An investigation of the effect of vitamin D intake on vitamin D status and functional health outcomes in healthy children

Neil R. Brett
School of Human Nutrition
McGill University, Montréal

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ABSTRACT

Vitamin D is important to maintain calcium homeostasis and musculoskeletal health. Current vitamin D intake guidelines for young children were based on studies in adults and adolescents and thus may not be appropriate. The Estimated Average Requirement (EAR: 400 IU/d) and the Recommended Dietary Allowance (RDA: 600 IU/d) for vitamin D were set so that 50% and 97.5%, respectively, of the healthy general population, would have vitamin D status (25-hydroxyvitamin D: 25(OH)D) of 40 nmol/L and 50 nmol/L, respectively. These concentrations of 25(OH)D are designed to support bone health and calcium homeostasis. However, in North American young children, the effect of vitamin D intakes on musculoskeletal health outcomes is unknown. Thus, the objectives of this thesis are: 1. determine the effect of 400 IU/d or 600 IU/d of vitamin D from fortified foods, on vitamin D status over 12 weeks (wk) in children 2-8 y; 2. determine the effect of 400 IU/d of vitamin D on vitamin D status and musculoskeletal health outcomes over 6 months (mo); 3. investigate the effect of vitamin D interventions worldwide on vitamin D status in children 2-18 y.

Study 1 was a randomized placebo-controlled trial (RCT) where children consumed control or vitamin D fortified yogurt and cheese (to reach 400 IU/d (EAR group) or 600 IU/d (RDA group)) over 12 wk, with vitamin D status, anthropometry and dietary intakes measured. Study 2 was a 6 mo RCT of similar design, except there was only one intervention group (400 IU/d) and additional measures included other vitamin D metabolites, body composition and bone mineral (dual-energy x-ray absorptiometry), biomarkers of bone metabolism as well as 3-dimensional bone geometry and muscle (peripheral quantitative computed tomography). Study 3 was a meta-analysis of RCTs with vitamin D interventions in healthy children 2-18 y. The

overall effect of interventions and subgroup analyses were investigated using PRISMA guidelines.

Results from study 1 (n=77) showed both interventions had significantly higher 25(OH)D concentration than control at 12 wk ($p < 0.05$), but there were no differences (mean ± SD) between intervention groups ($p > 0.05$) (control: 55.8 ± 12.3; EAR: 64.1 ± 10.0; and RDA: 63.7 ± 12.4 nmol/L). In both intervention groups, 96% of children maintained 25(OH)D ≥ 50 nmol/L. Study 2 (n=51) found differences in 25(OH)D between groups at 3 mo (control: 58.3 ± 15.3; intervention: 64.7 ± 12.2, $p < 0.05$), but not 6 mo (control: 56.6 ± 13.9; intervention: 58.4 ± 8.7, $p > 0.05$). Over the 6 mo, 85% of the intervention group and 70% of the control group maintained 25(OH)D ≥ 50 nmol/L. Lean mass accrual was greater in the intervention group ($p < 0.05$) whereas there were no differences between groups for bone health outcomes. Study 3 included 25 RCTs (n=5120 children) in the meta-analysis with a 25(OH)D weighted mean difference (23.2 nmol/L, 95% CI 20.6-25.8 nmol/L) with high heterogeneity among studies ($I^2 = 99.9\%$) that resulted in a 1.0 nmol/L increase for every 100 IU/d of vitamin D. The effect on vitamin D status was greater in subgroups with mean baseline 25(OH)D < 30 nmol/L, using fortified foods and with baseline vitamin D intakes < 100 IU/d.

In conclusion, these results suggest consuming 400 IU/d of vitamin D maintains 25(OH)D ≥ 50 nmol/L in almost all healthy young children. By measuring lean mass outcomes in study 2, results suggest vitamin D may have been taken up by muscle and used to increase lean mass accrual. Study 3 shows there was a strong effect of vitamin D interventions on 25(OH)D, however high heterogeneity remained in subgroup analyses. These studies will help to improve existing vitamin D recommendations for young children and food fortification guidelines.

RÉSUMÉ

La vitamine D est importante pour maintenir l'homéostasie du calcium et la santé musculo-squelettique. Les directives actuelles sur la consommation de vitamine D pour les jeunes enfants sont basées sur des études menées auprès d'adultes et adolescents, et ne sont peut-être pas appropriées. Les besoins moyens estimatifs (BME: 400 UI/j) et apports nutritionnels recommendés (ANR: 600 UI/j) pour la vitamine D ont été établis pour assurer que 50% et 97,5% de la population générale en bonne santé, auront respectivement un niveau de vitamine D (25-hydroxyvitamine D: 25(OH)D) de 40 nmol/L et 50 nmol/L. Ces concentrations de 25(OH)D ont été conçues pour le support de la santé osseuse et l'homéostasie du calcium. Pourtant, chez les jeunes enfants nord-Américains, l'impact de la prise de vitamine D sur la santé musculo-squelettique est inconnu. Ainsi, les objectifs de cette thèse sont: 1. déterminer l'effet de 400 UI/j ou 600 UI/j de vitamine D, provenant des aliments fortifiés, sur le niveau de vitamine D chez les enfants de 2 à 8 ans pendant une période de 12 semaines; 2. A) déterminer s'il existe un effet similaire sur la concentration de vitamine D après 6 mois, avec un apport de 400 UI/j; B) étudier l'effet de cet apport sur la santé musculo-squelettique; Et 3. En utilisant une méta-analyse, étudier l'effet des interventions de vitamine D sur le niveau de vitamine D chez les enfants de 2 à 18 ans.

La première étude était un essai randomisé contrôlé contre placebo (ECR) où les enfants consommaient du yogourt et du fromage enrichis avec vitamine D (pour atteindre 400 UI / j ou 600 UI / j) pendant 12 semaines, ; leurs niveaux de vitamine D, les mesures d'anthropométries et les apports alimentaires ont été mesurés. La deuxième étude était aussi un ECR de 6 mois conçu similairement en évaluant un seul groupe d'intervention (400 UI / j) suivi de mesures supplémentaires ; autres métabolites de vitamine D, la composition corporelle (absorptiométrie à

rayons X à double énergie), les biomarqueurs du métabolisme osseuse, ainsi que la géométrie osseuse et musculaire tridimensionnelle (tomodensitométrie quantitative périphérique). La troisième étude était une méta-analyse des ECR qui contenaient des interventions de vitamine D chez les enfants 2 à 18 ans, en bonne santé. L'effet global des interventions et des analyses de sous-groupes on été examinés en utilisant les directives de PRISMA.

Les résultats de la première étude (n=77) ont montré que les deux interventions avaient une concentration de 25(OH)D significativement plus élevée que le groupe contrôle à 12 semaines ($p < 0.05$), mais qu'il n'existait aucune différence entre les groupes d'intervention ($p < 0.05$) (contrôle: 55,8 ± 12,3; BME: 64,1 ± 10,0 et ANR: 63,7 ± 12,4 nmol/L). Quatre-vingt-six pourcent des enfants, dans les deux groupes d'intervention, avaient maintenu leur niveau de 25(OH)D ≥ 50 nmol/L. La deuxième étude (n=51) a démontré des différences dans les niveaux de 25(OH)D entre les groupes à 3 mois (contrôle: 58,3 ± 15,3; intervention: 64,7 ± 12,2, $p < 0,05$), mais pas à 6 mo (contrôle: 56,6 ± 13,9; intervention : 58,4 ± 8,7, $p > 0,05$). Au cours des 6 mois, 85% du groupe d'intervention ont conservé leurs niveaux de 25(OH)D ≥ 50 nmol/L. L'accumulation de masse maigre était plus élevée dans le groupe d'intervention ($p < 0,05$). Pourtant, aucune différence était présente entre les groupes concernant leurs résultats de santé osseuse. La troisième étude comprenait 25 ECR (n=5120 enfants) dans la méta-analyse avec une différence de moyenne pondérée de 25(OH)D (23,2 nmol/L, IC 95% 20,6-25,8 nmol / L, I^2 = 99,9%) qui a montré une augmentation de 1,0 nmol/L pour chaque 100 UI/j de vitamine D.

En conclusion, ces résultats suggèrent que la consommation de 400 UI/j de vitamine D dépasse la définition de l'BME, mais ne correspond pas à celle de l'ANR. Dans la deuxième étude, c'est possible que la vitamine D a été captée et utilisée par les muscles pour augmenter la quantité de masse maigre. Les interventions de vitamine D ont eu un effet important sur les

niveaux de 25(OH)D suivie d'une hétérogénéité élevée et démontrée dans les analyses de sous-groupes. Ces études aideront à améliorer les recommandations des apports en vitamine D pour les jeunes enfants, et de donner plus de directives sur la fortification alimentaire.

STATEMENT OF SUPPORT

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ADVANCE OF SCHOLARLY KNOWLEDGE

I. Original contribution to knowledge

This doctoral dissertation provides the first vitamin D fortified foods trials in children in North America. Study 1 tested current vitamin D intake recommendations in a dose-response manner. Study 2 tested the intake of 400 IU/d over the entire winter period. By quantifying multiple vitamin D metabolites, this study was uniquely able to examine the relationship of vitamin D intake and status. Importantly, this study also compared 25(OH)D measurement methods to explore the bias of the Liaison chemiluminescent assays. Both trials used novel fortified foods that were well accepted by children and families and demonstrated that using yogurt and cheese products did not affect energy or macronutrient intakes. Studies showed that vitamin D intakes were comparable between food frequency questionnaires and 24 h recalls. By using whole body, lumbar spine and forearm DXA scans, study 2 quantified whole body and regional body composition and bone parameters providing novel lean mass and bone mineral accrual results. Lean mass accrual results brought forward the important question if other measures of vitamin D status are needed besides 25(OH)D. For the first time in a population as young as 2 y, pQCT scans measured 3-dimensional bone and muscle outcomes, providing unique data in this age group. By complimenting these measures with multiple biomarkers of bone health, this study provided an extremely detailed data-set of musculoskeletal health outcomes.

With no vitamin D intake and status meta-analyses undertaken before in children, study 3 provided truly novel data. By combining supplementation, food fortification and bolus injection studies, these vitamin D intake methods could be compared. By comparing linear and non-linear regression models, this meta-analysis demonstrated that the effect of vitamin D intake on status was not linear, as previously thought in adults. Subgroup analyses showed important factors

affecting the relationship between vitamin D intake and status. Using qualitative and quantitative methods of assessing bias (Cochrane bias assessment tool and Jadad scale) highlighted areas where trials consistently needed improvement in design. Results of this thesis provide detailed evidence of the effect of vitamin D intake on status and functional outcomes that had not previously been investigated in Canadian children.

