecular mechanism by which $1,25(OH)_2D$ modulates $1,25(OH)_2D$ targets gene expression through VDR (100).

The involvement of the vitamin D-VDR system in bone metabolism is shown in VDR knockout mice in which bone development is impaired (103). Unless high calcium intake is provided to help prevent skeletal impairments, conditions similar to vitamin D deficiency rickets ensue. In addition, in vitro, higher VDR expression occurs subsequent to treatment of osteogenic cells with active vitamin D (104). Given that VDR expression in the placenta was shown to be associated with infant vitamin D status and femur length; higher VDR expression may be expected to positively impact bone postnatally (105).

Depending on the stage of bone development and type and maturity of bone cell, binding of $1,25(OH)_2D$-VDR complex to VDRE promotes bone formation by inducing the expression of

different proteins and factors that participate in bone formation such as calbindin 9K, osteocalcin and osteopontin, type I collagen, and RANKL in chondrocytes (106, 107) and by promoting growth plate expansion. The bone matrix proteins osteocalcin and osteopontin, are secreted by osteoblasts and help calcium bind to the bone matrix during the bone mineralization process (108); and type I collagen, which is also secreted by osteoblasts forms the primary component of the bone matrix and undergoes calcification to form mature bone (108). In addition, VDR suppresses bone resorption by suppressing transcription of genes that encode for PTH which itself mobilizes calcium from bone (109).

RANKL is an essential factor for osteochondroclast differentiation and cartilaginous tissue resorption and its mRNA expression in chondrocytes is induced by $1,25(OH)_2D$ (93, 107). In vitro, VDR signaling in chondrocytes induces RANKL expression and directly regulates osteoclastogenesis (110). Findings from a mouse model show that in growth plates, osteochondroclasts are formed and RANKL is expressed in nearby hypertrophic chondrocytes. In contrast, in permanent cartilage, RANKL is not expressed and osteochondroclasts are not formed (107). It is therefore plausible that $1,25(OH)_2D$ and VDR may contribute to growth plate development and endochondral bone formation by regulating RANKL gene expression (110, 111).

In addition to the genomic action of calcitriol in bone cells via VDR, vitamin D may also affect bone cells at the molecular level via IGF-1. IGF-1 is a mitogenic hormone mainly produced by the liver that drives fetal growth (112) and regulates infant and child growth (113). It is also produced in smaller amounts in other tissues such as bone. Indeed, deleterious mutations of IGF-1 or of its receptor have been shown to result in intrauterine growth restriction and impaired post-partum linear growth (114). During gestation, IGF-1 concentrations are the highest in the third trimester given the high growth rate characteristic of this gestational period (112) while at birth,

cord IGF-1 concentrations have been shown to be lower in case of intrauterine growth restriction and preterm birth in comparison with term birth highlighting the association between IGF-1 and growth (115). In term infants, serum IGF-1 concentrations peak at 1.5 months of life and then start to decrease and reach a nadir at 8 months of life (116).

IGF-1 has also been shown to influence postnatal bone growth. Both endocrine and autocrine/paracrine actions of IGF-1 exist in the growth plate and perhaps in other sites as well like the periosteum (117). In addition, IGF-1 has been shown to be positively associated with bone-specific alkaline phosphatase (118) and treatment of cell cultures with IGF-1 has been shown to enhance expression of early and late phase osteoblasts (119). Whole body (WB) and lumbar spine (LS) bone mineral content (BMC) have been found to be positively associated with IGF-1 in pre-pubertal girls (120). In a study by Soleman et al. (2007) (121), serum 25(OH)D and IGF-1 concentrations were measured in 46 infants/children up to 3 years of age and diagnosed with rickets, before and after the administration of a single intramuscular dose of vitamin D_3 (300,000 IU). A significant increase in IGF-1 concentrations occurred following supplementation (52.2 ±18.9 ng/mL after supplementation and 26.6 ± 12.8 ng/mL before supplementation) and a significant correlation was found to exist between serum 25(OH)D concentrations and serum IGF-I concentrations prior to and following treatment ($r=0.60$ and $r=0.59$, respectively; $p<0.001$) (121). Linear growth velocity standard deviation scores significantly increased following supplementation (2.76 ± 0.45) in comparison with baseline (0.25 ± 0.18) and were correlated with the increase in IGF-1 and 25(OH)D concentrations ($r=0.33$ and $r=0.31$, respectively; $p<0.01$). A possible mechanistic explanation for the increase in linear growth following vitamin D supplementation could be mediated by IGF-1 (121). The correction of vitamin D status provides the precursor of calcitriol: 25(OH)D. This would allow for conversion of 25(OH)D to $1,25(OH)_2D$

that would in turn normalize IGF-1 levels by increasing insulin-like growth factor binding protein-3 production which maintains a longer half-life of IGF-1 in circulation (122). Indeed, insulin-like growth factor binding protein-3 gene has two VDREs which are induced by $1,25(OH)_2D$ (123). In addition, IGF-1 may also increase $1,25(OH)_2D$ concentrations by upregulating the activity of CYP27B1 (124).

Overall, vitamin D exerts pleiotropic effects in calcium homeostasis and bone growth via a combination of molecular and endocrine actions. In addition to the use of 25(OH)D as a functional indicator of bone health, direct assessment of bone health can be performed using imaging methods such as dual-energy X-ray absorptiometry (DXA). The following section provides an overview of this method and its application in infants.

2.9 Assessment of bone using dual energy x-ray absorptiometry

In infants and children, bone mineral accretion can be measured using various techniques, the most feasible of which is DXA. DXA or bone densitometry is considered the gold standard for assessing bone health and fracture risk in adults (125). This imaging technique measures attenuation of X-ray beams of two different energy levels: high energy and low energy levels. X-rays of low energy level are attenuated by soft tissues only while X-rays of high energy level are attenuated by both soft tissues and bone (126). Therefore, a calculation of the difference between the attenuations resulting from the use of the two different energy levels enables estimation of the amount of BMC present.

Two main surrogate markers are estimated by DXA: BMC (g) and bone mineral density (BMD) (g/cm^2). Bone mineral content is defined as the amount of mineral matter contained in the skeleton (g) while BMD is the amount of mineral matter per square centimeter of the projected

bone area (g/cm^2) (127). Using Hologic DXA scanners and QDR operating system, BMC and BMD are estimated and areal BMD is calculated by dividing BMC by bone area (cm^2). In children and infants, relatively smaller BMC and/or BMD are observed than adult individuals given the smaller size of their bones and accordingly the smaller amount of mineral (126). The use of DXA in infant scanning has been shown to be of high reliability (0.99) for the two main scanning modes: lumbar spine and total body. At the lumbar spine site, both BMC and BMD are recommended to be measured by the International Society for Clinical Densitometry. As for total body measurements, only BMC is recommended to be measured as standardizing infant positioning is difficult and can lead to errors if WB BMD were to be measured. In addition, it is also recommended to look at WB less head BMC in infants given the relatively large BMC of the head (125). Scanning of the hip is not common practice in infants and children as proximal femur development varies greatly among growing individuals (126).

DXA scanners are common worldwide (126) and the duration needed to operate a scan is relatively short (few minutes). In addition, this imaging technique has a high precision and accuracy and low radiation exposure (1–6 μSv) which is equivalent to a 24 hour natural background exposure (cosmic and terrestrial radiations and inhalation and consumption of naturally occurring radionuclides) in Canada (4.9 μSv) (118, 119). One of the limitations of DXA is that it provides an areal 2 dimensional estimate of BMD instead of a 3 dimensional volumetric BMD and therefore it does not account for bone depth and might result in an overestimation and underestimation of BMD in large and small bones, respectively (126). In addition, reference data for bone mineral accretion in infancy have not been well-established and the main limiting error for DXA scanning of infants is motion artefacts (126, 128).

Despite DXA being the overall best method for assessment of bone in infancy, reference data on bone development in infancy are extremely scarce. A study by Gallo et al. (2012) (12), reporting such data in addition to other studies are discussed in the following section.

2.10 DXA and normative data on bone development in infancy

Reference data on bone growth in the first year of life are limited. In a study by Gallo et al. (2012) (12), bone mass of 63 healthy, term infants, with weight appropriate for gestational age was prospectively followed from birth until 12 months of age. Of the recruited infants, 18 were formula fed and 45 were breastfed. Formula fed infants did not receive vitamin D supplementation while those breastfed were provided with 400 IU of vitamin D daily. Cord plasma 25(OH)D was measured using a radioimmunoassay while BMC of the WB, LS (1-4), and femur were measured using DXA. Areal and volumetric BMDs were also calculated and provided by DXA. While limited to a small sample size, results showed that whole body BMC and femur BMC increase faster during the first 6 months of life (123% and 90%, respectively) than during the period from 6-12 months old (34% and 52%, respectively). Spine BMC was shown to increase in a more linear pattern over the first 12 months of life. Spine areal BMD showed a decrease during the first 6 months of life (5%) followed by an increase (21%) during the six months to follow. Volumetric BMD was found to decrease during the first 6 months of life and to stabilize up to 1 year (12). Several other studies measured bone outcomes using DXA in infancy and reported on WB and LS bone outcomes (129-132).

Other studies have also assessed bone mineralization in infancy with a focus on the relation between different doses of vitamin D supplementation and bone outcomes. These studies are presented below.

2.11 Vitamin D supplementation and bone mass in infants

A few older studies have compared the impact of the standard of care of vitamin D supplementation to placebo (133, 134). Supplementation of 400 IU/day was found to positively impact forearm BMC after 3 months of supplementation in healthy, term, breast-fed infants (134) as shown by the significantly lower serum 25(OH)D and distal forearm BMC g/cm for the placebo group (n=9) in comparison with the supplemented group (n=9). In a similarly designed study of shorter duration (9 weeks), (133) the impact of supplementation with 400 IU/day of vitamin D on bone outcomes was compared to placebo. In this study, only exclusively breast-fed infants were recruited and were randomized to receive vitamin D supplements (400 IU/day) or placebo for 9 weeks, then followed until 12 months of age. At 12 months of age, the vitamin D supplemented group was found to have a significantly higher distal forearm BMC g/cm and 25(OH)D concentrations in comparison with the placebo group. Results from these two studies show the positive impact of supplementation with a 400 IU/day of vitamin D on infantile forearm BMC but whether this site is representative of the others including WB is not clear. In addition, a 9-week duration might be too short to observe an effect in bone and thus it is likely that image interpretation may have been impacted by positioning artifacts of the forearm relative to the x-ray beam.

Other studies compared the impact of the standard of care of vitamin D supplementation in infants to higher daily supplementation doses. In 2013, Gallo et al. (1) randomized 132 healthy, term, singleton, breastfed infants with weight appropriate for age to receive 400, 800, 1200, or 1600 IU/d of vitamin D_3 from age of 1 month until the age of 1 year. Higher supplementation doses than 400 IU/d were shown to be capable of achieving the 75 nmol/L cut-off among a larger number

of infants than the 400 IU/d. Nonetheless the impact of different supplementation doses on bone outcomes measured at the level of the WB (BMC) and LS (BMC and BMD) as per the recommendations of the International Society for Clinical Densitometry (125) at 3 months of age and up to 3 years of age did not significantly differ between the doses. This study suggests that achieving 50 nmol/L of 25(OH)D is sufficient to support normal bone growth and mineralization. Similarly, in a study by Holmlund-Suila (2012) (84) in which 113 term newborns with body weight appropriate for gestational age were recruited in a 10 week trial from the age of 2 weeks to 3 months old and were randomized to receive 400, 1200, or 1600 IU/d of vitamin D per day. All infants reached a serum 25(OH)D concentration above 50 nmol/L when compliance was greater than 80%. No significant correlations were found to exist between peripheral quantitative computed tomography (pQCT) measured parameters of tibia and 25(OH)D. A trend towards better stress strain index (p=0.070), larger total bone (p=0.069), and cortical bone area (p=0.053) was noted with higher vitamin D doses when accounting for sex and quality pQCT measurement as covariates p=0.053, respectively). Although the authors mentioned that scans with no movement artifacts or minor ones were included in the analysis and those with major movement artifact were disregarded (25/106); adjusting for the quality of the measurement in the analysis may be questionable. Use of pQCT in younger children and infants in debatable. A summary of these 5 studies on bone mass in infancy in response to vitamin D supplementation is provided in **Table 2.2** and their quality is assessed using the Jadad scale(135). The latter is a scoring tool designed to assess the quality of randomized controlled trials based on the appropriateness of randomization and blinding as well as clear statement of reasons for participants' withdrawal.

In addition to bone imaging techniques bone biomarkers may also provide additional and more dynamic information in assessing bone health. A brief overview of these biomarkers is provided in the following section.

Table 2.2 Vitamin D supplementation and bone mass in infancy.

First author, year	Population characteristics	Doses of vitamin D supplementation, duration of intervention, type of instrument	Bone outcomes measured by treatment group and by age		Jadad scale
	Age at enrolment; n; infant or parent ethnicity, country; type of feeding		**BMC (g)**	**BMD (g/cm^2) or other**	
Greer, 1981[133]	0.75 month; 18; white (infant), UK; breastfed	Placebo (n=9) and 400 IU/day (n=9); 9 weeks; single photon absorptiometry	N/A		2/5
Greer, 1989[132]	0 month; 46; white (mothers and fathers except for two father), UK; breastfed	Placebo (n=22) and 400 IU/day (n=24); 6 months; single photon absorptiometry	Forearm: 1.5 months: - Placebo: 83.8 ± 12.4 - 400 IU/d: 81.2 ± 12.5 3months: - Placebo: 88.7 ± 13.8 - 400 IU/d: 80.6 ± 12.7 6 months: - Placebo: 101.0 ± 17.9 - 400 IU/d: 89.5 ± 12.5		2/5
Holmlund-Suila, 2012[84]	0.5 months; 113; Finland;	400 IU/d (n=38), 1200 IU/d (n=38), 1600 IU/d (n=37); 2.5 months;		BMD, total and trabecular bone: - 400 IU/d: 448 ±13 - 1200 IU/d: 430 ± 12 - 1600 IU/d: 451 ± 12	2/5

	predominantly breastfed by 3 months of age	pQCT (XCT-2000; Stratec, Birkenfeld, Germany)		BMD, cortical bone: - 400 IU/d: 724 ± 8 - 1200 IU/d: 716 ± 7 - 1600 IU/d: 726 ± 7 Area, total and trabecular bone: - 400 IU/d: 72 ± 3 - 1200 IU/d: 77 ± 3 - 1600 IU/d: 81 ± 3 Area, cortical bone: - 400 IU/d: 31 ± 1 - 1200 IU/d: 32 ± 1 - 1600 IU/d: 34 ± 1 Stress and strain index: - 400 IU/d: 48 ± 2 - 1200 IU/d: 48 ± 2 - 1600 IU/d: 54 ± 2	
Gallo, 2013[1]	1 month; 132; 84% white (infants), Canada; predominantly breastfed (88% at 6 months of age)	400 IU/d (n=39), 800 IU/d (n=39), 1200 IU/d (n=38), 1600 IU/d (n=16); 11 months; DXA (Hologic 4500A Discovery, APEX software version 13.2:1, Hologic Inc.)	Whole body: 1 month: - 400 IU/d: 102.37 ± 19.18 - 800 IU/d: 95. 58 ± 17.61 - 1200 IU/d: 102.69 ± 15.04 - 1600 IU/d: 97.22 ± 13.72 3 months: - 400 IU/d: 136.65 ± 18.20 - 800 IU/d:135.42 ± 24.61 - 1200 IU/d:133.02 ± 19.74	Lumbar spine BMD: 1 month: - 400 IU/d: 0.281 ± 0.051 - 800 IU/d:0.256 ± 0.059 - 1200 IU/d:0.264 ± 0.056 - 1600 IU/d: 0.268 ± 0.065 3 months: - 400 IU/d: 0.246 ± 0.046 - 800 IU/d:0.239 ± 0.033 - 1200 IU/d:0.243 ± 0.030	5/5

- 1600 IU/d: 128.71 ± 13.78

6 months:
- 400 IU/d:179.93 ± 28.02
- 800 IU/d:172.55 ± 30.93
- 1200 IU/d:178.26 ± 23.68
- 1600 IU/d: 166.47 ± 27.26

9 months:
- 400 IU/d: 200.60 ± 30.28
- 800 IU/d:200.74 ± 30.08
- 1200 IU/d: 203.27 ± 26.11
- 1600 IU/d: 203.03 ± 31.79

12 months:
- 400 IU/d: 235.93 ± 37.93
- 800 IU/d: 235.97 ± 29.89
- 1200 IU/d: 235.36 ± 29.23
- 1600 IU/d: 243.23 ± 29.82

Lumbar spine:

1 month:
- 400 IU/d: 2.88 ± 0.53
- 800 IU/d: 2.61 ± 0.65
- 1200 IU/d: 2.81 ± 0.63
- 1600 IU/d: 2.75 ± 0.73

3 months:
- 400 IU/d: 2.95 ± 0.70
- 800 IU/d: 2.91 ± 0.60
- 1200 IU/d: 2.95 ± 0.38
- 1600 IU/d: 2.95 ± 0.40

- 1600 IU/d: 0.248 ± 0.036

6 months:
- 400 IU/d: 0.263 ± 0.037
- 800 IU/d:0.258 ± 0.035
- 1200 IU/d: 0.275 ± 0.050
- 1600 IU/d: 0.258 ± 0.035

9 months:
- 400 IU/d: 0.296 ± 0.046
- 800 IU/d:0.292 ± 0.031
- 1200 IU/d: 0.298 ± 0.045
- 1600 IU/d: 0.304 ± 0.047

12 months:
- 400 IU/d: 0.337 ± 0.051
- 800 IU/d:0.320 ± 0.036
- 1200 IU/d: 0.336 ± 0.052
- 1600 IU/d: 0.343 ± 0.037

			6 months: - 400 IU/d: 3.55 ± 0.62 - 800 IU/d:3.51 ± 0.67 - 1200 IU/d: 3.71 ± 0.63 - 1600 IU/d: 3.55 ± 0.53 9 months: - 400 IU/d:4.54 ± 0.95 - 800 IU/d:4.43 ± 0.70 - 1200 IU/d: 4.66 ± 0.97 - 1600 IU/d: 4.61 ± 0.79 12 months: - 400 IU/d:5.68 ± 1.13 - 800 IU/d: 5.36 ± 0.81 - 1200 IU/d:5.62 ± 1.03 - 1600 IU/d: 5.76 ± 0.80		
Ziegler, 2017[217]	2 months; 194; Caucasian, Iowa, USA; breastfed	200 IU/d (n=56), 400 IU/d (n=60), 600 IU/d (n=56), 800 IU/d (n=41); 7 months; DXA (Hologic QDR 4500 Delphi A, Hologic Inc., Bedford, MA)	Whole body: 2 months: - 200 IU/d: 109.6 ± 15.9 - 400 IU/d: 105.4 ± 21.1 - 600 IU/d: 117.4 ± 19.3 - 800 IU/d: 109.4 ± 15.8 5.5 months: - 200 IU/d: 159.4 ± 20.1 - 400 IU/d: 161.9 ± 28.0 - 600 IU/d: 157.6 ± 18.7 - 800 IU/d: 144.7 ± 29.3	BMD: 2 months: - 200 IU/d: 0.2369 ± 0.0210 - 400 IU/d: 0.2274 ± 0.0258 - 600 IU/d: 0.2400 ± 0.0270 - 800 IU/d: 0.2353 ± 0.0204 5.5 months: - 200 IU/d: 0.2645 ± 0.0217 - 400 IU/d: 0.2586 ± 0.0287 - 600 IU/d: 0.2491 ± 0.0289 - 800 IU/d: 0.2477 ± 0.0136	2/5

	7.5 months: - 200 IU/d: 172.6 ± 20.4 - 400 IU/d: 183.2 ± 16.5 - 600 IU/d: 175.8 ± 26.1 - 800 IU/d: 182.1 ± 10.3	7.5 months: - 200 IU/d: 0.2709 ± 0.0196 - 400 IU/d: 0.2847 ± 0.0177 - 600 IU/d: 0.2475 ± 0.0489 - 800 IU/d: 0.2732 ± 0.0180
	9 months: - 200 IU/d: 181.3 - 400 IU/d: 212.3 ± 39.5 - 600 IU/d: 184.3 ± 12.1 - 800 IU/d: 180.2 ± 22.7	9 months: - 200 IU/d: 0.2730 - 400 IU/d: 0.2933 ± 0.0329 - 600 IU/d: 0.2695 ± 0.0064 - 800 IU/d: 0.2755 ± 0.0236

Abbreviations: BMC: bone mineral content, BMD: bone mineral density, WB: whole body, LS: lumbar spine, IU: international units.

2.12 Bone biomarkers

Despite DXA being the gold standard in assessment of bone health, BMC and BMD estimations only provide partial information about bone. More dynamic information about bone can be obtained using bone biomarkers. These are produced during bone modeling and remodelling and therefore considered to be biomarkers of bone metabolism. They include bone formation biomarkers, bone resorption biomarkers, and regulatory biomarkers.

Bone formation biomarkers include osteocalcin, procollagen type 1 N-terminal propeptide (P1NP), procollagen type 1 C-terminal propeptide, total alkaline phosphatase, bone-specific alkaline phosphatase (136). Bone resorption biomarkers include C-telopeptide, osteopontin, carboxy-terminal crosslinked telopeptide of type 1 collagen (CTX-I), amino-terminal crosslinked telopeptide of type 1 collagen, deoxypyridinoline, pyridinoline, hydroxyproline, hydroxylysine, bone sialoprotein, and cathepsin K. Bone remodeling regulators include RANKL, osteoprotegerin, dickkopf-1 (136). The advantage of biomarkers is it may allow for early diagnosis of osteoporosis in adults and of bone modeling in growing infants and children. Concentrations of bone biomarkers in infants are high given the relatively high rate of growth that occurs during early life.

According to the International Osteoporosis Foundation, it is recommended to measure P1NP and CTX-I as biomarkers of bone formation and degradation, respectively (137). P1NP is produced by osteoblasts and fibroblasts and is converted to type 1 collagen which constitutes >90% of bone matrix. Therefore, P1NP in infants and children is considered to be a biomarker of type 1 collagen deposition and of bone formation (138). Common assays used for measurement of P1NP include radio-immunoassays or enzyme-linked immunosorbent assay (136). CTX-I is released from type 1 collagen breakdown and thus is an indicator of bone resorption. Two isomers exist: alpha and beta which are derived from newly formed and old bones, respectively.

Different methods and biomarkers allow the assessment of infantile bone health. The latter is related to numerous factors including maternal ones as well as others inherent to the infant. Studies describing these factors are summarized in the section below.

2.13 Maternal and infant factors and infant bone outcomes

Several maternal and gestational factors are related to offspring bone outcomes. These include maternal supplementation, ethnicity, level of education, family income, 25(OH)D concentrations, pre-gravid body mass index (BMI), gestational weight gain (GWG), age, parity, smoking, and alcohol consumption. Smoking for instance, is known to impair linear growth as well as skeletal growth of the fetus by impairing the absorption of calcium in the mother and the transport of nutrients to the fetus in addition to the toxicity resulting from exposure to the metal cadmium. Indeed, maternal smoking during pregnancy is associated with lower BMC at the lumbar spine adjusted for size and lower femoral neck (139). Alcohol consumption during pregnancy may also result in impaired skeletal growth. In animal models it has been shown to result in shorter bones, reduced bone strength, and delays in ossification. The latter occurs even in the case of uncompromised linear growth indicating a response specific to bone (140).

Other factors including pre-gravid BMI may also impact on skeletal growth. According to a study by Macdonald-Wallis (2010) (141), there was a positive association between maternal pre-gravid BMI and different offspring bone outcomes including total body less head BMC and BMD, spine BMC and BMD. In a study by Monjardino et al. (2018) (142), an association was found to exist between GWG and offspring bone outcomes (BMC and BMD) in mothers with pre-gravid BMI <25 kg/m^2. In mothers with pre-gravid BMI ≥ 25 kg/m^2, there was no relation of GWG to offspring bone outcomes. Evidence on the impact of maternal vitamin D status on offspring bone

outcomes remains controversial although maternal vitamin D status is well known to relate to infantile vitamin D status. One interventional study (143) showed that supplementation of the mother with 1000 IU/d of vitamin D resulted in infants being born during the winter season to have higher BMC than infants to mothers not receiving supplementation. This was not the case for infants born during the summer season. Other observational studies point to an association between maternal 25(OH)D status and offspring bone outcomes. For instance, in a study by Joannou et al. (144), serum 25(OH)D concentrations of the mother were found to relate to fetal femoral size. In addition, in the Southamptom Women's Survey study, a negative relation was found to exist between maternal 25(OH)D concentrations and femoral metaphyseal cross-sectional area and femoral splaying index (145). The relation of maternal 25(OH)D concentrations to neonatal bone outcomes is not clear and further research is required to further clarify this relation. Moreover, maternal level of physical activity has also been shown to relate to offspring bone outcomes.

Infant gestational age and birth weight positively related to BMD and BMC in some studies but not in others. In addition, season of birth may relate to neonatal bone outcomes.

In Korea, infants born during the winter season were found to have lower BMC compared to infants born during the summer season (146). Differences in bone mass of infants have also been noted to vary according to type of feeding. Infants who are breastfed tend to have lower BMC versus formula fed infants (147). Given the lower bioavailability of minerals in formula versus breast milk, this effect could potentially be attributed to a behavioral factor whereby infants are forced to finish their bottle versus those who are breastfed directly. This leads to a higher consumption by those formula fed which then translates into a greater overall intake and a bigger bone mass. Similarly in a study by Butte et al., (2000) (147), weight velocity, total energy, and macronutrient intake was found to be higher in formula fed infants compared to breastfed infants

at 3 and 6 months of age. In addition, BMC was higher in formula-fed infants compared to breastfed infants at 12 months of age. Other infant factors that have been shown to relate to infant bone mass include their vitamin D status. A study by Weiler et al. (6) showed that in newborn infants (15 days of age), vitamin D deficiency relates to a lower WB BMC/kg compared to infants born non-deficient. Whether infants born with vitamin D deficiency would benefit from higher intakes of vitamin D to overcome the effects of fetal exposure to vitamin D deficiency is not known.

2.14 Conclusion

This literature review emphasized the tremendous amount of mineralization that takes place during infancy and the first year of life as being a critical period for bone growth. In addition, it highlighted the classic function of vitamin D in calcium homeostasis and its action on bone including bone modeling. Different knowledge gaps have been identified in this review of the literature and will be addressed in the following three manuscript chapters.

Bridge statement 1

As highlighted in the literature review, infancy is characterized by a rapid rate of mineral deposition in bone whereby by the end of infancy, bone mineral content (BMC) more than triples. The use of densitometry in infancy has been proven to be valid and accurate, and recommendations for dual energy x-ray absorptiometry scans in infants have been issued by the International Society for Clinical Densitometry whereby whole body BMC and lumbar spine BMC and bone mineral density (BMD) were recommended to be measured. Nonetheless, longitudinal studies reporting on bone mineral accretion throughout the length of infancy are scarce and reference data for infant BMC and BMD are still lacking. Such data would help define what normal mineralization patterns in infancy are and would help assist clinicians in assessment of skeletal health of infants when needed. One of the studies available in the literature that provided reference data for bone mass in infancy was conducted using a relatively small sample size especially at 12 months of age and provided growth charts for lumbar spine BMD only. Therefore, in the following chapter, we aimed to generate reference data for bone mineral accretion at the level of the whole body (BMC) and the lumbar spine site (BMD) using a larger sample size (n=63 infants 1 to 12 mo of age). Knowing the implications of vitamin D status to bone health, and given that the Dietary Reference Intakes for vitamin D have been set in accordance to concentrations that support bone health, reference data described in the following chapter were generated from a sample of vitamin D sufficient infants only.

Chapter 3: Manuscript 1

Patterns of bone mineral accretion and sex differences in healthy term vitamin replete and breastfed infants from Montreal, Canada: bone mass reference data

Patterns of bone mineral accretion and sex differences in healthy term vitamin D replete and breastfed infants from Montreal, Canada: bone mass reference data

Journal of Clinical Densitometry, S1094-6950(21)00058-5, 2021.

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3.1 Abstract

Introduction: Infancy is a period of rapid bone growth and mineral accretion; nonetheless, reference data remain scarce for this age group. The purpose of this report is to generate reference data for bone mass in breastfed vitamin D replete infants and investigate patterns of bone mineral accretion and sex differences.

Methodology: This is a secondary analysis from a double-blinded randomized controlled trial (NCT00381914). Healthy term breastfed (exclusively or mixed) infants were randomized to different doses of oral vitamin D supplementation (400-1600 IU/d) and followed prospectively from 1 to 12 months. Plasma 25-hydroxyvitamin D (LC-MS/MS), bone mineral content (BMC; whole body (WB) and lumbar spine (LS)) and bone mineral density (BMD; LS) were measured at 1, 3, 6, 9, and 12 months by dual-energy x-ray absorptiometry (Hologic Discovery 4500A) with no effect of supplementation on bone outcomes. For the purpose of this analysis, 63 infants with adequate plasma 25-hydroxyvitamin D ≥ 50 nmol/L at baseline, were included. Differences over time and between sexes were tested using mixed model repeated measures ANOVA.

