Vitamin D status in early life and body composition: Implications to programming of a lean body phenotype

Maryam Razaghi, PhD Candidate
School of Human Nutrition
McGill University, Montreal

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ABSTRACT

Vitamin D is required for growth and development, with implications in health and body composition in infancy. The Institute of Medicine's (IOM) expert committee set an Adequate Intake (AI) value for vitamin D at 400 IU (10 µg)/day across infancy to achieve and maintain vitamin D status in the range of 40 to 50 nmol/L of 25-hydroxyvitamin D [25(OH)D] to support health outcomes. Concentrations at or above 50 nmol/L are considered sufficient for individuals to optimize bone health, whereas evidence regarding the benefit to other health outcomes beyond bone is limited. In Canada, based on reports from provincial cohort studies, 16-32 % of pregnant women do not achieve the cut-point for sufficient vitamin D status in the third trimester and consequently, infants can be born with insufficient stores. Additionally, the proportion of mothers meeting vitamin D sufficiency is lower with pre-pregnancy BMI above the recommended range of 25 kg/m^2. This is thought to be due to vitamin D sequestration in maternal adipose tissue that consequently hinders placental transfer of vitamin D. This is of importance as recent evidence from pregnancy cohort studies suggest that fetal exposure to insufficient maternal vitamin D status is associated with elevated fat mass and lower lean mass in childhood. Whether these observations reflect maternal factors, such as adiposity, or vitamin D status is not clear and is addressed in this thesis. To eliminate the potential confounding associated with low vitamin D status and body composition in human neonates, the first study objective was to test the effect of dietary vitamin D_3 on body composition and growth hormones (IGF-1, IGFBP-3, and leptin) in weanling rats. Female Sprague Dawley rats (n=18) with vitamin D deficiency (25(OH)D <30 nmol/L) were randomized to diets with incremental amounts of vitamin D (1, 2, or 4 IU/g diet) over an 8-week period, from 4 to 12 weeks of age. No differences were observed in baseline body composition, however, at week 8 lean mass and lean mass

accretion were significantly higher ($P<0.05$) in rats that received 2 and 4 IU/g diet compared to those provided with 1 IU/g diet (control). Serum IGF-1 concentration decreased over time ($P<0.001$) with smaller declines in rats that received 4 IU/g diet at week 8 ($P< 0.05$). Serum IGFBP-3 concentration was lower ($P<0.05$) at week 4 in group 2 compared to groups 1 and 4 IU/g diet. Serum leptin concentration and fat mass were not affected by diet. These results suggest that in vitamin D deficient growing mammals, achievement of higher vitamin D status through enhanced vitamin D intakes supports a lean body phenotype without altering weight gain. The second study was designed to explore whether the relationship between maternal vitamin D status and offspring body composition is modified by maternal adiposity or is solely reflective of maternal vitamin D status. The objective was to investigate the correlates of maternal and neonatal vitamin D status and to investigate the interrelationships among maternal and neonatal vitamin D status and body composition. In this study, 142 mother-infant dyads were studied at birth and at 1 month postpartum and categorized into 1 of 4 groups based on vitamin D status above or below the cut-point for sufficiency (50 nmol/L 25(OH)D), and pre-pregnancy BMI within or above the recommended range of 18.5 to 25 kg/m^2. Infants of mothers with elevated pre-pregnancy BMI and insufficient vitamin D status had higher fat mass versus those of mothers with elevated pre-pregnancy BMI, yet vitamin D sufficiency ($P=0.04$). A difference in fat mass between infants of mothers with healthy pre-pregnancy BMI and sufficient vitamin D status and those of mothers with elevated BMI and insufficient vitamin D status was not observed ($P=0.09$). These findings showed that, maternal adiposity and compromised maternal-fetal vitamin D transfer are important dual-exposures associated with neonatal adiposity. To date, no study has tested for a dosage of postnatal vitamin D supplementation that mitigates vitamin D insufficiency and results in a leaner body phenotype. Therefore, the third study objective was to

test whether correction of insufficient vitamin D status using 400 or 1000 IU/d of vitamin D, early in the neonatal period, normalizes body composition (lean and fat mass) across infancy from birth to 1 year in a double-blinded randomized parallel group, controlled trial. Healthy term breastfed infants of appropriate weight for gestational age were recruited at birth and serum 25(OH)D was measured (24-36 h postnatally). Infants with serum 25(OH)D <50 nmol/L were randomized to vitamin D supplementation at 400 (n=49) or 1000 IU/d (n=49) until 12 months; a reference group (n=41) with 25(OH)D ≥50 nmol/L received 400 IU/d. This trial revealed that on average, both vitamin D dosages achieved and maintained vitamin D sufficiency (25(OH)D ≥50 nmol/L) from 3 to 12 months. Across the trial, ionized calcium was within normal limits. Even though no difference was observed in baseline lean mass, lean mass was higher in the 1000 IU/d group compared to the 400 IU/d group (P=0.008) by 12 months of age. Body weight, length and body fat mass were not different over the trial. According to this trial, the Adequate Intake of 400 IU/d supports building vitamin D stores of infants born with serum 25(OH)D <50 nmol/L (<10-49 nmol/L) when adherence is high. In this context, increasing intakes to 1000 IU/d does not seem to provide additional advantages. However, based on the primary outcome the achievement of higher vitamin D status modestly increases whole body lean mass without altering weight gain or linear growth. Whether the benefits of higher dose supplementation to lean mass early in life would extend into childhood with possible implications in reduced risk of excess adiposity, requires further research with longer term follow-up. Overall, this thesis research provides objective novel evidence that vitamin D is implicated in programming of a leaner body phenotype. Additionally, in the event of low maternal-fetal transfer of vitamin D, postnatal supplementation with high adherence appears to readily build vitamin D stores and in

doing so limits the impact of fetal exposures to maternal vitamin D insufficiency regardless of maternal weight status.

RÉSUMÉ

La vitamine D est nécessaire à la croissance et au développement, ce qui a des répercussions sur la santé et la composition corporelle des nourrissons. Le comité d'experts de l'Institute of Medicine (IOM) a fixé la valeur de l'apport suffisant (AI) en vitamine D à 400 IU (10 µg)/jour pendant la petite enfance afin d'atteindre et de maintenir un statut en vitamine D compris entre 40 et 50 nmol/L de 25-hydroxyvitamine D [25(OH)D] pour favoriser la santé. Des concentrations égales ou supérieures à 50 nmol/L sont considérées comme suffisantes pour optimiser la santé osseuse des individus, alors que les preuves concernant les avantages pour d'autres résultats de santé que les os sont limitées. Au Canada, d'après les rapports d'études de cohortes provinciales, 16 à 32 % des femmes enceintes n'atteignent pas le seuil d'un statut suffisant en vitamine D au cours du troisième trimestre et, par conséquent, les nourrissons peuvent naître avec des réserves insuffisantes. En outre, la proportion de mères présentant une carence en vitamine D est plus faible lorsque BMI avant la grossesse est supérieur à la fourchette recommandée de 25 kg/m^2. On pense que cela est dû à la séquestration de la vitamine D dans le tissu adipeux maternel, ce qui entrave le transfert placentaire de la vitamine D. Ceci est important car des preuves récentes provenant d'études de cohortes de grossesses suggèrent que l'exposition du fœtus à un statut maternel insuffisant en vitamine D est associée à une masse grasse élevée et à une masse maigre plus faible pendant l'enfance. La question de savoir si ces observations reflètent des facteurs maternels, tels que l'adiposité, ou le statut en vitamine D n'est pas claire et est abordée dans cette thèse. Afin d'éliminer les facteurs de confusion potentiels associés à un faible statut en vitamine D et à la composition corporelle chez les nouveau-nés humains, le premier objectif de l'étude était de tester l'effet de la vitamine D_3 alimentaire sur la composition corporelle et les hormones de croissance (IGF-1, IGFBP-3 et leptine) chez des rats sevrés. Des rats Sprague Dawley

femelles (n=18) présentant une carence en vitamine D (25(OH)D <30 nmol/L) ont été soumis de manière aléatoire à des régimes contenant des quantités croissantes de vitamine D (1, 2 ou 4 UI/g de régime) sur une période de 8 semaines, de 4 à 12 semaines d'âge. Aucune différence n'a été observée dans la composition corporelle de base, cependant, à la semaine 8, la masse maigre et l'accrétion de la masse maigre étaient significativement plus élevées (P<0,05) chez les rats ayant reçu une alimentation à 2 et 4 IU/g par rapport à ceux ayant reçu une alimentation à 1 IU/g (contrôle). La concentration sérique d'IGF-1 a diminué au fil du temps (P<0,001) avec des baisses plus faibles chez les rats ayant reçu un régime alimentaire de 4 IU/g à la semaine 8 (P<0,05). La concentration sérique d'IGFBP-3 était plus faible (P<0,05) à la semaine 4 dans le groupe 2 par rapport aux groupes 1 et 4 IU/g de régime. La concentration sérique de leptine et la masse grasse n'ont pas été affectées par le régime alimentaire. Ces résultats suggèrent que chez les mammifères en croissance déficients en vitamine D, l'obtention d'un statut plus élevé en vitamine D par le biais d'apports accrus en vitamine D soutient un phénotype de corps maigre sans altérer la prise de poids. La deuxième étude a été conçue pour explorer si la relation entre le statut maternel en vitamine D et la composition corporelle de la progéniture est modifiée par l'adiposité de la mère ou si elle est uniquement le reflet du statut maternel en vitamine D. L'objectif était d'étudier les corrélations du statut maternel et néonatal en vitamine D et d'étudier les interrelations entre le statut maternel et néonatal en vitamine D et la composition corporelle. Dans cette étude, 142 dyades mère-nourrisson ont été étudiées à la naissance et à un mois du post-partum et classées dans l'un des 4 groupes en fonction du statut en vitamine D supérieur ou inférieur au seuil de suffisance (50 nmol/L 25(OH)D), et de BMI avant la grossesse situé dans ou au-dessus de la fourchette recommandée de 18,5 à 25 kg/m^2. Les nourrissons de mères ayant un BMI élevé avant la grossesse et un statut insuffisant en vitamine D avaient une masse grasse plus

importante que ceux de mères ayant un BMI élevé avant la grossesse, mais un statut suffisant en vitamine D (P=0,04). On n'a pas observé de différence de masse grasse entre les nourrissons de mères ayant un BMI sain avant la grossesse et un statut suffisant en vitamine D et ceux de mères ayant un BMI élevé et un statut insuffisant en vitamine D (P=0,09). Ces résultats montrent que l'adiposité maternelle et un transfert maternel-fœtal de vitamine D compromis sont des expositions doubles importantes associées à l'adiposité néonatale. À ce jour, aucune étude n'a testé le dosage de la supplémentation postnatale en vitamine D qui atténue l'insuffisance en vitamine D et entraîne un phénotype corporel plus maigre. Par conséquent, le troisième objectif était de vérifier si la correction d'un statut insuffisant en vitamine D à l'aide de 400 ou 1000 IU/j de vitamine D, au début de la période néonatale, permet de normaliser la composition corporelle (masse maigre et masse grasse) chez le nourrisson, de la naissance à 1 an, dans le cadre d'un essai contrôlé randomisé en double aveugle avec groupes parallèles. Des nourrissons en bonne santé, nés à terme et allaités au sein, dont le poids était adapté à l'âge gestationnel, ont été recrutés à la naissance et le taux sérique de 25(OH)D a été mesuré (24-36 heures après la naissance). Les nourrissons présentant un taux sérique de 25(OH)D <50 nmol/L ont été randomisés pour recevoir une supplémentation en vitamine D à raison de 400 (n=49) ou 1000 IU/j (n=49) jusqu'à 12 mois; un groupe de référence (n=41) présentant un taux de 25(OH)D ≥50 nmol/L a reçu 400 IU/j. Cet essai a révélé qu'en moyenne, les deux dosages de vitamine D permettaient d'atteindre et de maintenir la suffisance en vitamine D (25(OH)D ≥50 nmol/L) de 3 à 12 mois. Tout au long de l'essai, le calcium ionisé se situait dans les limites normales. Bien qu'aucune différence n'ait été observée dans la masse maigre de départ, la masse maigre était plus élevée dans le groupe 1000 IU/j que dans le groupe 400 IU/j (P=0,008) à l'âge de 12 mois. Le poids corporel, la longueur et la masse grasse corporelle n'étaient pas différents au cours de

l'essai. Selon cet essai, l'apport adéquat de 400 IU/j favorise la constitution de réserves de vitamine D chez les nourrissons nés avec un taux sérique de 25(OH)D <50 nmol/L (<10-49 nmol/L) lorsque l'observance est élevée. Dans ce contexte, l'augmentation des apports à 1000 IU/j ne semble pas apporter d'avantages supplémentaires. Cependant, d'après le résultat primaire, l'obtention d'un statut plus élevé en vitamine D augmente modestement la masse maigre du corps entier sans modifier la prise de poids ou la croissance linéaire. La question de savoir si les avantages d'une dose plus élevée de supplémentation de la masse maigre au début de la vie s'étendent à l'enfance avec des implications possibles dans la réduction du risque d'excès d'adiposité, nécessite des recherches supplémentaires avec un suivi à plus long terme. Globalement, cette thèse fournit des preuves objectives que la vitamine D est impliquée dans la programmation d'un phénotype corporel plus maigre. De plus, dans le cas d'un faible transfert maternel-fœtal de vitamine D, la supplémentation postnatale avec une forte adhésion semble construire facilement les réserves de vitamine D et, ce faisant, limite l'impact des expositions fœtales à l'insuffisance de vitamine D maternelle, indépendamment du statut pondéral de la mère.

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PREFACE

This thesis is presented in manuscript format and consists of six chapters. Chapter 1 presents a general introduction and provides a brief overview of the research focus, explains the thesis rationale and objectives of the research. Chapter 2 presents a current and in-depth literature review of the topic covering different aspects of vitamin D metabolism in body composition in infancy, the mechanism of actions in skeletal muscle development and adipose tissue formation, current evidence from observational and interventional studies on the role of vitamin D on body composition. Chapter 3 through 5 are research-based manuscripts that are bridged sequentially through connecting statements. Amongst these three manuscripts, Chapter 3 has been published in Nutrition Research. Chapters 4 and 5 will be duly submitted for publication in BMC Pregnancy and Childbirth and American Journal of Clinical Nutrition, respectively. Finally, the last chapter presents an overall discussion and conclusion of the research presented throughout the thesis, along with contributions of knowledge and recommendations for future research. This dissertation is in accordance with guidelines for thesis preparation as published by the Graduate and Postdoctoral Studies of McGill University.

Research articles (published) or submitted to peer-reviewed journals

- **Razaghi M,** Djekic-Ivankovic M, Agellon S, Mak I, Lavery P, Weiler HA. Lean body mass accretion is elevated in response to dietary vitamin D: a dose-response study in female weanling rats (Chapter 3). Published in Nutrition Research 2019, 68:92-100.
- **Razaghi M,** Gharibeh N, Vanstone CA, Sotunde OF, Wei SQ, McNally D, Rauch F, Jones G, Weiler HA. Maternal excess adiposity and serum 25-hydroxyvitamin D <50 nmol/L are associated with elevated whole body fat mass in healthy breastfed neonates (Chapter 4). Under consideration by BMC Pregnancy and Childbirth.
- **Razaghi M,** Gharibeh N, Vanstone CA, Sotunde OF, Wei SQ, McNally D, Rauch F, Jones G, Kimmins S, Weiler HA. Correction of insufficient neonatal vitamin D status using 1000 IU/d of vitamin D increased lean body mass by 12 months of age compared to 400 IU/d: a randomized controlled trial (Chapter 5). Under consideration by American Journal of Clinical Nutrition.
- Hope A. Weiler, Catherine A. Vanstone, **Maryam Razaghi**, Nathalie Gharibeh, Sharina Patel, Shuqin Wei, Dayre McNally. The odds of achieving adequate vitamin D status in newborn infants are higher with maternal use of multivitamin supplements preconception and continued during pregnancy (Appendix 4). Published in Journal of Nutrition 2021, nxab344.

Abstracts

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- **Razaghi M,** Vanstone CA, Sotunde OF, Gharibeh N, Wei SQ, McNally D, Rauch F, Jones G, Kimmins S, Weiler HA. Interrelationships among vitamin D status and body composition in mother-infant dyads. American Society for Nutrition, online meeting due to COVID 19 outbreak, 2020 (Finalist for the American Society for Nutrition's Emerging Leaders in Nutrition Science Abstract Recognition Award Program).
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- **Razaghi M,** Vanstone CA, Gharibeh N, Sotunde OF, Wei SQ, McNally D, Rauch F, Jones G, Kimmins S, Weiler HA. Insufficient vitamin D status at birth is corrected by vitamin D supplementation (1000 IU/day) with increases in lean mass evident at 12 months of age in healthy term infants. American Society for Nutrition, online meeting due to COVID 19 outbreak, 2021 (Oral presentation, finalist for the American Society for Nutrition's Emerging Leaders in Nutrition Science Abstract Recognition Award Program).
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CONTRIBUTION OF AUTHORS

Author study involvement for Manuscripts 1, 2, and 3:

Manuscript 1: Dr. HA Weiler designed the weanling rat study and all of the analyses; the primary objective of the study was on bone outcomes (M Djekic-Ivankovic et al. Journal of Nutrition, 2016) and lean mass was the secondary outcome. Dr. Weiler, M Djekic-Ivankovic, P Lavery and S Agellon conducted the study; Dr. Weiler, M Djekic-Ivankovic, P Lavery, S Agellon, and I Mak collected the data including all body composition measurements and blood collection; the candidate, Maryam Razaghi, measured insulin-like growth factor binding protein-3 and leptin concentrations using enzyme-linked immunosorbent assays under the supervision of Mrs. S Agellon; Dr. Weiler and the candidate, analyzed and interpreted the data for the secondary outcome, lean mass. The candidate was the primary author who drafted the manuscript. The candidate edited all subsequent drafts and all of the authors (Dr. Weiler, M Djekic-Ivankovic, P Lavery, S Agellon, and I Mak) aided with revisions to the manuscript. This study was supported by a Collaborative Research and Development grant from Dairy Farmers of Canada and the Natural Sciences and Engineering Research Council of Canada and by infrastructure grants from the Natural Sciences and Engineering Research Council of Canada and the Canada Foundation for Innovation.

Manuscript 2 and 3: Dr. HA Weiler is the primary investigator of the Infant Vitamin D study and designed the research project in collaboration with Dr. Shu Qin Wei, Dr. Dayre McNally, Dr. Frank Rauch, Dr. Glenville Jones, and Dr. Sarah Kimmins that all contributed to the development of overall research plan, and study oversight. The candidate, Maryam Razaghi, was trained under the supervision of Dr. HA Weiler and the guidance of Mrs. Catherine Vanstone for clinical work. This study was funded by the Canadian Institutes of Health Research, Canada

Research Chairs Program, and Graduate Excellence Fellowships from McGill University. The candidate, M Razaghi, actively participated in participant recruitment at the Lakeshore General Hospital. The candidate accompanied by N Gharibeh, OF Sotunde and CA Vanstone conducted the study visits, performed anthropometric assessments, bio-electrical impedance analyses, skin pigmentation assessment. The candidate was trained by CA Vanstone on performing dual-energy x-ray absorptiometry as well as analyzing the scans followed by blood sample procurement. Additionally, the candidate, N Gharibeh, and CA Vanstone led in the data entry and data auditing. The candidate was also involved in dietary analysis, conducting the chemiluminescence immunoassay via an in-house automated analyzer to assess vitamin D status, assisted by Mrs. Maggie Yuan and under the supervision of Mrs. Sherry Agellon. In addition, the candidate performed the enzyme-linked immunosorbent assays for assessment of Insulin-like growth factor 1 and the main carrier protein-3 across the trial. During the period of the trial, the candidate was also actively involved in DNA extraction and samples preparation for epigenetic mechanisms testing although the results are outside the scope of this dissertation. The candidate was the primary author who drafted the manuscript 2 and 3, conducted all statistical analyses with the intellectual aid of Dr. HA Weiler. The candidate edited all subsequent drafts. All the co-authors (CA Vanstone, N Gharibeh, OF Sotunde, SQ Wei, D McNally, F Rauch, G Jones, S Kimmins, and HA Weiler aided with revisions to the manuscript. Dr. Kimmins provided guidance and feedback throughout this research project.

STATEMENT OF ORIGINALITY

Vitamin D supplementation is well-established within the health care domain in Canada and is well received by parents and caregivers. The Adequate Intake recommendation for vitamin D supplementation with 400 IU (10 μg) /day is applicable to all healthy breastfed infants and in fact regardless of the amount of maternal-fetal transfer of vitamin D and the status at birth. Many health professional society recommendations suggest that higher vitamin D dosage is required for some population groups. Whether this holds true on the basis of being born with insufficient vitamin D status has not been tested in a controlled manner in Canada. The objective of the overall research thesis is to test for the benefits of improving vitamin D status (in neonates) born with insufficient vitamin D stores on the development of lean body mass. The trial concurs with observations in weanling rats and provides high quality information to help guide health policy in Canada. It provides information regarding building of vitamin D stores after birth, and the possible functional outcomes including benefits to growth and lean mass phenotype.

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LIST OF ABBREVIATIONS

Δ	Delta
µCT	Micro-computed tomography
µg	Microgram
µSv	Microsievert
%BF	Percentage body fat
1,25$(OH)_2$D	1,25-dihydroxyvitamin D
24,25$(OH)_2$D	24,25-dihydroxyvitamin D
25(OH)D	25-hydroxyvitamin D
3-epi-25$(OH)D_3$	3-epimer-25-hydroxyvitamin D_3
7-DHC	7-dehydrocholesterol
Adipoq	Adiponectin
AI	Adequate Intake
ADP	Air displacement plethysmography
ANOVA	Analysis of variance
BIA	Bioelectrical impedance analysis
BL	Baseline
BMI	Body mass index
Ca	Calcium
CFG	Canada's Food Guide
CHMS	Canadian Health Measures Survey
CI	Confidence interval

CT	Computed tomography
CV	Coefficient of variation
CYP	Cytochrome P450 monooxygenases
CYP27A1	Cytochrome P450 27A1
CYP24A1	Cytochrome P450 24A1
CYP2R1	Cytochrome P450 2R1
d	Day
DEQAS	Vitamin D External Quality Assessment Scheme
DMRs	Differentially methylated regions
DXA	Dual-energy x-ray absorptiometry
EAR	Estimated Average Requirement
ELISA	Enzyme-linked immunosorbent assay
Fabp4	Fatty acid binding protein 4
FFQ	Food frequency questionnaire
FGF23	Fibroblast growth factor 23
FMI	Fat mass index
FSR	Fractional synthesis rate
g	Gram (s)
GH	Growth hormone
h	Hour (s)
HAZ	Height-for-age z-score
iCa	Ionized calcium

IGF-1	Insulin-like growth factor-1
IGFBP-3	Insulin-like growth factor binding protein-3
IOM	Institute of Medicine
I-OW/O	Insufficient-overweight/obese
I-RW	Insufficient-recommended weight
IU	International unit
kcal	Kilocalorie
kg	Kilograms
L	Liter (s)
LLOQ	Lower limit of quantification
m	Meter (s)
mg	Milligram (s)
min	Minute (s)
mL	Milliliter (s)
mo	Month (s)
MRI	Magnetic resonance imaging
NIST	National Institute of Standards and Technology
OR	Odds ratio
Pparg2	Peroxisome proliferator-activated receptor gamma 2
PTH	Parathyroid hormone
RCT	Randomized controlled trial
RDA	Recommended Dietary Allowance
RXR	Retinoid x receptor

SD	Standard deviation
S-OW/O	Sufficient-overweight/obese
SRC-3	Steroid receptor co-activator 3
S-RW	Sufficient-recommended weight
TBW	Total body water
UCP2	Uncoupling protein 2
UL	Tolerable upper intake level
UVB	Ultraviolet B
VDBP	Vitamin D binding protein
VDR	Vitamin D receptor
VDRE	Vitamin D response element
WAZ	Weight-for-age z-score
WB	Whole body
WC	Waist circumference
WHO	World Health Organization
wk	Week (s)
y	Year (s)

CHAPTER 1: THESIS RATIONALE

1.1 Thesis rationale

Infancy is one of the most sensitive periods in the human life span to environmental influences, particularly nutritional status (1). Growth and musculoskeletal development *in utero* and infancy occurs at a rapid rate unparalleled by other life stages (2). At birth, infants are relatively lean and have acquired approximately 3 kg of lean mass (3). In healthy full-term infants, muscular development continues to be rapid with lean mass almost doubling by 12 months of age (4, 5). In contrast, fat mass increases rapidly from birth to approximately 6 months of age, and then plateaus (6). This high accretion rate for lean and fat mass in early infancy makes it a critical period for programming of body composition. This is of significance as according to the developmental origins of pediatric obesity theory, the etiology of obesity can begin very early, *in utero*. For instance, altered maternal nutrition along with genetic and epigenetic influence may delay fetal growth resulting in accelerated postnatal growth that may predispose one to childhood obesity with long-lasting effects on health (7, 8). There is an emerging body of evidence from infancy to late childhood suggesting a positive association between vitamin D status and lean mass and an inverse association between vitamin D status and adiposity, (4, 9). Therefore, it appears that achievement of sufficient vitamin D status has a function in reprogramming a leaner body phenotype and thus may have implications in early life prevention of obesity.

In 2011, the Institute of Medicine's (IOM) expert committee set an Adequate Intake (AI) value for vitamin D at 400 IU (10 μg)/day across infancy to achieve and maintain vitamin D status in the range of 40 to 50 nmol/L of 25-hydroxyvitamin D [25(OH)D] and support healthy outcomes (10). As per the IOM, concentrations at or above 50 nmol/L of 25(OH)D are considered sufficient for individuals to optimize bone health, whereas, other health agencies such

as Canadian Paediatric Society and the Endocrine Society (11, 12) have suggested a higher target of 75 nmol/L of 25(OH)D in support of skeletal and extra-skeletal health outcomes. However, evidence regarding the benefits to other health outcomes beyond bone is limited. While serum 25(OH)D represents a continuum of risk, for the purpose of this thesis values below 50 nmol/L will be termed insufficient.

In Canada, not all pregnant women meet sufficient vitamin D status and consequently infants can be born with insufficient vitamin D stores (13-18). According to a large pregnancy cohort study in Quebec City, Canada; 44% of mothers in the first to second trimester had 25(OH)D below 50 nmol/L and subsequently 24% of their infants were born with 25(OH)D below 50 nmol/L based on cord blood assessments (19). Recent evidence from international pregnancy cohort studies (20-22) suggests that fetal exposure to insufficient maternal vitamin D status associates with changes in body composition across a wide age range during childhood. For instance, studies from New Zealand and India, indicate that lower maternal vitamin D status below 50 nmol/L in the beginning of second (weeks 15) and third trimesters (weeks 28-32) is associated with an elevated percentage body fat in children at the age of 5 to 9.5 years (20, 21). In addition, a study in the United Kingdom indicates that mothers in the lowest quartile of vitamin D status later in gestation (weeks 34) have children with lower percentage lean mass at the age of 4 years (22). Furthermore, in the Netherlands, severe maternal vitamin D deficiency, defined as serum 25(OH)D <25 nmol/L, in the second trimester (weeks 18.5–23.4) was associated with a lower percentage of lean mass in children at the age of 6 years (23). This body of evidence suggests that fetal exposure to maternal vitamin D status below sufficiency has a long-lasting impact on body composition. Due to the observational nature of these cohorts, causal relationships cannot be established, additionally, the studies in New Zealand and India

relied upon bioelectrical impedance analysis to estimate body composition in children and not multi-compartmental models that more accurately delineate lean and fat mass partitioning. In the aforementioned cohorts maternal body mass index (BMI) was used as a proxy for maternal adiposity, which is subject to underestimation bias. Whether these observations are confounded by sociodemographic factors, or reflect maternal factors, such as adiposity, or vitamin D status is not clear suggesting the need for highly controlled studies.

The only dose-response trial reporting changes in body composition according to vitamin D supplementation was in infants with plasma 25(OH)D on average ≥50 nmol/L at inception (4). In this study plasma 25(OH)D concentrations were positively associated with a higher percentage of lean body mass and lower fat mass at 12 months of age. Thus, the overarching goal of this thesis is to advance our understanding of how improving vitamin D status impacts lean body mass. As such, one of the aims was to conduct a highly controlled study in an experimental animal model with controlled diets, to test the effectiveness of increasing vitamin D intakes and status on body composition. Use of an animal model removes the confounding factors associated with low vitamin D status and body composition in human neonates such genetic variability, skin tone, season of birth, as well as maternal factors and sociodemographic status. Moreover, given the scarcity of data on the associations between vitamin D status and body composition in mother-infant dyads in early postnatal life, a second aim was to identify other influential factors in pregnancy that are important to enhance understanding of the interrelationships among maternal and neonatal vitamin D status and body composition. Considering that no study has tested for a dosage of postnatal vitamin D supplementation that mitigates low vitamin D stores acquired *in utero*, and supports a leaner body phenotype, a third aim was to conduct a randomized controlled trial in human infants to test whether correction of low neonatal vitamin

D status using incremental dosage of vitamin D would impact on body composition during infancy.

1.2 Research objectives

1) To establish if increased intake of vitamin D would result in a leaner body phenotype, greater lean mass accretion and lower fat mass accretion, in a dose–response study in a rapidly growing animal model. Secondly, since the positive effects of vitamin D on body composition are shown to be mediated by IGF-1, IGFBP-3 and leptin, this study aimed to establish whether increasing vitamin D intake also affects these growth-related biomarkers.

   It was **hypothesized** that low vitamin D status in weanling rats programs for higher fat mass accretion, and that achievement of vitamin D status above 50 nmol/L of $25(OH)D_3$ resets the trajectory to a leaner body phenotype and lower fat mass.

2) To explore the correlates of maternal and neonatal vitamin D status and to assess whether the association between maternal vitamin D status and neonatal adiposity is modified by maternal adiposity preconception.

   It was **hypothesized** that exposures to maternal excess adiposity preconception and maternal insufficient vitamin D status adversely affects neonatal vitamin D status and body composition.

3) To test whether correction of insufficient vitamin D status early in the neonatal period improves whole body lean mass in infancy. A secondary trial objective tested whether correction of vitamin D status below 50 nmol/L of serum 25(OH)D early in infancy using 1000 IU/d of vitamin D would also improve infant growth and fat mass accretion rate.

Since the positive effects of vitamin D on body composition are assumed to be mediated by IGF-1, IGFBP-3, this study aimed to compared insulin-like growth factor-1 (IGF-1) and its main carrier protein (IGFBP-3) among groups as these have established functions in growth and development of skeletal muscle across infancy.

It was **hypothesized** that neonates provided with the standard of care, 400 IU/d vitamin D_3, would have lower lean mass and lean mass accretion by 3 months of age and thereafter by 12 months compared to infants provided with higher dosage of 1000 IU/d vitamin D_3.

CHAPTER 2: LITERATURE REVIEW

2.1 An overview of vitamin D metabolism

Humans mainly obtain vitamin D through endogenous dermal synthesis when ultraviolet B (UVB) solar radiation is strong enough to elicit photoconversion of 7-dehydrocholesterol (7-DHC) to vitamin D_3 (cholecalciferol) and as such reflect seasonal variations in vitamin D status in the Northern and Southern hemispheres (24). In addition, vitamin D can be obtained from exogenous sources through natural and fortified food or supplements and in the form of vitamin D_2 or D_3 isomers (25). These isomers eventually undergo hydroxylation during metabolism as a function of several 25-hydroxylases such as cytochrome P450 CYP27A1 and CYP2R1. The first hydroxylation occurs in the liver to form 25(OH)D_3 or calcifediol. In humans, CYP2R1 is thought to be the main enzyme required for 25-hydroxylation of vitamin D_3 and the process is not tightly regulated nor rate limiting (26). Depending on calcium homeostasis, 25(OH)D_3 undergoes another hydroxylation via 1α-hydroxylase, CYP27B1, in the kidney to be converted to 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$) or calcitriol which is the hormonal and biological active form of vitamin D_3 with an established role in maintenance of appropriate blood calcium and phosphorus concentrations (27). Unlike the first hydroxylation in the liver, the hydroxylation process in the kidney has been shown to be rate limiting and tightly regulated via a variety of hormones including parathyroid hormone, bone-derived fibroblast growth factor-23 (FGF23) and 1,25$(OH)_2D_3$ (28). Liver is the main organ responsible for catabolism of vitamin D and its metabolites; this is also ascribed to cytochrome P450 enzymes (27). CYP24A1, is a mitochondrial cytochrome P450-containing hydroxylase enzyme, that degrades 1,25$(OH)_2D_3$ by formation of calcitrioic acid; this enzyme has an important role in regulation of vitamin D metabolism (29). Calcitriol exerts its biological actions by binding to its nuclear receptor (VDR). VDR is present in the nucleus and cytosol of the cell and has both ligand and DNA binding

domains (30). Vitamin D actions are mediated through VDR, which associates with a retinoid x receptor (RXR) in cytosol to form VDR/RXR heterodimers then transverse into the nucleus (31). The $1,25(OH)_2D$/VDR/RXR heterodimers bind to DNA sequence of the genes possessing vitamin D response elements (VDREs) in the promoter region and therefore initiate genomic pathways via increased expression of various genes in the body such as those encoding for peptides like collagen, osteocalcin, as well as muscle contractile proteins (32). VDR can be also found in the membrane of target cells such as intestinal enterocytes, pancreatic islets, renal distal tubules, osteoblasts (33) as well as skeletal muscle cells (34); and $1,25(OH)_2D$ by binding to this membrane receptor generates rapid actions and activates intracellular signal transduction involved in broad physiological actions through rapid and non-genomic pathways (25). During the first weeks of postnatal life, infants experience the transition from fetal to neonatal life which requires physiological changes that occur at a rapid rate as part of the developmental process to support subsequent growth (35). Most organs including liver and kidneys undergo maturation processes that extend beyond the neonatal period. At birth, liver and renal immaturity may affect vitamin D activation pathways (35). It has been shown that $1,25(OH)_2D_3$ coupled with its nuclear VDR promotes differentiation of renal cells, induces the activity of several brush border enzymes, and thus plays an integral role in renal tissue development (36). In addition, $1,25(OH)_2D_3$ has been shown to be essential for normal hepatocytes growth (26). Collectively, since the fetus and newborn are entirely dependent upon maternal supply of vitamin D (37), adequate maternal-fetal-infant transfer of vitamin D is important to enhance the quality of growth and overall health.

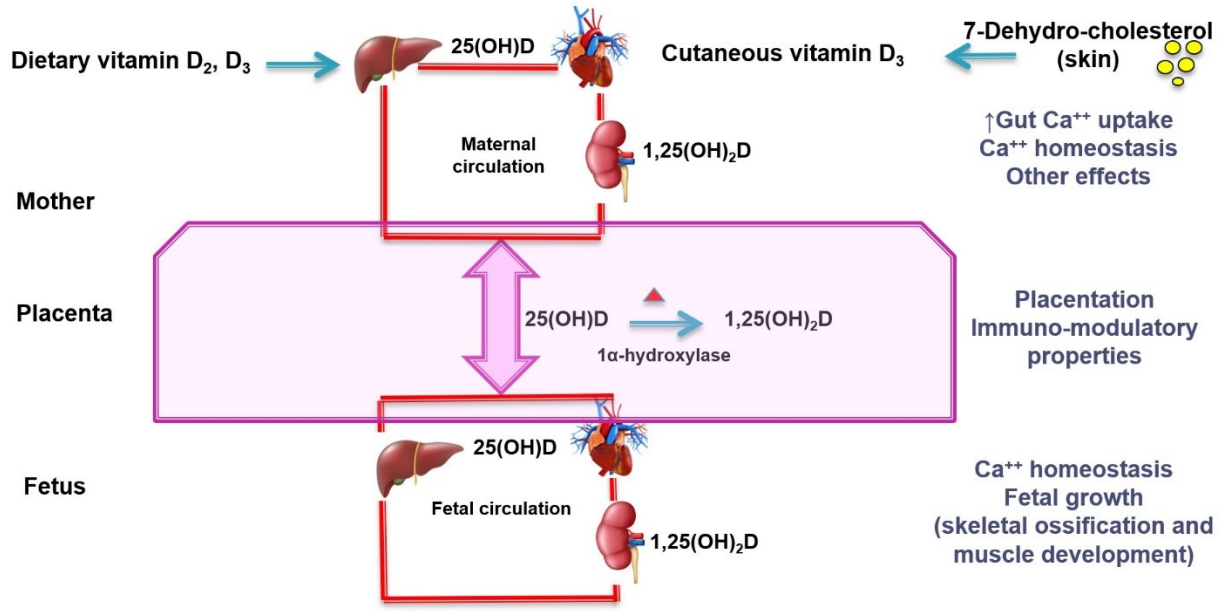
2.2 Maternal-fetal metabolism of vitamin D

Cord blood concentrations of vitamin D metabolites are highly correlated with maternal concentrations. However, the 25(OH)D values of newborns are consistently lower than those measured in the mother (38, 39). Maternal vitamin D stores are considered the major source of vitamin D for the developing fetus and newborn infant. Placenta is a vital organ that mediates transfer of vitamin D and other nutrients (40). Even though cholecalciferol (vitamin D_3) crosses the placenta, both maternal and fetal vitamin D_3 concentrations are considered to be low (41). However, maternal 25(OH)D can readily cross the placenta with fetal concentrations reaching ~75% of maternal concentrations by term. Therefore, maternal circulating 25(OH)D is the main source of vitamin D for the fetus as well as the newborn as until they commence taking vitamin D supplements or obtain vitamin D from other dietary sources. Maternal and fetal vitamin D status are highly correlated; this underscores the importance of adequate maternal-fetal transfer of 25(OH)D (42). The biological active metabolite, $1,25(OH)_2D$, does not readily cross the placenta, however, the placenta can synthesize $1,25(OH)_2D$ directly via the enzyme 1-α-hydroxylase, and this partly contributes to the concentration of circulating $1,25(OH)_2D$ in the fetus in addition to renal conversion of 25(OH)D to $1,25(OH)_2D$ (Figure 2.1). In both mother and fetus, the rise in $1,25(OH)_2D$ is dependent on substrate availability, which is 25(OH)D. Therefore, infants with vitamin D deficiency can also have lower $1,25(OH)_2D$ as it relies on availability of precursor (43). Moreover, placenta can also convert 25(OH)D to 24,25-dihydroxyvitamin D ($24,25(OH)_2D$) (44). Although the contribution of the placenta to $24,25(OH)_2D$ concentrations in humans is not fully elucidated, it is believed that $24,25(OH)_2D$ accumulates in bone and contribute to fetal skeletal formation and development (45). Evidence has been shown that in full term human placental tissue, the promoter region of the *CYP24A1*

gene is methylated and thus highly epigenetically regulated (46) which favors activity of $1,25(OH)_2D$ in placental tissue development and function and is reflective of local regulation of vitamin D metabolism within the placental tissue.

Figure 2.1 Maternal-fetal transfer of vitamin D

Adapted from (42).



Among other vitamin D metabolites, C3-epimers have received attention recently by researchers. Epimers are formed through C-3 epimerization pathway likely in extra-renal tissues and dissimilar to the classical metabolic pathway (47). Even though the biological function and clinical significance of the epimers are not fully identified, they may have important regulatory role(s) that support normal growth with possible implications in lean mass early in infancy (from 1 to 3 months of age) (4, 48). Furthermore, newborns are known to have relatively higher C3-epimer concentrations compared to adults (48, 49).

2.3 Response of vitamin D metabolites to supplementation

Infants metabolize 25(OH)D and $1,25(OH)_2D$ into the inactive metabolite $24,25(OH)_2D$, in response to supplementation dosage and based on vitamin D status (50). This suggests the initiation of the catabolic pathway to regulate both 25(OH)D and $1,25(OH)_2D$ concentrations within the circulation. Infants with lower vitamin D status have lower $24,25(OH)_2D$, indicative of increased response of 25(OH)D to vitamin D supplementation in low status states due to lower CYP24A1 activity. This is in favor of maintaining circulating 25(OH)D and $1,25(OH)_2D$ (51). In addition, according to studies in weanling rats and human infants, 3-epi-$25(OH)D_3$ follows a dose-response to vitamin D intake and demonstrate a positive correlation with $25(OH)D_3$ (4, 49).

In breastfed infants receiving a 400 IU/d supplement versus no supplement from birth, 25(OH)D was almost 50% higher and $1,25(OH)_2D$ 29% higher after 1.5 months (43). Values at 3 months for $1,25(OH)_2D$ were similar between groups whereas the 25(OH)D remained different, indicating that $1,25(OH)_2D$ utilization for tissue growth appears to take precedence over building vitamin D stores. That underscores the importance of neonatal stores to maintain $1,25(OH)_2D$ concentration within normal ranges to support functional outcomes in target tissues such as lean tissue.

2.4 Indicator of vitamin D status and the definitions of vitamin D status

Based on vitamin D metabolism and knowledge to date, the best biomarker of vitamin D is total 25(OH)D or calcifediol as it is a composite marker of both endogenous and exogenous sources (52-54). Total 25(OH)D concentration has relatively stable concentrations in the blood with a half-life of ~ 15-21 days and therefore is not significantly influenced by transient changes in dietary sources of vitamin D or excessive UVB exposure (54). This is in contrast to $1,25(OH)_2D$ or calcitriol, the active hormonal form of vitamin D, with a half-life of ~15 hours (55). In North America, the IOM set various vitamin D status cut-points, of relevance to the studies in infancy, deficiency is defined when 25(OH)D concentrations fall below 30 nmol/L. In 2011, the IOM published an updated Adequate Intake (AI) value of 400 IU/day vitamin D for infants up to 12 months to support vitamin D status in the adequate range of 40-50 nmol/L (10). Concentrations at or above 50 nmol/L are considered sufficient for individuals to optimize bone health (10), whereas evidence regarding the benefit to other health outcomes beyond bone particularly to musculoskeletal system is limited. For instance, the Canadian Paediatric Society similarly defines vitamin D deficiency as <30 nmol/L but recommends serum 25(OH)D between 75 to 225 nmol/L for skeletal and extra-skeletal health outcomes (56). This target is set based on bone health and according to trials in human adults when parathyroid hormone (PTH) plateaus (57, 58). In Canada, in the absence of national surveillance data on vitamin D status in infancy the prevalence of adequate vitamin D status is unknown. However, a few studies report upon vitamin D status in newborn infants. For instance, based on a pregnancy cohort study in Quebec City, 24% of infants had cord 25(OH)D below 50 nmol/L; in another study in Winnipeg, 36% of newborns had 25(OH)D below 30 nmol/L (16, 17). Additionally, according to another report from Calgary, 28% of newborns had serum 25(OH)D below 50 nmol/L (18). These are

concerning proportions and of importance as the amount of vitamin D required to improve stores in infants born with vitamin D deficiency or below the suggested range of 40-50 nmol/L is unclear.

Infants have a unique source of vitamin D. The fetus is entirely dependent upon the mother's supply of vitamin D as 25(OH)D readily crosses the placental barrier and is reflective of maternal concentrations (39). Even though transplacental maternal vitamin D can build fetal stores, un-supplemented newborn infants born to vitamin D-replete mothers may present vitamin D deficiency after 8 weeks of life during early infancy (59) as breast milk does not contain adequate vitamin D to meet the needs of infants (60). According to IOM, the Adequate Intake (AI) value for vitamin D at 400 IU/d across infancy is believed to support vitamin D status in the range of 40 to 50 nmol/L of 25(OH)D (61). In view of limited high-quality evidence in infancy, the IOM was unable to specify separate recommendation on amounts of vitamin D required to improve stores in infants born with deficiency (<30 nmol/L). The Endocrine Society recommends that infants with vitamin D deficiency may need to be supplemented with at least 1000 IU of vitamin D per day to rapidly correct deficiency (62). According to a systematic review and meta-analysis conducted in 2014, vitamin D supplementation with 1000 to 4000 IU/d in children with vitamin D deficiency, improves vitamin D stores based on serum 25(OH)D concentrations above 75 nmol/L within 1 month (63). Results from a dose-response study in Montreal (50) demonstrate that 1200 IU/d of vitamin D supplementation in infants with vitamin D insufficiency at 1 month of age rapidly improves vitamin D status of healthy term breastfed infants. In the same study, lower dosages of 400 IU/d, the standard of care, maintains vitamin D status to meet the IOM target, however more than half of these infants were below 75 nmol/L the target suggested by the Canadian Paediatric Society

and the Endocrine Society for skeletal and extra-skeletal health outcomes (56, 62). Thus, it appears that even though correction of vitamin D deficiency using 400 IU/d eventually builds vitamin D stores, it may be too low to support status associated with extra-skeletal health outcomes particularly lean body mass accretion.

Adequately well-powered trials are thus required to determine whether correction of low vitamin D stores with a higher dosage of vitamin D than the standard of care corrects the stores with additional advantages to programming of body composition.

2.5 Measurement of total serum 25(OH)D concentration

For the measurement of 25(OH)D, different assay techniques are currently available including competitive binding-protein assays, immunoassays such as chemiluminescent immunoassay (CLIA), high performance liquid chromatography (HPLC), as well as liquid chromatography-tandem mass spectrometry (LC-MS/MS), which is considered the gold standard for the measurement of vitamin D metabolites simultaneously including 25(OH)D_2, 25(OH)D_3, and 24,25$(OH)_2$D (64). Due to variations among vitamin D measurement methods, divergent results can be obtained from different laboratory procedures that confound the comparison of vitamin D status across different research laboratories and thus different population groups (65). This underscores the need for standardized circulating 25(OH)D measurement in both clinical and research settings (66). The Vitamin D Standardization Program (VDSP) was established to address this issue by endorsing the standardized laboratory measurement of serum total 25(OH)D, in order to promote accurate and comparable results via calibration of 25(OH)D assays (67). As part of the VDSP initiatives, in order to ensure the reliability of performance of the methods, certified materials from international quality control program such as Vitamin D External Quality Assessment Scheme (DEQAS) and standard reference materials from National

Institute of Standards and Technology (NIST) are distributed across participating laboratories around the world to establish precise values for total 25(OH)D ($25(OH)D_2$ and $25(OH)D_3$) in each measured sample (68).

2.5.1 Chemiluminescent immunoassay (CLIA)

The CLIA is a simple and sensitive assay technique, and one of the most commonly used methods in clinical and research laboratories for high-throughput measurement of serum 25(OH)D concentration (69). The LIAISON (DiaSorin) is an automated analyzer that maintains a certificate of proficiency from Vitamin D Standardization Certification Program (VDSCP) and uses chemiluminescent immunoassay technology to quantify serum total 25(OH)D (70). The assay comprises specific antibody against 25(OH)D. The antibody is used for coating magnetic particles and 25(OH)D is conjugated to an isolumino tracer. During the first incubation, 25(OH)D is detached from its binding protein and competes with labelled vitamin D for binding sites on the antibody at the solid phase. After the second incubation, the unbound material is removed after a wash cycle. Thereafter, the reagents are added, to induce a flash chemiluminescent reaction. The light signal is measured by a photomultiplier as relative light units (RLU) and is inversely proportional to the concentration of 25(OH)D present in calibrators, controls, or samples (70). The quality of the method is assessed using blinded samples with varying 25(OH)D concentrations from DEQAS, distributed across participating laboratories for comparison within and between various methods. In addition, in order to evaluate the reliability of vitamin D measurements, the NIST standard reference materials (SRM) with provided target values are implemented in routine measurements (71). Some of the limitations of immunoassays that may raise the risk of bias include inability to detect 3-epi-vitamin D metabolites, as well as cross-reactivity with the $24,25(OH)_2D_3$ and other vitamin D metabolites. In addition,

immunoassays mostly measure total 25(OH)D as they cannot differentiate between 25(OH)D_3 and 25(OH)D_2. Despite these limitations, the current automated immunoassay techniques have shown acceptable overall correlation with LC-MS/MS methods (72).

2.6 Vitamin D status and body composition

2.6.1 Evidence from observational studies

Vitamin D is required for growth and development (73), with implications in body composition in infancy (74). There is an emerging body of evidence suggesting that maternal vitamin D status is implicated in programming of body composition by influencing the development of muscle and fat tissue (4, 9, 75, 76). Even though these studies have linked vitamin D status and body composition in infancy and childhood, some provided divergent results (39, 77). Overall findings from observational studies of pregnancy cohorts suggest that intrauterine exposure to deficient or insufficient maternal vitamin D status, associates with elevated fat mass (20, 21, 23, 77-81) and lower lean mass (20, 22, 23, 82) in neonates and children. This indicates the importance of vitamin D-associated fetal programming of body composition and that fetal exposure to maternal vitamin D status has a long-lasting impact on body composition (83). Collectively, the present observational evidence as detailed in Table 2.1 suggests that maternal vitamin D insufficiency and altered vitamin D metabolism might have implications in the development of childhood obesity and may place infants at greater risk of adverse metabolic consequences later in life. All of these observational studies were well-designed with relatively high quality according to Newcastle-Ottawa quality assessment scale (Appendix 1), nonetheless these are cohort studies and limited by possible residual confounding factors that have not been considered in the analyses despite adjustment for multiple covariates. In addition, due the nature of these studies a causal relationship cannot be established. Therefore,

these findings reinforce the need for further work focusing on early life interventions to reset the adiposity trajectory to reprogram for a leaner body phenotype.