II. Research publications in peer-reviewed scientific journals

1. **Brett, N.R**, Gharibeh, N, Weiler H.A. The effect of vitamin D supplementation, food fortification or bolus injection on vitamin D status in children 2-18 years: a meta-analysis. *Submitted to* Nutr Rev.
2. **N. R. Brett**, C.A. Parks, S. Agellon, P. Lavery, C. Vanstone, M. Kaufmann, G. Jones, J. L. Maguire, F. Rauch, H. A. Weiler. Vitamin D status and functional health outcomes in children 2-8 y: A 6-month vitamin D randomized controlled trial. *Revisions submitted to* Am J Clin Nutr.
3. C.A. Parks, **N. R. Brett**, S. Agellon, P. Lavery, C. Vanstone, , J. L. Maguire, F. Rauch, H. A. Weiler. Omega-3 long-chain polyunsaturated fatty acids in red blood cell membranes are associated with dietary intakes of omega-3-rich fish in healthy children. *Revisions submitted to* Prostaglandins Leukot Essent Fatty Acids.
4. **N. R. Brett**, P. Lavery, S. Agellon, C. Vanstone, J. Maguire, F. Rauch, H. A. Weiler. Dietary vitamin D dose-response in healthy children 2 to 8 y of age: a 12-wk randomized controlled trial using fortified foods. Am J Clin Nutr 2016;103:144–52.

III. Abstracts and presentations

1. **Brett, N.R**, Weiler H.A. Vitamin D intake and status in children 2-18 years: a meta-analysis. 8th International Conference on Children's Bone Health, June 10-13, 2017,

Wurzburg, Germany. (poster)

2. **Brett, N.R**, Vanstone, C.A, Weiler, H.A. Bone mineral accretion is increased during winter and is positively related to lean mass accretion and calcium intake in healthy children 2-8 y. 8th International Conference on Children's Bone Health, June 10-13, 2017, Wurzburg, Germany. (poster)
3. **Brett, N.R**, Vanstone, C.A, Weiler, H.A. Lean mass accretion increases during summer and positively associates with vitamin D status in healthy children 2-8 y. 8th International Conference on Children's Bone Health, June 10-13, 2017, Wurzburg, Germany. (oral)
4. **Brett, N.R**, Weiler H.A. Vitamin D intake and status in children 2-18 years: a meta-analysis. Canadian Nutrition Society Annual Conference, Montreal, May 25-27, 2017. (poster)
5. Patel, S, Vanstone, C.A, **Brett, N.R**, Mullahoo, K, Glenn, L, Laliberte, A, Razaghi, M, Yuan, Y, Gharibeh, N, Wei, S, Weiler, H.A. Prenatal supplements are required to meet the Recommended Dietary Allowance for vitamin D but elevated intakes above the upper level for iron and folate in the majority of pregnant women. Canadian Nutrition Society Annual Conference, Montreal, May 25-27, 2017. (poster)
6. **Brett, N.R**, Vanstone, C.A, Weiler, H.A. Bone mineral accretion is increased during winter and is positively related to lean mass accretion and calcium intake in healthy children 2-8 y. Experimental Biology, Chicago, April 22-27, 2017. (oral)
7. **Brett, N.R**, Vanstone, C.A, Weiler, H.A. Lean mass accretion increases during summer and positively associates with vitamin D status in healthy children 2-8 y. Experimental Biology, Chicago, April 22-27, 2017. (oral)
8. **Brett, N.R**, Vanstone, C.A, Weiler, H.A. The 6-month change in bone mineral density

measured by a portable device has poor agreement with dual-energy x-ray absorptiometry at the forearm in 2-8 y olds. Canadian Musculoskeletal Conference, Toronto, Ontario, October 14-15th 2016. (poster)

9. **Brett N. R**, Miller, F, Cohen, T.R, Agellon, S, Vanstone, C.A, Weiler, H. A. Irisin is proportionally lower in obese compared to healthy body mass index 6-9 y olds. Canadian Musculoskeletal Conference, Toronto, Ontario, October 14-15th 2016. (oral)
10. **Brett**, **N. R,** Parsons, K.E. Vanstone, C.A, Weiler, H.A. Energy and protein intakes above recommendations inversely correlate with lean mass indices in 2-8 y olds. American Society for Bone and Mineral Research Annual Meeting, Atlanta, Georgia, September 16-19th 2016. (poster)
11. **Brett**, **N. R,** Parsons, K.E. Vanstone, C.A, Weiler, H.A. Lean mass indices: An alternative to BMI for assessing growth outcomes in young children. American Society for Bone and Mineral Research Annual Meeting, Atlanta, Georgia, September 16-19th 2016. (poster)
12. **Brett, N. R**, Parks, C.A, Agellon, S, Lavery, P, Vanstone, C.A, Maguire, J. L, Rauch, F, Weiler H. A. Vitamin D status and markers of bone metabolism: a 6 month randomized vitamin D trial in healthy 2-8 y olds using fortified foods. Canadian Nutrition Society Annual Conference, Ottawa, May 5-7, 2016. (poster)
13. Parks, C.A, **Brett, N. R**, Agellon, S, Lavery, P, Vanstone, C.A, Maguire, J. L, Rauch, F, Weiler H. A. Long-chain polyunsaturated fatty acids in erythrocyte membranes are associated with biomarkers of bone metabolism in healthy children. Canadian Nutrition Society Annual Conference, Ottawa, May 5-7, 2016. (poster)

14. **Brett, N. R**, Parks, C.A, Agellon, S, Lavery, P, Vanstone, C.A, Maguire, J. L, Rauch, F, Weiler H. A. Lean mass accretion associates with vitamin D intake: A 6 month randomized controlled trial in 2-8 y olds using fortified food. Experimental Biology, San Diego, April 1-4, 2016. (oral and poster)
15. **Brett, N. R**, Vanstone, C.A, Maguire, J. L, Rauch, F, Weiler H. A. Parental knowledge, perceptions and consumption of milk and alternatives relates to intakes of young children 2-8 y of age. Experimental Biology, San Diego, April 1-4, 2016. (oral)
16. Parks, C.A, **Brett, N. R**, Agellon, S, Lavery, P, Vanstone, C.A, Maguire, J. L, Rauch, F, Weiler H. A. Erythrocyte omega-3 long chain polyunsaturated fatty acids are associated with lumbar spine but not whole body bone mineral density in healthy children. Experimental Biology, San Diego, April 1-4, 2016. (oral)
17. **Brett, N. R**, Parks, C.A, Agellon, S, Lavery, P, Vanstone, C.A, Maguire, J. L, Rauch, F, Weiler H. A. The effect of diet and seasonal ultraviolet beta radiation exposure on serum 25(OH)D in 2-8 y olds. 9th International Symposium on Nutritional Aspects of Osteoporosis, Montreal, June 17-20th 2015. (poster)
18. **Brett, N. R**, Agellon, S, Lavery, P, Vanstone, C.A, Maguire, J. L, Rauch, F, Weiler H. A. Vitamin D status and functional health outcomes: A randomized vitamin D dose-response trial in 2-8 y olds. 2nd Annual Perform Centre Research Conference, Montreal, May 15th 2015. (poster)
19. **Brett, N. R**, Lavery, P, Vanstone, C.A, Maguire, J. L, Rauch, F, Weiler H. A. Distal one-third forearm bone mineral density of healthy young children is more highly related to lumbar spine than whole body bone mineral density. McGill Biomedical Graduate Conference, Montreal, March 19th 2015. (poster)

20. **Brett, N. R**, Agellon, S, Lavery, P, Vanstone, C.A, Maguire, J. L, Rauch, F, Weiler H. A. Vitamin D status and functional health outcomes: A randomized vitamin D dose-response trial in 2-8 y olds. Experimental Biology, Boston, March 28- April 1st 2015. (oral)
21. **Brett, N. R**, Agellon, S, Lavery, P, Vanstone, C.A, Maguire, J. L, Rauch, F, Weiler H. A. Milk product intake may not associate with body fat percentage or bone density in young children. Experimental Biology, Boston, March 28- April 1st 2015.(poster)
22. **Brett, N. R**, Agellon, S, Lavery, P, Vanstone, C.A, Maguire, J. L, Rauch, F, Weiler H. A. Vitamin D dose-response in young children 2 to 8 y of age: a 12 wk randomized clinical trial to establish requirements in the absence of ultra-violet beta solar radiation. Research Feeding Industry Symposium, Montreal, September 17th 2014. (oral)
23. **Brett, N. R**, Lavery, P, Vanstone, C.A, Maguire, J. L, Rauch, F, Weiler H. A. Distal one-third forearm bone mineral density of healthy young children is more highly related to lumbar spine than whole body bone mineral density. American Society for Bone and Mineral Research Annual Meeting, Houston Texas, September 12-15th 2014. (poster)
24. **Brett, N. R**, Agellon, S, Lavery, P, Vanstone, C.A, Maguire, J. L, Rauch, F, Weiler H. A. Vitamin D dose-response in young children 2 to 8 y of age: a 12 wk randomized clinical trial to establish requirements in the absence of ultra-violet beta solar radiation. American Society for Bone and Mineral Research Annual Meeting, Houston Texas, September 12-15th 2014. (poster)
25. **Brett, N. R**, Agellon, S, Vanstone, C.A, Weiler H. A. Vitamin D status positively associates with interleukin-6 and tumour necrosis factor alpha in healthy young children. Experimental Biology, San Diego California, April 26-30th 2014. (oral)

CONTRIBUTION OF AUTHORS

For manuscript 1, the candidate was the primary author, wrote the first draft of the manuscript, performed statistical analyses and made major data interpretations. The candidate was also involved with participant recruitment (with Catherine Vanstone and Paula Lavery) and with data acquisition along with Catherine Vanstone, Paula Lavery and Sherry Agellon. Dr. Weiler, Dr. Maguire and Dr. Rauch provided consultation for statistical analyses, data interpretation and revisions for the manuscript. This research was funded by an investigator initiated grant (by Dr. Weiler) from Dairy Farmers of Canada as well as from Canada Research Chairs and Canada Foundation for Innovation programs. The candidate was supported by the Donald Mackenzie Munroe Fellowship

For manuscript 2, the candidate was the primary author, was involved with designing methodology for the vitamin D intervention (with Catherine Vanstone and Dr. Weiler) and pQCT measurements (with Catherine Vanstone, Dr. Weiler and Dr. Rauch), participant recruitment (with Catherine Vanstone), collected data (with Colleen Parks, Catherine Vanstone, Paula Lavery, Sherry Agellon and Dr. Weiler) conducted biochemical analyses (with Sherry Agellon, Dr. Jones, Dr. Kaufmann and Dr. Rauch), DXA assessments (with Catherine Vanstone, Paula Lavery and Colleen Parks), performed statistical analyses and wrote the first draft of the manuscript. Dr. Weiler, Dr. Rauch, Dr. Maguire, Dr. Jones and Dr. Kaufmann all made contributions to some aspect of statistical analyses or data interpretations as well as providing critical revisions to the manuscript. This research was funded by the same grant and sources as study 1.