Results: Infants (31 males, 32 females) were 39.5 ± 1.1 wk gestational age at birth and appropriate for gestational age. WB BMC, LS BMC, and LS BMD increased by 143.2%, 116.8%, and 31.1%, respectively, across infancy. WB BMC was higher (4.2 - 9.4%; p=0.03) in males than in females across the study. After adjusting WB BMC for weight, length or head BMC, sex differences were not evident. LS BMC and LS BMD did not vary by sex. LS BMD growth charts for both sexes combined, were generated using LMS chartmaker.

Conclusions: WB BMC more than doubles during the first year of life confirming the importance of skeletal growth and the need for age-specific reference data in infancy. Sex differences in BMC, if any, are mostly driven by differences in body size.

3.2. Introduction

In infantile clinical conditions where bone health is a concern such as preterm birth, fractures, rickets or the investigation of non-accidental trauma, the availability of reference bone mineral accretion data is critical (148, 149). Such data would also provide us with a better understanding of bone accrual in the first year of life, a period of rapid bone growth (150), and of influencing factors including nutrition.

Dual-energy x-ray absorptiometry (DXA), the gold standard for the assessment of bone health, is currently being used in the infant population given its non-invasiveness, minimal radiation dose, and proven reliability despite inconsistencies in infant positioning (132). A high quality DXA scan requires a skilled operator to keep the infant still throughout scan acquisition (151). Otherwise, if motion artifacts are detected, they must be removed from scans when feasible (125) using advanced analysis techniques (152, 153). Bone outcomes of interest in infants, as recommended recently (2013) by the International Society for Clinical Densitometry (ISCD) include bone mineral content (BMC, g) of the whole body (WB) and the lumbar spine (LS) as well as bone mineral density (BMD, g/cm^2) of the LS (125). The ISCD advises against measurements of BMD of the WB due to difficulty in standardizing positioning (154). Reference BMC and BMD data during early life are particularly scarce in breastfed, vitamin D replete infants, using more modern DXA technology; and overall given that existing studies which are mainly of cross-sectional nature do not represent longitudinal skeletal growth (154).

Sex differences in skeletal growth in early life have been showcased across different studies (155-157). They are also reinforced by the fact that growth charts for linear growth, which reflects skeletal growth, are sex-specific (158). In addition, while current evidence as to whether differences in skeletal growth across ethnicities exist, remains conflicting (131, 159); it is accepted

that, in conditions of optimal nutrition, ethnic and racial differences in linear growth are minimal (158).

The objectives of this report are to provide reference data for bone mineral accretion, investigate patterns of bone mineral accretion and sex differences, and generate age specific WB BMC and LS BMD curves with respective percentiles for the purpose of informing clinical judgement of skeletal growth and mineralization of primarily breastfed, vitamin D sufficient infants.

3.3. Methods

Sample

This is a secondary analysis of data from a double-blind randomized controlled trial (NCT00381914), in which 132 healthy term infants (26-52 days) were randomized to one of four doses of oral vitamin D_3 supplementation (400 IU/d (n=39), 800 IU/d (n=39), 1200 IU/d (n=38), and 1600 IU/d (n=16)) (1). Infants were recruited from pediatric clinics and the Lakeshore General Hospital in Greater Montreal, Canada (2007-2010). All infants were healthy, term, singleton, breastfed (exclusively or mixed at baseline) and of appropriate weight for gestational age, born to mothers without malabsorptive syndromes, chronic alcohol consumption, gestational diabetes, or high blood pressure during pregnancy. Sociodemographic data including mother's self-identified ethnicity according to the Canadian Census criteria, education, and family income were collected. Infants on the 1600 IU arm had plasma 25-hdyroxyvitamin D (25(OH)D) concentrations ≥250 nmol/L by 3 months of age and were therefore switched to the standard of care (400 IU/d

) at 6-9 months of age. The 1600 IU/d treatment was consequently discontinued and the trial registry updated. Infants were followed prospectively throughout the first year of life at 1 (26-52

days), 3 (89-107 days), 6 (164-200 days), 9 (270-312 days), and 12 months (356-381 days) of age (1).

For the purpose of this secondary analysis, we included infants for whom: 1) bone outcomes of interest were successfully measured at 2 or more timepoints and 2) baseline plasma 25(OH)D concentrations were assessed using liquid chromatography tandem mass spectrometry (LC-MS/MS) and were ≥50 nmol/L, as per the Institute of Medicine (IOM)'s cut-point in support of bone health (160) (**Fig. 3.1**). A total of 63 infants met the inclusion criteria and were included in the analysis. Infants in the studied sample received varying doses of vitamin D supplementation in the primary study (1). The lack of dose response to supplementation reported in bone outcomes (WB BMC, LS BMC, and LS BMD) (1) corroborates the inclusion of infants from different intervention groups (1) for the purpose of generating growth charts for bone mass in infancy.

Ethical approval

Ethical approval for the present analysis was obtained from the Institutional Review Board of McGill University (A06-M-71-13A) and Health Canada (REB 2019-038H).

Plasma 25(OH)D

Plasma 25(OH)D was measured in capillary blood samples collected from heels or finger tips. Plasma 25(OH)D was measured using LC-MS/MS. The intra-assay coefficient of variation was less than 15% and the laboratory participated in the Vitamin D External Quality Assessment Scheme and obtained a certificate of proficiency. Details of the methodology have been published (1).

Anthropometry and diet

Infant length, nude weight, and head circumference were measured using a length board (O'Learly Length Board, Ellard Instrumentation Ltd), an infant scale (model SB 32000, Mettler-

Toledo Inc), and a non-stretchable tape, respectively, at each study visit. Respective z-scores (weight-for-age, length-for-age, and head-circumference-for-age z-scores) were calculated according to the World Health Organization growth standards (158). Information on whether infants were receiving mother's milk (any type: exclusive or mixed with alternative feeds) or not was extracted from the general health survey at each study visit and verified by 3-d diet records completed by the parents after each visit. In addition, parents also completed a milestone record starting at 6 months of age.

Bone measurements

Bone mineral content (BMC; WB and LS vertebrae 1 to 4) and BMD (LS vertebrae 1 to 4) were measured at each study visit using DXA (Hologic 4500A Discovery, APEX software version 13.2:1, Hologic Inc.) in array mode with a coefficient of variation of 1% for BMC and 0.3% for BMD based on the Hologic spine phantom (No.14774). Infant WB software and anterior posterior spine software were used, respectively, to obtain WB and LS scans. BMC of the WB and WB less head (WB LH) were both considered in the current analysis. Manual bone edge detection was applied to define regions of interest in LS site (vertebrae 1 to 4) and to allow for maximum accuracy. Infants were rocked to sleep prior to scanning in order to minimize motion artifacts and were not sedated.

Statistical analysis

Data are reported as mean ± SD (continuous variables) or percentages (categorical variables). Differences over time and between sexes in anthropometry (weight, length, head circumference, and respective z-scores), WB BMC, WB BMC/weight (WB BMC/wt), WB BMC/length (WB BMC/ln), WB BMC accretion rate, % increase WB BMC, WBLH BMC, % increase WBLH BMC, LS BMC, LS BMC accretion rate, %increase LS BMC, LS BMD, and % increase LS BMD were

tested over time using a linear mixed effects model. The variables tested as fixed effects included sex, time, sex*time, type of feeding (breastfed or not receiving any breast milk), type of feeding*time, family's yearly income (<75,000 CAD or ≥75,000 CAD), mother's educational level (high school, or vocational and/or apprenticeship, or college and/or university) and mother's self-reported ethnicity (white or non-white) and the random effect of infant ID. The same mixed models were used to test for differences over time for both sexes combined. Tukey's and Bonferroni tests were used for post hoc adjustments in case of parametric and non-parametric data, respectively. Analysis was conducted using SAS University Edition (SAS Institute Inc., Cary, N.C.) and statistical significance was set at $p<0.05$.

LMS chartmaker software light version (Medical Research Council, UK), was used for the purpose of generating WB BMC and LS BMD growth charts. Smoothed LMS centile curves (3^{rd}, 10^{th}, 25^{th}, 50^{th}, 75^{th}, 90^{th}, 97^{th}) were fitted to 1000 bootstrap replicates drawn from the original sample data using bootstrap resampling with replacement and 99% confidence intervals were calculated therefrom. Degrees of freedom for the Box-Cox transformation (L), the median (M), and the skewness (S) were chosen in accordance with the smallest model deviance and type of age scale was chosen according to the shape of the M curve. Best fit of the curves was verified graphically.

3.4 Results

Infants were term, appropriate for gestational age, with a similar distribution by sex (32 females and 31 males). A majority of the mothers were self-reported white (90.5%), held a college/university degree (95.2%), had parity >1 (61.9%) and a family income ≥75,000 CAD (58.7%) (**Table 3.1**). According to the general health survey data, 100%, 98.4%, 94.8%, 67.3%,

and 36.5% of infants were receiving mother's milk (any type: exclusive or mixed with alternative feeds) at 1, 3, 6, 9, and 12 months of age, respectively. 95.2%, 91.4%, 89.7%, 81.8%, and 75.0% of diet records were returned at each of 1, 3, 6, 9, and 12 months of age, respectively. Proportions of infants exclusively breastfed, formula-fed, or on mixed feeds at each timepoint, based on food records, are listed in **table 3.2**. Based on milestone records, medians (IQR) of the average age of introduction of formula, cow's milk, cereals, vegetables, fruits, and meat and alternatives were: 3.3 (1.1 - 6.4), 10.7 (9.2 - 11.3), 6.5 (6.1 - 7.0), 6.7 (6.2 - 7.1), 7.3 (6.7 - 7.5), and 7.8 (7.0 - 8.1) months, respectively.

Z-scores for infant weight, length, and head circumference data (weight-for-age z-score, length-for-age z-score, and head-circumference-for age z-score) were within expected ranges (Tables 3.1 and **3.3**). WB BMC increased by 143.2% between 1 and 12 months with a greater increase from 1 to 6 months (77.7%) vs 6 to 12 months (33.9%) ($p<0.0001$). LS BMC showed a smaller increase during the first 6 months of life (35.4%) vs 6 to 12 months of age (64.4%) with an overall increase of 116.8 % during infancy ($p<0.0002$). LS BMD increased by 2.1% during the first 6 months of life and then increased by 31.7% between 6 and 12 months of age with an overall 31.1% increase across infancy (**Supplementary Table S3.1**). There were no differences between male and female infants in mineral accretion rates of the WB and at the LS (**Table 3.4**). LS BMC and LS BMD did not vary by sex and LS BMD growth curves (**Figure 3.2**) for both sexes combined were generated. Degrees of freedom for the L, M, and S parameters were 1, 4, and 2, respectively, and a transformed age scale was chosen given the corresponding non-monotonic M curve. Modeled LMS parameters and selected percentiles are shown in **Supplementary Table S3.2**. As for WB BMC, males had consistently higher WB BMC than females (4.2% - 9.4%; $p=0.03$) across the study. Therefore, WB BMC growth curves for each sex separately were

considered. Due to the relatively small sample size of the female and male groups, corresponding centile means were not found to be good estimates of the population means as they did not fall within the 99% confidence intervals generated from the 1000 bootstrap replicates. Thus, generation of reference WB BMC growth curves could not be made with confidence. No differences between the sexes were observed in any of the adjusted models including WBLH BMC, WB BMC/wt, and WB BMC/ln at any of the timepoints studied.

From 6 to 12 months of age, WB BMC accretion rate was consistently lower (p=0.03) in breastfed infants (either exclusively or mixed with alternative feeds) compared to infants not receiving any breast milk (Table 3.5). WB BMC (p=0.07) and WB BMC/ln (p=0.07) did not vary by type of feeding. Mother's education was found to be a significant predictor of WB BMC/wt (college/university: 22.2 ± 2.26 g/kg, vocational/apprenticeship: 20.07 ± 1.57 g/kg; p=0.02). Other sociodemographic factors were not found to predict bone outcomes.

3.5 Discussion

Findings of this analysis confirm that infancy is a period of rapid bone mineral accretion. From 1 to 6 mo of age, BMC increased by 35.4% at the level of the LS vs 77.7% at the level of the WB; likely reflecting a slower crown rump rate of linear growth vs limbs in early infancy. Indeed throughout infancy, limbs are subject to an accelerated growth rate in comparison with the trunk as they are relatively underdeveloped at birth (161). WB BMC increased rapidly from 1 to 6 months of age (77.7%) and then continued to increase albeit at a slower rate during the second half of infancy (33.9%). This is in line with previously published findings (12) on a sample of term infants of appropriate weight for gestational age (n=52) where WB BMC increased by 123% and 34% during the first 6 months of life and from 6 to 12 months of age, respectively. While our study

was targeted to ~ 1 month old infants, the study by Gallo et al. (12) was targeted to newborns, which may explain the bigger percent increase in WB BMC reported by Gallo et al. (12) during the first half of infancy. Looking at the LS data, we could see that LS BMD decreased from 1 to 3 months of age followed by an increase thereafter to 12 months of age. The latter could be attributed to a faster rate of bone growth compared to bone mineralization (12) during the first 3 months of life. A similar pattern was reported previously by Gallo et al. (12); nonetheless 3 months data were not collected in this study (12) and 6 months of age may have appeared to be the turnover point for this reason. To the best of our knowledge, no other studies have explored LS BMD longitudinally and at every 3 months throughout the first 12 months of life.

Sex differences in BMC during infancy remain controversial. According to our data, WB BMC was found to be higher in males vs females throughout the first year of life. Our data support previous findings (17-19) as sex differences in WB BMC in our current study are small relative to differences over time and are most likely due to differences in head BMC and size, given that after adjusting for head BMC, weight or length, differences disappear. As for LS, the current paper shows no sex differences in LS BMC or LS BMD. Indeed, in a study by Kalkwarf et al. (2013) (17), sex differences in LS BMC, were found to be driven by weight and size emphasizing the fact that sex differences in bone mineral accretion during the first year of life are mostly driven by infant size and potentially growth rates (131).

Factors that may impact on skeletal growth throughout the first year of life include type of feeding and vitamin D status (17, 18). In this secondary analysis, only infants with baseline plasma 25(OH)D concentrations ≥50 nmol/L were included, as per the IOM's cut-point in support of bone health and given that these levels meet the needs of at least 97.5% of the population and thus are more likely to be reflective of a good overall nutritional status. In addition, although no differences

in WB BMC, LS BMC, and LS BMD were found amongst the different vitamin D supplementation groups in the trial, the response to supplementation (i.e. accretion rates) may have varied according to the baseline vitamin D status of the infants. For all these reasons, only infants with sufficient vitamin D status at baseline were considered for the purpose of generating reference bone growth data for infancy.

Findings from our study showed that WB BMC accretion rate was consistently higher in infants not receiving any breast milk compared to breastfed infants (exclusive or mixed) over the period of 6 to 12 mo. Nonetheless, WB BMC ($p=0.07$) and WB BMC/ln ($p=0.07$) were not different across the study between these 2 groups; suggesting that mineralization is not different in breastfed infants compared to infants not receiving any breast milk. Previously Butte et al. (147) observed differences in WB BMC at 12 months of age in formula-fed infants compared to breastfed (exclusively from birth to 4 months of age) infants. These differences were however small and transient. At 6 months, WB BMC or WB BMC/wt values from our study were relatively similar to those reported in the Gallo study (WB BMC: 169.48 ± 29.01 g; WB BMC/wt: 21.41 ± 1.89 g/kg) (16) although only 71.4% of the infants were breastfed from birth in the Gallo study (12). A plausible explanation for this is the better overall vitamin D status of infants in our study (100% sufficient) vs that in the Gallo study (30.2% of the infants being vitamin D deficient) (12). No effect of type of breastfeeding was detected on LS BMC and respective accretion rate or LS BMD. Nonetheless, LS BMD z-scores have previously been shown to be negatively associated with breastfeeding duration and to be lower in infants who received breast milk compared to those who never did (131).

Currently, assessment of growth in infancy mainly relies on anthropometric measurements and corresponding World Health Organization growth charts (162) generated using the same LMS

chartmaker as used in our study. Unfortunately, anthropometric indicators do not inform on bone health (152) and the generation of reference bone growth data in this age group is needed to help identify infants at higher risk of fracture or impaired accretion. Today, the proven validity (152) and reliability (132) of DXA use in infants, in addition to the fast scan acquisition and high resolution of the newer generation of fan beam DXA technology (163), made the use of densitometry in infants possible despite the challenges imposed by the relatively small bone size and low BMD at this age (131, 164). Consistency in image acquisition (i.e. positioning of infants) and analysis remain key factors in the generation of meaningful data in small subjects (153).

This study has extended our knowledge on patterns of skeletal mineralization in early life. One of the main strengths of our data is that it consists of longitudinal data collected on a sample of infants who were, for the majority, breastfed until 3 and 6 months of age. Whether these findings apply to formula fed infants is not clear. Moreover, infant skin color was not measured and maternal self-reported ethnicity was limited and predominantly white. In consequence, not much can be inferred from our data concerning potential differences in skeletal growth in infancy across different ethnicities. DXA measurements were obtained using a Hologic fan beam DXA machine (Discovery 4500A), which belongs to the new generation of DXA machines. Despite previously proven validity of Hologic fan-beam devices for measurement of BMC in small animals, a variability of DXA measurements across different X-ray sources and different manufacturers exists today as each has a different algorithm, bone detection software, and a unique calibration specific to the manufacturer standards (24). The latter makes comparing DXA measurements across studies difficult. This limitation applies to the field in general and emphasizes the need for the generation of reference data specific to the type of DXA manufacturer.

3.6 Conclusion

This paper generated reference data for bone mass accretion in breastfed vitamin D replete infants, shed the light on the infantile patterns of skeletal growth, and showed that sex differences in BMC in infancy are mainly driven by size. By generating LS BMD growth curves, physicians will be able to refer to reference bone values and have better informed judgement of bone health of their infantile patient population. Efforts should now be allocated to the utilization of reference data for bone mineral accretion in infancy.

3.7 Acknowledgments

This study was supported by the Canadian Institutes of Health Research, Nutricia Research Foundation, and the Canada Foundation for Innovation.

3.8 Authors' contributions

NG analyzed and interpreted the data, and elaborated and revised the manuscript. OFS helped guide analyses and sample estimates. CAV and SG carried out the data collection process of the original trial (NCT00381914). HAW and CJR were involved in the design, conduct, guidance, and supervision of the original trial and secondary analyses. All authors have approved the manuscript for publication.

Table 3.1. Infant and maternal characteristics

Infant characteristics at birth (n=63)	
Male, n (%)	31 (49.2)
Synthesizing period[a], n (%)	35 (55.6)
Gestational age (wk)	39.5 ± 1.1
Weight (kg)	3.5 ± 0.5
WAZ	0.40 ± 0.97
HCAZ	0.31 ± 1.29
Maternal characteristics (n=63)	
Age (y)	32.7 ± 3.9
Parity >1, n (%)	39 (61.9)
Education: college/university, n (%)	60 (95.2)
Family income ≥75,000 CAD, n (%)	37 (58.7)
White (self-reported), n (%)[b]	57 (90.5)

Data are reported as mean ± SD or n (%).
[a] Vitamin D - synthesizing period starts on April 1st and ends on October 31st.
[b] The remaining 9.5% of the maternal population included the following self-reported ethnicities: Black, Hispanic, First Nation, Asian, Hawaiian/Pacific Islander, and others.
Abbr: WAZ: weight-for-age z-score and HCAZ: head-circumference-for-age z-score.

Table 3.2. Type of infant feeding

	1 mo (n=63)	3 mo (n=63)	6 mo (n=58)	9 mo (n=55)	12 mo (n=52)
Breast milk exclusively	59/60 (98.3)	45/53 (84.9)	3/52 (5.8)	0/45 (0)	0/39 (0)
Breast milk mixed and formula	1/60 (1.7)	6/53 (11.3)	0/52 (0)	0/45 (0)	0/39 (0)
Breast milk and solids	0/60 (0)	0/53 (0)	30/52 (57.7)	10/45 (22.2)	1/39 (2.6)
Breast milk, formula, and solids	0/60 (0)	1/53 (1.9)	13/52 (25)	15/45 (33.3)	1/39 (2.6)
Breast milk, cow's milk, and solids	0/60 (0)	0/53 (0)	0/52 (0)	3/45 (6.7)	4/39 (10.3)
Formula only	0/60 (0)	1/53 (1.9)	0/52 (0)	0/45 (0)	0/39 (0)
Formula and solids	0/60 (0)	0/53 (0)	5/52 (9.6)	16/45 (35.6)	13/39 (33.3)
Formula, cow's milk, and solids	0/60 (0)	0/53 (0)	0/52 (0)	0/45 (0)	7/39 (17.9)
Cow's milk and solids	0/60 (0)	0/53 (0)	0/52 (0)	1/45 (2.2)	13/39 (33.3)
Solid foods only	0/60 (0)	0/53 (0)	1/52 (1.9)	0/45 (0)	0/39 (0)

Data are reported as proportions of records returned (%).

Table 3.3 Anthropometry reported for males and females

Variable	Sex	1 mo	3 mo	6 mo	9 mo	12 mo
Age (mo)	Female	1.11 ± 0.16 (n=32)	3.12 ± 0.15 (n=32)	6.07 ± 0.21 (n=30)	9.14 ± 0.27 (n=28)	12.03 ± 0.21 (n=26)
	Male	1.15 ± 0.20 (n=31)	3.10 ± 0.17 (n=31)	6.06 ± 0.11 (n=28)	9.08 ± 0.13 (n=27)	11.98 ± 0.17 (n=26)
Weight (kg)	Female*	4.4 ± 0.5[a] (n=32)	5.9 ± 0.6[b] (n=32)	7.4 ± 0.6[c] (n=30)	8.5 ± 0.8[d] (n=28)	9.4 ± 1.0[e] (n=26)
	Male	4.8 ± 0.7[a] (n=31)	6.4 ± 0.8[b] (n=31)	8.0 ± 1.0[c] (n=28)	9.2 ± 1.2[d] (n=27)	10.1 ± 1.3[e] (n=26)
WAZ	Female	0.05 ± 0.91[a] (n=32)	-0.02 ± 0.74[b] (n=32)	0.04 ± 0.71[c] (n=30)	0.22 ± 0.76[d] (n=28)	0.30 ± 0.83[e] (n=26)
	Male	0.13 ± 0.95[a] (n=31)	-0.07 ± 1.07[b] (n=31)	0.02 ± 1.17[c] (n=28)	0.17 ± 1.19[d] (n=27)	0.31 ± 1.21[e] (n=26)
Weight velocity (kg/mo)	Female*	N/A	0.782 ± 0.180[a] (n=32)	0.500 ± 0.317[b] (n=30)	0.358 ± 0.351[c] (n=28)	0.259 ± 0.405[d] (n=26)
	Male	N/A	0.849 ± 0.219[a] (n=31)	0.549 ± 0.476[b] (n=28)	0.373 ± 0.116[c] (n=27)	0.333 ± 0.550[d] (n=26)
Length (cm)	Female	54.0 ± 2.0[a] (n=32)	60.6 ± 1.7[b] (n=32)	66.2 ± 2.2[c] (n=30)	70.9 ± 2.1[d] (n=28)	75.0 ± 2.3[e] (n=26)
	Male	55.3 ± 2.6[a] (n=31)	61.7 ± 2.3[b] (n=31)	67.4 ± 2.6[c] (n=28)	71.8 ± 2.8[d] (n=27)	75.9 ± 3.2[e] (n=26)

LAZ	Female	-0.06 ± 1.03 (n=32)	0.27 ± 0.76 (n=32)	0.17 ± 0.97 (n=30)	0.25 ± 0.86 (n=28)	0.39 ± 0.85 (n=26)
	Male	1.00 ± 1.21 (n=31)	0.02 ± 1.05 (n=31)	-0.17 ± 1.21 (n=28)	-0.12 ± 1.24 (n=27)	0.07 ± 1.32 (n=26)
Linear velocity (cm/mo)	Female	N/A	3.3 ± 0.6 [a] (n=32)	1.9 ± 0.4[b] (n=30)	1.5 ± 0.4[c] (n=28)	1.4 ± 0.5[c] (n=26)
	Male	N/A	3.3 ± 0.5 [a] (n=31)	1.9 ± 0.5[b] (n=28)	1.5 ± 0.3[c] (n=27)	1.4 ± 0.5[c] (n=26)
HC (cm)	Female*	37.3 ± 1.2[a] (n=32)	40.4 ± 1.0[b] (n=32)	43.2 ± 1.0[c] (n=30)	44.8 ± 1.1[d] (n=28)	45.7 ± 1.2[e] (n=26)
	Male	38.4 ± 1.3[a] (n=31)	41.3 ± 1.3[b] (n=31)	44.3 ± 1.2[c] (n=28)	45.9 ± 1.5[d] (n=27)	47.0 ± 1.5[e] (n=26)
HCAZ	Female	0.46 ± 0.97 (n=32)	0.56 ± 0.76 (n=32)	0.70 ± 0.82 (n=30)	0.66 ± 0.85 (n=28)	0.62 ± 0.88 (n=26)
	Male	0.74 ± 1.03 (n=31)	0.59 ± 1.04 (n=31)	0.75 ± 1.03 (n=28)	0.761± 1.17 (n=27)	0.71 ± 1.20 (n=26)
BAZ	Female	0.11 ± 0.91[a] (n=32)	-0.22 ± 0.64[b] (n=32)	-0.07 ± 0.63[ab] (n=30)	0.11 ± 0.83[ab] (n=28)	0.11 ± 0.87[ab] (n=26)
	Male	0.19 ± 0.94[a] (n=31)	-0.12 ± 1.18[b] (n=31)	0.16 ± 1.03[ab] (n=28)	0.34 ± 1.02[ab] (n=27)	0.38 ± 1.14[ab] (n=26)

Data are reported as mean ± SD.
*indicate statistically significant differences between sexes at all timepoints ($p<0.05$, post hoc adjustment).
Distinct letter superscripts ([a,b,c,d,e]) indicate statistically significant differences across time ($p<0.05$, post hoc adjustment); values that share a common superscript are not different from one another.

Abbr: WAZ: weight-for-age z-score, LAZ: length-for-age z-score, HC: head circumference, HCAZ: head-circumference-for-age z-score, and BAZ: BMI-for-age z-score.