Table 2.1 Characteristics of cohort studies on the association between vitamin D status and body composition in infancy and childhood[1]

Study; Year Country	Study Design; n[2]	Participants	25(OH)D (nmol/L)	Vitamin D Assessment Technique	Age at Visit	Body Composition Outcome	Newcastle-Ottawa Quality Assessment Scales		
							Selection	Comparability	Outcome
Gale et al; 2008 (84) UK	Prospective cohort 596	Mother–child pairs	Median serum 25(OH)D at ~32-week gestation: 50.0 (IQR: 30-75.3 nmol/L	RIA	9 years	Lean and fat mass DXA	****	**	***
Krishnaveni et al; 2011 (20) India	Cohort 568	Mother–child pairs	Median serum 25(OH)D at birth: 39.0 (IQR: 24.0-58.0 nmol/L)	RIA	5 and 9.5 years	Percentage body fat and fat-free mass BIA	****	**	***
Crozier et al; 2012 (85) UK	Prospective cohort 977	Mother–child pairs	Mean serum 25(OH)D at 34-week gestation: 62.0 (IQR: 43–89 nmol/L)	RIA	4 and 6 years	Lean and fat mass DXA	****	**	***
Harvey et al; 2014 (22) UK	Prospective cohort 678	Mother–child pairs	Median serum 25(OH)D at 34-week gestation: 61.0 (IQR: 43-88 nmol/L)	RIA	4 years	Lean and fat mass and relative percentages DXA	****	**	***
Godang et al; 2014 (39)	Sub-cohort 202	Mother-neonate pairs	Mean serum 25(OH)D at 30–32-week gestation:	RIA	4 days	Lean and fat mass	****	*	**

Norway			45.0 (SD: ±17 nmol/L)			DXA			
Hrudey et al; 2015 (81) Netherlands	Cohort 1882	Mother–child pairs	Median 25(OH)D at ~16-week gestation: 60.4 (IQR:18.9-102.7 nmol/L)	EIA	5-6 years	Percentage body fat BIA	****	*	***
Boyle et al; 2017 (21) New Zealand	Prospective cohort 922	Mother–child pairs	Mean 25OHD at 15-week gestation: 73.0 (SD: ±26.9 nmol/L)	LC-MS/MS	5-6 years	Percentage body fat BIA	****	**	***
Sauder et al; 2017 (79) USA	Prospective cohort 605	Mother-neonate pairs	Total 25(OH)D in cord blood: 56.0 (SD ±21.2 nmol/L)	CLIA	At birth & 5 months	Fat mass and fat-free mass and relative percentages ADP	***	**	***
Daraki et al; 2018 (78) Greece	Prospective cohort 532	Mother–child pairs	Maternal mean 25(OH)D at week 14 gestation: 46.3 (SD±15.7 nmol/L)	CLIA	6 years	Fat mass and fat-free mass and percentage body fat BIA	****	**	***
Hyde et al; 2018 (82) Australia	Prospective cohort 402	Mother–child pairs	Maternal median 25OHD at 28–32 week: 56.1(IQR: 43.6–73.9 nmol/L)	RIA	11 years	Lean and fat mass and relative percentages DXA	***	**	***

Tint et al; 2018 (80) Singapore	Prospective cohort 292	Mother-neonate pairs	Maternal plasma 25(OH)D at 26–28-week gestation: NA 46.6% ≤ 75.0 nmol/L	LC-MS/MS	~10 days	Abdominal adipose tissue MRI	***	**	***
Miliku et al; 2018 (23) Netherlands	Prospective cohort 4903	Mother–child pairs	Maternal median plasma 25(OH)D at ~ 20.4-week gestation: 50.4 (95% range: 7.5, 122.5)	LC-MS/MS	6 years	Lean and fat mass and relative indices DXA	****	**	***

[1]25(OH)D, 25-hydroxivitamin D; [2]n, sample size; ADP, air displacement plethysmography; BIA, bioelectrical impedance analysis; DXA, dual-energy X-ray absorptiometry; CLIA, chemiluminescent immunoassay; IQR, interquartile range; LC-MS/MS, liquid chromatography with tandem mass spectrometry; MRI, magnetic resonance imaging; RIA, radioimmunoassay; UK, United Kingdom; US, United States of America.

2.6.2 Evidence from randomized controlled trials

While observational studies have suggested that low vitamin D status during pregnancy is associated with altered body composition in infancy and beyond, interventional studies have reported inconclusive results. Overall, very few randomized controlled trials (RCT) have tested the effectiveness of vitamin D supplementation early in life on body composition (lean and fat mass partitioning) of infants and young children (4, 9, 86-90). As such, vitamin D repletion during pregnancy or early in infancy has not yet been proven to improve offspring body composition. Based on the results of a recent systematic review and meta-analysis of RCT (74), vitamin D supplementation early in pregnancy or infancy has a consistent trend to decrease adiposity in children, however the null results were due to insufficient numbers of participants studied in each RCT (detailed in Table 2.2). The only randomized controlled trial in infancy that reported a positive association between vitamin D status and lean body mass in response to vitamin D supplementation is from Hazell et al (4, 9). The majority of infants included in this study were healthy vitamin D-replete and breastfed at inception that were randomly assigned to incremental dosage of oral cholecalciferol supplements (400, 800, 1200, 1600 IU/d) from 1 to 12 month of age. There was no effect of vitamin D dosage group on body composition at 12 months or at 36 months follow-up. However, Hazell's findings showed that plasma 25(OH)D concentration were positively associated with percentage lean mass and inversely associated with percentage fat mass at the age of 12 months that sustained to 36 months.

There is some evidence in older age groups including children, adolescents, and adults suggesting additional benefits of improving vitamin D status on the development of lean body mass. For instance, in healthy young children 2 to 8 years of age, vitamin D intake of 400 IU/d over 6 months improved lean mass accretion (91). Consistently, in premenarchal girls, increasing

serum 25(OH)D concentration from 35 to 70 nmol/L over 12 months of vitamin D supplementation (14000 IU/week) improved lean body mass with no difference in body weight compared to the placebo group (92). Further, in healthy adults, one year cholecalciferol supplementation with 420 IU/day effectively improved lean body mass (93). Recent evidence in healthy monozygotic adult twins confirmed previous findings and demonstrated that, increasing serum 25(OH)D through cholecalciferol supplementation at 2000 IU/d over 60 days increased gynoid lean mass (94). This body of evidence provides further proof that improving vitamin D status has a function in programming a leaner body phenotype.

Considering the lack of a well-designed trial of vitamin D supplementation in infants born with low vitamin D status to specifically target body composition outcomes, the aforementioned systematic review and meta-analysis (74) suggest the need for more high quality RCTs to assess the causal relationship between vitamin D supplementation and lean mass in early life.

Table 2.1 Characteristics of the RCTs on vitamin D supplementation early in life and body composition changes in infancy and childhood[1].

Study; Year Country	Study Design; n[2]	Participants	25(OH)D (nmol/L)	Vitamin D Assessment Technique	Duration of Supplementation	Interventions; Compliance	Body Composition Outcome; Method
Brustad et al; 2020 (86) Denmark	RCT 517	Mother-child pairs	High dose, mean 76.6±25 Placebo, mean: 76.4±25 nmol/L	LC-MS/MS	24-week gestation to 1-week postpartum	Cholecalciferol 2800 vs. 400 IU/day; 74%	No differences in lean and fat mass At 3 and 6 years old; DXA
Cooper et al; 2016 (87) UK	RCT 965	Mother-infant pairs	High dose, mean: 46.7± 17.7 Placebo, mean: 45.9± 17.0 nmol/L	RIA	~14-week gestation until delivery	Cholecalciferol, 1000 IU/day vs. placebo; 70%	No difference in lean and fat mass within 2 weeks of birth; DXA
Czech-Kowalska et al; 2014 (88) Poland	RCT 137	Mother-infant pairs	Median: 37.9 nmol/L	CLIA	At delivery for 6 months	Mothers: Cholecalciferol 1200 vs. 400 IU/day Infants: 400 IU/d; 82%	No difference in lean and fat mass At 6 months old; DXA
Hazell et al; 2014 (4) Canada Hazell et al; 2017 (9)	RCT 132	Infants	Mean: 62.1 nmol/L	LC-MS/MS	1 -12 month for 11months	Cholecalciferol 800, 1200 or 1600 vs. 400 IU/day; 84%	Plasma 25(OH)D was (+) associated with lean and (-) related to fat mass; DXA
Sahoo et al; 2016 (89) India	RCT 52	Mother-infant pairs	Mean: 28.2 nmol/L	RIA	14–20 weeks of gestation until delivery	Cholecalciferol 60,000 IU/4 weeks or 60,000 IU/8 weeks vs. 400 IU/day; NA	No differences in lean and fat mass at 12-16 months old; DXA
Trilok-Kumar et al; 2015 (90) India	RCT 229	Infants	Mean: 36 nmol/L	RIA	Infants at 7 days of age for 6 months	Cholecalciferol 1500 IU/week vs. placebo; NA	No difference in fat and fat-free mass in children at age 3–6 years; Deuterium dilution

[1]25(OH)D, 25-hydroxivitamin D; [2]n, sample size; ADP, air displacement plethysmography; DXA, dual-energy X-ray absorptiometry; CLIA, chemiluminescent immunoassay; LC-MS/MS, liquid chromatography with tandem mass spectrometry; RCT, randomized controlled trial; RIA, radioimmunoassay; UK, United Kingdom

2.7 The combined influence of maternal adiposity and vitamin D status on offspring body composition

According to the developmental origins of health and disease theory, childhood obesity can develop in relation to early life events with negative metabolic consequences and susceptibility to adverse health outcomes (7, 8). This is true in the context of vitamin D as a new player in inclusion in this theory, as the amount of vitamin D an infant is exposed to could have genetic and epigenetic effects with implications on growth and body composition of the fetus and subsequently in infancy and beyond into childhood (95). Whether fetal programming of body composition or excess adiposity could be reversed or mitigated by early life interventions is unclear. This theory thus emphasizes the need for early life interventions to reprogram body composition. Among influential factors associated with maternal or neonatal vitamin D status such as season of birth and skin pigmentation (96), maternal adiposity appears to be an effect modifier of the association between maternal and neonatal vitamin D status. In overweight/obese condition, expanded adipose tissue readily absorbs vitamin D through chemical affinity (97). Vitamin D is a fat-soluble vitamin that tightly binds to fat depots, in this context fat tissue serves as a reservoir for vitamin D, making it less available to the circulation to support serum 25(OH)D concentrations (97). Evidence has been shown that in the obesity condition, the expression of VDR and enzymes involved in vitamin D metabolism are altered in adipose tissue (98). This condition results in lower circulating 25(OH)D concentrations at the maternal-fetal interface and consequently, lower 25(OH)D transferred to the fetus (99, 100).

Studies have demonstrated that offspring born to mothers with excess adiposity prior to pregnancy have higher fat mass and less lean mass compared to those born to mothers within the recommended BMI range (18.5 to 24.9 kg/m^2) (101, 102). Consistently, infants born to mothers

with elevated BMI have lower 25(OH)D concentrations in the cord blood compared to those born to mothers within the recommended BMI range (100). These findings demonstrate that the combined exposure of maternal excess adiposity and vitamin D deficiency/insufficiency acquired *in utero* are modifiable and co-associated with health condition and may result in long-term changes in offspring body composition with possible adverse metabolic health complications. Given the public health concerns regarding vitamin D deficiency/insufficiency in pregnant women and their offspring and the combined prevalence of overweight and obesity among children, clinical trials are warranted to determine the role of vitamin D in early-life programming of body composition and prevention of obesity.

2.8 Molecular mechanisms through which vitamin D impacts muscle mass

The growth rate of skeletal muscle is the fastest in the neonatal period, and unparalleled by any other stages of postnatal life (103). This is mainly due to an exceptionally higher rate of protein synthesis in this particular period versus protein degradation yielding a high accretion mass that occurs along with accumulation of nuclei in muscle fibers (103). The regulation of skeletal muscle growth is complex and dependent upon the stage of development and under the influence of numerous factors. Skeletal muscle is the target tissue of a range of hormones including growth hormone, insulin, Insulin-like growth factor-1 (IGF-1), thyroid hormones, and $1,25(OH)_2D$, among many others which exert effects on skeletal muscle growth and function via various established and non-established mechanisms (32). Vitamin D has been recently endorsed for its broad range of effects in skeletal muscle beyond its established role in calcium homeostasis for bone metabolism and health (32). Skeletal muscle cells also require calcium for normal development and function (104). Moreover, VDRs and CYPs are shown to be expressed in skeletal muscle cells (105-108). The expression of VDR and the CYPs in skeletal muscle

indicates that vitamin D can directly influence skeletal muscle tissue (34). Vitamin D influences skeletal muscle through genomic and non-genomic pathways. At the nucleus, $1,25(OH)_2D$ forms a heterodimer with nuclear VDR-RXR and influence transcription of various genes involved in cellular proliferation and differentiation of muscle cells (109). Through a non-genomic pathway, $1,25(OH)_2D$ binds to its cell membrane VDR and induces a rapid intracellular calcium handling. This suggests a potential role for rapid calcium influx in muscle cell differentiation and contraction as it is shown that in vitamin D deficiency, the uptake of Ca^{2+} ions into the sarcoplasmic reticulum is decreased, this affects muscle contraction and results in extends relaxation (110, 111). Further details about direct and indirect effects of vitamin D on muscle are depicted in Figure 2.2.

In a study in vitamin D deficient mouse model, an association between reduced grip strength and downregulation of genes involved in calcium-handling was reported (112) suggesting that the role of vitamin D on muscle mass is partly mediated via activation of calcium channels. There is some evidence suggesting that phosphate uptake in muscle could be also mediated through vitamin D. In a study in vitamin D deficient and phosphate-deplete rats, 25(OH)D administration increased phosphate concentration in the muscle cells and induced *adenosine triphosphate* (ATP) synthesis required for muscle contraction (32).

Figure 2.2 Direct and indirect effects of vitamin D on skeletal muscle

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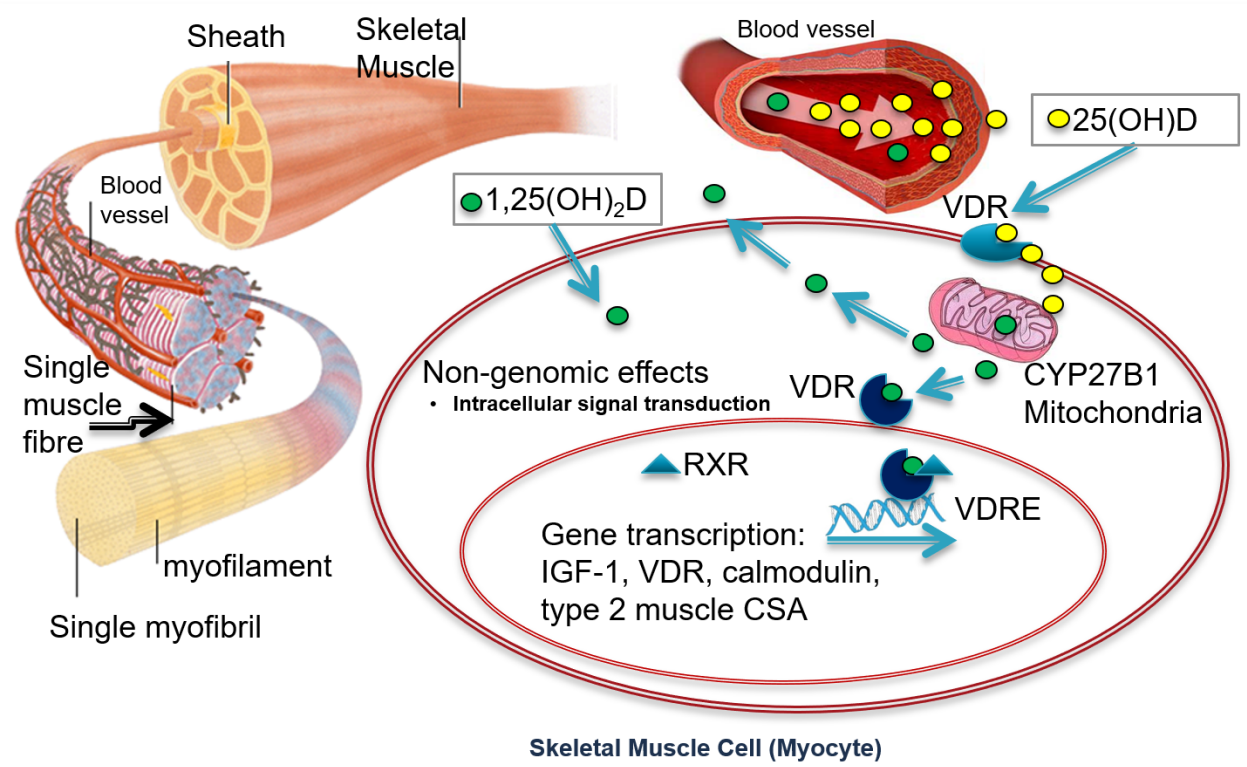


There is strong mechanistic evidence from animal models and cell culture studies regarding the effects of vitamin D on muscle mass. For instance, in skeletal muscle-specific VDR knockout mice (VDR -/-), significant reduction in type II muscle fiber diameter was observed, suggesting a direct role of vitamin D on muscle fiber size perhaps via downregulation of myostatin expression as a negative regulator of myogenesis (113). In addition, deletion of the VDR gene (VDR -/-) in mice deregulates myogenic transcription factors and impairs muscle growth and development (114). Human muscle biopsies reveal that 16 weeks supplementation with 4000 IU/d vitamin D_3 increases VDR mRNA expression and related protein in the treatment group of adults compared to the placebo group (115). Additionally, results from human myoblasts cultured with incremental $1,25(OH)_2D$ concentrations demonstrate that both VDR and the enzyme CYP24A1 mRNA increased in a dose-related manner (116). In an adjacent animal study, higher levels of VDR transcript and protein in quadriceps were observed in newborn wild type mice (>50%) compared to weaning and adult counterparts signifying higher VDR expression in young mice (116). In a C2C12 mouse myoblast cell line, adding either $25(OH)D_3$ or $1,25(OH)_2D$ alters cell proliferation and differentiation (117), suggesting that vitamin D has a direct role in muscle cell development through autocrine and paracrine signaling resulting in an increase in the diameter and size of myotubules. In an *in vivo* study in aging Wistar rats, vitamin D deficiency reduced muscle size (-25%) and markers of cell proliferation (118), confirming that vitamin D is required for normal muscle cell proliferation and size. In terms of body composition, lean mass was 12% lower and proportionately fat mass was 43% higher in the vitamin D deficient rats compared to a control group. This body of evidence supports a role for vitamin D in the development and function of skeletal muscle and underscore the need for well-

controlled trial to correct for low vitamin D status early in life as a novel approach to improve quality of growth and body composition and particularly a lean body mass phenotype.

2.9 Molecular mechanisms through which vitamin D impacts adipose tissue

Human infants are born with three types of fat tissue: brown, beige, and white adipose tissue. Brown adipose tissue is rich in mitochondrial uncoupling protein 1 (UCP1) which is a unique carrier protein specific to brown adipocytes and plays a critical role in thermoregulation and energy balance (119). In contrast, white adipose tissue beyond its capacity in storing excess energy as triglycerides, functions more like an endocrine tissue by secreting a number of adipokines, as well as interacting with numerous hormones and bioactive proteins (120). Beige adipose tissue has similar morphologic and thermogenetic properties as brown adipose tissue but contains only 10% of the amount of UCP1 of brown adipose tissue and depending on the environmental condition, it also has energy storing capacity (121, 122).

Recent evidence suggests that white adipose tissue might be a direct target of vitamin D signaling pathways; likely due to its ability to express VDR (123), whether vitamin D metabolism also modulates brown adipose tissue formation or function has been the subject of debate. Some evidence from a murine brown adipocyte cell line suggested suppressive effects of $1,25(OH)_2D_3$ and its nuclear VDR on the differentiation of brown adipocytes possibly through altered *UCP1* expression (124), however the results require further confirmation *in vivo*.

Accumulating evidence has shown the effects of vitamin D in adipogenesis and lipid metabolism in white adipose tissue (123). Human preadipocytes and adipocytes express CYP27B1 and CYP24 genes, encoding the enzymes that activate and deactivate $1,25(OH)_2D_3$. Therefore, adipocytes are locally and tightly involved in the synthesis and degradation of the hormonally active form of vitamin D (123). Once liganded with $1,25(OH)_2D_3$, VDR forms a

heterodimer complex with RXR and subsequently binds to the VDREs within the promoter of target genes, thus inducing a molecular cascade that regulates gene expression via activating or suppressing vitamin D target genes (125). Thus, at the tissue level, $1,25(OH)_2D_3$ is thought to be involved in adipose tissue energy homeostasis, modulation of adipogenesis, as well as adipocyte formation and secretion (126). In a C2C12 mouse myoblast cell line cultured in incremental concentrations of $1,25(OH)_2D_3$, the lowest $1,25(OH)_2D_3$ concentration, representing vitamin D deficiency, altered the capacity of these cells to transdifferentiate into mature adipocytes rather than myofibers (127). This thus resulted in fat accumulation and further induced expression of the genes involved in adipogenesis such as peroxisome proliferator-activated receptor gamma 2 (*Pparg2*) as a key regulator of adipocyte differentiation and lipid storage as well as fatty acid binding protein 4 (*Fabp4*) and adiponectin (*Adipoq*) (128). In contrast, highest $1,25(OH)_2D_3$ concentration, representing vitamin D sufficiency, induced myogenesis in a dose-response manner, reversed fat accumulation, and limited the expression of the above-mentioned genes (127). These findings suggest that improving vitamin D status has the potential to prevent trans-differentiation of muscle precursor cells into adipocytes and in favor of optimizing muscle health and function. A study on weanling mice demonstrated that offspring born to vitamin D deficient dams gained weight more rapidly, acquired greater visceral fat and were more susceptible to high fat diet-induced adipocyte hypertrophy compared to offspring born to vitamin D sufficient dams (129). In addition, the expression of *Pparg* was also higher in visceral adipose tissue, suggesting that vitamin D deficiency has a long-term effect on adipogenesis and increased risk of obesity and health related consequences later in life. In male mice provided a normal or high fat diet for a duration of 9 weeks, cholecalciferol administration reduced body weight, weight gain, as well as white adipose tissue weight. In addition, treatment with vitamin D_3 also enhanced expression

of *UCP3* gene in C2C12 cell lines (130). These findings are indicative of anti-obesity properties of vitamin D and that vitamin D limits fat accumulation and weight gain possibly through engagement with nuclear VDR, binding to functional VDREs in the promoter of the *UCP3* gene and activation of the gene which is involved in energy and lipid metabolism (130). Vitamin D appears to have implications in reduced risk of excess adiposity by inhibiting adipogenesis during early differentiation of adipocytes (131). In the event of low vitamin D status, secondary hyperparathyroidism results in increased calcium influx into the adipocytes, that eventually increases lipogenesis and upregulates the expression of fatty acid synthase and reduces lipolysis activity (132). Therefore, low circulating vitamin D concentrations in overweight/obese conditions may contribute to elevated metabolic risk associated with inflammation. Further studies are required to address the role of vitamin D in preventing excess adiposity as well as the effectiveness of supplementation in human adipose tissue and its relevance in the associated health outcomes.

2.10 The interaction between vitamin D and insulin-like growth factor-1

In addition to the role of vitamin D during normal postnatal muscle growth and developmental processes, several other growth regulatory hormones including growth hormone (GH) and IGF-1 are implicated in skeletal muscle formation and development (133). IGF-1 is mainly produced in the liver and is transported to muscle tissues within the circulation in a ternary complex with its predominant binding protein-3, IGFBP-3, and an acid labile subunit. This complex prolongs the half-life of IGF-1 as it prevents its clearance from the circulation (134). In addition, IGF-1 can be locally secreted by muscle cells during rapid growth and hypertrophy and enhances proliferation of satellite cells which are responsible for skeletal muscle regeneration (135).

The mechanisms that explain enhanced muscle mass include a mutual engagement in skeletal muscle growth and metabolism exerted by both vitamin D and IGF-1; this is believed to be through molecular and cellular pathways that regulate cell differentiation and growth in skeletal muscle mass (136). Previous studies have proposed a role for vitamin D in modulating IGF-1 and IGFBP-3 in the circulation. It has been suggested that vitamin D stimulates hepatic production of IGF-1 and IGFBP-3 (137). Even though the molecular mechanisms through which vitamin D regulates IGF-1 and IGFBP-3 concentrations have not been fully elucidated, it could be through enhancing transcription of *IGFBP-3* gene that contains VDREs in the regulatory regions which subsequently increases synthesis of IGFBP-3. In addition, pituitary cells that secrete GH, express VDR; VDR is one of multiple transcriptional factors controlling *GH* gene transcription; the liganded VDR can thus stimulate expression of *GH* gene by binding to VDRE in the promoter of *GH* gene (137-139).

In children with vitamin D deficiency, supplementation with high dose (300,000 IU) vitamin D significantly increased IGF-1 and IGFBP-3 concentrations (140, 141). Positive correlations between serum concentrations of IGF-1, 25(OH)D and $1,25(OH)_2D$ have been also observed in healthy subjects (142, 143). In a rodent study, VDR knockout mice presented reduced circulating IGF-1 compared to their wild type counterparts (144). Reports from animal models indicated that unlike hepatocytes that are very low in VDR content, non-parenchymal hepatic cells including sinusoidal endothelial, Kupffer, and stellate cells express VDR to a greater extent, these cells are likely vitamin D target cells as they also largely express IGF-1 and IGFBP-3 (145-147). IGFBP-3 gene promoter contains VDREs; liganded VDR binds to VDRE and induces the transcription activity of the gene and promotes the synthesis IGFBP-3. Findings from a steroid receptor coactivator-3 (SRC-3) knockout mice model indicated a significant

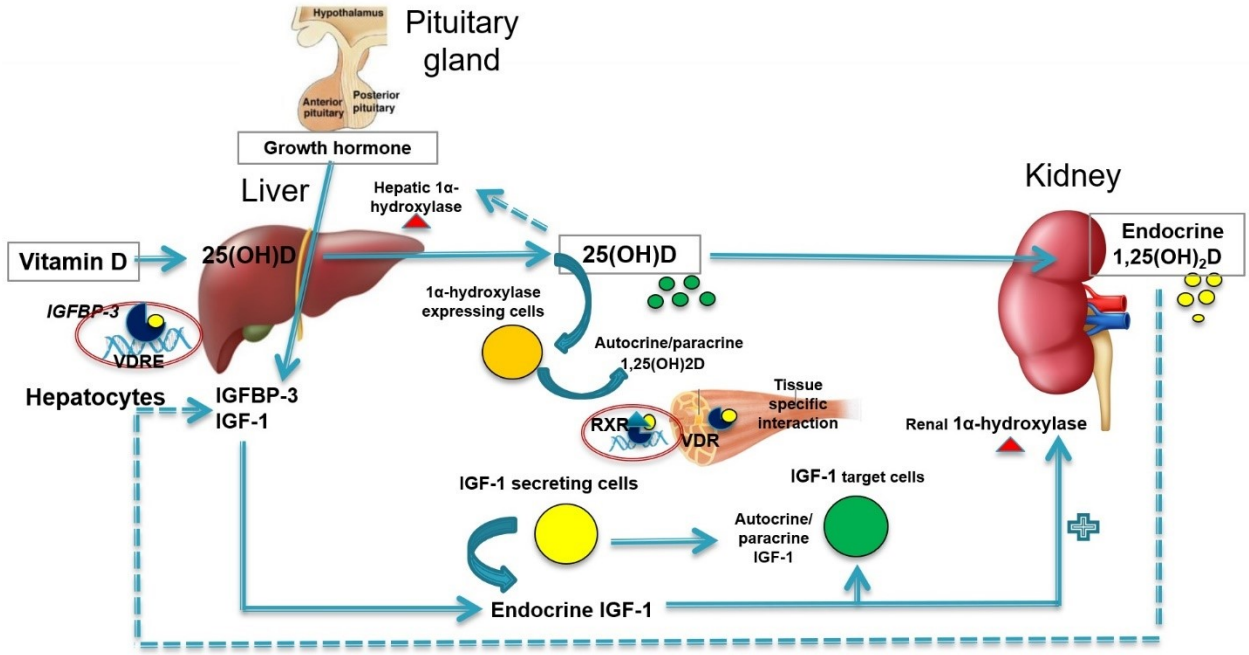
decline in circulating IGF-1 and IGFBP-3. This is thought to be due to SRC-3 ablation and reduced VDR function that consequently downregulates IGFBP-3 and results in an increased degradation of IGF-1 from the circulation (148).

There is well-established experimental evidence suggesting that GH and IGF-1 are positive regulators of the synthesis and activity of the enzyme CYP27B1 which is responsible for hydroxylation and activation of vitamin D (137, 149-151). Consistently, short-term administration of both GH and IGF-1 increased $1,25(OH)_2D$ concentrations in healthy elderly and younger subjects (150, 152). These studies underscore the interaction between vitamin D metabolism, IGF-1 and its binding protein-3 and are indicative of molecular mechanisms reflective of vitamin D and IGF-1 mutual engagement in the skeletal muscles; details about vitamin D and IGF-1-IGFBP-3 interaction are depicted in Figure 2.3.

Given the scarcity of data on the importance of vitamin D as a modifiable factor in the pediatric domain, it is an opportune moment to test low vitamin D status as a possible etiological factor in the early life origins of adiposity and also to investigate whether improving the vitamin D stores would result in a leaner body phenotype in association with higher IGF-1 and IGFBP-3.

Figure 2.3 Interactions between vitamin D, IGF-1, and IGFBP-3

Modified from (137) with permission of Dr. Ameri to reprint (Appendix 3)



2.11 Body composition assessment techniques in infancy

The pace of growth and development during infancy and particularly the neonatal period is unparalleled to any other stages of life (103). Growth involves induced changes in body size as well as body composition, (5) and nutrition is considered essential to influence the quality of growth and development (153). Anthropometric measurements such as body weight, length, and head circumference and relative indices as well as skin folds are largely used as proxy measures of growth (154), however little is known of the partitioning of nutrients to fat mass or fat-free mass and the changes in the chemical composition of the body during this rapid period of growth (155). As both lean and fat mass have important implications to benefit health and development during infancy, more specific methods of growth measurements provide adequate knowledge of the changes in body composition and advances the understanding of nutritional requirements of this age group. There are various techniques to assess body composition that are appropriate for use in human infants including, bioelectrical impedance analysis (BIA), dual energy X-ray absorptiometry (DXA), air displacement plethysmography (ADP), magnetic resonance imaging (MRI), and isotope dilution.

Bioelectrical impedance analysis (BIA)

Bioelectrical Impedance Analysis or Bioimpedance Analysis (BIA) is a practical, simple, and non-invasive method of assessing body fat in relation to lean body mass in clinical, and particularly in large-scale research settings. The device uses a small electrical current and measures how this current is impeded while passing through different types of tissue in the body. The electrolyte and water content of tissues influence the flow of the current. Tissues with higher amount of fluid such as fat-free mass have higher conductivity compared to fat mass (154). BIA provides indirect estimates of body composition based on prediction equations of reference

methods and with the assumption of constant tissue hydration (156). Body water content is the highest in neonates, fat-free mass fluid content changes rapidly during infancy, water and electrolyte concentrations are higher in extracellular and proportionately lower in intracellular compartments. Therefore, these rapid changes in body water content make the estimates of body composition obtained from BIA less reliable (157).

Dual-energy x-ray absorptiometry (DXA)

Dual-energy x-ray absorptiometry (DXA) is the gold standard for measuring bone mineral density for diagnosis of osteoporosis and fracture risk in adults (158) owing to its high reproducibility and precision (159). DXA is generally accepted as a precise and noninvasive method of body composition measurement and is widely used as a reliable technique in the pediatric population due to its availability, ease of use, as well as low radiation exposure (160). The effective radiation dose from an infant whole body DXA scan is minimal and approximately 7.5 microSv for a 1-year-old infant and 8.9 microSv for a neonate, being equivalent to approximately less than one day background radiation an infant would receive from all natural sources (161). DXA procedure is simple, and the actual scan time takes 3-6 minutes, however, from start to finish considering time for getting changed and infant positioning, it takes ~10 minutes so it is readily feasible. For all the above-mentioned reasons, DXA is frequently used by research and medical facilities.

A DXA scanning system consists of an X ray source, a detector array, radiolucent examination table and a computer workstation for data analysis. DXA is considered a three-compartment (3-C) model that measures bone and lean mass and estimates fat mass accordingly by using proprietary algorithms (162). The fundamental principle of DXA is the measurement of transmission of x-rays with high- and low-energy photons depending on density and chemical

composition of the tissue while passing throughout the body. X-ray beam energy is attenuated with passage through different types of tissue, where each has a unique attenuation property. Thus, the extent of attenuation varies with the energy of the photons and the density and thickness of the material through which x-rays pass (162).

Body composition measurement by DXA in neonates and infants introduces some technical difficulties concerning data acquisition and data analysis, some of these challenges include small body mass in infants and insufficient DXA sensitivity to detect small changes in body composition, movement artifacts are typical of pediatric body composition measurements and may decrease the precision of results considerably, additionally, non-standardized positioning and technical challenges related to infants positioning may influence the performance of the scan (157). Other important considerations include lack of standardized normative data that is a major barrier to routine clinical body composition assessment in infancy (163), in addition, infant whole body phantoms with adapted and correct anatomical structure are not commercially available (164), and DXA has a standard assumption on the hydration status of lean mass thus rapid changes in fluid content of the tissue in infancy would be interpreted as changes in lean mass (165). Collectively, all these factors are integral and should be considered prior to interpretation of the results obtained from DXA.

Air-displacement plethysmography (ADP)

Air displacement plethysmography (ADP) is a practical assessment tool that has been broadly used to measure body composition due to its reliability, validity, and acceptability (154). Pea Pod is the infant version of ADP that can assess body composition in full-term as well as pre-term infants (166). ADP measures body volume by detecting air pressure differences between two enclosed chambers, in one chamber human infant is located, and the other is a

reference chamber with controlled air pressure. Unlike DXA, ADP is a two-compartment (2-C) model that divides body weight into fat mass and fat-free mass and does not delineate variation in fat-free mass (166). The PEA POD procedure is easy to perform, and it only takes a few minutes to complete the measurements. One significant advantage of ADP is the absence of radiation exposure, that allows researchers to get serial body composition measurements on an infant and over time, serial measurements nullify the movement artifact which is a significant factor in DXA body composition assessments (167). The PEA POD device is only suitable for infants with body weight of ~8 kg and therefore it can only provide estimates of body composition for infants up until 6 months of age; this limits its applicability in longitudinal studies in infancy and beyond (128).

Magnetic resonance imaging (MRI)

Magnetic resonance imaging (MRI) is a non-invasive, radiation-free technique which is considered a reference method for total and regional body composition measurement due to its capability to generate high resolution images (154). MRI visualizes total and regional adipose tissue depots and in contrast to ADP, it is able to produce detailed information regarding body fat distribution (168). MRI utilizes the properties of the hydrogen contained in water and fat to produce quantitative images of soft tissues (169). MRI procedure is quick, and it takes approximately 5 min to perform the whole scanning and thus readily feasible for the infant population. Similar to ADP, MRI does not use ionizing radiation making it a suitable method for use in neonates and infants; however, movement artifacts in MRI reduce image quality and lead to misinterpretation of the results therefore infants are preferably scanned while sleeping. Due to challenges in obtaining scan in older infants, it is more feasible to perform MRI scanning in infants 6 months of age or younger (170).

Isotope dilution

Total body water (TBW) assessment by isotope dilution technique is a precise method of body composition measurement at a molecular level and can be used from birth to old ages (5). The fundamental principle of isotope dilution is that water is distributed equally in all parts of the body except body fat. Water is found exclusively within the fat-free mass. At birth, the body contains ~70-80% water, however as an infant grows, this proportion gradually declines. TBW can be estimated by administration of labeled water with stable isotopes such as ^{18}O or ^{2}H, and subsequently measurement of the isotope concentrations in the saliva or urine after a period of equilibrium. Fat-free mass can thus be estimated from TBW and fat mass is basically calculated from the difference between body weight and fat-free mass (171). Isotope dilution method can be very challenging to apply in neonates and infants as the technique is time-consuming due to the nature of the equilibration period (172), the potential for failure to administer an accurate dose due to spillage, difficulty in collection of serial samples from infants, and the sensitivity to fat-free mass hydration state for estimation of body composition (154).

As mentioned in the above section, each of these techniques offer significant advantages to infant body composition measurement, however, infancy present considerable challenges to these assessment processes mainly due to rapid growth rate, and insufficient sensitivity of some of these methods to detect small changes in infants body composition. Therefore, to mitigate this it's essential to design the study to have measurements time-points far enough apart in order to capture the real changes in body composition over time. In addition, in some of these measurement methods such as DXA and MRI the lack of control in body movements in infants invalidate scan results making the data acquisition cumbersome. Establishing a soothing environment for infants, involvement of parents to comfort them, and avoidance of any

distraction such as loud sounds and bright lights in the examination room can significantly improve the success rate

2.12 Epigenetics and DNA methylation-the role of vitamin D as an essential nutrient

Epigenetics refers to a series of modifications beyond the nucleotide sequence that affects gene activity without altering the underlying DNA sequence (173). Unlike genetic variations, epigenetic alterations are reversible. An individual's epigenetic patterns are established early in life and are shaped further through the lifetime by interacting with a variety of environmental and behavioral factors including nutritional exposures (174). These modifications might be transmitted by the gametes transgenerationally (175). There are three major mechanisms involved including DNA methylation, histone modification, and non-coding RNAs (176). Across these mechanisms, nutrients appear to play important role in DNA and histone methylation.

In the context of vitamin D metabolism, $1,25(OH)_2D$, is thought to be epigenetically active and the key catalyzing enzymes, 1α-hydroxylase and 24-hydroxylase are epigenetically regulated (177). In addition, VDR as the main part of a transcriptional complex, $1,25(OH)_2D_3$–VDR–RXR, modifies epigenetic status at the binding sites of various genes (178). Therefore, the vitamin D system is not only regulated by epigenetic mechanisms, but also is implicated in epigenetic events as epigenetic regulators (179).

Many of the stable alterations in DNA expression and related musculoskeletal changes are ascribed to the $1,25(OH)_2D_3$–VDR–RXR complex; this complex binds to VDRE located on the regulatory regions, recruits co-regulatory nuclear proteins and thus impacts targeted gene transcription activity (180). Transcriptional regulation is mostly affected by the methylation pattern of the genome. Therefore, analysis of the methylation patterns is the main approach in different studies that focuses on gene regulation. In this context, the activity of the gene can be

regulated by the covalent attachment and removal of some methyl groups to the DNA sequence (181). DNA methylation takes place at cytosine residues that commonly occur at CpG islands where DNA sequences are enriched in cytosine and guanine nucleotides. As a general rule, methylation silences the gene and demethylation activates the gene expression (176) however, methylation has been also implicated in activation of the transcription in some cases (182).

Genes involved in vitamin D metabolism with CpG islands in their promoter region include *VDR*, *CYP2R1*, *CYP27B1*, *CYP24A1*, and *RXR*; and each can be silenced via methylation processes (183). In vitamin D deficient adolescents, it is shown that *CYP24A1* is hypermethylated and silenced (184). In a study on adults, higher baseline DNA methylation levels in the promoter region of *CYP24A1* favors the response to vitamin D supplementation (185). Results from another study of responder and non-responder women to vitamin D supplementation revealed that the baseline DNA methylation ratio of *CYP2R1* and *CYP24A1* genes are negatively associated with individual's response to vitamin D supplementation. In this context, hypermethylation of these genes was mostly seen in individuals with low response to vitamin D supplementation (186). These epigenomic approaches underline the crucial roles played by these genes in vitamin D metabolism.

Epigenetic regulation induced by vitamin D deficiency might also influence IGF-1, IGFBP-3, and muscle mass. In a recent study, RXR (alpha) methylation in umbilical cord was associated with offspring obesity later in life (187). In addition, lower serum IGFBP-3 in children with vitamin D deficiency rickets (140), is explained to some extent by the presence of a functional VDRE in the IGFBP-3 promoter region (188). An *in vitro* study demonstrated that, binding of $1,25(OH)_2D$ to VDR, followed by formation of the VDR-RXR heterodimer and binding to the VDRE of *IGFBP-3*, results in increased IGFBP-3 mRNA expression (189).

Additionally, periconceptional variation in methyl-donor dietary intakes modulates DNA methylation of epialleles in children confirming that maternal nutritional status during early pregnancy results in persistent epigenetic alterations in the offspring (190). In a recent cohort study, an 87% concordance between differentially methylated regions (DMRs) in adipose tissue from a high fat diet in obese mouse model was found with DMRs in adipose tissue of obese men. Based on previous epigenome-wide studies in adults, prenatal malnutrition may alter epigenetic pathways resulting in adverse metabolic phenotypes in later life and even in subsequent generations.

In a comprehensive epigenetic epidemiologic study, the importance of the *in utero* dietary exposure and its long-lasting effects throughout life span is well emphasized. Together these findings support that apart from vitamin D role in skeletal muscles and adipose tissue through molecular mechanisms, the key genes involved in vitamin D metabolism are highly responsive to epigenetics modulations. Epigenetics is critically important during developmental stages of life and thus the alteration of genome-wide DNA methylation may predispose one to chronic conditions such as obesity later in life. Therefore, targeted vitamin D supplementation may support proper epigenetic programming of body composition with possible implications to child and public health.

BRIDGE STATEMENT 1

The literature review presented in Chapter 2 has outlined the increasing recognition of extra-skeletal effects of vitamin D beyond bone health and in early life programming of body composition and has discussed vitamin D metabolism and possible mechanisms of action through which it impacts on growth, as well as muscle and adipose tissue development. The existing evidence from observational studies suggest that maternal vitamin D status during gestation may influence body composition in offspring while interventional studies have not established any causal relationship.

It has been shown that maternal vitamin D deficiency/insufficiency during gestation contributes to excess adiposity and reduction in lean mass in infancy, which may then persist into childhood and possibly increase the risk of chronic health conditions later in life. This is of high importance as early development *in utero* and the first postnatal year of life is exceptionally rapid and particularly sensitive to nutrient deficiencies. Despite multiple adjustment for potential confounders, as in any other observational studies that are subject to confounding, it is highly possible that residual confounders may have affected these observations and therefore it is unclear whether the relationship observed between maternal vitamin D status and offspring body composition solely reflect vitamin D status. The study in Chapter 3 was conducted in a carefully controlled experimental animal model and was designed in order to test the impact of vitamin D on body composition without the interference of potential confounders associated with low vitamin D status and body composition in human infants including sex, skin color, season of birth as well as maternal and sociodemographic factors. Chapter 3 has been published in Nutrition Research.

CHAPTER 3: RESEARCH PAPER 1

Lean body mass accretion is elevated in response to dietary vitamin D: a dose-response study in female weanling rats

Maryam Razaghi[1], Marija Djekic-Ivankovic[2], Sherry Agellon[1], Ivy Mak[1], Paula Lavery[1], Hope A. Weiler[1]

[1]School of Human Nutrition, McGill University, Macdonald-Stewart Building, Macdonald Campus, 21111 Lakeshore Road, Ste-Anne-de-Bellevue, Quebec, Canada, H9X 3V9

[2]Centre for Outcomes Research and Evaluation, Research Institute of the McGill University Health Centre, Montreal, Quebec, Canada, H4A 3S5

Abstract

Background: Vitamin D status positively relates to lean body mass in infants.

Objective: This study tested the effect of vitamin D on body composition and growth-related hormones. It was hypothesized that low vitamin D status programs for higher fat mass accretion.

Methods: Female weanling Sprague-Dawley rats (4 wk; n=6/diet) were randomized to AIN-93G diets with modified vitamin D contents for 8 wk: group 1 (1 IU vitamin D_3/g diet), group 2 (2 IU vitamin D_3/g diet), and group 3 (4 IU vitamin D_3/g diet). At wk 0, 4 and 8 of study, measurements included: serum 25(OH)D_3, IGF-1, IGFBP-3, leptin, and whole body composition assessed with DXA. Differences among groups were tested using a linear mixed effect model with Tukey's *post hoc* t-tests.

Results: No differences were observed in baseline body composition and biomarkers, nor did body weight and food intake differ over the study. At wk 8, serum 25(OH)D_3 in group 3 was higher ($P<0.0001$) compared to groups 1 and 2. At 8 wk, lean mass ($P<0.05$) and lean mass accretion ($P<0.05$) were significantly higher in groups 2 and 3 compared to group 1. Serum IGF-1 concentration declined over time ($P<0.001$) with smaller declines at wk 8 in group 3 ($P<0.05$). Serum IGFBP-3 concentration was lower at wk 4 in group 2 compared to groups 1 and 3. Serum leptin concentration and fat mass were not affected by diet.

Conclusion: These results suggested that the achievement of higher vitamin D status may support a lean body phenotype without altering weight gain.

Key words: lean mass; vitamin D; IGF-1 and IGFBP-3; leptin; female weanling rats

3.1 Introduction

Early postnatal life represents a period of rapid growth which is influenced by many genetic factors as well as environmental exposures, particularly nutrition (191). Numerous hormones, including growth hormone, insulin-like growth factor-1 (IGF-1), leptin, and 1,25-dihydroxyvitamin D ($1,25(OH)_2D$), also have significant influences on growth (141, 192-194). The developmental origins of health and disease concept postulates that the development of overweight and obese patterns observed later in life might stem from early life exposures (195). In addition, there is substantial evidence that prenatal and early postnatal nutrition have key roles in determining susceptibility to developing metabolic disorders such as obesity, impaired glucose tolerance, insulin resistance and type 2 diabetes mellitus (176). The first few weeks postnatally may thus offer a window of opportunity for implementing some preventive nutritional strategies to support healthy body composition and long-term health.

In human infants, fat mass increases across the first 9 months of life and tapers off thereafter. Conversely, muscle mass increases constantly across the first year of life, resulting in a higher accretion rate compared to that of fat mass (5, 196). Hence, the high rate of lean mass accretion in infancy suggests this as a period that is important for muscular growth and development. Serum 25-hydroxyvitamin D_3 ($25(OH)D_3$) concentration above 75 nmol/L supports a lean body mass phenotype across infancy (4), evident by higher lean mass and lower fat mass while following a normal growth trajectory. Additionally, some evidence from human and murine studies demonstrates the link between vitamin D insufficiency and increased adiposity (197, 198).

To date, there is evidence that improving vitamin D status leads to improved musculoskeletal outcomes through mechanisms involving vitamin D receptors (VDRs) and

cytochrome P-450 proteins (CYPs) that are widely expressed in myocytes (105, 199, 200). Deletion of VDR in mice deregulates myoregulatory transcription factors and impairs skeletal muscle growth and development (201). In a human trial where participants were given vitamin D supplementation (4000 IU/d) for 16 weeks, skeletal muscle biopsies demonstrate higher VDR mRNA and protein levels compared to controls (202). Data in human adults indicates that adequate vitamin D intake to meet the Dietary Reference Intake (DRI) suppresses fatty acid synthase activity and decreases calcium influx in adipocytes, which leads to reduced adiposity (203, 204). Hence, vitamin D insufficiency may result in fat accumulation and contribute to the etiology of obesity (205). These studies reinforce the need to test interventions to reverse low vitamin D status as a novel approach to improve quality of growth and to achieve a leaner body phenotype. We hypothesized that low vitamin D status in weanling rats programs for higher fat mass accretion, and that achievement of vitamin D status above 50 nmol/L of $25(OH)D_3$ resets the trajectory to a leaner body phenotype and lower fat mass. The primary objective of this study was to establish if increased intakes of vitamin D would result in a leaner body phenotype, greater lean mass accretion and lower fat mass accretion. Secondly, since the positive effects of vitamin D on body composition are assumed to be mediated by IGF-1, IGFBP-3 and leptin, this study aimed to establish whether increasing vitamin D intake also affects these growth-related biomarkers. The weanling Sprague-Dawley rat model provides an appropriate experimental system for investigating the effect of dietary vitamin D on body composition and enabled us to test the hypothesis by removing the potential confounding factors associated with low vitamin D status and body composition in human neonates such as skin color, sex and season of birth (206).

3.2 Methods and materials

3.2.1 Animals and study design

Three-wk old weanling female Sprague-Dawley rats (n=18, Charles River Laboratories, St-Constant, QC) were housed under standard conditions (23°C, 50% humidity, 12 h:12 h light-dark cycle). Female rats were studied to minimize confounding effects of testosterone as a stimulus for lean mass accretion (207) that is not usually a prominent factor in young children. In order to be able to relate to the infancy period of human life and more specifically to the weaning age in humans (at 6 months of age on average), we chose to study 3-4 wk old rats as weaning in rats occurs at this age (208). After 1 week of adaptation during which rats were fed a commercial AIN-93G chow-based diet (Teklad Diets, Madison WI, Envigo, 2920X), 4-wk-old rats were block-randomized to one of three AIN-93G diets modified in vitamin D_3 content (n = 6 in each group). The diet groups were masked by the use of color coding. The study spanned an 8-wk period based on the half-life of vitamin D_3 (15 to 21 d), and to correspond to up to four half-lives (55). Moreover, the beneficial effect of vitamin D_3 supplementation on the rapid recovery and protection against exercise-induced muscle damage has been demonstrated over a period of 8 wk in growing rats (209).

Body weight was recorded (to the nearest 0.1 g) weekly using a weigh scale (Mettler, PM4600, Greifensee-Zurich, Switzerland) with a built-in vibration adapter to provide accurate weights of moving animals. Food intake was estimated twice per week by subtracting food remaining from food provided. Both food and distilled water were provided *ad libitum*. Animals were fasted 8 hours prior to blood sampling at wks 0, 4 and 8 following experimental diets were provided. Following blood collection, anaesthesia was induced using isoflurane. Whole body scans were obtained using dual-energy x-ray absorptiometry (DXA; Hologic 4500, QDR Version

12.5; Hologic Incorporated, Bedford, USA). To evaluate abdominal lean and fat mass, *in vivo* micro-computed tomography (μCT; Aloka; LaTheta LCT-200; Japan) was performed at wk 8. After performing DXA and μCT scans at wk 8, rats were placed under deep anesthesia and then euthanized via exsanguination by cardiac puncture. The study protocol was reviewed and approved by the ethical committee of the Macdonald Campus Facility Animal Care Committee, McGill University, and carried out in an ethical manner following the guidelines of Canadian Council on Animal Care standards (210).