For manuscript 3, the candidate was primary author, conducted the literature search and data synthesis (with Nathalie Gharibeh), performed statistical analyses, made major data

interpretations and wrote the first draft of the manuscript. The candidate and Dr. Weiler were responsible for study concept and design. Nathalie Gharibeh and Dr. Weiler provided edits for the manuscript and Dr. Weiler was consulted for statistical analyses. The candidate was supported by the Donald Mackenzie Munroe Fellowship.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

intervention: n=26). All spearman correlations were not significant ($R^2 < 0.10$, $p > 0.05$) except for the control group in Panels A, C and D ($R^2 = 0.18$-0.26, $p < 0.05$).

Figure 6.6 Comparison of the mean total 25(OH)D measured by Liaison with $25(OH)D_3$ (Panel A) and total 25(OH)D (Panel B) measured by LC-MS/MS in children 2-8 y. Different superscripts depict significant differences between time-points using a mixed model ANOVA with post hoc Bonferroni correction. No differences between measurement methods. Oct: n=51, Jan: n=49, Apr: n=49.

Figure 6.7 Bland-Altman plots of agreement between total 25(OH)D measured by Liaison and $25(OH)D_3$ (Panel A) and total 25(OH)D (Panel B) measured by LC-MS/MS at all 3 time-points (Oct: n=51, Jan: n=49, Apr: n=49) in children 2-8 y.

LIST OF ABBREVIATIONS

Abbreviation	Full text
$1,25(OH)_2D$	1,25-dihydroxyvitamin D
3-epi-25(OH)D	C3 epimer of 25-hydroxyvitamin D
$24,25(OH)_2D$	24,25-dihydroxyvitamin D
25(OH)D	25-hydroxyvitamin D
µSV	Microsieverts
AAP	American Academy of Pediatrics
AI	Adequate Intake
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BMC	Bone mineral content
BMD	Bone mineral density
BMI	Body mass index (kg/m^2)
$Ca2^+$	Calcium 2^+
CCHS	Canadian Community Health Survey
CHMS	Canadian Health Measures Survey
CLIA	Chemiluminescent immunoassay
cm	Centimeters
CONSORT	Consolidated Standards of Reporting Trials
CPBA	Competitive protein binding assay
CpG	Cytosine followed by guanine nucleotides

CPS	Canadian Paediatric Society
CSA	Cross-sectional area
CTX	C-terminal telopeptide of type 1 collagen
CYP2R1	Vitamin D_3-25-hydroxylase
CYP24A1	24-hydroxylase (for 25(OH)D or 1,25$(OH)_2$D
CYP27B1	25$(OH)D_3$-1-hydroxylase
d	Day
DBP	Vitamin D binding protein
DEQAS	Vitamin D External Quality Assessment Scheme
DIN	Drug Identification Number
DRI	Dietary Reference Intakes
DXA	Dual-energy x-ray absorptiometry
EAR	Estimated Average Requirement
ELISA	Enzyme-linked immunosorbent assay
EU	European Union
FFQ	Food frequency questionnaire
FGF23	Fibroblast growth factor 23
g	Grams
GRAS	Generally regarded as safe
HPLC	High performance liquid chromatography
HRpQCT	High resolution peripheral quantitative computed tomography
I^2	I-squared statistic
IGF-1	Insulin-like growth factor 1

IGFBP3	Insulin-like growth factor binding protein three
IOM	Institute of Medicine (US)
IQR	Interquartile range
ISCD	International Society for Clinical Densitometry
ITA	Individual typological angle
IU	International Unit [1 IU vitamin D = 0.025μg]
kg	Kilogram
LC-MS/MS	Liquid chromatography tandem mass spectrometry
MAPK	Mitogen-activated protein kinase
mg	Milligrams
min	Minutes
mo	Month
mRNA	Messenger ribonucleic acid
NIH	National Institutes of Health (US)
NHANES	National Health and Nutrition Examination Survey (US)
NIST	National Institute for Standards and Technology
nmol/L	Nanomoles per litre
NOAEL	No Observed Adverse Effect Level
nVDRE	Vitamin D receptor negative response elements
P1NP	Procollagen type 1 N-terminal propeptide
pQCT	Peripheral quantitative computed tomography
PRISMA	Preferred reporting items for systematic reviews
PTH	Parathyroid hormone

PTHrP	Parathyroid hormone related protein
RANKL	Receptor activator of nuclear factor kappa-B ligand
RIA	Radioimmunoassay
RCT	Randomized controlled trial
RDA	Recommended Dietary Allowance
RXR	Retinoid X receptors
SD	Standard deviation
SEM	Standard error of the mean
SNP	Single nucleotide polymorphism
SPF	Sun protection factor
UL	Tolerable Upper Intake Level
US	United States of America
USDA	United States Department of Agriculture
UVB	Ultraviolet beta
Vitamin D_2	Ergocalciferol
Vitamin D_3	Cholecalciferol
VDIR	Vitamin D interacting repressor
VDR	Vitamin D receptor
VDRE	Vitamin D receptor elements
wk	Week
WHO	World Health Organization
y	Year

CHAPTER 1: INTRODUCTION

1.1 BACKGROUND AND RATIONALE

Vitamin D is well accepted for its function in normal bone mineral accretion (8) as well as having roles in muscle function and general health. Vitamin D is considered an essential nutrient (8) as it cannot be endogenously synthesized year round in regions above 40^{o} N latitude. This is due to the limited ultraviolet beta (UVB) solar radiation from the months of October through to end of March (9). Canada's Food Guide recommends children age 2 to 8 y consume 2 servings of milk and alternatives per day (10, 11). Though average dietary calcium intakes for Canadian children 4-8 y of age (~1005 mg, SE 41) exceed the Institute of Medicine (IOM) Estimated Average Requirement (EAR, 800 mg), the average vitamin D intake (244 IU, SE 16 IU) is only slightly more than half of the EAR (400 IU/d) (12, 13). This is most likely because vitamin D fortification regulations were set when recommended intakes were lower and are for year-round, not just UVB void periods (14-16).

In Canada, data shows that individuals relied on vitamin D fortified foods for between 65-85% of their total daily vitamin D intake (17). Though vitamin D began being added to certain ready to eat cereals, biscuits and cacao mixes in Canada in 1942, due to risk of excess vitamin D intakes, this practice was halted by the mid-1960's (18). However, in an effort to decrease rickets and vitamin D deficiency, vitamin D began being added to fluid milk in 1975. Currently, fluid milk and margarine (100 IU/250 mL and 530 IU/100 g, respectively) are mandatorily fortified with vitamin D and are allowed to be within a range of ± 15% of their fortification targets. Though vitamin D is also voluntarily added to orange juices and cereals, fortified fluid milk accounts for between 40-65% of total intake of vitamin D from fortified foods (17). According to national surveillance data, there is a positive association between fluid

milk intake and vitamin D status (7). However, meeting the food group recommendation for milk and alternatives by only consuming vitamin D fortified fluid milk provides only half of the EAR and a third of the Recommended Dietary Allowance (RDA) of 600 IU/d. Furthermore, consuming yogurt or cheese instead of fluid milk increases the risk of diets containing lower amounts of vitamin D intakes as they have < 30% of the vitamin D content compared to that in fluid milk (8).

The IOM dietary vitamin D intake recommendations were designed to align with serum 25-hydroxyvitamin D (25(OH)D: a composite reflection of total intake from the diet, supplements and endogenous synthesis) concentration of 40 nmol/L (EAR) and 50 nmol/L (RDA) (8). Given these recommendations, Canadian children are at risk of not meeting the target 25(OH)D concentrations during the UVB void periods (14). However, indication of low vitamin D status based on national surveillance data is not evident (7). Importantly though, there is a lack of confidence in the interpretation of the percentage of Canadian children who are vitamin D deficient due to the small sample size and high variance in the data (7). National data also only depicts year-round vitamin D status of children (7), suggesting further work is needed to both assess the prevalence of vitamin D deficiency and how vitamin D status differs between the UVB void period versus other months of the year.

Since the IOM recommendations set for children were based on results in adults and older children (8), it is important to assess the EAR for young children as it is not only required to derive the RDA value (EAR+2 SD), but it is also used in establishing food fortification policies. Interestingly, in two Canadian cross sectional studies (2, 19) the average vitamin D status was above 50 nmol/L (88.9 nmol/L, 95% C.I. 88.0-90.5 nmol/L and 75.0 nmol/L, 95% CI 70.3-79.7 nmol/L), for children 1-5 y of age, and < 5% of both populations had 25(OH)D < 40

nmol/L even though dietary intakes of vitamin D were well below the EAR for almost all children. In both studies, vitamin D status was however, lower by an average of 4.0 nmol/L (95%CI 1.0-7.0 nmol/L) and 10.1 nmol/L, respectively in children measured in winter compared to those measured in summer. With data depicting vitamin D insufficiency, a study in Montreal showed almost twice as many children insufficient in the UVB void period compared to the synthesizing period (UVB void: 14.0% (95% CI 9.3-18.6%) of children, synthesizing: 8.2% (95% CI: 5.0-11.3% of children). Therefore, the amount of dietary vitamin D necessary to reach the status target ranges for young children remains unclear.

Cross-sectional studies have shown significant associations between vitamin D intake and status (1, 20, 21) including in 6-12 y old American children (n=140, r=0.44, p=0.004) (11). Vitamin D supplementation trials have shown that over a period as short as 7-8 wk, vitamin D status can significantly increase (22). However, since only ~30% of Canadian children consume vitamin D supplements and young children (2-8 y) consume ≥ 2 servings/d of milk and alternatives, on average, fortification seems a more realistic method of increasing vitamin D intake instead of supplementation. In winter food fortification trials in young children in Denmark, Germany as well as in Mongolia, fortified food products were well accepted by children. In these interventions, for every 100 IU increase in vitamin D intake per day, 25(OH)D concentrations increased ~7.5-10 nmol/L (23-25). Similarly in Canadian national surveillance data, children 3-5 y and 6-11 y who consumed 1 or more glasses of milk per day had 15 nmol/L and 8 nmol/L, respectively, higher 25(OH)D concentration than those who consumed < 1 glass per day (6).

Research not only needs to focus on current dietary intakes of vitamin D, but also needs to investigate the effect of vitamin D intake on musculoskeletal functional health outcomes. The

IOM vitamin D intake recommendations were set with bone health outcomes in mind (8). With regard to bone, Vitamin D has been shown to help ensure healthy parathyroid hormone concentrations, a hormone involved in calcium and bone metabolism (26). Vitamin D acts to maintain calcium homeostasis; this is a composite result of improved calcium absorption in the duodenum, action in bone cells, osteoblasts and osteoclasts to mobilize calcium and on cells in the proximal tubules of the kidney to regulate calcium reabsorption (27, 28). Cross-sectional research focused on bone health in pre-school age children in Montreal (n=504), showed that vitamin D intake was positively associated with whole body and radial bone mineral density (BMD) (29). Vitamin D trials in children 3-5 y from Sri Lanka, and 10 y from China corroborate this, with results showing vitamin D food fortification leading to increased bone mineral accrual (30, 31). However, a meta-analysis of vitamin D related bone outcomes in children showed that the relationship of bone health outcomes and vitamin D is unclear (32). This highlights that vitamin D research with bone health outcomes is needed in Canadian children to further elucidate the vitamin D and bone development relationship.