Table 3.4. Bone parameters reported for males and females

Variable	Sex	1 mo	3 mo	6 mo	9 mo	12 mo
WB BMC (g)	Female*	94.38 ± 16.12[a] (n=32)	128.97 ± 17.07[b] (n=31)	164.89 ± 21.22[c] (n=27)	189.48 ± 28.66[d] (n=25)	224.69 ± 32.37[e] (n=20)
	Male	102.04 ± 18.80[a] (n=31)	134.43 ± 22.82[b] (n=30)	180.42 ± 33.01[c] (n=26)	201.73 ± 31.14[d] (n=23)	242.29 ± 39.23[e] (n=23)
WB BMC/wt (g/kg)	Female	21.56 ± 1.90[ab] (n=32)	21.72 ± 2.39[a] (n=31)	22.33 ± 2.43[b] (n=27)	22.16 ± 2.08[ab] (n=25)	23.87 ± 1.93[c] (n=20)
	Male	21.37 ± 1.95[ab] (n=31)	20.89 ± 1.96[a] (n=30)	22.50 ± 2.75[b] (n=26)	21.93 ± 1.94[ab] (n=23)	23.89 ± 1.52[c] (n=23)
WB BMC/ln (g/cm)	Female	1.74 ± 0.27[a] (n=32)	2.12 ± 0.25[b] (n=31)	2.48 ± 0.30[c] (n=27)	2.67 ± 0.37[d] (n=25)	3.00 ± 0.38[e] (n=20)
	Male	1.84 ± 0.29[a] (n=31)	2.17 ± 0.32[b] (n=30)	2.67 ± 0.43[c] (n=26)	2.80 ± 0.39[d] (n=23)	3.17 ± 0.43[e] (n=23)
WB BMC accretion rate (g/mo)	Female	N/A	17.05 ± 6.55[a] (n=31)	11.93 ± 7.10[ab] (n=26)	7.55 ± 7.76[c] (n=24)	10.63 ± 6.09[bc] (n=20)
	Male	N/A	16.48 ± 6.55[a] (n=30)	14.94 ± 8.07[ab] (n=26)	5.89 ± 9.13[c] (n=22)	12.75 ± 8.52[bc] (n=19)
WBLH BMC (g)	Female	49.76 ± 8.28[a] (n=32)	65.41 ± 8.33[b] (n=31)	82.90 ± 12.68[c] (n=27)	93.52 ± 15.49[d] (n=25)	119.41 ± 29.32[e] (n=20)
	Male	54.19 ± 9.21[a] (n=31)	68.44 ± 11.87[b] (n=30)	89.99 ± 21.33[c] (n=26)	101.41 ± 18.77[d] (n=23)	129.43 ± 25.00[e] (n=23)
LS BMC (g)	Female	2.81 ± 0.71[a] (n=32)	2.85 ± 0.47[b] (n=32)	3.55 ± 0.60[a] (n=30)	4.41 ± 0.73[c] (n=28)	5.52 ± 94[d] (n=26)
	Male	2.58 ± 0.46[a] (n=31)	2.96 ± 0.58[b] (n=31)	3.46 ± 0.72[a] (n=28)	4.61 ± 0.76[c] (n=27)	5.64 ± 1.13[d] (n=26)

LS BMC accretion rate (g/mo)	Female	N/A	0.01 ± 0.35[a] (n=32)	0.24 ± 0.18[ab] (n=30)	0.31 ± 0.26[b] (n=28)	0.37 ± 0.30[b] (n=26)
	Male	N/A	0.19 ± 0.27[a] (n=31)	0.15 ± 0.24[ab] (n=28)	0.38 ± 0.28[b] (n=27)	0.32 ± 0.28[b] (n=25)
LS BMD (g/cm^2)	Female	0.281 ± 0.065[a] (n=32)	0.244 ± 0.035[b] (n=32)	0.267 ± 0.034[ab] (n=30)	0.298 ± 0.038[c] (n=28)	0.339 ± 0.040[d] (n=26)
	Male	0.249 ± 0.039[a] (n=31)	0.244 ± 0.040[b] (n=31)	0.255 ± 0.037[ab] (n=28)	0.298 ± 0.033[c] (n=27)	0.330 ± 0.048 [d] (n=26)
				1 to 6 mo		6 to 12 mo
% Increase WB BMC	Female			77.7 (65.0 to 90.4)[a] (n=27)		31.7 (24.4 to 38.9)[b] (n=19)
	Male			77.7 (67.3 to 88.1)[a] (n=26)		35.8 (28.5 to 43.1)[b] (n=23)
% Increase WBLH BMC	Female			69.3 (57.9 to 80.8)[a] (n=27)		32.8 (22.9 to 42.7)[b] (n=19)
	Male			64.5 (53.1 to 75.9)[a] (n=26)		40.9 (32.9 to 48.9)[b] (n=23)
% Increase LS BMC	Female			33.4 (22.1 to 44.7)[a] (n=30)		64.0 (54.1 to 74.0)[b] (n=25)
	Male			37.5 (24.9 to 50.1)[a] (n=28)		64.7 (49.6 to 79.8)[b] (n=26)
% Increase LS BMD	Female			0.2 (-7.8 to 8.2)[a] (n=30)		31.3 (25.2 to 37.4)[b] (n=25)
	Male			4.1 (-2.9 to 11.2)[a] (n=28)		32.1 (20.5 to 43.6)[b] (n=26)

Data are reported as mean ± SD or (95%CI).

Individual % increase for each bone parameter (X) was calculated using: $\frac{\text{Final X} - \text{Initial X}}{\text{Initial X}} \times 100$

*indicate statistically significant differences between sexes at all timepoints ($p<0.05$, post hoc adjustment).

Letter superscripts [(a,b,c,d,e)] indicate statistically significant differences across time ($p<0.05$, post hoc adjustment); values that share a common superscript are not different from one another.

Abbr: WB BMC: whole body bone mineral content, WBLH BMC: whole body less head bone mineral content, LS BMC: lumbar spine bone mineral content, and LS BMD: lumbar spine bone mineral density.

Table 3.5. Bone parameters reported according to type of feeding

		BL	3mo	6mo	9mo	12mo
WB BMC (g)	Breastfed	98.15±17.77[a] (n=63)	131.8±20.26[b] (n=60)	171.80± 28.82[c] (n=49)	191.15±29.84[d] (n=33)	225.01±35.42[e] (n=13)
	Not receiving any breast milk	- (n=0)	122.31±0.00[b] (n=1)	181.25±25.34[c] (n=4)	204.59±29.85[d] (n=15)	236.29±36.76[e] (n=29)
WB BMC/wt (g/kg)	Breastfed	21.46±1.91[ab] (n=63)	21.32±2.23[a] (n=60)	22.38±2.55[b] (n=49)	21.97±2.05[ab] (n=33)	23.86±1.49[c] (n=13)
	Not receiving any breast milk	- (n=0)	20.97±0.00[a] (n=1)	3.20±1.60[b] (n=4)	22.24±1.93[ab] (n=15)	23.91±1.89[c] (n=29)
WB BMC/ln (g/cm)	Breastfed	1.79±0.28[a] (n=63)	2.15±0.29[b] (n=60)	2.56±0.38[c] (n=49)	2.68±0.38[c] (n=33)	2.98±0.37[d] (n=13)
	Not receiving any breast milk	- (n=0)	2.04±0.00[b] (n=1)	0.38±0.19[c] (n=4)	2.85±0.36[c] (n=15)	3.12±0.43[d] (n=29)
WB BMC accretion rate (g/mo)	Breastfed*	N/A	16.82±6.52[a] (n=60)	13.08±7.68[b] (n=48)	5.79±9.08[c] (n=33)	8.70±6.30[bc] (n=12)
	Not receiving any breast milk	N/A	13.93±0.00[a] (n=1)	17.65±7.13[b] (n=4)	9.20±5.91[c] (n=13)	12.53±7.28[bc] (n=26)
WBLH BMC (g)	Breastfed	51.94±8.96[a] (n=63)	67.02±10.30[b] (n=60)	85.99±17.95[c] (n=49)	95.01±16.05[d] (n=33)	116.24±25.13[e] (n=13)
	Not receiving any breast milk	- (n=0)	59.93±0.00[b] (n=1)	91.12±14.72[c] (n=4)	102.34±19.76[d] (n=15)	123.83±27.33[e] (n=29)
LS BMC (g)	Breastfed	2.70±0.61[a] (n=63)	2.91±0.52[a] (n=62)	3.51±0.67[b] (n=54)	5.54±0.75[c] (n=37)	5.58±1.18[d] (n=19)
	Not receiving any breast milk	- (n=0)	2.25±0.00[a] (n=1)	3.58±0.63[a] (n=4)	4.45±0.73[c] (n=18)	5.60±0.96[d] (n=32)
LS BMC accretion rate (g/mo)	Breastfed	N/A	0.10±0.32[a] (n=62)	0.19±0.03[ac] (n=54)	0.36±0.27[b] (n=37)	0.32±0.32[bc] (n=18)
	Not receiving any breast milk	N/A	0.16±0.00[a] (n=1)	0.26±0.06[ac] (n=4)	0.30±0.28[b] (n=17)	0.37±0.28[bc] (n=32)

LS BMD (g/cm^2)	Breastfed	0.266 ± 0.056^{a} (n=63)	0.244 ± 0.038^{b} (n=62)	0.261 ± 0.037^{ab} (n=54)	0.301 ± 0.036^{c} (n=37)	0.331 ± 0.056^{d} (n=19)
	Not receiving any breast milk	- (n=0)	0.226 ± 0.00^{b} (n=1)	0.25 ± 0.03^{ab} (n=4)	0.293 ± 0.034^{c} (n=18)	0.339 ± 0.035^{d} (n=32)

Data are reported as mean ± SD.

*indicate statistically significant differences between types of feeding: breastfed (exclusively or mixed with alternative feeds) vs not receiving any breast milk ($p<0.05$) at each of 6, 9, and 12 months old.

Distinct letter superscripts (a,b,c,d,e) indicate statistically significant differences across time ($p<0.05$, post hoc adjustment); values that share a common superscript are not different from one another.

Abbr: WB BMC: whole body bone mineral content, WB BMC/wt: whole body bone mineral content per weight, WB BMC/ln: whole body bone mineral content per length, WBLH BMC: whole body less head bone mineral content, LS BMC: lumbar spine bone mineral content, and LS BMD: lumbar spine bone mineral density.

Table S3.1. Bone parameters reported for males and females combined

Variable	1 mo	3 mo	6 mo	9 mo	12 mo
WB BMC (g)	98.15 ± 17.77[a] (n=63)	131.66 ± 20.13[b] (n=61)	172.51 ± 28.47[c] (n=53)	195.35 ± 30.19[d] (n=48)	234.10 ± 36.87[e] (n=43)
WB BMC/wt (g/kg)	21.46 ± 1.91[ab] (n=63)	21.31 ± 2.21[a] (n=61)	22.41± 2.57[bcd] (n=53)	22.05 ± 2.00[ac] (n=48)	23.88 ± 1.74[d] (n=43)
WB BMC/ln (g/cm)	1.79 ± 0.28[a] (n=63)	2.14 ± 0.29[b] (n=61)	2.57 ± 0.38[c] (n=53)	2.73 ± 0.38[d] (n=48)	3.09 ± 0.41[e] (n=43)
WBLH BMC (g)	51.94 ± 8.96[a] (n=63)	66.90 ± 10.25[b] (n=61)	86.38 ± 17.66[c] (n=53)	97.30 ± 17.42[d] (n=48)	122.36 ± 26.91[e] (n=43)
LS BMC (g)	2.70 ± 0.61[a] (n=63)	2.88 ± 0.48[a] (n=63)	3.51 ± 0.66[b] (n=58)	4.51 ± 0.74[c] (n=55)	5.58 ± 1.03[d] (n=52)
LS BMD (g/cm^2)	0.266 ± 0.056[a] (n=63)	0.244 ± 0.037[b] (n=63)	0.261 ± 0.036[ab] (n=58)	0.298 ± 0.036[c] (n=55)	0.335 ± 0.044[d] (n=52)
			1 to 6 mo		6 to 12 mo
% increase in WB BMC			77.7 (69.5 to 85.9)[a] (n=53)		33.9 (28.8 to 39.1)[b] (n=42)
% increase in WBLH BMC			67.0 (58.9 to 75)[a] (n=53)		37.2 (30.9 to 43.5)[b] (n=42)
% increase in LS BMC			35.4 (27.0 to 43.7)[a] (n=58)		64.4 (55.3 to 73.4)[b] (n=51)
% increase in LS BMD			2.1 (-3.3 to 7.4)[a] (n=58)		31.7 (25.2 to 38.2)[b] (n=51)

Data are reported as mean ± SD or (95% CI).

Individual % increase for each bone parameter (X) was calculated using: $\frac{\text{Final X} - \text{Initial X}}{\text{Initial X}} \times 100$.

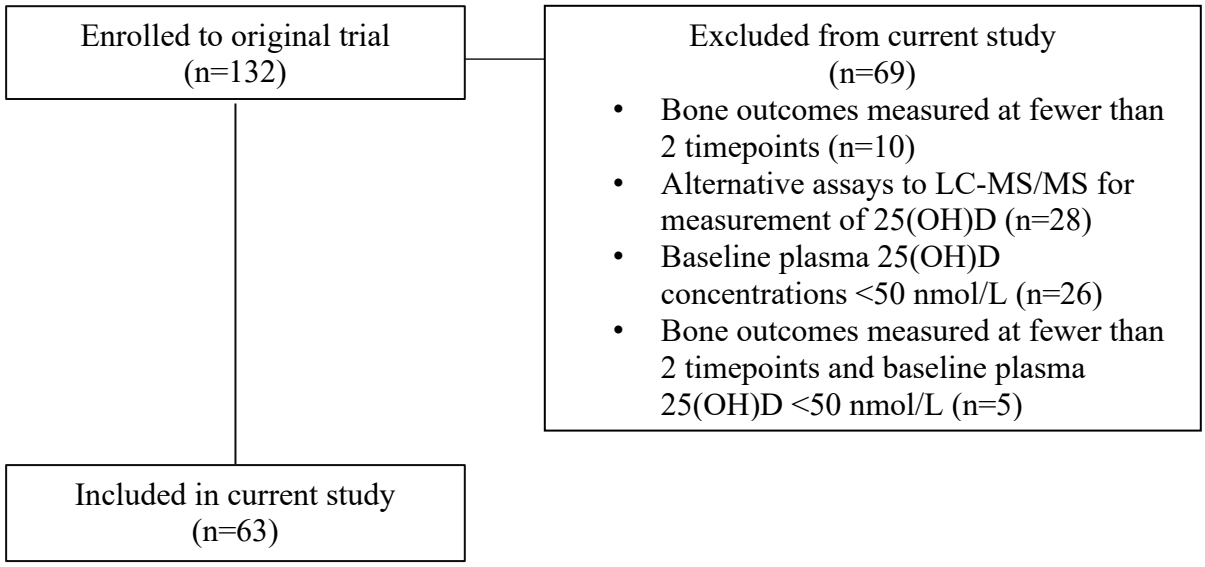
Abbr: WB BMC: whole body bone mineral content, WB BMC/wt: whole body bone mineral content per weight, WB BMC/ln: whole body bone mineral content per length, WBLH BMC: whole body less head bone mineral content, LS BMC: lumbar spine bone mineral content, and LS BMD: lumbar spine bone mineral density.

Table S3.2. L, M, S parameters and modeled percentiles for LS BMD (g/cm^2) by age: 1-12 mo for females and males combined

	LMS parameters*		Percentiles						
	L	S	3	10	25	M (50)	75	90	97
Z-score:			-2.00	-1.33	-0.67	0	0.67	1.33	2.00
Age (mo)									
0.85	-0.009	0.203	0.175	0.201	0.230	0.263	0.301	0.345	0.395
1	-0.009	0.199	0.175	0.200	0.228	0.260	0.297	0.339	0.387
2	-0.009	0.178	0.173	0.194	0.219	0.246	0.277	0.313	0.352
3	-0.009	0.165	0.174	0.194	0.216	0.242	0.270	0.301	0.336
4	-0.009	0.155	0.179	0.198	0.220	0.244	0.270	0.300	0.333
5	-0.009	0.147	0.186	0.205	0.227	0.250	0.276	0.304	0.336
6	-0.009	0.141	0.196	0.215	0.236	0.260	0.285	0.313	0.344
7	-0.009	0.135	0.207	0.227	0.248	0.271	0.297	0.325	0.355
8	-0.009	0.130	0.219	0.238	0.260	0.283	0.309	0.337	0.367
9	-0.009	0.125	0.230	0.250	0.272	0.295	0.321	0.349	0.380
10	-0.009	0.121	0.242	0.262	0.284	0.308	0.333	0.361	0.392
11	-0.009	0.117	0.253	0.273	0.295	0.319	0.345	0.373	0.403
12	-0.009	0.113	0.263	0.284	0.306	0.330	0.356	0.384	0.414
12.53	-0.009	0.111	0.269	0.290	0.312	0.336	0.362	0.390	0.420

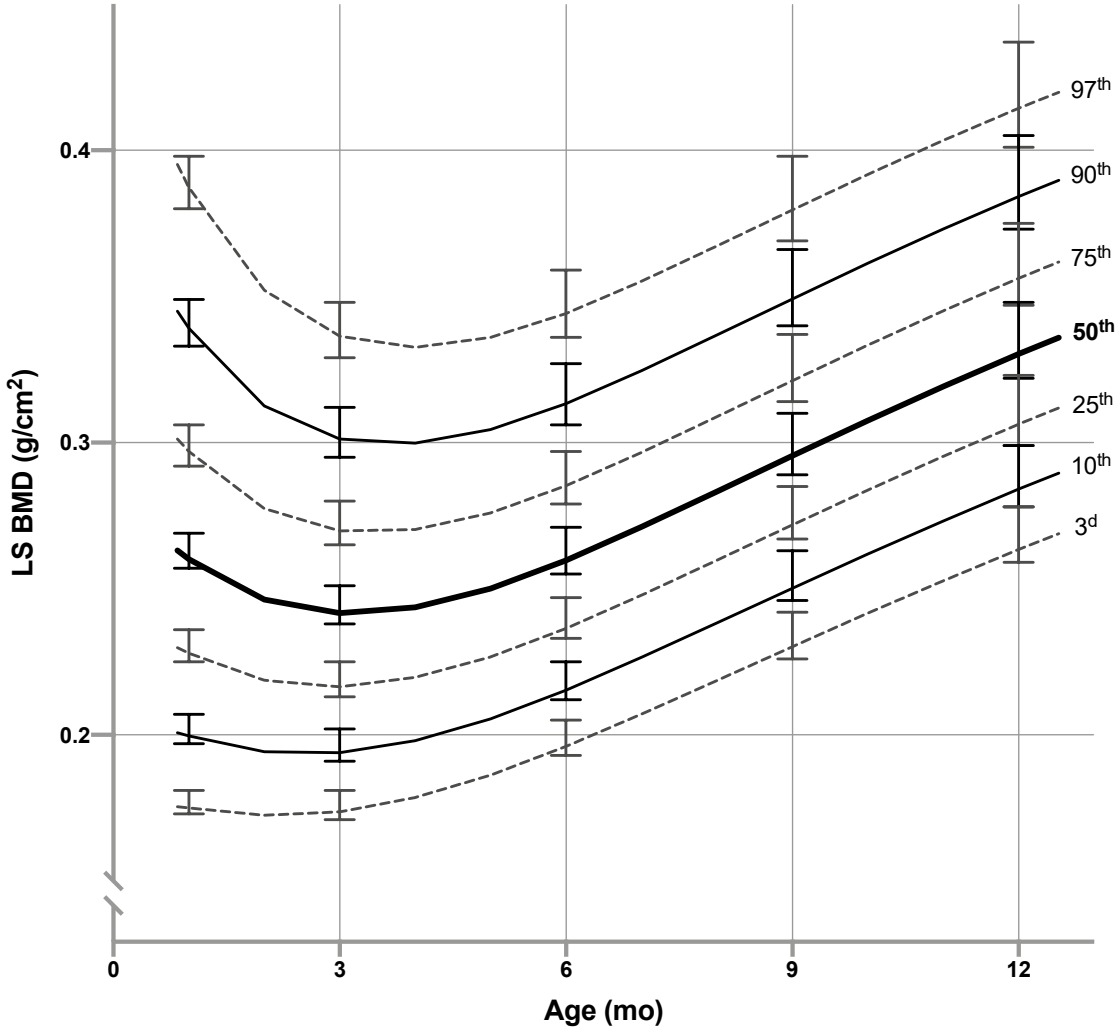
*Age-specific median (M), skewness (S), and power (L).
Abbr: LS BMD: lumbar spine bone mineral density.

Figure 3.1. Participant disposition chart



Participant disposition chart illustrating the number of infants enrolled into the original trial (NCT00381914), number of participants included in the current study and reasons for exclusion from the current study. LC-MS/MS: liquid chromatography tandem mass spectrometry and 25(OH)D: 25-hydroxyvitamin D.

Figure 3.2. Lumbar spine BMD-for-age smoothed centiles curve



Lumbar spine BMD-for-age smoothed centiles curve fitted to the original data of male and female infants (n=63), 1 (26-52 d) to 12 mo (356-381 d). 99% confidence intervals (denoted as error bars) were calculated from the bootstrapped samples (n=1000) analyzed using the same L (Box-Cox transformation), M (median), and S (skewness) parameters as for the original data.
Abbr: LS BMD: lumbar spine bone mineral density.

Bridge statement 2

Chapter 3 generated reference data and explored patterns of bone mineral accretion in infancy showing that the first half of infancy is characterized by a faster bone mineral accretion rate compared to 6 to 12 months of age. It also explored sex differences in skeletal mineralization throughout infancy showing that those differences are not evident throughout but are actually driven by size. Growth charts for lumbar spine bone mineral density but not whole body bone mineral content were generated from 1 to 12 months of age using LMS chartmaker, a tool used by the World Health Organization for generation of weight-for-age, length-for-age and head-circumference-for-age growth charts. Percentile means calculated for LS BMD growth charts from the available infant sample were found to be a good representation of the population means using 95% CI calculated using bootstrapping methods. This was not the case for whole body bone mineral content percentile means due to the relatively small sample size available.

As periods of rapid growth are critical for programming of health and disease; ensuring healthy skeletal mineralization as early as during the neonatal phase may help set the trajectory for healthy bone mineral accretion for life. Several biological and environmental factors may impact on a neonate's bone mass including maternal factors. For instance, maternal smoking and alcohol consumption may negatively impact on offspring's bone mass. In addition, other factors including pre-gravid body mass index, gestational weight gain (GWG), and maternal vitamin D status may also relate to a neonate's bone mass. As described in the literature review, data on the relation of pre-gravid body mass index and GWG to neonatal bone mass are scarce and the relation of maternal vitamin D status to neonatal bone mass is unsettled. The following chapter will address maternal vitamin D status and GWG and their relation to neonatal bone mineral content and density.

Chapter 4: Manuscript 2

Maternal vitamin D status and gestational weight gain as correlates of neonatal bone mass in healthy term breastfed young infants from Montreal, Canada

Maternal vitamin D status and gestational weight gain as correlates of neonatal bone mass in healthy term breastfed young infants from Montreal, Canada

Nutrients, *13*(12), 4189, 2021.

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4.1 Abstract

Implications of maternal gestational weight gain (GWG) and vitamin D status to neonatal bone health are unclear. We tested whether maternal 25-hydroxyvitamin D (25(OH)D) and GWG relate to neonatal bone mineral content (BMC) and bone mineral density (BMD). Healthy term appropriate for gestational age breastfed neonates (n=142) and their mothers were recruited 24-36 hours after delivery and followed at 1.0±0.5 month. At birth, obstetric data were collected and newborn serum 25(OH)D was measured. At 1 month, neonatal whole-body (WB) BMC, WB BMC relative to body weight (WB BMC/kg), lumbar spine BMC and BMD, maternal and neonatal 25(OH)D concentrations, and anthropometry were measured. Infant BMC and BMD between maternal 25(OH)D (<50, ≥50 nmol/L) and GWG (insufficient, adequate, excessive) categories were compared. Maternal 25(OH)D was not related to whole body BMC, BMC/kg, lumbar spine BMC, and BMD. Infants in the excessive maternal GWG category had greater (p=0.0003) whole body BMC and BMC/kg, lumbar spine BMC and BMD than inadequate GWG, and greater (p=0.0063) whole body BMC/kg and lumbar spine BMC and BMD than adequate GWG. These results suggest that maternal GWG, but not vitamin D status, modestly relates to bone mass in neonates.

4.2 Introduction

A significant amount of variance in peak bone mass remains unexplained by genetic and lifestyle factors (165, 166), and is postulated to be attributed to skeletal programming in utero (13). This is exemplified in the Avon Longitudinal Study of Parents and Children in which maternal exposure to ultraviolet B (UVB) radiation and folate intake during pregnancy were both positively related to childhood (9 years old) bone mineral content (BMC) and density (BMD) (167). Other maternal factors related to an offspring's bone include maternal smoking (168) and alcohol consumption (169) that are negatively related to bone outcomes, whereas the relation of maternal vitamin D supplementation (170-172), vitamin D status (6, 145, 173, 174), pre-pregnancy body mass index (BMI), and gestational weight gain (GWG) (142) to infant bone outcomes is equivocal.

Indicators of nutritional status of the mother, including pre-gravid BMI and GWG, have been emphasized in the US Institute of Medicine (IOM) guidelines for a healthy pregnancy (175). In order to optimize health of infants and their chances of being born appropriate for gestational age (AGA), GWG targets have been set by the IOM according to pre-gravid BMI. Accordingly, GWG is described as being insufficient, adequate, and excessive. Nonetheless, the impact of maternal pre-gravid BMI and GWG on offspring's growth parameters including skeletal growth remains inconclusive. One study from the UK suggests that the positive associations between pre-gravid BMI and child BMC and BMD at 9 years of age are possibly due to genetic and environmental influences throughout childhood rather than intrauterine exposures (141). Another study found that GWG positively associates with offspring BMC at 7 years of age in mothers with a pre-gravid BMI <25 kg/m^2, but not in mothers with pre-gravid BMI ≥ 25 kg/m^2 (142). As such, the relation of pre-gravid BMI and excessive GWG to offspring bone outcomes is unclear. Excessive GWG essentially translates into excess adiposity; which is well known to be associated with higher BMC

and BMD in children (176) and adults (177), but may limit the availability of vitamin D and potentially bone health of the offspring. Sequestration of vitamin D in adipose tissue (178) could result in lower circulating serum 25(OH)D concentrations in the mother and thus a decreased transfer of 25(OH)D from the mother to the fetus.

Vitamin D stores of newborn infants are a function of maternal-fetal transfer of 25(OH)D during gestation (179) and are usually lower in the infant compared to the mother (4-6). While some epidemiological studies report a positive relationship between maternal 25(OH)D status and bone outcomes in infancy (145, 173, 180, 181), childhood (182), and adulthood (183), others do not (143, 184-187). In a cohort of 50 newborn infants and their mothers in Winnipeg, Canada, neonates with insufficient vitamin D status at birth have lower whole-body bone mineral content (BMC) relative to weight (BMC/kg) (6), suggesting that maternal vitamin D status may affect offspring bone health.

To our knowledge, previous studies have not explored how both maternal vitamin D status and GWG relate to neonatal bone outcomes. The objective of this study was to determine whether neonatal bone mass in healthy term, breastfed infants differs according to maternal vitamin D status and GWG. We hypothesized that sufficient maternal vitamin D status and healthy pregnancy weight gain associate with greater BMC/kg compared to insufficient and excessive GWG.

4.3 Methods

Sample and study design

This study was based on the newborn and 1 month postpartum data from a double-blinded randomized controlled trial on vitamin D supplementation in infancy registered with clinicaltrials.gov (NCT02563015). Mother-infant dyads were recruited within 24-36 hours after delivery at the Lakeshore General Hospital, Montreal, Canada (March 2016 - March 2019).

Inclusion criteria were healthy, term, AGA singleton infants of a healthy pregnancy and whose mothers had an intent to breastfeed for at least 3 months. Exclusion criteria were maternal diseases (diabetes (any type), pre-eclampsia, celiac disease, and inflammatory bowel disease), smoking during pregnancy, and the use of prescription medication(s) that alter vitamin D and/or bone metabolism and/or fetal growth. At the hospital, neonatal capillary blood was sampled for subsequent measurement of serum 25(OH)D concentrations. The sample collection occurred during clinical screening for inborn errors of metabolism (e.g., phenylketonuria) in order to minimize frequency of blood sampling.

At the hospital, data on obstetric history, anthropometric measurements at birth, and demographics were collected. The bone health of the neonate, nutritional intake of the mother during gestation, serum 25(OH)D concentrations, and anthropometry of the neonate and mother were assessed at 1.0±0.5 months postpartum at the Mary Emily Clinical Nutrition Research Unit of McGill University, Montreal.

Obstetric history, anthropometric, and demographic data

Obstetric history including mother's age, height, weight (prior to conception and at delivery), gravida, and pre-gravid BMI were collected from the medical records and confirmed by a maternal postpartum survey. GWG was calculated as: weight at delivery minus weight prior to conception (kg) and the categories (inadequate, adequate, and excessive) were defined according to the IOM's classification (175). Based on pre-gravid BMI <18.5 kg/m^2, the recommended adequate GWG is 12.7 to 18.2 kg; if BMI was between 18.5 to 24.9 kg/m^2, adequate GWG is 11.4 to 15.9 kg; if BMI was between 25.0 and 29.9 kg/m^2, adequate GWG is 6.8 to 11.4 kg; and if BMI was 30 kg/m^2, adequate GWG is defined as 5 to 9.1 kg. Values for GWG below the lower range for each BMI category are considered inadequate; and values for GWG above the upper range are considered

excessive (**Supplementary Figure S4.1).** Mothers were also grouped into two categories according to their pre-gravid BMI: <25 kg/m^2 and ≥25 kg/m^2.

Data on date of birth, sex, gestational age at birth, and birth weight of the infants were obtained from the medical charts. Birth length and head circumference data were not used as the measurements were not standardized and thus were not reliable. At the postnatal study visit, infant nude weight (dynamic scale, Mettler-Toledo Inc.) was measured to the nearest gram and length (infantometer, O'Learly Length Boards, Ellard Instrumentation Ltd.) and head circumference (non-stretchable tape, Perspective Enterprises) were measured to the nearest 0.1 cm. Corresponding z-scores (World Health Organization AnthroPlus Software 2009, Geneva, Switzerland) were calculated. The mother's weight (balance-beam scale, Detecto; Webb) and height (stadiometer, Seca Medical Scales and Measuring Systems), were also measured once to the nearest 0.1 kg and 0.1 cm, respectively.

Demographic data were collected using a postpartum survey designed according to Statistics Canada descriptors (188, 189) and for the purpose of better understanding health needs of different populations. Data on the mother's self-reported population group (white, all other: South Asian, Chinese, Black, Filipino, Latin American, Arab, Southeast Asian, West Asian, Korean, Japanese, and other), education (high school, college, or university), in addition to annual family income (<70,000 CAD, ≥70,000 CAD, or not reported) were obtained.