3.2.2 Diets

Vitamin D_3 was purchased from Isosciences (King of Prussia, PA) and supplied to Harlan Laboratories (Madison, WI) for diet production. The AIN-93G diet was the base for all diets (control and 2 experimental diets), and only the vitamin D_3 content was different in the experimental diets. The control diet was a standard AIN-93G diet containing 1 IU/g (0.025 μg/g) vitamin D_3. The two experimental diets (Table 3.1) differed only by vitamin D_3 content with 2 and 4 IU/g (0.05 and 0.1 μg/g) diet, as previously described (49).

3.2.3 Sample collection

Food was removed at 2400 h on the night prior to blood sampling at wk 0, 4 and 8. Samples were collected between 0800 and 1000 h from the saphenous vein (400 μl) into micro tubes with or without heparin (Becton Dickinson, New Jersey, US), centrifuged at 2000g for 10 min at 4 °C to isolate plasma and serum, which were stored at -80 °C until analysis.

3.2.4 Biochemical measurements

Serum 25(OH)D_3 was measured using high performance liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) (Warnex Inc., Laval, QC; GLP laboratory), as described (49). The inter-assay precision value for the 25(OH)D_3 calibration standard was 3.0%.

The lower limit of quantification (LLOQ) was 12.5 nmol/L for 25(OH)D_3. The National Institute of Standards and Technology (NIST) reference values were used to assess the accuracy of measurements, with errors of ± 7% for NIST 968e level 2 (32.3 nmol/L) and ± 2.5 % for NIST 968e level 3 (57.7 nmol/L) standards. The epimer of 25(OH)D was excluded from the analyses. Serum IGF-1, IGFBP-3 and leptin concentrations were measured at wk 0, 4 and 8 of the study using mouse/rat specific enzyme-linked immunosorbent assays (Alpco 180513 USA; Mediagnost 160216 Germany; Millipore 2586298 USA). The intra-assay coefficient of variation for IGF-1, IGFBP-3 and leptin were 3.8%, 3.9% and 7.8%, respectively.

3.2.5 Body composition analysis

Whole body lean mass and fat mass (excluding bone mineral content) were obtained via DXA (Hologic QDR-4500 version 12.5, Incorporated Bedford, USA) using the manufacturer's software for small animal imaging (49). Anaesthetized rats were placed in a prone position for the scan. To measure abdominal lean and fat mass, *in vivo* μCT (LaTheta LCT-200; Aloka; Japan) was performed subsequent to DXA scans. Images were captured using an isometric pixel size of 120 μm, slice thickness of 120 μm and slice pitch of 120 μm. Abdominal fat analysis was performed using the manufacturer's proprietary software. The acquisition region of interest was consistently located at lumbar vertebra 3 and comprised of 9 slices. The recognition of visceral versus subcutaneous fat was based on detection of the abdominal muscle wall and verified slice-by-slice and corrected as necessary. All μCT analyses were performed by a single operator.

3.2.6 Statistical analyses

The sample size estimated to detect a 10% difference in lean mass in a 2-sided t test was n = 5 with an αof 0.05 and β of 0.2. An additional animal per group was added to account for the possible loss of an animal or a smaller size effect. A procedure described by Kononoff was used

(211) to estimate the statistical power of the study and sample size for data sets including random effects with repeated measurements in a linear mixed effect model. Differences in actual values as well as changes in values over the course of study among groups (group 1, 2 and 3) were tested using a linear mixed effect model for repeated measures with fixed effects of diet groups and time, and random effect of rat nested within study block. Group differences were determined using Tukey's *post hoc* t-tests with $P<0.05$ accepted as statistically significant. Normality and homogeneity of variances were tested with Shapiro Wilk normality test and Levene's test respectively. All testing was two-tailed to enable acceptance or rejection of the null hypothesis. All data was analyzed with SAS version 9.3 (SAS institute, Cary NC) and are presented as means±SD.

3.3 Results

3.3.1 Dietary intake, anthropometric and body composition measurements

There were no significant differences in food intake or body weight and fat mass among the groups at baseline or over the course of the study, although values increased significantly ($P<0.0001$) over time (Figure 3.1). Lean mass was not different among groups at baseline; however, lean mass and lean mass accretion were significantly higher ($P<0.05$) in groups 2 and 3 at wk 8 compared to group 1 (control). There were no differences among groups in abdominal lean mass and fat mass and the percentage change over the course of the study, while values significantly increased ($P<0.0001$) over time (Figure 3.2).

3.3.2 Biochemical measurements

No differences in vitamin D status were observed among groups at baseline with all rats having serum $25(OH)D_3$ concentration below 30 nmol/L. Serum $25(OH)D_3$ in group 3 was significantly higher compared to groups 1 ($P<0.05$) and 2 ($P<0.0001$) at wk 8 of the study (Figure 3.3). There were no statistical differences in serum $25(OH)D_3$ between groups 1 and 2 at any time point while values significantly increased ($P<0.0001$) over time in all groups. Serum IGF-1 was not different among groups, however, there was a significant effect of time ($P<0.0001$). Smaller declines were observed in changes in IGF-1 from wk 4 to wk 8 in group 3 compared to groups 1 and 2 ($P<0.05$) (Figure 3.4). There was a significant difference in IGFBP-3 concentration over time ($P<0.0001$). Serum IGFBP-3 was significantly lower in group 2 compared to groups 1 and 3 at wk 4 of the study. There was no significant difference among groups in changes in IGFBP-3. Leptin concentration also increased significantly over time ($P<0.0001$), although this change was not affected by diet group.

3.4 Discussion

The most prominent observation in this study is that increasing dietary vitamin D_3 content resulted in increased accretion of lean mass without any changes to weight gain during an 8-wk period from weanling age to maturation in female Sprague-Dawley rats. Our study is the first highly controlled study investigating the effect of increasing vitamin D intake on lean body mass in weanling rats and is unique in that the benefits were observed into young adulthood. These findings are in line with the results reported by Hazell et al. (4) in human infants, where higher vitamin D status was associated with a lean body phenotype in infancy (birth to 1 y) that was sustained to 3 y of age (9). Studies in adolescent girls who were vitamin D deficient and randomized to vitamin D supplementation (low-dose 200 IU/d, or high-dose vitamin D 2,000 IU/d) also showed a positive effect on lean mass and greater lean mass accretion over one year compared to the control group (placebo oil) (212). Collectively, with our reported results, we accept our hypothesis that improving vitamin D status above 50 nmol/L during growth favors the development of a lean body mass phenotype.

The IOM defines vitamin D deficiency as <30 nmol/L of serum 25(OH)D_3 and recommends at least 50 nmol/L as a target for vitamin D sufficiency and healthy status in human populations (61). The present study was designed to mimic the response to vitamin D supplementation in newborn infants and to examine whether increasing vitamin D status would confer benefits to lean mass accretion. The animals had serum 25(OH)D_3 <30 nmol/L at baseline and only group 3 achieved concentrations >50 nmol/L after wk 4 of the study, which was sustained to wk 8. Interestingly, both groups 2 and 3 responded with greater lean mass accretion suggesting that moderately increased dosages of dietary vitamin D improved body composition in weanling rats and that utilization for tissue growth appears to take precedence over forming

vitamin D stores. With our established model, future studies will enable us to further explore molecular mechanisms by which vitamin D exerts its beneficial effects on lean mass during a period of rapid growth.

Similar to the marked role of vitamin D during normal postnatal muscle growth and development (213), other hormones including IGF-1 and its binding protein IGFBP-3 as well as leptin appear to be determinative factors in lean mass accretion. Circulating IGF-1, which mainly originates from the liver and is transported to muscle tissues, induces muscle hypertrophy and proliferation of satellite cells; it is noteworthy that IGFBP-3 stabilizes IGF-1 in the circulation and therefore extends its half-life and supply to target cells (135, 137). Following periods of rapid somatic growth, IGF-1 concentrations taper off but continue to have anabolic effects in adulthood (214). In the current study, natural declines in IGF-1 over time were significantly lower in group 3 versus group 1, suggesting that IGF-1 concurrent with increased dietary vitamin D stimulated lean mass accretion. A study in piglets showed that IGF-1 infusion stimulated protein synthesis in skeletal muscle and elevated fractional synthesis rates by 25 to 60%; however, this response was more pronounced in the early suckling period compared to the weanling age (215). In the present study, increases in lean mass accretion from weanling to young adult age were greater in rats receiving 4 IU vitamin D_3/g diet in line with lower declines in serum IGF-1 concentrations, both of which were likely due to rapid improvements in vitamin D status.

Vitamin D has also been hypothesized to increase circulating IGF-1 and IGFBP-3 by stimulating hepatic production; however, this might be through inducing the transcription of IGF-1 and IGFBP-3 genes in nonparenchymal hepatic cells (137). $1,25(OH)_2D$ is known to induce liver production of IGFBP-3 through a steroid receptor co-activator 3, which activates

nuclear receptors, particularly VDRs (216). On the other hand, IGF-1 is also able to affect renal production of $1,25(OH)_2D$ by stimulating the enzyme 1-alpha-hydroxylase (CYP27A1), therefore, it would be important to consider IGF-1 in further studying the health benefits of vitamin D in skeletal muscle (137). These studies are consistent with our findings and demonstrate the interactions among vitamin D status, IGF-1 and IGFBP-3, however further investigations are required to characterize these complicated pathways.

Fat mass accretion occurred uniformly over the course of the study which aligned with increasing leptin concentrations. Leptin, which was originally described as a nutrient-related hormone, originates from adipocytes, and is mainly required to regulate energy homeostasis and appetite. However, the abundant distribution of its receptors in different tissues, including skeletal muscle, liver, and bone (217), indicates its broad endocrine effects (218). In our study leptin concentrations increased over time and this agrees with some studies that suggest leptin is required during periods of rapid growth and development (219-221). However, our data in growing rats demonstrates that leptin is not affected by modest increases in vitamin D intakes (1 to 4 IU vitamin D_3/g of food) and achievement of healthy vitamin D status.

Although a limitation of our study was small sample size and a retrospective power analysis showed 65% for the lean mass outcomes, the sample size of 6 rats per each group was sufficient to identify changes in lean mass. We thus expect that a larger sample size of 9 rats per each group would increase the power of the study above 80% to confirm the same effects. The generalizability of this study is limited by the sex of the animals, as female weanling rats were used to minimize confounding effects of testosterone as a stimulus for lean mass accretion. Furthermore, since muscle mass is our primary outcome of interest, information regarding whole body and tissue specific protein kinetics would be of importance to study as both techniques that

we used (DXA and μCT) to measure body composition do not provide us further information in this regard.

3.5 Summary and conclusion

We undertook a highly controlled study in a rapidly growing animal model using randomized diets containing incremental dosages of vitamin D_3 from 4 to 12 wk of age. Lean mass and serum 25(OH)D_3 increased over time in group 3 compared to group 1. The declines over time in IGF-1 concentrations were lower in group 3 versus group 1, suggesting the interaction between circulating vitamin D and IGF-1 as stimulus for lean mass accretion. The effect of vitamin D on lean mass accretion in a young animal model proposes that vitamin D supports a lean body phenotype without altering weight gain during rapid periods of growth. Further studies are required to determine the molecular mechanisms underlying this process.

Acknowledgments

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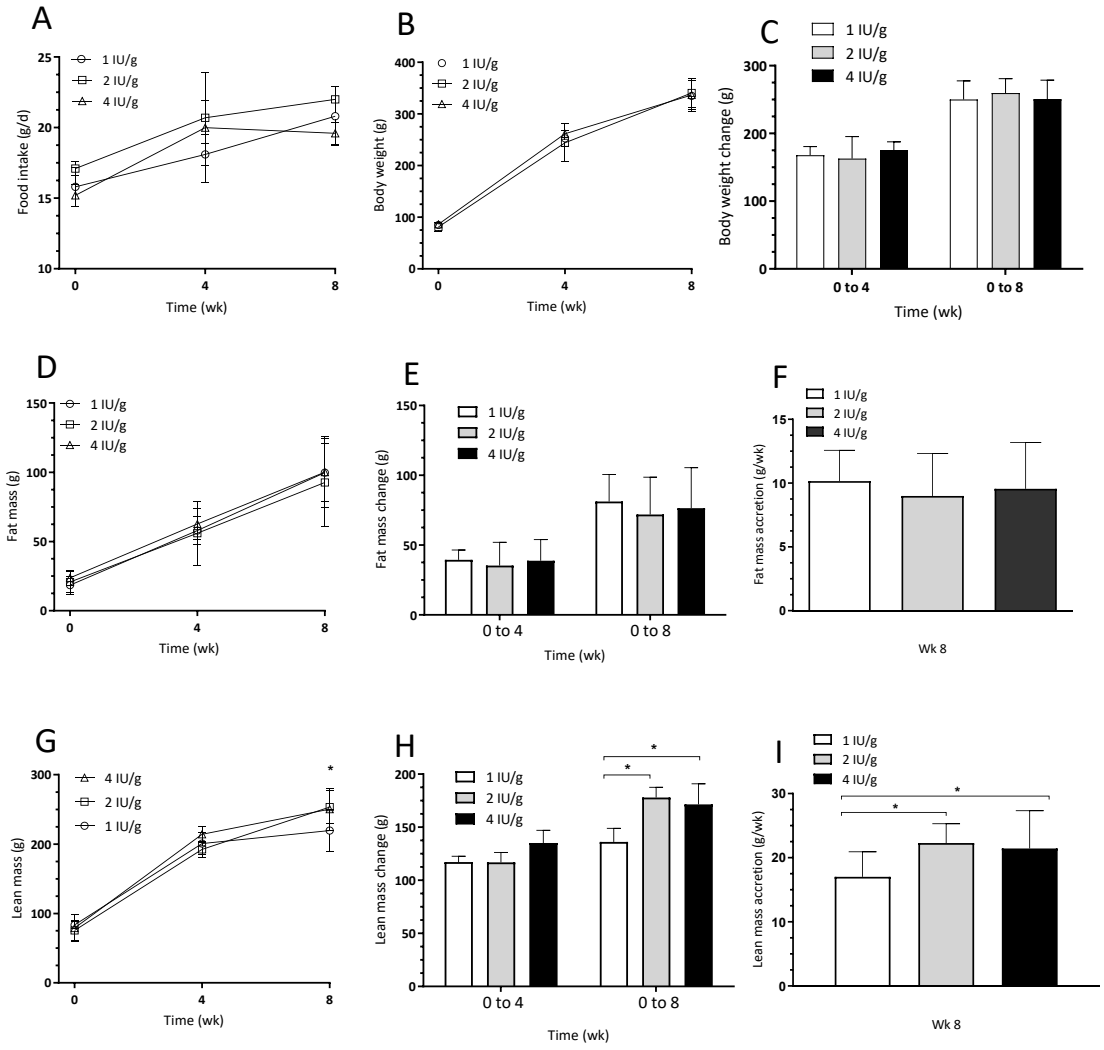
Table 3.1 Ingredients composition of the diets fed to rats[1]

Ingredient	**g/kg of diet**
Casein, "Vitamin-Free"	200
L-Cystin	3
Corn starch	397
Maltodextrin	132
Sucrose	99.70
Soybean oil	70
Cellulose	50
Mineral Mix, AIN-93G-MX	35
Vitamin Mix, AIN-93 without A, D, E	10
Vitamin A Palmitate (500, 000 IU/g)	0.008
Vitamin E, d,l-alpha tocopheryl acetate (500 IU/g)	0.15
Cholecalciferol (50,000 IU/g in sucrose)[2]	0.02
Choline bitartrate	2.5
TBHQ, antioxidant	0.014
Food color[3]	0.1

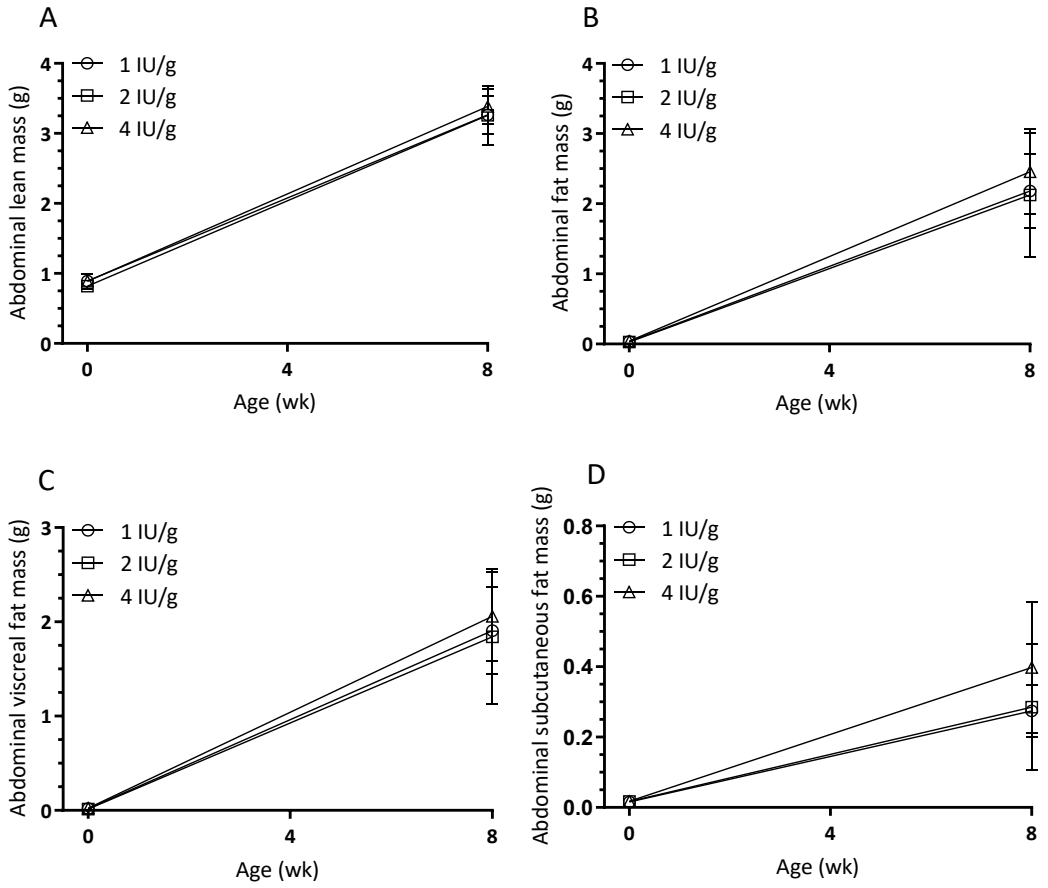
[1]Containing 63.8% carbohydrate, 16.7% fat, and 19.4% protein per g of diet as per AIN-93G.
[2]Experimental diets had altered cholecalciferol (vitamin D_3) content with 2 and 4 IU/g (0.05 and 0.1 μg/g) diet as described [27]. [3]Food color added to diets to enable blinding during the feeding trial.

Figure 3.1 Food intake, body weigh, and body composition



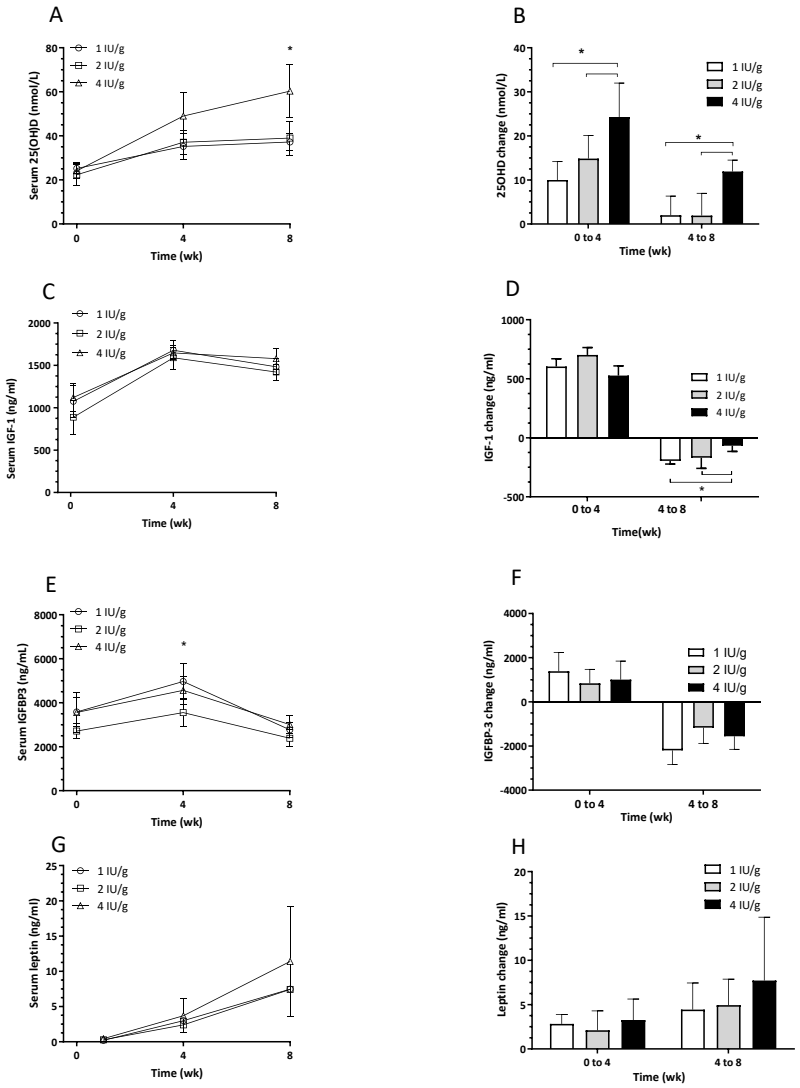
Food intake (panel A), body weight (panel B), change in body weight (panel C), fat mass (panel D), change in fat mass (panel E), fat mass accretion (panel F), lean mass (panel G), change in lean mass (panel H) and lean mass accretion (panel I) in female Sprague Dawley rats fed 1, 2 or 4 IU vitamin D_3/g food at wk 0, after 4 and 8 wks. Differences were identified using repeated measures linear mixed effect model with Tukey's post hoc t testing; n=6/diet. Data are means±SD. *Denotes significance with $P<0.05$.

Figure 3.2 Abdominal microcomputed tomography lean-fat partitioning



Abdominal lean mass (panel A), abdominal fat mass (panel B), abdominal visceral fat mass (panel C), and abdominal subcutaneous fat mass (panel D), in female Sprague Dawley rats fed 1, 2 or 4 IU vitamin D_3/g food at wk 0, after 4 and 8 wks. Differences identified using repeated measures linear mixed effect model with Tukey's post hoc t testing; n=6/diet. Data are means±SD.

Figure 3.3 Serum concentrations of 25(OH)D_3, IGF-1, IGFBP-3, and leptin



Serum concentrations of 25(OH)D_3 (panel A), change in 25(OH)D_3 (panel B), IGF-1 (panel C), change in IGF-1 (panel D), IGFBP-3 (panel E), change in IGFBP-3 (panel F), leptin (panel G) and change in leptin (panel H). Sprague Dawley rats fed 1, 2 and 4 IU vitamin D_3/g diet at wk 0, after 4 and 8 wks. Differences were identified using repeated measures linear mixed effect model with post-hoc testing; n=6/diet. Data are means±SD. * Denotes significance with $P<0.05$.

BRIDGE STATEMENT 2

The study presented in Chapter 3 with a highly controlled design addressed an important research gap and demonstrated promising results regarding the effectiveness of dietary vitamin D on building vitamin D stores and improving lean body mass in early life. This study in a young rapidly growing animal model was designed to mimic the response to vitamin D supplementation in newborn infants and proposes that vitamin D does have a link to body composition and supports a leaner body phenotype. Since this study was conducted in a controlled environment, variables associated with vitamin D status or body composition in human infants including sex, skin color, season of birth, as well as sociodemographic and cultural determinants, were not factors in the positive causal relationship observed between vitamin D status and lean mass accretion.

As discussed in chapter 2, maternal vitamin D status and maternal adiposity may influence offspring body composition. The combined influence of excess adiposity and low maternal 25(OH)D concentration on newborns body composition outcomes is unclear. Therefore, the study in chapter 4 investigated the role of maternal excess adiposity and potentially other important factors in pregnancy in the association between maternal and neonatal 25(OH)D concentration and neonatal body composition outcomes.

CHAPTER 4: RESEARCH PAPER 2

Maternal excess adiposity and serum 25-hydroxyvitamin D <50 nmol/L are associated with elevated whole body fat mass in healthy breastfed neonates

Maryam Razaghi[1], Nathalie Gharibeh[1], Catherine A. Vanstone[1], Olusola F. Sotunde[1], Shu Qin Wei[2], Dayre McNally[3], Frank Rauch[4], Glenville Jones[5], Hope A. Weiler[1,6]

[1]School of Human Nutrition, McGill University, Ste-Anne-de-Bellevue, Québec.

[2]Institut national de santé publique du Québec, Montréal, Québec.

[3]Department of Pediatrics, Children's Hospital of Eastern Ontario, University of Ottawa, Ottawa, Ontario.

[4]Shriners Hospital for Children, Montréal, Québec.

[5] Department of Biomedical and Molecular Sciences, Queen's University, Kingston, Ontario.

[6]Nutrition Research Division, Bureau of Nutritional Sciences, Food Directorate, Health Products and Food Branch, Health Canada, Ottawa, Ontario.

Abstract

Background: Vitamin D status of pregnant women is associated with body composition of the offspring. The objective of this study was to assess whether the association between maternal vitamin D status and neonatal adiposity is modified by maternal adiposity preconception.

Methods: Healthy mothers and term appropriate weight for gestational age (AGA) infants (n=142; 59% male, Greater Montreal, March 2016-2019) were studied at birth and 1 month postpartum (0.2-1.5 mo). Newborn (24-36 hour) serum was collected to measure total 25-hydroxyvitamin D [25(OH)D] (immunoassay); maternal pre-pregnancy BMI was obtained from medical records. Anthropometry, body composition (dual-energy X-ray absorptiometry) and serum 25(OH)D were measured at 1 month postpartum in mothers and infants. Mothers were classified into 4 categories based on their vitamin D status (sufficient 25(OH)D ≥50 nmol/L vs. at risk of being insufficient <50 nmol/L) and pre-pregnancy BMI (<25 vs. ≥25 kg/m^2): insufficient-recommended weight (I-RW, n=24); insufficient-overweight/obese (I-OW/O, n=21); sufficient-recommended weight (S-RW, n=69); and sufficient overweight/obese (S-OW/O, n=28).

Results: At birth, on average, infant serum 25(OH)D concentrations were below the cut-point for sufficiency of 50 nmol/L for both maternal pre-pregnancy BMI categories; 47.8 [95%CI: 43.8, 51.9] nmol/L if BMI <25 kg/m^2 and 38.1 [95%CI: 33.5, 42.7] nmol/L if BMI ≥25 kg/m^2. Infant serum 25(OH)D concentrations at birth and (r=0.77; P<0.0001) and 1-month postpartum (r=0.59, P<0.0001) were positively correlated with maternal serum 25(OH)D concentrations. Maternal serum 25(OH)D concentration was weakly correlated with maternal percent whole body fat mass (r=-0.26, P=0.002). Infants of mothers in I-OW/O had higher fat mass versus those of mothers in S-OW/O (914.0 [95%CI: 766.4, 1061.6] vs. 780.7 [95%CI: 659.3, 902.0] g; effect

size [Hedges' *g*: 0.42]; P=0.04) with magnitude of difference of 220.4 g or ~28% difference (adjusting for covariates).

Conclusions: Maternal vitamin D status is positively correlated with neonatal vitamin D. In this study, maternal adiposity and serum 25(OH)D <50 are dual exposures for neonatal adiposity. These results reinforce the importance of vitamin D supplementation early in infancy irrespective of vitamin D stores acquired *in utero* and maternal weight status.

Keywords: Mother-infant dyads, body composition, vitamin D status

4.1 Introduction

Adverse nutritional exposures *in utero* and in infancy impair growth (73) and increase the risk of chronic conditions later in life (222, 223). A burgeoning body of evidence suggests that maternal-fetal transfer of vitamin D is associated a lean body mass phenotype in childhood (74). This is a very complex physiological phenomenon with multiple factors to consider, some of which are modifiable. Maternal excess adiposity preconception is a modifiable correlate of low vitamin D status in the neonate (100, 224). This is thought to be due to vitamin D sequestration in maternal adipose tissue as well as volumetric dilution (99) and consequently hindered placental transfer of vitamin D (100). Lower 25-hydroxyvitamin D [25(OH)D] concentrations in cord blood are observed in newborns of mothers with BMI over 30 kg/m^2 compared to those born to mothers with recommended BMI range (18.5 to 24.9 kg/m^2), even though the mothers, on average, had sufficient vitamin D status (25(OH)D ≥50 nmol/L) in the third trimester (100). In addition, neonates born to mothers with pre-gravid BMI over 25 kg/m^2 (101) and those with gestational weight gain above the Institute of Medicine (IOM) guidelines (225) have higher neonatal whole body fat mass and percentage body fat, particularly in infants born to mothers with pre-pregnancy BMI over 25 kg/m^2 (226).

In Canada, not all women are vitamin D sufficient according to the 50 nmol/L 25(OH)D cut-point with as many as 10-15% <50 nmol/L (13, 14, 227). Based on a birth cohort study in Singapore, inadequate maternal 25(OH)D at 26-28 weeks gestation associate with higher abdominal adiposity in neonates (80). In addition, in two pregnancy cohort studies (New Zealand and India) maternal vitamin D status above 50 nmol/L of 25(OH)D associate with a lower percentage of body fat in children at 5 to 9.5 years of age (20, 228). Similarly, in a study from the United Kingdom, mothers in the highest quartile of vitamin D status in the third trimester had

greater percent lean mass in children at 4 years of age compared to those of mothers in the lowest quartile (229). These patterns prevailed even after adjusting for sociodemographic factors as well as maternal BMI before (80, 229), during (20, 228), and after pregnancy (229).

This growing body of evidence suggests that fetal exposure to both low maternal vitamin D status and excess maternal adiposity is associated with body composition of the offspring. The majority of these studies used BMI as a proxy measure of adiposity and studies reporting upon lean and fat mass partitioning in both mother and neonate are scarce. Therefore, the aim of the current study was to explore the correlates of maternal and neonatal vitamin D status and to assess whether the association between maternal vitamin D status and neonatal adiposity is modified by maternal adiposity preconception.

4.2 Materials and methods

4.2.1 Study design and population

Participants included mother-infant pairs (n=142) who were recruited at the Lakeshore General Hospital, located in greater Montréal, Québec, Canada as part of a trial of vitamin D supplementation in breastfed infants, from March 2016 through to March 2019. The present study includes data collected prior to hospital discharge and data from the baseline visit (0.2-1.5 months postpartum) before entering the trial. The inclusion criteria were healthy, singleton, term born infants of appropriate weight for gestational age (AGA) according to a Canadian growth reference (230) and born to mothers intending to breastfeed for at least 3 months. Exclusion criteria for the present analysis included infants born to mothers with gestational diabetes or hypertension in the present pregnancy, comorbidities (liver, renal, celiac and Crohn's diseases), medications that are known to impact vitamin D metabolism or limit growth as well as any smoking or illicit drugs (231-233).

Prior to collection of data and blood samples, parents provided written informed consent. The study was approved by the St. Mary's Hospital Research Ethics Committee (Montréal, Québec, Canada) that oversees research at the Lakeshore General Hospital. Both ethics approval (REB# 15-34) and trial registration with clinicaltrials.gov (NCT02563015) were completed prior to beginning recruitment. This study was also approved by the Health Canada Research Ethics Board (REB 2019-033H) and Privacy Management Division (HC-PR-2019-000024).

4.2.2 Obstetric history, demographic and lifestyle surveys

Prior to hospital discharge, the obstetric history including maternal pre-pregnancy weight, weight at delivery, parity, and type of delivery were obtained from the medical record. The mother's pre-pregnancy BMI was then calculated using mothers' weight prior to pregnancy and measured height at the postpartum visit. Demographic information surveyed included: maternal age, self-reported population group (white/all other groups) which was used according to the proposed guideline from Canadian Institute for Health Information (234), and in doing so any mixed ancestry were categorized within the other groups. In addition, the highest level of education completed (elementary/high school, college/vocational school, or university), household income was surveyed according to the median annual income for Canadian families with children (235) and collapsed into ≥70,000, <70,000 Canadian dollars (CAD), or not reported. Lifestyle factors were surveyed as reported previously (236) including maternal multivitamin supplement use (yes/no) and the frequency (every day, almost every day, 2-3/week or less), exercise habits (yes/no), typical frequency (none, 1-2 h/wk, ≥ 3 h/wk), and intensity (low, moderate or high), assessed 3 months prior to pregnancy and then separately for across pregnancy. In addition, whether the infants had received vitamin D supplements containing 400

IU/d prior to the follow-up visit was surveyed. Measurements at the postpartum visit included anthropometry, body composition, and blood samples from each infant and their mother.

4.2.3 Biochemistry measurements

Capillary blood was sampled from the neonates between 24 and 36 h of life (236). Mothers and their infants participated in a postnatal visit at 1 month (± 0.5 month) at the Mary Emily Clinical Nutrition Research Unit, McGill University. In the non-fasted state, capillary blood samples (0.5 ml) were collected from infants by heel lance; and a maternal venous sample (5 ml) was taken to assess vitamin D status which does not vary significantly between delivery and 1 mo postpartum (237, 238). Samples were centrifuged (4000 x g for 20 min at 6°C) to obtain serum to assess vitamin D status by measuring total serum 25(OH)D using an automated chemiluminescence immunoassay (Liaison, Diasorin Inc.). The laboratory maintained a certificate of proficiency from the Vitamin D External Quality Assessment Scheme. Vitamin D control samples from the National Institute of Standards and Technology quality assurance program were implemented in routine quality control measures. The inter-assay coefficient of variation for NIST972a (levels 1 to 4) was on average <10% and the accuracy 97.4% of certified values. The inter-assay coefficient of variation for an internal laboratory control (62.8 nmol/L) human serum sample was 8.2% across all assays. Deming regression was used to standardize the original measured 25(OH)D values to NIST reference measurements: standardized concentration=0.9634 (Liaison concentration + 3.122 nmol/L). In a subgroup of mothers and infants (n= 83), total 25(OH)D was in agreement (mean difference= -0.8) with liquid chromatography tandem mass spectroscopy (Queen's University, Kingston, Ontario, Canada) using an assay certified by the Vitamin D Standardization Certification Program. In the current study, the cut-point for sufficiency of vitamin D status in otherwise healthy mother-infant dyads

was set at ≥50 nmol/L of serum 25(OH)D in accordance with the IOM (10) and since vitamin D status above this cut-point also positively relates to lean mass at 3-4 y (9, 229). For ease of readability, individuals with serum 25(OH)D concentration <50 nmol/L, were termed as insufficient to reflect the increasing risk of being insufficient as serum 25(OH)D falls below 50 nmol/L. The population cut-point of 40 nmol/L of serum 25(OH)D was not used in the present analysis since the assessment was at the level of individual mother-infant dyads.

4.2.4 Skin pigmentation and UVB exposure

Skin tone of the infant was measured at the research facility by taking the average of three measurements at the inner upper arm for constitutive pigmentation (basal color uninfluenced by UV light or other environmental factors) using a spectrophotometer (CM-700d/600d, Konica Minolta, USA). Individual typological angle (ITA^{o}) was calculated with the L* and b* values using published equations (239). Infants were classified into two skin tone groups (F I-III; F IV-VI) based on Fitzpatrick scales (240, 241). Based on the strength of solar UVB, vitamin D synthesizing /vitamin D non-synthesizing periods (April 1st-October 31st/ November 1st-March 31st) at birth were used as a proxy for potential vitamin D synthesis (242). Season at birth (winter, spring, summer, fall) was also explored as previously reported (236).

4.2.5 Anthropometric measurements

At the research facility, infant weight, length and head circumference were measured using standard methodology (243). Weight, length, and BMI for age z-scores were calculated using WHO growth standards and software (WHO AnthroPlus, Switzerland). Maternal anthropometric measurements, including weight using a balance-beam scale (Detecto; Webb, US) to the nearest 0.1 kg while wearing light clothing and no shoes, and height to nearest 0.1 cm using a wall-mounted stadiometer (Seca Medical Scales and Measuring Systems, US) were used

to calculate BMI (kg/m^2). Additionally, total weight gain during pregnancy was estimated by subtracting pre-pregnancy weight from the weight obtained at delivery (244) and classified into 3 categories (inadequate, adequate, and excess weight gain) according to pre-pregnancy BMI and IOM guidelines (225).

4.2.6 Body composition measurements

Body composition of infant (infant whole body software) and mothers (whole body software) was assessed using a fan-beam dual-energy X-ray absorptiometer (DXA; APEX version 13.3:3, Hologic 4500A Discovery Series, Bedford, MA) that provides a three-compartment model of body composition as reported in detail (243). For quality control and quality assurance purposes, a spine phantom (Hologic phantom; No. 14774) was used at each study visit and the coefficient of variation for bone mineral content, bone mineral density, and bone area were <1%; the radiographic uniformity tests were within established limits across the study. Whole body scans provided lean mass (g) excluding bone mineral content, fat mass (g and %); from these values, lean mass index (LMI, lean body mass (kg)/stature (m)^2), and fat mass index (FMI, fat mass (kg)/stature (m)^2) were then calculated using standing height for mothers and crown heel length for infants.

4.2.7 Power analysis and sample size estimation

This was a convenience sample, 142 mother-infant dyads were included in a cross-sectional analysis of data at birth and 1 month postpartum as part of a randomized trial (245) and thus a retrospective power was calculated based on changes in the primary outcome (fat mass) between mothers with pre-pregnancy ≥25 kg/m^2 and 25(OH)D <50 (n=21) and those with pre-pregnancy ≥25 kg/m^2 and 25(OH)D ≥50 (n=28). Power was estimated to be 75% using the procedure described by Kononoff (211) for specific data sets accounting for the fixed effects of

gestational weight gain, neonatal sex, gestational age, UVB period at birth, actual age of infant at the postnatal visit, and infant length in a linear fixed effects model design.

4.2.8 Statistical analysis

Data were analyzed using Statistical Analysis System (SAS; version 9.4, SAS Institute Inc., Cary, NC). Descriptive characteristics for mothers and infants were expressed as mean (95% confidence interval) or n (%). In order to describe the population, mothers were classified into 1 of 4 groups according to serum 25(OH)D concentrations (insufficient: 25(OH)D <50, sufficient: 25(OH)D ≥50 nmol/L) and pre-pregnancy BMI (healthy BMI: <25, overweight/obese: ≥25 kg/m^2). The 2×2 design of maternal vitamin D status and pre-pregnancy BMI, formed 4 groups of interest, I-RW: insufficient-recommended weight (25(OH)D <50 and BMI <25 kg/m^2), I-OW/O: insufficient-overweight/obese (25(OH)D <50 and BMI ≥25 kg/m^2), S-RW: sufficient-recommended weight (25(OH)D ≥50, BMI: <25 kg/m^2), and S-OW/O: sufficient-overweight/obese (25(OH)D ≥50 nmol/L, BMI ≥25 kg/m^2). Vitamin D status and body composition were also compared among maternal postpartum FMI categories (low to normal: 4-9, excess fat: >9-13, and obese: >13 kg/m^2) (246), as well as gestational weight gain (225) categories (inadequate, adequate, and excess weight gain).

Chi-square or Fisher's exact tests (frequency analysis) were used to test for differences in proportions. Correlation tests were used to identify linear relationships between continuous variables including maternal and neonatal vitamin D status and maternal vitamin D status and body composition (SAS PROC CORR and PROC GLM using MANOVA option). According to the product of the interaction effect of maternal vitamin D status and pre-pregnancy BMI, the 4 groups of interests (I-RW, I-OW/O, S-RW, and S-OW/O), frequency analysis creates 3-way crosstabulation tables. Each of the categorical variables was used in the model to stratify the

crosstabulation tables followed by the interaction term (maternal 25(OH)D*pre-pregnancy BMI) creating two 2-way tables of 25(OH)D and pre-pregnancy BMI, for each levels of the categorical variables.

For maternal and neonatal characteristics at delivery and 1 mo postpartum, continuous data were compared using a linear fixed effects model followed by post hoc Tukey's tests with Tukey-Kramer adjustment for multiple comparisons. The interrelationship among maternal predictors of infant body composition (pre-pregnancy BMI, maternal 25(OH)D, and the interaction effect of these two variables) and neonatal body composition (lean and fat mass, related percentages, and indices) was tested using a linear fixed effects model and *post hoc* tests adjusted for multiple comparisons using Tukey-Kramer adjustment. Additional fixed effects modelled included: gestational weight gain, neonatal sex, gestational age (GA) at birth, UVB period at birth, actual age of infant at the postnatal visit, and infant length. Additional variables that were considered in these analyses were: family income, maternal age, self-reported population group, education, multivitamin supplement use, exercise habits during pregnancy, season at birth as well as changes in neonatal serum 25(OH)D concentration during postpartum. These variables did not improve the model as judged by Bayesian information criterion (BIC) and were not statistically significant ($P>0.05$), thus these were removed from the final model using backward elimination method.

In order to determine factors associated with serum 25(OH)D concentrations in mother-infant dyads, maternal and neonatal serum 25(OH)D were separately modelled against maternal indicators of adiposity including BMI and FMI, and other important lifestyle or demographic factors including exercise prior to as well as during pregnancy (yes/no) as two separate variables, multivitamin supplement use prior to/during pregnancy (yes/no), UVB period at birth/at delivery

(vitamin D synthesizing, non-synthesizing period), parity (primiparous, multiparous), maternal education (elementary/high school, college/vocational school, university), and family income (≥70,000, <70,000 CAD, or not reported). In these regression models, the frequency of multivitamin supplements, the frequency and intensity of the exercise prior to/during pregnancy as well as season at birth did not appear to improve the models and were thus removed. For the regression analysis of neonatal 25(OH)D, neonatal sex (male, female) and skin tone (F I-III, F IV-VI) were additional fixed factors included in the model. Data was tested using Kolmogorov-Smirnov and Shapiro-Wilk for normality and the residuals were normally distributed; and Levene and Bartlett tests of homogeneity of variances to meet the assumptions of the post-hoc testing. For all tests, a P-value of <0.05 was used to guide interpretation of the results.

4.3 Results

4.3.1 Maternal characteristics according to vitamin D status and pre-pregnancy BMI categories

Groups categorized based on maternal 25(OH)D and pre-pregnancy BMI were not different in terms of maternal age, country of birth, self-reported population group and education (Table 4.1). Although the majority of mothers (92.3%) in all groups took a multivitamin supplement during pregnancy, the proportion was lower in I-RW compared to the rest of the groups. No differences were observed in the dose of vitamin D taken. A higher proportion of mothers in I-OW/O had annual household income <70,000 CAD compared to the other groups. At the postpartum visit 98.6% of mothers were breastfeeding, 2 mothers discontinued breastfeeding between recruitment at birth and the baseline visit due to milk insufficiency.

4.3.2 Neonatal characteristics at birth and postpartum according to maternal vitamin D status and pre-pregnancy BMI

Newborns (59% male) were not different among groups in terms of GA, age at the postpartum visit, UVB period at birth, and anthropometric measurements (Table 4.2). At birth, on average, infant serum 25(OH)D concentrations were below the cut-point for sufficiency of 50 nmol/L for both maternal pre-pregnancy BMI categories of within or above the recommended range (47.8 [95%CI: 43.8, 51.9] vs. 38.1 [95%CI: 33.5, 42.7]). At birth and 1 month of age, infants of mothers in I-RW and I-OW/O had significantly lower serum 25(OH)D concentrations compared to infants born to mothers in S-RW and S-OW/O. In addition, more infants in I-OW/O were male compared to the other groups whereas a higher proportion of infants in S-RW had skin tone F I-III compared to the rest of the groups.

Overall, the majority (95.0%) of infants received daily vitamin D supplements (containing 400 IU vitamin D) between discharge from hospital and the follow-up visit. Infant mean serum 25(OH)D concentrations significantly increased during the postnatal period (birth: 44.5 [95%CI: 41.3, 47.6] vs. 1 month: 54.7 [95%CI: 51.9, 57.5] nmol/L; $P<0.0001$). Infants born with serum 25(OH)D ≥50 nmol/L had significantly lower ($P<0.0001$) mean change in serum 25(OH)D concentration (1.39, 95%CI: -3.0, 5.8 nmol/L) compared to neonates born with 25(OH)D 30-49.9 nmol/L (12.9, 95%CI: 10.2, 15.7 nmol/L), and those deficient <30 nmol/L (17.7, 95%CI: 12.5, 22.8 nmol/L). Infant serum 25(OH)D concentrations at birth ($r=0.77$; $P<0.0001$) and at one month ($r=0.59$, $P<0.0001$) were positively correlated with maternal serum 25(OH)D concentrations. These correlations remained evident after adjusting for parity, maternal multivitamin supplement use, gestational age at birth, infant sex, UVB period at birth, infant skin tone.

4.3.3 Maternal vitamin D status, indicators of adiposity and whole-body lean mass

In mothers, serum 25(OH)D concentrations were weakly, positively correlated with whole body lean mass (r=0.23, P=0.006) and weakly, inversely correlated with percent whole body fat mass (r=-0.26, P=0.002). Maternal serum 25(OH)D was on average higher in mothers with pre-pregnancy BMI <25 kg/m^2 (Figure 4.1.A) compared to ≥25 kg/m^2. Similarly, serum 25(OH)D was lower with postpartum BMI <25 kg/m^2 or 25-29.9 kg/m^2 (Figure 4.1.B) compared to mothers with BMI ≥30 kg/m^2. However, on average, maternal serum 25(OH)D concentrations were ≥50 nmol/L in all BMI categories (68.3% and 57.1% of mothers with BMI <25 and ≥25 kg/m^2 had 25(OH)D concentrations ≥50 nmol/L, respectively). Likewise, serum 25(OH)D of mothers with low to normal or excess FMI categories was higher compared to mothers with FMI in the obese range (Figure 4.1.C). Appropriateness of gestational weight gain was not related to maternal serum 25(OH)D (Supplemental Table 4.1). Among other covariates included in the statistical analysis, mothers who self-reported being physically active (indoor and outdoor combined) 3 months prior to conception or during pregnancy had higher 25(OH)D concentrations postpartum versus mothers who were not active. Additionally, self-reported population group (white/all other groups) was a prominent factor related to maternal serum 25(OH)D, however other covariates such as parity, multivitamin supplement use, education level and family income were not related (Supplemental Table 4.1).

4.3.4 Neonatal vitamin D status and maternal indicators of adiposity

The mean serum 25(OH)D was on average higher in infants of mothers with pre-pregnancy BMI <25 kg/m^2 compared to ≥25 kg/m^2 (Figure 4.1.D). Infant 25(OH)D was also higher in infants of mothers with postpartum BMI <25 kg/m^2 or 25-29.9 kg/m^2 (Figure 4.1.E) compared to mothers with BMI ≥30 kg/m^2. Similarly, serum 25(OH)D concentrations of infants

born to mothers in the low to normal category of FMI were higher compared to infants born to mothers in the obese category (Figure 4.1.F). However, on average, infant serum 25(OH)D concentrations were below the cut-point for sufficiency of 50 nmol/L for all maternal BMI (38.7 and 20.4% of infants of mothers with <25 and ≥25 kg/m^2 had vitamin D sufficiency) or FMI categories (39.2, 30.8, and 0% of infants of mothers with all FMI categories of low to normal: 4-9, excess fat: >9-13, and obese: >13 kg/m^2 had vitamin D sufficiency respectively). The relationship among neonatal vitamin D status and maternal indicators of adiposity and other maternal factors is shown in detail (Supplemental Table 4.2).

Maternal pre-pregnancy BMI (<25 kg/m^2 or ≥25 kg/m^2) and maternal serum 25(OH)D (>50 or <50 nmol/L) as independent categorical variables were not associated with any of neonatal indicators of adiposity (Table 4.3 and Supplemental Table 4.3). However, a significant interaction effect of maternal pre-pregnancy BMI and 25(OH)D concentration was observed in neonatal fat mass (Table 4.3), fat percentage and FMI adjusting for multiple covariates (Supplemental Table 4.3). After pairwise comparison tests, infants of mothers in the I-OW/O group with elevated pre-pregnancy BMI (≥25 kg/m^2) and vitamin D insufficiency (25(OH)D <50 nmol/L) had significantly higher fat mass (Figure 4.2.A), fat percentage (Figure 4.2.B), and FMI (Figure 4.2.C) compared to infants born to mothers in the S-OW/O group with BMI >25 kg/m^2 but vitamin D sufficiency (25(OH)D ≥50 nmol/L). The magnitude of difference in whole body fat mass observed between I-OW/O and S-OW/O groups was 220.4 g (95% CI: 56.4, 384.3) representing ~28% difference with effect size of 0.42. Whole body fat mass, fat percentage, and FMI were also higher in female infants versus males (P<0.05) (Supplemental Figure 1A, B, C) and in infants born in vitamin D synthesizing period (UVB period) versus those born in vitamin D non-synthesizing period (P<0.05) (Supplemental Table 3). Other covariates linked to greater

infant adiposity indicators were infant age and length (Table 3 and Supplemental Table 3). In these adjusted linear fixed effects models, gestational weight gain was not associated with neonatal fat mass or any other adiposity indicators (Table 4.3 and Supplemental Table 4.3).