Though the IOM, as well as the Endocrine Society, took bone health outcomes into consideration when deriving vitamin D intake recommendations, they did not take parameters of muscle health into consideration (8, 33). Vitamin D has been shown to have many functions in muscle, mediated through the vitamin D receptor (34), including inducing protein synthesis, affecting cellular proliferation and differentiation and improving muscular contraction (35). Keeping in mind that in young children, because of rapid development, lean mass accretion occurs at an average rate of 2 kg/y (children 5-10 y) (36), it is essential to investigate how muscle development in young children relates to vitamin D.

Associations between vitamin D status and lean mass accretion in children are not well understood, however, a study of children 3 y in Montreal showed that having a vitamin D status ≥ 75 nmol/L from infancy to 3 y, led to a leaner body phenotype (37). Agreeing with this, a study of 15 y old Chinese girls (n=323), showed that lean body mass positively associated with 25(OH)D ($r=0.446$, $p < 0.001$) (38). However, trials of vitamin D in young children investigating lean mass outcomes are very limited. In a 1 y trial of vitamin D deficient pre-pubertal girls in Lebanon (n=34), percent change in lean mass was significantly higher in girls supplemented with 200 IU/d or 2000 IU/d of vitamin D compared with control ($p=0.04$) (39). Thus, a vitamin D intervention, with lean mass outcomes, is well warranted in young Canadian children.

There has been an increased interest in vitamin D research in children because of the 2011 IOM vitamin D recommendations. Since the vitamin D RDA for children is an extrapolation from studies mostly in adults, recent trials have attempted to elucidate the dose of vitamin D needed for 97.5% of children to maintain 25(OH)D ≥ 50 nmol/L during winter or the absence of UVB exposure (consistent with the RDA). Results in children 4-10 y from Denmark and the USA show 800 IU/d and 1500 IU/d of vitamin D, from supplements, were needed to achieve the vitamin D status target of 50 nmol/L (40, 41). In children 14-18 y, it was not known how much vitamin D was needed for 97.5% of children to maintain 25(OH)D ≥ 50 nmol/L because the dose-response effect of vitamin D intake on status plateaued at an intake of 1200 IU/d (40). Without a meta-analysis of vitamin D intake and status having been done in children, these results show a need for this type of analysis so that factors affecting the relationship of vitamin D intake and status can be elucidated. A 2012 meta-analysis of vitamin D_3 food fortification studies in adults found a dose dependent increases of 3.0 nmol/L for every increase of 100 IU/d in vitamin D (42). This analysis also found that living at ≥ 40 ^{o} N latitude and having

a baseline vitamin D status < 50 nmol/L significantly increased the effect of vitamin D interventions. However, because these results were only from food fortification trials, it would be efficacious for a meta-analysis in children to include supplementation trials as well to be able to compare the effects of various administration modalities such as bolus oral or injection dosages. Also, by including both young children and teenagers, it can be investigated if vitamin D intake guidelines should differ based on age of children.

1.2 STATEMENT OF PURPOSE

In North American children, there is a lack of data regarding how intakes at the level of the current DRI values for vitamin D affect vitamin D status and functional health outcomes in months with minimal UVB exposure. The aim of this thesis was to longitudinally test, using randomized placebo-controlled trials, the effect of increasing vitamin D intake on status using experimentally vitamin D fortified milk products, and how this effect compares to previous supplementation and fortification vitamin D trials in children around the world. In addition, these trials were designed *a priori* to test whether vitamin D status ≥ 50 nmol/L, compared to below 50 nmol/L, confers musculoskeletal benefits.

1.3 OBJECTIVES AND HYPOTHESES

Study 1: January to April 2014 (12 wk duration)

Primary Objective: Determine whether vitamin D intakes consistent with the EAR or RDA, through fortification of additional dairy products, will result in higher vitamin D status in young children.

Secondary Objective: Confirm if vitamin D intakes reaching the RDA will sustain a serum 25(OH)D concentration of 50 nmol/L in young children during winter and early spring months.

Hypotheses: During this 12 wk randomized controlled dose-response trial, it was hypothesized that fortified cheese and yogurt (providing 254 to 420 IU/d), when compared to regular milk products, will significantly increase serum 25(OH)D concentrations by 25-42 nmol/L. Also, it was hypothesized that winter 25(OH)D values will be ~ 50-65 nmol/L and will drop throughout the study in the control group and that dietary intakes reaching the EAR and RDA (400 IU/d and 600 IU/d) will both lead to 25(OH)D concentrations significantly higher than the control group and that the RDA intake will lead to 25(OH)D concentrations that exceed 75 nmol/L.

The results of study one will determine the amount of vitamin D_3 added to cheese and yogurt products for study two, to ensure serum 25(OH)D concentration in the intervention group will be sustained over 50 nmol/L.

Study 2: October 2014 to April 2015 (6 mo duration)

Primary Objective: To test how much vitamin D intake from food is required to maintain healthy vitamin D status from the beginning of the UVB-void period (end of October) to the end of the winter period (March).

Secondary Objectives: Explore the effects on lean mass, bone mineral accrual, bone geometry and biomarkers of bone mass and mineral metabolism.

Hypotheses: It was hypothesized that the average 25(OH)D concentration at the end of summer will be ~75 nmol/L and concentrations will significantly drop throughout the study in the control group. However, it was thought that the increased vitamin D intakes of the intervention group would sustain serum 25(OH)D concentrations of 75 nmol/L throughout the study duration.

Over the 6 month study period, it was thought that children in the intervention group would have: Significantly increased bone mineral accrual and lean mass accrual, procollagen type 1 N-terminal propeptide (P1NP) osteocalcin and significantly decreased C-terminal telopeptide of type 1 collagen (CTX) and parathyroid hormone (PTH).

Study 3: Vitamin D meta-analysis

Primary objective: In a meta-analysis, investigate the effect of vitamin D interventions on vitamin D status, in children 2-18 years of age.

Secondary objective: To explore if the effect of vitamin D intake on status is linear and if factors including age, sex, latitude, baseline vitamin D status and measurement method have a significant role in the relationship between exogenous intakes and status.

Hypotheses: It was hypothesized that there would be a dose dependant increase of 25(OH)D based on vitamin D intake. It was thought that baseline 25(OH)D, vitamin D intervention method, age of children and location of the intervention will significantly effect this dose-response relationship.

CHAPTER 2: REVIEW OF LITERATURE

2.1 INTRODUCTION

It is well accepted that vitamin D has functions in calcium homeostasis and bone health (43). Deficient vitamin D status can lead to rickets, reduced bone mineralization which could result in bone fragility, osteoporosis and an increased risk of fractures later in life (44). Since endogenous synthesis of vitamin D is minimal in Canada during winter months due to northern latitude (45), it is essential to consume vitamin D from dietary sources. In 2011, the harmonized Canada and US dietary vitamin D recommendations were updated to an Estimated Average Requirement (EAR) of 400 IU/d and a Recommended Dietary Allowance (RDA) of 600 IU/d (12). The vitamin D status recommendations of 40 nmol/L and 50 nmol/L of 25-hydroxyvitamin D (25(OH)D) were set to align with the vitamin D EAR and RDA, respectively, based on adequacy of markers of bone health and calcium homeostasis (12).

For young children, it is unclear however, if these recommendations are appropriate as they were set based on studies in adults and adolescents (12). Illustrating this, though the majority of young children (2-8 y) in Canada do not meet vitamin D intake recommendations (mean intake: 244 IU/d (± SE 16 IU/d)), the average year-round vitamin D status is ≥ 50 nmol/L in 78% of children 3 to 11 y of age (6). During winter months specifically however, decreases in 25(OH)D may lead to twice as many children having inadequate vitamin D status (1). It is not known if this has a detrimental effect on bone development in young children. Since vitamin D receptors are present in many tissues, including muscle (46), investigation of muscle as a functional outcome of vitamin D intake in young children is also needed. This comprehensive review of the literature seeks to highlight the vitamin D intake and status of young Canadian

children and identify current gaps in knowledge about vitamin D and musculoskeletal health outcomes.

2.1.1 DEFINITIONS

For exogenous vitamin D intakes in children 2-8 years old, there are three values that are used to determine daily intake. The Estimated Average Requirement (EAR), which is set to meet 50% of the populations needs, the Recommended Dietary Allowance (RDA), which is set to meet 97.5% of the populations needs and the Tolerable Upper Intake Level (UL), which is the highest level of nutrient intake likely to pose no adverse health effect. The respective EAR and RDA for vitamin D daily intake for this age group are 400 IU (10 µg) and 600 IU (15 µg) (8). The vitamin D UL for children is differentiated by age groups 1-3 years and 4-8 years, for which it is 2500 IU (62.5 µg) and 3000 IU (75 µg), respectively (8).

Vitamin D status is commonly assessed by analyzing the serum concentration of 25(OH)D. This biomarker is a composite measure of vitamin D acquired from all sources. Vitamin D deficiency is a state that causes obvious abnormalities in bone metabolism and growth as well as inefficiency of calcium intestinal absorption (33). This state is associated with diseases including rickets in children, osteoporosis, autoimmune diseases and inflammatory bowel diseases (47). Insufficiency of vitamin D is a state associated with possible disease risks without the obvious bone abnormalities seen in deficiency(8). Vitamin D sufficiency is the range of 25(OH)D concentration least associated with disease risk and negative health outcomes. The Institute of Medicine (8) and the Canadian Paediatric Society (CPS) (47) both agree that vitamin D deficiency is defined as having 25(OH)D concentrations below 30 nmol/L (**Table 2.1**). There is debate about the concentrations ranges that should fit under the definition of insufficiency, the CPS and the Endocrinology Society (33) both state that vitamin D sufficiency is having

25(OH)D concentrations above 75 nmol/L. However, both Canada and USA use the IOM vitamin D intake and status sufficiency recommendations of 50-125 nmol/L.

2.2 VITAMIN D SOURCES

2.2.1 Endogenous synthesis

2.2.1.1 Sun Exposure

Radiation from the sun is emitted across numerous electromagnetic spectrums, but ultraviolet radiation (UVR) is the only radiation of significance to vitamin D in the skin (48). UVR can have a number of functional purposes besides those related to vitamin D, it is used in some water purifiers as a viricide and bacteriocide and it is used as a treatment method for certain autoimmune disorders (49). Health Canada states that UV radiation can also have negative health effects as it has been linked to premature aging, skin cancer, eye problems, sun burns and weakening of the immune system (48).

There are a number of subtypes of UVR (100-400 nm wavelength), these include: ultraviolet A (UVA), ultraviolet B (UVB) and ultraviolet C (UVC) with wavelengths of 315-400 nm, 280-315 nm, 100-280 nm, respectively (50). The Earth's ozone layer, when the sun is at its zenith, is able to absorb a minimum of 77% of the UV rays from sunlight. About 97-99% of the UV rays that do make it to the Earth's surface are UVA because both the dioxygen and the ozone in the atmosphere are able to block all of the UVC and almost all of the UVB radiation (51). When UV rays from sunlight do hit the Earth's surface, about 5% are UVB and 95% are UVA. Standard glass windows are able to block roughly 90% of UV rays below 300 nm but about 90% of UVA above 350 nm would be able to pass through window panes (52).