Dietary intake, multivitamin use, and physical activity

Nutritional intake during all three trimesters of pregnancy combined was assessed using a validated semi-quantitative food frequency questionnaire (190) given the potential relation of maternal intake and GWG during pregnancy to offspring bone outcomes (191, 192). The data were analyzed using the Canadian Nutrient File. Data on intake of total energy, protein, carbohydrates,

fat as well as calcium, vitamin D, magnesium, and phosphorous from both food and supplements were explored as continuous variables. Daily intake of these nutrients (g or mg per 1000 kcal) normalized for energy intake was also tested. Data on use of multivitamins and physical activity during pregnancy (Yes/No) were collected using the maternal postpartum survey.

Serum 25(OH)D concentration

Infant capillary blood samples (0.5 mL) were collected (at birth and at 1.0±0.5 months of age) by heel lance. Maternal venous blood (5 mL) was collected at the postnatal visit. Samples were centrifuged (4000xg, 6°C) for 20 min and serum was collected and stored at -80°C until 25(OH)D concentrations were measured. The serum 25(OH)D concentrations were measured using an automated chemiluminescent immunoassay (Liaison, Diasorin Inc.) and standardized to National Institute of Standards and Technology (NIST) reference measurements (193) using Deming regression (standardized concentration (nmol/L) = 0.9634 measured concentration (nmol/L) + 3.122). The serum 25(OH)D concentrations were classified as sufficient (≥50 nmol/L) or insufficient (<50 nmol/L) as per the IOM's recommendations (2). The laboratory participated in Vitamin D External Quality Assessment Scheme and obtained a certificate of proficiency. Using the NIST Standard Reference Materials 972a Level 1-4 quality control samples, the accuracy was 97.4%. Precision was measured using both NIST972a and internal laboratory controls; with inter-assay % CV <10% for both. The total serum 25(OH)D from a subgroup of mother-infant dyads (n=83) measured using chemiluminescent immunoassay agreed well (mean difference = - 0.75 nmol/L) with concentrations obtained using liquid chromatography tandem mass spectrometry from a laboratory (Queen's University, Kingston, Canada) certified by the DEQAS Certification Program.

Bone outcomes

Whole body BMC, lumbar spine (1-4) BMC, and BMD were measured using dual-energy X-ray absorptiometry (DXA, fan beam, APEX 13.3:3, Hologic 4500A Discovery Series, Hologic Inc., Bedford, MA, USA) in array mode as recommended (125). Whole body and lumbar spine scans were obtained using infant whole-body mode and anterior posterior (AP) spine mode, respectively. The regions of interest, including head and lumbar spine (1-4), were defined using manual bone edge detection. The precision was measured using a Hologic spine phantom (No. 14774) and % CV for each of BMC, BMD, and bone area were <1%. Neonates were dressed in light gowns and diapers, covered with light blankets, and rocked to sleep prior to scan acquisition.

Ethical approval

This study was reviewed and approved by the St. Mary's Hospital Research Ethics Committee which oversees ethics at the Lakeshore General Hospital (SMHC 15-34). The study was also reviewed and approved by the Health Canada Research Ethics Board (REB 2019-033H) and the Privacy Management Division (HC-PR-2019-000024). The study was conducted in accordance with the Declaration of Helsinki. All of the study materials were available in English and French, the two official languages in Canada. Written informed consent was provided by the families at the hospital prior to participation in the newborn screening and at McGill University prior to enrolment in the vitamin D trial.

Statistical analysis

The sample size was calculated based on differences in neonatal whole-body BMC using 5% significance level (α=0.05), power of 95%, an effect size of 0.2, and considering a total of 9 variables included as fixed effects in the models tested: infant: sex, age, length-for-age z-scores (LAZ) at the 1 month postpartum visit; maternal: 25(OH)D categories, pre-pregnancy BMI

categories, GWG categories, education, self-reported population group, and annual family income. The minimum sample size required was 127. To account for the risk of motion artifacts, given that keeping infants still during scans is often challenging, an additional 10% was considered and a total of 140 mother-infant dyads was set as the target sample size.

Data are reported as mean ± SD or mean (95% CI) or median (IQR) for continuous variables or percentages for categorical variables. In addition to the mean ± SD, the full data range (minimum-maximum) was also provided for the 25(OH)D concentrations of infants and mothers (nmol/L) in different 25(OH)D categories and for GWG (kg) in each GWG category. Infant vitamin D status reflects maternal fetal transfer and vitamin D supplementation, and we therefore tested the relation between maternal and neonatal vitamin D status at birth and 1 month using a linear mixed model (PROC MIXED). The differences between maternal 25(OH)D categories and GWG categories in whole-body BMC (g) and BMC per body weight (BMC/kg; g/kg), lumbar spine BMC (g) and BMD (g/cm^2) were tested using a linear mixed model. The variables tested as fixed effects were considered based on previous research, including infant sex (155, 156), age (12), and length-for-age z-scores (LAZ) (194); maternal characteristics: 25(OH)D categories (6, 181), pre-pregnancy BMI categories (142), GWG categories (2), gravida (195), age (196); and sociodemographic characteristics (197-199): maternal education and self-reported population group, and annual family income. Given that GWG may be an effect modifier of the maternal vitamin D status-infant bone outcomes association (178), both factors were tested in the same model. The regression coefficients (95% CI) for these variables are reported. The model fit was evaluated using the Bayesian information criterion (BIC) and R-squared values. The testing of normality of residuals was done using Shapiro-Wilk test. Tukey-Kramer tests were used for post hoc comparisons with adjustment for multiple comparisons (i.e., three GWG categories) and Chi

square or Fisher's exact test for categorical variables. While the recommended ranges for maternal serum 25(OH)D, pre-gravid BMI, and GWG formed the main analysis, these variables were also tested as continuous data. The means of the different macro and micronutrient intakes consumed by the mothers during gestation were compared between the GWG categories in a subgroup analysis using a linear mixed model with the data expressed as absolute values and normalized for energy. All statistical analyses were conducted using SAS University Edition (SAS Institute Inc., Cary, N.C.) and the statistical significance was set at $p<0.05$ after adjustment for multiple comparisons.

4.4 Results

Out of 1035 infants tested for newborn vitamin D status, a total of 142 mother-infant dyads participated in the postnatal assessment (**Figure 4.1**); the comprehensive participant flow diagram is published elsewhere (200). The infants were born at 39.2 ± 1.1 weeks of gestation to mothers 32.2 ± 4.4 years of age, with 58.5% of the infants born between April 1 and October 31. The maternal and infant characteristics were not different among groups of maternal 25(OH)D concentrations except for maternal self-reported population group and education (**Table 4.1**). Overall, 92.2% (n=131) of the mothers reported they took multivitamins, and 49.3% (n=70) exercised during pregnancy. In terms of infant characteristics as per GWG categories, at birth, infants of mothers with inadequate GWG had lower weight-for-age z-scores (WAZ) compared to those in the adequate and excessive GWG categories. Differences between inadequate and excessive GWG categories in WAZ were carried over to the neonatal phase. In addition, at 1 month, infants born to mothers with excessive GWG had higher LAZ compared to mothers with inadequate GWG. As for maternal characteristics, the proportions of mothers with pre-pregnancy

BMI <25 and ≥25 kg/m^2 varied among groups of GWG (Table 4.1). No differences in sociodemographic characteristics were noted between the GWG categories. Intake of energy, protein, fat, carbohydrates, vitamin D, calcium, phosphorous, and magnesium did not vary according to GWG categories (**Supplementary Table S4.1)**. Similarly, when normalized for energy intake (per 1000 kcal), intake of these macronutrients and micronutrients did not vary according to GWG (p>0.69).

Maternal serum 25(OH)D concentrations were associated with infant serum 25(OH)D concentrations at birth and at 1 month postpartum (**Figure 4.2**). Serum 25(OH)D concentrations of the mothers were 39.9 ± 8.6 nmol/L (range: 15.0- 49.9 nmol/L) and 80.0 ± 21.4 nmol/L (range: 51.2-155.3 nmol/L) in the vitamin D insufficient and vitamin D sufficient groups, respectively. In the vitamin D insufficient group, infant 25(OH)D concentrations at birth and at 1 month of age were 28.0±9.4 nmol/L and 44.2±14.5 nmol/L. In the vitamin D sufficient group, infant 25(OH)D concentrations at birth and at 1 month of age were 52.1±17.4 nmol/L and 59.8±16.0 nmol/L.

At birth, amongst infants born to mothers with 25(OH)D <50 nmol/L, 53.3% and 97.8% had 25(OH)D concentrations <30 nmol/L (range: 7.9–29.6 nmol/L) and <50 nmol/L (range: 7.9–48.3 nmol/L), respectively. At birth, 46.4% of infants born to mothers with 25(OH)D ≥50 nmol/L had 25(OH)D concentrations ≥50 nmol/L (range: 50.0-100.4 nmol/L). At the postnatal visit, 20.0% and 66.7% and of infants born to mothers with 25(OH)D <50 nmol/L had 25(OH)D concentrations <30 nmol/L (range: 15.9-29.9 nmol/L) and <50 nmol/L (range: 15.9 – 49.8 nmol/L), respectively. The majority (73.2%) of infants born to mothers with 25(OH)D ≥50 nmol/L had 25(OH)D concentrations ≥50 nmol/L (51.2-106.2 nmol/L) at the postnatal visit. Maternal GWG was 7.1±3.4 (0-11.7), 12.6±2.6 (7.2-18.2), and 18.3±4.1 (9.2-29.1) kg in the insufficient, adequate, and excessive GWG categories, respectively.

Using a linear mixed model analysis, GWG but not maternal 25(OH)D was related to neonatal whole-body BMC and BMC/kg (**Figure 4.3**), and lumbar spine BMC and BMD (**Figure 4.4**). Infants of mothers with excessive GWG had greater whole-body BMC (101.40 ± 12.47 g), whole-body BMC/kg (25.27 ± 3.29 g/kg), lumbar spine BMC (2.34 ± 0.38 g), and lumbar spine BMD (0.239 ± 0.048 g/cm^2) than those with inadequate GWG (whole-body BMC: 89.41±13.62 g; whole-body BMC/kg: 23.47±1.92 g/kg; lumbar spine BMC: 1.99±0.34 g; lumbar spine BMD: 0.206±0.032 g/cm^2; all $p<0.01$). Additionally, infants of mothers with excessive GWG had greater whole-body BMC/kg, lumbar spine BMC, and lumbar spine BMD than those with adequate GWG (whole body BMC/kg: 23.93±2.41 g/kg, lumbar spine BMC: 2.11±0.40 g, and lumbar spine BMD: 0.217±0.036 g/cm^2) (Figure 4.3-4). Other correlates of these bone outcomes included infant sex, age, LAZ, and family income (**Supplementary Table S4.2**). Using adequate GWG as referent, regression coefficients for inadequate GWG are listed in Table S4.2. Regression coefficients for excessive GWG with adequate GWG as the referent are: whole-body BMC: 4.32 g (95%CI: -0.08 to 8.72), whole-body BMC/kg: 1.52 g/kg (95%CI: 0.50 to 2.53), lumbar spine BMC: 0.20 g (95%CI: 0.06 to 0.34), and lumbar spine BMD: 0.023 g/cm^2 (95%CI: 0.008 to 0.038). All of the traits had normally distributed residuals except for LS BMD. A $\log_{10}$ transformation of this variable rendered the residuals normally distributed but did not change any of the results and conclusions. Thus, for simplicity and consistency with the other traits, the untransformed data for this variable were reported. For all of these models, use of continuous data for maternal vitamin D status, pre-gravid BMI, and pregnancy weight gain results in the same interpretation (Supplementary Table S4.3).

4.5 Discussion

This study was undertaken to simultaneously test whether maternal GWG and vitamin D status relate to bone mass of the newborn using internationally accepted guidelines for classification of GWG, vitamin D status, and DXA scanning of infants (2, 175, 201). We observed that excessive GWG related to greater BMC and BMD in healthy, term, AGA infants. These differences remained after adjustment for infant weight, suggesting that these differences are not driven by body size alone. In contrast, maternal vitamin D status did not relate to infant bone mass, only to neonatal 25(OH)D concentrations from birth to ~1 month of age. To our knowledge, no similar studies have been reported in the literature which makes this study unique and provides insight on novel correlates for bone health in infancy that trace back to maternal exposures both preconception and during the gestational phase; an understudied area of research.

Our results show that infant whole body BMC did not vary across the spectrum of maternal GWG in AGA infants. This is reassuring as infants born to mothers who were not able to meet the minimum GWG recommended for their pre-gravid BMI, do not seem to experience adverse bone mass outcomes at this early point in life. On the other end of the spectrum of GWG, differences between excessive and inadequate GWG categories were in part driven by body size, given the respective differences in WAZ and LAZ between these 2 categories. As for the lumbar spine site, while differences were reported between the different GWG categories; the clinical significance of this measurement remains to be defined. Differences observed in our study in the patterns between the whole body and the regional lumbar spine scan could be attributed to the fact that the whole body measurement is more function of body size versus the LS site. Indeed, after adjusting for the weight of the infant, excessive GWG was associated with greater neonatal whole body BMC/kg compared with inadequate and adequate GWG groups. Moreover, in our study, the

association of appropriateness of GWG to bone outcomes was irrespective of pre-pregnancy BMI highlighting the importance of the mother's weight gain during pregnancy regardless of her bodily stores. In a study by Monjardino et al. (142), the association between GWG and bone outcomes varied according to the early pregnancy BMI categories. They (142) noted a positive association between whole body less head BMC and GWG in women with early pregnancy BMI <25 kg/m^2 but not in women with BMI ≥25 kg/m^2. Additionally, they reported that in both BMI categories, weight gain in amounts exceeding the IOM's recommendation was not shown to present any advantages for childhood bone mass. The study by Monjardino et al. may include preterm infants with smaller size at birth as the inclusion criteria specified 24 weeks of gestational age as the cut-off for recruitment. In addition, mothers were not necessarily healthy as some had gestational diabetes and others were smokers. In contrast, our study was restricted to term AGA infants born to healthy mothers. Nonetheless, overall, both studies show modest incremental increases in BMC and BMD according to GWG categories. Interpretation of these findings should be cautious given the negative implications of excessive GWG on overall pregnancy outcomes (202).

Infant 25(OH)D concentration at birth and during the neonatal period are related to maternal vitamin D status (4, 5), in addition to supplementation of the neonate (203). The influence of maternal-fetal transfer of vitamin D becomes weaker with time, i.e. 1 month versus at birth. Counterintuitive to the role of vitamin D in calcium homeostasis, maternal 25(OH)D was not found to be a correlate of newborn bone outcomes. A study by Dror et al. (2012) (204) reported similar findings while several other studies reported a positive relation (6, 173, 180, 181) between maternal 25(OH)D concentration and fetal/neonatal/childhood bone outcomes. This could be explained as a function of vitamin D metabolism and exposures prenatally compared to postnatally. During gestation, intestinal calcium absorption of the mother is dependent on the

active form of vitamin D or calcitriol (144), which is supported as long as 25(OH)D concentrations are not severely deficient (202), which is the case for all mothers in our sample. The transfer of minerals including calcium from the mother to the fetus is also a function of other hormones including parathyroid hormone-related protein and parathyroid hormone (205), in addition to placental and umbilical cord regulations (206). The intrauterine metabolism of calcium contrasts that of the newborn. At birth, calcium absorption mainly occurs through a passive intestinal process that becomes more vitamin D-dependent near weaning (205). Thus, maternal vitamin D status has implications to offspring bone outcomes, but perhaps adverse outcomes are evident only when maternal concentrations are below the threshold of deficiency. The studies by Viljakainen et al. (173, 181) and by Weiler et al. (6) were of a full spectrum of vitamin D status from deficient up to sufficient but with greater proportions of mothers who were vitamin D insufficient (46.0-60.4%) compared to our study (31.7%). In our study, only a small proportion of mothers were vitamin D deficient (4.9%) and 92.2% of the mothers took multivitamins during pregnancy; a proportion comparable to the Canadian population (89.7%) (207).

This study presents certain advantages and limitations. In addition to the unique findings reported in this study, one of its strengths is the standardization of 25(OH)D concentrations to NIST reference measurements (193) and assessment of infant bone mass according to ISCD (201). In addition, this study shows that CLIA is suitable for measurement of total 25(OH)D in newborn serum from capillary samples. This is important in view of recent findings on overestimation of 25(OH)D measured by CLIA in cord blood samples (208). On the other hand, some of the limitations of this study include that maternal serum 25(OH)D concentrations were not measured at delivery. In addition, GWG was calculated for term deliveries and uncomplicated pregnancies as the difference between weight at delivery and pre-conception weight. This calculation overlooks

the variability within trimesters as well as in late gestation, the length of which varied between 37.1 and 41.9 weeks in our study. While the IOM provides recommendations for total GWG and weekly weight gain; methods of calculation of GWG have not been standardized (209). The latter calls to action for a better comparison of GWG related findings across studies and for a more accurate assessment of GWG in clinical practice. Exercise was not further explored as prospectively collected data would better serve that analysis. Maternal dietary intake during gestation was estimated using a food frequency questionnaire which presents inherent limitations including misreporting and recall bias given its retrospective nature. Moreover, it would have been of added value if neonatal bone outcomes of interest were measured at birth. However, this was not feasible due to many family factors including cultural views on research in newborns (210), fear especially in first-time parents, as well as difficulties in managing to attend to a study visit and caring for the other children at once (211).

4.6 Conclusion

Our findings suggest that GWG is a modest determinant of neonatal bone mass even when interpreted relative to body size. Additionally, although maternal 25(OH)D predicts neonatal 25(OH)D, it is not related to neonatal bone mass of healthy term AGA infants born to predominantly vitamin D sufficient mothers. Nonetheless, given reliance in the extra-uterine environment on vitamin D stores acquired through maternal-fetal transfer, a longer-term follow-up is required to elucidate the impact of insufficient maternal vitamin D status on offspring's bone outcomes.

4.7 Acknowledgements

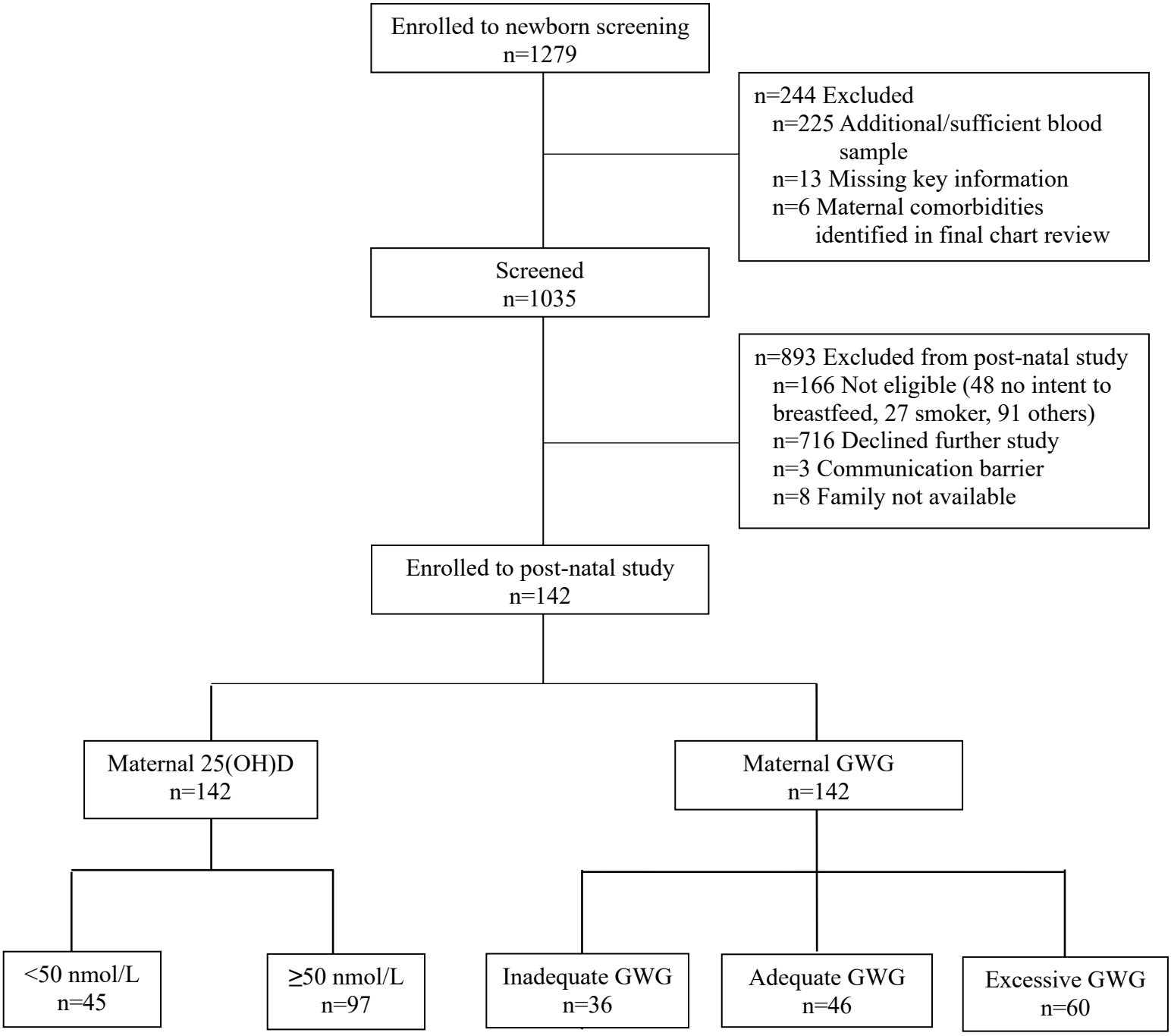
This study was funded by the Canadian Institutes of Health Research (grant #MOP-142391) and the Canada Foundation for Innovation (Project 202820).

The authors thank Sherry Agellon, Paula Lavery, Olusola Sotunde, and Maggie Yuan from McGill University for their contribution to the laboratory work as well Sharina Patel, Veronique Menard, Erika de Risi, and Zahra Farahnak for their involvement in the recruitment of participants at Lakeshore General Hospital, Montreal, Canada.

4.8 Authors' contributions

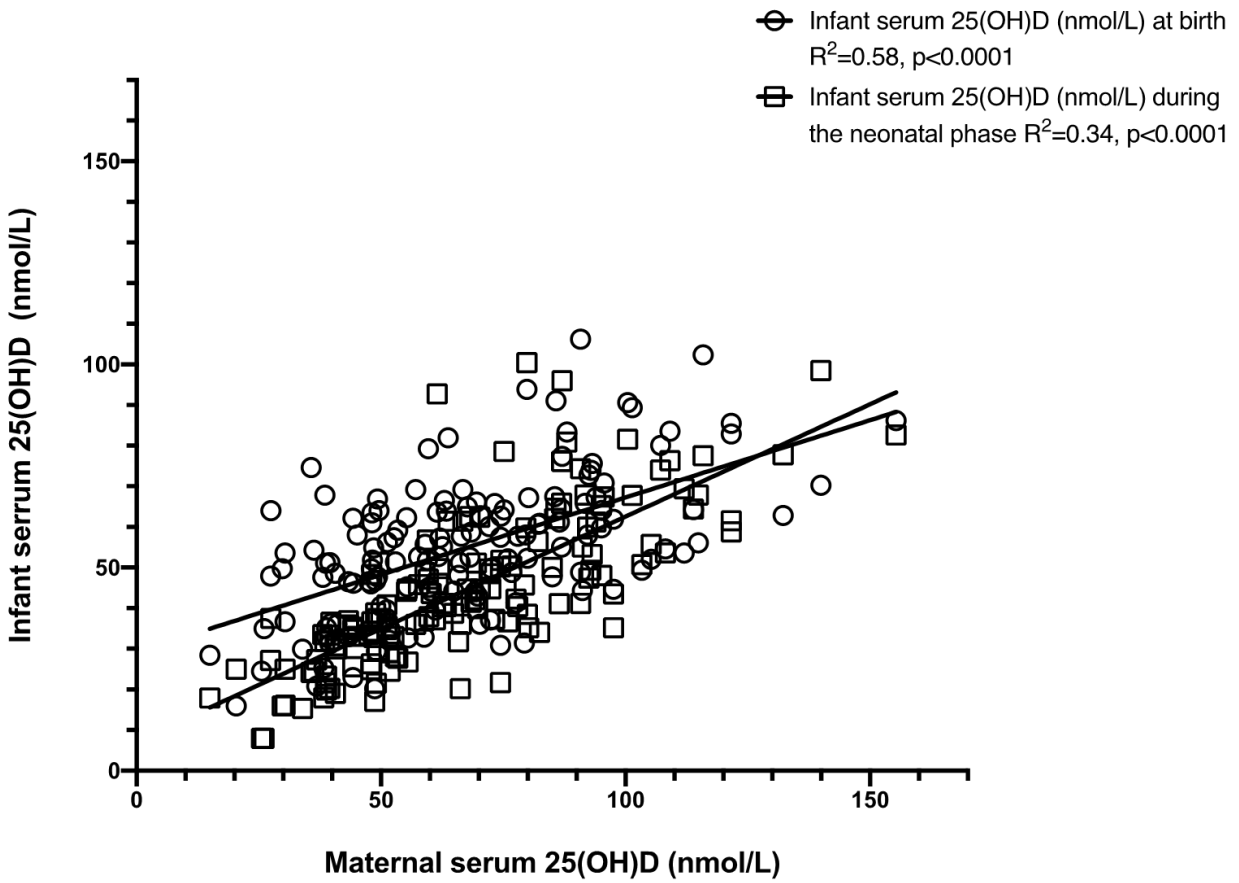
NG, MR, CAV, and HAW contributed to the data collection. NG analyzed and interpreted the data, and elaborated and revised the manuscript. SQW, DM, and FR were the steering committee in terms of recruitment and served as data safety monitors. GJ and MK were the expert analytical chemists collaborators. HAW, SW, DM, FR, and GJ were involved in the design. H.A.W. was involved in the conduct, guidance, and supervision of the study, staff, graduate trainees, and analyses. All authors have approved the manuscript for publication.

Figure 4.1. Participant flow diagram



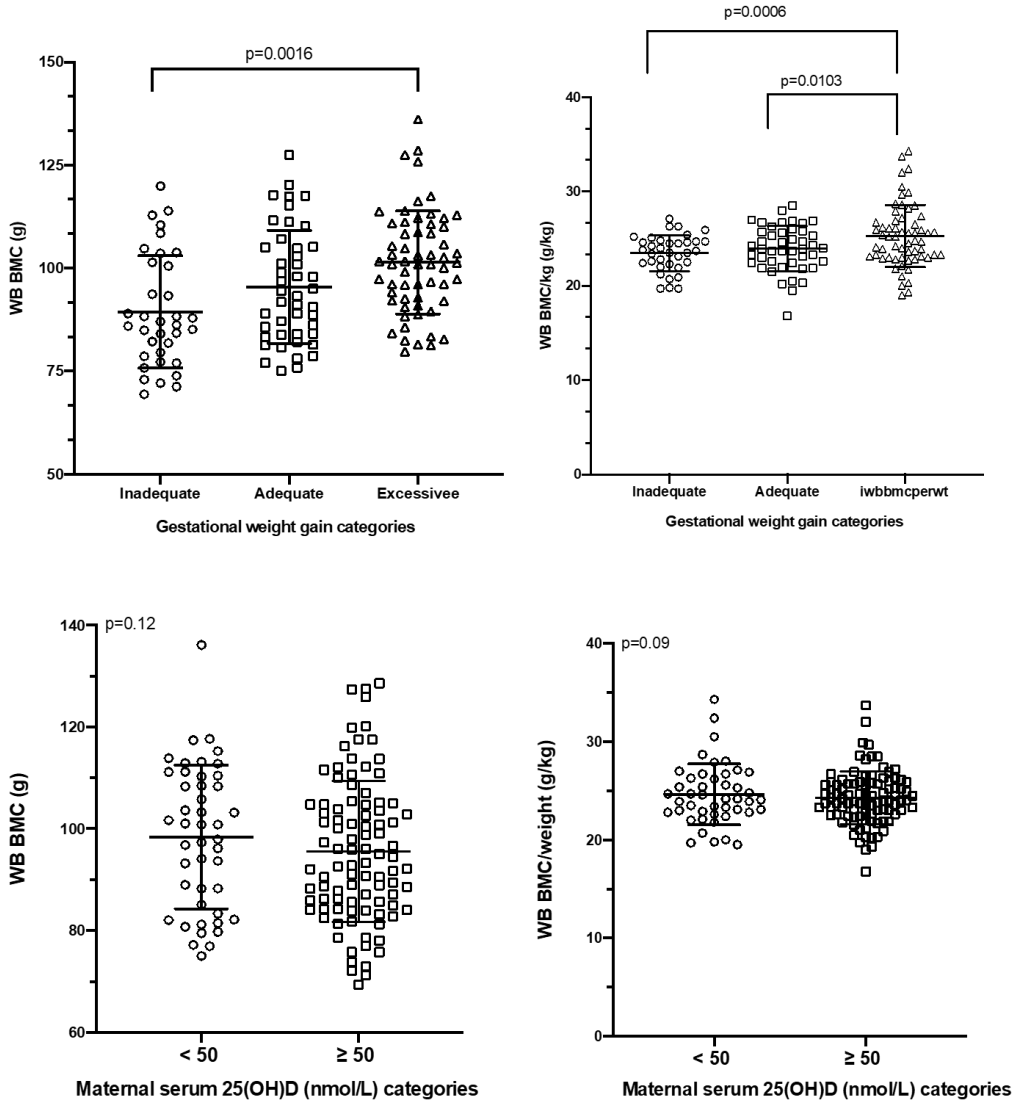
Participant flow diagram illustrating the number of mother-infant dyads assessed for eligibility 24-36 hours after delivery, enrolled to newborn screening, screened, and enrolled to post-natal study with distribution according to maternal vitamin D status (insufficient vs sufficient) and GWG categories (inadequate, adequate, and excessive). Abbreviations: SGA: small for gestational age, LGA: large for gestational age, 25(OH)D: 25-hydroxyvitamin D, and GWG: gestational weight gain.