Similar to neonatal indicators of adiposity, maternal pre-pregnancy BMI (<25 kg/m^2 or ≥25 kg/m^2) and maternal serum 25(OH)D (>50 or <50 nmol/L) were not independently linked to lean mass, lean percentage and LMI (Table 4.4 and Supplementary Table 4.4). A significant interaction effect of maternal pre-pregnancy BMI and 25(OH)D concentration was observed on neonatal lean mass (Table 4.4 & Figure 4.2.D) and lean percentage (Figure 4.2.E) but not LMI (Figure 4.2.F). However, after pairwise comparison tests, only infants of mothers in I-OW/O with BMI ≥25 kg/m^2 before pregnancy and vitamin D insufficiency (25(OH)D <50 nmol/L) had significantly lower lean percentage compared to infants born to mothers in S-OW/O with BMI ≥25 kg/m^2 but with vitamin D sufficiency 25(OH)D ≥50 nmol/L (P=0.05) (Supplemental Table 4.4). Whole body lean mass, percentage lean mass, and LMI were also observed to be higher in male infants versus females (P<0.05) (Supplemental Figure 1D, E, F). In addition, only percentage lean mass appeared to be lower in infants born in vitamin D synthesizing period (UVB period) versus those born in vitamin D non-synthesizing period (P=0.0482) (Supplemental Table 4.4). Infant age and length were important correlates associated with 295 (P=0.0215) and 102 grams (P<0.0001) higher lean mass and percentage lean mass respectively (Table 4.3 and Supplemental Table 4.3).

4.4 Discussion

According to our data, healthy neonates dually exposed to insufficient maternal vitamin D status and elevated pre-pregnancy BMI had higher whole body fat mass (Δ 220.4 g, ~28% difference) compared to those of mothers with elevated pre-pregnancy BMI yet vitamin D

sufficient. Our results are unique in simultaneously examining maternal adiposity and vitamin D status as dual exposures in programming of neonatal body composition and build upon other reports linking maternal vitamin D status to adiposity in the neonatal period (80) or in childhood (20, 228), and maternal overweight/obese pre-gravid BMI with neonatal adiposity (101, 247).

Most national guidelines for a healthy pregnancy (248, 249) do not have a specific recommendation on vitamin D supplementation for pregnant women with an elevated BMI; nor their newborn. Although the majority of mothers took multivitamin supplements containing vitamin D during pregnancy, 64.3% of the infants of mothers with elevated pre-pregnancy BMI, had serum 25(OH)D <50 nmol/L. If maternal serum 25(OH)D was <50 nmol/L all infants had 25(OH)D <50 nmol/L and the majority (71.4%) were vitamin D deficient. These observations reinforce the importance of encouraging overweight/obese women to seek nutrition counselling prior to conception (250) and if already pregnant to initiate the consultation and multivitamin supplementation as soon as possible to help establish vitamin D stores in the fetus and ultimately in the newborn (100). This should be followed by vitamin D supplementation of the infant (251).

This study adds that maternal 25(OH)D concentration is an influential factor in neonatal vitamin D status at birth and within the neonatal period. We observed a positive correlation between maternal and neonatal vitamin D status at birth, which decreases 23.4% through the neonatal period in line with the other reports (252, 253). This is attributed to the fact that the developing fetus is fully reliant on maternal-fetal transfer of vitamin D (254), however, shortly after birth, the majority of infants commenced routine supplements containing 400 IU/d vitamin D and were breastfed. Overall, the majority of infants at birth (67.6%) had had serum 25(OH)D <50 nmol/L, whereas at the postpartum visit, this declined to 40%. Even though 4~6 week is not long enough to see a plateau in the response to vitamin D supplementation (50, 255, 256), the

increments in vitamin D status of infants born with 25(OH)D <50 nmol/L were in agreement with another study (257). The response of infants to vitamin D supplementation inversely relates to their basal status resulting in a greater increment in serum 25(OH)D in neonates with vitamin D deficiency. Thus, it appears that the standard of care of 400 IU/d of vitamin D is suitable for healthy term-born infants, even if born with vitamin D deficiency.

Vitamin D status of mothers could be a proxy for other healthy behaviors, reflect quality of diet, or time spent outside. In our study exercise (combined indoor and outdoors) in pregnancy was associated with 14.8 nmol/L higher 25(OH)D concentration in mothers of all BMI ranges. This is of relevance since much of exercise tends to take place in outdoor (258) with a positive association between exercise and time spent outside (259, 260). Outdoor activity and being more exposed to sunlight promote vitamin D synthesis as well as vitamin D mobilization from expanded adipose tissue (261) and consequently support both achievement and maintenance of higher vitamin D status. Activity is encouraged as part of a healthy lifestyle in pregnancy (262).

The genes that regulate fat distributions, adiposity (263), as well as skeletal muscle phenotypes (264) are in turn responsive to a variety of environmental and lifestyle exposures. For instance, achieving appropriate body weight and being physically active preconception and during pregnancy can determine body composition in the offspring (265). Regular physical activity as part of a healthy lifestyle shifts the increased energy demands to maternal muscle mass and away from the adipocytes of the fetus leading to offspring with proportionately increased lean mass and decreased adipose tissue (265). This pattern is consistent with the evidence observed in the present study and is likely due to vitamin D mobilization from fat tissue into circulation (261). Therefore, it can be inferred that genetic linkages in conjunction with

important environmental and behavioral factors contribute to variations in fat-muscle mass partitioning in early development.

In this study, we did not observe an association between vitamin D status and infant lean body mass, potentially due to all infants being born term and AGA. Recent evidence illustrates that in comparison to infants born AGA, body composition partitioning differs among infants at the extremes of birth weight such as those born small or large for gestational age (266). Infants born large for gestational age tend to associate more with vitamin D insufficiency (267, 268), possibly due to entrapment of vitamin D in fat tissue compared to AGA or counterparts born small for gestational age. In addition, by including only non-smoking mothers we eliminated smoking as a confounder as neonates born to smoking mothers appear to have lower vitamin D status (269) as well as lower lean body mass (270, 271). Lack of association between vitamin D and lean body mass might be due to the rapid growth spurt occurring in the first month of life.

In the current study, sex differences in infant body composition emerged early in the postnatal period. This effect was independent of the UVB period at birth, GA, maternal supplement use, and maternal and neonatal vitamin D status. Male infants have more muscle mass due to the anabolic effect of testosterone which temporarily surges postnatally within 1~3 months postpartum (272, 273). In contrast, in our study and others, females have greater stores of fat mass irrespective of their vitamin D status (274).

4.5 Strengths, limitations, and further research

Strengths of this study are inclusion of infants of a diverse ancestry which aids the generalizability of the findings to multi-ethnic populations and assessment of body composition early in postnatal period in mother infant dyads. There are however some limitations including that pre-pregnancy weight was obtained from medical records, which could be self-reported. Pre-

pregnancy BMI as a proxy for overweight and obesity may underestimate or misclassify adiposity (275), however, the three-compartmental model of DXA confirmed excess adipose tissue. We used an immunoassay to measure total serum 25(OH)D which is not a gold standard technique, nonetheless, the manufacturer (Liaison, Diasorin Inc.) is certified by the Vitamin D Standardization Certification Program (276). Furthermore, we used rigorous quality assurance measures and standardized 25(OH)D concentrations to NIST standard reference materials. Given the design of the study, maternal 25(OH)D concentrations were only measured at the postpartum visit and not at delivery. This may not be a major limitation as maternal 25(OH)D concentrations are not significantly different between 36 weeks of GA and 1 month postpartum (237, 238). Additionally, our analysis might have been statistically underpowered to detect relationships such as the association between gestational weight gain and maternal or neonatal vitamin D status, future larger studies are needed. Lastly, whether the body composition pattern extends later into childhood requires a longitudinal study.

4.6 Conclusion

In otherwise healthy mother-infant dyads, maternal overweight/obesity and serum 25(OH)D <50 nmol/L are dual exposures that associate with neonatal serum 25(OH)D <50 nmol/L as well as higher adiposity. More concerning, 71.4% of neonates in this cohort were vitamin D deficient. These results reinforce the importance of postnatal vitamin D supplementation in infants born to mothers with BMI ≥25 kg/m^2. In the event of low maternal-fetal transfer of vitamin D, postnatal supplementation with 400 IU/d of vitamin D readily builds vitamin D stores and in doing so limits the impact of fetal exposures.

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Authors' contributions

HAW, SW, DM, FR, and GJ designed the study. HAW and CAV supervised the study. HAW, CAV, MR, NG, OFS conducted the study; HAW, CAV, MR, NG, OFS collected the data; MR, NG and OFS performed the laboratory analyses. MR performed statistical analysis; MR wrote the final manuscript with the intellectual aid and comments of HAW. All authors (MR, NG, CAV, OFS, SW, DM, FR, GJ, SK, and HAW) have read and approved the manuscript.

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Availability of data

The data described in the manuscript, code book and analytic code will not be made available because permission to share data was not requested at the time of obtaining participant consent.

Table 4.1 Maternal characteristics according to maternal pre-pregnancy BMI and vitamin D status postpartum

Characteristic[1]	**All**	**25(OH)D < 50**		**25(OH)D ≥ 50**		**P-value**[2]		
		BMI <25 (*n*=24)	**BMI ≥25 (*n*=21)**	**BMI <25 (*n*=69)**	**BMI ≥25 (*n*=28)**	**25(OH)D**	**BMI**	**25(OH)D* BMI**
Age at delivery, y	32.2 (31.4, 32.9)	31.0 (28.9, 33.0)	32.7 (30.1, 35.2)	31.9 (31.0, 32.8)	33.5 (31.7, 35.2)	0.29	0.05	0.93
Parity, n (%)								
Primiparous (1)	44 (31.0)	12 (50.0)	6 (28.6)	21 (30.4)	5 (17.9)	0.23	0.0009	0.28
Multiparous (≥2)	98 (69.0)	12 (50.0)	15 (71.4)	48 (69.6)	23 (82.1)	<0.0001	0.03	0.04
Pre-pregnancy BMI, kg/m^2	24.6 (23.8, 25.3)	22.4 (21.6, 23.2)	30.0 (28.1, 31.8)	21.8 (21.4, 22.2)	29.2 (27.3, 31.0)	0.22	<0.0001	0.85
Gestational weight gain, kg	13.6 (12.6, 14.6)	13.3 (10.2, 16.5)	12.0 (9.3, 14.6)	14.8 (13.5, 16.1)	12.0 (9.7, 14.4)	0.49	0.07	0.53
Serum 25(OH)D, nmol/L	67.4 (63.1, 71.7)	40.0 (36.2, 43.9)	40.4 (36.8, 43.9)	83.3 (78.1, 88.6)	71.8 (64.8, 78.8)	<0.0001	0.10	0.08
Maternal birthplace, n (%)								
Canada	90 (63.4)	13 (54.2)	8 (38.1)	50 (72.5)	19 (67.9)	<0.0001	0.0001	0.35
Elsewhere	52 (36.6)	11 (45.8)	13 (61.9)	19 (27.5)	9 (32.1)	0.58	0.27	0.11
Self-reported population group, n (%)								
White	79 (55.6)	8 (37.5)	7 (33.3)	47 (71.0)	17 (60.7)	<0.0001	0.0002	0.16
All other groups[3]	63 (44.4)	16 (62.5)	14 (66.7)	22 (29.0)	11 (39.3)	0.80	0.20	0.32
Supplement use[4], n (%)								
Yes	131 (92.3)	20 (83.3)	20 (95.2)	65 (94.2)	26 (92.9)	<0.0001	0.0007	0.02
No	11 (7.7)	4 (16.7)	1 (4.8)	4 (5.8)	2 (7.1)	0.76	0.13	0.62
Vitamin D dosage, IU/d	501.5 (478.6, 524.3)	470 (402.8, 537.2)	513.2 (458.8, 567.5)	504.6 (472, 537.1)	510.9 (453.7, 568.1)	0.53	0.34	0.48
Education, n (%)								
Elementary/high school	13 (9.2)	4 (17.8)	4 (19.0)	4 (5.8)	1 (3.6)	0.41	0.41	0.28
College/vocational school	30 (21.1)	5 (13.3)	1 (4.8)	15 (21.7)	9 (32.1)	0.001	0.07	0.33
University	99 (69.7)	15 (68.9)	16 (76.2)	50 (72.5)	18 (64.3)	0.0002	0.002	0.01
Family yearly income[5], n (%)								
≥70,000 CAD	80 (56.3)	15 (62.5)	5 (23.8)	42 (60.9)	18 (64.3)	<0.0001	0.0001	0.67
<70,000 CAD	41 (28.9)	4 (16.7)	13 (61.9)	18 (26.1)	6 (21.4)	0.27	0.64	0.001
Not reported	21 (14.8)	5 (20.8)	3 (14.3)	9 (13.0)	4 (14.3)	0.28	0.13	1.00

Table 4.1 Maternal characteristics according to maternal pre-pregnancy BMI and vitamin D status postpartum

[1]Data are mean (lower and upper 95% confidence limits) or n (%); [2]Data were compared using a linear fixed effects model for continuous variables followed by post hoc Tukey's tests with Tukey-Kramer adjustment for multiple comparisons and Chi-square or Fisher exact tests for categorical variables (using frequency procedure to create 3-way crosstabulation tables; categorical variables were used in the model to stratify the crosstabulation tables followed by the last two variables: maternal 25(OH)D*pre-pregnancy BMI, creating two 2-way tables of 25(OH)D and pre-pregnancy BMI, for each level of the categorical variables); [3]other groups included: South Asian, Chinese, Black, Filipino, Latin American, Arab, Southeast Asian, West Asian, Korean, Japanese, or other; [4]use of prenatal supplement containing vitamin D during pregnancy; [5]the median income (in Canadian dollars) for Canadian families with children. Abbreviations: 25(OH)D: 25-hydroxyvitamin D; BMI: body mass index; CAD: Canadian dollar.

Table 4.2 Neonatal characteristics according to maternal pre-pregnancy BMI and vitamin D status postpartum

Characteristic[1]	**All**	**25(OH)D <50**		**25(OH)D ≥50**		**P-value**[2]		
		BMI <25 (*n*=24)	**BMI ≥25 (*n*=21)**	**BMI <25 (*n*=69)**	**BMI ≥25 (*n*=28)**	**25(OH)D**	**BMI**	**25(OH)D*BMI**
Birth								
Gestational age, (wk)	39.64 (39.5, 39.8)	39.9 (39.4, 40.4)	39.7 (39.2, 40.1)	39.7 (39.4, 39.9)	39.3 (38.9, 39.8)	0.13	0.13	0.90
Sex, n (%)								
Male	83 (58.5)	13 (54.2)	14 (66.7)	40 (58.0)	16 (57.1)	0.002	0.01	0.04
Female	59 (41.5)	11 (45.8)	7 (33.3)	29 (42.0)	12 (42.9)	0.003	0.01	0.47
UVB period[3], n (%)								
Synthesizing	83 (58.5)	14 (58.3)	9 (42.9)	43 (62.3)	17 (60.7)	<0.0001	0.001	0.34
Non-synthesizing	59 (41.5)	10 (41.7)	12 (57.1)	26 (37.7)	11 (39.3)	0.05	0.09	0.06
Weight (kg)	3.4 (3.3, 3.5)	3.4 (3.3, 3.6)	3.5 (3.3, 3.7)	3.3 (3.3, 3.4)	3.4 (3.2, 3.6)	0.12	0.44	0.69
Weight z score	0.2 (0.0, 0.3)	0.3 (-0.1, 0.7)	0.3 (-0.1, 0.7)	0.1 (-0.1, 0.2)	0.2 (-0.1, 0.5)	0.20	0.68	0.54
Serum 25(OH)D, nmol/L	44.5 (41.3, 47.6)	29.3 (24.8, 33.7)	26.6 (23.0, 30.2)	54.3 (50.0, 58.6)	46.7 (40.8, 52.5)	<0.0001	0.07	0.38
Postnatal visit								
Age, mo	0.7 (0.6, 0.8)	0.7 (0.6, 0.8)	0.6 (0.5, 0.8)	0.7 (0.7, 0.8)	0.7 (0.6, 0.8)	0.37	0.25	0.51
Weight, kg	4.0 (3.9, 4.1)	4.1 (3.8, 4.3)	3.9 (3.7, 4.2)	3.9 (3.8, 4.1)	4.0 (3.8, 4.2)	0.61	0.68	0.29
Weight z score	-0.1 (-0.2, 0.0)	0.1 (-2.0, 0.5)	-0.01 (-0.4, 0.4)	-0.2 (-0.4, -0.03)	-0.02 (-0.4, 0.3)	0.23	0.86	0.28
Length, cm	53.1 (52.7, 53.4)	53.8 (52.9, 54.8)	53.0 (52.2, 53.8)	52.9 (52.4, 53.3)	53.0 (52.1, 53.9)	0.22	0.34	0.19
Length z score	-0.01 (-0.2, 0.1)	0.4 (-0.1, 0.8)	0.1 (-0.3, 0.4)	-0.2 (-0.4, 0.1)	-0.03 (-0.4, 0.3)	0.06	0.57	0.20
Head circumference, cm	36.4 (36.2, 36.6)	36.5 (35.9, 37.0)	36.5 (35.9, 37.0)	36.3 (36.1, 36.6)	36.7 (36.2, 37.2)	0.95	0.40	0.43
Head circumference z score	0.1 (-0.02, 0.3)	0.2 (-0.3, 0.6)	0.3 (-0.1, 0.7)	-0.03 (-0.2, 0.2)	0.3 (0.01, 0.7)	0.71	0.13	0.43
Serum 25(OH)D, nmol/L	54.7 (51.9, 57.5)	45.1 (40.3, 49.9)	42.3 (34.5, 50.1)	61.9 (57.9, 65.9)	54.5 (49.3, 59.7)	<0.0001	0.08	0.42

Skin tone[4], n (%)								
F I-III	110 (77.5)	17 (70.8)	14 (66.7)	59 (85.5)	20 (71.4)	<0.0001	<0.0001	0.04
F IV-VI	32 (22.5)	7 (29.2)	7 (33.3)	10 (14.5)	8 (28.6)	0.48	0.72	0.75

[1]Data are mean (lower and upper 95% confidence limits) or n (%); [2]Data were compared using a linear fixed effects model for continuous variables followed by post hoc Tukey's tests with Tukey-Kramer adjustment for multiple comparisons; and Chi-square or Fisher exact tests for categorical variables (using frequency procedure to create 3-way crosstabulation tables; categorical variables were used in the model to stratify the crosstabulation tables followed by the last two variables: maternal 25(OH)D*pre-pregnancy BMI, creating two 2-way tables of 25(OH)D and pre-pregnancy BMI, for each level of the categorical variables); [3]vitamin D synthesizing: April 1st-October 31st or vitamin D non-synthesizing: November 1st-March 31st; [4]classified based on Fitzpatrick descriptions: F I-III (light) and F IV-VI (dark) (240, 241). Abbreviations: 25(OH)D: 25-hydroxyvitamin D; F: Fitzpatrick; UVB: ultraviolet B.

Table 4.3 Correlates of neonatal body fat mass

Fixed effects model	Regression coefficients	95% Confidence intervals	P-value	Adjusted P-value
Neonatal whole-body fat mass g (R^2 0.38, R^2_{adj} 0.37)[1]				
Sex[2] of infant (Ref: female)	-119.85	-219.24, -20.45	0.02	
Gestational age at birth, wk	-15.38	-67.13, 36.38	0.56	
Infant age, mo	435.35	187.62, 683.09	0.001	
Infant length, cm	58.80	29.99, 87.57	<0.0001	
UVB period at birth[3] (Ref: non-synthesizing period)	105.28	8.94, 201.63	0.03	
Gestational weight gain, kg	6.57	-1.61, 14.76	0.11	
Maternal pre-pregnancy BMI[4] (Ref: <25 kg/m^2)	-45.26	-173.54, 83.02	0.12	
Maternal 25(OH)D[5] (Ref: ≥50 nmol/L)	-40.57	-176.04, 94.91	0.09	
BMI*25(OH)D interaction (pairwise comparisons)			0.02	
BMI≥25, 25(OH)D<50 vs BMI≥25, 25(OH)D≥50	220.40	56.19, 384.60	0.009	0.04
BMI≥25, 25(OH)D<50 vs BMI<25, 25(OH)D<50	215.70	44.73, 386.68	0.01	0.07
BMI≥25, 25(OH)D<50 vs BMI<25, 25(OH)D≥50	175.14	28.38, 321.89	0.02	0.09
BMI≥25, 25(OH)D≥50 vs BMI<25, 25(OH)D<50	-4.70	-161.54, 152.15	0.95	0.99
BMI≥25, 25(OH)D≥50 vs BMI<25, 25(OH)D≥50	-45.26	-173.54, 83.02	0.49	0.90
BMI<25, 25(OH)D<50 vs BMI<25, 25(OH)D≥50	-40.57	-176.04, 94.91	0.55	0.93

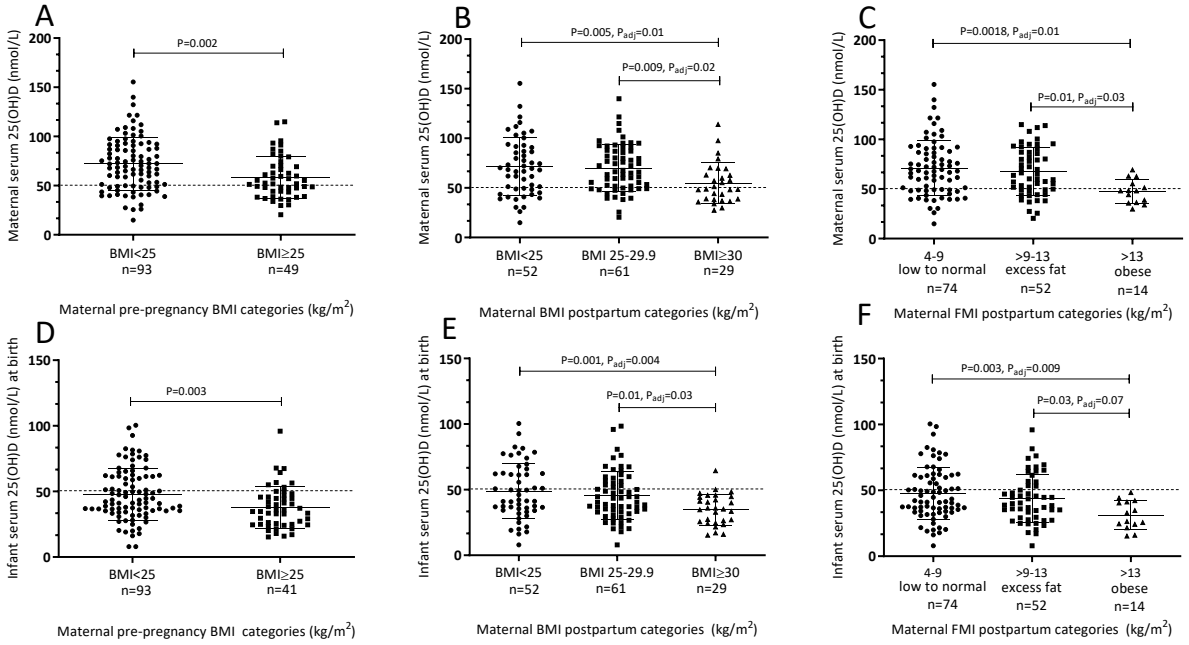
[1]Data were compared using a linear fixed effects model for continuous variables followed by post hoc Tukey's tests with Tukey-Kramer adjustment for multiple comparisons; [2]Sex of infant (male vs. female); [3]UVB period (April 1st-October 31st or November 1st-March 31st); [4]Maternal pre-pregnancy BMI (BMI <25 kg/m^2 or BMI ≥25 kg/m^2); [5]Maternal serum 25(OH)D (≥ or <50 nmol/L). Abbreviations: 25(OH)D: 25-hydroxyvitamin D; BMI: body mass index; UVB: ultraviolet B.

Table 4.4 Correlates of neonatal body lean mass

Fixed effects model	Regression coefficients	95% Confidence intervals	P-value	Adjusted P-value
Neonatal whole-body lean mass g (R^2 0.53, R^2_{adj} 0.52)[1]				
Sex[2] of infant (Ref: female)	174.52	73.41, 275.64	0.0009	
Gestational age at birth, wk	32.29	-20.36, 84.94	0.23	
Infant age, mo	294.98	42.95, 547.00	0.02	
Infant length, cm	102.26	72.97, 131.55	<0.0001	
UVB period[3] (Ref: non-synthesizing period)	-68.86	-166.88, 29.16	0.17	
Gestational weight gain, kg	-4.56	-12.89, 3.76	0.28	
Maternal pre-pregnancy BMI[4] (Ref: <25 kg/m^2)	124.67	-5.83, 255.17	0.06	
Maternal 25(OH)D[5] (Ref: ≥50 nmol/L)	55.67	-82.15, 193.49	0.43	
Maternal pre-pregnancy BMI*25(OH)D			0.04	
BMI≥25, 25(OH)D<50 vs BMI≥25, 25(OH)D≥50	-164.58	-384.34, 55.19	0.05	0.21
BMI≥25, 25(OH)D<50 vs BMI<25, 25(OH)D<50	-95.57	-324.39, 133.24	0.28	0.70
BMI≥25, 25(OH)D<50 vs BMI<25, 25(OH)D≥50	-39.91	-236.31, 156.50	0.60	0.95
BMI≥25, 25(OH)D≥50 vs BMI<25, 25(OH)D<50	69.00	-140.90, 278.91	0.39	0.83
BMI≥25, 25(OH)D≥50 vs BMI<25, 25(OH)D≥50	124.67	-47.01, 296.35	0.06	0.24
BMI<25, 25(OH)D<50 vs BMI<25, 25(OH)D≥50	55.67	-125.64, 236.97	0.43	0.85

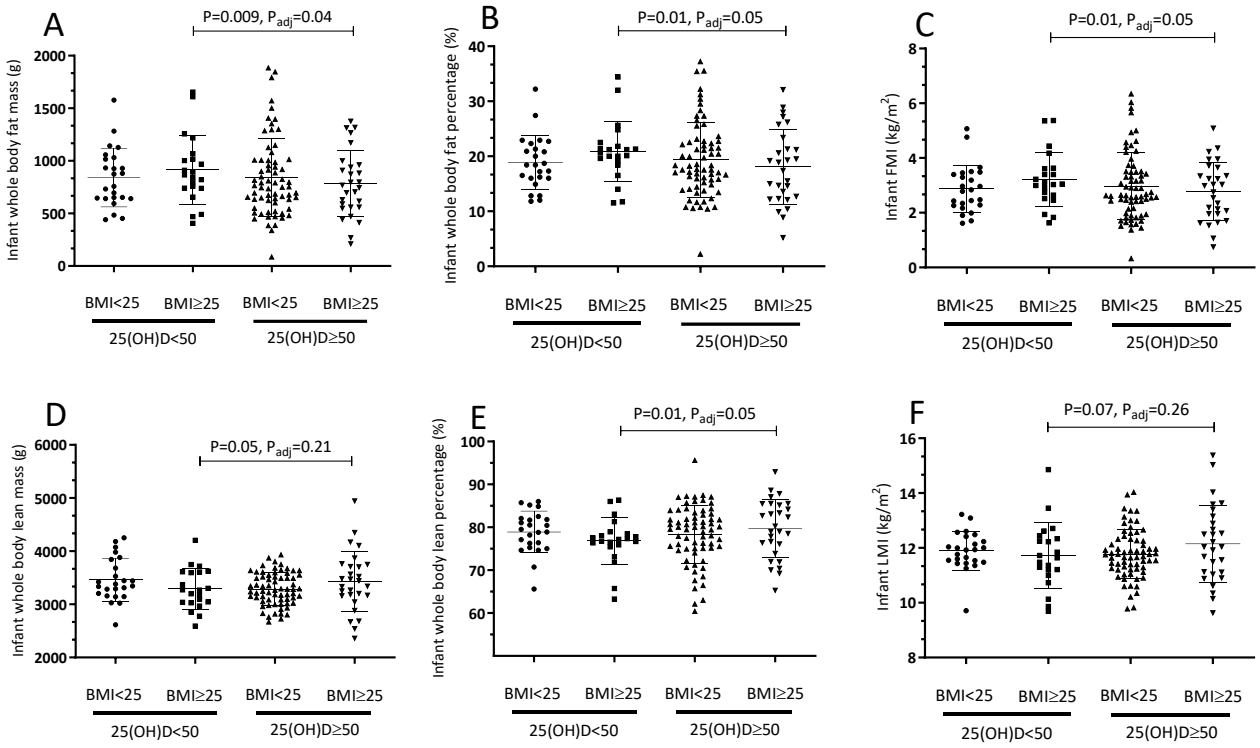
[1]Linear fixed effects model; [2]Sex of infant (male vs. female); [3]UVB period (April 1st-October 31st or November 1st-March 31st); [4]Maternal pre-pregnancy BMI (BMI<25 or BMI>25 kg/m^2); [5]maternal serum 25(OH)D (≥ or <50 nmol/L. Abbreviations: 25(OH)D: 25-hydroxyvitamin D; BMI: body mass index; UVB: ultraviolet B.

Figure 4.1 Maternal and neonatal serum 25(OH)D according to maternal BMI and FMI categories



Serum 25(OH)D concentrations of A) mothers according to pre-pregnancy BMI categories (healthy: BMI <25 or overweight/obese: BMI ≥25 kg/m^2), B) Serum 25(OH)D concentrations of mothers according to postpartum BMI categories (healthy: BMI <25, overweight: BMI 25-29.9, obese: BMI ≥ 30 kg/m^2, C) mothers according to their postpartum fat mass index (FMI) categories (low to normal: 4-9, excess fat: >9-13, and obese: >13 kg/m^2). Serum 25(OH)D concentrations of infants at birth according to mothers, D) pre-pregnancy BMI categories, E) postnatal BMI categories and, F) FMI categories. Data were compared using a linear fixed effects model, maternal pre-pregnancy, postpartum BMI and FMI as categorical fixed effects followed by *post hoc* Tukey's tests with Tukey-Kramer adjustment for multiple comparisons. Data are mean ± SD.

Figure 4.2 The interaction effect of maternal pre-pregnancy BMI and maternal 25(OH)D status with neonatal body composition



The interaction effect of maternal pre-pregnancy BMI and maternal 25(OH)D status with neonatal: A) whole-body fat mass, B) whole-body fat percentage, C) fat mass index (FMI), D) whole-body lean mass, E) whole-body lean percentage, and F) lean mass index (LMI). Data were compared using a linear fixed effects model, maternal pre-pregnancy BMI and 25(OH)D interaction as a categorical fixed effect followed by *post hoc* Tukey's tests with Tukey-Kramer adjustment for multiple comparisons. Data are mean ± SD.

Supplemental Table 4.1 Maternal serum 25(OH)D postpartum based on maternal characteristics

Explanatory variables[1]	***n***	**Maternal serum 25(OH)D**[3]	**Adjusted P-value**[2]
Pre-pregnancy BMI, kg/m^2			0.003
<25	93	72.2^{a} (66.6, 77.8)	
25.0-29.9	34	61.1^{ab} (53.1, 69.0)	
≥30	15	50.0^{bc} (41.6, 58.3)	
Postpartum BMI, kg/m^2			0.007
<25	52	71.4^{a} (63.2, 79.6)	
25.0-29.9	61	70.0^{a} (63.9, 76.1)	
≥30	29	53.9^{b} (46.1, 61.6)	
Postpartum FMI, kg/m^2			0.02
Low to normal: 4-9	74	71.1^{a} (64.6, 77.5)	
Excess fat: >9-13	52	67.5^{a} (60.7, 74.3)	
Obese: >13	15	47.5^{b} (40.6, 54.4)	
Gestational weight gain, kg			0.41
Inadequate	35	63.0^{a} (54.8, 71.1)	
Adequate	47	71.9^{a} (64.1, 79.7)	
Excess	59	66.1^{a} (59.1, 73.1)	
Exercise prior conceiving			0.03
Yes	95	72.3 (67.1, 77.4)	
No	47	56.9 (49.7, 64.2)	
Exercise during pregnancy			0.007
Yes	70	74.9 (68.5, 81.3)	
No	72	59.7 (54.2, 65.1)	
Multivitamin use prior conceiving			0.72
Yes	65	71.4 (65.2, 77.6)	
No	77	63.6 (57.6, 69.6)	
Multivitamin use during pregnancy			0.20
Yes	131	67.7 (63.3, 72.3)	
No	11	59.2 (40.5, 77.9)	
Canadian-born			0.006
Yes	90	72.2 (67.4, 78.0)	
No	52	58.2 (51.2, 65.2)	
Self-reported population group			0.0002
White	82	74.2 (68.7, 79.6)	
All other groups	60	58.1 (51.7, 64.6)	
UVB period at delivery			0.11
Vitamin D synthesizing	83	70.1 (64.7, 75.5)	
Vitamin D non-synthesizing	59	63.0 (55.9, 70.1)	
Parity			0.17
Primiparous (1)	44	62.3 (54.5, 70.0)	
Multiparous (≥2)	98	69.3 (64.1, 74.6)	
Education			0.16
Elementary/High school	13	55.2^{a} (40.3, 70.1)	
College/vocational school	30	71.5^{a} (60.4, 82.6)	
University degree	99	67.8^{a} (62.8, 72.7)	
Family income, CAD			0.35
≥70,000	80	71.3^{a} (65.3, 77.3)	
<70,000	41	61.3^{a} (54.1, 68.5)	
Refused to answer	21	62.9^{a} (50.6, 75.2)	

[1]Linear fixed effects model, additional fixed effects: maternal age; [2]adjusted for Tukey-Kramer post-hoc testing; [3]data are mean (lower and upper 95% confidence limits). The means with different superscript letters a, b, and c are significantly different. Abbreviations: 25(OH)D: 25-hydroxyvitamin D; BMI: body mass index; CAD: Canadian dollar; F: Fitzpatrick; FMI: fat mass index; UVB: ultraviolet B.

Supplemental Table 4.2 Neonatal serum 25(OH)D at birth based on maternal characteristics

Explanatory variables[1]	***n***	**Neonatal serum 25(OH)D**[3]	**Adjusted P-value**[2]
Maternal 25(OH)D, nmol/L			<0.0001
≥50	97	52.1 (48.6, 55.6)	
<50	45	28.0 (25.2, 30.7)	
Maternal pre-pregnancy BMI, kg/m^2			0.004
<25	93	47.8^{a} (43.8, 51.9)	
25.0-29.9	34	40.1^{ab} (34.5, 48.9)	
≥30	15	31.2^{bc} (25.0, 37.4)	
Maternal postpartum FMI, kg/m^2			0.02
Low to normal: 4-9	74	47.5^{a} (43.0, 52.1)	
Excess fat: >9-13	52	43.8^{a} (38.7, 50.0)	
Obese: >13	14	31.2^{b} (30.2, 38.9)	
Gestational weight gain, kg			0.47
Inadequate	35	40.6^{a} (34.4, 46.8)	
Adequate	47	48.3^{a} (42.4, 54.1)	
Excess	59	43.7^{a} (38.8, 48.5)	
Infant sex			0.77
Male	83	43.6 (39.6, 47.6)	
Female	59	45.7 (40.5, 50.9)	
Skin tone			0.06
F I-III	110	46.9 (43.3, 50.6)	
F IV-VI	32	36.0 (30.2, 41.8)	
UVB period at birth			0.02
Synthesizing period	83	46.9 (42.8, 51.0)	
Non-synthesizing period	59	41.1 (36.2, 45.9)	
Maternal exercise prior conceiving			0.11
Yes	95	48.1 (44.1, 52.1)	
No	47	37.2 (32.6, 41.7)	
Maternal exercise during pregnancy			0.03
Yes	70	48.5 (44.1, 52.9)	
No	72	40.5 (36.1, 44.9)	
Maternal supplement use prior conceiving			0.38
Yes	65	48.7 (43.9, 53.5)	
No	77	40.9 (36.8, 45.0)	
Maternal supplement use during pregnancy			0.31
Yes	131	44.9 (41.7, 48.1)	
No	11	39.2 (22.5, 56.0)	
Canadian-born			0.0003
Yes	90	48.7 (44.7, 52.7)	
No	52	37.1 (32.4, 41.9)	
Self-reported population group			<0.0001
White	37.0	49.9 (45.7, 54.1)	
All other groups		37.0 (32.9, 41.2)	
Education			0.18
Elementary/High school	13	35.2^{a} (24.8, 45.5)	
College/vocational school	30	45.0 ^{a} (37.4, 52.6)	
University degree	99	45.5 ^{a} (41.8, 49.3)	
Family income, CAD			0.03
≥70,000	80	48.0^{a} (43.6, 52.5)	
<70,000	41	38.4^{b} (33.4, 43.4)	
Refused to answer	21	42.7^{ab} (34.3, 51.2)	

[1]Linear fixed effects model, additional fixed effect: gestational age; [2]adjusted for Tukey post-hoc testing; [3]data are mean (lower and upper 95% confidence limits). The means with different superscript letters a, b, and c are

significantly different. Abbreviation: 25(OH)D: 25-hydroxyvitamin D; BMI: body mass index; CAD: Canadian dollar; F: Fitzpatrick; FMI: fat mass index; UVB: ultraviolet B.

Supplemental Table 4.3 Correlates of neonatal body fat mass

Fixed effects model	Regression coefficients	95% Confidence intervals	P-value	Adjusted P-value
Neonatal whole-body fat mass g (R^2 0.38, R^2_{adj} 0.37)[1]				
Sex[2] of infant (Ref: female)	-119.85	-219.24, -20.45	0.02	
Gestational age at birth, wk	-15.38	-67.13, 36.38	0.56	
Infant age, mo	435.35	187.62, 683.09	0.001	
Infant length, cm	58.80	29.99, 87.57	<0.0001	
UVB period at birth[3] (Ref: non-synthesizing period)	105.28	8.94, 201.63	0.03	
Gestational weight gain, kg	6.57	-1.61, 14.76	0.11	
Maternal pre-pregnancy BMI[4] (Ref: <25 kg/m^2)	-45.26	-173.54, 83.02	0.12	
Maternal 25(OH)D[5] (Ref: ≥50 nmol/L)	-40.57	-176.04, 94.91	0.09	
BMI*25(OH)D interaction (pairwise comparisons)			0.02	
BMI≥25, 25(OH)D<50 vs BMI≥25, 25(OH)D≥50	220.40	56.19, 384.60	0.009	0.04
BMI≥25, 25(OH)D<50 vs BMI<25, 25(OH)D<50	215.70	44.73, 386.68	0.01	0.07
BMI≥25, 25(OH)D<50 vs BMI<25, 25(OH)D≥50	175.14	28.38, 321.89	0.02	0.09
BMI≥25, 25(OH)D≥50 vs BMI<25, 25(OH)D<50	-4.70	-161.54, 152.15	0.95	0.99
BMI≥25, 25(OH)D≥50 vs BMI<25, 25(OH)D≥50	-45.26	-173.54, 83.02	0.49	0.90
BMI<25, 25(OH)D<50 vs BMI<25, 25(OH)D≥50	-40.57	-176.04, 94.91	0.55	0.93
Neonatal fat percentage % (R^2 0.26, R^2_{adj} 0.25)				
Sex of infant (Ref: female)	-3.34	-5.40, -1.36	0.002	
Gestational age at birth, wk	-0.37	-1.43, 0.68	0.49	
Infant age, mo	5.97	0.91, 11.0	0.02	
Infant length, cm	0.67	0.08, 1.26	0.03	
UVB period at birth (Ref: non-synthesizing period)	2.04	0.08, 4.00	0.04	
Gestational weight gain, kg	0.14	-0.03, 0.31	0.10	
Maternal pre-pregnancy BMI (Ref: <25 kg/m^2)	-1.02	-3.64, 1.60	0.44	
Maternal 25(OH)D (Ref: ≥50 nmol/L)	-0.89	-3.65, 1.88	0.53	
BMI*25(OH)D (pairwise comparisons)				0.02
BMI≥25, 25(OH)D<50 vs BMI≥25, 25(OH)D≥50	4.38	-0.03, 8.79	0.01	0.05

BMI≥25, 25(OH)D<50 vs BMI<25, 25(OH)D<50	4.25	-0.34, 8.83	0.02	0.08
BMI≥25, 25(OH)D<50 vs BMI<25, 25(OH)D≥50	3.36	-0.58, 7.30	0.03	0.12
BMI≥25, 25(OH)D≥50 vs BMI<25, 25(OH)D<50	-0.13	-4.34, 4.08	0.93	0.99
BMI≥25, 25(OH)D≥50 vs BMI<25, 25(OH)D≥50	-0.89	-4.46, 2.43	0.44	0.87
BMI<25, 25(OH)D<50 vs BMI<25, 25(OH)D≥50	-3.34	-4.5, 2.75	0.53	0.92
Neonatal fat mass index kg/m^2 (R^2 0.53, R^2_{adj} 0.52)				
Sex of infant (Ref: female)	-0.23	-0.42, -0.05	0.02	
Gestational age at birth, wk	-0.03	-0.12, 0.07	0.12	
Infant age, mo	1.81	0.34, 1.27	0.001	
Infant length, cm	0.08	0.03, 0.14	0.003	
UVB period at birth (Ref: non-synthesizing period)	0.19	0.01, 0.37	0.04	
Gestational weight gain, kg	0.01	-0.003, 0.03	0.12	
Maternal pre-pregnancy BMI (Ref: ≥25 kg/m^2)	-0.08	-0.32, 0.16	0.51	
Maternal 25(OH)D (Ref: ≥50 nmol/L)	-0.08	-0.33, 0.18	0.55	
BMI*25(OH)D (pairwise comparisons)				0.02
BMI≥25, 25(OH)D<50 vs BMI≥25, 25(OH)D≥50	0.40	-0.0001, 0.81	0.01	0.05
BMI≥25, 25(OH)D<50 vs BMI<25, 25(OH)D<50	0.40	-0.02, 0.82	0.01	0.07
BMI≥25, 25(OH)D<50 vs BMI<25, 25(OH)D≥50	0.32	-0.04, 0.68	0.02	0.10
BMI≥25, 25(OH)D≥50 vs BMI<25, 25(OH)D<50	-0.002	-0.39, 0.38	0.99	1.00
BMI≥25, 25(OH)D≥50 vs BMI<25, 25(OH)D≥50	-0.08	-0.39, 0.23	0.51	0.91
BMI<25, 25(OH)D<50 vs BMI<25, 25(OH)D≥50	-0.08	-0.41, 0.26	0.55	0.93

[1]Data were compared using a linear fixed effects model for continuous variables followed by post hoc Tukey's tests with Tukey-Kramer adjustment for multiple comparisons; [2]Sex of infant (male vs. female); [3]UVB period (April 1st-October 31st or November 1st-March 31st); [4]Maternal pre-pregnancy BMI (BMI <25 kg/m^2 or BMI ≥25 kg/m^2); [5]maternal serum 25(OH)D (≥ or <50 nmol/L). Abbreviations: 25(OH)D: 25-hydroxyvitamin D; BMI: body mass index; UVB: ultraviolet B.

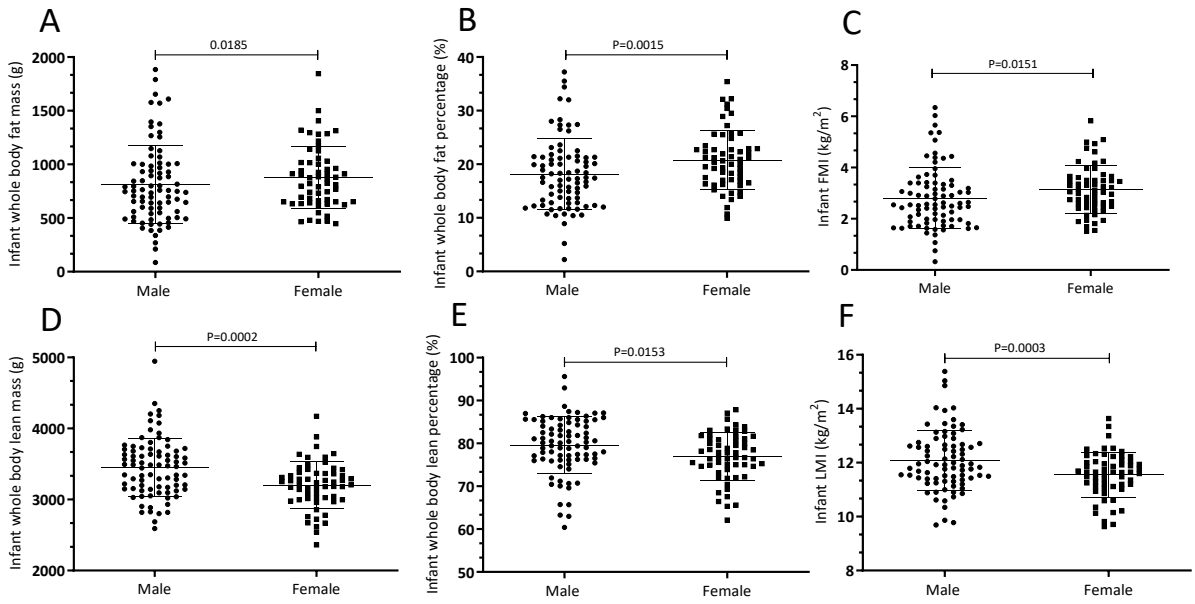
Supplemental Table 4.4 Correlates of neonatal body lean mass

Fixed effects model	Regression coefficients	95% Confidence intervals	P-value	Adjusted P-value
Neonatal whole-body lean mass g (R^2 0.53, R^2_{adj} 0.52)[1]				
Sex[2] of infant (Ref: female)	174.52	73.41, 275.64	0.0009	
Gestational age at birth, (wk)	32.29	-20.36, 84.94	0.23	
Infant age, (mo)	294.98	42.95, 547.00	0.02	
Infant length, (cm)	102.26	72.97, 131.55	<0.0001	
UVB period[3] (Ref: non-synthesizing period)	-68.86	-166.88, 29.16	0.17	
Gestational weight gain, (kg)	-4.56	-12.89, 3.76	0.28	
Maternal pre-pregnancy BMI[4] (Ref: <25 kg/m^2)	124.67	-5.83, 255.17	0.06	
Maternal 25(OH)D[5] (Ref: ≥50 nmol/L)	55.67	-82.15, 193.49	0.43	
Maternal pre-pregnancy BMI*25(OH)D			0.04	
BMI≥25, 25(OH)D<50 vs BMI≥25, 25(OH)D≥50	-164.58	-384.34, 55.19	0.05	0.21
BMI≥25, 25(OH)D<50 vs BMI<25, 25(OH)D<50	-95.57	-324.39, 133.24	0.28	0.70
BMI≥25, 25(OH)D<50 vs BMI<25, 25(OH)D≥50	-39.91	-236.31, 156.50	0.60	0.95
BMI≥25, 25(OH)D≥50 vs BMI<25, 25(OH)D<50	69.00	-140.90, 278.91	0.39	0.83
BMI≥25, 25(OH)D≥50 vs BMI<25, 25(OH)D≥50	124.67	-47.01, 296.35	0.06	0.24
BMI<25, 25(OH)D<50 vs BMI<25, 25(OH)D≥50	55.67	-125.64, 236.97	0.43	0.85
Neonatal lean percentage % (R^2 0.03, R^2_{adj} 0.03)				
Sex of infant (Ref: female)	3.32	1.28, 5.35	0.002	
Gestational age at birth, (wk)	0.34	-0.72, 1.40	0.52	
Infant age, (mo)	-5.78	-10.85, -0.70	0.03	
Infant length, (cm)	-0.65	-1.24, -0.06	0.03	
UVB period (Ref: non-synthesizing period)	-2.0108	-3.98, -0.04	0.0459	
Gestational weight gain, (kg)	-0.15	-0.32, 0.02	0.08	
Maternal pre-pregnancy BMI (Ref: <25 kg/m^2)	1.00	-1.62, 3.63	0.45	
Maternal 25(OH)D (Ref: ≥50 nmol/L)	0.85	-1.92, 3.62	0.54	
Maternal pre-pregnancy BMI*25(OH)D			0.02	
BMI≥25, 25(OH)D<50 vs BMI≥25, 25(OH)D≥50	-4.4178	-8.84, 0.01	0.01	0.05

BMI≥25, 25(OH)D<50 vs BMI<25, 25(OH)D<50	-4.2678	-8.88, 0.34	0.02	0.08
BMI≥25, 25(OH)D<50 vs BMI<25, 25(OH)D≥50	-3.4141	-7.37, 0.54	0.03	0.12
BMI≥25, 25(OH)D≥50 vs BMI<25, 25(OH)D<50	0.1500	-4.08, 4.38	0.93	0.99
BMI≥25, 25(OH)D≥50 vs BMI<25, 25(OH)D≥50	1.0037	-2.45, 4.46	0.45	0.87
BMI<25, 25(OH)D<50 vs BMI<25, 25(OH)D≥50	0.8537	-2.80, 4.50	0.54	0.93
Neonatal lean mass index kg/m^2 (R^2 0.13, R^2_{adj} 0.12)				
Sex of infant (Ref: female)	0.33	0.14, 0.52	0.001	
Gestational age at birth, (wk)	0.06	-0.04, 0.16	0.24	
Infant age, (mo)	0.56	0.09, 1.03	0.02	
UVB period (Ref: non-synthesizing period)	-0.12	-0.31, 0.06	0.19	
Gestational weight gain, (kg)	-0.01	-0.02, 0.01	0.32	
Maternal pre-pregnancy BMI (Ref: <25 kg/m^2)	0.22	-0.02, 0.46	0.08	
Maternal 25(OH)D (Ref: ≥50 nmol/L)	0.09	-0.16, 0.35	0.47	
Maternal pre-pregnancy BMI*25(OH)D			0.06	
BMI≥25, 25(OH)D<50 vs BMI≥25, 25(OH)D≥50	-0.29	-0.70, 0.12	0.07	0.26
BMI≥25, 25(OH)D<50 vs BMI<25, 25(OH)D<50	-0.16	-0.59, 0.26	0.32	0.75
BMI≥25, 25(OH)D<50 vs BMI<25, 25(OH)D≥50	-0.07	-0.44, 0.30	0.62	0.96
BMI≥25, 25(OH)D≥50 vs BMI<25, 25(OH)D<50	0.13	-0.27, 0.52	0.40	0.84
BMI≥25, 25(OH)D≥50 vs BMI<25, 25(OH)D≥50	0.22	-0.10, 0.54	0.08	0.29
BMI<25, 25(OH)D<50 vs BMI<25, 25(OH)D≥50	0.09	-0.25, 0.43	0.47	0.89

[1]Linear fixed effects model; [2]Sex of infant (male vs. female); [3]UVB period (April 1st-October 31st or November 1st - March 31st); [4]Maternal pre-pregnancy BMI (BMI<25 or BMI>25 kg/m^2); [5]maternal serum 25(OH)D (≥ or <50 nmol/L. Abbreviations: 25(OH)D: 25-hydroxyvitamin D; BMI: body mass index; UVB: ultraviolet B.