Table 2.1 Recommended vitamin D status categorizations as defined by 25(OH)D concentrations

	Deficiency (nmol/L)	**Insufficiency (nmol/L)**	**Sufficiency (nmol/L)**
Canadian Paediatric Society (47)	< 25	25-74.9	75-225
Endocrinology Society (33)	< 50	50-74.9	≥ 75
Institute of Medicine (8)	< 30	30-49.9	50-125

When the sun is at a more oblique angle towards the earth, light has to pass through more of the atmospheric layer and thus, more UVB gets absorbed in the atmosphere (53). Living further from the equator means that there are fewer months of the year that sunlight contains sufficient quantities of UVB rays to support vitamin D synthesis in the skin. For example, in the city of Edmonton (52° N) cutaneous vitamin D synthesis is basically non-existent from October-April but living within 37° of the equator, people are able to synthesize vitamin D year round (54). Time of day is also an important factor to sun exposure, because of oblique angles of the sun during the early morning and evening hours, vitamin D synthesis is only possible when exposed to sunlight between 10 am and 3 pm (54). People over the age of 70 y have been shown to synthesize 25% less vitamin D from exposure to sunlight when compared to 20 y olds (54), whilst 8 y old children were shown to synthesize 20% more vitamin D compared to 18 y olds (55). Interestingly, based on Canadian national surveillance data, it was shown that non-white Canadians did not have a significant 25(OH)D response to summer UVB exposure (56). Health Canada recommends that children limit UV exposure and take measures of photoprotectivity when in the sun, including the use of sunscreen (57). By following these recommendations, children would be able to synthesize very minimal amounts of vitamin D from exposure to UVB from sunlight and as a result would be getting the majority of their vitamin D from dietary sources year round.

2.2.1.2 Pre-Vitamin D(7-Dehydrocholesterol) and the skin

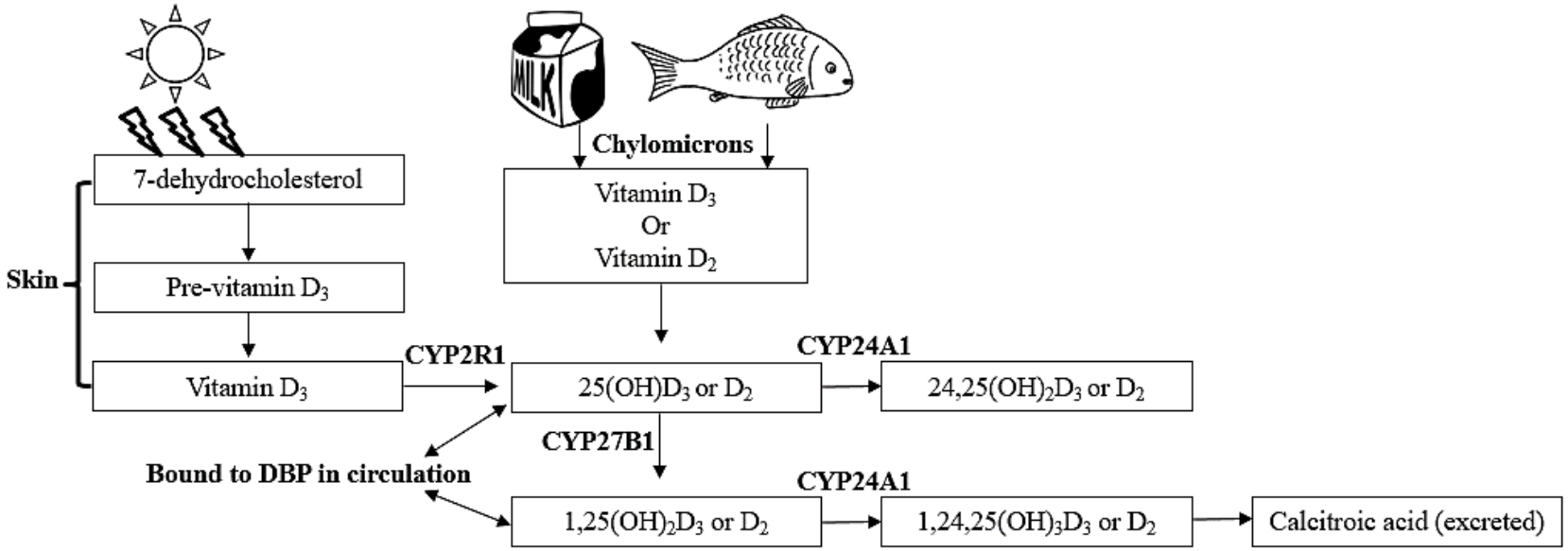
The skin is composed of two primary layers, the outer epidermis, which is thinner than the inner layer called the dermis and is mostly composed of connective tissue (58). Vitamin D is synthesized in the epidermis, which has multiple layers, the two most inner layers (stratum basale and stratum spinosum) being responsible for the majority of vitamin D synthesis (**Figure**

2.1) (58). These two layers have the highest concentration of 7-dehydrocholesterol, also called pre-vitamin D. When 7-dehydrocholesterol gets irradiated by UVB light, it gets activated, the bond between carbon 9 and 10 breaks and it becomes pre-vitamin D_3 through a long temperature dependent thermal isomerization (hydrogen transfer from carbon 19 to carbon 9) to vitamin D_3 (also called cholecalciferol) (59). Vitamin D_3 binds to the vitamin D binding protein (DBP), found in the capillary bed of the dermis, and enters the circulatory system headed for the liver where it is metabolized to 25(OH)D_3. The vitamin D_3 binding protein allows continued production of vitamin D_3 by ensuring the equilibrium of the synthesis reaction is shifted so that pre-vitamin D_3 continues to isomerize. The slow heat dependent isomerization process allows the release of vitamin D_3 into circulation up to 3 days after being exposed to sunlight (59).

2.2.1.3 Regulation of the synthesis of vitamin D_3

Melanin is a chief factor that regulates the extent to which vitamin D_3 is synthesized(43). The pigment melanin is produced by melanocyte cells, mostly located in the stratum basale in the epidermis, and is responsible for the colour of the skin. Melanin pigment is transferred throughout epidermal cells so that it is present in all epidermal layers (43). Melanin functions to absorb UVB light and because of this, acts to limit the amount of UVB that is able to reach 7-DHC in the lower levels of the epidermis. In the skin, after about 15% of the available 7-DHC gets converted to pre-vitamin D_3, isomerizations to physiologically inert stereoisomers of pre-vitamin D_3 and vitamin D_3 begin (43). The isomers produced from pre-vitamin D_3 include lumisterol and tachysterol and this controlled process of photoisomerization explains the mechanism behind why prolonged sun exposure does not induce vitamin D toxicity.

Figure 2.1 Vitamin D synthesis and metabolism from sunlight and dietary sources



2.2.2 Exogenous intake

2.2.2.1 Naturally occurring in food

Cholecalciferol and ergocalciferol (vitamin D_2) are the two naturally occurring forms of vitamin D. Vitamin D_3 is naturally most abundantly found in fish (**Table 2.2**) (33). Wild caught fish including salmon, cod and mackerel can have as much as 1000 IU of vitamin D in 100 g, whilst farmed fish will usually only contain 100-250 IU vitamin D for the same sized serving (6, 60). Vitamin D seems to be a relatively heat stable molecule in fish as it is 50-100% preserved through cooking, even after the high temperatures of frying (61). Fungi and yeast also produce vitamin D_2 from the conversion of ergosterol to ergocalciferol in the presence of UVB light (62), often during processing (63). According to the USDA nutrient database (63), portobello mushrooms exposed to UVB light for 15-20 seconds during processing can contain up to 1000 IU of vitamin D_2 per 100 g after grilling.

2.2.2.2 Fortified food sources and bioenrichment

In Canada and the USA, NHANES data show that individuals relied on vitamin D fortified foods for between 65-85% of their total daily vitamin D intake and that fortified fluid milk accounted for between 40-65% of that amount (17). Vitamin D_3 is used to fortify milk sources and most other fortified products including orange juices, margarine and cereals, and is produced by irradiating 7-dehydrocholesterol found in lanolin in sheep's wool (64). Since this process is deriving vitamin D from an animal source, some vegetarians will not eat foods fortified in this manner and many milk alternatives are fortified with Vitamin D_2 as a result (64). Though some eggs are bioenriched with vitamin D_3, it is common for eggs to be bioenriched with vitamin D_2 as hens are given yeast derived vitamin D_2 in their feed that results in the enrichment of eggs (egg yolk) with vitamin D (65).

Table 2.2 Common foods containing Vitamin D in Canada (66)

Food	Serving size[1]	Vitamin D (IU)	
		Total/serving	IU/100 g
Fortified orange juice	250 mL	100	40
Fluid milk	250 mL	100	40
Fortified yogurt	175 g	58-71	33-41
Fortified soy beverage	250 mL	100	40
Egg yolk (cooked)	2, large[2]	57-88	163-262
Margarine	5 mL	25-36	500-720
Salmon (red or pink, cooked)	75 g	392-636	521-846
Whitefish (cooked)	75 g	135	180
Halibut (cooked)	75 g	144	192
Mackerel (cooked)	75 g	78	104

[1] Canada's Food Guide Serving Size.
[2] 1 large egg yolk = 17 g.

Health Canada regulates that all milk, including fluid milk, powdered milk and infant formula in Canada be fortified with vitamin D to 100 IU/250mL (40 IU/ 100ml) (15). Before 2011 when the new Health Canada DRIs for vitamin D were introduced, the AI for vitamin D was 200 IU/d for all infants and children based on the 1997 Institute of Medicine report (67). Since the introduction of the RDA for vitamin D for children of 600 IU/d, Health Canada has not increased the fortification of vitamin D in fluid milk nor have they increased the recommended daily amount of milk consumed. With the new vitamin D recommendations in mind, there is now a 90% prevalence of inadequate intake of vitamin D from food sources across most age and gender groups (68). However, very few Canadian children are vitamin D deficient and 78% of children 3-11 y have average year-round vitamin D status that is ≥ 50 nmol/L (6).

In many countries in Europe, milk is still not fortified with vitamin D because of an intoxication of children that happened in the 1950's. More northerly countries, including Finland and Sweden have recently started fortifying milk with vitamin D as well as adding it to margarine, cereals and breads (69). Vitamin D in the USA is classified as generally regarded as safe (GRAS) and is regulated as a food additive through good manufacturing procedures (GMP) (70). Under government legislation it is not mandatorily added to milk or milk products, unless the milk product specifically states "fortified" on the label. It is also added to the fat substitute Olestra to aid in compensating for the disruption of digestion of fat soluble vitamins (70). Currently, on the labels of products in the USA, vitamin D is presented as a percentage of the daily value (DV) of 400 IU, whilst in Canada, the daily value for vitamin D on food labels is only 200 IU (70). However, new harmonized food labels in the USA and Canada will soon be used, as Canada will update its food labels to match the 2011 vitamin D recommendations.