Figure 4.2. Maternal 25(OH)D concentration as a correlate of vitamin D status of neonates at birth and 1 month of age



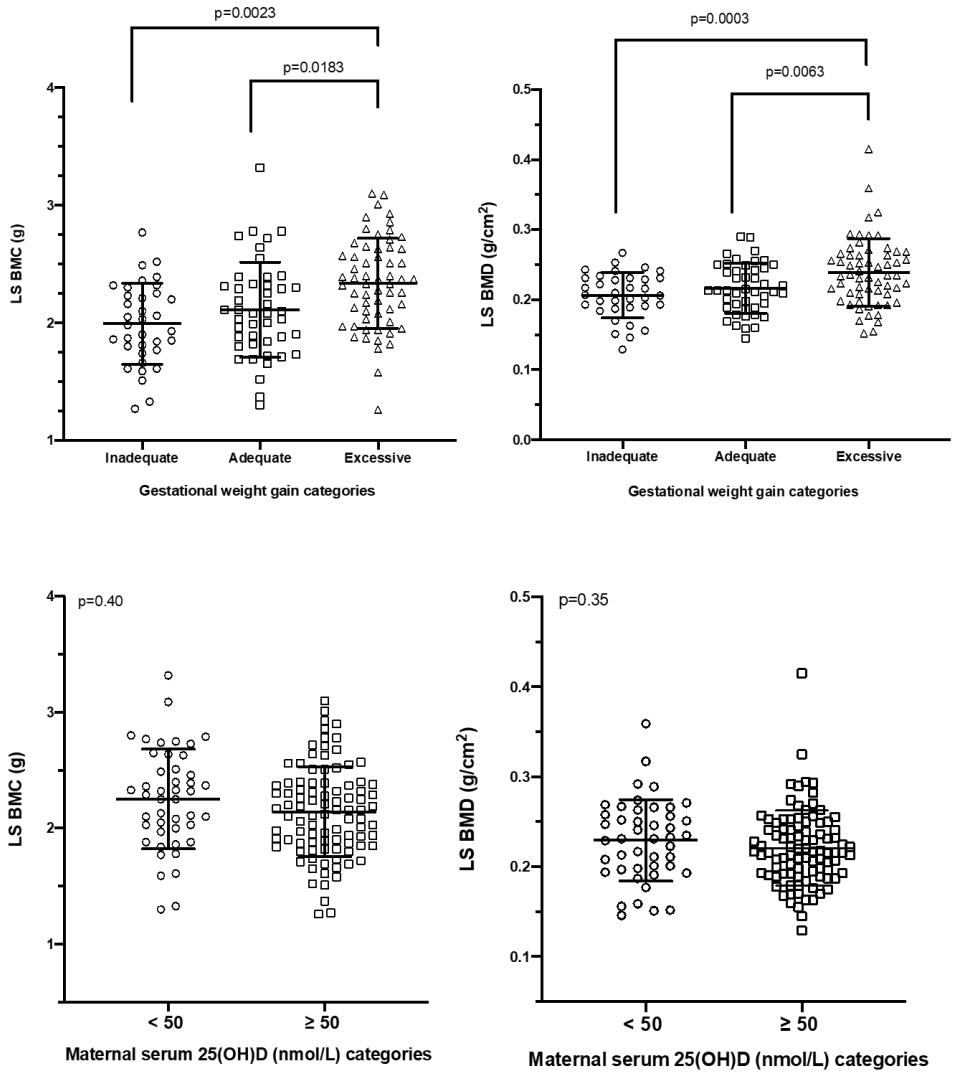
Linear regression of infant birth (7.9-100.4 nmol/L, n=142) and neonatal (15.9-106.2 nmol/L, n=142) serum 25(OH)D concentrations on maternal (15.0-155.3 nmol/L, n=142) serum 25(OH)D concentrations. Data were analyzed using linear mixed model ($p<0.05$). Abbreviations: 25(OH)D: 25-hydroxyvitamin D.

Figure 4.3. Whole body bone mass in the neonatal period according to gestational weight gain categories and maternal 25(OH)D status



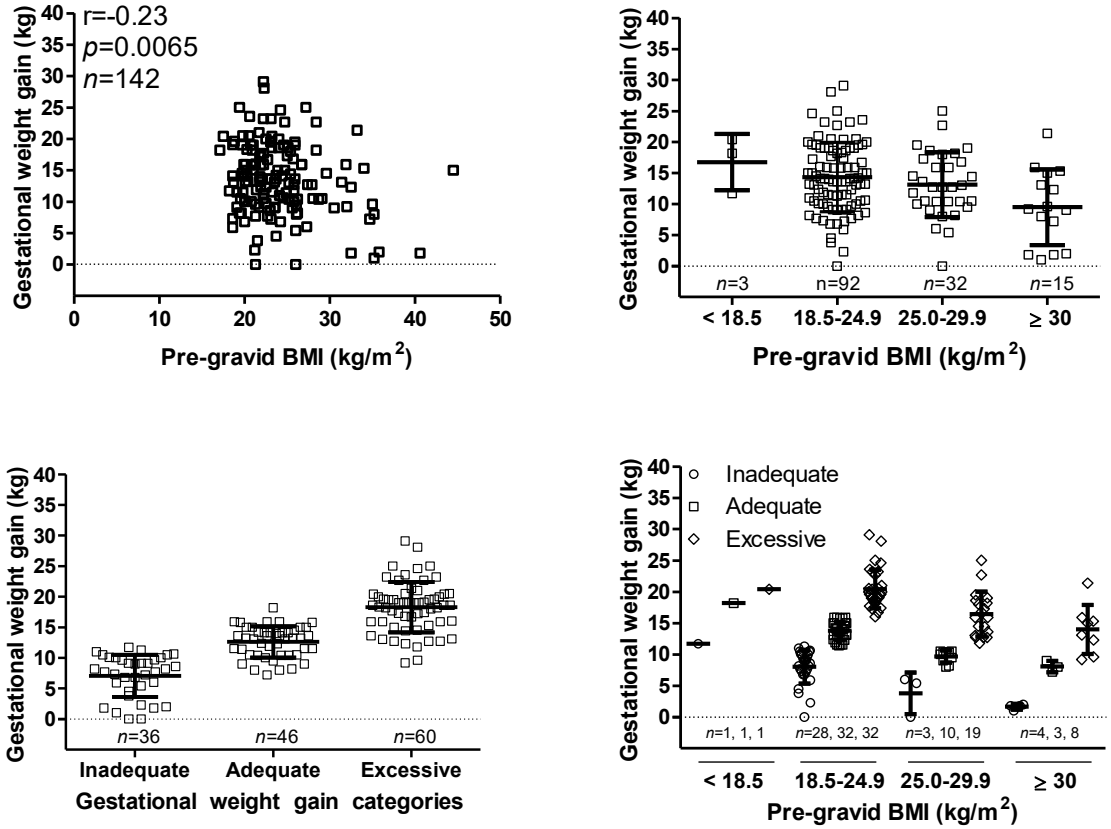
Data are reported as mean ± SD and were analyzed using linear mixed model accounting for the fixed effects of infant's sex, age, and LAZ-score and mother's 25(OH)D categories, pre-pregnancy BMI categories, gestational weight gain categories, education, self-reported population group as well as family income ($p<0.05$, post hoc adjustment). Abbreviations: 25(OH)D: 25-hydroxyvitamin D, WB BMC: whole body bone mineral content, WB BMC/kg: whole body bone mineral content per kilogram body weight.

Figure 4.4 Lumbar spine bone mass in the neonatal period according to gestational weight gain categories and maternal 25(OH)D status



Data are reported as mean ± SD and were analyzed using linear mixed model accounting for the fixed effects of infant's sex, age, and LAZ-score and mother's 25(OH)D categories, pre-pregnancy BMI categories, gestational weight gain categories, education, self-reported population group as well as family income ($p<0.05$, post hoc adjustment). Abbreviations: 25(OH)D: 25-hydroxyvitamin D, LS BMC: lumbar spine bone mineral content, and LS BMD: lumbar spine bone mineral density.

Supplementary Figure S4.1. Gestational weight gain relative to pre-gravid BMI expressed as continuous data and according to recommended categories.



Data are reported as mean ± SD. Data were analyzed using Pearson correlation for gestational weight gain (kg) according to pre-gravid BMI (kg/m^2). Pregnancy weight gain (kg) is descriptively shown relative to pre-gravid BMI categories and expressed as inadequate, adequate or excessive according to pre-gravid BMI categories.

Table 4.1. Maternal and infant characteristics according to maternal 25(OH)D and gestational weight gain categories.

	Maternal 25(OH)D (nmol/L)			Gestational Weight Gain			
	<50 (n=45)	**≥50 (n=97)**	**p-value**	**Inadequate (n=36)**	**Adequate (n=46)**	**Excessive (n=60)**	**p-value**
Infant							
Sex, n (%)			0.80				0.71
Male	27 (19.0)	56 (39.4)		21 (14.8)	29 (20.4)	33 (23.2)	
Female	18 (12.7)	41 (28.9)		15 (10.6)	17 (12.0)	27 (19.0)	
WAZ at birth	0.3 ± 0.8	0.1 ± 0.7	0.12	−0.3 ± 0.7 [a]	0.2 ± 0.8 [b]	0.4 ± 0.7 [b]	0.0002
Age (mo) at follow-up	0.7 ± 0.2	0.7 ± 0.2	0.31	0.7 ± 0.3	0.7 ± 0.2	0.7 ± 0.2	0.83
WAZ at follow-up	0.1 ± 0.8	−0.2 ± 0.8	0.13	−0.4 ± 0.8 [a]	−0.0 ± 0.8 [ab]	0.1 ± 0.7 [b]	0.02
LAZ at follow-up	0.2 ± 1.0	−0.1 ± 0.9	0.05	−0.4 ± 0.9 [a]	−0.0 ± 0.9 [ab]	0.2 ± 0.9 [b]	0.02
Mother							
Age at delivery (y)	31.8 ± 5.3	32.4 ± 4.0	0.45	31.9 ± 5.0	33.0 ± 3.8	31.7 ± 4.5	0.32
Gravida, n (%)			0.11				0.34
1	18 (12.7)	26 (18.3)		14 (9.9)	11 (7.8)	19 (13.4)	
>1	27 (19.0)	71 (50.0)		22 (15.5)	35 (24.6)	41 (28.8)	
Self-reported population group, n (%)			0.0003				0.12
White	15 (10.6)	64 (45.1)		15 (10.6)	26 (18.3)	38 (26.8)	
All other[†]	30 (21.1)	33 (23.2)		21 (14.8)	20 (14.1)	22 (15.4)	
Education, n (%)			0.0266				0.97
University	31 (21.8)	68 (47.9)		24 (16.9)	33 (23.2)	42 (29.6)	
College/Vocational	6 (4.2)	24 (16.9)		9 (6.3)	9 (6.3)	12 (8.5)	
Elementary/High school	8 (5.6)	5 (3.6)		3 (2.1)	4 (2.8)	6 (4.3)	
Family income, n (%)			0.15				0.37
≥70,000 CAD	20 (14.1)	60 (42.3)		20 (14.1)	26 (18.3)	34 (23.9)	
<70,000 CAD	17 (12.0)	24 (16.9)		9 (6.3)	11 (7.8)	21 (14.8)	
Not reported	8 (5.6)	13 (9.1)		7 (4.9)	9 (6.3)	5 (3.6)	
Pre-pregnancy BMI			0.05				0.03
<25 kg/m^2	25 (17.6)	70 (49.3)		29 (20.4)	33 (23.2)	33 (23.2)	

≥25 kg/m^2	20 (14.1)	27 (19.0)	7 (5.0)	13 (9.2)	27 (19.0)

Data are reported as mean ± SD or n (%) and were analyzed using a linear mixed model for continuous variables accounting for the fixed effect studied and using Chi square or Fisher's test for categorical variables. Distinct letter superscripts (^{a,b}) indicate statistically significant differences between gestational weight gain categories ($p<0.05$, post hoc adjustment); values that share a common superscript are not different from one another. † All other self-reported population groups include South Asian, Chinese, Black, Filipino, Latin American, Arab, Southeast Asian, West Asian, Korean, Japanese, and other. Abbreviations: 25(OH)D: 25-hydroxyvitamin D, GA: gestational age, WAZ: weight-for-age z-scores, LAZ: length-for-age z-scores, CAD: Canadian dollars

Table S.4.1. Maternal nutrient intake from food and supplements during pregnancy according to gestational weight gain categories

	Gestational weight gain			
	Inadequate (n=31/35)	**Adequate (n=42/45)**	**Excessive (n=51/59)**	**p-value**
Energy (kcal)	2170 ±735	2161±952	2080±787	0.86
	1954 (1782-2426)	1964 (1508-2744)	1831 (1458-2466)	
Protein (g)	98±33	97±45	92±35	0.71
	87 (80-114)	90 (67-114)	84 (65-118)	
Carbohydrates (g)	292±134	280±141	270±105	0.74
	262 (228-310)	251 (178-336)	252 (186-338)	
Fat (g)	74±22	79±33	76±34	0.80
	70 (58-83)	72(54-92)	66 (50-96)	
Vitamin D (IU)	615 ± 252	646±285	613±229	0.80
	593 (503-766)	635 (468-809)	586 (479-788)	
Calcium (mg)	1359±426	1399±587	1364±503	0.93
	1233 (974-1673)	1289 (943-1695)	1287 (1034-1747)	
Magnesium (mg)	471±152	486±165	476±149	0.91
	431 (370-548)	444 (350-608)	459 (351-599)	
Phosphorus (mg)	1563±519	1571±710	1471±578	0.69
	1426 (1197-1898)	1388 (1040-1947)	1401(1003-1812)	

Data are reported as mean±SD and median (IQR). Differences between the means of the different GWG categories were analyzed using linear mixed model accounting for the fixed effect studied.
Distinct letter superscripts (^{a,b,c}) indicate statistically significant differences between gestational weight gain categories ($p<0.05$, post hoc adjustment); values that share a common superscript are not different from one another.
Abbreviations: IU: international units.

Table S4.2. Correlates of neonatal bone mass tested using linear mixed model analysis

	Regression coefficients	p-values	95% CI
Infant WB BMC g (R^2 0.43, R^2_{adj} 0.42)			
Infant Factors			
Sex (male; referent: female)	5.70	0.0035	1.91, 9.48
Age (mo)	16.64	<0.0001	8.66, 24.62
LAZ	5.09	<0.0001	2.94, 7.24
Maternal Factors			
Serum 25(OH)D (<50 nmol/L; referent: ≥50 nmol/L)	2.96	0.19	-1.49, 7.40
GWG (insufficient; referent: adequate)	-5.14	0.0010	-10.22, -0.06
Pre-pregnancy BMI (<25 kg/m^2; referent: ≥25 kg/m^2)	2.43	0.25	-1.72, 6.59
Self-identified population group (all others; referent: white)	1.92	0.36	-2.36, 6.19
Education (elementary/high school; referent: university)	-1.61	0.13	-8.36, 5.13
Family yearly income (<70,000 CAD; referent: ≥70,000 CAD)	-1.91	0.0161	- 6.54, 2.72
Infant WB BMC/kg g/kg (R^2 0.27, R^2_{adj} 0.26)			
Infant Factors			
Sex (male; referent: female)	0.25	0.57	-0.61, 1.12
Age (mo)	-3.99	<0.0001	-5.81, -2.16
LAZ	-0.72	0.0044	-1.21, -0.23
Maternal Factors			
Serum 25(OH)D (<50 nmol/L; referent: ≥50 nmol/L)	0.82	0.11	-0.19, 1.84
GWG (insufficient; referent: adequate)	-0.77	0.0002	-1.93, 0.40
Pre-pregnancy BMI (<25 kg/m^2; referent: ≥25 kg/m^2)	0.50	0.29	-0.44, 1.45
Self-identified population group (all others; referent: white)	0.01	1.00	-0.97, 0.99
Education (elementary/high school; referent: university)	-0.70	0.0823	-2.25, 0.84
Family yearly income (<70,000 CAD; referent: ≥70,000 CAD)	-0.28	0.19	-1.34, 0.78
Infant LS BMC g (R^2 0.28, R^2_{adj} 0.28)			
Infant Factors			
Sex (male; referent: female)	-0.06	0.35	-0.18, 0.06
Age (mo)	-0.34	0.0104	-0.60, -0.08

LAZ	0.11	0.0024	0.04, 0.18
Maternal Factors			
Serum 25(OH)D (<50 nmol/L; referent: ≥50 nmol/L)	0.08	0.28	-0.07, 0.22
GWG (inadequate; referent: adequate)	-0.07	0.0051	-0.24, 0.09
Pre-pregnancy BMI (<25 kg/m^2; referent: ≥25 kg/m^2)	-0.05	0.43	-0.19, 0.08
Self-identified population group (all others; referent: white)	-0.02	0.79	-0.16, 0.12
Education (elementary/high school; referent: university)	-0.13	0.43	-0.35, 0.09
Family yearly income (<70,000 CAD; referent: ≥70,000 CAD)	-0.03	0.56	-0.19, 0.12
Infant LS BMD g/cm^2 (R^2 0.29, R^2_{adj} 0.29)			
Infant Factors			
Sex (male; referent: female)	-0.01	0.0236	-0.03, -0.00
Age (mo)	-0.06	<0.0001	-0.09, -0.04
LAZ	-0.00	0.74	-0.01, 0.01
Maternal Factors			
Serum 25(OH)D (<50 nmol/L; referent: ≥50 nmol/L)	0.01	0.21	-0.01, 0.02
GWG (inadequate; referent: adequate)	-0.01	0.0018	-0.03, 0.01
Pre-pregnancy BMI (<25 kg/m^2; referent: ≥25 kg/m^2)	-0.00	0.69	-0.02, 0.01
Self-identified population group as (all others; referent: white)	-0.01	0.28	-0.02, 0.01
Education (elementary/high school; referent: university)	-0.01	0.38	-0.04, 0.01
Family yearly income (<70,000 CAD; referent: ≥70,000 CAD)	0.00	0.82	-0.01, 0.02

Data are regression coefficients (95% CI) calculated using linear mixed model accounting for the fixed effects of infant's sex, age, and LAZ-score and mother's 25(OH)D categories, pre-pregnancy BMI categories, gestational weight gain categories, education, self-reported population group as well as family income. Abbreviations: WB BMC: whole body bone mineral content, LAZ: length-for-age z-score, 25(OH)D: 25-hydroxyvitamin D, GWG: gestational weight gain, CAD: Canadian dollars, WB BMC/kg: whole body bone mineral content per kilogram body weight, LS BMC: lumbar spine bone mineral content, and LS BMD: lumbar spine bone mineral density

Table S4.3. Correlates of neonatal bone mass tested using linear mixed model analysis

	Regression coefficients	p-values	95% CI
Infant WB BMC g (R^2 0.42, R^2_{adj} 0.42)			
Infant Factors			
Sex (male; referent: female)	5.57	0.0043	1.78, 9.36
Age (mo)	18.63	<0.0001	10.67, 26.58
LAZ	5.67	<0.0001	3.60, 7.74
Maternal Factors			
Serum 25(OH)D (per each nmol/L)	-0.04	0.32	-0.12, 0.04
GWG (per each kg)	0.64	0.0005	0.29, 0.99
Pre-pregnancy BMI (per each kg/m^2)	0.39	0.0778	-0.04, 0.82
Self-identified population group (all others; referent: white)	2.47	0.26	-1.83, 6.78
Education (elementary/high school; referent: university)	-1.41	0.16	-8.11, 5.30
Family yearly income (<70,000 CAD; referent: ≥70,000 CAD)	-2.34	0.0051	-6.99, 2.30
Infant WB BMC/weight g/kg (R^2 0.26, R^2_{adj} 0.25)			
Infant Factors			
Sex (male; referent: female)	0.20	0.65	-0.67, 1.07
Age (mo)	-3.51	0.0002	-5.33, -1.68
LAZ	-0.57	0.0174	-1.05, -0.10
Maternal Factors			
Serum 25(OH)D (per each nmol/L)	-0.01	0.20	-0.03, 0.01
GWG (per each kg)	0.16	0.0001	0.08, 0.24
Pre-pregnancy BMI (per each kg/m^2)	0.09	0.07	-0.01, 0.19
Self-identified population group (all others; referent: white)	0.14	0.78	-0.85, 1.13
Education (elementary/high school; referent: university)	-0.63	0.10	-2.17, 0.90
Family yearly income (<70,000 CAD; referent: ≥70,000 CAD)	-0.35	0.08	-1.41, 0.72
Infant LS BMC g (R^2 0.28, R^2_{adj} 0.27)			
Infant Factors			
Sex (male; referent: female)	-0.06	0.33	-0.18, 0.06
Age (mo)	-0.29	0.0289	-0.55, -0.03
LAZ	0.13	0.0002	0.06, 0.20

Maternal Factors			
Serum 25(OH)D (per each nmol/L)	-0.0002	0.85	-0.003, 0.002
GWG (per each kg)	0.02	0.0031	0.01, 0.02
Pre-pregnancy BMI (per each kg/m^2)	0.02	0.0045	0.01, 0.03
Self-identified population group (all others; referent: white)	0.01	0.85	-0.13, 0.15
Education (elementary/high school; referent: university)	-0.13	0.43	-0.34, 0.09
Family yearly income (<70,000 CAD; referent: ≥70,000 CAD)	-0.04	0.34	-0.19, 0.12
Infant LS BMD g/cm^2 (R^2 0.30, R^2_{adj} 0.29)			
Infant Factors			
Sex (male; referent: female)	-0.02	0.0201	-0.03, 0.00
Age (mo)	-0.06	<0.0001	-0.08, -0.03
LAZ	-0.001	0.83	-0.006, 0.008
Maternal Factors			
Serum 25(OH)D (per each nmol/L)	0.00004	0.75	-0.0003, 0.0002
GWG (per each kg)	0.002	0.0005	0.001, 0.003
Pre-pregnancy BMI (per each kg/m^2)	0.002	0.0034	0.001, 0.002
Self-identified population group as (all others; referent: white)	-0.004	0.58	-0.019, 0.010
Education (elementary/high school; referent: university)	-0.01	0.38	-0.04, 0.01
Family yearly income (<70,000 CAD; referent: ≥70,000 CAD)	0.003	0.63	-0.013, 0.019

Data are regression coefficients (95% CI) calculated using linear mixed model accounting for the fixed effects of infant's sex, age, and LAZ-score and mother's 25(OH)D concentration, pre-pregnancy BMI, gestational weight gain, education, self-reported population group as well as family income.

Abbreviations: WB BMC: whole body bone mineral content, LAZ: length-for-age z-score, 25(OH)D: 25-hydroxyvitamin D, GWG: gestational weight gain, CAD: Canadian dollars, WB BMC/kg: whole body bone mineral content per kilogram body weight, LS BMC: lumbar spine bone mineral content, and LS BMD: lumbar spine bone mineral densit

Bridge statement 3

The previous chapter is focused on the neonatal phase, a phase of rapid growth and transition from the intra-uterine to the extra-uterine environment. Gestational weight gain (GWG) was found to relate to infantile bone mass. While WB BMC, LS BMC, and LS BMD were not found to be compromised in infants born to mothers with inadequate GWG compared to mothers with adequate GWG; they were found to be greater in infants born to mothers who experienced excessive GWG compared to others (insufficient and adequate GWG). These effects were partially driven by size. As for maternal vitamin D status, it was not found to relate to neonatal bone mass which may be suggestive of non-compromised bone mineral accretion in the neonate unless in the case of severe maternal vitamin D deficiency. Vitamin D may have a stronger role to play postnatally compared to the intra-uterine period given all the different placental regulations that take place during gestation and the role other hormones play in mineral transfer to the fetus including parathyroid hormone and parathyroid hormone related peptide. As described in the literature review, vitamin D supplementation in amounts equivalent to what is found in 1 teaspoon of cod liver oil (400 IU/d) is required in infants to help prevent vitamin D deficiency rickets. This supplementation is supported and endorsed by multiple nutrition societies and regulatory bodies. However, it is not clear if higher doses of vitamin D supplementation may be beneficial in infants with serum 25-hydroxyvitamin D concentrations that do not meet the cut-point for sufficiency needed to support bone health. The latter is explored in the next chapter.

Chapter 5: Manuscript 3

Effect of vitamin D supplementation (400 vs 1000 IU/d) on bone health parameters: a double-blinded randomized controlled trial in infants born with insufficient vitamin D status

Effect of vitamin D supplementation (400 vs 1000 IU/d) on bone health parameters: a double-blinded randomized controlled trial in infants born at elevated risk of insufficient vitamin D status

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5.1 Abstract

Introduction: An Adequate Intake (AI) of 400 IU/d of supplemental vitamin D was set to maintain serum 25-hydroxyvitamin D (25(OH)D) concentration in the 40 to 50 nmol/L range and in support of bone health. This study tested whether a higher supplemental dose (1000 vs. 400 IU/d) is required in infants born with 25(OH)D <50 nmol/L (at elevated risk of insufficient status) to support bone mineral accretion across infancy.

Methods: Term-born, healthy, breastfed infants were recruited from Montreal, Canada (March 2016-March 2019) to participate in a double blinded randomized parallel group controlled trial. Capillary blood was collected 24-36 hours after birth to measure serum total 25(OH)D. Infants with 25(OH)D concentrations <50 nmol/L were randomized to one of two trial groups (400 or 1000 IU/d of oral vitamin D_3 supplementation) and those with birth serum 25(OH)D concentrations ≥50 nmol/L were allocated to a non-randomized reference group (400 IU/d of oral vitamin D_3 supplementation). Infants were followed at 1, 3, 6, and 12 months of age for whole body bone mineral content (BMC), lumbar spine BMC and bone mineral density (BMD), serum $25(OH)D_3$, $24,25(OH)_2D_3$, 3-epi-$25(OH)D_3$, $1,25(OH)_2D_3$, and bone biomarkers (plasma procollagen Type-I N-terminal propeptide and urinary alpha telopeptide of Type-I collagen).

Results: Neonates (n=81 male; n=58 female) were born at 39.7±1.0 weeks of gestation and of appropriate birth weight (3.4±0.4 kg). Whole body BMC, lumbar spine BMC and BMD, and bone biomarkers were not different ($p \geq 0.05$) among groups across the study. Both trial groups had lower serum $25(OH)D_3$ at baseline in comparison with the reference group ($p<0.01$). Serum $25(OH)D_3$ were higher in the 1000 IU/d group compared to the 400 IU/d group at 3, 6, and 12 months of age and compared to the reference group at 3 and 6 months of age ($p<0.002$). Concentrations of $24,25(OH)_2D_3$ were higher at baseline in the reference group

compared to both trial groups ($p<0.01$). At 3, 6, and 12 months of age, the1000 IU/d trial group had higher $24,25(OH)_2D_3$ concentrations than the 400 IU/d trial group ($p<0.0001$). In addition, the 1000 IU/d trial group had higher $24,25(OH)_2D_3$ concentrations at 3 and 6 months of age ($p<0.0001$) compared to the reference group.

Conclusion: A higher dose of vitamin D supplementation in infants born at elevated risk of insufficient vitamin D status does not present advantages in bone outcomes in infancy. This study supports the AI of 400 IU/d currently endorsed by public health policies in North America is thus suitable for breastfed infants born with 25(OH)D <50 nmol/L.

5.2 Introduction

Vitamin D status at birth spans the spectrum from deficiency to sufficiency (4, 5, 203) and is reflective of maternal-fetal transfer of 25-hydroxyvitamin D (25(OH)D) during gestation (212). When the expecting mother has 25(OH)D below 50 nmol/L, infants are born with elevated risk of vitamin D insufficiency (25(OH)D <50 nmol/L) or are deficient (25(OH)D <30 nmol/L) (213). In addition, mother's milk, the best source of nutrition for infants, does not provide vitamin D in amounts consistent with the level of the Adequate Intake (AI: 400 IU/d) (214) unless mothers are supplemented with relatively high doses of vitamin D (>1000 IU/d), a practice not currently endorsed by public health policies (215). Therefore, public health policies and interventions in North America, which are inherently population-based, recommend that all breastfed infants begin vitamin D supplementation (400 IU/d) shortly after birth (2, 23). The supplemental dose of vitamin D is set in accordance with intakes that are safe and maintain serum 25(OH)D concentration, the best marker of vitamin D status, in the range of 40 to 50 nmol/L in support of bone health.