Supplemental Figure 4.1 Sex dimorphism in neonatal body composition



Infant sex differences in A) whole body fat mass B) whole body percentage fat mass, and C) fat mass index (FMI), D) whole body lean mass E) whole body percentage lean mass, and F) lean mass index (LMI). Data are mean ± SD; n= 83 male, n= 59 female. Data was compared using a linear fixed effects model for continuous variables (body composition) as outcome variables and sex as fixed effect, additional fixed factors included in the model were: GA, infant age at postpartum visit, length, gestational weight gain, and the interaction effect of maternal pre-pregnancy BMI and 25(OH)D concentration followed by *post hoc* Tukey's tests with Tukey-Kramer adjustment for multiple comparisons.

BRIDGE STATEMENT 3

The findings from Chapter 4 showed that in healthy term-born neonates of AGA born to mothers of all BMI ranges, maternal 25(OH)D concentration is an influential factor in vitamin D status at birth and through the neonatal period. Compared to neonates born to mothers with elevated BMI preconception and sufficient vitamin D status, intrauterine exposure to both lower 25(OH)D concentration and maternal overweight/obesity was associated with increased whole body fat mass in neonates. The results of this study demonstrate that the early-life programming of adipose tissue appears to be mediated by maternal vitamin D status and thus suggest a potential protective role for vitamin D in resetting the adiposity trajectory early in neonatal period.

Whether improvements in vitamin D status through interventions early in life would overcome the combined effects of exposures to maternal adiposity and low vitamin D status *in utero* and result in a leaner body phenotype has not been tested in a controlled manner. Therefore, the research study in Chapter 5 tested whether correction of compromised neonatal vitamin D status using the standard of care, or 1000 IU/d of vitamin D would normalize body composition and result in a leaner body composition during infancy in a double-blinded randomized parallel controlled group trial.

CHAPTER 5: RESEARCH PAPER 3

Correction of neonatal vitamin D status using 1000 IU/d of vitamin D increased lean body mass by 12 months of age compared to 400 IU/d: a randomized controlled trial

Maryam Razaghi[1], Nathalie Gharibeh[1], Catherine A. Vanstone[1], Olusola F. Sotunde[1], Ali Khamessan[3], Shu Qin Wei[4], Dayre McNally[5], Frank Rauch[6], Glenville Jones[7], Sarah Kimmins[2], Hope A. Weiler[1,8]

[1]School of Human Nutrition, McGill University, Ste-Anne-de-Bellevue, Québec.

[2]Animal Sciences, Faculty of Agricultural & Environmental Sciences, McGill University, Ste-Anne-de-Bellevue, Québec.

[3]Europharm International Canada Inc, Montréal, Québec.

[4]Institut national de santé publique du Québec, Montréal, Québec.

[5]Department of Pediatrics, Children's Hospital of Eastern Ontario, University of Ottawa, Ottawa, Ontario.

[6]Shriners Hospital for Children, Montréal, Québec.

[7]Department of Biomedical and Molecular Sciences, Queen's University, Kingston, Ontario.

[8]Nutrition Research Division, Bureau of Nutritional Sciences, Food Directorate, Health Products and Food Branch, Health Canada, Ottawa, Ontario.

Abstract

Background: Intrauterine exposure to maternal vitamin D status <50 nmol/L of serum 25-hydroxyvitamin D (25(OH)D) may adversely impact infant body composition. Whether postnatal interventions can reprogram for a leaner body phenotype is unknown.

Objective: The primary objective was to test whether 1000 IU/d of supplemental vitamin D (vs. 400 IU/d) improves lean mass in infants born with serum 25(OH)D <50 nmol/L.

Design: Healthy term breastfed infants (Montréal, Canada, March 2016-2019) were assessed for serum 25(OH)D (immunoassay) 24-36 h postpartum. Infants with serum 25(OH)D <50 nmol/L were eligible for the trial and randomized at baseline (1 month postpartum) to 400 (29 males, 20 females) or 1000 IU/d (29 males, 20 females) vitamin D until 12 months. Infants (23 males, 18 females) with 25(OH)D ≥50 nmol/L (sufficient) formed a non-randomized reference group provided 400 IU/d. Anthropometry, body composition (dual-energy x-ray absorptiometry) and serum 25(OH)D concentrations were measured at 1, 3, 6 and 12 months.

Results: At baseline, mean serum 25(OH)D concentration in infants allocated to the 400 and 1000 IU/d vitamin D groups were 45.8 ± 14.1 and 47.6 ± 13.4, respectively, and the reference group 69.2 ± 16.4 nmol/L. Serum 25(OH)D concentration increased on average to ≥50 nmol/L in the trial groups at 3 to 12 months. Lean mass varied differently among groups over time; at 12 months it was higher in the 1000 IU/d vitamin D group compared to the 400 IU/d group (7013 ± 904.6 vs. 6690.4 ± 1121.7 g, P=0.0428), but not the reference (6715 ± 784.6 g, P=0.19). Whole body fat mass was not different among groups over time.

Conclusion: Vitamin D supplementation (400 or 1000 IU/d) during infancy readily corrects vitamin D status, whereas 1000 IU/d modestly increases lean mass by 12 months of age. The long-term implications require further research.

Keywords: Infant, vitamin D status, vitamin D supplementation, lean mass, randomized controlled trial

5.1 Introduction

Vitamin D is required for growth and development (73), with a well-established role in bone health through calcium and phosphate homeostasis (277). There is an emerging body of evidence beyond bone indicating that vitamin D status is implicated in programming of body composition (4, 9, 75). Recent evidence from large pregnancy cohort studies in India, New Zealand, and United Kingdom (20-22) suggest that intrauterine exposure to insufficient vitamin D status, defined as serum 25-hydroxyvitamin D (25(OH)D) <50 nmol/L, associates with elevated percentage fat mass and lower percentage lean mass in the offspring at 4 to 9.5 years of age. Additionally in the Netherlands, maternal severe vitamin D deficiency, defined as serum 25(OH)D <25 nmol/L, associates with lower percentage lean mass in children at 6 years of age (23). This implies that fetal exposure to insufficient maternal vitamin D status may have a long-lasting impact on body composition. According to the developmental origins of pediatric obesity, the etiology of excessive adiposity and its metabolic consequences can begin very early in life (7, 8), suggesting that early life interventions may reprogram body composition.

A recent systematic review and meta-analysis of randomised controlled trials on the effects of vitamin D supplementation early in life on children's body composition proposes that vitamin D supplementation in pregnancy and early during the neonatal period has a consistent trend to decrease adiposity early in life (74). The Institute of Medicine (IOM) expert committee set an Adequate Intake (AI) value for vitamin D at 400 IU (10 μg)/day across infancy to maintain vitamin D status in the range of 40 to 50 nmol/L of 25(OH)D (10) and to support bone health outcomes. To the best of our knowledge, the only dose-response study reporting body composition according to vitamin D supplementation is in infants with plasma 25(OH)D on average ≥50 nmol/L at baseline (2). Plasma 25(OH)D concentrations were positively associated

with higher percentage lean body mass and lower fat mass at 12 months (4). In Canada, insufficient vitamin D status is prevalent among newborns (17, 18, 278). For instance, based on a large pregnancy cohort study in Quebec City, 24% of infants had vitamin D insufficiency (17). To date, no study has determined a dosage of postnatal vitamin D supplementation that mitigates vitamin D insufficiency and results in a lean body phenotype to overcome exposures to low maternal-fetal transfer of vitamin D *in utero*.

The primary objective of this trial was to test whether correction of insufficient vitamin D status early in the neonatal period improves whole body lean mass in infancy. It was hypothesized that neonates born with insufficient vitamin D status and provided the standard of care, 400 IU/d vitamin D_3, would have lower lean mass and lean mass accretion by 3 months of age and thereafter by 12 months compared to infants provided with higher dosage of 1000 IU/d vitamin D_3. A secondary objective tested whether correction of insufficient vitamin D status early in infancy using 1000 IU/d of vitamin D would also improve infant growth, fat mass accretion rate. Additionally, we compared insulin-like growth factor-1 (IGF-1) and its main carrier protein (IGFBP-3) among groups as these have established functions in growth and development of skeletal muscle across infancy.

5.2 Methods

5.2.1 Study design and participants

This is a double-blinded, randomized, controlled parallel group trial comparing 1000 to 400 IU/d of supplemental vitamin D in infants from 1 to 12 months of age. Healthy term breastfed infants (n=139; 81 males, 58 females) were recruited at the Lakeshore General Hospital, located in greater Montréal (Québec, Canada) from March 2016 through March 2019. The inclusion criteria for the trial were healthy term singleton infants of appropriate weight for gestational age (AGA; 10th to 90th percentile, according to Canadian birth reference values (279)) born to healthy mothers who intend to breastfeed for at least 3 months. For the reference group, infants were recruited if their mothers had a pre-pregnancy body mass index (BMI) between 18.5 and 27.0 kg/m^2 to help limit maternal excess adiposity preconception as a confounding factor associated with adverse infant body composition, whereas pre-pregnancy BMI was not an exclusion criterion for infants in the trial groups. The exclusion criteria for both trials and the reference groups were maternal smoking, as well as maternal comorbidities including type 1, type 2, and gestational diabetes, hypertension, preeclampsia, malabsorption syndromes such as celiac disease, Crohn's disease, as well as taking any medication that alters vitamin D metabolism except for vitamin/mineral supplements.

Prior to discharge from hospital, infant capillary blood samples were collected by heel lance within 24-36 hours postnatally for measurement of serum 25(OH)D using a chemiluminescence immunoassay (CLIA, Liaison, Diasorin Inc.). Blood samples were collected at the same time as the routine blood collection for newborn screening (e.g., phenylketonuria). Families subsequently attended the Mary Emily Clinical Nutrition Research Unit, McGill University (greater Montréal, Québec) for a baseline visit at 1 month (0.2-1.5 mo) and were

followed at 3, 6, and 12 months of age. At the baseline visit, infants born with serum 25(OH)D <50 nmol/L (n=98) were randomized to receive either 400 or 1000 IU/d of vitamin D supplementation until 12 months of age. Randomization was stratified by infants measured skin tone using a spectrophotometer (CM-700d/600d, Konica Minolta, USA) at baseline. Infants born with 25(OH)D ≥50 nmol/L (n=41) formed a non-randomized reference group and received 400 IU/d, the standard of care, with all of the same follow-up measurements identical to the trial. Participant enrollment and assignment to the trial groups based on skin tone blocks were performed by the research team.

5.2.2 Ethics approval and trial registration

The research protocol was reviewed and approved by St. Mary's Hospital Research Ethics Committee which oversees research approvals of the Lakeshore General Hospital. Trial registration at clinicaltrials.gov was completed prior to the beginning of recruitment (NCT02563015). Prior to collection of data or blood samples at the hospital or at the baseline visit, parents provided written informed consent in either official language (English or French). The trial was also reviewed and approved by Health Canada Research Ethics Board (REB 2019-033H) and Privacy Management Division (HC-PR-2019-000024).

5.2.3 Demographic data/obstetric history and lifestyle survey

Demographic information was surveyed including maternal age, maternal self-reported population group (white/all other groups), as well as maternal education level (elementary/high school, college/vocational school, university), maternal country of birth (Canada/all other countries) and family income (<70,000, ≥70,000 Canadian dollars (CAD), or not reported) according to the median income for Canadians (280). Type of delivery (caesarean/vaginal), parity, infant gestational age at birth (GA), and birth weight were obtained from hospital records.

Weight for age and sex z-scores were calculated using the growth standards from the World Health Organization (281).

Pre-pregnancy weight and weight at delivery were extracted from medical record. Gestational weight gain was also calculated by subtracting pre-pregnancy weight from weight measured at delivery. Maternal use of vitamin supplement in 3 months prior to conception and during pregnancy were surveyed separately, along with frequency of use. Lifestyle factors surveyed included smoking history (never, past, current), alcohol consumption during pregnancy (yes/no), and physical activity 3 months prior to conception and separately during pregnancy (yes/no) along with frequency/intensity of activity. Sun exposure including the time spent outdoors between 10 am and 4 pm with hands and/or face exposed (yes/ no), and the use of sun protection factor (SPF) products including make-up creams (yes/no) in the last trimester were also surveyed according to questions adapted from the Canadian Health Measures Survey (282). In view of the potential for endogenous synthesis of vitamin D, date of birth was converted into a theoretical vitamin D synthesizing/non-synthesizing period based on solar ultraviolet B (UVB) radiation strength (April 1st-October 31st/ November 1st-March 31st) for Canada (283) and season of birth as defined by equinox and solstice dates. Study data were collected and managed using REDCap (Research Electronic Data Capture, v7.4).

5.2.4 Skin tone and UVB exposure

Skin tone of the infant was measured at the research facility by taking the average of three measurements at the inner upper arm for constitutive pigmentation (basal skin color) using a spectrophotometer (CM-700d/600d, Konica Minolta, USA). Individual typological angle (ITA^{o}) was calculated with the L* and b* values using published equations (239). Infants were classified into two skin tone types (FI-III; FIV-VI) based on Fitzpatrick descriptions (240, 241).

5.2.5 Supplements, randomization, masking, and adherence

Study products containing 400 or 1000 IU vitamin D_3 (cholecalciferol) were formulated by Euro-Pharm (Euro-Pharm International Canada Inc). The products were externally verified (Sandoz, Novartis division) to be within 5% of target and stable up to 12 months. All supplements for the trial and reference groups were provided in identical bottles (50 mL), color (brown liquid), taste (cherry flavour), smell, and texture. Each bottle had a unique code to enable tracing back to the product dosage in the case of an adverse event (e.g., 25(OH)D concentrations ≥225 nmol/L associated with hypercalcemia) without unblinding the study. Both study products were blinded to participating families and all researchers across the entire study and only unblinded after all data was double-audited.

The allocation was at the end of the initial baseline visit and parents were educated by a registered nurse on how to properly give the supplement in 1-mL/day volume using a dropper provided by the company. Randomization (allocation ratio 1:1) was set according to block sizes of 4 (2 x 400 IU and 2 x 1000 IU), using Random Allocation Software (http://mahmoodsaghaei.tripod.com/Softwares/randalloc.html) with stratification based on measured skin tone (FI-III and FIV-VI) to create two trial arms. At the baseline visit, families were provided with a vitamin D compliance calendar in order to record the number of missed dosages; this was reviewed and recorded at each follow-up visit to estimate adherence to supplementation.

5.2.6 Body composition assessment

Body composition was assessed in infants using a fan-beam dual-energy x-ray absorptiometer (DXA; APEX version 13.3:3, Hologic 4500A Discovery Series, Bedford, MA) and using infant whole-body mode. At each study visit, infants were scanned while wearing a

single light gown with no metal or plastic components and a diaper. To minimize the potential for movement artefact, infants were wrapped in a single receiving blanket, and most were scanned asleep. Output from whole-body scan included lean mass (g), fat mass (g and %), and total body mass (g). For quality control purposes, a spine phantom (Hologic phantom; No. 14774) was used and the coefficient of variation (CV%) for bone mineral content, bone mineral density, and bone area were <1% across the study. Values for lean mass and fat mass accretion (g/mo) were calculated as change in lean mass or fat mass from baseline to 3, 3 to 6, or 6 to12 months and adjusted for time between visits. Lean and fat percentage (%) were calculated using lean and fat mass (kg) divided by total mass (kg) times 100. Lean mass index (LMI) and fat mass index (FMI) were also calculated using lean and fat mass relative to length (kg/m^2).

5.2.7 Anthropometric measurements and dietary assessments

Infant nude weight was measured to the nearest gram using an electronic scale with a dynamic weighing program (Mettler-Toledo Inc., Switzerland). Crown heel length was measured to the nearest 0.1 cm using an infant length board (infantometer; O'Learly Length Boards, Ellard Instrumentation Ltd., US). Head circumference was measured to the nearest 0.1 cm using a non-stretchable tape (Perspective Enterprises, US). Weight, length and head circumference z-scores for age and sex were calculated using World Health Organization software (WHO AnthroPlus, Switzerland). Maternal anthropometric measurements included weight using a balance-beam scale (Detecto; Webb, US) to the nearest 0.1 kg while wearing light clothing and no shoes, and height to nearest 0.1 cm using a wall-mounted stadiometer (Seca Medical Scales and Measuring Systems, US). Mother's pre-pregnancy weight from the medical record and measured height at baseline were used to calculate pre-pregnancy BMI (kg/m^2). All infants were discharged from the hospital with a prescription for vitamin D supplements (400 IU/d) from their physician and

compliance surveyed at the baseline visit. At each study visit, information regarding breastfeeding status (yes/no) and the type of breastfeeding (exclusively/mixed) as well as infant's age at the introduction of solid food, and when they start crawling or walking were surveyed. In addition, infant dietary intake over the study period was assessed using 3-day diet records completed by parents after each study visit. To estimate nutrient intake from breastmilk, total number of feeds for each infant over 24 h was multiplied by the average feed volume depending on infant age (1, 3, 6, or 12 months) and based on test-weighing as previously reported (50). Nutrient intake was created using Nutritionist Pro software version 5.4.0 (Axxya Systems LLC) and the 2010b Canadian Nutrient File database (Health Canada) (284). Moreover, maternal supplement use over pregnancy was surveyed.

5.2.8 Biochemistry measurements

Capillary blood samples (0.5 ml; Capiject, Terumo Corp.) were collected at newborn screening (within 24-36 hours of birth) and thereafter at baseline (1 mo), 3, 6, and 12 months of age via infant heel/finger lance into micro tubes, one with and one without heparin (Becton Dickinson, New Jersey, US). Samples were centrifuged (4000 x g for 20 min at 6°C) in order to obtain plasma and serum. Serum that was not analyzed immediately and all plasma samples were stored at -80 °C until batch analysis at McGill University. One 5 ml venous sample was taken from mothers at the baseline visit (non-fasted state) for measurement of serum 25(OH)D. Total serum 25(OH)D was measured using an automated chemiluminescent immunoassay (25 µL; CLIA, Liaison, Diasorin Inc.). Newborn samples (n=2) with 25(OH)D below the lower limit of quantification of 10 nmol/L were assigned a value of 5 nmol/L (285); concentration at baseline and thereafter were all above 10 nmol/L. The laboratory obtained a certificate of proficiency from the Vitamin D External Quality Assessment Scheme to facilitate comparison with other laboratories. For quality assurance purposes, vitamin D control samples from the National Institute of Standards and Technology (NIST) were implemented in routine quality control measures. The inter-assay coefficient of variation for NIST972a (levels 1 to 4) was on average <10% and the accuracy was 97.4%. The inter-assay CV% for an internal laboratory control human serum sample (62.8 nmol/L) was 8.2% across all assays. In addition, in a subset of mothers and infants (n= 83) total serum 25(OH)D was in agreement (mean difference= -0.8) with liquid chromatography tandem mass spectrometry (Queen's University, Kingston, Ontario, Canada), as certified by the Vitamin D Standardization Certification Program. Whole blood ionized calcium was measured immediately after a blood draw at each follow-up visit using a portable blood gas analyzer (65 µL; ABL80 FLEX Radiometer Medical A/S, Denmark) and

values were compared to the age-specific (2.5th to 97.5th percentile) reference ranges of 1.32-1.47 mmol/L for 1 month, 1.31-1.46 mmol/L for 3 months, 1.29-1.41 mmol/L for 6 months, and 1.25-1.39 mmol/L for 12 months of age (286). Plasma samples were used to measure insulin-like growth factor 1 (IGF-1) and its binding protein 3 (IGFBP-3) using quantitative sandwich enzyme immunoassay technique (10 µl; Quantikine® ELISA R&D Systems, Inc., USA; CAT# DG100B and SG100B respectively). An internal laboratory pooled sample was implemented for quality assurance yielding intra-assay CV% of less than <8% and 7% for IGF-1 and IGFBP-3, respectively. The minimum detectable concentration of human IGF-1 and IGFBP-3 were 0.004 and 0.02 ng/mL, respectively.

5.2.9 Sample size estimation

As indicated in the trial registration, the aim was to recruit up to 74 infants per trial group in order to account for possible dropouts or missing data (no whole body scan due to movement artifact, or failure to cooperate) or the maximum sample recruited over 3 years to reflect all seasons equally. The minimum estimated sample size (n=46) per trial arm is based on the primary objective, lean mass in grams at 3 months of age and an effect size of 0.59, SD of 670 g, an allocation ratio of 1:1, power of 80% and alpha of 5%. The estimated effect size of 0.59 was based on a subgroup analysis of vitamin D dose-response trial of breastfed infants with healthy status, supplemented with 400 IU/d and increased lean mass between 1 to 3 months by 670 grams and with the mean change of 400 grams (4). We aimed to recruit a similar number of infants to the non-randomized reference group in order to help guide interpretation of the data; over the 3 years of recruitment n=41 agreed to participate.

5.2.10 Statistical analyses

Characteristics at birth and continuous data for outcome measurements are expressed as n (%) or mean ± SD, unless otherwise noted. The effect of vitamin D dosage on lean body mass was evaluated among groups (trial 400 IU/d and 1000 IU/d, and reference group) over time as the primary analysis using a linear mixed effects model (SAS PROC MIXED). This procedure provides unbiased estimates using the method of restricted/residual maximum likelihood (RELM) and uses all available data, thus missing data does not result in omission of an infant's existing data (287). In evaluating our primary outcome, differences among groups over time were tested using the existing data and without imputation. As stipulated a priori, baseline was included as a time point (as part of the outcome vector), yielding four repeated measurements (baseline, 3, 6, and 12 months). This was important since the non-randomized reference group had different inclusion criteria by design (i.e. sufficient vitamin D status at birth). The mixed effect model tested for fixed effects of group by time interaction, sex (male, female), skin tone (FI-III or FIV-VI) based on design, as well as participant characteristics, infant actual age at each visit, gravida (first child, second, or third and more), maternal and paternal age at delivery, maternal BMI preconception (<25, ≥25 kg/m^2), and family annual income (≥70,000, <70,000 CAD, or not reported). Additional variables that were considered as fixed were: self-reported population group (white, any other groups), education (elementary/high school, college/vocational school, or university), as well as breastfeeding status (yes, no). These variables did not improve the model as judged by Akaike information criterion (AIC), thus were removed from the final model using the backward elimination method. Each model included the random effect of an individual infant (ID) modeled as variance covariance, as well as a repeated statement for time using first order autoregressive or AR(1) covariance structure selected based

on the correlation matric and the lowest Akaike information criterion (287). In order to test for effects within group*time the SLICE statement was used to test for differences of least-square means followed by Tukey-Kramer post-hoc adjustment for multiple comparisons. For each model normality of the residuals was evaluated using normality tests (Kolmogorov-Smirnov and Shapiro-Wilk), the histogram and Q-Q plots. Homogeneity of variances was checked using Levene or Bartlett tests. The same model was used for the analysis of the secondary outcomes including fat mass, anthropometry, and serum biochemistry. Baseline values of the outcome measurements were not included as covariates in the model, as inclusion was not specified a priori in the protocol or statistical analysis plan (288). However, a post hoc sensitivity analysis of our primary outcome with adjustment for baseline values of whole body lean mass was conducted. Sensitivity analyses were also conducted for the trial groups and the reference group based on maternal pre-pregnancy BMI 18.5-27.0 kg/m^2 as per inclusion criterion for the reference group.

Differences in categorical data among groups over time, including the proportion meeting the sufficient cut-point of 50 nmol/L (20 ng/mL) of 25(OH)D according to the IOM definition for individuals (10), adherence to infant vitamin D supplementation (proportion of supplements taken) and breastfeeding status (yes/no) among groups, were compared at each time-point using Chi-square (χ^2) and Fisher's exact tests for proportions. All data were analyzed using Statistical Analysis System (SAS; version 9.4, SAS Institute Inc., Cary, NC). Data were interpreted according to $P<0.05$, including after adjustment for multiple comparisons where applicable.

5.3 Results

Of the mother-infant dyads screened for eligibility, 2959 were eligible. Of 866 with serum 25(OH)D concentration available and being eligible for the trial, 139 families consented to the infant vitamin D study. Ninety-eight infants with serum 25(OH)D <50 nmol/L at 24-36 h were eligible for the trial and randomized at baseline to 400 (29 males, 20 females) or 1000 IU/d (29 males, 20 females) of vitamin D until 12 months. Infants (23 males, 18 females) with 25(OH)D ≥50 nmol/L formed the non-randomized reference group provided 400 IU/d (Figure 5.1). Maternal and neonatal characteristics for the groups are shown in Table 5.1.

5.3.1 Effect of vitamin D supplementation on infant body composition and anthropometry

Lean body mass of the infants differed among groups over time (Figure 5.2A), at 12 months of age, lean body mass was higher in the 1000 IU/d trial group compared to the 400 IU/d trial group (7013 ± 905 vs. 6690 ± 1122 g, P=0.0428; 4.8% difference), but not different from the reference group (7013 ± 905 vs. 6715 ± 785 g, P=0.19). Lean body mass was also observed to be higher in male infants versus females (P<0.0001) (Supplemental Figure 5.1A, B) and in infants born to mothers with pre-pregnancy BMI ≥25 kg/m^2 versus those born to mothers with BMI <25 kg/m^2 (P=0.0222). In a sensitivity analysis according to maternal pre-pregnancy BMI (18.5-27.0 kg/m^2) as per inclusion criterion for the reference group, the interpretation was the same in terms of the difference between the trial 1000 IU/d compared to trial 400 IU/d group in lean body mass at 12 months (7049 ± 870 vs. 6448 ± 965 g, P=0.0005) and not different from the reference group (7049 ± 870 vs. 6715 ± 785 g, P=0.11). Post hoc sensitivity analyses of the primary outcome at 12 months with adjustment of baseline lean body mass values as a covariate, did not change the interpretation with respect to a difference between the trial 1000 IU/d compared to trial 400 IU/d group in lean body mass (P=0.0402) and not different from the

reference group (P=0.35). No difference was observed among groups over time for LMI and percentage lean body mass (Figure 5.2B, C).

There were no differences in fat mass, FMI, as well as fat percentage among groups at any time across the study (Figure 5.2D, E, F) with no sex differences in fat mass and percentage body fat after accounting for multiple comparison tests and adjusting for covariates (Supplemental Figure 5.1C, D). Fat mass increased over time from baseline to 6 months of age and then plateaued thereafter. Similarly, FMI and fat percentage values increased over time, but the values slightly decreased after 6 months of age.

All of the infants were healthy term and AGA and growing well. Infant weight, length, and head circumference increased in all groups over time. Weight, length, and head circumference for age and sex z-scores were within normal ranges according to the WHO growth standard. Overall, there was no interaction effects of group and time on all standard anthropometry measurements except body weight over the course of the study; body weight was higher in the 1000 IU/d group by 12 months (296.05 g, 3.0% difference; P= 0.0298) compared to the 400 IU/d group and not different from the reference group (P=0.37) (**Figure 5.3**).

5.3.2 Effect of vitamin D supplementation on infant serum 25(OH)D concentration

At birth, by design, on average the reference group had serum 25(OH)D concentration at or above 50 nmol/L and different from the pool of infants eligible for the trial (68.0 ± 13.7 nmol/L vs. 32.6 ± 10.8 nmol/L) (Figure 5.4A). Similarly, measurements done at the baseline visit showed that infants in the reference group on average had higher serum 25(OH)D concentrations compared to 400 and 1000 IU/d vitamin D supplement groups (69.2 ± 16.4 nmol/L vs. 45.8 ± 14.1 and 47.6 ± 13.4, respectively, P<0.0001). Thereafter, the trial group receiving 1000 IU/d vitamin D had higher concentrations compared to the reference group and the trial 400 IU/d at

each time from 3-12 months of the study (Figure 5.4B). Across the trial, ionized calcium was within normal limits (Figure 5.4C). At the baseline visit, in both trial 400 and 1000 IU groups, 42.9 and 40.8 % of infants had sufficient concentrations of 25(OH)D ≥50 nmol/L whereas from 3 to 12 months of the study the percentage of infants achieved the cut-point for sufficiency of vitamin D increased over time (Figure 5.4D). Only in the 1000 IU/d group did 100% of infants achieve sufficient 25(OH)D concentrations at 3 and 6 months.

5.3.3 Effect of vitamin D supplementation on plasma IGF-1 and IGFBP-3

There was no difference among groups in IGF-1 and IGFBP-3 at baseline or during the trial, regardless of whether shown as concentrations (Figure 5A, B) or the change between each time-point (Figure 5C, D). IGF-I and IGFBP-3 concentrations had different developmental patterns over the first 12 months of life; IGF-1 concentration declined from baseline and 6 months, with an increase with age thereafter. However, a less prominent change was observed in the IGFBP-3 concentrations overtime. The main effect of sex was not significant for IGF-1 (P=0.41) or IGFBP-3 (P=0.0721) (Supplemental Figure 5.2).

5.3.4 Compliance to vitamin D supplementation and nutritional data

Between birth and the baseline visit, all of the infants in the reference group received 400 IU/d of vitamin D supplementation, whereas those in the pool for the trial were 96.4% adherent. For trial and reference groups, the median compliance with supplementation was ≥ 91% from baseline until 3 months, ≥ 92% from 3 to 6 months and ≥ 85% from 6 to 12 months follow-up (Supplemental Table 5.1).

In the trial groups, all of infants were predominantly breastfed and 81.6% in the trial 400 and 85.7% in the trial 1000 IU/d were exclusively breastfed at the initial baseline visit. At 3 months of age >90% received breast milk, >80% at 6 months of age, and 40% at 12 months were

still receiving some breast milk (Supplemental Table 5.2). The median age at solid food introduction was 5 months (inter-quartile range: 4-5.5) with no difference among groups. Likewise, the median ages for crawling and walking were 6 and 10 months (inter-quartile range: 5-7 and 9-11 months for age at crawling and walking, respectively) with no difference among groups. No differences were observed in nutrient intakes, including energy, carbohydrate, fat, vitamin D, and calcium among vitamin D supplementation groups over time; however, protein intake was higher in the reference group compared to the trial 400 IU/d group and not different from the 1000 IU/d group; overall, the values for all nutrient intakes increased over time (Supplemental Table 5.3).

Among trial groups, 78 families completed the 12-month trial, and 20 families were lost to follow-up mainly due to family circumstances (e.g., time constraints or moving out from the area); furthermore, due to movement artifact, 4.6% (16/348) whole body scans were missing over the course of the trial. Similarly, in the reference group 9 families were lost to follow-up and 3.5% (5/142) whole body scans were missing across the study. Neonatal and maternal characteristics of completers and dropouts are shown in Supplemental Table 5.4. Among trial groups a higher proportion of the dropouts were born in the vitamin D synthesizing period ($P=0.0238$) and were multiparous vs. completers ($P=0.0290$). Family income was lower in dropouts vs completers ($P=0227$), and dropouts had lower gestational weight gain vs completers ($P<0.0001$).

5.4 Discussion

This trial was designed to test whether infants who were born with serum 25(OH)D <50 nmol/L, would benefit from a higher dose of vitamin D supplementation. Interestingly, body composition did not vary in neonates according to vitamin D status at birth, whereas divergent patterns were observed later in infancy. Specifically, a dosage of 1000 IU/d was shown to modestly increase whole body lean mass (322.1 g; 4.8% difference compared to 400 IU/d) without altering weight or length z-scores for age and sex. Thus, our research hypothesis was not fully accepted, as a difference in lean body mass was evident only at 12 months and not at 3 months of age. No differences were observed in whole body fat mass across the trial; likely due to normal developmental variation in infants and the limitation of the technology in estimating fat mass.

In accordance with a recent systematic review and meta-analysis of intervention trials (289), in our study achieving the AI of 400 IU/d of vitamin D through supplementation supports recovery of vitamin D status in infants born with serum 25(OH)D <50 nmol/L. This research is novel in that infants allocated to 1000 IU/d of vitamin D not only had increased serum 25(OH)D but also had greater lean body mass, the magnitude of which parallels that of Hazell et al (4). Unlike our trial, most infants in Hazell's study had 25(OH)D ≥50 nmol/L at inception. Therefore they used a higher cut-point of 75 nmol/L (12, 227) and reported that plasma 25(OH)D was positively associated with percentage lean mass at 12 months (Δ 3.89%, 5.41% difference; P=0.006). In the present trial, lack of differences in fat mass and a higher body weight of the 1000 IU/d vitamin D group at 12 months (296.05 g; 3.0% difference compared to 400 IU/d) suggests increments in lean body mass are more prominent than decreases in fat mass. This agrees with observations in female weanling rats with vitamin D deficiency (75). Rats that

received 4 IU vitamin D_3/g diet have an increase in 25(OH)D from 24.1 to 60.3 nmol/L within 8 weeks and had higher lean mass (250.4 vs. 219.6 vs g, P=0.01) compared to the control (1 IU vitamin D_3/g diet). These controlled studies in human infants and weanling rats suggest that increased dietary vitamin D modestly improves lean body mass (75) and complement the aforementioned cohorts in pregnancy where maternal vitamin D sufficiency associates with a lean body phenotype of the child (20, 22, 23). Collectively, the evidence is consistent for sufficient vitamin D status having a role to play in healthy body composition of children.

In line with reports of body composition in healthy term infants (6, 196), fat mass increased rapidly during the first 6 months. The plateau observed in fat mass after 6 months can be ascribed to postnatal fat mass being served as an energy reserve. As infants accomplish energy-demanding developmental milestones such as crawling and walking, they require additional energy to support growth (196, 290, 291). Since age at crawling and walking, and nutrient intakes were not different among groups, this might help to explain lack of differences in fat mass.

The mechanisms that explain enhanced lean mass include a mutual engagement in skeletal muscle growth and metabolism exerted by vitamin D and IGF-1; this is through molecular and cellular pathways that regulate cell differentiation and growth in skeletal muscle (136). In weaning rats (75) higher dietary vitamin D_3 maintained plasma IGF-1 longer during growth compared to the control group, suggesting a potential mechanism by which IGF-1 and vitamin D stimulate lean mass accretion. Consistent with other report in healthy infants (292), different patterns were observed in terms of IGF-1 and IGFBP-3 concentrations. IGF-1 declined over time from baseline to 6 months in a well-accepted physiological pattern, followed by an increase with age (from 6 to 12 months); this could be ascribed to increased dietary protein

intake as a strong positive predictor of IGF-1 concentration (293). Less notable changes were observed in IGFBP-3 with relatively higher concentrations compared to IGF-1. Both trial groups overlapped in IGF-1 and IGFBP-3 across the study and the values were within the anticipated range judged by our reference data.

In line with other reports where sexual dimorphisms in IGF-1 and IGFBP-3 concentrations appear at birth (294) or from 3-12 months (295), we observed similar patterns, but only appeared to diverge at 12 months. Lack of statistical differences between the sexes could be explained by our smaller sample size. Higher concentrations of IGF-1 and IGFBP-3 are usually observed in female infants compared to males likely due to differentials in the IGF-1/GH axis over the first 12 months of life. Early postnatally, IGF-1 is more responsive to nutritional intake (breastfeeding status) and insulin secretion whereas later in infancy growth hormone is the predominant regulator of IGF-1 (296). Growth hormone sensitivity to changes in IGF-1 is more pronounced in females than males (294). Overall, the results suggest that increments in lean body mass are not necessarily ascribed to plasma IGF-1 and IGFBP-3, hence other mechanisms need to be explored considering the dynamics of vitamin D status and free IGF-1 utilization for tissue (lean mass) growth in infancy.

The major implication of our trial is that vitamin D has a role in establishing a healthy body composition in infants and specifically a leaner body phenotype. Vitamin D supplementation of 400 IU/d for healthy breastfed infants is suitable to compensate for serum 25(OH)D <50 nmol/L at birth. Increasing vitamin D intakes to 1000 IU/d is not required to support serum 25(OH)D in the range of 50-125 nmol/L (10). This counters some professional society recommendations (227, 297). Whether the modest benefits to lean mass evident at 12 months would extend into childhood with possible implications in reduced risk of excess

adiposity requires further research with longer term follow-up. In doing so, unraveling epigenetic mechanisms such as DNA methylation of the genes involved in vitamin D metabolism (*VDR*, *DBP*, *RXR*, and *CYPs*), would aid to better understand how improving vitamin D status with a higher dosage of supplementation results in a change in lean body phenotype.

We acknowledge the limitations of this study. We recruited our minimum sample size needed to ensure the specified precision and while we observed differences in lean mass, the dropout rate may have biased our results. Nonetheless by using a mixed model all the available data was used in our analyses including sociodemographic covariates. Additionally, numerous assessments were tested across 4 time-points posing risk of Type I errors. To alleviate this concern, post hoc testing was limited to differences among groups over time and adjusted for multiple comparisons. Serum 25(OH)D was measured using an immunoassay which is not a gold standard technique, although the one we used is certified by the Vitamin D Standardization Certification Program (298). In addition, we implemented rigorous quality assurance measures across the study using Vitamin D External Quality Assessment Scheme as well as the NIST standard reference materials. Moreover, we verified in a subgroup of mothers and infants (n= 83), that total serum 25(OH)D was in agreement (mean difference= -0.8) with liquid chromatography tandem mass spectrometry. Another limitation is that normative data for body composition in infancy is limited (196) to infants with different demographic characteristics from the present study. To help overcome this, patterns of growth and body composition in trial groups were compared to the reference group tracked at the same time. Lastly, parent self-report compliance was a limitation and pre and post weighing of used bottles of supplement was not feasible; those that were returned had often been cleaned for recycling. Nonetheless, the biological response to supplementation was monitored using serum 25(OH)D concentration.

5.5 Conclusion

Overall, this trial provides high level of evidence suggesting that postnatal vitamin D supplementation (400 and 1000 IU/d) with high adherence compensates for low maternal-fetal transfer of vitamin D in otherwise healthy AGA infants. Increasing supplemental vitamin D intakes to 1000 IU/d appears to have implications in programming of a leaner body phenotype without altering other patterns of growth. Further investigations are required to explore the underlying mechanisms, with possible implications to child and public health.

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Authors' contributions to the manuscript: *1*) HAW, SW, DM, FR, GJ, and SK designed the study. *2*) HAW supervised the study. *3*) AK designed the product, performed the external testing, and randomization scheme *4*) HAW and CAV managed the study project. *5*) HAW, CAV, MR, NG, OFS collected the data; *6*) MR, NG and OFS performed the laboratory analyses. *7*) HAW and MR performed statistical analysis; *8*) MR wrote the final manuscript with the intellectual aid and comments of HAW. *9*) All authors (MR, NG, CAV, OFS, AK, SW, DM, FR, GJ, SK, and HAW) have read and approved the manuscript.

Table 5.1 Neonatal and maternal characteristics at birth and at baseline

Parameters[1]	Reference 400 IU/d (n=41)	Trial 400 IU/d (n=49)	Trial 1000 IU/d (n=49)
Infants			
At birth (24-36 h postpartum)			
Sex, n (%)			
Male	23 (56.1)	29 (59.2)	29 (59.2)
Female	18 (43.9)	20 (40.8)	20 (40.8)
UVB period[2], n (%)			
Synthesizing period	25 (61.0)	30 (61.2)	26 (53.1)
Non-synthesizing period	16 (39.0)	19 (38.8)	23 (49)
Gestational age, wk	39.6 ± 1.0	39.7 ± 1.0	39.6 ± 1.1
Weight, kg	3.5 ± 0.3	3.4 ± 0.4	3.4 ± 0.4
Weight for age z-score	0.3 ± 0.7	0.1 ± 0.8	0.1 ± 0.8
Serum 25(OH)D, nmol/L	68.0 ± 13.2	30.8 ± 9.2	34.4 ± 12.0
At baseline (1 month postpartum)			
Skin tone[3], n (%)			
F I-III	38 (92.7)	35 (71.4)	35 (71.4)
F IV-VI	3 (7.3)	14 (28.6)	14 (28.6)
Weight, kg	4.1 ± 0.5	3.9 ± 0.5	3.9 ± 0.5
Weight for age z-score	0.03 ± 0.8	-0.2 ± 0.9	-0.1 ± 0.7
Length, cm	53.5 ± 2.0	52.7 ± 1.8	52.9 ± 2.2
Length for age z-score	0.1 ± 1.0	-0.2 ± 0.9	0.03 ± 0.9
HC, cm	36.5 ± 1.1	36.6 ± 1.2	36.1 ± 1.3
HC for age z-score	0.1 ± 0.9	0.3 ± 0.9	-0.02 ± 0.8
Serum 25(OH)D, nmol/L	69.2 ± 16.4	45.8 ± 14.1	47.6 ± 13.4
Mothers			
At delivery (24-36 h postpartum)			
Mother's age, y	32.3 ± 4.0	32.8 ± 4.3	31.2 ± 4.8
Father's age, y	35.0 ± 4.9	35.8 ± 5.4	33.2 ± 5.3
Self-reported population[5], n (%)			
White	31 (75.6)	22 (44.9)	24 (49.0)
All other groups	10 (24.4)	27 (55.1)	25 (51.0)
Family income, n (%)			
≥70,000 CAD	29 (70.7)	27 (55.1)	22 (44.9)
<70,000 CAD	7 (17.1)	15 (30.6)	18 (36.7)
Not reported	5 (12.2)	7 (14.3)	9 (18.4)
Education, n (%)			
Elementary/high school	1 (2.4)	8 (16.3)	4 (8.2)
College/vocational school	10 (24.4)	8 (16.3)	11 (22.4)
University	30 (73.2)	33 (67.4)	34 (69.4)

Pre-pregnancy BMI, kg/m^2	23.1 ± 2.6	24.6 ± 4.4	25.8 ± 5.9
Supplement use[6], n (%)	38 (92.7)	44 (89.8)	46 (93.9)
Parity, n (%)			
Primiparous	13 (31.7)	12 (24.5)	19 (38.8)
Multiparous ≥ 2	28 (68.3)	37 (75.5)	30 (61.2)
At baseline (1 month postpartum)			
Serum 25(OH)D, nmol/L	94.3 ± 23.3	51.6 ± 14.5	60.0 ± 22.9

[1]Data are mean ± SD or n (%); [2]infants born in vitamin D synthesizing period: April 1st- October 31st or vitamin D non-synthesizing period: November 1st -March 31st; [3]skin tone based on Fitzpatrick descriptions (FI-III or FIV-VI); [4]self-reported population group (white or all other groups including: South Asian, Chinese, Black, Filipino, Latin American, Arab, Southeast Asian, West Asian, Korean, Japanese, and other); [5]maternal multivitamin use during pregnancy (yes/no). Abbreviations: 25(OH)D: 25-hydroxyvitamin D; CAD: Canadian dollar; F: Fitzpatrick; IU: International Unit; UVB: ultraviolet B.