Fortified milk is not consumed uniformly across the population in Canada and the United States (71). Recent research in Vancouver looking at 104 fluid milk products found that 54% of them were under-fortified with vitamin D (vitamin D content below 35 IU/100 ml) (72). However, national data from 2001 and 2007 suggests < 20% of fluid milk was under-fortified with vitamin D (73). With children in Canada, on average, consuming 230 IU/d (19) of vitamin D from milk products and a 90% prevalence of inadequate intake of vitamin D from food sources in most age groups in Canada, strategies for increasing vitamin D intake to reach the EAR and RDA still need to be investigated.

2.2.2.3 Differences between vitamin D_2 and vitamin D_3

Structurally speaking, vitamin D_2 and D_3 differ due to the double bond between carbon 22 and 23 on the side chain of D_2 as well as the addition of a methyl group on carbon 24 (74). Because of research done in the first half of the 20th century, vitamin D_2 and D_3 were deemed to have identical levels of biological activity by the World Health Organization (62).

When using 25(OH)D as an indicator of vitamin D potency, it was shown that vitamin D_3 could be as much as 2-10 times as effective for raising 25(OH)D as D_2 (75). Most of this difference is explained by vitamin D_3 having a greater affinity for DBP, possibly due to the methyl group on carbon 24 of vitamin D_2 decreasing its binding affinity (76). Due to the difference in affinity, vitamin D_2 gets removed from circulation more rapidly and has a shorter half-life than D_3 (76). Though a 2008 study showed no differences in 25(OH)D increases between vitamin D_2 and D_3 supplementation(77), a 2012 meta-analysis of randomized controlled trials in adults showed significantly greater increases in 25(OH)D from vitamin D_3 supplementation compared to D_2 (78). Studies are now underway that look at specific biological

responses of the two vitamin D molecules, in an effort to further elucidate evidence for differences in action between vitamin D molecules (79).

2.3 VITAMIN D METABOLISM AND EXCRETION

After ingestion, dietary vitamin D (~50%) is absorbed from the small intestinal lumen as part of the micelle complex and repackaged, along with triglycerides and other lipid soluble compounds, in the enterocyte into chylomicrons. To reach circulation, vitamin D is transported through the lymphatic system via the thoracic duct as part of a chylomicron complex that is taken up by liver, muscle and adipose tissues (80). Some free vitamin D may enter into the circulation bound to DBP or albumin for transport to adipose tissue for storage if not to the liver (81).

DBP, also known as GC-Globulin has multiple roles including transporting vitamin D metabolites, binding certain fatty acids as well as implications in roles in inflammatory pathways related to the immune system (82). The serum concentration of DBP is 0.6–11 μmol/L (83) and DBP transports up to 99% of 25(OH)D in the blood and about 90% of all vitamin D metabolites (84). Albumin and lipoproteins are responsible for the remaining small fraction of vitamin D metabolite transport. DBP has a binding affinity for 25(OH)D that is between 1 and 2 orders of magnitude higher than its binding affinity for $1,25(OH)_2D$ (85).

Once vitamin D gets to the liver from chylomicrons, DBP or other transport proteins, vitamin D enters the mitochondria where it undergoes hydroxylation at the 25th carbon to become 25(OH)D (81). This is catalyzed by the enzyme vitamin D_3-25-hydroxylase called CYP2R1 (86), which has very high specificity to 25(OH)D as it binds it in an elongated form in a hydrophobic pocket where the hydrocarbon side chain of 25(OH)D is orientated near the heme section for the addition of the hydroxyl group through catalysis (87). Even though all vitamin D metabolites can undergo epimerization, 25(OH)D is the best substrate for this process (88). The

proportion of 25(OH)D made up of the C-3α epimer of 25(OH)D (3-epi-25(OH)D) is higher in infants (up to 41%) compared to adults (up to 17%). It is possible that 3-epi-25(OH)D may have similar physiological effects to 25(OH)D, but with less effectiveness due to it's lower affinity for DBP (89). Whilst recent work in adult rats suggests that the effect of 3-epi-25(OH)D may not differ from 25(OH)D for bone density outcomes (88), a study in infants suggests that 25(OH)D may be more related to lean mass than 3-epi-25(OH)D (89). Thus, this epimer should continue to be measured in vitamin D studies to gain a better understanding of its role in growth and development.

Once back into the circulation, 25(OH)D travels to tissues where it undergoes transformation to $1,25(OH)_2D$ and $24,25(OH)_2D$ (90). The main endocrine tissue capable of synthesizing $1,25(OH)_2D$ is the kidney, specifically the mitochondria and endoplasmic reticulum in cells of the proximal tubules of the kidney. Found in these cells are the enzymes responsible for this including $25(OH)D_3$-1-hydroxylase (CYP27B1) and the $25(OH)D_3$-24-hydroxylase, which are both cytochrome P450 mixed function oxidases (90). $1,25(OH)_2D$ (calcitriol) production is the most important point of regulation in this pathway and the enzyme renal-1-hydroxylase modulates production through the influence of parathyroid hormone (PTH), calcium, phosphate and insulin-like growth factor-1 (IGF-1) (91).

There are numerous sites of extra-renal $1,25(OH)_2D$ synthesis that include lymph nodes, myocytes, activated monocytes and macrophages (92). It is difficult to separate the activities of circulating $1,25(OH)_2D$ from that of $1,25(OH)_2D$ synthesized in extra-renal tissues (93). Using mouse models, a chondrocyte specific knockout of CYP27B1 resulted in bone and growth plate abnormalities whilst the opposite phenotype was observed in mice overexpressing CYP27B1

(94). *In vivo* studies like this one are what strengthen the argument for CYP27B1 function in extra-renal tissues leading to vitamin D function in cells including osteoblasts.

2.3.1 1,25$(OH)_2$D and Calcium

Even through early research in the twentieth century, it was clear that 1,25$(OH)_2$D had an important role in modulating and controlling serum calcium and PTH (91). 1,25$(OH)_2$D increases uptake of calcium by the enterocyte and increases renal tubular reabsorption of calcium so as to increase circulating blood calcium concentration (95). In animal models of vitamin D deficiency, the intestinal absorption of calcium can be decreased by as much as 75% (95). In children, prolonged vitamin D deficiency leads to low vitamin D absorption and periosteal calcium deposition in bone, resulting in insufficient bone mineral accretion, also known as rickets (96).

The vitamin D receptor (VDR) is a nuclear receptor that is a mediator for the majority of the action of calcitriol and the receptor is present diversely through all tissues in humans (82). It mediates action to stimulate the majority of calcium absorption in both the proximal duodenum and proximal jejunum, but also seems necessary for active calcium absorption in other sections of the small intestine (82). VDR, once activated, performs functions to modify transcriptional output through binding to VDR response elements (VDRE) and specific sequences near promoter regions of genes (97). It has a role in mediating renal-1-hydroxylase expression by forming a complex with 1,25$(OH)_2$D (91). Once this complex has been formed, it is postulated that the expression for the gene for renal-1-hydroxylase becomes down regulated by the VDR negative response element (nVDRE) (98). There has been some evidence to suggest that due to aging and estrogen depletion, individuals will have low VDR levels in the intestine and decreased active transport of calcium as a consequence (99).

There is some debate as to if $1,25(OH)_2D$ is the only vitamin D metabolite that influences calcium absorption or if 25(OH)D has an effect as well (27). Studies that have shown 25(OH)D to have an independent effect on calcium absorption (27, 100) believe that the mechanism behind the effect is related to the activity of $25(OH)D_3$-1-hydroxylase. It is thought that this enzyme does not only operate in the kidney, but also in the duodenum (98). Studies that argue this hypothesis, have data from wide ranging human population groups showing no significant effect of 25(OH)D on calcium absorption independent of calcitriol (101, 102). It has been postulated that individuals with vitamin D deficiency may show larger effects of 25(OH)D on calcium absorption levels, possibly because of increased VDR in vitamin D deficiency (103).

Calcium measured in serum exists in three forms: protein bound, complexed and ionized (45). Protein bound calcium is linked to albumin and globulins and is non-diffusible. Complexed and ionized calcium make up the diffusible fraction of calcium, where complexed calcium is coupled with anions and ionized calcium is in the free ionic state (45). Ionized calcium is the biologically active fraction of calcium that is able to bind to negatively charged sites on protein molecules, and thus, is measured in serum as the physiologically relevant calcium form (104). Both PTH and $1,25(OH)_2D$ have important functions in regulating serum calcium concentrations, with PTH acting directly on bone and kidney cells (105). When serum calcium concentrations are low, increases in PTH concentrations act to mobilize calcium from bone through increased stimulation of osteoclastogenesis and osteoclastic resorption (105).

Since calcitriol synthesis in the kidneys depends upon PTH concentration, for healthy people, calcitriol concentration in the blood can be thought of as an average of the PTH secretion over the previous hours (105). Increases in serum calcitriol will be proportional to increases in

calcium and decreases in circulating PTH, thus, increasing osteoblastic activity and decreasing osteoclastic bone resorption (106).

2.4 25(OH)D CONCENTRATION

Serum 25(OH)D is the best clinically measurable indicator of vitamin D status and is preferred over $1,25(OH)_2D$ because of the short half-life, the more rapid removal from circulation and the lower serum concentration of $1,25(OH)_2D$ (76). Venipuncture and capillary blood sampling are the two ways that serum can be collected from humans; venipuncture is usually preferred because capillary sampling has been shown to overestimate actual 25(OH)D levels in adults (107). Though the differences between blood sampling methods are challenging to explain, it is possible that cell rupture of leukocytes and other cells in capillary sampling tubes could inflate 25(OH)D measured (108). However, recent work in preschool age children (n=20) suggests that LC-MS/MS methods may potentially have minimal bias when comparing capillary and serum 25(OH)D concentrations (109). It has been shown that 25(OH)D is very stable at freezer temperatures of -80 degrees Celsius and can be freezer stored for > 10 years during lengthy studies because of this (110).

With the introduction of a competitive protein binding assay (CPBA) in the 1970's, 25(OH)D was able to be measured quantitatively with validity (111). The assay that gained popularity was the DBP assay using rat serum vitamin D binding protein (82). These assays utilized column chromatography for purification and were used until UV quantitative HPLC was introduced (112). A radioimmunoassay (RIA) was also introduced and became popular for use (43). The problem with early RIAs and CPBAs was that their specificity was low and thus they also measured other vitamin D metabolites in serum. This gave the possibility of overestimating 25(OH)D by up to 20% (43). Though more recent RIA methods claim increased precision and

accuracy for 25(OH)D measurement (43), before the release of the standard 25(OH)D reference material (NIST SRM 972) in 2008, there was disagreement in results produced between RIA and LC-MS/MS methods (113). As well, from results submitted to the International Quality Assessment Scheme (DEQAS), it was shown that there was interlaboratory imprecision due to poor assay standardization (114). The introduction of this standard reference material increased the precision of LC-MS/MS methods and demonstrated the accuracy of these methods across physiological ranges. Immunoassays from multiple manufacturers have been shown to bind to 24,25$(OH)_2$D (RIA and competitive protein chemiluminescence assays (CLIA)) and have problematic detection of epimers of 25(OH)D including 3-epi-25$(OH)D_3$ and 3-epi-25$(OH)D_2$ that may account for up to 17% of total 25(OH)D. These assays also cannot distinguish between 25$(OH)D_2$ and 25$(OH)D_3$, and below a concentration of 20 nmol/L, have been shown to have poor performance with biases as high as 35% (113). Novel methods of ultra-HPLC-MS/MS have been recently used to more rapidly analyze both 25$(OH)D_3$ and 25$(OH)D_2$ with accuracy of 96-102% (115).