Serum 25(OH)D responds to supplementation (84) and shows a heightened response in infants with lower vitamin D status (80). Adherence to 400 IU/d of vitamin D supplementation during the first year of life has been established to prevent vitamin D deficiency rickets (216). Early studies by Greer et al. (133, 217) reported on the absence of response of forearm bone mineral content (BMC) to supplementation (400 IU/d) compared to placebo in infants using single photon absorptiometry. A double blinded randomized controlled parallel group trial conducted in infants with sufficient vitamin D status, showed that 25(OH)D concentrations ≥50 nmol/L support bone health with no differences in whole body (WB) BMC, lumbar spine (LS) BMC, and LS bone mineral density (BMD) in response to different doses of vitamin D supplementation (1). One trial (84) showed minimal differences in tibial total bone and cortical bone area in response to higher

doses of vitamin D supplementation; use of peripheral quantitative tomography in infants is however debatable. A third trial in infants with insufficient vitamin D status at 28 days of age (218) showed that 25(OH)D concentrations responded to supplementation from 2 to 9 months of age. WB BMC was an optional measurement and not standardized by age, limiting interpretation of the data. Therefore, it remains unclear whether BMC of infants born with insufficient vitamin D status is compromised, and whether a higher dose of vitamin D supplementation is required to build tissue vitamin D stores and to support bone health across infancy.

The objective of the current study was to investigate whether correction of insufficient vitamin D status early in infancy using 1000 IU/d of vitamin D normalizes bone mass across infancy. Our hypothesis was that neonates with insufficient vitamin D status, provided with a supplement of 400 IU/d (compared to 1000 IU/d), have lower bone mineral accretion by 3 months without resolution at 12 months of age.

5.3 Methods

Study design

This is a double blinded randomized controlled parallel group trial registered with clinicaltrials.gov (NCT02563015). The trial was designed with lean body mass as the primary outcome and assessment of bone mass among the secondary outcomes.

Mothers and their infants were screened during the first 24-36 hours after delivery at the Lakeshore General Hospital, Montreal, Canada (March 2016 - March 2019). They were then recruited to the postnatal study at the Mary Emily Clinical Nutrition Research Unit of McGill University, Montreal, Canada which included a total of 4 study visits at each of 1, 3, 6, and 12 months of age.

Only healthy, term, appropriate weight for gestational age singleton infants of a healthy pregnancy and whose mothers had an intent to breastfeed for at least 3 months were recruited at birth. Medical diseases/conditions (diabetes, pre-eclampsia, celiac disease, and inflammatory bowel disease), smoking during pregnancy, and the use of prescription medication(s) that could impact on vitamin D and/or bone metabolism and/or fetal growth were reasons for exclusion. Capillary blood was sampled from newborns for subsequent measurement of serum 25(OH)D concentrations. At the 1 month postnatal visit at McGill University, infants with birth serum 25(OH)D concentrations <50 nmol/L (labeled as insufficient; in contrast with sufficient status defined as serum 25(OH)D ≥50 nmol/L by the Institute of Medicine) were randomized to one of two trial groups and those with birth serum 25(OH)D concentrations ≥50 nmol/L (sufficient vitamin D status) were allocated to a non-randomized reference group. Infants in the trial groups were randomized 1:1 to receive either 400 or 1000 IU/d of oral vitamin D_3 supplementation from 1 to 12 months of age. Randomization was stratified by Fitzpatrick skin type (FI-III and FIV-VIl) according to individual typology angle (ITA°) calculated using ITA° = arc tan (L*-50)/b*) x 180/3.14159. Colorimetric parameters: luminance, L* and the yellow chroma component, b* (219) were measured using a spectrophotometer (CM-700d/600d, Konica Minolta, USA) at the inner upper arm site for constitutive pigmentation. Skin type was classified from very light, type I (ITA°>55), type II (41<ITA°<55), type III (28<ITA°<41), type IV (10<ITA°<28), type V (-30<ITA°<10), to dark, type VI (ITA°< -30). Participant ID numbers were allocated to 400 or 1000 IU dosage groups within blocks of 4 for each strata. Infants in the reference group received the standard of care of vitamin D supplementation (400 IU/d). This group served as a reference group for bone measurements (dual energy x-ray absorptiometry (DXA) and biomarkers of bone metabolism) given the lack of reference data in infancy.

Supplements, blinding, randomization, and adherence

Water-based vitamin D_3 supplements were provided by Euro-Pharm International Canada Inc. Both the 400 and the 1000 IU/d doses were packaged in indistinguishable bottles and shared the same physical characteristics (color, texture, taste, and smell). Doses were verified by external testing (Sandoz, Novartis division) and confirmed to have a 95% accuracy and were shelf stable for 12 months. A code was provided on the bottle by Euro-Pharm which allowed unblinding of its dose if needed for safety purposes. Blinding codes were also provided in sealed envelopes by Euro-Pharm to each of the principal investigator and the medical team. At each of the 1 and 6 months study visits, families were provided with 4 bottles of supplements of 50 mL each and a 1 mL dropper. A block randomization was done using block sizes 152 of 4 (2 for the 400 IU trial group and 2 for the 1000 IU group) and Random Allocation Software 153.

At the 1 month visit, they were instructed by a registered nurse to provide the infants with 1 mL of supplement daily. They were also asked to fill out a compliance calendar on which they were asked to mark the days on which vitamin D was given to the infant. Adherence to supplementation was then estimated at each study visit. Researchers and families were blinded throughout the study until its completion and after the data were audited independently by two investigators.

Obstetric history and anthropometry

At the hospital, data on date of birth, sex, gestational age, and birth weight of the infants in addition to obstetric history including mother's age, height, weight (pre-gravid and at delivery), and parity (primiparous/multiparous) were obtained from the medical records. Maternal pre-gravid BMI was calculated using the pre-gravid weight and height obtained from the medical records. Infant birth weight-for-age z-scores were calculated using World Health Organization AnthroPlus

Software. Infant length and head circumference measurements at birth were not standardized and therefore were not used in this report.

At each study visit, infant nude weight (dynamic scale, Mettler-Toledo Inc.) was measured to the nearest gram and length (infantometer, O'Learly Length Boards, Ellard Instrumentation Ltd.) and head circumference (non-stretchable tape, Perspective Enterprises) were measured to the nearest 0.1 cm; and corresponding z-scores (World Health Organization AnthroPlus Software) were calculated.

Demographic data

During the newborn screening at the hospital, information on mother's self-reported population group (white, or all others), education (elementary/high school, college/vocational school, or university), as well as annual family income (<70,000 CAD, ≥70,000 CAD, or not reported) were surveyed (189). Participant family demographics were collected to help improve health equity among different population groups.

Dietary and lifestyle data

At the hospital, use of multivitamins and exercise 3 months prior to and during pregnancy was surveyed and studied using categorical variables (Yes, No). At the 1 month visit, nutritional intake during pregnancy from food and supplements was assessed using a validated 9-months semi-quantitative food frequency questionnaire (FFQ) (190). Estimations of total energy, protein, carbohydrates, fat as well as calcium, vitamin D, magnesium, and phosphorous from both supplements and food were obtained using the Canadian Nutrient File. All data from the FFQ were tested as continuous and quartile variables.

In addition, at each study visit, breastfeeding status of the mother (Yes: exclusive or mixed, or No) was surveyed. Age of introduction of solid foods was collected as part of a milestone questionnaire provided to the parents at the 6 months visit.

Bone outcomes

BMC (WB and LS 1-4)) and BMD (LS, 1-4) of the infants were assessed using dual-energy x-ray absorptiometry (DXA) (fan beam, APEX 13.3:3, Hologic 4500A Discovery Series). Infant WB mode and anterior posterior spine mode were used, respectively, for WB and LS scans and scans were obtained using array mode and as per the recommendations of the International Society for Clinical Densitometry (125). Manual bone edge detection was used if needed to define regions of interest including head and LS (1-4). WB BMC and LS BMC accretion rates (baseline to 3 months, 3 months to 6 months, and 6 months to 12 months) were calculated as: $\frac{\Delta \text{ BMC}}{\Delta \text{ age}} \times 100$. Coefficient of variation (% CV) for each of BMC, BMD, and bone area were <1% based on Hologic spine phantom No. 14774. Infants were scanned by a certified DXA operator and in minimal clothing (diaper and light gown). In order to help standardize their positioning and avoid movement artifacts, they were wrapped in a light flannel and rocked to sleep.

Biochemistry

Blood and urinary collection

Within 24-36 h after birth and at each postnatal visit, infant capillary blood samples (0.5 mL) were obtained using heel lance. Maternal venous blood (5 mL) was collected at the baseline postnatal visit. All blood samples were centrifuged (4000xg, 6°C) for 20 min and serum and plasma were collected and kept at -80°C until analysis of vitamin D metabolites and biomarkers of bone metabolism. Spot urine samples were collected from the infant using a pediatric urine-

bag at each postnatal visit and stored at -80°C until analyzed for determination of calcium to creatinine ratio, phosphate to creatinine ratio, and biomarkers of bone metabolism.

Serum 25(OH)D: 24-36 h after birth

At the hospital, infant capillary blood samples (0.5 mL) were collected during clinical newborn phenylketonuria screening in order to minimize frequency of blood sampling.

For facilitated recruitment to the trial early postnatally, serum 25(OH)D concentrations at birth were assessed through an automated chemiluminescent immunoassay (CLIA, Liaison, Diasorin Inc.) and standardized to National Institute of Standards and Technology (NIST) reference measurements (193) using Deming regression (standardized concentration (nmol/L) = 0.9634 measured concentration (nmol/L) + 3.122). Serum 25(OH)D greater or equal to 50 nmol/L was considered sufficient while serum 25(OH)D below 50 nmol/L was classified as insufficient (2). The laboratory participated in Vitamin D External Quality Assessment Scheme and received a certificate of proficiency. National Institute of Standards and Technology (NIST) Standard Reference Materials 972a Level 1-4 quality control samples controls were used to measure accuracy which was: 97.4%. Testing for precision was done using both NIST972a and internal laboratory controls; with inter-assay %CV <10% for both. Total 25(OH)D values were in good agreement with concentrations measured by liquid chromatography tandem mass spectrometry (LC-MS/MS) (Queen's University, Kingston, Ontario, Canada) with a mean difference of -0.8 nmol/L in 83 mother-infant samples.

Vitamin D metabolites: Postnatal study visits

Infant serum $25(OH)D_3$, 24,25-dihydroxyvitamin D_3 ($24,25(OH)_2D_3$) and 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) concentrations were measured using LC-MS/MS at Queen's University, Kingston, Canada. Using 100 μL of serum, $25(OH)D_3$ and $24,25(OH)_2D_3$ were

measured according to methods described elsewhere (220). In a subgroup with adequate sample volume remaining, $1,25(OH)_2D_3$ (100 µL) was measured using an adapted LC-MS/MS method (221). The laboratory participated in the the Vitamin D Standardization-Certification Program and the Vitamin D External Quality Assessment Scheme and obtained a certificate of proficiency. Accuracy was within 5% of the NIST standard reference materials with inter-assay %CV <10%.

Biomarkers of calcium homeostasis and bone metabolism

Plasma procollagen type I N-terminal propeptide (P1NP) and urinary alpha telopeptide of collagen I (CTX-I) were measured as the recommended reference markers for bone formation and resorption according to the International Osteoporosis Foundation (222). Plasma P1NP (Human P1NP ELISA, Creative Diagnostics, USA), CTX-I (CTX-I EIA, Immunodiagnostic Systems, UK), and parathyroid hormone (1-84 PTH; Human PTH EIA, Quidel, MicroVue, USA) were measured using immunoassays. All three analytes had an inter-assay %CV of <10%. Urinary calcium:creatinine and phosphate:creatinine ratios were measured in spot urine samples using an auto-analyzer (Beckman Coulter UniCel DxC600) at McGill University Health Centre clinical chemistry laboratory, certified by the International Organization for Standardization. Urinary CTX-I:creatinine ratios were also calculated. Infant blood-ionized calcium (iCa) was measured immediately in whole blood (65 µL) using blood gas analyzer (ABL80 FLEX Radiometer Medical A/S, Denmark) calibrated daily.

Ethical approval

Ethical approval was obtained from St. Mary's Hospital Research Ethics Committee which handles ethics at the Lakeshore General Hospital (SMHC 15-34), where newborn screening and recruitment of mother-infants dyads took place. The study was thus considered to be approved by McGill University's Institutional Review Board. The study was also reviewed and approved by

Health Canada Research Ethics Board (REB 2019-033H) and Privacy Management Division (HC-PR-2019-000024). Written consent was obtained from the parents at both the newborn screening and the baseline visit of the trial.

Statistical analysis

Sample estimate

This study is based on the secondary objective of the trial. The primary objective was focused on lean mass outcomes with the aim of recruiting a minimum of 46 infants per trial group and up to 74 to account for dropouts. For the current objective, using an alpha of 0.05 and power of 80%, an effect size of 0.68 based on differences in WB BMC/kg body weight between infants with serum 25(OH)D below 27.5 nmol/L (deficient at the time) and those with adequate serum 25(OH)D (147), the sample size required per trial group was 28.

Data are reported as mean ± SD or mean (95% CI) (continuous variables) or percentages (categorical variables). Proportions of infants with sufficient vitamin D status across time were compared between the groups using Chi square or Fisher's exact test. Differences in breastfeeding status, age of introduction of solid foods, maternal dietary intake from food and supplements during pregnancy between the groups were tested using linear fixed effect model (continuous variables) and using Chi square or Fisher's exact test (categorical variables).

Differences between the three groups over time in WB BMC, WB BMC accretion rate, LS BMC, LS BMC accretion rate, LS BMD, serum $25(OH)D_3$, $24,25(OH)_2D_3$, $25(OH)D:24,25(OH)_2D_3$, $1,25(OH)_2D_3$, and 3-epi-$25(OH)D_3$; plasma PTH and P1NP; blood-iCa, and urinary calcium:creatinine, phosphate:creatinine, and CTX-I:creatinine were tested using a linear mixed model. The variables tested as fixed effects included: group, timepoint, timepoint*group, and infant's sex. Random effect of the participant was accounted for in the

model. The Bayesian information criterion (BIC) and R-squared values were used to examine model fit. Tukey-Kramer tests were used for post-hoc comparisons with adjustment for multiple comparisons. Normality of the residuals was tested using Shapiro Wilk test. All statistical analyses were conducted using SAS University Edition (SAS Institute Inc., Cary, N.C.) and statistical significance was set at $p<0.05$ after adjustment for multiple comparisons. An intention to treat analysis was used.

5.4 Results

Out of 139 infants who met the inclusion criteria and were enrolled into the study, 49 were randomized to each of the 400 or 1000 IU trial groups **(Figure 5.1)** and 41 infants were allocated to the reference group. All infants were term and born of appropriate weight for gestational age as per the study inclusion criteria. The majority of infants in both groups were males, born during the vitamin D-synthesizing period, and to mothers who were highly educated. (**Table 5.1**). Weight, length, and head circumference increased with age and were not different among the groups **(Figure 5.2)**.

WB BMC was not different among groups at baseline and across all timepoints (**Figure 5.3**). WB BMC accretion rate (g/month) was also not found to differ among groups and both WB BMC and accretion rates differed by time (Figure 5.3). A total of 469 WB scans out of a possible 489 were obtained across all timepoints. The remaining 20 were not obtained due to movement artifact issues. As for the LS site, which is less likely to be subject to movement artifacts, a total of 484 scans were obtained out of a possible 489. LS BMC did not respond to supplementation but was shown to increase over time (Figure 5.3). Similarly, LS BMC accretion rate did not vary according to supplementation dose but varied overall according to time (Figure 5.4). LS BMD was also found

to vary over time with no differences between groups (Figure 5.3). Males had consistently higher WB BMC compared to females (p=0.0061). No sex differences were noted in LS BMC, and LS BMD.

Birth total serum 25(OH)D concentrations (CLIA) (unadjusted and adjusted) were not different among the trial groups, but were lower compared to the reference group as per the group allocation criteria. As for the LC-MS/MS data, at the 1 month visit, there were no differences in serum $25(OH)D_3$ among the randomized trial groups. Both trial groups had lower serum $25(OH)D_3$ in comparison with the non-randomized reference group at baseline (~1 month) (Table 5.1). Higher serum $25(OH)D_3$ concentrations were found to occur in the 1000 IU/d trial group at 3, 6, and 12 months of age compared to the 400 IU/d trial group (**Figure 5.4**). Higher serum $25(OH)D_3$ were also reported for the 1000 IU/d trial group compared to the reference group at 3 and 6 months of age ($p<0.0001$). Proportions of infants with sufficient vitamin D status were not different at any of the timepoints among the groups after adjustment for multiple comparisons ($p\geq0.05$) except for baseline (p=0.0003).

Concentrations of $24,25(OH)_2D_3$ were higher at baseline in the reference group compared to both trial groups ($p<0.01$). At 3, 6, and 12 months of age, the1000 IU/d trial group had higher $24,25(OH)_2D_3$ concentrations than the 400 IU/d trial group ($p\geq0.0040$) (Figure 5.4). In addition, the 1000 IU/d trial group had higher $24,25(OH)_2D_3$ concentrations at 3 and 6 months of age ($p<0.0001$) compared to the reference group. $25(OH)D_3$:$24,25(OH)_2D_3$ was found to be lower at 1 month of age in the reference group ($p<0.0001$) compared to both trial groups. At 3 months and 6 months of age, the 400 IU/d trial group and the reference group had higher $25(OH)D_3$:$24,25(OH)_2D_3$ compared to the 1000 IU/d trial group ($p<0.04$) (Figure 5.4). At 12 months of age, the reference groups had higher $25(OH)D_3$:$24,25(OH)_2D_3$ compared to the 1000

IU/d trial group (p=0.04). Serum 3-epi-25(OH)D_3 concentrations were higher in the 1000 IU/d trial group at each of 3 and 6 months of age compared to the 400 IU/d trial group (p<0.0001) and the reference group (p<0.0003) (Figure 5.4). Calcitriol concentrations, using a subgroup analysis, **(Table 5.2)**, were not different between groups (p=0.37) but were found to be lower at 1 month compared to 12 months of age (p=0.0012). When log_{10} PTH was added to the model, it was found to be a positive correlate of 1,25(OH)$_2D_3$ concentrations (ß=50.0; 95% CI: 22.56-77.41; p=0.0004).

As for biomarkers of bone formation and resorption, a log_{10} transformation was used given that the residuals were not normally distributed and an improvement in their distribution was observed with the transformed data. There were no differences between groups (urinary CTX-I:creatinine, p:0.36; plasma P1NP, p=0.85) and both biomarkers were found to vary with time (**Figure 5.5**).

A log_{10} transformation was also used for plasma PTH values given that the transformation improved the distribution of the residuals. Blood-iCa concentrations and log_{10} PTH did not differ among groups (iCa: p=0.73, log_{10} PTH: p=0.10) but did vary by time (**Figure 5.6**). Log_{10} PTH was lower at each of 1 and 3 months compared to 6 and 12 months (p<0.0001). Urinary calcium: creatinine and phosphate: creatinine were also not different among the three groups (calcium:creatinine: p=0.94, phosphate:creatinine : p=0.61) but did vary by time.

Proportions of infants who were breastfed (any type: exclusive or mixed with alternative feeds) at each timepoint and average age of introduction of solid foods are reported in **Table 5.3**. Maternal dietary intakes during pregnancy did not vary by group except for vitamin D intake which was lower in the 400 IU/d trial group compared to the reference group (Table 5.3**)**. Compliance to vitamin D supplementation was ≥85% in all groups **(Supplementary Table S5.1).** A total of 29 dropouts occurred throughout the study (20.9%). A comparison of infant and maternal

characteristics of those who completed the study compared to those who dropped out is provided in **Supplementary Table S5.2.** Characteristics at birth of infant-mother dyads who accepted to participate in the vitamin D study and those who did not are reported in **Supplementary Table S5.3.**

5.5 Discussion

In the absence of previously reported robust trials investigating the impact of a dose of vitamin D supplementation higher than the standard of care on bone mineral accretion in infants born with 25(OH)D <50 nmol/L, the present trial provided much needed information to help guide recommendations for vitamin D supplementation in this understudied infantile population. Using currently accepted methods of assessment of bone mineralization (DXA), neonates with insufficient vitamin D status provided with a daily supplement of 400 IU (compared to 1000 IU/d) did not have lower bone mineral accretion or density across infancy. Therefore, we reject our research hypothesis and conclude that daily supplementation with a 1000 IU of vitamin D does not present detectable advantages in bone mineral accretion and density at this early point in life.

Across the trial, infants born with serum 25(OH)D <50 nmol/L and receiving 400 IU/d did not have compromised bone outcomes (WB BMC, LS BMC, and LS BMD) compared to those receiving 1000 IU/d or our reference group. This in line with findings from a study by Ziegler et al. (2014) (218), in which different doses of vitamin D supplementation ranging from 200 to 800 IU/d did not show an impact on bone mineral content measured using DXA from 2 to 9 months of age, in a small subsample of exclusively breastfed infants. In their study (218), supplementation was only started at 2 months of age and as DXA scans were optional, the subsample was too small to evaluate the dose response in bone. The present research is to our knowledge the only trial that was conducted in infants born vitamin D insufficient. Two other trials that investigated bone

outcomes in response to vitamin D supplementation include a trial by Gallo et al. (2013) (1) and one by Holmlund-Suila (2012) (84). Both were conducted predominantly in vitamin D sufficient infants and showed no differences in BMC according to dose of vitamin D supplementation ranging from 400 to 1600 IU/d. The consistent absence of dose-response in bone mass in all of these studies could be due to a saturable effect in bone mineral accretion when vitamin D is supplemented at 400 IU/d.

No differences in biomarkers of bone formation and resorption were noted between the groups. These markers lack reference data in infancy as they have not been commonly measured and efforts on standardizing measurements of biomarkers of bone metabolism are needed (222). Findings by Bayer (2013) (223) and Choi et al. (2019) (224) showed that during infancy, levels of blood P1NP are higher than in childhood and adolescence, indicative of the high bone turnover and rapid linear growth that takes place during the first year of life. While bone outcomes measured by DXA inform us about the impact of the in utero and postnatal environments, bone biomarkers are more dynamic and have a relatively short half-life and therefore reflect acute changes in physiology. Findings on both biomarkers of bone metabolism and bone mineral accretion rates from DXA were consistent; showing no differences among groups and confirming that in healthy term born infants, 400 IU/d is enough to support bone health.

This study provides extensive data on different vitamin D metabolites assayed using LC-MS/MS, a worldwide analytical gold-standard for vitamin D assessment. Differences in $25(OH)D_3$, $24,25(OH)_2D_3$, $25(OH)D_3$:$24,25(OH)_2D_3$, and 3-epi-$25(OH)D_3$ concentrations were noted between the groups. It was previously shown that in conditions of low vitamin D status, there is a decreased activity of the 24-hydroxylase enzyme (CYP24A1). This helps to explain the lower concentrations of $24,25(OH)_2D_3$ in the trial groups compared to the reference group at 1

month of age. Consistently, higher $25(OH)D_3$:$24,25(OH)_2D_3$ were also observed at baseline in the trial groups compared to the reference group, but not after 3 months of supplementation. Even so, $25(OH)D_3$:$24,25(OH)_2D_3$ responds well to supplementation given the higher ratio observed in the 400 IU/d compared to the 1000 IU/d trial group. To our knowledge, the only other study reporting on different vitamin D metabolites in infancy is a trial by Gallo et al. (1) in which higher doses of vitamin D supplementation also resulted in greater concentrations of different vitamin D metabolites including 3-epi-$25(OH)D_3$ and $24,25(OH)_2D_3$. Variations in these metabolites over time were similar to the ones reported in our study.

In terms of $1,25(OH)_2D_3$, the lack of dose response to supplementation is likely due to the fact that calcitriol concentrations are tightly controlled within the picomolar range (225) and decrease only when the precursor 25(OH)D concentrations are lower than 20 nmol/L (226); which was the case in a total of 6 infants at the 1 month visit only. Interestingly, PTH concentrations were a positive correlate of calcitriol concentrations. Perhaps, the relatively high $1,25(OH)_2D_3$ concentrations observed throughout infancy is related to the continued high calcium demand in developing infants and the reduced consumption of mother's milk which has a greater calcium bioavailability compared to solid foods (227). The decrease observed over the 12 months could be indicative of the maturation of the liver function and higher circulating albumin concentrations which binds calcium (18). This is in line with previously reported findings by Sharma et al. (2016) (18). Nonetheless, the lack of differences between groups in iCa confirms that calcium homeostasis is maintained in infancy, even when vitamin D concentrations are below 40 to 50 nmol/L, the desirable concentrations in infancy according to which the Adequate Intake was defined (2). Given that iCa is the principal determinant of PTH concentrations, it is not surprising that no differences

were observed in $\log_{10}$ PTH concentrations between the trial groups or in comparison with the reference.

Proportions of infants who were breastfed and age of introduction of solid foods did not vary by group indicating that differences in dietary sources of vitamin D other than supplementation are unlikely among the groups. To further understand the characteristics of the population that may have impacted participation, a comparison of infant and mother factors between those who accepted to take part in the study and those who did not are listed in **Supplementary Table S5.3.** Interestingly, a higher proportion of mothers were highly educated (university) among those who consented to the participation of their infants to the study. That could be due to better knowledge and a better understanding of the risks and benefits of participating (228). Similar findings on volunteer bias have been previously reported in the literature (229). Another maternal factor was smoking postpartum which was found to be higher in those who declined participation. This could indicate that parents of infants enrolled to the trial engaged in healthier behaviors compared to those who did not. In a previous study (230), it was shown that the main reason for parents to consent for the participation of their children to a clinical research study is their interest in learning more about the health of their children and secondly to help advance medical research. In the case of our trial, it is possible that parents were initially interested in learning about the vitamin D status of their infants at birth, their growth and body composition throughout infancy, and their chance of becoming vitamin D sufficient faster (if born with serum 25(OH)D <50 nmol/L).

Strengths of this study are its double blinded randomized controlled parallel group design and its longitudinal nature that provide valuable data on bone mass in infancy assessed at more than one site and as per the recommendations of the International Society for Clinical Densitometry. Moreover, this trial used LC-MS/MS, the gold standard for measurement of different vitamin D

metabolites from a laboratory with certifications from the Vitamin D External Quality Assessment Scheme and Vitamin D Standardization-Certification Program (193). The use of such analytical technique helped provide a better understanding of physiological ranges of different vitamin D metabolites which are understudied including 3-epi-25(OH)D_3. In addition, this trial added to the scarce body of literature on infant physiology including dynamics of blood iCa and plasma PTH in infants. A total of 44.6% of the maternal population studied was self-reported to belong to a population group other than white which indicates that visible minorities which constitute 22.3% of the Canadian population (231) were well represented in our study. It would have been of added value to have vitamin D metabolites measured at birth using LC-MS/MS given that CLIA may have underestimated 25(OH)D concentrations. Other limitations to the study include the attrition rate (20.9%); which was often due to the busy schedules of the parents.

5.6 Conclusion

In conclusion, in neonates with low 25(OH)D concentrations at birth, both doses of vitamin D supplementation (400 and 1000 IU/d) normalized and maintained 25(OH)D concentrations that align with skeletal health. The 1000 IU/d dose of vitamin D supplementation while safe, did not lead to measurable improved bone outcomes. Evidence from this study reinforces that the standard of care of 400 IU/d is enough to support bone health of infants born with 25(OH)D <50 nmol/L.

5.7 Acknowledgements

This study was supported by the Canadian Institutes of Health Research, Nutricia Research Foundation, and the Canada Foundation for Innovation. The authors thank Sherry Agellon, Paula Lavery, and Maggie Yuan from McGill University for their contribution to the laboratory work

as well Sharina Patel, Veronique Menard, and Erika de Risi for their involvement in the recruitment of participants at Lakeshore General Hospital, Montreal, Canada; and Dr. Katherine Gray-Donald for her contribution to the study design.

5.8 Authors' contributions

NG, MR, CAV, OFS, LG, KM, ZF and Dr. HAW contributed to the data collection. NG analyzed and interpreted the data, and elaborated and revised the manuscript. SQW, DM, and FR were the steering committee in terms of recruitment and served as data safety monitors. GJ and MK were the expert analytical chemists collaborators. HAW, SQW, DM, FR, and GJ were involved in the design. AK designed the product, performed the external testing, and randomization scheme. HAW was involved in the conduct, guidance, and supervision of the study, staff, graduate trainees, and analyses. All authors approved the manuscript for publication.