Figure 5.1 CONSORT Flow Diagram

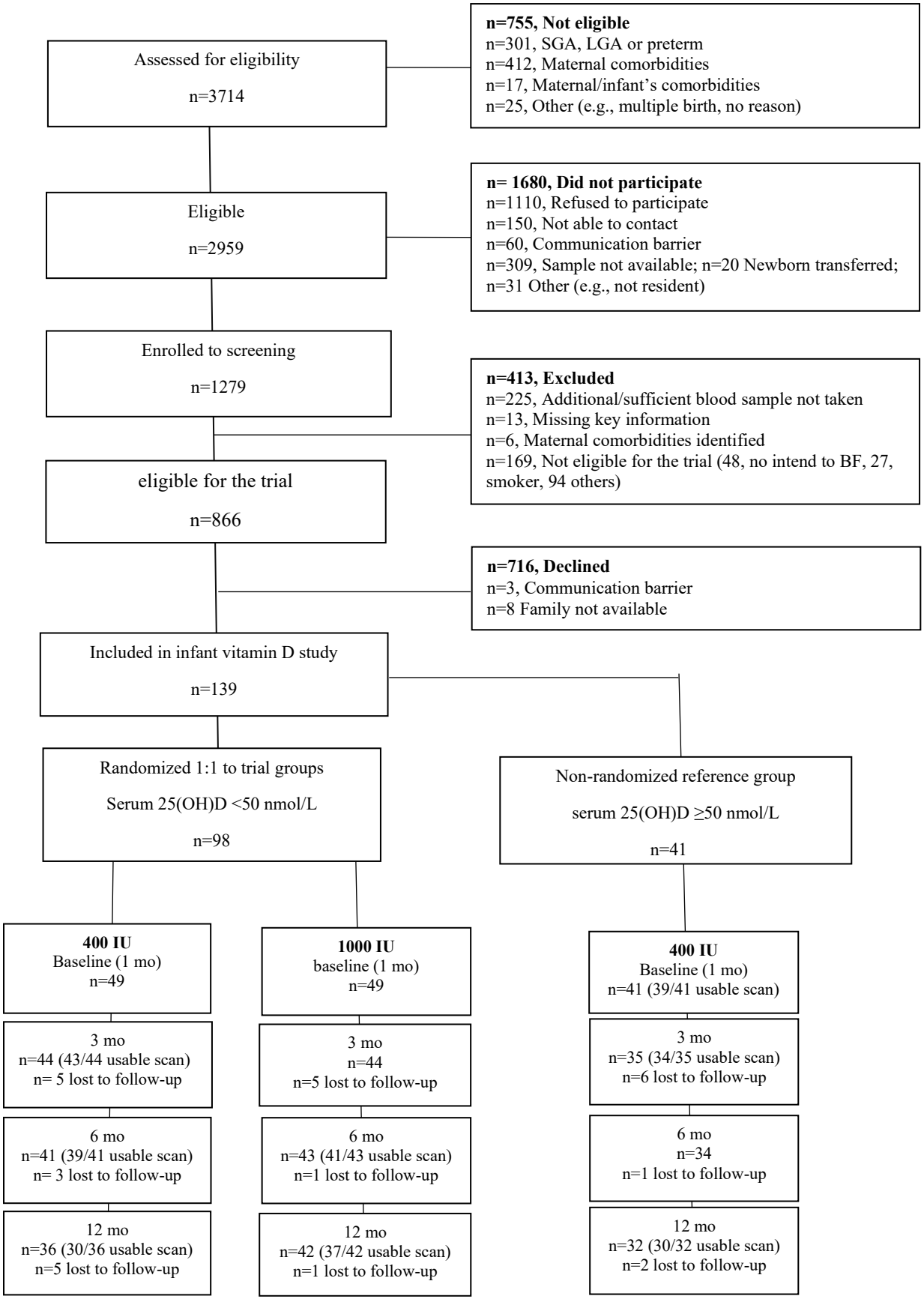
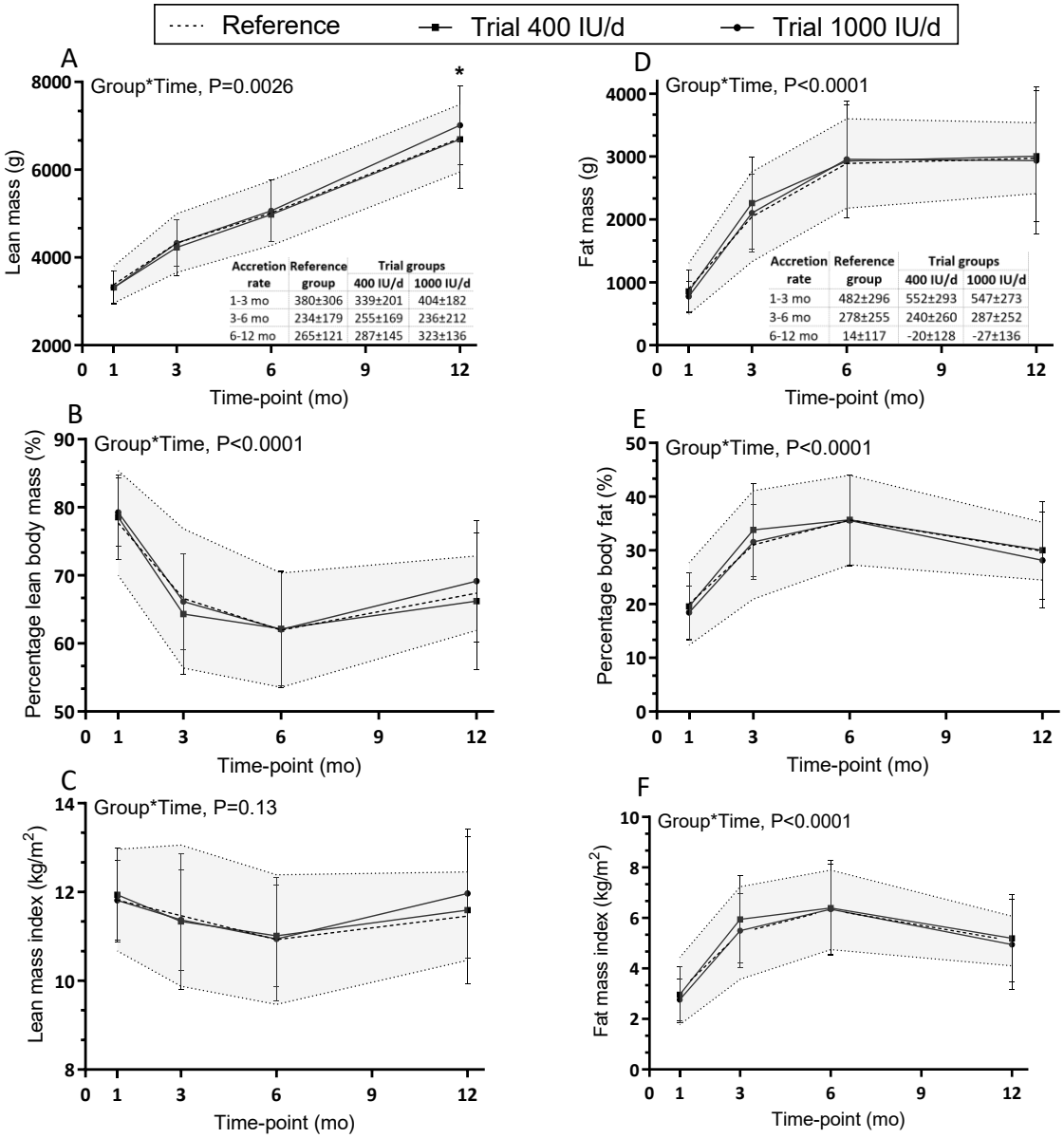


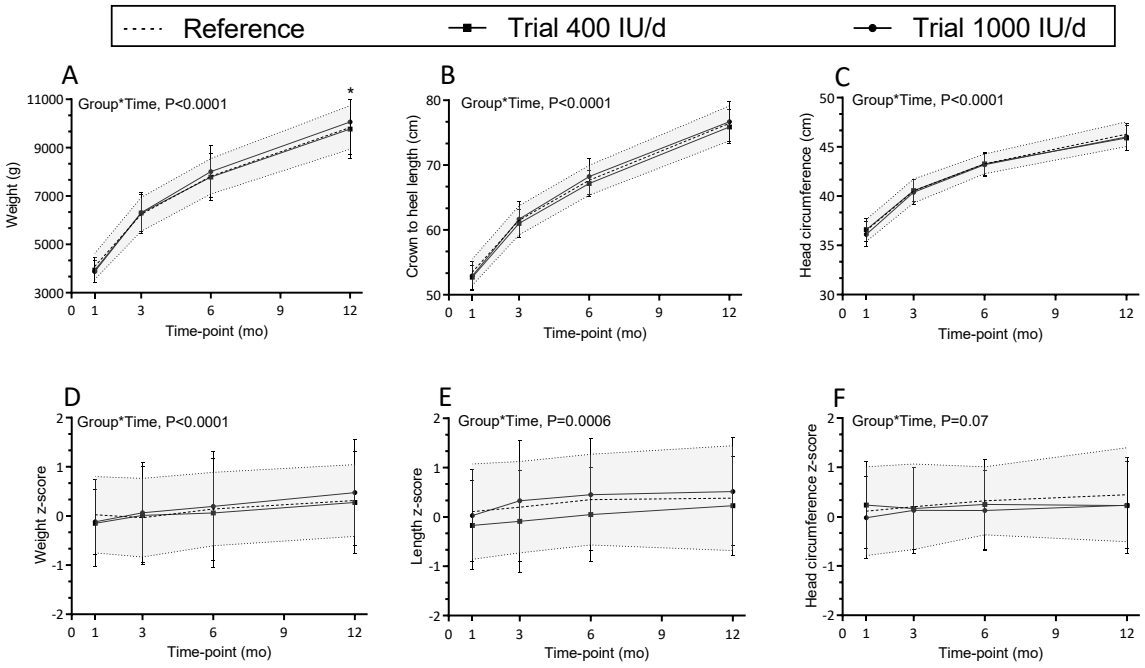
Figure 5.2 Infant body composition



Infant body composition over time: (A) lean mass and lean mass accretion, (B) percentage lean mass, (C) lean mass index (LMI), (D) fat mass and fat mass accretion, (E) percentage fat mass, and (F) fat mass index (FMI) among groups over time. Data are mean ± SD. Sample sizes are: reference group (baseline n=39; 3 mo n=34; 6 mo n=34; 12 mo n=30); 400 IU/d group (baseline n=49; 3 mo n=43; 6 mo n=39; 12 mo n=30); and 1000 IU/d group (baseline n=49; 3 mo n=44; 6 mo n=41; 12 mo n=37). Data were compared using a linear mixed effects model (SAS PROC MIXED) for fixed effects of group x time, sex, skin tone, infant actual age at each visit, gravida, parental age at delivery, maternal BMI preconception, as well as family income. Participant (ID)

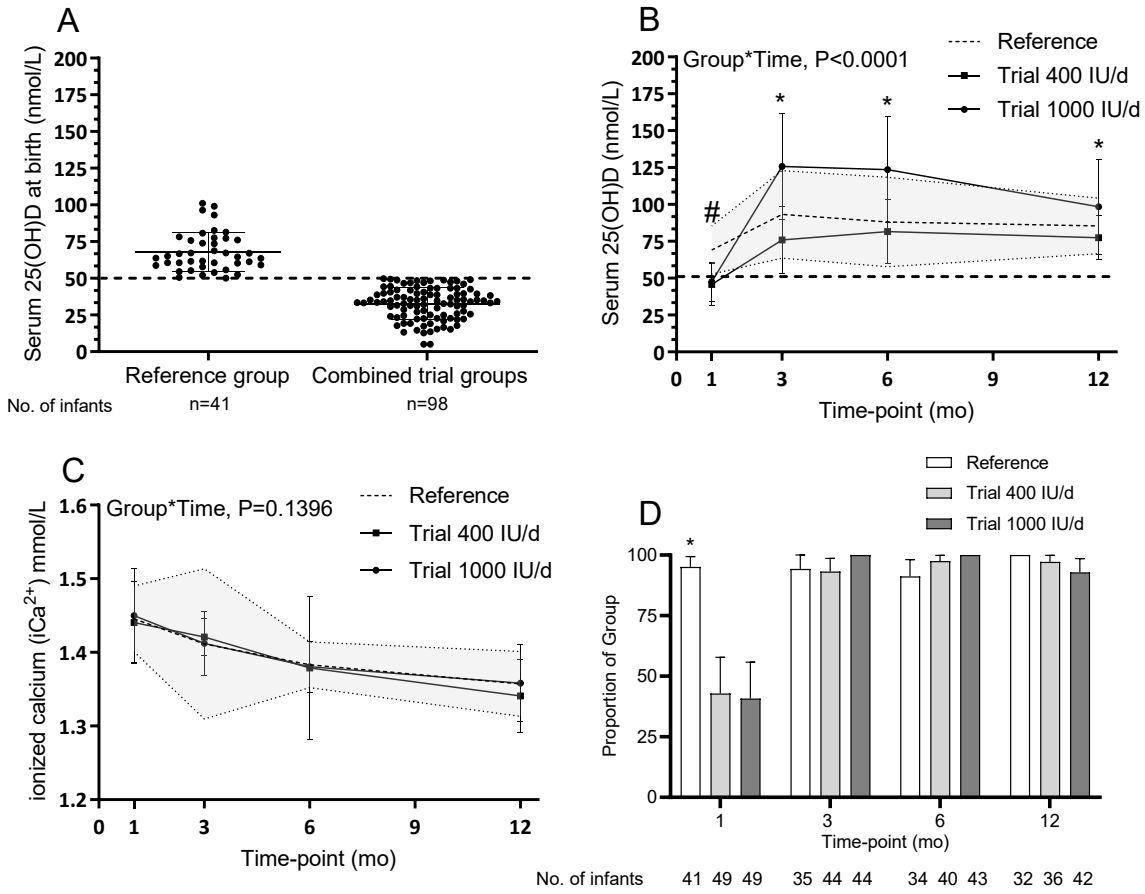
modelled as a random effect. P-values reflect Tukey's post hoc tests with Tukey-Kramer adjustment for multiple comparisons. The shaded area reflects reference ± SD. *P<0.05 1000 IU/d group vs 400 IU/d group at 12 months for lean mass; no other differences among groups over time were observed.

Figure 5.3 Infant anthropometry



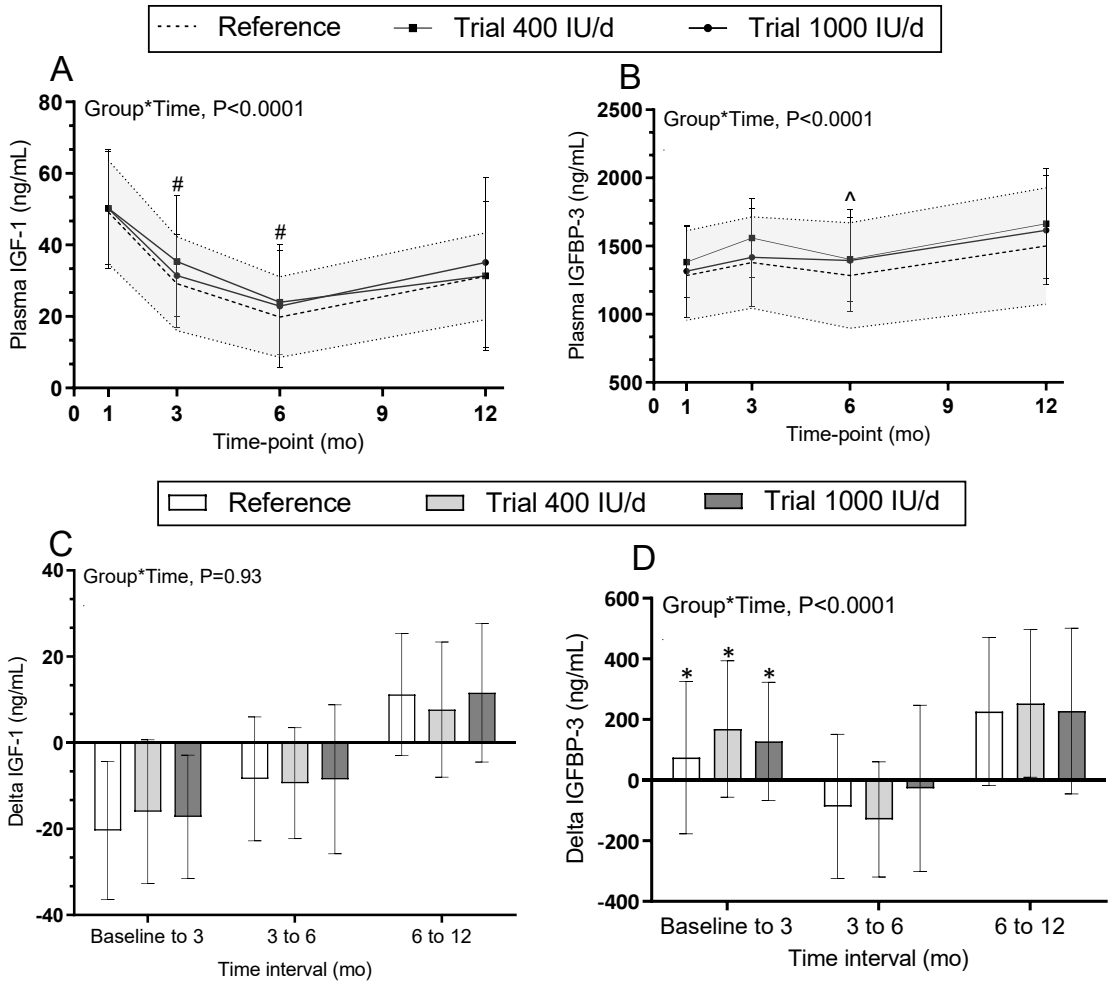
Infant growth over time: (A) weight, (B) crown to heel length, (C) head circumference measurements, (D) weight for age, (E) length for age, and (F) head circumference for age z-scores, among groups over time. Data are mean ± SD. Sample sizes are: reference group (baseline n=41; 3 mo n=35; 6 mo n=34; 12 mo n=32); 400 IU/d group (baseline n=49; 3 mo n=44; 6 mo n=40; 12 mo n=36); and 1000 IU/d group (baseline n=49; 3 mo n=44; 6 mo n=43; 12 mo n=42). Data were compared for weight, length, and head circumference measurements using a linear mixed effects model (SAS PROC MIXED) tested for fixed effects of group x time, sex, skin tone, infant actual age at each visit, gravida, parental age at delivery, maternal BMI preconception, as well as family income. Participant (ID) modelled as a random effect; and for weight, length, and head circumference for age z-scores sex and infant actual age at each visit were removed from the mixed model. P-values reflect Tukey's *post hoc* tests with Tukey-Kramer adjustment for multiple comparisons. The shaded area reflects reference ±SD. *P<0.05 1000 IU/d vs 400 IU/d at 12 months for body weight, no other differences in growth among groups over time were observed.

Figure 5.4 Infant serum 25(OH)D and iCa, and the proportion meeting vitamin D sufficiency



Infant biochemistry (A) Infant serum 25(OH)D concentrations of the reference group (n=41) and those in the pool for the trial (n=98) at birth (24-36 h postpartum), (B) infant serum 25(OH)D concentrations among groups over the first 12 mo of life (baseline, 3, 6, and 12 mo), (C) infant whole blood ionized calcium among groups, and (D) Proportion of infants in the reference group, trial 400 IU/d, and trial 1000 IU/d groups achieving the cut-point for sufficiency for vitamin D status (25(OH)D ≥50 nmol/L) at each time-point. Panels A-C data are mean ± SD and panel D data are mean and 95% CIs. Sample sizes are: reference group (baseline n=41; 3 mo n=35; 6 mo n=34; 12 mo n=32); 400 IU/d group (baseline n=49; 3 mo n=44; 6 mo n=40; 12 mo n=36); and 1000 IU/d group (baseline n=49; 3 mo n=44; 6 mo n=43; 12 mo n=42). Continuous data were compared using a linear mixed effects model (SAS PROC MIXED) tested for fixed effects of group x time, sex, skin tone, infant actual age at each visit, gravida, parental age at delivery, maternal BMI preconception, as well as family income. Participant (ID) modelled as a random effect. P-values reflect Tukey's post hoc tests with Tukey-Kramer adjustment for multiple comparisons. The shaded area is reference ± SD. #P<0.05 reference group vs trial 400 and 1000 IU/d groups; *P<0.05 trial 1000 IU/d vs trial 400 IU/d and the reference group. Categorical data were compared at each time-point using Chi-square analyses, *P<0.05 reference group vs trial 400 IU/d and 1000 IU/d groups at baseline only. Abbreviations: 25(OH)D indicates 25-hydroxyvitamin D.

Figure 5.5 Infant plasma concentration of IGF-1 and IGFBP-3



Infant plasma (A) IGF-1, (B) IGFBP-3 concentrations, C) delta IGF-1, and D) delta IGFBP-3. Data are mean ± SD. Sample sizes are: reference group (baseline n=41; 3 mo n=35; 6 mo n=34; 12 mo n=32); 400 IU/d group (baseline n=49; 3 mo n=44; 6 mo n=40; 12 mo n=36); and 1000 IU/d group (baseline n=49; 3 mo n=44; 6 mo n=43; 12 mo n=42). Data were compared using a linear mixed effects model (SAS PROC MIXED) tested for fixed effects of group x time, sex, skin tone, infant actual age at each visit, gravida, parental age at delivery, maternal BMI preconception, as well as family income. Participant (ID) modelled as a random effect. P-values reflect Tukey's post hoc tests with Tukey-Kramer adjustment for multiple comparisons. The shaded area is reference ± SD. $^{\#}$P<0.05 baseline vs 3 mo and 6 mo for all groups; $^{\wedge}$P<0.05 3 mo vs 6 mo for all groups except trial 1000 IU/d; *P<0.05 baseline to 3 mo vs 3 mo to 6 mo for all groups.

Supplemental Table 5.1 Compliance to vitamin D supplementation among groups overtime

Time interval	Reference 400 IU/d	Trial 400 IU/d	Trial 1000 IU/d
1 to 3 mo	0.91 (0.80, 0.95)	0.91 (0.84, 0.98)	0.93 (0.84, 0.97)
3 to 6 mo	0.93 (0.82, 0.98)	0.92 (0.85, 0.97)	0.93 (0.79, 0.98)
6 to 12 mo	0.93 (0.79, 0.98)	0.88 (0.81, 0.98)	0.85 (0.65, 0.94)

Data are median (interquartile range). Sample sizes are: reference group (1 to 3 mo n=32; 3 to 6 mo n=31; 6 to 12 mo n=27); 400 IU/d group (1 to 3 mo n=44; 3 to 6 mo n=35; 6 to 12 mo n=32); and 1000 IU/d group (1 to 3 mo n=42; 3 to 6 mo n=41; 6 to 12 mo n=41).

Supplemental Table 5.2 Breastfeeding status among groups from baseline to 12 months of trial

Time-point	Feeding status	Reference 400 IU/d	Trial 400 IU/d	Trial 1000 IU/d
Baseline	Any breastfeeding[1]	41 (100.0)	49 (100.0)	49 (100.0)
(*n*=139)	Exclusive[2]	31 (75.6)	40 (81.6)	42 (85.7)
	Mixed[3]	10 (24.4)	9 (18.4)	7 (14.3)
3 mo	Any breastfeeding	30 (85.7)	41 (93.2)	41 (93.2)
(*n*=123)	Exclusive	25 (83.3)	35 (85.4)	33 (80.5)
	Mixed	5 (16.7)	6 (14.6)	8 (19.5)
6 mo	Any breastfeeding	27 (79.4)	34 (85.0)	36 (83.7)
(*n*=117)	Exclusive	3 (11.1)	7 (20.6)	5 (13.9)
	Mixed	24 (88.9)	27 (79.4)	31 (86.1)
12 mo	Any breastfeeding	12 (37.5)	16 (44.4)	17 (40.5)
(*n*=110)	Exclusive	0 (0.0)	0 (0.0)	0 (0.0)
	Mixed	12 (100.0)	16 (100.0)	17 (100.0)

Data are n (%). Sample sizes are: reference group (baseline n=41; 3 mo n=30; 6 mo n=27; 12 mo n=12); 400 IU/d group (baseline n=49; 3 mo n=41; 6 mo n=34; 12 mo n=16); and 1000 IU/d group (baseline n=49; 3 mo n=41; 6 mo n=36; 12 mo n=17). [1]Any breastfeeding: the proportion of infants receiving any breast milk (either exclusive or mixed with alternative feeds); [2]exclusive: the proportion of infants receiving breast milk only; [3]mixed: the proportion of infants receiving breast milk mixed with formula and/or cow's milk and/or solid food.

Supplemental Table 5.3 Infant nutrient intake among groups over time

		Vitamin D supplementation groups			P-value
Parameters[1]	**All**	**Reference**	**Trial 400 IU/d**	**Trial 1000 IU/d**	**Group x Time**
Energy, kcal					<0.0001
1 mo (n=121)	661.6 ± 203.8[a]	682.6 ± 169.0	608.3 ± 150.4	696.3 ± 263.2	
3 mo (n=103)	670.0 ± 206.4[a]	676.5 ± 206.7	642.8 ± 177.0	688.7 ± 231.2	
6 mo (n=89)	670.0 ± 229.3[a]	663.6 ± 205.5	626.6 ± 165.9	707.3 ± 281.8	
12 mo (n=64)	1063.1 ± 550.7[b]	1089.4 ± 521.0	1024.4 ± 690.2	1070.8 ± 469.0	
Protein, g					<0.0001
1 mo (n=121)	10.4 ± 6.8[a]	11.9 ± 11.2	9.3 ± 2.4	10.2 ± 3.9	
3 mo (n=103)	11.0 ± 6.8[a]	12.2 ± 10.6	10.2 ± 3.6	10.9 ± 5.0	
6 mo (n=89)	14.4 ± 8.4[a]	14.9 ± 8.3	12.0 ± 4.6	15.9 ± 10.2	
12 mo (n=64)	41.8 ± 23.6[b]	47.7 ± 31.9	37.3 ± 19.3	40.2 ± 17.2	
CHO, g					<0.0001
1 mo (n=121)	65.0 ± 19.6[a]	66.4 ± 15.0	60.1 ± 14.7	68.5 ± 25.9	
3 mo (n=103)	67.5 ± 22.5[ab]	68.2 ± 19.6	64.3 ± 17.6	69.9 ± 28.0	
6 mo (n=89)	77.6 ± 32.3[b]	72.8 ± 20.7	73.0 ± 24.0	84.9 ± 42.9	
12 mo (n=64)	122.7± 60.0[c]	118.5 ± 45.0	119.2 ± 74.4	129.0 ± 60.9	
Fat, g					0.0036
1 mo (n=121)	41.1 ± 12.7[a]	42.0 ± 10.1	37.8 ± 9.7	43.6 ± 16.5	
3 mo (n=103)	40.6 ± 11.7[ab]	40.5 ± 11.9	39.3 ± 11.2	41.7 ± 12.2	
6 mo (n=89)	34.6 ± 11.0[c]	35.8 ± 11.8	32.8 ± 8.9	35.0 ± 11.8	
12 mo (n=64)	46.7 ± 31.0 [ad]	48.6 ± 33.0	46.1 38.5	45.6 ± 22.7	
Vitamin D, IU					<0.0001
1 mo (n=121)	51.0 ± 51.2[a]	63.4 ± 64.8	51.1 ± 59.0	39.8 ± 15.0	
3 mo (n=103)	77.5 ± 101.9[ab]	92.6 ± 111.2	71.5 ± 92.7	71.2 ± 103.5	
6 mo (n=89)	114.8 ± 135.9[b]	126.7 ± 153.1	94.4 ± 108.7	120.4 ± 141.4	
12 mo (n=64)	255.0 ± 320.1[c]	256.9 ± 251.0	266.2 ± 448.8	244.6 ± 260.8	
Calcium, mg					<0.0001
1 mo (n=121)	313.7 ± 109.4[a]	331.5 ± 119.6	293.4 ± 85.3	318.3 ± 120.3	
3 mo (n=103)	352.4 ± 195.0[a]	361.8 ± 163.1	327.7 ± 124.0	366.7 ± 260.0	
6 mo (n=89)	471.2 ± 382.4[b]	421.8 ± 199.6	423.1 ± 240.1	546.5 ± 543.1	
12 mo (n=64)	864.0 ± 499.6[c]	871.6 ± 388.8	816.0 ± 599.4	895.3 ± 517.7	

[1]Data are mean. Sample sizes are: reference group (baseline n=37; 3 mo n=30; 6 mo n=28; 12 mo n=21); 400 IU/d group (baseline n=42; 3 mo n=34; 6 mo n=26; 12 mo n=19); and 1000 IU/d group (baseline n=42; 3 mo n=39; 6 mo n=35; 12 mo n=24). [2]Data were compared using a linear mixed effects model (SAS PROC MIXED) including fixed effects of group x time interaction, and the random effect of an individual infant (ID) with repeated measurements followed by post hoc Tukey's tests with Tukey-Kramer adjustment for multiple comparisons;

participant modelled as a random variable, the overall means over time with different superscript letters a, b, c, d are significantly different $P<0.05$. Abbreviations: CHO: carbohydrate; IU; International Unit.

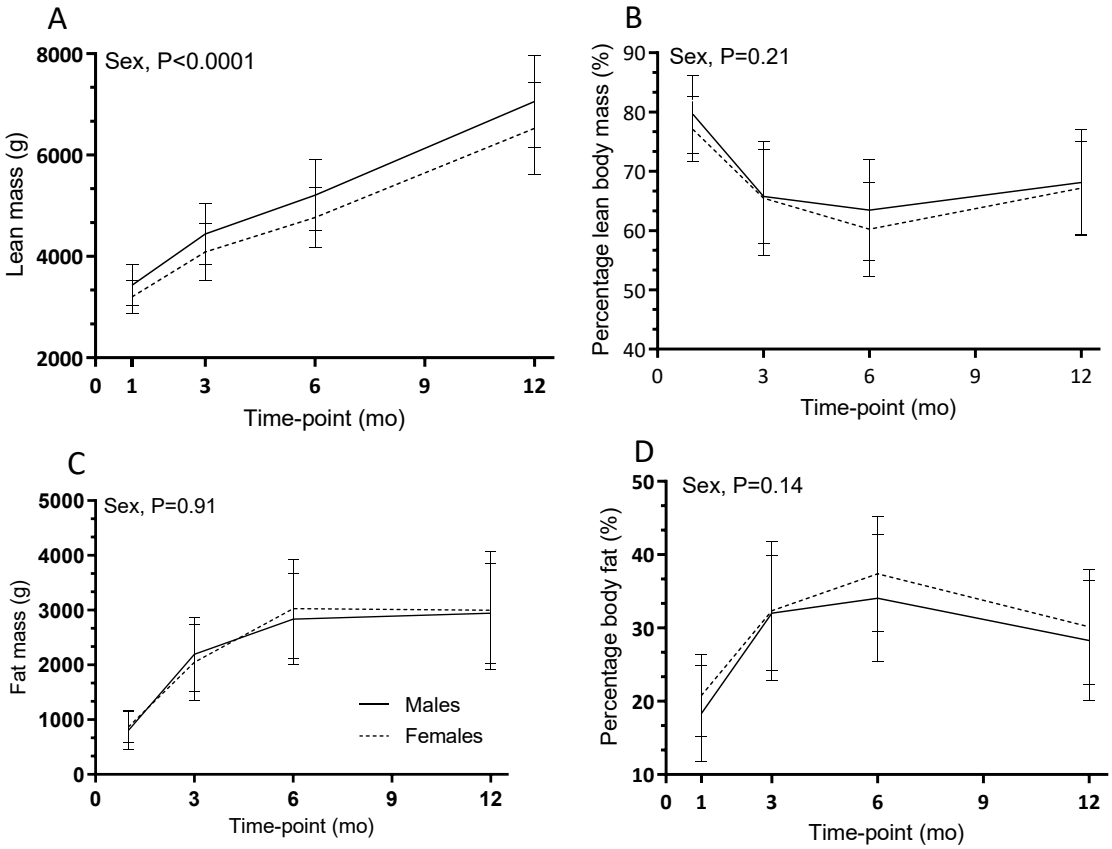
Supplemental Table 5.4 Neonatal and maternal characteristics of those who competed the 12-month trial vs. dropouts

Parameters[1]	Reference completers (*n*=32)	Reference dropouts (*n*=9)	P-value	Trial completers (*n*=78)	Trial dropouts (*n*=20)	P-value[2]
Infants						
Gestational age, wk	39.6 (39.3, 39.9)	39.8 (38.9, 40.7)	0.63	39.7 (39.5, 39.9)	39.6 (39.1, 40.1)	0.67
Sex, n (%)			0.47			0.55
Male	19 (59.4)	4 (44.4)		45 (57.7)	19 (65.0)	
Female	13 (40.6)	5 (55.6)		33 (42.3)	7 (35.0)	
UVB period[3], n (%)			0.44			0.02
Synthesizing	18 (56.3)	7 (77.8)		40 (51.3)	16 (80.0)	
Non-synthesizing	14 (43.8)	2 (22.2)		38 (48.7)	4 (20.0)	
Weight, kg	3.4 (3.3, 3.6)	3.6 (3.3, 3.7)	0.57	3.4 (3.3, 3.5)	3.3 (3.1, 3.4)	0.15
Serum 25(OH)D, nmol/L	67.7 (62.5, 72.9)	68.9 (62.2, 75.6)	0.82	32.2 (29.8, 34.7)	34.1 (29.3, 38.9)	0.49
Mothers						
Age at delivery, y	32.6 (31.2, 33.9)	31.5 (27.4, 35.6)	0.49	32.1 (30.9, 33.1)	31.9 (29.9, 33.8)	0.99
Gravida, n (%)			0.69			0.02
Primiparous	11 (34.4)	2 (22.2)		29 (37.2)	2 (10.0)	
Multiparous	21 (65.6)	7 (77.8)		49 (62.8)	18 (90.0)	
PP-BMI[4], kg/m^2	23.4 (22.4, 24.3)	22.1 (20.2, 24.0)	0.19	25.2 (24.0, 26.4)	25.1 (23.0, 27.2)	0.96
GWG[5], kg	13.6 (12.1, 15.2)	16.0 (13.1, 18.8)	0.15	14.3 (12.9, 15.7)	10.3 (7.7, 12.9)	0.01
Serum 25(OH)D, nmol/L	92.6 (85.1, 100.0)	100.5 (76.4, 124.6)	0.37	56.0 (51.4, 60.6)	54.9 (47.1, 62.7)	0.82
Self-reported population[6], n (%)			0.38			0.06
White	26 (81.3)	6 (66.7)		42 (53.8)	6 (30.0)	
All other groups	6 (18.7)	3 (33.3)		36 (46.2)	14 (70.0)	
Family income, n (%)			0.43			0.03

≥70,000 CAD	24 (75.0)	5 (55.6)		44 (56.4)	5 (25.0)	
<70,000 CAD	5 (15.6)	2 (22.2)		24 (30.7)	9 (45.0)	
Not reported	3 (9.4)	2 (22.2)		10 (12.8)	6 (30.0)	
Education, n (%)			0.02			0.51
Primary/high school	0 (0.0)	1 (11.2)		8 (10.3)	4 (20.0)	
College/vocational school	6 (18.7)	4 (44.4)		15 (19.2)	4 (20.0)	
University	26 (81.3)	4 (44.4)		55 (70.0)	12 (60.0)	

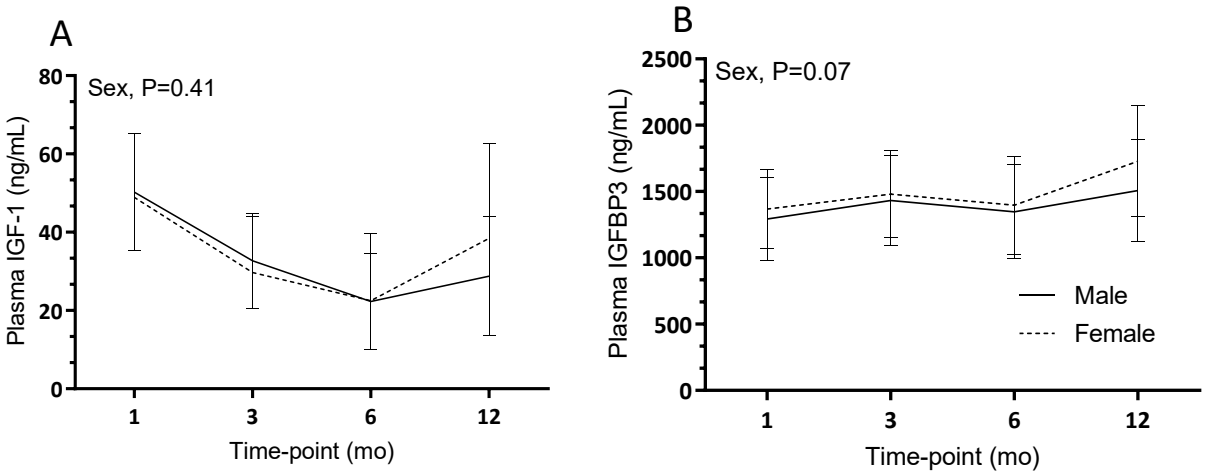
[1]Data are mean (lower and upper 95% confidence limits) or n (%); [2]Data were compared using a linear fixed effects model (SAS PROC MIXED) for continuous variables followed by post hoc Tukey's tests with Tukey-Kramer adjustment for multiple comparisons; and Chi-square or Fisher exact tests for categorical variables; [3]Infants born in vitamin D synthesizing period: April 1- October 31st or vitamin D non-synthesizing period: November 1st - March 31st; [4]PP-BMI: Pre-pregnancy BMI; [5]GWG: Gestational weight gain; [6]Self-reported population group (white/all other groups including: South Asian, Chinese, Black, Filipino, Latin American, Arab, Southeast Asian, West Asian, Korean, Japanese, and other); Abbreviations: 25(OH)D: 25-hydroxyvitamin D; CAD: Canadian dollar; UVB: ultraviolet B.

Supplemental Figure 5.1 Infant sex differences in body composition



Sex differences in infant: (A) lean mass (B) percentage lean body mass (C) fat mass (D) percentage body fat. Data were mean ± SD; n= 81 males, n= 58 females. Data were compared using a Data were compared using a linear mixed effect model (SAS PROC MIXED) for fixed effects of group x time, sex, skin tone, infant actual age at each visit, gravida, parental age at delivery, maternal BMI preconception, as well as family income. Participant (ID) modelled as a random effect with repeated measurements followed by post hoc Tukey's tests with Tukey-Kramer adjustment for multiple comparisons; *P<0.05 males vs females.

Supplemental Figure 5.2 Infant sex differences in IGF-1 and IGFBP-3 concentrations



Sex differences in infant: (A) IGF-1 (B) IGFBP-3 concentrations. Data are mean ± SD; n= 81 males, n= 58 females. Data were compared using a Data were compared using a linear mixed effect model (SAS PROC MIXED) for fixed effects of group x time, sex, skin tone, infant actual age at each visit, gravida, parental age at delivery, maternal BMI preconception, as well as family income. Participant (ID) modelled as a random effect with repeated measurements followed by post hoc Tukey's tests with Tukey-Kramer adjustment for multiple comparisons.

CHAPTER 6: GENERAL DISCUSSION AND CONCLUSIONS

6.1 General Discussion

The overall objective of this dissertation was to assess whether building of healthy vitamin D stores early in life normalizes body composition (lean and fat mass) and improves quality of growth by programming for a leaner body phenotype and lower fat mass accretion. This research also aimed to test whether correction of vitamin D status alters circulating growth hormones including IGF-1 and IGFBP-3 as stimulus for lean tissue accretion with established roles in growth and musculoskeletal health outcomes in growing animal models and human infants with vitamin D deficiency/insufficiency. The impact of maternal excess adiposity preconception as an effect modifier of the association between maternal vitamin D status and body composition in offspring was explored.

Collectively, this series of research studies was designed to broaden evolving knowledge of the role of vitamin D in body composition and particularly in the development of lean body mass (muscle mass). There is no national surveillance data on vitamin D status of newborn infants. Based on regional studies, vitamin D deficiency/insufficiency is a prevalent condition among pregnant women and their newborns in Canada (13-15) and worldwide (299-302). Even though there is some observational evidence linking compromised maternal vitamin D status to lower offspring lean body mass and excess adiposity, there is not enough evidence to establish a cause-and-effect link with vitamin D and body composition.

There is only one randomized controlled trial reporting upon changes in body composition in response to vitamin D status in infancy (4). This could be due to the challenges in pediatric research domain in the assessment of body composition using the reference standard methods that accurately delineate lean-fat mass partitioning as these assessment techniques

require active cooperation of the subject. It could be also due to publication bias (i.e., negative trials are not always published). To the best of the author's knowledge there are now at least five pregnancy cohorts looking at the relationship between maternal vitamin D status and body composition in children, however, the impact of vitamin D on body composition without the interference of potential confounders remains unclear. This underscores the importance for more randomized controlled trials in human infants as well as highly controlled animal studies, which has been met by the research in the present thesis.

At the time that this thesis was constructed, there were important research gaps that were addressed and closed in part as reported in detail in the previous chapters. This chapter will highlight the general findings from the thesis objectives and discuss additional considerations as they relate to the study findings. In spite of limitations noted, this research has important implications to infant, child and public health and provides insight for future related investigation.

Hypothesis 1:

> *Low vitamin D status in weanling rats, programs for higher fat mass accretion, and that achievement of vitamin D status above 50 nmol/L of 25(OH)D_3 resets the trajectory to a leaner body phenotype and lower fat mass. Increased intakes of dietary vitamin D alter circulating of growth hormones (IGF-1, IGFBP-3, and leptin).*

Study one (chapter 3) was a highly controlled study in a rapidly growing animal model that provided a unique opportunity to explore the impact of incremental dosage of dietary vitamin D (1, 2 or 4 IU vitamin D_3/g) on body composition using two assessment methods of DXA that accurately delineates lean-fat partitioning as well as micro computed tomography

(μCT) in order to capture cross sectional views of subcutaneous and omental adipose tissue. Weanling female Sprague Dawley rats (4 to12 weeks of age) were studied to remove the interference of potential confounders and influential factors associated with low vitamin D status in human infants including sex, skin color, season of birth, as well as socio-demographic, cultural impacts and maternal factors such as adiposity. Female rats were included as in males the effect of testosterone might have overpowered the potential benefits of vitamin D. In the present study, the characteristics at baseline were balanced out among groups via randomization. No differences in vitamin D status were observed among groups at baseline with all rats having low basal vitamin D status (<30 nmol/L). The rise in $25(OH)D_3$ was higher in 4 IU/g vitamin D_3. Interestingly, rats in 2 and 4 IU/g vitamin D_3 groups responded with greater lean mass and lean mass accretion indicating that increased dietary vitamin D above the recommended dosage (1 IU/g vitamin D_3) improved body composition in weanling rats and that lean mass accretion appears to be a priority even over building storage pools of $25(OH)D_3$ in rats receiving 2 IU/g vitamin D_3. The effect of vitamin D on lean mass accretion in growing mammals in a controlled environment provided evidence that vitamin D does have a link to body composition and supports a leaner body phenotype. Collectively, with the reported results, the hypothesis that improving vitamin D status at or above 50 nmol/L during growth favors lean mass development is accepted.

According to these findings, the increments in lean body mass could be also ascribed to greater IGF-1 concentration, as the highest dietary vitamin D_3 maintained IGF-1 concentration longer in association with greater lean mass accretion. This appears to be through complex mechanisms as skeletal muscle is shown to be responsive to both IGF-1 and $1,25(OH)_2D$ for

tissue growth and metabolism (32). As discussed in detail in chapter 2, this could be through indirect (epi)genomic mechanism that enhances IGF-1 production and increases its concentration in the blood; likely via the role of $1,25(OH)_2D$ in regulation of the pituitary gland and growth hormone production (303). Pituitary cells secreting GH express VDR, and *GH* gene contains VDREs in the promoter (139), suggesting a direct role for $1,25(OH)_2D$ in GH production and consequently induction of GH-stimulated IGF-1. In addition, $1,25(OH)_2D$ increases circulating IGFBP-3 via direct transcriptional induction of its gene. Accordingly, IGFBP-3 extends IGF-1 stability and prevents its clearance from the circulation (304).

Aside from vitamin D and IGF-1 intertwined actions in skeletal muscle, IGF-1 itself is a mediator of growth and promotes pre- and postnatal linear growth (295). IGF-1 has been shown to have growth stimulating effects independent from GH, particularly early during development (305). In a rodent study, IGF-1 receptor (IGF-1R) knockout mice model presented smaller muscles and decreased cross-sectional area of myofiber compared to their wild-type counterparts (306). Using C2C12 myoblast cell line, adding IGF-1 increased proliferation, and further induced fusion and hypertrophy of myotubes (307). This body of evidence suggests a direct role for IGF-1 in skeletal muscle development through autocrine/paracrine signalling rather than systemic and endocrine pathways.

According to the findings of the present study, fat mass accretion occurred consistently over the course of the study and aligned with increases in leptin concentrations. However, serum leptin and fat mass were not affected by modest increases in dietary vitamin D and achievement of healthy vitamin D status. Leptin is a hormone that is predominantly secreted by adipocytes. Expression of leptin in human fetal and placental tissues is indicative of its essential role during

early development (308). As part of normal postnatal growth pattern, leptin concentrations increase in relation to normal acquisition of adipose tissue (309). In mice, $1,25(OH)_2D$ has been shown to promote differentiation of preadipocyte into adipocytes, it also stimulates the expression of leptin at the tissue level.

In contrast, leptin limits the activation of 25(OH)D into $1,25(OH)_2D$, by inhibiting 1-α hydroxylase activity (310). According to findings from a systematic review and meta-analysis of observational studies and randomized controlled trials, megadose and long-term vitamin D supplementation were associated with increased leptin concentrations in young human adults (311). However, in an adult animal model, high dose oral administration of vitamin D reduced leptin in visceral adipose tissue of 6 to 9 months old obese female Wistar rats suggestive of a role for vitamin D in reducing markers of inflammation in obesity condition (312). This body of evidence demonstrates an interaction between vitamin D and leptin in adipose tissue. In the present study, serum leptin concentrations were not affected by dietary vitamin D intake. Since leptin secretion is directly related to adipose tissue mass accretion, lack of difference in fat mass and fat mass accretion might help to explain partly the lack of differences in leptin concentration among groups. In addition, this could be ascribed to other important mediators influencing early life growth and development and that the interaction of vitamin D and leptin may become more evident over time and likely in a more mature system. Moreover, based on early reports on tissue distribution of 25(OH)D (313, 314), it appears that a significant proportion of circulating 25(OH)D is taken up by skeletal muscle mass and this is perhaps more pronounced in early rapid period of life due to accelerated muscle growth rates and exceptionally high rate of protein

synthesis, (103) and vitamin D direct involvement in muscle tissue growth and development (104).

Some of the limitations of this research are the secondary use of data and small sample size. Inherent to the nature of the secondary analysis, the available data were not collected to address this particular research question regarding the effectiveness of dietary vitamin D_3 on lean mass. The original study by Djekic-Ivankovic et al (49) was powered at 80% for the primary outcome, which was bone density, however the post hoc power calculation of this study (using the procedure described by Kononoff (211)), was at 65%. Even though, the current study design with six animals per group was sufficient to detect changes in lean mass and lean mass accretion by week 8 of the study, there is a need for replication of this study with a larger sample size to confirm these findings, especially for the intermediate vitamin D dose. In addition, as mentioned earlier in chapter 3, all rats had basal serum $25(OH)D_3$ concentration <30 nmol/L, thus this study design is lacking a control group with sufficient vitamin D status at baseline. The presence of a comparable control group with vitamin D sufficiency could reduce bias and help limit the influence of other unmeasured variables associated with vitamin D deficiency and improve the quality of the inferences of (possible) post-intervention trajectories in body composition in response to dietary vitamin D supplementation. Even though the assay technique releases IGF-1 from its carrier protein and thus measures total serum IGF-1 concentration (bound and free IGF-1), the present study lacks a more detailed assessment of the GH/IGF-1 axis, including measurements of GH as the main regulator of IGF-1 in tissues including liver, lean mass and bone periosteum (305). Additionally, the study is lacking direct measurements on $1,25(OH)_2D$

metabolite and thus was unable to provide more insight into the dynamics of $1,25(OH)_2D$ and IGF-1 in the context of lean tissue growth.

These findings have important implications. This animal model was designed to mimic the response to vitamin D supplementation in newborn infants born with insufficient vitamin D status (study 3, chapter 5) and provided objective evidence that improving vitamin D status confers benefits to accretion of lean mass during rapid period of growth. The findings of the current study provided insights into the opportunity to replicate the results in human infants using more rigorously designed trial in order to understand how these findings would translate into human research.

Hypothesis 2:

Exposures to maternal excess adiposity preconception and maternal insufficient vitamin D status adversely affect neonatal vitamin D status and body composition.

Study two (chapter 4) demonstrated that in healthy mother-infant dyads, maternal pre-gravid and postpartum adiposity have important implications in both maternal and neonatal vitamin D status. Pre-pregnancy BMI ≥25 kg/m^2 was associated with 13.8 nmol/L and 13.3 nmol/L lower maternal and neonatal serum 25(OH)D concentration, respectively. This is of high importance as according to the data from the retrospective cohort of the current study consisting of 1035 healthy mother-infant dyads, 20.8% of newborn infants were born with vitamin D deficiency (315) and in fact the odds for vitamin D deficiency were higher with maternal BMI >25 kg/m^2 (OR_{adj}: 1.82, 95%CI: 1.18, 2.81). Similarly, according to the same report, the odds for inadequate vitamin D were also higher with pre-pregnancy BMI >25 kg/m^2 (OR_{adj}: 1.70, 95%CI: 1.20, 2.41) (236) (Appendix 4).

These findings add to the body of evidence that serum 25(OH)D concentration of pregnant women with elevated BMI is lower compared to the leaner counterparts (19, 316-318) and subsequently their offspring are born with lower vitamin D status (38, 319). Healthy term born infants dually exposed to insufficient maternal vitamin D status and elevated pre-pregnancy BMI had 220.4 grams higher whole body fat mass in the early postnatal period compared to infants of mothers with elevated pre-pregnancy BMI yet vitamin D sufficiency. This difference is of particular interest as infants included in this analysis were all healthy term and AGA, with no differences in type of feeding with almost all receiving breastmilk at the postpartum visit, thus the divergent pattern in fat accumulation observed in neonatal period is mainly reflective of intrauterine environment that is influenced by maternal nutrition and weight status. Based on these findings the hypothesis that the association between maternal vitamin D status and elevated fat mass in neonates is modified by maternal excess adiposity preconception, is thus accepted.

Based on food frequency questionnaire (FFQ) data analysis, surveyed at initial baseline visit to reflect pregnancy, and consistent with national datasets (320), vitamin D intake from food was approximately 200 IU/d on average among all mothers in the present study suggesting that food sources of vitamin D are inadequate to meet the Estimated Average Requirement (EAR) needs of 400 IU/d, however total vitamin D intake from food and supplements was on average above 400 IU/d. These results, in line with other Canadian reports (318, 321, 322), suggest that the primary source of maternal vitamin D intake is prenatal supplementation that largely contributes to meet the requirements. In the present study the majority of mothers (92.3%) within all BMI ranges took a multivitamin supplement containing vitamin D with a median dose of 600

IU (95%CI: 400, 600) during pregnancy. The compliance was equally high, with 90.1% taking the supplements daily or almost daily.

More than half (57.1%) of the mothers with elevated BMI had sufficient 25(OH)D concentrations and the mean (SD) serum 25(OH)D of those mothers classified as below <50 nmol/L was 40.4 nmol/L (7.6) with only two mothers being classified as vitamin D deficient (25(OH)D <30 nmol/L). This is likely due to high adherence to prenatal supplementation containing vitamin D. These results are in line with recent pregnancy reports showing that women with elevated BMI and taking multivitamins have adequate vitamin D status (323, 324). However, among infants born to mothers with elevated BMI, 79.6% had vitamin D insufficiency, and a concerning proportion of 36.7% were vitamin D deficient. This could be partly due to limited placental transfer of 25(OH)D in pregnant women with overweight/obesity condition due to increased sequestration of 25(OH)D in excess adipose tissue and subsequently decreased 25(OH)D bioavailability to the circulation (205). In addition, maternal obesity stimulates low-grade inflammation that may result in placental dysfunction due induced accumulation of pro-inflammatory mediators and macrophage population at the maternal-fetal interface (325).

Recent evidence suggests that excessive fat mass accretion in the developing fetus and newborn are associated with elevated fat, altered lipid metabolism and markers of adiposity in childhood (326) and demonstrated that early life is a critical period of programming for childhood obesity and cardiometabolic conditions later in life. Many studies have demonstrated links between pre-pregnancy BMI and later adiposity in newborn infants (101, 247, 327, 328).

Consistently, pregnancy cohorts have linked maternal vitamin D deficiency/insufficiency with fetal body composition programming, and have shown that low maternal-fetal transfer of vitamin D is associated with higher fat mass and lower lean mass in the neonatal period (80) and later on during childhood (20, 21). Even though due to the cross-sectional nature of the present study, cause and effect relationship could not be established, the findings of chapter 4 are essentially indicative of a protective effect of maternal sufficient vitamin D status in relation to neonatal adiposity. The results point out that maternal serum 25(OH)D <50 nmol/L is not a factor necessarily linked to higher fat mass in neonates, except if the effects are combined with maternal excess adiposity. The findings did not show any links to neonatal lean mass; it appears changes in lean tissue may evolve over time, however due to the nature of the present research, it was not possible to explore patterns or within-individual changes in body composition over time. Additionally, a rapid growth spurt occurring in the first postnatal month can obscure minor compositional changes that become more evident over time later in infancy and childhood. Hence, longitudinal studies are required to investigate natural patterns of changes in fat and lean mass over time and to see whether the observed differences in neonatal fat mass persist into older ages in infancy. It is also essential to explore whether achievement of sufficient serum 25(OH)D early in life through postnatal vitamin D supplementation, would overcome the intrauterine exposures to maternal pre-pregnancy weight status and vitamin D insufficiency and reset the adiposity trajectory with long-term benefits in body composition and overall health.

Hypothesis 3:

Neonates born with insufficient vitamin D status and provided with the standard of care, 400 IU/d vitamin D_3, would have lower lean mass and lean mass accretion by 3 months

of age and thereafter by 12 months compared to infants provided with higher dosage of 1000 IU/d vitamin D_3.

Study three (chapter 5) showed a positive dose-response relationship of serum 25(OH)D concentrations relative to vitamin D dosage. Supplementation with 400 or 1000 IU/d improved vitamin D stores, achieved healthy target of serum 25(OH)D ≥50 nmol/L and sustained from 3 to 12 months of age in both trial groups. The finding from current trial in line with health guideline recommendations (61, 329) confirm that the Adequate Intake value of 400 IU is sufficient to support recovery of building vitamin D status even in infants born with low stores. However, the higher dosage of 1000 IU/d, modestly increased whole body lean mass without altering weight or length z-scores for age and sex by 12 months of age. Since the difference in lean body mass was only evident at 12 months of age, the hypothesis is partly accepted. These results indicate a role for vitamin D in enhancing lean body composition in infancy.