Since the year 2000, competitive protein chemiluminescence assays (CLIA) and liquid chromatography tandem mass spectroscopy (LC-MS/MS) techniques have been primarily used for 25(OH)D assessment (112). LC-MS/MS has multiple impracticalities including the cost and the low throughput capacity (116, 117) . CLIA methods now use the automated chemiluminescence LIAISON 25(OH)D assay system (118) and because of the practicality of this system, it is now the most popularly used 25(OH)D assay clinically (118).

It would be expected that the mean measured 25(OH)D concentration of Canadian children would be < 50 nmol/L, due to multiple risk factors as previously reviewed; including practices of sun protection, the northern latitude of Canada and low dietary intake of vitamin D.

However, from the largest cross-sectional assessment of Canadian children 1-5 y (n=1898), Maguire *et al* (2) found the average 25(OH)D concentration was 87.4 nmol/L (95% CI, 87-89 nmol/L). Using data on 25(OH)D concentrations during winter or early spring months from different cities in Canada (**Figure 2.2**), (1-5) the percentage of children with 25(OH)D $\geq$ 50 nmol/L ranged from 42-86%. The study with the highest percentage of children with 25(OH)D $\geq$ 50 nmol/L was a representative cross-sectional study of pre-school age children attending daycares in Montreal (1). Similarly, results from the Canadian Health Measures Survey (CHMS 2009-2011 and 2012-2013) (7, 19) showed that 76-89% of children 3-11 y had 25(OH)D of $\geq$ 50 nmol/L with 3-5 y olds having the highest proportion (89%) (**Figure 2.3**).

However, overall there were 15% less people with 25(OH)D $\geq$ 50 nmol/L in the winter compared to the summer. Children who consumed 1 or more serving of milk/d had 8-15 nmol/L higher mean 25(OH)D than children who did not. As shown by NHANES 2001-2004 data (119), obesity in children (1-21 y) is also a risk factor for vitamin D status < 50 nmol/L with an odds ratio of 1.9 (95% CI: 1.5-2.9) compared to non-obese children. With latitude also being a factor influencing 25(OH)D concentration, a study of Inuit children in nothern Canada found 78.6% and 96.8% of children in the summer and winter had 25(OH)D concentrations < 50 nmol/L (120). It has also been found that immigrant and refugee children, from countries closer to the equator than Canada, have significantly lower mean vitamin D status (41-52 nmol/L) (121, 122) compared to average Canadian children. For policy making, it is important to realize that there are many factors along with latitude including diet, age, socioeconomic status and religious practices that may effect the vitamin D status of children in Canada and therfore, specific policies and strategies need to be addressed to support sufficent vitamin D status in differing subpopulations.

Figure 2.2 Percentage of children who had Vitamin D status <50 nmol/L in cross-sectional studies in Montreal (1), Toronto (2), Newfoundland (3), Quebec (4), and Edmonton (5) during winter or early spring. [1] Sample size of study in Toronto was for children year round, sample size for winter only was not provided.

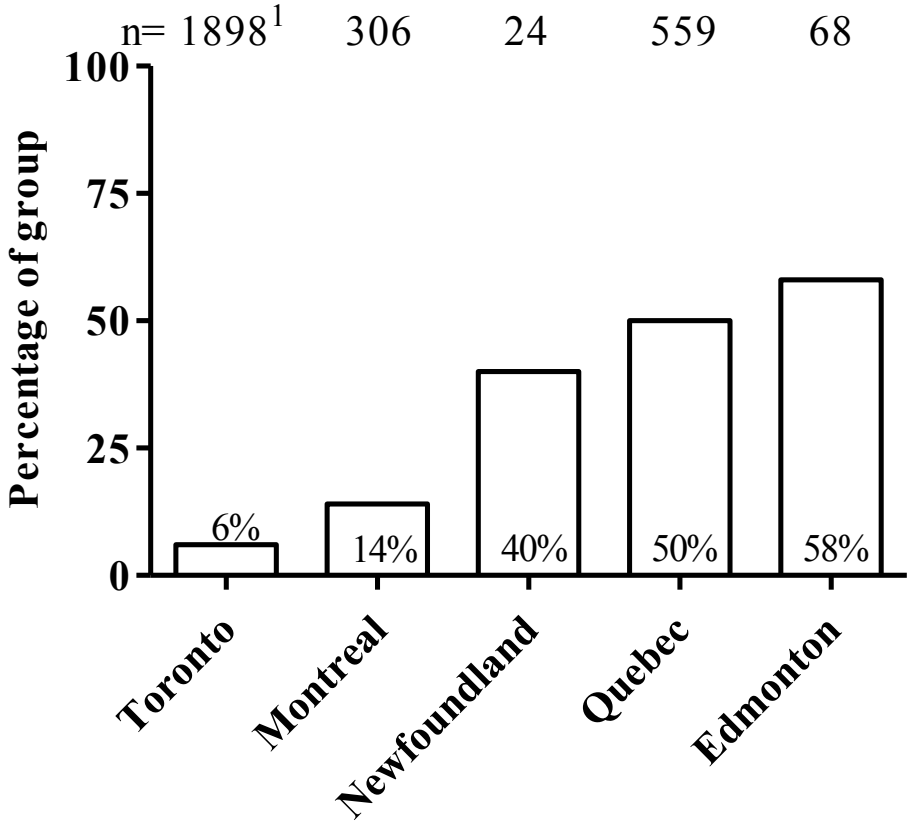
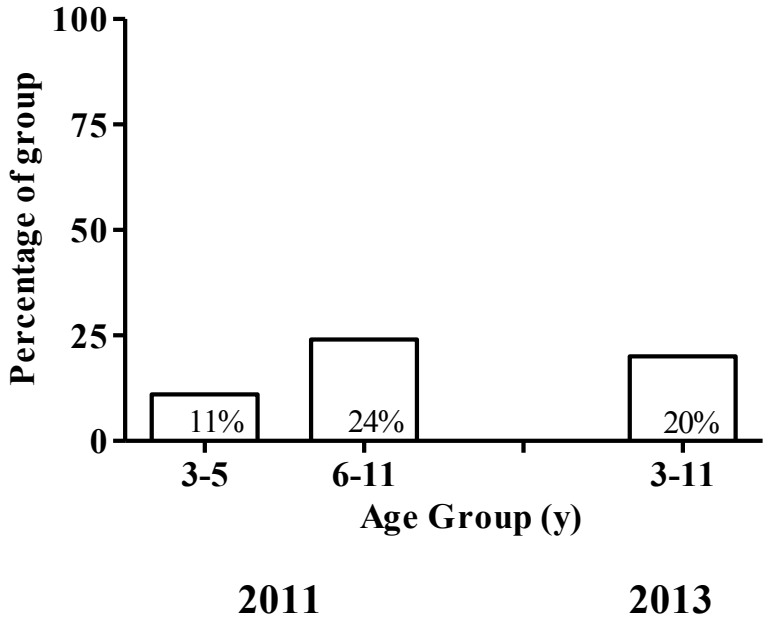


Figure 2.3 Percentage of children from Canadian Health Measures Surveys in 2011 (6) and 2013 (7) who had year-round vitamin D status between 30-49.9 nmol/L. In both 2011 and 2013, there were not enough data to capture the percentage of children with status <30 nmol/L.



A number of genetic variants, due to single nucleotide polymorphisms (SNPs), may affect vitamin D status. First, the two DBP SNPs generate differing proteins that have effects on the concentration of circulating 25(OH)D (123). Homozygotes for the alleles *DBP*-1 and *DBP*-2 have been shown to have decreased circulating concentrations of DBP and decreased circulating 25(OH)D (123). This is because of an increased conversion of 25(OH)D to its inactive metabolites, which leads to a decrease in the circulating concentration of 25(OH)D (124). Confirming this, a recent trial in Danish adults showed homozygotes for DBP had the smallest increases in 25(OH)D concentrations to both UVB irradiation and the consumption of vitamin D fortified foods (125).

Secondly, DHCR7 removes 7-DHC from vitamin D metabolism by converting it to cholesterol. Two meta-analyses prior to 2011 identified SNPs of DHCR7 as significantly effecting 25(OH)D (126), however, this is still contested because a 2014 vitamin D RCT of 1800 healthy adults did not show an association between 25(OH)D and DHCR7 polymorphisms (126). CYP2R1 is more widely accepted to have SNPs affecting 25(OH)D, since one genetic variation can cause vitamin D deficiency and meta-analyses (127) and trials (125, 126) have shown polymorphisms affecting 25(OH)D. Interestingly, a 2013 trial found that CYP2R1 SNPs were only associated to 25(OH)D if vitamin D intake was ≥ 400 IU/d or endogenous synthesis was high because of summer sun exposure (128), indicating that during winter with normal vitamin D intake, these polymorphisms may not be important. Though VDR is widespread in tissues in the body and vital to the biological roles of vitamin D, a review states that SNPs of VDR are not related to 25(OH)D concentration because VDR expression does not regulate the production of 25(OH)D or its conversion to inactive metabolites (127). Lastly, highly active CYP24A1 has

been linked to lower 25(OH)D concentration (129), but CYP27B1 has not, due to the tight regulation of $1,25(OH)_2D$ (126).

Young children living in countries around the world with similarly distant latitude from the equator as Canada, also have lower vitamin D status in the winter. In the US, average 25(OH)D concentration for children between 1-8 years of age is 67.2-69.5 nmol/L (23, 130). In a national cross-sectional study of children in Great Britain (n= 1102)(131), the average 25(OH)D concentration was found to be 62.1 nmol/L (95%CI 60.4-63.7) with 35% of individuals having concentrations lower than 50 nmol/L. During winter months in Denmark (September to March), researchers found that the average 25(OH)D in families dropped from 71.1 nmol/L to 41.7 nmol/L (23). This large decrease in vitamin D status over winter may be linked to the low mean vitamin D intake of 85 IU/d in the population.