Table 5.1. Characteristics at birth and at baseline

	400 IU/d trial (n=49)	1000 IU/d trial (n=49)	Reference (n=41)
Infant			
Season of birth			
Synthesizing[1]	30 (21.6)	26 (18.7)	25 (18.0)
Non-synthesizing	19 (13.7)	23 (16.6)	16 (11.5)
Sex			
Male	29 (20.9)	29 (20.9)	23 (16.6)
Female	20 (14.4)	20 (14.4)	18 (13.0)
Type of birth			
Vaginal	30 (21.6)	34 (24.5)	29 (20.9)
Vaginal after C-section	1 (0.7)	1 (0.7)	0 (0)
C-section	11 (7.9)	7 (5.0)	10 (7.2)
Repeat C-section	7 (5.0)	7 (5.0)	2 (1.4)
Gestational age at birth, wk	39.7 ± 1.0 (49)	39.6 ± 1.1 (49)	39.6 ± 1.0 (41)
WAZ at birth	0.1 ± 0.8 (49)	0.1 ± 0.8 (49)	0.3 ± 0.7 (41)
WAZ at baseline	-0.2 ± 0.9 (49)	-0.1 ± 0.7 (49)	0.0 ± 0.8 (41)
WLZ at baseline	-0.2 ± 1.0 (49)	-0.4 ± 0.9 (49)	-0.3 ± 0.9 (41)
LAZ at baseline	-0.2 ± 0.9 (49)	0.0 ± 0.9 (49)	0.1 ± 1.0 (41)
BMIAZ at baseline	-0.1 ± 1.0 (49)	-0.2 ± 0.7 (49)	-0.0 ± 0.7 (41)
HCAZ at baseline	0.3 ± 0.9 (49)	-0.0 ± 0.8 (49)	0.1 ± 0.9 (41)
Total 25(OH)D (nmol/L) at birth[2]	30.8 ± 9.2 (49)	34.4 ± 12.0 (49)	68.0 ± 13.2 (41)
Total 25(OH)D (nmol/L) at birth (adjusted)[3]	32.8 ± 8.9 (49)	36.3 ± 11.5 (49)	68.6 ± 12.8 (41)
Total 25(OH)D (nmol/L) at baseline	45.8 ± 14.1 (49)	46.4 ± 14.4 (48)	60.9 ± 14.9 (41)
Total 25(OH)D_3 (nmol/L) at baseline	44.3 ± 14.3 (49)	44.7 ± 15.3 (48)	59.5 ± 15.3 (41)
Inner arm ITA° at baseline	30.0 ± 19.8 (49)	31.2 ± 18.1 (49)	42.0 ± 8.6 (41)
Mother			
Age at delivery, y	32.8 ± 4.3 (49)	31.2 ± 4.8 (49)	32.3 ± 4.0 (41)
Gravida			
1	12 (8.6)	19 (13.7)	13 (9.4)
>1	37 (26.6)	30 (21.6)	28 (20.1)

Gestational weight gain class			
Inadequate	14 (10.1)	13 (9.4)	9 (6.5)
Adequate	14 (10.1)	18 (12.9)	14 (10.1)
Excessive	21 (15.1)	18 (12.9)	18 (12.9)
Pre-gravid BMI, kg/m^2	24.0 ± 5.1	24.0 ± 4.3	24.0 ± 4.3
Multivitamin use			
In the 3 months before pregnancy			
Yes	19 (13.7)	20 (14.4)	25 (18.0)
No	30 (21.6)	29 (20.9)	16 (11.5)
During pregnancy			
Yes	44 (31.7)	46 (33.1)	38 (27.3)
No	5 (3.6)	3 (2.2)	3 (2.2)
Exercise			
In the 3 mo before pregnancy			
Yes	30 (21.6)	31 (22.3)	33 (23.7)
No	19 (13.7)	18 (13.0)	8 (5.8)
During pregnancy			
Yes	18 (13.0)	25 (18.0)	26 (18.7)
No	31 (22.3)	24 (17.3)	15 (10.8)
Highest education			
Elementary/ High school	8 (5.8)	4 (2.9)	1 (0.7)
College/ Vocational	8 (5.8)	11 (7.9)	10 (7.2)
University	33 (23.7)	34 (24.5)	30 (21.6)
Self-reported population group			
White	22 (15.8)	24 (17.3)	31 (22.3)
All other	27 (19.4)	25 (18.0)	10 (7.2)
Serum 25(OH)D (nmol/L) at baseline	53.6 ± 14.1 (n=49)	59.9 ± 22.1 (n=49)	90.5 ± 20.4 (n=41)
Family income			
<70,000 CAD/y	15 (10.8)	18 (13.0)	7 (5.0)
≥70,000 CAD/y	27 (19.4)	22 (15.8)	29 (20.9)
Not reported	7 (5.0)	9 (6.5)	5 (3.6)

Data are reported as mean±SD and n(%). [1]Synthesizing season is the vitamin D synthesizing period that extends from April 1 to October 31. [2]Serun 25(OH)D concentrations prior to adjustment using Deming regression. [3]Serun 25(OH)D concentrations after adjustment using Deming regression. Abbreviations: WAZ: weight-for-age z-score, 25(OH)D: 25-hydroxyvitamin D, CAD: Canadian dollars, WLZ: weight-for-length z-score, LAZ: length-for-age-z-score, BMIAZ: BMI-for-age z-score, HCAZ: head-circumference-for-age z-score, ITA°: individual typology angle, F: Fitzpatrick scale.

Table 5.2. Serum $1,25(OH)_2D_3$ of groups over time using a sub-group analysis

	Serum $1,25(OH)_2D_3$ (pmol/L)		
Timepoint (mo)	400 IU/d trial	1000 IU/d trial	Reference
1	187.1 ± 74.3 (41/49)	160.3 ± 71.1 (41/49)	163.0 ± 72.6 (32/41)
3	185.3 ± 61.8 (39/44)	181.2 ± 58.2 (39/44)	178.5 ± 59.6 (33/35)
6	184.9 ± 87.6 (38/40)	184.9 ± 69.1 (42/43)	179.3 ± 63.2 (32/34)
12	204.2 ± 50.1 (32/36)	199.8 ± 82.4 (39/42)	198.0 ± 53.0 (30/32)

Data are reported as mean ± SD (proportion of infants with $1,25(OH)_2D_3$ measured). Differences between the trial groups were tested using a linear mixed model accounting for the fixed effects of group (p=0.37), timepoint (p=0.0025), timepoint*group (p=0.95), infant sex (p=0.34) and the random effect of infant ID. Abbreviations: $1,25(OH)_2D_3$: 1,25-dihydroxyvitamin D_3, IU: international units.

Table 5.3. Infant breastfeeding status and age of introduction of solid foods and maternal nutrient intake from food and supplements during

	400 IU/d trial	1000 IU/d trial	Reference	p-value
Infant				
Breastfeeding status				
1 mo	49/49 (100%)	49/49 (100%)	41/41 (100%)	1.00
3 mo	41/44 (93.2%)	41/44 (93.2%)	30/35 (85.7%)	0.56
6 mo	34/40 (85.0 %)	36/43 (83.7%)	27/34 (79.4%)	0.80
12 mo	16/36 (44.4%)	17/42 (40.5%)	12/32 (37.5%)	0.84
Age of introduction of solid foods (mo)	5.0 ± 0.9 (range: 3-7) (n=40)	4.9±0.9 (range: 3 to 6) (n=43)	4.9±0.7 (range: 4 to 7) (n=33)	0.93
Mother				
Energy (kcal)	2137 ± 777 1892 (1010-4600)	2191 ± 982 1955 (1179-6382)	2091 ± 697 1914 (1018-3805)	0.87
Protein (g)	92 ± 35 87 (33-192)	98 ± 44 85 (47-278)	96 ± 36 89 (42-182)	0.76
Carbohydrates (g)	291 ± 130 266 (134-852)	286 ± 147 249 (142-964)	261 ± 83 254 (132-455)	0.52
Fat (g)	73 ± 29 63 (31-141)	78 ± 33 75 (35-181)	79 ± 32 72 (31-161)	0.61
Vitamin D (IU)	552 ± 222.0[a] 578(75-1240)	610 ± 241[ab] 589 (100-1132)	730 ± 289[b] 762 (100-1282)	0.0075
Calcium (mg)	1318 ± 444 1250 (614-2253)	1350 ± 554 1304 (621-3511)	1500 ± 529 1317 (718-2786)	0.25
Magnesium (mg)	475 ± 161 419 (239-930)	478 ± 173 441 (178-1079)	491 ± 119 482 (271-748)	0.89
Phosphorus (mg)	1512 ± 547 1411 (673-2783)	1556 ± 700 1363 (668-4384)	1548 ± 577 1405 (682-2685)	0.94

Distinct letter superscripts (a,b,c) indicate statistically significant differences between groups ($p<0.05$);Data are reported as mean±SD and median (IQR) for maternal intake during pregnancy. Breastfeeding status is reported as proportions (%) and age of introduction of solid foods as mean±SD (minimum-maximum) (n). Differences between the means of the three groups were tested using linear fixed effects model. Abbreviations: IU: international unit

Supplementary Table S5.1. Compliance to vitamin D supplementation

	Trial 400 IU/d	Trial 1000 IU/d	Reference 400 IU/d
1 to 3 mo	0.91 (0.84, 0.98)	0.93 (0.84, 0.97)	0.91 (0.80, 0.95)
	n=44	n=42	n=32
3 to 6 mo	0.92 (0.85, 0.97)	0.93 (0.79, 0.98)	0.93 (0.82, 0.98)
	n=35	n=41	n=31
6 to 12 mo	0.88 (0.81, 0.98)	0.85 (0.65, 0.94)	0.93 (0.79, 0.98)
	n=32	n=41	n=27

Data are median (interquartile range).

Supplementary Table S5.2. Characteristics of study completers versus non-completers

Parameters	**Trial completers** (n=78)	**Trial dropouts** (n=20)	p-value	**Reference completers** (n=32)	**Reference dropouts** (n=9)	p-value
Infants at birth						
Gestational age, wk	39.7 ± 1.1	39.6 ± 1.0	0.67	39.6 ± 0.9	39.8 ± 1.2	0.63
Sex, n (%)			0.55			0.47
Male	45 (57.7)	19 (65.0)		19 (59.4)	4 (44.4)	
Female	33 (42.3)	7 (35.0)		13 (40.6)	5 (55.6)	
Season of birtth[1], n (%)			0.0238			0.44
Synthesizing	40 (51.3)	16 (80.0)		18 (56.3)	7 (77.8)	
Non-synthesizing	38 (48.7)	4 (20.0)		14 (43.8)	2 (22.2)	
Weight, kg	3.4 ± 0.4	3.3 ± 0.3	0.15	3.4 ± 0.4	3.5 ± 0.3	0.57
Serum 25(OH)D, nmol/L	32.2 ± 10.9	34.1 ± 10.3	0.49	67.7 ± 14.4	68.9 ± 8.7	0.82
Mothers						
Age at delivery, y	32.0 ± 4.7	31.9 ± 4.3	0.99	32.6 ± 3.7	31.5 ± 5.3	0.49
Gravida, n (%)			0.0290			0.69
Primiparous	29 (37.2)	2 (10.0)		11 (34.4)	2 (22.2)	
Multiparous	49 (62.8)	18 (90.0)		21 (65.6)	7 (77.8)	
Self-reported population, n (%)			0.06			0.38
White	42 (53.8)	6 (30.0)		26 (81.3)	6 (66.7)	
All other groups[2]	36 (46.2)	14 (70.0)		6 (18.7)	3 (33.3)	
Family income, n (%)			0.0227			0.43
≥70,000 CAD	44 (56.4)	5 (25.0)		24 (75.0)	5 (55.6)	

<70,000 CAD	24 (30.7)	9 (45.0)		5 (15.6)	2 (22.2)	
Not reported	10 (12.8)	6 (30.0)		3 (9.4)	2 (22.2)	
Education, n (%)			0.51			0.0222
Primary/high school	8 (10.3)	4 (20.0)		0 (0.0)	1 (11.2)	
College/vocational school	15 (19.2)	4 (20.0)		6 (18.7)	4 (44.4)	
University	55 (70.0)	12 (60.0)		26 (81.3)	4 (44.4)	

Data are reported as mean±SD. Differences between the means of the two trial groups were tested using repeated measures linear mixed model (continuous variables) and Chi square test or Fisher's exact test (categorical variables). [1]Birth in the vitamin D synthesizing period: April 1- October 31st; [2]all other groups include: South Asian, Southeast Asian, Middle Eastern, Latino, Black, and others. Abbreviations: 25(OH)D: 25-hydroxyvitamin D; CAD: Canadian dollar; GWG: gestational weight gain; UVB: ultraviolet B.

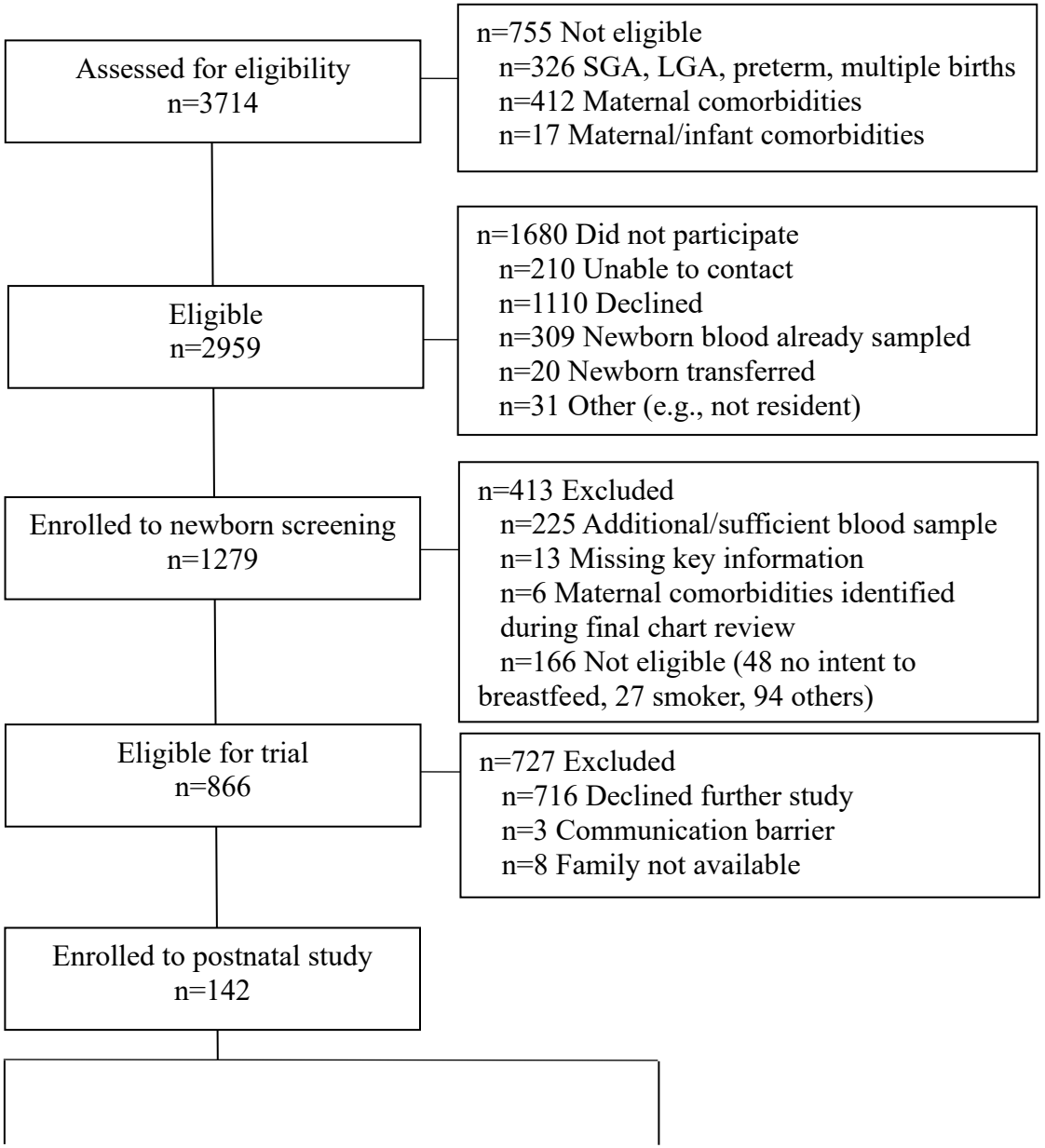
Supplementary Table S5.3. Characteristics at birth of infants-mother dyads who accepted to participate in the vitamin D study and those who did not

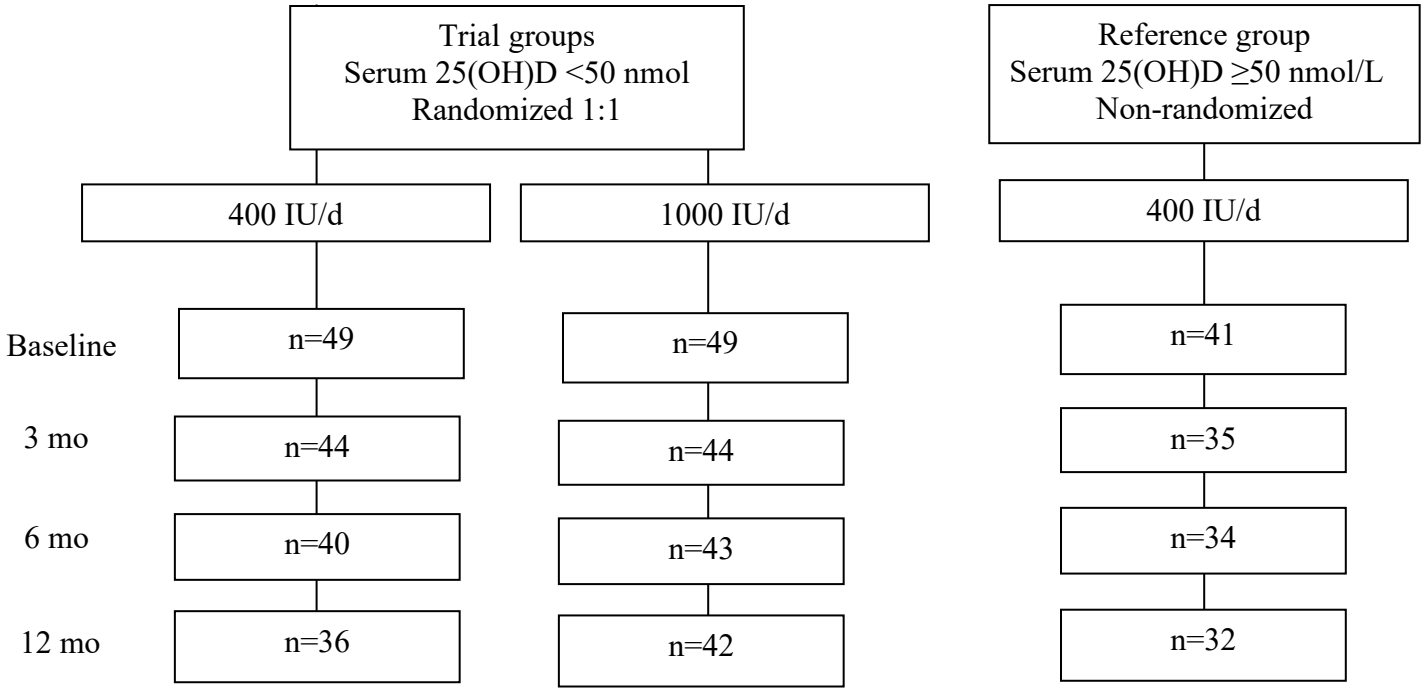
	Did not participate (n=1140)	Participated (n=139)	p-value
Season of birth			0.0001
Synthesizing*	689 (54.0)	58 (4.5)	
Non-synthesizing	449 (35.2)	81 (6.3)	
Sex			0.06
Male	565 (44.3)	81 (6.4)	
Female	572 (44.8)	58 (4.6)	
Type of birth			0.46
Vaginal	827 (64.9)	93 (7.3)	
Vaginal after C-section	9 (0.7)	2 (0.2)	
C-section	198 (15.5)	28 (2.2)	
Repeat C-section	102 (8.0)	16 (1.3)	
Gestational age, wk	39.7 ± 1.1	39.7 ± 1.0	1.00
Birth WAZ	0.2 ± 0.8	0.1 ± 0.8	0.42
Infant birth 25(OH)D (nmol/L)	44.5 ± 19.0	43.0 ± 19.9	0.41
Mother's age at delivery	32.2 ± 4.5	32.1 ± 4.4	0.74
Mother's gravida			0.56
1	333 (26.1)	44 (3.4)	
>1	806 (63.1)	95 (7.4)	
Mother's pregnancy weight gain	13.9 ± 5.9	13.7 ± 6.0	0.60
Mother's pre-pregnancy BMI	24.8 ± 4.9	24.1 ± 4.7	0.11
Exercise			
In the 3 mo before pregnancy			0.12
Yes	665 (52.4)	94 (7.4)	
No	462 (36.4)	45 (3.6)	
Not reported	2 (0.2)	0 (0)	
During pregnancy			0.78
Yes	538 (42.4)	69 (5.4)	
No	591 (46.5)	70 (5.5)	

Not reported	2 (0.16)	0 (0)	
Mother's highest education			0.0048
Elementary/High school	197 (15.5)	13 (1.0)	
College/Vocational school	302 (23.8)	29 (2.3)	
University	638 (49.8)	97 (7.6)	
Mother's self-reported population group			0.49
White	449 (35.1)	59 (4.6)	
All other	690 (54.0)	80 (6.3)	
Family income			0.27
<70,000 CAD/y	308 (24.1)	40 (3.1)	
≥70,000 CAD/y	592 (46.3)	78 (6.1)	
Not reported	239 (18.7)	21 (1.6)	
Smoker after delivery			0.0083
Yes	50 (3.9)	0 (0.0)	
No	1059 (83.5)	138 (10.9)	
Not reported	20 (1.6)	1 (0.1)	
Smoker before pregnancy			0.15
Yes	119 (9.5)	10 (0.8)	
No	995 (79.3)	128 (10.2)	
Not reported	2 (0.2)	1 (0.1)	

Data are reported as mean±SD and n(%). Differences between the groups were tested using a linear fixed effect model (continuous variables) and using Chi square or Fisher's exact test (categorical variables). *Synthesizing season is the vitamin D synthesizing period that extends from April 1 to October 31. Abbreviations: WAZ: weight-for-age z-score, 25(OH)D: 25-hydroxyvitamin D, CAD: Canadian dollars.

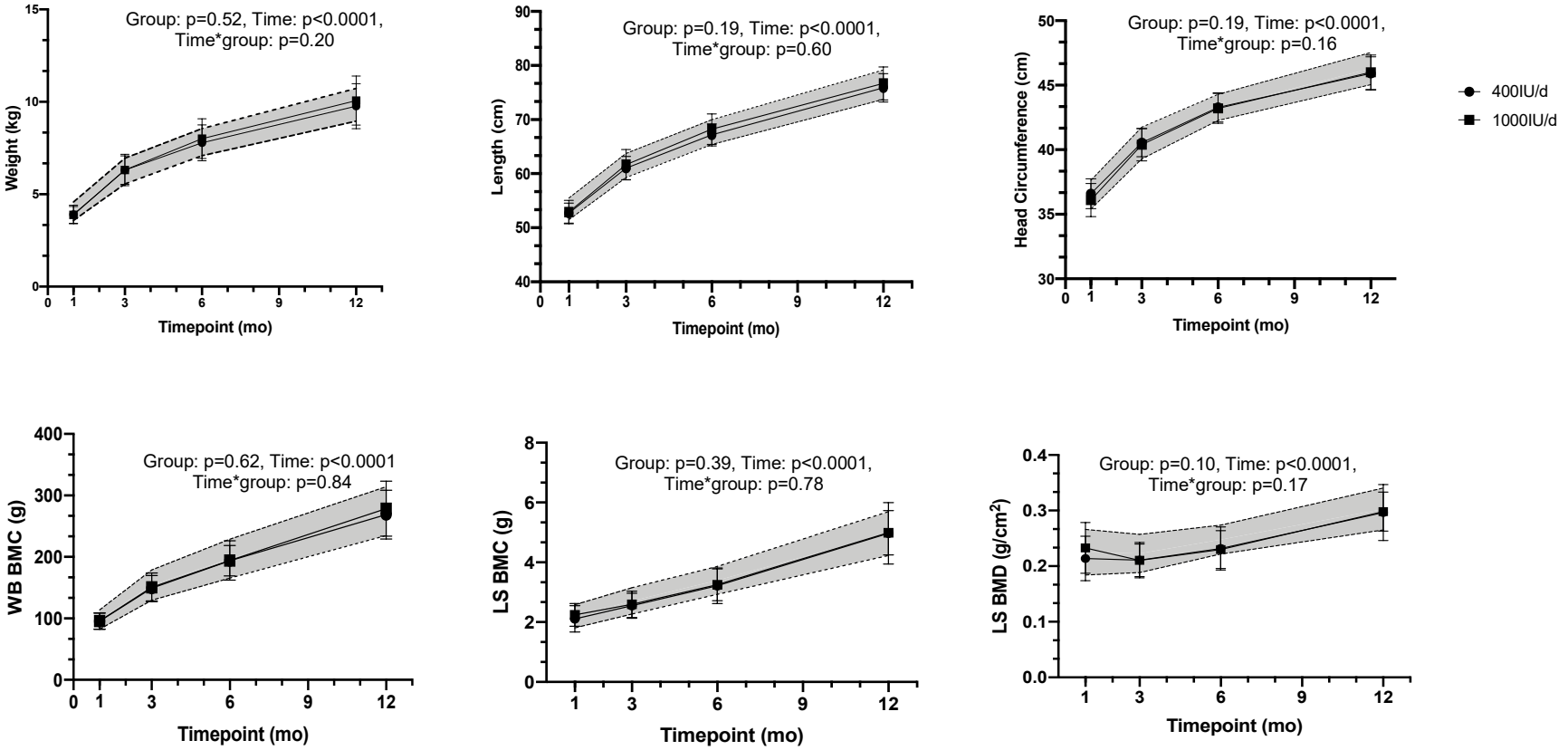
Figure 5.1. Participant flow diagram





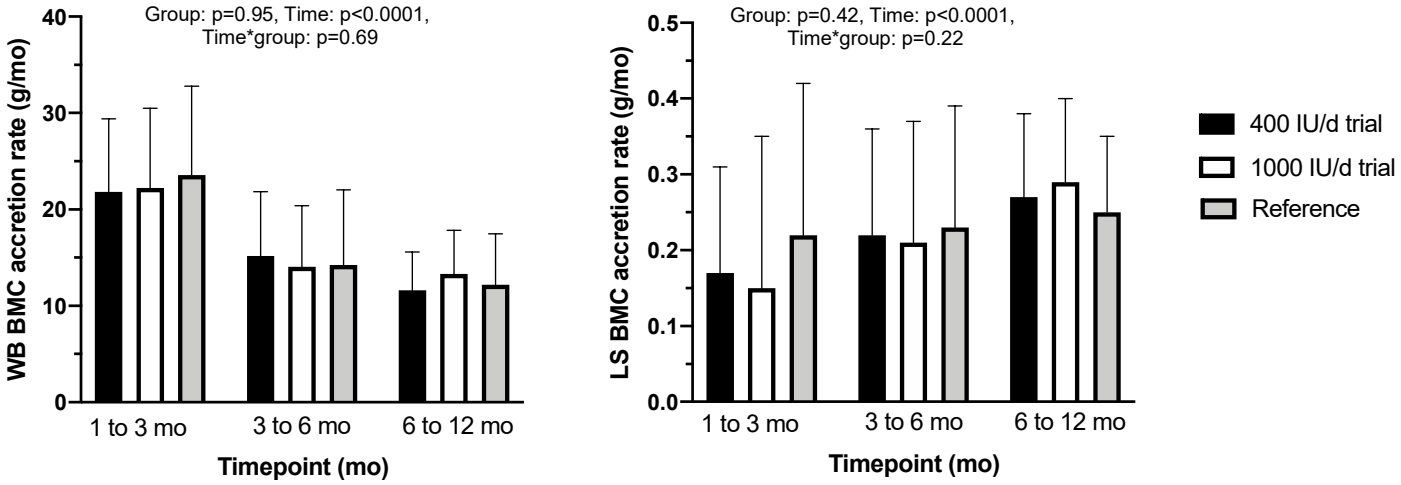
Participant's flow diagram showing number of mother-infant dyads assessed for eligibility 24-36 hours after delivery, enrolled to newborn screening, screened, enrolled to the postnatal study and allocated to either trial (serum 25(OH)D <50 nmol/L) or reference group (serum 25(OH)D ≥50 nmol/L). Infants allocated to the trial group were randomized to 1 of 2 trial groups receiving either 400 or 1000 IU/d. Infants in the reference group received 400 IU/d. Abbreviations: SGA: small for gestational age, LGA: large for gestational age, IU: international units.