Previous work by Hazell et al. (4) was the first to report changes in body composition in infancy based on vitamin D status. They studied infants who were mostly vitamin D-replete at 1 month postpartum and supplemented with incremental dosage of vitamin D (400, 800, 1200 and 1600 IU/d) for 11 months. Even though no differences were observed across groups in body composition, Hazell et al. demonstrated a positive relationship between plasma 25(OH)D_3 concentrations and lean mass at 12 months (4) that was sustained at 36 months of age (9). The present trial is novel in that the difference in lean mass was observed between the trial groups. It is worth mentioning that the Hazell et al. (4) study consisted of a large proportion of infants with baseline 25(OH)D concentrations ≥50 nmol/L who were born to well-educated, high-income mothers of European ancestry. The present trial of infants from a lower and more diverse

sociodemographic population highlights that even though trial infants were born with insufficient vitamin D status, the sociodemographic differences between Hazell's study and the present trial was not a factor in adherence or the response to supplementation. As mentioned in detail in chapter 5, both trial groups responded well to vitamin D supplementation with high adherence, thus the findings of the present trial better address sociodemographic diversity than prior studies and aid the generalizability of the findings to multi-ethnic populations to improve health equity and equality.

Although the evidence is limited in infants, there are RCTs suggesting that vitamin D supplementation increases lean soft tissue in younger children, adolescents and adults. For instance, in healthy young children 2 to 8 years of age, vitamin D intake of 400 IU/d over 6-month RCT improved lean mass accretion suggesting that vitamin D confers an advantage in body composition and particularly in lean mass development in children (91). In addition, in a 12-month RCT in prepubertal girls with vitamin D insufficiency, the percentage change in lean mass was greater in those receiving a 14000 IU/week vitamin D supplements compared to the placebo group; even though no difference was observed in body weight between groups (212). These results are in line with the observations from study 1 (chapter 3) where vitamin D deficient weanling rats that received 4 IU vitamin D_3/g diet responded with higher 25(OH)D concentrations and greater lean mass accretion compared to the control group at week 8 of the study (75). In addition, in the 24-month post-trial follow-up of the present study, forty-six children (33.1%) who completed the original trial and returned for the 24-month follow-up had available whole body DXA scan (23 males, 23 females) and were included in the analysis. In consistent with the 12-month trial results, fat mass did not emerge as a factor in the response of

serum 25(OH)D to supplementation. While the mean serum 25(OH)D was maintained ≥75 nmol/L, interestingly, children in the trial 1000 IU/d group had higher whole body lean mass compared to their counterparts in the 400 IU/d group, with the magnitude of difference of 381.3 grams (5.1% difference), which is similar to 322.1 grams (4.8% difference) observed at 12 months of age. To the best of author's knowledge, this trial is one of the first studies that focused on body composition and provides novel findings that suggest correction of serum 25(OH)D concentrations early in life programs for a leaner body composition at 12 months of age which appears to be sustained into second year of life.

The results of the present trial in human infants concur with observations in weanling rats (chapter 3), provide objective evidence that vitamin D has a role to play in programming body composition. The observed link between vitamin D and body composition is mainly ascribed to differences in lean mass rather than fat mass. Even though the present trial could not provide a possible mechanism for the role of vitamin D in lean body composition, lean tissue appears to be the main target for vitamin D actions. This is perhaps due to the role of the biologically active $1,25(OH)_2D$ in regulating the transcription activity of the genes involved in proliferation and differentiation of skeletal muscle cells (113) and thus promoting muscle formation in response to increased needs for tissue growth particularly in early period of life. In addition, $1,25(OH)_2D$ coupled with VDR-RXR promotes IGFBP-3 gene transcription, and thus stimulates IGFBP-3 production and activity (137). IGFBP-3 is essential for controlling delivery of IGF-1 to its receptors at the tissue level. IGF-1 has well-established role in pre- and postnatal muscle growth and development by promoting the uptake of amino acids into the muscle cells and enhancing protein synthesis (330). In addition, early during postnatal life IGF-1 is more responsive to

nutritional intake and insulin secretion whereas growth hormone turns to have a stronger influence on IGF-1 later in infancy (296) this could help to better explain a higher lean body mass phenotype observed later in infancy at 12 months. As discussed in detail in chapter 2, earlier research demonstrated that skeletal muscle cells express VDR (116, 202, 331), mainly during early stages of muscle formation and development, or muscle regeneration after an acute injury (32). In skeletal muscle cell line, adding either 25(OH)D or $1,25(OH)_2D$, increased myofiber diameter and altered gene expression confirming the autocrine and paracrine actions of vitamin D in skeletal muscle (113). In addition, in murine C2C12 myotubes, adding $1,25(OH)_2D_3$ increased expression of insulin receptor and VDR mRNA and sensitized the Akt/mTOR pathway and enhanced protein synthesis in skeletal muscles which is essential for muscle growth during development (332). Consistently, the vitamin D signaling pathway plays an integral role in maintaining normal concentrations of calcium and phosphate in the blood and skeletal muscle cells require calcium for normal development and function (333).

As mentioned earlier in chapter 5, the data demonstrated a positive dose-response relationship of serum 25(OH)D concentrations relative to vitamin D dosage. Overall, serum 25(OH)D increased from baseline to 3 months and plateaued or slightly declined afterwards, this pattern could be ascribed to increased tissue demand and enhanced utilization of vitamin D metabolites for postnatal growth (50). In the present analysis, sex of infant, breastfeeding status, skin tone, and UVB period at birth were also explored as important covariates when comparing serum 25(OH)D among vitamin D supplement groups. Based on the present trial data neither infant sex nor breastfeeding status emerged as a factor in the response of serum 25(OH)D to supplementation. Even though overall, FI-III skin tone and vitamin D synthesizing period at birth

were positive predictors of on average higher serum 25(OH)D concentrations, these factors did not appear significant after pair-wise comparison tests. At baseline, 3-, 6-, and 12-months follow-up, serum 25(OH)D concentrations of infants born in vitamin D synthesizing period were 4.5, 10.9, 10.9, and 8.3 nmol/L higher compared to infants born in non-synthesizing seasons and reached significance only at 3 and 6 months. Similar magnitude of differences was observed in infants with FI-III compared with IV-VI skin tone at each time-point, although this trend did not reach statistical significance at any time-point and suggesting that the response of 25(OH)D concentration to vitamin D supplementation is not affected by skin tone of infants.

Strengths of this study are the longitudinal RCT design and strict compliance with CONSORT (Appendix 5), inclusion of ethnicity diverse infants, and rigorous assessment of body composition. Body composition was evaluated using whole body DXA scan which provides measures of infant bone-free lean tissue and fat mass content with a high degree of accuracy (154). This method is highly useful for tracking changes in lean and fat mass accretion over time (157) and in contrast to methods using predictive equations, DXA has shown sufficient reliability and reproducibility in measurement of lean and fat mass (159). However, due to unique challenges that were discussed in detail in chapter 2, the use of DXA in studies in infants is limited, and thus normative body composition data in this population group is lacking. To mitigate this, outside of the present trial, a healthy reference group with sufficient vitamin D status was established and were given the AI of 400 IU/d with all of the same measurements as the trial groups. To help limit the effect of maternal adiposity preconception as a confounding factor associated with adverse infant body composition, only infants were included in the reference group if their mothers had a pre-gravid maternal BMI between 18.5 and 27.0 kg/m^2.

Thus, the reference group provided important data regarding normal growth and body composition in healthy infants born to healthy women, and the data was used to track growth pattern consistently along with the trial.

The present trial employed randomization and double blinding techniques to ensure random allocation to trial groups and to avoid the influence of outcome bias and to make the reference and both trial groups as balanced as possible. The randomization was unique as infants were stratified by their measured skin tone (based on Fitzpatrick descriptor: I-III vs. IV-VI) which is reflective of endogenous synthesis of vitamin D_3 and helps to understand the biological response to vitamin D supplementation among groups. Skin tone was established using a spectrophotometer by measuring constitutive pigmentation (334). In contrast to other alternative and commonly used subjective methods of skin tone assessment such as visual inspection by trained individuals, comparative color tiles, as well as written skin phototyping guidelines (335), this method is accurate and allows for more reliable quantification of skin pigmentation.

In the present trial, the higher dosage of 1000 IU/d of vitamin D was used as a safe (10, 61) and accepted dosage (12) as the tolerable upper intake level (UL) of vitamin D for infants from birth to 6 months of age was set at 1000 IU/d, and 1500 IU/d from 6 to 12 months (61). Nonetheless, vitamin D status was closely monitored until 6 months of age as the UL for vitamin D thereafter is higher and none of the safety assessments including measures of ionized calcium (iCa), serum 25(OH)D as well as urine calcium and phosphate concentrations indicated adverse effects of the 1000 IU/d vitamin D supplement.

Even though at each time-point some infants were lost to follow up, from the reference, 400 IU/d and 1000 IU/d groups, 78%, 73.5%, and 86% returned for the follow-up at 12 months

with attrition rate 22%, 26.5%, and 14.3% respectively, and the sample size was sufficient for a repeated measures design. In evaluating body composition over time, differences among trial groups were tested using the existing data and without imputing the number of missing observations. A thorough comparison of infants with missing DXA scan and those with completed data were consistent with data missing at random.

The present study provides unique sex-specific information regarding body composition over the first 12 months of life. The observed sex differences in body weight and length consistent with previous reports (336, 337) indicate that male infants are generally heavier and taller compared to female infants. Overall, lean body mass differed between male and female infants at all time points, with males having higher lean mass compared to female counterparts; this divergent pattern in body composition is mainly ascribed to anabolic effects of testosterone as a key male hormone with established roles in limiting fat accumulation and inducing protein synthesis and lean mass accretion (336, 338) in infants and later in adult men (274). Even though on average fat percentage was higher in female infants compared to males, this difference appeared significant only at baseline and 6 months and no sex difference was observed in terms of fat mass at any time-point. These findings are suggesting that sex differences in body weight is mainly attributed to sex dimorphism in lean mass and fat mass does not appear as a major component.

In the present study, as discussed earlier in this section, despite slightly higher proportion of males in the trial groups (59%), the change in serum 25(OH)D concentration in response to vitamin D supplementation did not differ between male and female infants with high proportion (93%) of infants in both trial groups achieving and maintaining the cut-point for sufficiency from

3 to 12 months. Consistent with previous reports by Gallo et al. (257), which indicated that the response of healthy term born infants to vitamin D supplementation (at 400 IU/d) is inversely associated with basal vitamin D status (257), in the present study, the higher increases were observed at lower basal 25(OH)D concentrations. The fact that ~35% of infants in combined trial groups were born with vitamin D deficiency and started supplementation with serum 25(OH)D on average <50 nmol/L and receiving at least 400 IU/day supplementation with high compliance likely contributed to rapid improvement of serum 25(OH)D concentration in response to vitamin D supplementation.

The current randomized controlled trial was limited by selective drop-out. Families in the trial groups who completed follow-ups had higher household income, with borderline higher proportion self-identified as white and being primiparous mothers who were more likely to attend and complete the study. In addition, higher proportion of completers were born in vitamin D non-synthesizing period and had higher gestational weight gain compared to those who were lost to follow-up, although there were no differences between completers and dropouts in meeting the IOM recommendations for weight gain throughout gestation. In the reference group however, the only difference observed was that higher proportion of completers attained a university degree versus the dropouts. All these differences may have affected the strength of the observed effect size. Furthermore, the present study lacks a more detailed assessment of key vitamin D metabolites including 24,25$(OH)_2$D, and 3-epimer-25(OH)D in the response to vitamin D supplementation. Mechanistic inferences were also limited due to lack of data on serum concentrations of 1,25$(OH)_2$D and VDBP. One of the most important limitations of the present study was that the additional follow up of 24- and 36-month post-intervention were

underway once COVID-19 pandemic happened. That affected the final number of infants available for the follow-up analyses and thus due to closure of the study, full capture of longitudinal data to assess the long-term effects of supplementation on the functional changes in body composition was not possible.

6.2. Implications to policy and public health

According to the findings already presented in this dissertation, multiple important maternal, demographic, and lifestyle factors linked to maternal and neonatal vitamin D status have been explored including maternal excess adiposity, maternal self-reported population group (white/other groups), maternal physical activity prior to/during pregnancy, as well as maternal multivitamin supplementation use. Some of these important correlates that are presently within health policy include weight-management strategies for women of reproductive age or those who commence pregnancy with overweight/obesity condition and recommendations on appropriate dietary modifications, promoting physical activity while reducing sedentary behaviours (248, 262, 339). Even though most national health guidelines emphasize the importance of nutrition counseling prior to conception for women who commence pregnancy with overweight/obese condition, they do not provide targeted recommendations for vitamin D supplementation in this population group (248, 249). According to the findings presented in this thesis, it can be inferred that women who commence pregnancy with an overweight/obese condition should seek nutrition counselling regarding the required dosage of supplementation and to be advised to initiate taking of a multivitamin supplement containing vitamin D as soon as possible, ideally prior to conception to ensure sufficient maternal-fetal transfer of vitamin D. In addition, more educational strategies need to be developed to reinforce vitamin D supplementation

recommendation of women at risk of reduced cutaneous vitamin D synthesis due to having darker skin pigmentation, for those who cover their skin, or those residing in far north latitude, in clinical practice guidelines, to ensure health equity and equality.

In view of limited high-quality trials, the IOM was unable to establish an EAR or Recommended Dietary Allowance (RDA) for infants younger than 12 months of age, and thus the AI of 400 IU/d has been developed for vitamin D intake across infancy (61). In the present trial, the 400 IU/d dosage of vitamin D was based on the IOM recommendation to maintain serum 25(OH)D concentration in the range of 40 to 50 nmol/L and appears to be desirable for infants (61). According to IOM guidelines (54), a value of 40 nmol/L, is established consistent with median dietary requirement and aligned with EAR; however, the target of serum 25(OH)D concentration of 50 nmol/L is adequate for bone health optimization in almost all individuals and is consistent with the RDA and that 97.5% of the population achieving adequacy. The AI of 400 IU/d value is set for all healthy breastfed or partially breastfed infants across infancy and in fact regardless of vitamin D status at birth. The present trial showed that 400 IU/d supports recovery of building vitamin D stores even if infants are born with insufficient vitamin D status, darker skin tone categories (FIV-VI) or those born in vitamin D non-synthesizing period (340, 341).

Due to the randomized controlled design this study provides valuable information on the effects of vitamin D supplementation in populations with variable vitamin D status ranging from vitamin D deficiency to sufficient status. The implications of the present research are important as the findings add to the existing evidence to help health agencies in derivation an EAR from AI for vitamin D across infancy (0-12 months). The findings are of high significance as the observed benefits in lean mass in human infants evident at 12 months even in small magnitude

has the potential for large population benefits and may contribute to early prevention of childhood obesity and subsequent health outcomes. Vitamin D supplementation is a modifiable factor that is already within health care recommendations for all breastfed term born infants. These findings indicate that 400 IU/d vitamin D is sufficient in building of healthy vitamin D status, but in terms of functional outcomes, higher amounts of 1000 IU/d supplementation may better support leaner body phenotype.

6.3 Future directions

According to the present trial data and findings from weanling rats, important outputs are in hand regarding correction of neonatal vitamin D status and the impacts on body composition and growth pattern. Using samples already collected from infants during the study follow-ups for this purpose, further characterization of the epigenetic mechanisms related to vitamin D metabolism and body composition will help to explain how improving vitamin D status with 1000 IU/d of supplementation enhanced accretion of lean body mass. Even though the epigenetic component of the present trial is beyond the scope of this dissertation, the results will specifically inform the researchers of the mechanisms responsible for the observations in lean body mass and vitamin D metabolism and will offer novel explanations for the phenotypic persistence.

These mechanisms will focus on epigenetic regulation of the genes and enzymes involved in vitamin D metabolism as well as IGF-1 and IGFBP-3 that have well-known functions in growth and musculoskeletal health outcomes. In the development of high throughput methods for assessment of DNA methylation, 850K array technique provides comprehensive genome-wide methylation pattern and offers an overall insight into epigenetic changes by examining over 850,000 CpGs using whole-genome bisulfite sequencing (342). Unlike commonly used

pyrosequencing method that only targets short regions (80-200 bp long), 850K array provides not only an overall overview of the methylation status across the genome, but also methylation at the level of each CpG and this method has potential of revealing novel vitamin D responsive genes. This epigenetic assay technique will thus offer novel explanations for the observed phenotypic changes in lean mass. To expand the epigenetic research, it is also important to examine functional variants in genes involved in the vitamin D metabolism pathway such as *VDR*, *CYP2R1*, *CYP24A1*, *GC* (vitamin D binding protein), and *CYP27B1* that had been previously recognized by genome-wide association studies or population studies to be linked to altered 25(OH)D concentrations (343-346). In the event of low maternal-fetal-infant transfer of vitamin D these findings will be valuable to the researchers and will help to broaden the knowledge of the involved mechanisms.

6.4 General conclusion

In summary, this dissertation has tested the effects of vitamin D_3 on body composition and growth-related hormones in weanling Sprague Dawley rats fed diets with different vitamin D content (1, 2, or 4 IU/g) from 4 to 12 weeks of age, explored the influential factors related to maternal and neonatal vitamin D status and neonatal body composition, and also tested whether correction of insufficient vitamin D status using 400 or 1000 IU/day vitamin D_3 early in the neonatal period, normalizes body composition (lean and fat mass) across infancy in a well-controlled trial. The results presented throughout the dissertation suggested that in weanling rats improving low vitamin D status results in a lean body phenotype. Natural declines over time in IGF-1 were lower in the 4 IU versus control group, suggesting better stimulus for lean mass accretion. Further, maternal adiposity and compromised maternal-fetal vitamin D transfer were

associated with greater whole body fat mass in the neonates, this is mainly reflective of intrauterine environment that is influenced by maternal nutrition and weight status. This dissertation confirmed that in infants born with vitamin D insufficiency, supplementation with 400 or 1000 IU/d improved vitamin D status. In different terms, this dissertation provided evidence that the AI value of 400 IU/d seems sufficient to compensate for inadequate maternal-fetal transfer of vitamin D when there is high adherence to supplementation. Lastly, this research project is one of the first trials to focus on body composition and provided valuable information on the effect of vitamin D_3 supplementation on lean soft tissue acquisition and indicated that the higher dosage of 1000 IU/d, enhanced whole body lean mass without altering growth pattern by 12 months of age that was sustained into second year of life, all of which favorably contributes to body composition and overall health. Whether the benefit to lean mass is maintained in long-term in relation to reduced risk of excess adiposity in children requires further research.

6.5 General References

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APPENDIX

Appendix 1

NEWCASTLE - OTTAWA QUALITY ASSESSMENT SCALE

COHORT STUDIES

Note: A study can be awarded a maximum of one star for each numbered item within the Selection and Outcome categories. A maximum of two stars can be given for Comparability

Selection

1) Representativeness of the exposed cohort

a) truly representative of the average _____ (describe) in the community □

b) somewhat representative of the average _____ in the community □

c) selected group of users eg nurses, volunteers

d) no description of the derivation of the cohort

2) Selection of the non-exposed cohort

a) drawn from the same community as the exposed cohort □

b) drawn from a different source

c) no description of the derivation of the non-exposed cohort

3) Ascertainment of exposure

a) secure record (eg surgical records) □

b) structured interview □

c) written self-report

d) no description

4) Demonstration that outcome of interest was not present at start of study

a) yes □

b) no

Comparability

1) Comparability of cohorts on the basis of the design or analysis

a) study controls for _____ (select the most important factor) □

b) study controls for any additional factor □ (This criterion could be modified to indicate specific control for a second important factor.)

Outcome

1) Assessment of outcome

a) independent blind assessment □

b) record linkage □

c) self-report

d) no description

2) Was follow-up long enough for outcomes to occur

a) yes (select an adequate follow up period for outcome of interest) □

b) no

3) Adequacy of follow up of cohorts

a) complete follow up - all subjects accounted for □

b) subjects lost to follow up unlikely to introduce bias - small number lost - > ____ % (select an adequate %) follow up, or description provided of those lost) □

c) follow up rate < ____% (select an adequate %) and no description of those lost

d) no statement

CODING MANUAL FOR COHORT STUDIES

Selection

1) Representativeness of the Exposed Cohort

Item is assessing the representativeness of exposed individuals in the community, not the representativeness of the sample of women from some general population. For example, subjects derived from groups likely to contain middle class, better educated, health-oriented women are likely to be representative of postmenopausal estrogen users while they are not representative of all women (e.g. members of a health maintenance organisation (HMO) will be a representative sample of estrogen users. While the HMO may have an under-representation of ethnic groups, the poor, and poorly educated, these excluded groups are not the predominant users of estrogen).

Allocation of stars as per rating sheet

2) Selection of the Non-Exposed Cohort

Allocation of stars as per rating sheet

3) Ascertainment of Exposure

Allocation of stars as per rating sheet

4) Demonstration That Outcome of Interest Was Not Present at Start of Study

In the case of mortality studies, outcome of interest is still the presence of a disease/ incident, rather than death. That is to say that a statement of no history of disease or incident earns a star.

Comparability

1) Comparability of Cohorts on the Basis of the Design or Analysis

A maximum of 2 stars can be allotted in this category

Either exposed and non-exposed individuals must be matched in the design and/or confounders must be adjusted for in the analysis. Statements of no differences between groups or that differences were not statistically significant are not sufficient for establishing comparability. Note: If the relative risk for the exposure of interest is adjusted for the confounders listed, then the groups will be considered to be comparable on each variable used in the adjustment.

There may be multiple ratings for this item for different categories of exposure (e.g. ever vs. never, current vs. previous or never)

Age = ☆, Other controlled factors = ☆

Outcome

1) Assessment of Outcome

For some outcomes (e.g. fractured hip), reference to the medical record is sufficient to satisfy the requirement for confirmation of the fracture. This would not be adequate for vertebral fracture outcomes where reference to x-rays would be required.

a) Independent or blind assessment stated in the paper, or confirmation of the outcome by reference to secure records (x-rays, medical records, etc.) ☆
b) Record linkage (e.g. identified through ICD codes on database records) ☆
c) Self-report (i.e. no reference to original medical records or x-rays to confirm the outcome)
d) No description.

2) Was Follow-Up Long Enough for Outcomes to Occur

An acceptable length of time should be decided before quality assessment begins (e.g. 5 yrs. for exposure to breast implants)

3) Adequacy of Follow Up of Cohorts

This item assesses the follow-up of the exposed and non-exposed cohorts to ensure that losses are not related to either the exposure or the outcome.

Allocation of stars as per rating sheet

Appendix 2

Permission to use Copyrighted Material in a Doctoral Thesis

Name: Maryam Razaghi
Email address: maryam.razaghi@mail.mcgill.ca
Date: 8/10/2021

Dear Dr. Weiler,

I would like your permission to use your modified figure on "Vitamin D in Skeletal Muscle" in my PhD thesis entitled "*Vitamin D status in early life and body composition: Implications to programming of a lean body phenotype*". I am requesting to grant your permission for use of the above material in both print and electronic formats of my dissertation to be archived at McGill University library. Proper acknowledgement of your copyright of the reprinted figure will be given in the thesis.

If these arrangements meet with your approval, please sign where indicated below. Thank you very much.
Kind regards,
Maryam Razaghi

Permission Granted for the Use Requested Above

Authorized by: Hope Weiler, Adjunct Professor, School of Human Nutrition, McGill University

Signature/date:

Hope Weiler Digitally signed by Hope Weiler
Date: 2021.08.10 15:38:43 -04'00'

Appendix 3

R: Permission to use a figure from a publication

pietro.ameri@unige.it <pietro.ameri@unige.it>
Sat 7/10/2021 8:54 AM
To: Maryam Razaghi <maryam.razaghi@mail.mcgill.ca>

Dear Maryam,

Yes you can. Good luck with your dissertation!

Best regards,

Pietro Ameri

Da: Maryam Razaghi <maryam.razaghi@mail.mcgill.ca>
Inviato: venerdì 9 luglio 2021 18:26
A: pietroameri@unige.it
Oggetto: Permission to use a figure from a publication

Hi Dr. Ameri,

This is Maryam Razaghi from McGill University, Canada. I am writing to you today to request permission to use your figure from the Clinical Endocrinology (2013) 79, 457–463 a publication titled: ***Interactions between vitamin D and IGF-I: from physiology to clinical practice*** in my PhD thesis. I would appreciate it if I can cite **Figure 2** (Schematic representation of vitamin D-IGF-I interactions) and use it in my dissertation.
Thank you in advance,

Kind regards,
Maryam

Appendix 4

Disparities in vitamin D status of newborn infants from a diverse sociodemographic population in Montreal, Canada

Hope A. Weiler[1,2], Catherine A. Vanstone[2], Maryam Razaghi[2], Nathalie Gharibeh[2], Sharina Patel[2], Shu Qin Wei[3], Dayre McNally[4]

[1]Nutrition Research Division, Bureau of Nutritional Sciences, Health Products and Food Branch, Health Canada, Ottawa, ON

[2]School of Human Nutrition, McGill University, Ste Anne de Bellevue, QC

[3]Institut national de santé publique du Québec, Montréal, QC

[4]Department of Pediatrics, Division of Critical Care, University of Ottawa, Ottawa, ON

Corresponding author: Hope Weiler, PhD, Research Scientist

Nutrition Research Division, Health Products and Food Branch, Health Canada

251 Sir Frederick Banting Driveway, Room E338

Ottawa, Ontario, Canada, K1A 0K9

e-mail: hope.weiler@hc-sc.gc.ca

T: 613-297-6158

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The authors state no conflicts of interest.

Hope Weiler is a member of the Journal's Editorial Board

The data described in the manuscript will not be made available because permission to share data was not requested at the time of obtaining participant consent.

Word count: 5000

Figures: 2

Tables: 3

Supplementary data submitted

Running title: Vitamin D status of newborn infants

Abbreviations: AGA: appropriate for gestational age; BMI: body mass index; CLIA: chemiluminescence immunoassay; DEQAS: Vitamin D External Quality Assessment Scheme; GA: gestational age; NIST: National Institutes of Standards and Technology; SPF: sun protection factor; 25OHD: 25-hydroxyvitamin D; UVB: ultraviolet beta; VDSCP: Vitamin D Standardization Certification Program.

Abstract

Background: Vitamin D status at birth is reliant on maternal-fetal transfer of vitamin D during gestation.

Objective: To examine vitamin D status of newborn infants in a diverse population and to subsequently identify the modifiable correlates of vitamin D status.

Design: In this cross-sectional study, healthy mother-infant dyads (*n*=1035) were recruited within 36 h after term delivery (March 2016 to March 2019). Demographic and lifestyle factors were surveyed. Newborn serum 25-hydroxyvitamin D (25OHD) was measured (standardized chemiluminescence immunoassay) and categorized as deficient (serum 25OHD <30 nmol/L) or adequate (≥40 nmol/L). Serum 25OHD was compared among categories of maternal characteristics using ANOVA. Subgroups (use of multivitamins preconception and continued in pregnancy vs. during pregnancy only) were matched (*n*=352/group) for maternal factors (ancestry, age, income, education, parity and pre-pregnancy BMI) using propensity scores; logistic regression models were generated for odds of deficiency, or adequacy.

Results: Mean serum 25OHD was 45.9 (95% CI: 44.7, 47.0) nmol/L (*n*=1035), with 20.8% (95% CI: 18.3, 23.2) deficient and 60.7% (95% CI: 55.2, 66.2) adequate. Deficiency ranged from 14.6% of white infants to 41.7% of black infants. Serum 25OHD was higher (*P*<0.0001) in infants of mothers with higher income, BMI <25 kg/m^2, exercise and sun exposure in pregnancy and use of multivitamin preconception. In the matched-subgroup, multivitamin supplementation preconception plus during pregnancy relative to only during pregnancy associated with lower odds for vitamin D deficiency (OR_{adj}: 0.55, 95% CI: 0.36, 0.86) and higher odds for adequate vitamin D status (OR_{adj}: 1.47, 95% CI: 1.04, 2.07).

Conclusions: In this study most newborn infants had adequate vitamin D status yet one-fifth were vitamin D deficient with disparities among population groups. Guidelines for a healthy

pregnancy recommend maternal use of multivitamins preconception and continued in pregnancy, emphasis on preconception use may help to achieve adequate neonatal vitamin D status.

Key words: vitamin D status, 25-hydroxyvitamin D, newborn infant, population groups

Introduction

National surveillance data on vitamin D status of newborn infants in Canada is not available. According to population standards for Canada and the USA, maintaining circulating 25-hydroxyvitamin D (25OHD) concentration in the range of 40 to 50 nmol/L is desirable for infants, whereas the risk of vitamin D deficiency symptoms increases as 25OHD falls below 30 nmol/L (1). Vitamin D status of the newborn infant is reliant on maternal-fetal transfer during gestation and achievement of maternal 25OHD of at least 50 nmol/L appears protective against neonatal vitamin D deficiency (2). Based on regional cohort studies in Canada, 16% to 32% of women in the third trimester of pregnancy have 25OHD <50 nmol/L (3-6). Similarly, 24% to 35% of infants are born with 25OHD <50 nmol/L (7-9), whereas the proportion of infants with 25OHD <30 nmol/L appears more variable ranging from 4% to 36% (7, 9-11). The majority of these studies reflect predominantly (87-98%) European ancestry (7-9), and thus it is not clear whether disparities in neonatal vitamin D status exist among other population groups. This is important to acknowledge as 22% of the Canadian population is of a visible minority (12), and 30% of births are to women born outside of Canada (13). Consequently, participant bias and associated validity of the modifiable determinants of neonatal vitamin D status and the relevance to all population groups in Canada are unclear. This makes it difficult to assess health equity and to achieve equality through improvements in public health policy by targeting modifiable factors.

Guidelines for a healthy pregnancy focus on modifiable factors for women of all population groups and include being physically active, achievement of a healthy pre-pregnancy BMI and appropriate weight gain during pregnancy (14, 15). Dietary guidance specifies that all women who could become pregnant or are pregnant take a daily multivitamin containing folic acid (16). These lifestyle recommendations are highly relevant to women of reproductive age in

Canada as 34% begin pregnancy with a BMI above the recommended range (17). Likewise, 40% of non-pregnant women have 25OHD <50 nmol/L (18) with evidence of racial disparities in vitamin D status (19). Use of a multivitamin containing vitamin D increases maternal vitamin D status, and consequently opportunity for maternal-fetal transfer of vitamin D (5, 7, 20). In a pan-Canadian pregnancy cohort study, adherence to multivitamin supplementation was high (87%) and accordingly >85% achieved 25OHD ≥50 nmol/L (6). Multivitamin use associated with higher vitamin D status across the range of pre-pregnancy BMI and appeared to be beneficial regardless of self-reported race. However, the sample was mainly of European ancestry and did not assess newborn vitamin D status, making it difficult to judge whether these modifiable lifestyle factors are influential in women and newborn infants from other population groups.

As highlighted above, pregnancy cohort studies are usually not representative of the general population of pregnant women (21). Recruitment of a representative longitudinal pregnancy and birth cohort is challenged by multiple barriers to participation including mistrust, cultural insensitivity and language barriers (22). To assess vitamin D status of newborn infants among population groups and identify tangible modifiable factors, alternative study designs are warranted. Such studies need to reflect representative diverse ancestry with the goal to inform the need for and design of more costly longitudinal cohort studies. Therefore, the objectives of this cross-sectional study were to examine vitamin D status among newborn infants of diverse population ancestry. We subsequently explored the relationships between neonatal vitamin D status and sociodemographic variables, focusing on population groups among the non-modifiable factors, as well as modifiable factors. We then aimed to determine whether the relationships between neonatal vitamin D status and the most prominent modifiable factor(s) persisted among population groups through use of propensity score matching and logistic regression.

Methods

Study population

All data were collected and analyzed as planned *a priori* to address the study objective and served as the newborn vitamin D screening phase of a double-blind randomized controlled parallel group trial on vitamin D supplementation (clinicaltrials.gov, NCT02563015). From March 2016 through March 2019, mother-infant dyads were screened for eligibility within 36 hours of delivery at the Lakeshore General Hospital in Montreal, QC. Recruitment details that were consistent with the recommended strategies to enhance cultural safety (22) included that a health care worker, typically a nurse, conducted an initial screen for eligibility criteria and accordingly requested permission for a researcher to discuss the study with the family. The study team included multilingual researchers of multicultural ancestry. Inclusion criteria for this study were singleton pregnancies of any ancestry, and healthy term infants (37-42 weeks) of appropriate weight for gestational age (GA) and sex (AGA: 10-90 percentiles) (23). Exclusion criteria were factors that relate to altered maternal-fetal transfer of vitamin D, including: maternal preeclampsia, gestational diabetes, Crohn's disease, celiac disease and liver or kidney disease, as well as infants born with small or large weight for GA and sex ($<10^{th}$ or $>90^{th}$ percentile) according to Canadian birth reference values (23). If an infant met the inclusion criteria, but already had blood sampled for newborn clinical screening for inborn errors of metabolism, they were ineligible for this study. This approach ensured only one heel lance and blood sampling occurrence for both clinical testing and the research as another approach to ensure cultural safety. Signed informed consent was obtained from mothers before administration of a questionnaire and collection of a capillary blood sample from the newborn. All study materials were available in English and French.

Ethical approval

This study was reviewed and approved by the St. Mary's Hospital Research Ethics Board that administers ethics at the recruiting hospital (SMHC 15-34). Health Canada Research Ethics Board (REB 2019-033H) and Privacy Management Division (HC-PR-2019-000024) also reviewed and approved the use of the data in this report.

Demographic/lifestyle questionnaire and pregnancy history

Population groups: Demographic information was surveyed before discharge from the hospital. Mothers self-reported which population group(s) they identify with; data were subsequently categorized into population groups using proposed standards for race-based data in Canada (24). Population groups were: white (European descent); black (African, Afro-Caribbean, African Canadian Descent); East/Southeast Asian (Chinese, Korean, Japanese, Taiwanese or Filipino, Vietnamese, Cambodian, Thai, Indonesian or other Southeast Asian Descent); Latino (Latin American, Hispanic descent); Middle Eastern (Arab, Persian, West Asian descent e.g. Afghan, Egyptian, Iranian, Lebanese, Turkish, Kurdish); South Asian (South Asian descent e.g. East Indian, Pakistani, Bangladeshi, Shri Lankan, Indo-Caribbean); or other (all others including mixed ancestry, and where mothers did not identify as white and stated other or unknown). Country of birth (Canada vs. elsewhere) was also surveyed as a factor related to vitamin D status in pregnancy (25, 26). These data were collected to better inform actions and to help reduce health inequities (24).

Other sociodemographic non-modifiable factors: Maternal age (actual years) was obtained from the hospital record whereas education and income were surveyed. Data were categorized as maternal: age (≤30, >30 y) to align with the Dietary Reference Intake life stage groups (1); highest level of education completed (high school, college, or university); and family income was categorized (<70,000, ≥70,000 Canadian dollars, or not reported) based on the median income of Canadian families/households with children (27).

History of the current pregnancy: Mother's pre-pregnancy weight, weight at delivery, and height from the hospital record were used to calculate pre-pregnancy BMI and gestational weight gain. Gestational weight gain was evaluated based on pre-pregnancy BMI status (underweight <18.5, healthy 18.5-24.9, overweight 25.0-29.9, and obese ≥30 kg/m^2) and according to weight gain guidelines (inadequate, adequate, excess) (28). Type of delivery, parity (1, ≥2), as well as infant GA at birth, birth weight, and Apgar scores were obtained from hospital records. Weight z-scores were calculated using the World Health Organization growth standards (29).

Lifestyle variables: Maternal vitamin and multivitamin supplement use (yes, no) during the 3 months prior to conception or during pregnancy were surveyed separately along with frequency of use and continuance thereafter categorized as compliant (every day, almost every day) or not compliant (2-3/week or less). Where brand was reported, the dose of vitamin D (IU) was extracted from the Drug Products, and Natural Health Products Databases of Health Canada. Other variables included smoking history (never, past, current), alcohol consumption during pregnancy (yes, no), and any indoor or outdoor physical activity during pregnancy (yes, no). Sun exposure in the third trimester was surveyed using questions adapted from the Canadian Health Measures Survey (19) including time spent outdoors between 10 am and 4 pm (0, ≤15, 16-30, >30 min/day) with skin directly exposed to sunlight. From this data a variable for exposure to ultraviolet beta (UVB) for ≥15 min/d with at least hands and face exposed was created (yes, no); if all the third trimester occurred between November 1 to March 31 the participant was assigned to the no category. Use of any sun protection factor (SPF) products, including make-up creams, was surveyed (yes, no); a response of yes was considered separately from no UVB exposure as use of SPF does not fully prevent synthesis of vitamin D (30). As a

proxy for maternal UVB exposure, a UVB category was created to represent the vitamin D synthesizing (April 1 through October 31) and non-synthesizing (November 1 to March 31) periods for Canada (31) based on date of birth. Season at birth was defined using yearly equinox and solstice dates for each year (e.g. winter (December 21-March 20), spring (March 21-June 20), summer (June 21-September 20), and fall (September 21-December 20).

Blood sample collection and 25OHD analysis

A 400-500 µl capillary blood sample (Capiject, Terumo Corp.) was collected from the heel of infants within 36 hours of birth. Samples were stored at 4 °C until transported to McGill University, then centrifuged (3000 *g* for 20 minutes at 6 °C) to obtain serum. Serum (175-250 µl) that was not analyzed immediately was stored at -80 °C until measurement of total 25OHD using an automated chemiluminescent immunoassay (CLIA; Liaison, Diasorin Inc.). Samples (*n*=12) with 25OHD below the lower limit of quantification of 10 nmol/L were assigned a value of 5 nmol/L (32). Based on a pooled laboratory sample, the mean intra- and inter-assay coefficient of variation across this study was <10%. The laboratory maintained certificates of proficiency from the Vitamin D External Quality Assessment Scheme (DEQAS) to ensure reliability of the assay and used National Institute of Standards and Technology (NIST) Standard Reference Materials (SRM) 972a Level 1-4 quality control samples to confirm accuracy of measurements during the study period. Across the study period, 75 NIST SRM concentrations were 98.2% (CV% 10.3) of certified values, and 190 DEQAS samples were 99.5% (CV% 12.4) of NIST certified values. The certified NIST concentrations were used to standardize infant serum 25OHD concentrations according to the Vitamin D Standardization Program (VDSP) protocols (33, 34), and using Deming regression (standardized concentration=0.9586 CLIA concentration + 3.399 nmol/L) to fit the CLIA measured 25(OH)D values to NIST reference measurement procedures. Neonatal serum 25OHD concentrations were then classified according

to recommendations (1) using the cut-point for adequacy of vitamin D status for infants at ≥40 nmol/L given the recommended range of 40-50 nmol/L, which aligns with targets for population assessments or groups of pregnant women. Infants were also categorized as being deficient (<30 nmol/L). The proportion (95% CI) of infants with serum 25OHD <30, 30-39.9, 40-49.9 and 50-125 nmol/L were used in describing the population distribution.

Statistical analyses

The sample size estimate for this study was *n*=2000 eligible infants invited to the study as the recruitment pool for a trial. As mentioned earlier, up to 35% of infants in studies of mainly European ancestry are born with 25OHD <50 nmol/L based on previous studies in Canada (3, 7, 9, 20). In the absence of data on the prevalence of vitamin D status at the recommended cut-point for adequacy (≥40 nmol/L) for population groups, a sample size of 576 would be sufficient to detect a prevalence of up to 60%, assuming 4% precision of the estimate and 95% CI (35).

To evaluate the potential for participant bias, continuous data of those included in the analysis were compared to those excluded (e.g., due to lack of a serum sample or missing information) using a mixed model ANOVA accounting for fixed effects of inclusion/exclusion and infant study number as a random variable. Differences in proportions among included/excluded groups were tested using Chi-square tests and when cell counts were <10, Fisher's exact test was reported.

The distribution of vitamin D status was described for the full sample using mean ± SEM (95% CI of the mean) as well as within the various vitamin D status categories using proportions (%, 95% CI). Vitamin D intake from supplements was described as median (95% range). Characteristics according to vitamin D status categories (<30, 30-39.9, and ≥40 nmol/L of 25OHD) were compared using a mixed model ANOVA accounting for fixed effects maternal characteristics (e.g., age ≤30 vs. >30 y) and infant study number as a random variable for

continuous variables and Chi-square or Fisher's exact tests for proportions. Data are reported as mean ± SEM, or *n* (%). For differences among characteristics with 3 or more categories (e.g., population groups), *post hoc* testing using Tukey's t-tests was conducted, and adjusted for multiple comparisons using Tukey-Kramer adjustment.

To help inform future studies and health policy, propensity score methodology was used to analyze the data as a nonrandomized study (36). Maternal use of multivitamins preconception *n*=404) vs. during pregnancy (*n*=561) was selected as the lifestyle-based treatment given high compliance with multivitamin use overall and because it is more readily modifiable than reducing pre-pregnancy BMI into the recommended range of 18.5-24.9 kg/m^2. Participants not taking multivitamins (*n*=70) were removed as were those with pre-pregnancy BMI <18.5 (*n*=23) prior to matching as these are not consistent with guidelines for a healthy pregnancy (14-16). Groups were then matched in a hierarchical process, first (*n*=291/group) using propensity scores generated on the basis of greedy matching of binary variables including maternal pre-pregnancy BMI (<25 vs. ≥25 kg/m^2), parity (1 vs. ≥2), age (≤30 vs. >30 y), population group (white vs. any other group), education (high school vs. greater) and income (<70,000 vs. ≥70,000 Canadian dollars). A separate matching procedure was conducted for participants where income was not reported (*n*=61/group) using the same approach; both datasets were subsequently combined to form a database with *n*=352/group. For both propensity score matches, a standardized difference (caliper) of 0 (exact) was applied for population group and pre-pregnancy BMI category and <0.1 accepted for the other variables. Differences among the two groups (*n*=352/group) of maternal multivitamin supplement use (preconception vs. pregnancy) were compared using mixed model ANOVA accounting for fixed effect of group and matched pair as a random variable. The same model was also conducted for serum 25OHD concentration according to

maternal characteristics as a fixed effect (age, actual maternal population group, pre-pregnancy BMI, income, education, season at delivery, exercise during pregnancy, pre-pregnancy BMI and parity). For differences among groups and according to maternal characteristics with 3 or more categories (e.g., population groups), *post hoc* testing using Tukey's t-tests was conducted, and adjusted for multiple comparisons using Tukey-Kramer adjustment. Differences in proportions among multivitamin use groups were tested using Chi-square or Fisher's exact test. Odds ratios (OR) for having infant serum 25OHD <30, or ≥40 nmol/L were calculated according to maternal multivitamin use groups using logistic regression models; crude and adjusted for multiple covariates. The reference group for each OR was based on either recommended practices (e.g. achievement of recommended BMI, exercise or multivitamin use in pregnancy) or a large dataset (e.g. postsecondary education). Covariates considered in adjusted OR included those associated with endogenous synthesis or metabolism of vitamin D (maternal population group, exercise during pregnancy, season at delivery, use of SPF products, and exposure to UVB), maternal sociodemographic factors (age, income, education, Canadian-born) and pregnancy factors (parity, pre-pregnancy BMI, weight gain). Data for the multivitamin use groups are reported as mean ± SEM, or *n* (%).

All statistical analyses were performed using SAS (v9.4, SAS Institute Inc., Carry, NC, US). A $P<0.05$ (2-sided) including Tukey-Kramer adjustments was used to guide the interpretation of the data.

Results

Summary of participants: Of the mother-infant dyads screened for eligibility (**Figure 1**), 79.7% were eligible. Of these, 35% gave consent and fully met the study inclusion criteria including that the neonatal blood sample volume was sufficient to test for 25OHD concentration. By design, all infants were born at term and of AGA weight (**Supplementary Table 1**). Mother-infant dyads excluded from this report due to incomplete data or a missed blood sample more frequently did not report income but were otherwise not different from those included.

Vitamin D status at birth: Mean neonatal (*n*=1035) serum 25OHD concentration was 45.9 ± 0.6 (95% CI: 44.7, 47.0) nmol/L with no differences among male and female infants (*P*=0.99). The distribution of serum 25OHD (**Figure 2**), spanned 8 to 124 nmol/L with 60.7% (95% CI: 55.2, 66.2) ≥40 nmol/L; the distribution of neonatal serum 25OHD (**Supplementary Figure 1)** and characteristics among population groups were explored (**Supplementary Table 2)**. The proportion of infants in these status categories (**Table 1**) varied as a function of all sociodemographic characteristics except for maternal age and education. Serum 25OHD was higher (*P*<0.0001) in infants of white mothers compared to those of all other groups. Higher 25OHD concentration was also observed in infants born to mothers born in Canada, or according to higher income. There were no differences according to family type (data not shown).

Among the obstetric and lifestyle variables (Table 1), those that associated with a lower proportion with 25OHD <30 nmol/L included: primiparous women, BMI in the recommended range, use of multivitamins preconception, summer season at birth, exposure to sun, use of SPF products and exercise in pregnancy resulting in higher serum 25OHD concentrations. Pregnancy weight gain did not associate with infant vitamin D status; nor did maternal smoking behavior, delivery mode or infant gestational age or weight at birth (data not shown).

Potential modifiable factors associated with vitamin D status: Of the lifestyle variables suitable for consideration in future studies, use of multivitamins was prominent (93.3%, 95% CI: 91.8, 94.9). The majority of mothers were of recommended pre-pregnancy BMI of 18.5-24.9 kg/m^2 (57.9%; 95% CI: 54.9, 60.9) with fewer in the 25.0-29.9 kg/m^2 range (24.4%; 95% CI: 21.8, 27.1) or ≥30 kg/m^2 (12.7%; 95% CI: 10.6, 14.7). In contrast, 47.1% (95% CI: 44.1, 50.2) were physically active during pregnancy. In the maternal multivitamin use subgroups, 6.8% (95% CI: 5.2, 8.3) did not adhere to multivitamin use in pregnancy, 54.2% (95% CI: 51.2, 57.2) of mothers took a multivitamin supplement during pregnancy, and 39% (95% CI: 36.1, 42.0) took a supplement in the 3 months before as well as during pregnancy. Compliance to daily multivitamin supplement use was high with 90.0% (95% CI: 87.5, 92.5) of the group taking supplements during pregnancy doing so daily/almost daily; in the group taking supplements before and during pregnancy, daily/almost daily use was 92.1% (95% CI: 89.5, 94.7) before and 93.8% (95% CI: 91.5, 96.2) during pregnancy. Multivitamin brand name was reported in 85.1% (*n*=821/965), whereas the remaining 14.9% who reported prenatal multivitamin use could not recall a specific brand name. Based on brand name and product composition, the median dose of vitamin D was 400 (95% range 250, 400) IU/d both before pregnancy and during pregnancy. Very few (5.0%, 95% CI: 3.7, 6.4) mothers took an additional vitamin D supplement during pregnancy.

Propensity score matched analyses: Among the propensity matched multivitamin supplement use groups (*n*=352/group), the mean propensity score was not different (*P*=0.97) between mothers using multivitamin supplements before and during pregnancy compared to those using multivitamins in pregnancy (**Table 2**). Mothers were evenly matched based on the criteria used in the propensity procedure (age, population group, education, income, pre-

pregnancy BMI and parity). However, mothers in the preconception group were more often born in Canada, and fewer delivered their infant during the summer season (Table 2). There were no differences in other maternal characteristics. Family structure (P=1.00), smoking history (P=0.26), type of delivery (P=0.92), and infant birth weight (P=0.62) were not different among maternal supplement use groups (data not shown).

Even though the multivitamin use groups were matched for multiple demographic factors, infant serum 25OHD varied according to maternal population group with white mothers having infants born with higher concentrations compared to Black, East/Southeast Asian, Middle Eastern, and South Asian, but not those of Latino or mixed/unknown heritage (Table 2). Other factors associated with higher serum 25OHD included Canadian born mothers, above median income, having a pre-pregnancy BMI in the recommended range, exercising in pregnancy along with use of SPF products, sun exposure and season of birth. Factors not associated with infant serum 25OHD included maternal age, education, weight gain during pregnancy, parity as well as when pre-pregnancy BMI was tested as 3 categories (18.5-24.9, 25-29.9, ≥30 kg/m^2). Use of multivitamins preconception associated with higher infant serum 25OHD (Table 2). It remained higher in the preconception group (P<0.0007) when tested in a multivariable model including maternal population group (P<0.0001), season at birth (P<0.0001), exercise in pregnancy (P=0.0475), recommended pre-pregnancy BMI (<25 kg/m^2 vs above, P=0.0271), pregnancy weight gain (P=0.07), age category (P=0.10), education (P=0.65), income (P=0.68), and parity (P=0.14). Maternal country of birth was explored but did not contribute to the model (P=0.59), thus it was excluded from the final analysis.

The proportion of infants with serum 25OHD <30 nmol/L (**Table 3**) in the matched subgroups was lower with maternal preconception use of multivitamins, pre-pregnancy BMI in

the recommended range, summer season at birth, white ancestry and above median income. Similarly, the proportion of infants with serum 25OHD ≥40 nmol/L was higher when maternal pre-pregnancy BMI was in the recommended range, with exercise in pregnancy, summer season at birth, primiparity and white ancestry. In univariate logistic regression, similar factors were observed to influence the odds of having infant serum 25OHD <30 (deficient), or ≥40 nmol/L (adequate). Of the modifiable factors tested together in the multivariable regression, use of prenatal multivitamins was protective against having an infant born with serum 25OHD <30 and improved the odds of having serum 25OHD ≥40 nmol/L as was achievement of pre-pregnancy BMI in the recommended range. Exercise was also a protective factor for achieving serum 25OHD ≥40 nmol/L. Other protective factors against serum 25OHD being <30 nmol/L, or in support of achieving serum 25OHD ≥40 nmol/L, that emerged in the multivariable models included white heritage, maternal age >30 y and summer season at birth; as well as primiparity for serum 25OHD ≥40 nmol/L.