Figure 5.2. Weight, length, head circumference, whole body BMC, lumbar spine BMC, and lumbar spine BMD of groups over time



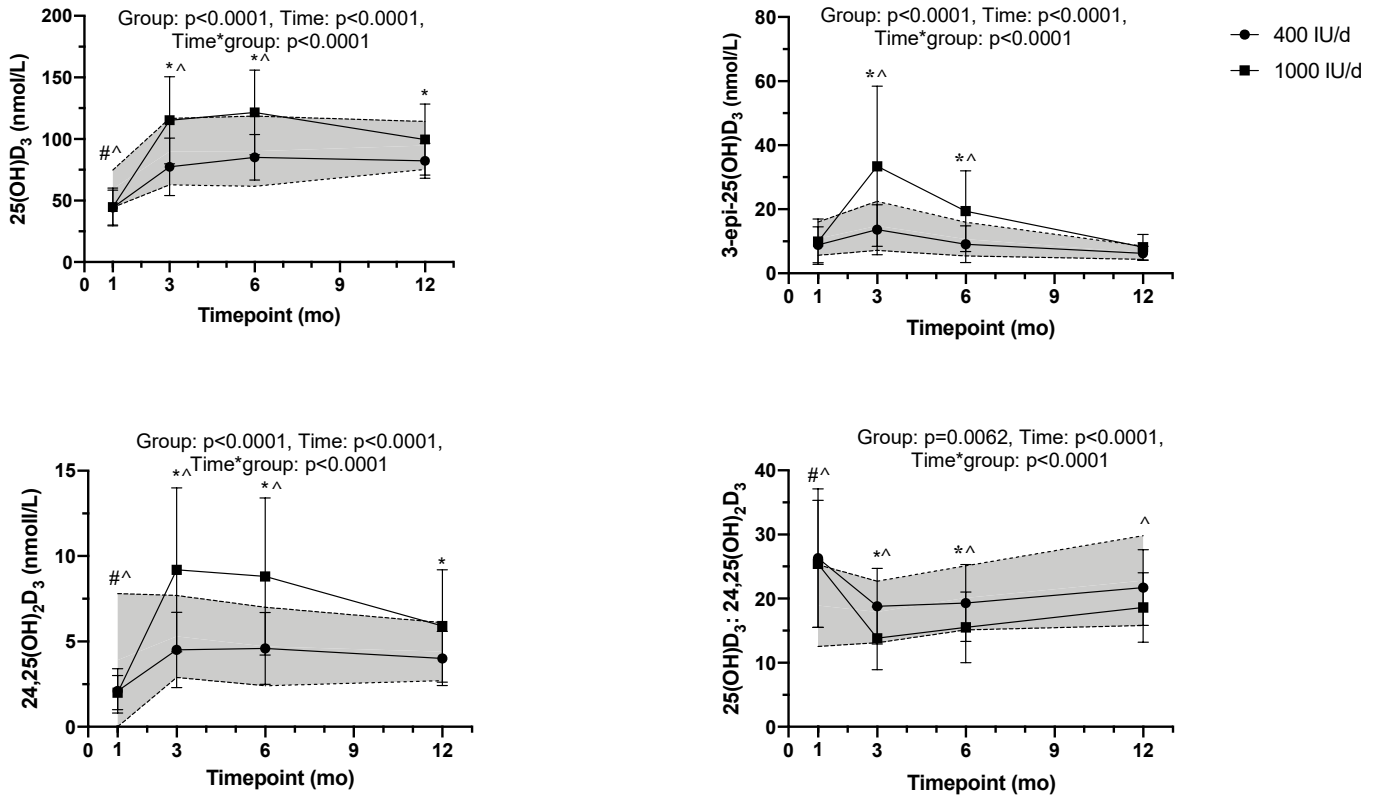
Data are reported as mean ± SD and were analyzed over time using a linear mixed effects model accounting for the fixed effect of group, timepoint, timepoint*group, infant's sex, and the random effect of the participant ($p<0.05$, post hoc adjustment). The shaded areas correspond to the reference group means ± SD. Abbreviations: IU: international units.

Figure 5.3. Whole body BMC and lumbar spine BMC accretion rates of groups over time



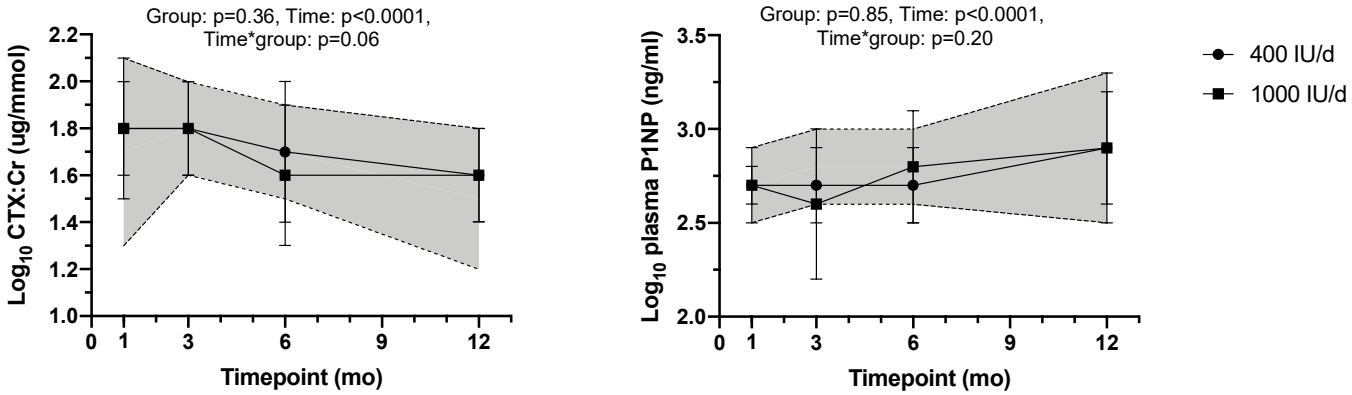
Data are reported as mean ± SD and were analyzed over time using a linear mixed effect model ANOVA accounting for the fixed effect of group, timepoint, timepoint*group, infant's sex, and the random effect of the participant ($p<0.05$, post hoc adjustment). Abbreviations: BMC: bone mineral content, BMD: bone mineral density, IU: international units, LAZ-score: length for age z-scor

Figure 5.4. Serum 25(OH)D, 24,25$(OH)_2D_3$, 24,25$(OH)_2D_3$:25$(OH)D_3$, and 3-epi-25$(OH)D_3$ concentrations of groups over time



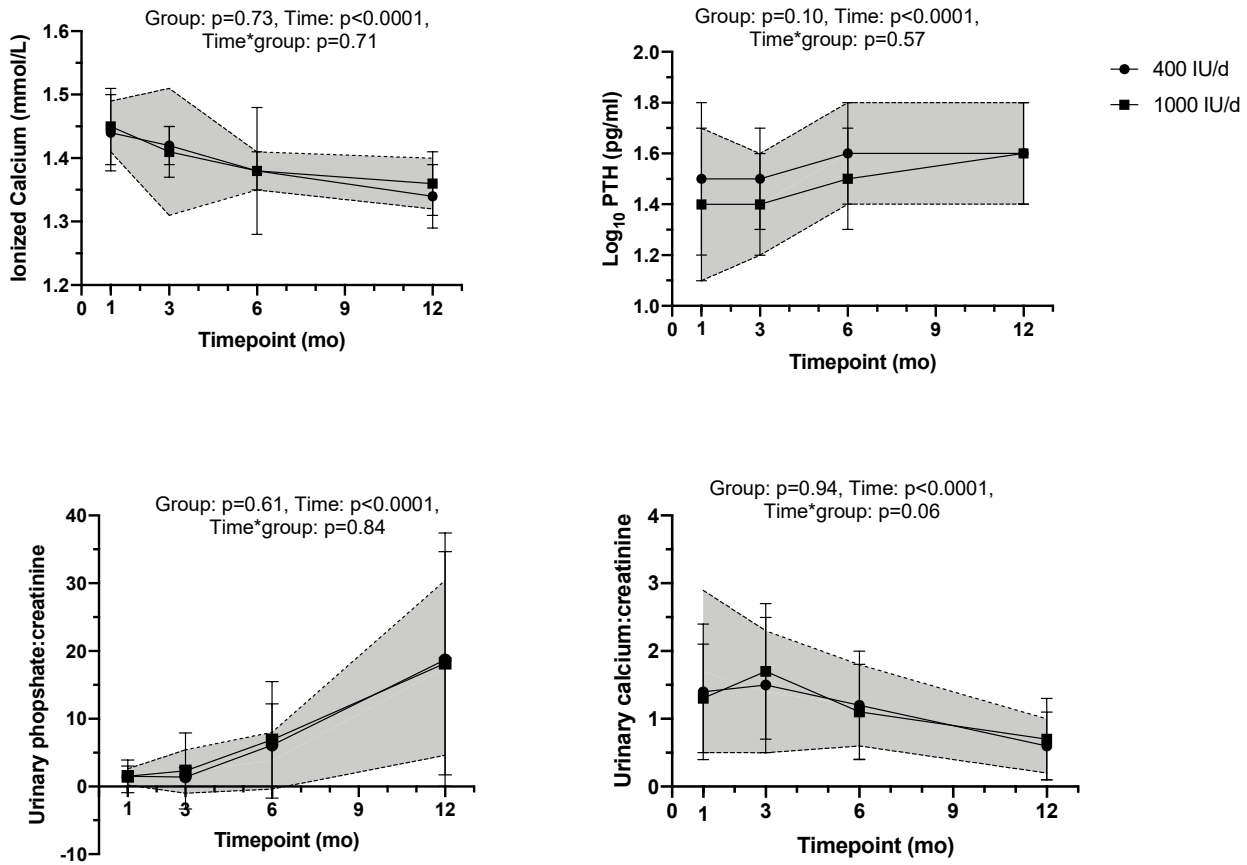
Data are reported as mean ± SD and were analyzed over time using a linear mixed effects model accounting for the fixed effect of group, timepoint, timepoint*group, infant's sex, and the random effect of the participant ($p<0.05$, post hoc adjustment). The shaded areas correspond to the reference group means ± SD. *$p<0.05$ 400 IU/d trial group vs 1000 IU/d trial group; #$p<0.05$ 400 IU/d trial group vs reference group; ^$p<0.05$ 1000 IU/d trial group vs reference group. Abbreviations: 25$(OH)D_3$: 25-hydroxyvitamin D_3, 24,25$(OH)_2D_3$: 24,25-dihydroxyvitamin D_3, IU: international unit

Figure 5.5. Urinary CTX-I:creatinine and plasma P1NP by groups over time



Data are reported as mean ± SD and were analyzed over time using a linear mixed effects model accounting for the fixed effect of group, timepoint, timepoint*group, infant's sex, and the random effect of the participant ($p<0.05$, post hoc adjustment). The shaded areas correspond to the reference group means ± SD. Abbreviations: P1NP: Procollagen type I N-terminal propeptide, CTX-I: urinary alpha telopeptide of collagen I, IU: international units.

Figure 5.6. Blood ionized calcium, plasma PTH, urinary phosphate:creatinine, and urinary calcium:creatinine



Data are reported as mean ± SD and were analyzed over time using a linear mixed effects model accounting for the fixed effect of group, timepoint, timepoint*group, infant's sex, and the random effect of the participant ($p<0.05$, post hoc adjustment). The shaded areas correspond to the reference group means ± SD. Abbreviations: PTH: plasma thyroid hormone, IU: international units.

Chapter 6: Discussion and conclusion

6.1 Findings and hypotheses

This dissertation focused on bone mass in infancy and aimed to fill multiple knowledge gaps ranging from the generation of reference data for bone mineral accretion in infants to providing a better understanding of its influencing factors and specifically vitamin D supplementation. Using three different studies including one randomized double blinded parallel controlled trial; three different research questions were addressed. In brief, study 1 provided reference data for bone mass throughout infancy and investigated sex differences, adding to the limited longitudinal evidence in the literature on what defines reference skeletal mineralization in infancy. The second study explored the relation of maternal vitamin D status and gestational weight gain (GWG) to neonatal bone outcomes; a research question which to the best of the author's knowledge has never been addressed before. This study has important implications to public health policies on optimal pregnancy outcomes that have etiology in pregnancy stages. The trial (study 3) investigated the impact of a higher dose of vitamin D supplementation than the standard of care in infants born with serum 25-hdroxyvitamin D (25(OH)D) concentrations <50 nmol/L. It is to my knowledge the first high quality study to provide solid results that present strong implications to worldwide public health policies on vitamin D supplementation in infancy in relation to bone health. The purpose of this chapter is to provide an overall interpretation of findings from all three manuscripts, position and relate these findings to existing relevant literature, point out their public health implications, and suggest additional research areas to be addressed.

In study 1, patterns of skeletal mineralization in infancy were explored and showed that infants undergo more rapid increases in bone mineral content (BMC) at the level of the whole body (WB) and lumbar spine (LS) during the first six months of life compared to 7 to 12 months of age. Growth charts for LS bone mineral density (BMD) from 1 to 12 months of age were also generated

using LMS chartmaker and showed that sex differences in skeletal mineralization in infants if any, are driven by size in a sample of breastfed, term, appropriate for gestational age infants with sufficient vitamin D status at baseline (1 month of age). Therefore, the hypothesis that BMC is at its fastest accretion rate within the first six months of life with no sex differences throughout infancy was partially accepted. These findings are in line with the literature whereby the first six months of life are known to be characterized by a higher rate of linear and skeletal growth and mineralization compared to the second half of infancy. In terms of sex differences, the findings indicate that differences observed in WB BMC are due to size. No sex differences in LS BMD were observed which suggests that protective effects against osteoporosis seen in adult males may not be evident since infancy. Similar findings were reported in a study by Kurl et al. (156) and Koo et al. (157) whereas in a study by Rupich et al. (232), it was shown that BMC and BMD were greater in male infants compared to female infants. When WB BMC/kg was compared between the sexes in the present study, differences were no longer found, confirming that these differences are related to size.

The LS BMD growth charts generated in this study are similar to those created previously (12). However, in the Gallo study, the sample did not consist exclusively of vitamin D sufficient infants and was relatively small in terms of whole-body measurements by 12 months (n=11) of age. Given that the Institute of Medicine (IOM) defined 25(OH)D concentrations in support of bone health at ≥50 nmol/L; this secondary analysis focused on infants with sufficient vitamin D status only for the purpose of generating reference data for bone mineral accretion in infancy. As previously described in the literature review section, vitamin D is involved in bone modeling and severe vitamin D deficiency can lead to vitamin D deficiency rickets, a condition in which bone mineralization is severely impaired. However, based on findings from the vitamin D trial (study

3), there are no overt differences in bone outcomes among sufficient and insufficient infants when 400 IU/d of vitamin D supplementation is received. Therefore, it could have been possible to include vitamin D insufficient infants for the generation of WB BMC growth charts using a bigger sample size and to then be able to generate sex specific curves for WB BMC (i.e. mean within the bootstrap-based 95%CI). As for the lack of sex differences (after adjusting for body size) in bone mineral accrual at this age, this could be explained by the fact that sex hormones, which are key players in sexual dimorphism, reach peak levels at later points in life and are not considered major players in infancy.

Study 2 explored the relation of maternal vitamin D status and GWG to neonatal bone outcomes. The alternative hypothesis was rejected given that healthy maternal GWG and vitamin D status were not found to be associated with greater BMC/kg compared to insufficient and excessive GWG. The relation of GWG to bone outcomes was modest and its clinical significance remains to be determined. The absence of relation between maternal vitamin D status and neonate bone outcomes could have a positive interpretation whereby compromised but not deficient maternal vitamin D status is not linked to sub-optimal neonatal bone outcomes. Alternatively, maternal vitamin D status in pregnancy was not available and is required to fully address this question in future studies. One other study reported on the relation of GWG to neonatal bone outcomes and showed that there is a positive relation between two of them in women with pre-gravid body mass index (BMI) <25 kg/m^2 but showed no relation in women with pre-gravid BMI ≥25 kg/m^2 (143). Higher BMC and BMD in the excessive GWG may happen through mechanical loading because of excessive body fat.

In study 3, the objective was to test whether providing a safe but higher dose of supplementation (1000 IU/d) than the standard of care to infants born vitamin D insufficient would

allow for rapid repletion of vitamin D stores and a better bone mineral accretion. The hypothesis was that neonates with low vitamin D status provided with a supplement of 400 IU/d (compared to 1000 IU/d) will have lower accretion of BMC by 3 months of age. This hypothesis was fully rejected as there were no differences in WB BMC, WB BMC accretion rate, LS BMC, LS BMC accretion rate, and LS BMD between groups of infants who received 400 or 1000 IU/d of vitamin D_3. The lack of response could be due to a saturable effect in bone accretion once supplementation of 400 IU/d is received postnatally. This finding is in line with previous studies that showed no impact of increased supplementation on bone outcomes in infants. The majority of these studies was conducted in vitamin D sufficient infants and had a poor overall quality assessed using Jadad scale (Table 2.1), except for one study by Gallo et al. (2013). The study by Holmlund-Suila (84) which was conducted in vitamin D sufficient infants showed that there was a trend towards a dose response to supplementation in bone outcomes measured using peripheral quantitative computed tomography. The use of this measure is debatable in infants given their small bone size and randomization, blinding, and attrition of participants in this trial were not well described. The one study that tested a dose response to supplementation in vitamin D insufficient infants by Ziegler et al. (218) also showed no dose response to supplementation in bone. Nonetheless, this study was limited by a small dual energy x-ray absorptiometry (DXA) output as it was optional and not adjusted for age in addition to an overall score of 2/5 on the Jadad scale.

6.2 Interpretation of our findings and implications

Differences between male and female infants in bone mass were not attributed to inherent differences in skeletal growth but instead to differences in linear growth and weight. In addition, findings from the first study highlight the first six months which represents a critical window for

skeletal growth. In this study, growth charts for WB BMC were not generated due to the relatively small sample size resulting in the mean being outside the 95%CI generated using bootstrapping. The extent to which the LS BMD growth charts generated may help guide clinical judgement may be limited given that infantile growth varies from one skeletal size to the other but can be useful when used in conjunction with other indicators of bone health. By looking at the LS site only, one might be missing impaired mineralization and skeletal issues at other sites like the limbs or head for example. Nevertheless, given that bone health has been used as an indicator for defining Dietary Reference Intake values, such data may help in determining nutrient recommendations in infancy, a phase during which Adequate Intakes but not Estimated Average Requirements or Recommended Dietary Allowances have been defined. Factorial models which have been previously used to generate Adequate Intakes for zinc, iron, and phosphorous may also be considered to confirm or adjust previously set Dietary Reference Intake values in infancy for different nutrients including vitamin D, calcium and phosphorous.

Study 2 sheds light on GWG as a maternal factor that relates to neonatal bone outcomes; a relationship that has not been thoroughly explored before. In fact, the body of literature includes only one study that evaluated the relation between GWG and neonatal bone outcomes. Given that GWG targets are set according to pre-gravid BMI, these findings highlighted the importance of gestational factors. In this study, 42.3% of women had excessive GWG, 32.4% had adequate GWG, and 25.4% had inadequate GWG. These proportions are similar to those reported in the US as per National Vital Statistics System birth whereby 32% of women gain an adequate amount of weight throughout gestation, 21% gain weight in insufficient amounts and 48% exceed the recommended weight gain (233). In Canada, numbers are provided according to age category. The average age of women at delivery is 30.2 years (234); among women of age 30-34 at delivery,

23% gain insufficient weight during pregnancy, 38% gain adequate amounts, and 39% gain excessive amounts (235). Knowing that a significant proportion of women do not gain enough weight to meet the recommendations, it is reassuring that infants born to mothers with inadequate GWG have normal bone mass compared to those born to mothers who gained sufficient weight.

As for those infants born to mothers with excessive GWG, it is not clear what is the driving factor behind the increased BMC of these infants as those differences cannot be explained fully by size in the present study. In the literature, excessive GWG is associated with different factors including nulliparous pregnancies, overweight pre-gravid BMI, lower socio-economic status as per a follow-up analysis from the Maternity Experiences Survey in Canada (207). Energy intake is also positively associated with excessive GWG while physical activity is inversely associated with it (236). Overeating when alone and eating in front of a screen have also been shown to be positively associated with excessive GWG (237). In addition, in the present study, 66.9% of mothers had a pre-gravid BMI <25 kg/m^2 and 33.1% had a pre-gravid BMI ≥25 kg/m^2. These proportions are similar to those reported in the general population (235). According to national survey data, 59.3% of expecting women have a normal pre-gravid BMI (18.5-24.9 kg/m^2), 6.1% are underweight (pre-gravid BMI: <18.5 kg/m^2), 21.0% are overweight (pre-gravid BMI: 25-29.9 kg/m^2) and 13.6% are obese (pre-gravid BMI ≥30 kg/m^2) (235). According to a study conducted in Canada (238), the majority of women do not receive enough guidance concerning their weight gain throughout gestation. Only 21% of healthcare professionals informed women of their recommended GWG as per their pre-gravid BMI and only 16% discussed their rate of weight gain. In order to help women achieve healthy pregnancy weight gain, further attention should be drawn to gestational weight gain counseling. As for maternal 25(OH)D status, our data shows no relation of maternal 25(OH)D to neonatal bone mass.

Findings from the trial (study 3) included in this thesis indicate that providing doses of supplementation as high as 1000 IU/d does not present benefits for bone mass of infants who are born vitamin D insufficient. Therefore, the current supplementation dose of 400 IU/d in North America (216), Europe (239), and Australia (240) is enough to support bone mass. The lack of dose response observed in bone could be attributed to a saturable effect whereby bone mineralization is only impaired in case of chronic vitamin D deficiency and doses of vitamin D supplementation beyond 400 IU/d cannot enhance it further.

All of these findings present clinical and policy implications. For instance, the growth charts created in the first study can help provide clinicians with a better-informed judgement on skeletal mineralization in infancy when used as part of a comprehensive assessment. In addition, these charts may also guide clinical decisions in pediatric cases of non-accidental trauma and response to treatment. Based on study 2, GWG category (inadequate, adequate, excessive) which is defined according to maternal pre-conception BMI has implications to offspring bone mass. Thus, this shows the importance of having public health policies and recommendations that focus on the pre-conception phase for optimal and healthy pregnancy outcomes. Moreover, study 3 confirms that current public health policies endorsed in North America on vitamin D supplementation of 400 IU daily in infancy are appropriate for infants born with vitamin D across the spectrum of serum 25(OH)D concentrations.

6.3 Strengths and limitations of our studies

Study 1 is one of the very few studies that have explored differences between sexes longitudinally throughout infancy. The results of this study are solidified by the use of liquid chromatography tandem mass spectrometry (LC-MS/MS) to assess vitamin D status in infants, the

gold standard for the assessment of vitamin D metabolites. The growth charts generated were created using the LMS chartmaker, the same software used by the World Health Organization for generation of weight for age, length for age, and head circumference for age growth charts. Using highly recognized tools and high-quality methodology made our findings more credible and useful at the clinical and policy level despite the relatively small sample size in comparison with the sample sizes used by the WHO in the generation of weight for age and length for age growth charts. In study 2, the investigation of the impact of maternal 25(OH)D and GWG on neonatal bone outcomes comes as an innovative addition to the literature. Another gap that this research has filled in the literature is the implementation of a trial (study 3) on vitamin D insufficient infants. As far as I know, this study was only preceded by one similar trial. The latter was a study by Ziegler et al. (218) which presented with several inherent limitations. A robust answer to the research question on whether a higher dose of vitamin D supplementation presents skeletal advantages in infants born vitamin D insufficient was still needed.

The present trial has many advantages including the use of LC-MS/MS for measurement of many vitamin D metabolites including 25(OH)D_3, 24,25-dihydroxyvitamin D_3 and 3-epi-25-hydroxyvitamin D_3 (3-epi-25(OH)D_3) in a Vitamin D External Quality Assessment Scheme certified laboratory. In addition, this study provides data on parathyroid hormone, 1,25-dihrdoxyvitamin D_3 and iCa in infancy; the dynamics of which are not fully explored and understood in the literature yet. This study also provides data on two bone biomarkers that were selected by the International Osteoporosis Foundation: Procollagen type I N-terminal propeptide and urinary alpha telopeptide of collagen I as the recommended biomarkers to be measured in clinical trials. Data on bone biomarkers in infancy in the literature are scarce and thus this study adds to an understudied area of research. In addition, this trial is valuable as clinical trials

conducted in infants and children are less common than those conducted in adults. The latter is due to a combination of factors (241). Enrolment of offspring to a trial may be compromised by the parents' concerns regarding long-term effect and discomfort or pain to their little ones (241) and their need to consent on behalf of their infants/children which has been addressed in the declaration of Helsinki (242). In addition, the pharmaceutical industry may be more interested in conducting trials in the adult population versus the pediatric one given the greater financial profits that could be generated from a population at greater health risk and with a higher prevalence of chronic diseases (241). Research in pediatrics may be more challenging in a sense that sample collection (eg. blood) may be restricted by volume (241). Furthermore, ethics framework in pediatrics has not been robustly defined yet which makes it more difficult to define what is and is not acceptable in terms of health risks (241).

Challenges experienced during the implementation of the trial include the ability to meet recruitment targets (i.e. 49 vs. 74/group) given that initial recruitment to the newborn screening took place at 24-36 h after delivery, a time when families are usually tired and emotional. Nonetheless, in order to circumvent this obstacle the hospital nurses on the delivery ward were asked to enquire from the families whether or not they would be interested in learning more about the vitamin D study before getting approached by the study team. In an effort to reduce the number of blood samplings and to make it easier for parents to accept enrollment of their infants to the newborn screening, blood was collected from the newborns at the time of blood withdrawal for phenylketonuria testing.

Another challenge was that measurement of 1,25-dihydroxyvitamin D_3 concentration was not possible in all participants due to low sample volumes. In addition, only total 25(OH)D was measured at birth using chemiluminescent immunoassay and not LC-MS/MS but values were

standardized using Deming regression, and accuracy (<5%) and precision (<10%) were within internationally accepted limits. In accordance with the assay used, other metabolites were not measured at birth. Conducting DXA scans in infants is always challenging due to potential movement artifacts which renders the quality of the scan poor and not fit for analysis. Rocking infants to sleep prior to scanning them is also challenging and not always feasible depending on the sleeping schedule of the infants, how easily they are disturbed by the sound of the DXA machine and its moving arm, and how well they cooperate with the study team. Nevertheless, in the trial, a high DXA output was achieved for both WB and LS scans. The success rate was 95.9% for WB scans and 99.0% for LS scans. This is ascribed to having a dedicated family-friendly facility and experienced staff. Risk of motion artefacts is lower with LS measurements compared to WB given its shorter time of acquisition and the lower risk of infants moving their lumbar spine compared to their extremities. As such, including both types of scans per infant increases the chances of collecting robust information on bone mineralization status of the infant. Moreover, the low bone density of infants makes it harder for the software to define their edges accurately and may require input from the technician.

Other factors impacting the results may have been related to the recruitment center and population. The choice of Lakeshore General Hospital as the recruitment site for this study versus private clinics was for the sole purpose of attempting to capture different population groups to ensure an equitable, diverse and inclusive research as much as possible. Although the majority of the study population recruited self-identified as white, other population groups were also included. In the Canadian population, the visible minority population constitutes 22.3% of the total population (Census 2016) (231). This group was well represented in the study sample given that a total of 44.6% of mothers self-identified as belonging to another population group than white.

Around 20% of the families studied did not disclose their salaries and the majority of infants with serum 25(OH)D <50 nmol/L were from these families. One speculation is that some families of lower socio-economic status may be reluctant to share their income. If so, insufficiency may be related to a lower level of education or due to difficulty securing supplements for financial or logistical reasons. Indeed, as per a recent study, multiparous mothers and family income that is below the median are associated with the lack of use of a prenatal supplement and a lower maternal education level was associated with lower 25(OH)D concentrations (243). Nonetheless, this statement highlights the need for public health policies to take into account populations at increased risk of insufficiencies and deficiencies given the health risks associated with it (notably, rickets).

6.4 Conclusion

This thesis helped further knowledge on bone health in infancy, a critical period of bone mineralization. It generated reference data for bone mass in infancy and growth charts for LS BMD and confirmed that sex differences in bone mass are due to body size. It also explored maternal determinants of bone mineralization and showed that GWG is a modest determinant of neonate BMC and BMD. Additionally, it confirmed that birth serum 25(OH)D concentrations below the cut-point for sufficiency (<50 nmol/L) and their negative impact on bone health can be avoided with 400 IU/d of vitamin D supplementation postnatally.

Additional research focused on the generation of growth charts for WB BMC in infancy is required given their clinical significance in assessment of bone mineralization. Moreover, further studies tailored to provide a better understanding of the mechanism by which GWG determines

neonate bone mass are needed. Last but not least, the implication of the pre-conception and gestational phases on infantile bone health ought to be explored.

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