Discussion

Vitamin D status of infants remains a public health concern in Canada and globally (37). Reports regarding vitamin D status of newborns in Canada reflect predominantly European heritage. The present report of infants from a diverse sociodemographic population highlights that the majority were born with adequate vitamin D stores, defined as a serum 25OHD ≥40 nmol/L (1). Nonetheless, a concerning proportion (20.8%) were born with serum 25OHD in the deficient range <30 nmol/L, with evidence of health inequity based on higher proportions of infants with vitamin D deficiency in all population groups compared to white. This is important as exposure to adequate vitamin D is implicated in neurodevelopment (38), tooth enamel formation (39), body composition (40) and preventing nutritional rickets (41).

Adherence to current public health recommendations for a healthy pregnancy including being physically active, achievement of the recommended BMI (14, 15) and use of multivitamin supplements (16) all associated with higher newborn vitamin D status in our study. The majority of mothers in all population groups had a pre-pregnancy BMI in the recommended range (52.6-63.8%) and showed higher receptivity for multivitamin use (86.1-96.9%) compared to exercise (38.9-56.1%). The US National Health and Nutrition Examination Survey is among few to report on vitamin D status of a large sample of pregnant women of diverse ancestry (42). While ethnic disparities were clearly evident, overall, the proportion of women using a multivitamin increased during pregnancy (61% to 86%) and by the third trimester, 82% had 25OHD ≥50 nmol/L; which has been shown to protect against neonatal vitamin D deficiency in other studies (2). The authors concluded that higher dosages or a longer duration of supplement may be required, although vitamin D status of the newborn infant was not assessed. The most prominent

modifiable factor that increases maternal vitamin D status, and consequently maternal-fetal transfer of vitamin D, is prenatal use of a multivitamin containing vitamin D (5, 7, 20).

In Canada, compliance with multivitamin use in pregnancy is high at 90% (43), and typically these contain 400 IU of vitamin D in our study, and others (7, 44). Less information is available regarding preconception use of multivitamins (43). Our study adds that use of multivitamins before pregnancy varied (29.2 to 43.6) according to population group. This practice was ~8-14% higher in white compared to all other population groups in association with 8-27% fewer infants with vitamin D deficiency; suggesting an advantage due to a longer exposure of infants to maternal supplementation and improvements in vitamin D status prior to pregnancy. In a recent trial, maternal vitamin D supplementation (400-800 IU/d) from ~13 weeks of pregnancy to term corresponded to cord serum 25OHD <30 nmol/L in the majority of pregnancies with baseline 25OHD <50 nmol/L (45). While multivitamins are well-received by women planning a pregnancy (46), the benefit to neonatal vitamin D status requires further study.

In the absence of a randomized controlled trial testing for the benefits of maternal multivitamin use preconception vs. during pregnancy on vitamin D status, we conducted a nonrandomized analysis using propensity scores to minimize the impact of sociodemographic characteristics (36). Compared to infants of mothers who used multivitamins during pregnancy, using a multivitamin both before and during pregnancy was more likely to protect against vitamin D deficiency and aligned more strongly with adequate vitamin D status. Protection remained evident in multivariable analyses that accounted for other important variables including maternal population group, pre-pregnancy BMI, exercise, age, parity and season at birth. This underscores the importance of national dietary guidance (16) that advises women who could become (i.e. preconception) or are pregnant to take a daily multivitamin.

Exogenous intakes of vitamin D are important when endogenous synthesis is limited (1) due to residing at higher latitudes (i.e. shorter summers) or higher amounts of melanin in the skin as is characteristic of many population groups (47). It is accepted that SPF product application does not fully prevent vitamin D synthesis (30), consistent with our observations that SPF product use and sun exposure associated with 5-7.5 nmol/L higher serum 25OHD. Serum 25OHD in summer born infants was ~10 nmol/L higher compared to all other seasons, and of similar magnitude to differences among population groups. Canadian surveys report 16 nmol/L differences in 25OHD among population groups, and 10 nmol/L differences in 25OHD from winter to summer (19). In agreement with pregnancy cohorts, (3, 7) maternal exercise (in or outdoors) was a correlate of neonatal vitamin D status. Exercise may reflect other healthy behaviors that cluster in pregnancy (48), potentially mobilization of vitamin D stores (49) and is encouraged as part of a healthy lifestyle in pregnant women without contraindications (14).

Most guidelines for a healthy pregnancy do not specifically recommend vitamin D supplementation for women with elevated BMI (15, 50), rather these suggest a nutritional consultation to establish if additional supplementation is required. There is evidence (51) that pregnant women with elevated BMI do not adhere to multivitamins, and that serum 25OHD is lower in both mother (5, 7, 52, 53) and infant (7, 54). In our study, pre-pregnancy BMI ≥25 kg/m^2 also associated with lower (4.9 nmol/L) neonatal 25OHD concentration. Recent studies demonstrate that women with elevated BMI and using multivitamins have adequate vitamin D status (55, 56). The benefits of initiating multivitamins preconception are further suggested in our study and confirmed in the matched-subgroup analysis where BMI was not different among groups. Thus, recommendations for a healthy pregnancy should include a multivitamin before or as soon as possible during pregnancy for women of all BMI categories (46).

This study has several limitations including a single center, healthy mothers and term born AGA infants. Infant birth weight approximated the mean birthweight for newborns 39 to 40 wk from Quebec (3489, 95% CI: 3489, 3491 g) and Canada (3509, 95% CI: 3509, 3510 g) (13). Maternal age and parity paralleled Canadian statistics (57), 52.7% had above median Canadian income at the time of the study (27), and the proportion of infants born to immigrant women in the present study and Canada in 2016 surpassed 30% (13). In contrast, mothers were more highly educated in our study than reported for Canada (57). Pregnancy history was from the medical record; while accuracy of maternal pre-pregnancy BMI is unclear, the proportion with a BMI ≥30 kg/m^2 (12.7%) is consistent with national data (12.1%, 95% CI: 10.9-13.4) (43). The retrospective survey precluded verification of supplement dose and exact timing of initiation and is thus subject to recall bias, and maternal vitamin D status was not assessed. The assay used to measure serum 25OHD is not the gold-standard and may overestimate 25OHD in cord blood samples (58). To mitigate this, we used rigorous quality assurance measures and standardized 25OHD concentrations to NIST reference materials (33, 34) and capillary sampling after birth.

This study of newborn infants from a large metropolitan center enabled us to evaluate multiple maternal and sociodemographic factors associated with neonatal vitamin D status. The mean serum 25OHD was within the recommended 40-50 nmol/L for infants and 20.8% were at risk of vitamin D deficiency symptoms (<30 nmol/L) (1). Maternal preconception use of a multivitamin supplement is a readily modifiable factor associated with greater odds of newborn infants having adequate vitamin D status. Studies to establish whether public health policy should specifically encourage multivitamins containing vitamin D in women of reproductive age prior to as well as during pregnancy would be of value to help improve vitamin D status of newborn infants and to reduce health inequity among all population groups.

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Authors' contributions to the manuscript: *1*) HAW, CAV, DM, SW designed research (study conception and design); *2*) all authors contributed to the development of overall research plan, and/or study oversight); *3*) HAW, CAV, NG, MR, SP conducted research (hands-on conduct of the experiments and data collection); *4*) HAW and CAV analyzed data or performed statistical analysis; *5*) HAW, DM, SW wrote the paper (only authors who made a major contribution); *6*) HAW had primary responsibility for final content. All authors approved the final content (HAW, CAV, NG, MR, SP, SW, DM)

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Table 1. Neonatal vitamin D status according to maternal demographic, obstetric and lifestyle characteristics[1]

Variable	Neonatal serum 25OHD (nmol/L)			*P*-value	*n*	Neonatal serum 25OHD (nmol/L)	*P*-Value
	< 30 *n*=215 (20.8%)	30.0 to 39.9 *n*=192 (18.6%)	≥ 40.0 *n*=628 (60.7%)				
Age, y	31.4 ± 0.3[7]	32.5 ± 0.3	32.5 ± 0.2)	0.0077			0.22
≤30 y, *n* (%)	74 (34.4)	59 (30.7)	178 (28.3)	0.24	311	44.8 ± 1.1	
>30 y, *n* (%)	141 (65.6	133 (69.3)	450 (71.7)		724	46.3 ± 0.7	
Maternal population group[2], *n* (%)				<0.0001			<0.0001
Black	30 (14.0)	15 (7.8)	27 (4.3)		72	36.2 ± 1.9	
East/Southeast Asian	18 (8.4)	14 (7.3)	26 (4.1)		58	38.4 ± 1.9	
Latino	11 (5.1)	15 (7.8)	21 (4.3)		47	41.5 ± 2.4	
Middle Eastern	27 (12.6)	22 (11.5)	47 (7.5)		96	40.6 ± 1.6	
South Asian	24 (11.2)	17 (8.9)	35 (5.6)		76	38.1 ± 1.7	
Mixed or Unknown Heritage	13 (6.1)	12 (6.3)	32 (5.1)		57	43.5 ± 2.3	
White	92 (42.8)	97 (50.5)	440 (70.1)		629	49.9 ± 0.7[7]	
Maternal country of birth, *n* (%)				<0.0001			<0.0001
Canada	97 (45.1)	105 (54.7)	437 (69.6)		639	49.1 ± 0.7	
Elsewhere	118 (54.9)	87 (45.3)	191 (30.4)		396	40.6 ± 0.9	
Education, *n* (%)				0.27			0.37
High school or less	46 (21.4)	30 (15.6)	95 (15.1)		171	44.1 ± 1.5	
College/undergraduate degree	51 (23.7)	46 (24.0)	167 (26.6)		264	46.0 ± 1.1	
Graduate degree	118 (54.9)	116 (60.4)	366 (58.3)		600	46.3 ± 0.7	
Family income, *n* (%)				<0.0001			<0.0001
<70,000 CAD	86 (40.0)	57 (29.7)	145 (23.0)		288	42.1 ± 1.1	
≥70,000 CAD	94 (43.7)	93 (48.4)	359 (57.2)		546	48.2 ± 0.8[7]	
Not reported/known	35 (16.3)	42 (21.9)	124 (19.8)		201	44.9 ± 1.2	
Pre-pregnancy BMI, kg/m^2	25.6 ± 0.4	25.0 ± 0.4	24.2 ± 0.2	0.0015			<0.0001
<18.5 kg/m^2, *n* (%)	8 (3.7)	5 (2.6)	20 (3.2)	0.0027	33	42.8 ± 2.6	
18.5-24.9 kg/m^2, *n* (%)	97 (45.1)	106 (55.2)	396 (63.1)		599	47.8 ± 0.7	
25.0-29.9 kg/m^2, *n* (%)	69 (32.1)	48 (25.0)	136 (21.7)		253	43.6 ± 1.2[8]	
≥30.0 kg/m^2, *n* (%)	35 (16.3)	29 (15.1)	67 (10.7)		131	42.7 ± 1.7[8]	
Data not available, *n* (%)	6 (2.8)	4 (2.1)	9 (1.4)		19	43.0 ± 4.9	
Weight gain in pregnancy, kg	13.7 ± 0.5	13.6 ± 0.4	14.0 ± 0.2	0.68			
Appropriate weight gain, *n* (%)				0.90			0.88
Below	41 (19.1)	41 (21.4)	124 (19.8)		206	46.7 ± 1.3	
Within	67 (31.2)	61 (31.8)	215 (34.2)		343	45.8 ± 1.0	
Above	99 (46.1)	86 (44.8)	272 (43.3)		457	45.5 ± 0.9	
Data not available	8 (3.7)	4 (2.0)	17 (2.7)		29	46.1 ± 4.0	
Parity, *n* (%)				0.0193			0.08

Primiparous	81 (37.7)	71 (37.0)	290 (46.2)		442	47.0 ± 0.8	
Multiparous	134 (62.3)	121 (63.0)	338 (53.8)		593	45.0 ± 0.8	
Multivitamin use[3], *n* (%)				<0.0001			<0.0001
None	32 (14.9)	16 (8.3)	22 (3.5)		70	35.4 ± 2.2[7]	
Pregnancy	126 (58.6)	106 (55.2)	329 (52.4)		561	44.9 ± 0.7[7]	
Pre-conception and pregnancy	57 (26.5)	70 (36.5)	277 (44.1)		404	49.0 ± 0.9[7]	
SPF products used[4], *n* (%)				<0.0001			<0.0001
Yes	16 (7.4)	26 (13.5)	127 (20.2)		169	52.1 ± 1.4	
No	199 (92.6)	166 (86.5)	501 (79.8)		866	44.6 ± 0.6	
Sun exposure (hands/face)[4], *n* (%)				0.0002			<0.0001
Yes	63 (29.3)	65 (33.9)	277 (44.1)		405	48.9 ± 0.9	
No	152 (70.7)	127 (66.1)	351 (55.9)		630	43.9 ± 0.7	
Exercised in pregnancy[5], *n* (%)				<0.0001			<0.0001
Yes	88 (40.9)	67 (34.9)	333 (53.0)		488	48.4 ± 0.8	
No	127 (59.1)	125 (65.1)	295 (47.0)		547	43.6 ± 0.8	
Season at birth[6], *n* (%)				<0.0001			<0.0001
Winter	70 (32.6)	46 (24.0)	135 (21.5)		251	43.5 ± 1.2	
Spring	59 (27.4)	68 (35.4)	158 (25.2)		285	43.7 ± 0.9	
Summer	33 (15.4)	35 (18.2)	200 (31.9)		268	53.0 ± 1.2[7]	
Fall	53 (24.7)	43 (22.4)	135 (21.5)		231	42.7 ± 1.0	
Born in UVB period, *n* (%)				0.0001			<0.0001
April 1 to October 31	101 (47.0)	110 (57.3)	397 (63.2)		608	48.0 ± 0.7	
November 1 to March 31	114 (53.0)	82 (42.7)	231 (36.8)		427	42.8 ± 0.9	

[1]Data are mean ± SEM, or *n* (%), BMI: body mass index; CAD: Canadian dollars; SPF: sun protection factor; 25OHD: 25-hydroxyvitamin D; UVB: ultraviolet beta. [2]Maternal self-reported population group according to ancestral origin.[3]Surveyed as multivitamin used in the 3 months prior to conceiving; and used during pregnancy.[4]Surveyed to reflect third trimester sun exposure behavior.[5]Any exercise during pregnancy included any indoor or outdoor activity.[6]Seasons according to equinox and solstice dates.[7]Significant difference $P<0.01$ vs. other age groups, population subgroup, lower income, other seasons, other multivitamin use groups. [8]Sgnificant difference $P<0.05$ vs. BMI 18.5-24.9 kg/m^2 category.

Table 2. Characteristics according to use of multivitamins preconception or during pregnancy in a subgroup matched based on propensity scores[1]

Variable	Pregnancy only (*n*=352)	Preconception+pregnancy (*n*=352)	*P*-value	*n*	Neonatal serum 25OHD (nmol/L)	*P*-value
Age, y	32.7 ± 0.2	33.2 ± 0.2	0.12			
≤30	81 (23.0)	87 (24.7)	0.60	168	47.2 ± 1.4	0.91
>30	271 (77.0)	265 (75.3)		536	48.0 ± 0.8	
Maternal population group[2], *n* (%)			0.87			<0.0001
Black	18 (5.1)	18 (5.1)		36	36.2 ± 2.3[6]	
East/Southeast Asian	18 (5.1)	17 (4.8)		35	37.4 ± 2.3[6]	
Latino	13 (3.7)	14 (4.0)		27	44.9 ± 3.4	
Middle Eastern	32 (9.1)	29 (8.2)		61	40.0 ± 1.9[6]	
South Asian	18 (5.1)	24 (6.8)		42	42.6 ± 2.1[6]	
Mixed or Unknown Heritage	20 (5.7)	17 (4.8)		37	47.4 ± 3.0	
White	233 (66.2)	233 (66.2)		466	51.3 ± 0.8	
Maternal country of birth, *n* (%)			0.0317			<0.0001
Canada	218 (61.9)	246 (69.9)		464	50.4 ± 0.8	
Elsewhere	134 (38.1)	106 (30.1)		240	42.9 ± 1.1	
Education, *n* (%)			0.81			0.82
High school or less	37 (10.5)	39 (11.1)		76	47.3 ± 2.2	
College/university degree	315 (89.5)	313 (88.9)		628	47.9 ± 0.7	
Family income, *n* (%)			1.00			0.0018
<70,000 CAD	67 (19.0)	67 (19.0)		134	43.5 ± 1.5	
≥70,000 CAD	224 (63.6)	224 (63.6)		448	49.5 ± 0.9[6]	
Not reported/known	61 (17.3)	61 (17.3)		122	46.4 ± 1.5	
Pre-pregnancy BMI, kg/m^2	24.6 ± 0.2	24.6 ± 0.2)	0.94			
<25 kg/m^2, *n* (%)	218 (61.9)	219 (62.2)	1.00	437	49.0 ± 0.8	0.0325
≥25 kg/m^2, *n* (%)	134 (38.1)	133 (37.8)		267	45.8 ± 1.1	
Pre-pregnancy BMI, *n* (%)			0.84			0.08
18.5-24.9 kg/m^2	218 (61.9)	219 (62.2)		437	49.0 ± 0.8	
25-29.9 kg/m^2	100 (28.4)	95 (27.0)		195	45.4 ± 1.2	
≥30.0 kg/m^2	34 (9.7)	38 (10.8)		72	47.0 ± 2.3	
Pregnancy weight gain, kg	14.3 ± 0.3	13.9 ± 0.3	0.33			
Appropriate weight gain, *n* (%)			0.56			0.18
Below	69 (19.6)	63 (17.9)		132	50.0 ± 1.6	
Within	109 (31.0)	122 (34.7)		231	48.1 ± 1.1	
Above	174 (49.3)	167 (47.4)		341	46.8 ± 1.0	
Parity, *n* (%)			0.60			0.41
Primiparous	159 (45.2)	167 (47.4)		326	48.7 ± 0.9	
Multiparous	193 (54.8)	185 (52.6)		378	47.0 ± 0.9	

SPF products used[3], *n* (%)			0.55			0.0004
Yes	56 (15.9)	63 (17.9)		119	53.4 ± 1.6	
No	296 (84.1)	289 (82.1)		585	46.7 ± 0.7	
Sun exposure (hands/face)[3], *n* (%)			0.25			0.0002
Yes	149 (42.3)	133 (37.8)		282	50.5 ± 1.1	
No	203 (57.7)	219 (62.2)		422	46.0 ± 0.8	
Exercised in pregnancy[4], *n* (%)			0.33			0.0073
Yes	171 (48.6)	185 (52.6)		356	49.9 ± 1.0	
No	181 (51.4)	167 (47.4)		348	45.7 ± 0.9	
Season at birth[5], *n* (%)			0.0203			<0.0001
Winter	75 (21.3)	88 (25.0)		163	46.6 ± 1.5	
Spring	84 (23.9)	104 (29.6)		188	44.9 ± 1.2	
Summer	110 (31.3)	75 (21.3)		185	54.6 ± 1.4[6]	
Fall	83 (23.6)	85 (24.2)		168	44.8 ± 1.1	
UVB exposure months at birth, *n* (%)			0.40			0.0010
April 1 to October 31	218 (60.8)	202 (57.4)		420	49.6 ± 0.9	
November 1 to March 31	138 (39.2)	150 (42.6)		288	45.3 ± 1.0	
Infant serum 25OHD, nmol/L	46.1 ± 0.9	49.6 ± 1.0	0.0083			<0.0001
<30 nmol/L, *n* (%)	68 (19.3)	46 (13.0)	0.07	114	23.1 ± 0.4	
30-39.9 nmol/L, *n* (%)	63 (17.9)	64 (18.2)		127	35.5 ± 0.2	
≥40 nmol/L, *n* (%)	221 (62.8)	242 (68.8)		463	57.3 ± 0.6	
Propensity score	0.4540 ± 0.0059	0.4543 ± 0.0059	0.97			

[1]Data are mean ± SEM, or *n* (%), BMI: body mass index; CAD: Canadian dollars; GA: Gestational age; SPF: sun protection factor; 25OHD: 25-hydroxyvitamin D; UVB: ultraviolet beta. [2]Maternal self-reported population group according to ancestral origin. [3]Surveyed to reflect third trimester sun exposure behavior.[4]Any exercise during pregnancy included any indoor or outdoor activity. [5]Seasons according to equinox and solstice dates. [6]Significant difference $P<0.05$ vs. white, lower income or other seasons.

Table 3. Protective factors against infants being born with serum 25OHD < 30 or < 40 nmol/L[1]

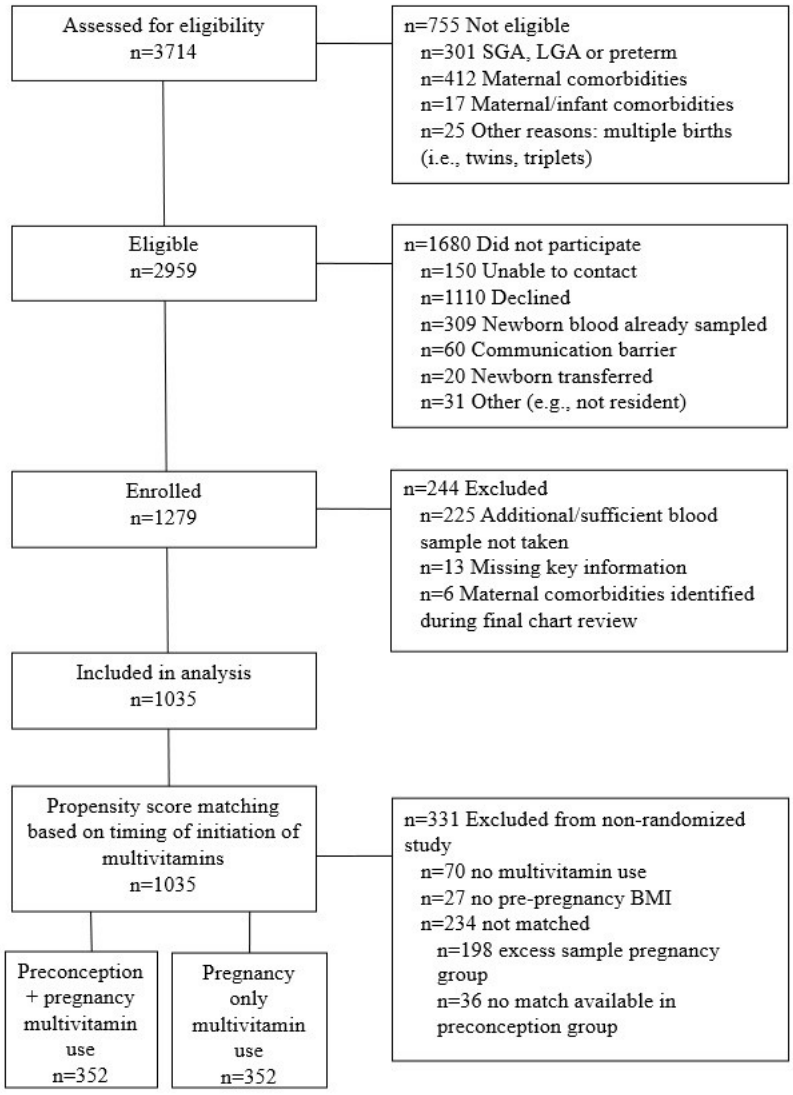
	n	Serum 25OHD <30 nmol/L				Serum 25OHD ≥40 nmol/L			
		%	*P*-value	Unadjusted OR (95% CI)	Adjusted OR (95% CI)[2]	%	*P*-value	Unadjusted OR (95% CI)	Adjusted OR (95% CI)[2]
Multivitamin use[3]			*P*=0.0244				*P*=0.10		
Preconception+pregnancy	352	13.1		0.63 (0.42, 0.94)	0.55 (0.36, 0.86)	68.8		1.30 (0.95, 1.78)	1.47 (1.04, 2.07)
Pregnancy only	352	19.3		Reference	Reference	62.8		Reference	Reference
Pre-pregnancy BMI			*P*=0.0152				*P*=0.0070		
<25 kg/m^2	437	13.5		Reference	Reference	69.6		Reference	Reference
≥25 kg/m^2	267	20.6		1.66 (1.11, 2.49)	1.82 (1.18, 2.81)	59.6		0.64 (0.47, 0.89)	0.59 (0.42, 0.83)
Exercise in pregnancy[4]			*P*=0.25				*P*=0.0007		
Yes	356	14.6		Reference	Reference	71.6		Reference	Reference
No	348	17.8		1.27 (0.85, 1.90)	1.08 (0.69, 1.68)	59.8		0.59 (0.43, 0.81)	0.69 (0.49, 0.97)
Season at birth[5]			*P*=0.0100				*P*=0.0002		
Winter	163	22.1		2.80 (1.51, 5.21)	3.58 (1.84, 6.94)	60.7		0.43 (0.27, 0.68)	0.34 (0.21, 0.57)
Spring	188	18.1		2.18 (1.17, 4.06)	2.61 (1.34, 5.09)	58.0		0.38 (0.24, 0.60)	0.31 (0.19, 0.51)
Summer	185	9.2		Reference	Reference	78.4		Reference	Reference
Fall	168	16.1		1.89 (0.99, 3.61)	2.40 (1.21, 4.79)	65.5		0.52 (0.33, 0.84)	0.39 (0.23, 0.65)
Parity			*P*=0.61				*P*=0.0468		
Primiparous	326	15.3		Reference	Reference	69.6		Reference	Reference
Multiparous	378	16.9		1.13 (0.75, 1.69)	1.27 (0.79, 2.05)	62.4		0.73 (0.53, 0.99)	0.67 (0.46, 0.96)
Maternal population group[6]			*P*<0.0001				*P*<0.0001		
Black	36	36.1		4.74 (2.26, 9.96)	5.58 (2.50, 12.47)	36.1		0.20 (0.10, 0.40)	0.16 (0.07, 0.34)
East/Southeast Asian	35	34.3		4.38 (2.05, 9.34)	5.22 (2.25, 12.10)	40.0		0.23 (0.11, 0.47)	0.20 (0.09, 0.42)
Latino	27	18.5		1.91 (0.69, 5.26)	2.09 (0.72, 6.04)	51.9		0.37 (0.17, 0.81)	0.38 (0.17, 0.87)
Middle Eastern	67	28.4		3.32 (1.81, 6.10)	3.81 (1.94, 7.46)	46.3		0.30 (0.18, 0.50)	0.25 (0.14, 0.45)
South Asian	42	19.1		1.97 (0.87, 4.50)	2.64 (1.10, 6.35)	59.5		0.51 (0.27, 0.97)	0.36 (0.18, 0.72)
Mixed or Unknown	37	21.6		2.31 (1.00, 5.34)	2.63 (1.08, 6.38)	64.9		0.64 (0.31, 1.29)	0.50 (0.24, 1.07)
White	460	10.7		Reference	Reference	74.4		Reference	Reference
Maternal age			*P*=0.19				*P*=0.3071		
≤30 y	168	19.6		1.37 (0.88, 2.15)	1.75 (1.03, 2.97)	26.1		1.21 (0.84, 1.73)	0.60 (0.39, 0.91)
>30 y	536	15.1		Reference	Reference	33.2		Reference	Reference
Maternal education			*P*=0.41				*P*=0.90		
High school or less	76	19.7		0.76 (0.42, 1.39)	0.84 (0.43, 1.65)	32.9		1.07 (0.65, 1.77)	0.84 (0.47, 1.48)
Postsecondary	628	15.8		Reference	Reference	34.4		Reference	Reference
Family income			*P*=0.0056				*P*=0.11		
<70,000 CAD	134	25.4		2.04 (1.27, 3.27)	1.11 (0.65, 1.91)	41.8		1.53 (1.03, 2.28)	1.17 (0.74, 1.85)
≥70,000 CAD	448	14.3		Reference	Reference	31.9		Reference	Reference

Not reported	122	13.1	0.91 (0.50, 1.63)	0.53 (0.28, 1.02)	34.4	1.12 (0.73, 1.71)	1.53 (0.94, 2.50)	

[1]Data are *n* and row % or odds ratios, BMI: body mass index; CAD: Canadian dollars; 25OHD: 25-hydroxyvitamin D.
[2]Adjusted for all other independent variables in the table. [3]Surveyed as multivitamin used in the 3 months prior to conceiving; and used during pregnancy. [4]Any exercise during pregnancy included any indoor or outdoor activity. [5]Seasons according to equinox and solstice dates. [6]Maternal self-reported population group according to ancestral origin.

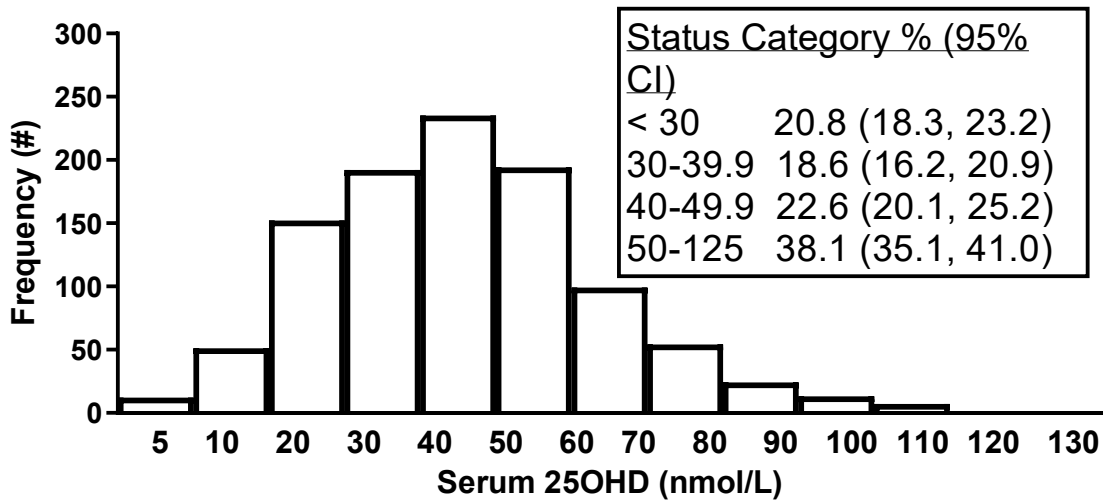
Figure legends

Figure 1. Participant flow diagram.



Participant flow diagram for all newborn infants assessed for study eligibility and participating in the assessment of vitamin D status as well as maternal use of multivitamin groups matched based on propensity scores.

Figure 2. Frequency distribution of serum 25OHD in newborn infants (n=1035)



Frequency distribution of serum 25OHD in newborn infants (n=1035); insert shows the proportion (95% CI) with serum 25OHD in each status category: <30 nmol/L (n=215), 30-39.9 (n=192), 40-49.9 (n=234), 50-125 (n=394) nmol/L.

Supplementary Table 1. Maternal and infant characteristics according to neonatal vitamin D status assessment[1]

Variable	*n*	Assessed	*n*	Not assessed	*P*-value
Age, y	1035	32.3 ± 0.1	244	31.9 ± 0.3	0.26
Self-reported as white, *n* (%)	1035	629 (60.8)	244	143 (58.4)	0.49
Canadian born, *n* (%)	1035	639 (61.7)	236	150 (63.6)	0.59
Education, *n* (%)	1035		236		0.56
High school or less		171 (16.5)		39 (16.5)	
College/undergraduate degree		264 (25.5)		68 (28.8)	
Graduate degree		39 (58.0)		129 (54.7)	
Family income, *n* (%)	1035		244		0.0228
<70,000 CAD		288 (27.8)		60 (24.5)	
≥70,000 CAD		546 (52.8)		118 (48.2)	
Not reported/known		201 (19.4)		67 (27.4)[2]	
Two-parent family, *n* (%)	1020	989 (97.0)	232	228 (98.3)	0.27
Pre-pregnancy BMI, kg/m^2	1016	24.6 ± 0.2	231	25.0 ± 0.3	0.26
Primiparous, *n* (%)	1035	442 (42.7)	241	106 (44.0)	0.72
Smoking, *n* (%)	1021		232		0.34
Never		922 (90.3)		202 (87.1)	
Quit before pregnancy		58 (5.7)		18 (7.8)	
Current		41 (4.0)		12 (5.2)	
No alcohol in pregnancy, *n* (%)	1035	967 (93.4)	245	229 (93.5)	0.98
Multivitamin during pregnancy *n* (%)	1035	965 (93.2)	244	213 (90.3)	0.10
GA at delivery, wk	1035	39.6 ± 0.1	242	39.7 ± 0.1	0.26
Infant male sex, *n* (%)	1035	526 (50.8)	244	121 (49.8)	0.77
Infant birth weight, kg	1035	3.41 ± 0.01	241	3.42 ± 0.03	0.59
Weight *z*-score	1035	0.22 ± 0.02	244	0.25 ± 0.05	0.55
Apgar at 1 min	1035	9 (9, 9)	244	9 (9, 9)	0.61
Apgar at 5 min	1035	9 (9, 10)	244	9 (9, 10)	0.97

[1]Data are mean ± SEM, or *n* (%), BMI: body mass index; CAD: Canadian dollars; GA: Gestational age. [2]60/67 incomplete survey following inadequate/missed blood sample.

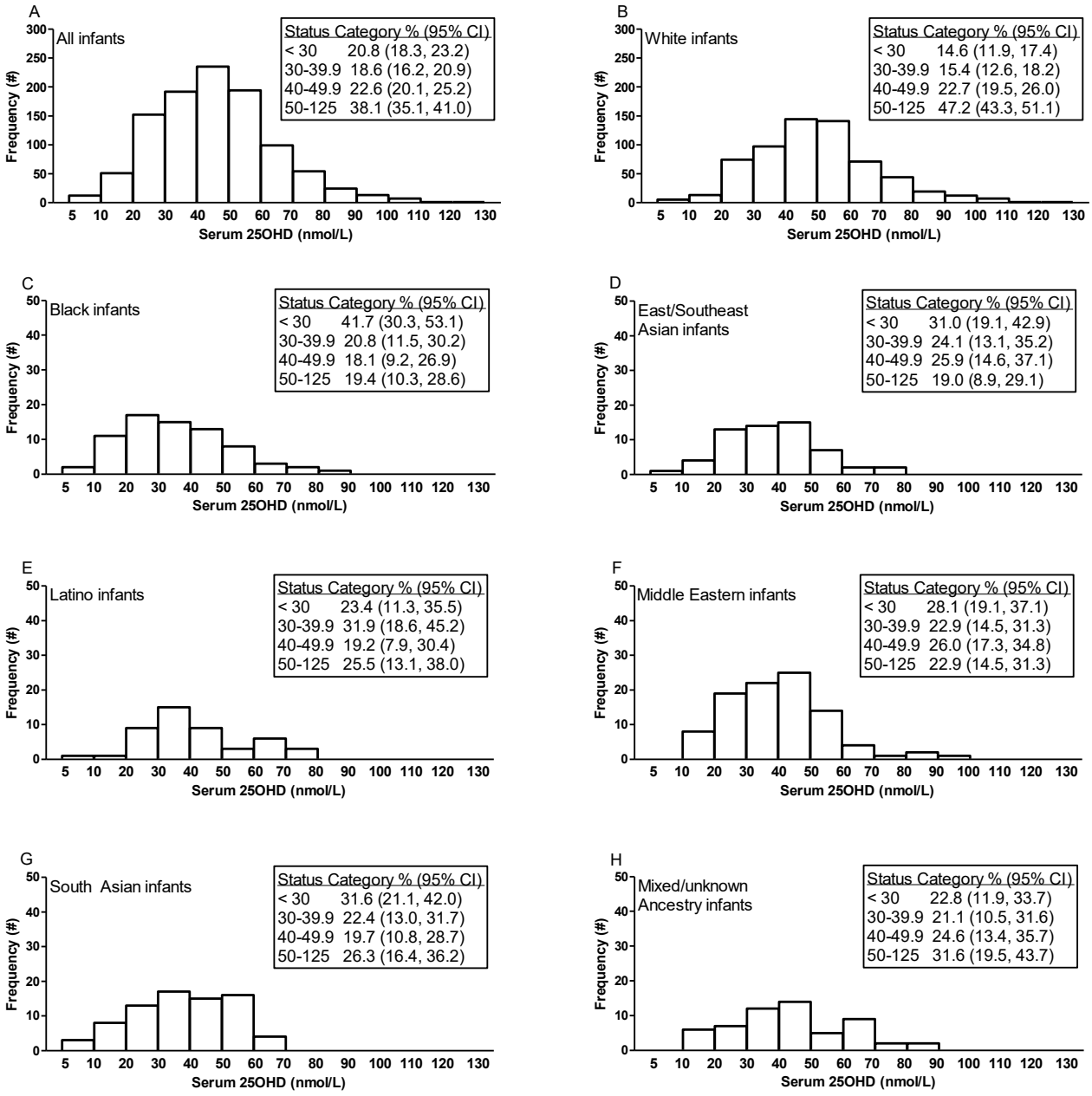
Supplementary Table 2. Maternal and infant characteristics among population groups[1]

Variable	Maternal Self-reported Population Group based on Ancestry[2]							*P*-value
	Black	**East/Southeast Asian**	**Latino**	**Middle Eastern**	**South Asian**	**Mixed or Unknown**	**White**	
n	72	58	47	96	76	57	629	
Age, y	32.4 ± 0.6	34.2 ± 0.6[7,8]	33.6 ± 0.6	31.4 ± 0.5	32.0 ± 0.5	32.3 ± 0.6	32.1 ± 0.2	0.0033
Maternal country of birth, *n* (%)								<0.0001
Canada	17 (23.6)	9 (15.5)	7 (14.9)	12 (12.5)	23 (30.3)	16 (28.1)	555 (88.2)	
Elsewhere	55 (76.4)	49 (84.5)	40 (85.1)	84 (87.5)	53 (69.7)	41 (71.9)	74 (11.8)	
Education, *n* (%)								<0.0053
High school or less	15 (20.8)	3 (5.2)	4 (2.3)	9 (5.3)	20 (26.3)	8 (14.0)	112 (17.8)	
College/university degree	57 (79.2)	55 (94.8)	43 (91.5)	87 (90.6)	56 (73.7)	49 (86.0)	517 (82.2)	
Family income, *n* (%)								<0.0001
<70,000 CAD	28 (38.9)	22 (37.9)	22 (46.8)	39 (40.6)	33 (43.4)	24 (42.1)	120 (19.1)	
≥70,000 CAD	21 (29.2)	17 (29.3)	18 (38.3)	25 (26.0)	24 (31.6)	22 (38.6)	419 (76.7)	
Not reported/known	23 (31.9)	19 (32.8)	7 (14.9)	32 (33.3)	19 (25.0)	11 (19.3)	90 (14.3)	
Pre-pregnancy BMI, kg/m^2	24.8 ± 0.6	22.2 ± 0.5[7,9]	25.6 ± 0.7	24.5 ± 0.4	24.4 ± 0.5	24..6 ± 0.6	24.8 ± 0.2	0.0089
Pre-pregnancy BMI, *n* (%)								0.30
<18.5 kg/m^2	1 (1.4)	6 (10.3)	0	2 (2.1)	4 (5.3)	1 (1.8)	19 (3.0)	
18.5-24.9 kg/m^2	44 (61.1)	37 (63.8)	28 (59.6)	60 (62.5)	40 (52.6)	34 (59.7)	356 (56.6)	
25-29.9 kg/m^2	17 (23.6)	11 (19.0)	9 (19.1)	23 (24.0)	21 (27.6)	15 (26.3)	157 (25.0)	
≥30.0 kg/m^2	8 (11.1)	2 (3.5)	10 (21.3)	10 (10.4)	9 (11.8)	6 (10.5)	86 (13.7)	
Not available	2 (2.8)	2 (3.5)	0	1 (1.0)	2 (2.6)	1 (1.8)	11 (1.8)	
Pregnancy weight gain, kg	13.0 ± 1.1	14.7 ± 0.7	12.2 ± 0.7	12.5 ± 0.6[7]	12.1 ± 0.7[7]	13.7 ± 0.7	14.4 ± 0.2	0.0007
Appropriate weight gain, *n* (%)								0.14
Below	17 (23.6)	10 (17.2)	13 (27.7)	24 (25.0)	20 (26.3)	9 (15.8)	113 (18.0)	
Within	20 (27.8)	23 (39.7)	17 (36.2)	35 (36.5)	27 (35.5)	22 (38.6)	199 (31.6)	
Above	33 (45.8)	20 (34.5)	17 (36.2)	35 (36.5)	27 (35.5)	25 (43.9)	300 (47.7)	
Not available	2 (2.8)	5 (8.6)	0	2 (2.0)	2 (2.6)	1 (1.7)	17 (2.7)	
Parity, *n* (%)								0.90
Primiparous	31 (43.1)	22 (37.9)	18 (38.3)	40 (41.7)	29 (38.2)	24 (42.1)	278 (44.2)	
Multiparous	41 (56.9)	36 (62.1)	29 (61.7)	56 (58.3)	47 (61.8)	33 (57.9)	351 (55.8)	
Multivitamin use[3], *n* (%)								0.0162
None	10 (13.9)	2 (3.5)	4 (8.5)	3 (3.1)	5 (6.6)	3 (5.3)	43 (6.8)	
Pregnancy	41 (56.9)	38 (65.5)	28 (59.6)	63 (65.6)	44 (57.9)	35 (61.4)	312 (49.6)	
Preconception and pregnancy	21 (29.2)	18 (31.0)	15 (31.9)	30 (31.3)	27 (35.5)	19 (33.3)	274 (43.6)	
Exercised in pregnancy[4], *n* (%)								0.0029
Yes	28 (38.9)	26 (44.8)	19 (40.4)	32 (33.3)	28 (36.8)	32 (56.1)	323 (51.4)	
No	44 (61.1)	32 (55.2)	28 (59.6)	64 (66.7)	48 (63.2)	25 (43.9)	306 (48.6)	

SPF products used[5], *n* (%)								0.10
Yes	8 (11.1)	7 (12.1)	7 (14.9)	8 (8.3)	13 (17.1)	7 (12.3)	119 (18.9)	
No	64 (88.9)	51 (87.9)	40 (85.1)	88 (91.7)	63 (82.9)	50 (87.7)	510 (81.1)	
Sun exposure[5], *n* (%)								0.54
Yes	30 (41.7)	21 (36.2)	18 (38.3)	28 (29.2)	31 (40.8)	22 (38.6)	255 (40.5)	
No	42 (58.3)	37 (63.8)	29 (61.7)	68 (70.8)	45 (59.2)	35 (61.4)	374 (59.5)	
Season at birth[6], *n* (%)								0.0162
Winter	14 (19.4)	16 (27.6)	11 (23.4)	24 (25.0)	24 (31.6)	10 (17.5)	152 (24.2)	
Spring	18 (25.0	21 (36.2)	22 (46.8)	29 (30.2)	12 (15.8)	16 (28.1)	167 (26.5)	
Summer	22 (30.6)	10 (17.2)	4 (8.5)	31 (32.3)	25 (32.9)	17 (29.8)	159 (25.3)	
Fall	18 (25.0)	11 (19.0)	10 (21.3)	12 (12.5)	15 (19.7)	14 (24.6)	151 (24.0)	
Gestational age, wk	39.4 ± 0.1	39.4 ± 0.1	39.4 ± 0.1	39.3 ± 0.1 [7,10]	39.6 ± 0.1	39.9 ± 0.1	39.7 ± 0.1	<0.0001
Birth weight, kg	3.36 ± 0.5	3.31 ± 0.05	3.40 ± 0.05	3.31 ± 0.04[7]	3.29 ± 0.05[7]	3.45 ± 0.05	3.44 ± 0.01	0.0004
Infant serum 25OHD, nmol/L	36.2 ± 1.9[7]	38.4 ± 1.9[7]	41.5 ± 2.4[7]	40.6 ± 1.6[7]	38.1 ± 1.7[7]	43.5 ± 2.3[7]	49.9 ± 0.7	<0.0001
Infant serum 25OHD, *n* (%)								<0.0001
<30 nmol/L	30 (41.7)	18 (31.0)	11 (23.4)	27 (28.1)	24 (31.6)	13 (22.8)	92 (14.6)	
30-39.9 nmol/L	15 (20.8)	14 (24.1)	15 (31.9)	22 (22.9)	17 (22.4)	12 (21.1)	97 (15.4)	
≥40 nmol/L	27 (37.5)	26 (44.8)	21 (44.7)	47 (49.0)	35 (46.0)	32 (56.1)	440 (70.0)	

[1]Data are mean ± SEM, or *n* (%), BMI: body mass index; CAD: Canadian dollars; SPF: sun protection factor; 25OHD: 25-hydroxyvitamin D; UVB: ultraviolet beta. [2]Maternal self-reported population group according to ancestral origin. [3]Surveyed as multivitamin initiated in the 3 months prior to conceiving; and initiated during pregnancy. [4]Any exercise during pregnancy included any indoor or outdoor activity. [5]Surveyed to reflect third trimester sun exposure behavior. [6]Seasons according to equinox and solstice dates. [7]Significant difference $P<0.05$ vs. white, [8]Significant difference $P<0.05$ vs Middle Eastern, [9]Significant difference $P<0.05$ vs. Latino, or [10]Significant difference $P<0.05$ vs. mixed/unknown population subgroups.

Supplementary Figure 1. A Frequency distribution of serum 25OHD in newborn infants (*n*=1035)



A Frequency distribution of serum 25OHD in newborn infants (*n*=1035), and those of specific population subgroups: B white (*n*=629), C black (*n*=72), D East/Southeast Asian (*n*=58), E Latino (*n*=47), F Middle Eastern (*n*=96), G South Asian (*n*=76) or H mixed or unknown (*n*=57). For each panel, the insert shows the proportion (95% CI) with serum 25OHD in each status category: <30 nmol/L, 30-39.9, 40-49.9, 50-125 nmol/L.

Appendix 5

CONSORT 2010 checklist of information to include when reporting a randomized trial

CONSORT 2010 checklist of information to include when reporting a randomised trial*

Section/Topic	Item No	Checklist item	Reported on page No
Title and abstract			
	1a	Identification as a randomised trial in the title	106
	1b	Structured summary of trial design, methods, results, and conclusions (for specific guidance see CONSORT for abstracts)	107
Introduction			
Background and objectives	2a	Scientific background and explanation of rationale	109-110
	2b	Specific objectives or hypotheses	110
Methods			
Trial design	3a	Description of trial design (such as parallel, factorial) including allocation ratio	111, 114
	3b	Important changes to methods after trial commencement (such as eligibility criteria), with reasons	111
Participants	4a	Eligibility criteria for participants	111
	4b	Settings and locations where the data were collected	111
Interventions	5	The interventions for each group with sufficient details to allow replication, including how and when they were actually administered	114
Outcomes	6a	Completely defined pre-specified primary and secondary outcome measures, including how and when they were assessed	114-115
	6b	Any changes to trial outcomes after the trial commenced, with reasons	N/A
Sample size	7a	How sample size was determined	118
	7b	When applicable, explanation of any interim analyses and stopping guidelines	N/A
Randomisation:			
Sequence generation	8a	Method used to generate the random allocation sequence	114
	8b	Type of randomisation; details of any restriction (such as blocking and block size)	114
Allocation concealment mechanism	9	Mechanism used to implement the random allocation sequence (such as sequentially numbered containers), describing any steps taken to conceal the sequence until interventions were assigned	114
Implementation	10	Who generated the random allocation sequence, who enrolled participants, and who assigned participants to interventions	112, 114
Blinding	11a	If done, who was blinded after assignment to interventions (for example, participants, care providers, those	114

		assessing outcomes) and how	114
	11b	If relevant, description of the similarity of interventions	114
Statistical methods	12a	Statistical methods used to compare groups for primary and secondary outcomes	119, 120
	12b	Methods for additional analyses, such as subgroup analyses and adjusted analyses	120
Results			
Participant flow (a diagram is strongly recommended)	13a	For each group, the numbers of participants who were randomly assigned, received intended treatment, and were analysed for the primary outcome	121, 133
	13b	For each group, losses and exclusions after randomisation, together with reasons	124, 133
Recruitment	14a	Dates defining the periods of recruitment and follow-up	111
	14b	Why the trial ended or was stopped	N/A
Baseline data	15	A table showing baseline demographic and clinical characteristics for each group	131-132
Numbers analysed	16	For each group, number of participants (denominator) included in each analysis and whether the analysis was by original assigned groups	133-138
Outcomes and estimation	17a	For each primary and secondary outcome, results for each group, and the estimated effect size and its precision (such as 95% confidence interval)	121-123
	17b	For binary outcomes, presentation of both absolute and relative effect sizes is recommended	N/A
Ancillary analyses	18	Results of any other analyses performed, including subgroup analyses and adjusted analyses, distinguishing pre-specified from exploratory	121-122
Harms	19	All important harms or unintended effects in each group (for specific guidance see CONSORT for harms)	N/A
Discussion			
Limitations	20	Trial limitations, addressing sources of potential bias, imprecision, and, if relevant, multiplicity of analyses	128
Generalisability	21	Generalisability (external validity, applicability) of the trial findings	125-126
Interpretation	22	Interpretation consistent with results, balancing benefits and harms, and considering other relevant evidence	125-126
Other information			
Registration	23	Registration number and name of trial registry	112
Protocol	24	Where the full trial protocol can be accessed, if available	112
Funding	25	Sources of funding and other support (such as supply of drugs), role of funders	130

*We strongly recommend reading this statement in conjunction with the CONSORT 2010 Explanation and Elaboration for important clarifications on all the items. If relevant, we also recommend reading CONSORT extensions for cluster randomised trials, non-inferiority and equivalence trials, non-pharmacological treatments, herbal interventions, and pragmatic trials. Additional extensions are forthcoming: for those and for up to date references relevant to this checklist, see www.consort-statement.org.