Interestingly, the 2005-2006 NHANES data showed that overweight/obese or non-hispanic black children were 4-5 times more likely to have vitamin D deficiency (132). This is likely because adipose tissue is thought of as the principal storage site of vitamin D because, in states of 25(OH)D > 15 nmol/L, the liver 25-hydroxylase (CYP2R1) becomes saturated and cholecalciferol can be absorbed into fat cells instead of being quickly hydroxylated to 25(OH)D (133). In adults, it was shown that 17% of orally administered radiolabelled vitamin D was stored in adipose tissue (134), however, similar work in children is lacking. Vitamin D storage in adipose tissue can happen within 24 hours (135) proportionally to the 25(OH)D concentration in circulation. Though adipose tissue has been shown to express both CYP27B1 and CYP24A1, half of the vitamin D stored in adipose is unmetabolized vitamin D_3 that is thought to be able to be slowly released back into circulation (136). The author, stipulated that the mobilization of vitamin D from adipose is thought to be proportional to the concentration already in the tissue.

Similar to cholesterol, vitamin D is likely released from adipose tissue by catecholamine induced lipolysis related to the β2 adrenergic receptor (137). The production of norepinephrine and other catecholamines in the adrenal gland is likely regulated by the presence of CYP27B1 and VDR, which upregulate the hydroxylase enzyme for producing catecholamines when vitamin D status is low (138).

It is possible that vitamin D mobilization is compromised with greater adiposity through a dysfunction of the β2 adrenergic receptor (137), illustrated by overweight and obese children consistently having vitamin D status lower (~ 10 nmol/L lower) than their healthy weight comparators (139-141). Complicating the relationship of obesity and vitamin D are recent results in mouse models showing that lower vitamin D status may contribute to adiposity through increased inflammatory cytokine production, adipogenesis and adipocyte secretion (142). This is because low $1,25(OH)_2D$ concentration will increase the expression of intracellular signalling molecules dickkopf1 and secreted frizzled-related protein 2, which in turn activate the adipogenic peroxisome proliferator-activated receptor gamma/retinoid X receptor complex, leading to increased preadipocyte differentiation into mature adipocytes (142). Corroborating these results in mouse models, a 30 mo study in children 5-12 y (n=479) showed children with 25(OH)D concentration < 50 nmol/L, compared to those > 75 nmol/L, had 19% greater increases in waist circumference and a 3 times greater change in subscapular-to-triceps skinfold ratio (143). Thus, it may be prudent to make efforts to have separate vitamin D recommendations for obese children in the future.

2.5 VITAMIN D SUPPLEMENTATION AND FORTIFICATION STUDIES

Cross-sectional studies in both the USA and Canada have concluded that current vitamin D food fortification does not adequately prevent the risk of vitamin D deficiency due to low

vitamin D intakes (144, 145). Though supplements can increase vitamin D intake, according to the 2011 Canadian Health Measures Survey, only 34% of Canadians take a vitamin D supplement with the 3-5 year old age group being the age group with the highest supplement intake (146). It has been recommended by the American Academy of Pediatrics (AAP) and CPS that breast fed infants, as well as mothers, should be taking vitamin D supplements of 400 IU/d (infants) and enough to reach a total daily intake of 600 IU/d for mothers (147). CPS also states that for young children, 400 IU-800 IU/d of vitamin D supplementation is safe but due to the prevalence of vitamin D deficiency, higher doses may be needed. Vitamin D supplementation most commonly uses vitamin D_3, but before supplements in the form of pills, cod liver oil was traditionally used as a vitamin D supplement and was noticed as early as the 19th century to reverse rickets in children (148).

Vitamin D supplementation trials have shown that 1000 IU/d of vitamin D can significantly increase 25(OH)D concentrations in children over an 8 wk period (22). A 6 mo trial in Denmark (**Table 2.3**) (n=130, 4-8 y) found that baseline 25(OH)D (56.9 ± 12.7 nmol/L) was maintained with a 400 IU/d supplement (61.8 ± 10.6 nmol/L) and increased (75.8 ± 11.5 nmol/L) with an 800 IU/d supplement, whilst significantly decreased in the control group (31.1 ± 7.5 nmol/L) (40). These results put forward the question of: is taking vitamin D supplements more sustainable/realistic for children to increase their vitamin D intake than it would be to increase their intake of vitamin D through increasing the fortification of milk products? Canadian young children (2-8 y) are, on average, consuming more than the 2 recommended servings of milk products per day. Thus, increasing fortification of these products or their alternatives would seem more effective to increase vitamin D intake in this age group compared to trying to increase supplement intake of children.

Table 2.3 Vitamin D supplementation or fortification randomized controlled trials in young children

Author	Population, duration	Vitamin D Intervention	25(OH)D (nmol/L) increase/ 100 IU/d
Abrams et al. (22) (Houston, USA)	n=130 (4-8 y) 8 wk (season not reported)	**Supplement**: Tablet, 1000 IU/d	1.6
Mortensen et al. (Denmark)	n=130 (4-8 y) Oct. to March	**Supplement**: Tablet, 400 IU/d 800 IU/d	400IU/d: 7.2 800 IU/d: 5.2
Madsen et al. (23) (Denmark)	n=323 (4-17 y) Sept. to April	**Fortified**: Milk and bread, 320 IU/d	7.8
Camargo et al. (25) (Mongolia)	n=247 (8-10 y) Jan. to March	**Fortified:** Milk, 300 IU/d	10.0
Hower et al. (24) (Germany)	n= 80 (2-6 y) Nov. to March	**Fortified:** Milk, 280 IU/d	8.8

A 2012 meta-analysis of vitamin D_3 food fortification studies in adults, found a dose dependent increases of 3.0 nmol/L for every increase of 100 IU/d in vitamin D (42). In the largest Canadian assessment of vitamin D status in preschool age children (n=1898) (2), a positive association between milk intake and vitamin D status was found regardless of season or of the skin pigmentation of the individual. This demonstrates the importance of dietary intake as a modifiable factor for vitamin D status. These results are also supported by cross-sectional studies (2, 19) and randomized control trials (22-24) by which for every 100 IU increase in vitamin D intake per day, 25(OH)D concentrations increase ~7.5-10 nmol/L in children (**Table 2.3**). Interestingly, in these Canadian cross-sectional studies (2, 19) the average vitamin D status was above 50 nmol/L (88.9 nmol/L, 95% C.I. 88.0-90.5 nmol/L and 75.0 nmol/L, 95% CI 70.3-79.7 nmol/L) for the children but dietary intakes of vitamin D were well below the EAR for almost all children. Therefore, the amount of dietary vitamin D necessary to reach the target ranges for young children remains unclear.

Since very few randomized controlled trials (RCTs), have been performed looking at the effect of vitamin D fortified foods on 25(OH)D levels in children, the best fortified foods to use are not known. Due to the fact that Canadian children get the majority of their vitamin D intake from milk products (19), targeting milk products for fortification is logical. In the RCT involving 323 children in Denmark by Madsen *et al.* (23), fortifying milk and bread to increase vitamin D intake by 320 IU/d was sufficient to ameliorate the decreases in serum 25(OH)D during winter. From a study in young Mongolian children (25), similar daily fortification (~300 IU/d) saw significant increases in 25(OH)D status compared to children in the control group, over just 7 wk (**Table 2.3**).

2.6 BONE PHSYIOLOGY AND THE ROLE OF VITAMIN D

2.6.1 Bone Anatomy and Physiology

Bone health outcomes are the major functional outcomes linked to the IOM vitamin D recommendations (8). Bone is one of the two types of connective tissue in the human body, along with cartilage, and together they form the skeleton (149). Bones are classified as either long (femur, humerus, etc.), flat (skull, mandible, etc.) or irregular (vertebral column, the carpal and tarsal bones, etc). The outer layer of the bone is called the periosteum, which can be split into two separate sublayers; an outer fibrous layer and an inner layer involved in osteogenic growth called the cambium (150). The central cavity of the diaphysis (the central narrower portion of long bones), which contains arterial blood supply and fatty bone marrow, is called the medullary cavity and has an outer lining layer called the endosteum (149). The compact cortical bone of the diaphysis is made of up bone matrix in the form of concentric rings, which surround blood vessels. In the trabecular bone of the epiphysis, which has also been called spongy bone, is red bone marrow responsible for the production and formation of blood cells (hematopoesis) (151).

Long bones consist of wider sections on each end called epiphyses, a cylindrical shaft called the diaphysis and a section for development inbetween called the metaphysis (151). The growth plates of long bones are situated between the epiphysis and metaphysis and are made up of a cartilage matrix. The diaphysis consists mostly of cortical bone whilst the epiphysis and metaphysis are primarily a meshwork of trabecular bone surrounded by an outer layer of denser cortical bone (151). The adult human skeleton, overall, is made up of about 80% cortical bone and 20% trabecular bone(149). In children, since the skeleton is still undergoing modelling and

remodelling, there will be a greater percentage of trabecular bone and a smaller percentage of cortical bone.

In children, it was shown that 57% of growth of long bones was at the proximal end with 43% being at the distal end (152). As children continue to grow, the contribution to growth by the diaphysis decreases whilst the contribution by the proximal eiphysis increases. A longitudinal study on growth of the tibia and radius (long bones in the lower leg and lower arm, respectively), from age 1 mo to 18 y in healthy children (n=156) (153) showed there were no sex differences in tibia length for children 2-13 y with length increasing relatively uniformly (~ 20 mm/y) across this age range. However, in this same age range, growth of the radius (~ 6 mm/y) occurred at a rate of 1/4 to 1/3 of that of the tibia and tibial length was significantly greater in boys then girls at each 1 y increment of age. These results show that the tibia may be an ideal location to measure bone outcomes in young children

Healthy children are not expected to undergo any significant weight loss and should undergo bone mineral accrual throughout childhood. Healthy babies are born with roughly 75 g of bone mineral content (154). By 5 years this reaches 500 g, by 6-10 years 1200 g and by 11-16 years 2200 g (155). Bone mineral accretion rates, as a percent of total BMC, are similar throughout childhood and adolescence (156), meaning that bone mineral accretion is equally important throughout these years. This bone mineral accretion occurs until peak bone mass is reached in early adulthood through a dynamic process of modeling and remodeling. Bone modelling happens only in growing skeletons whilst remodelling occurs throughout life.

In long bones, bone modelling (also called endochondral ossification) involves mesenchymal cells differentiating into prechondroblasts and then chondroblasts. Around the growth plate of long bones, chondrocytes continue to proliferate and divide to produce an

extracellular collagen containing matrix and will continue to do so if the growth factor parathyroid hormone related protein (PTHrP) is low (157). Increases in PTHrP will induce apoptosis of chodrocytes and this zone of growth will then calcify and undergo partial resorption of the collagen matrix through osteoclast activity. These formation and resorption activities allow the longitudinal growth of bone through the continued epiphysis formation as well as moving the growth plate upwards and reforming the diaphysis from the prior epiphysis areas (158).

Bone remodeling involes the replacement of old bone with packets of new bone matrix to prevent the build up of microdamage within bone (151). Remodelling involves four cycles, which are called activation, resorption, reversal and formation (159). Activation and resorption steps are resopnsible for bone resorption (160, 161) whilst reversal and formation steps are responsible for bone formation (162). Skeletal mineral deposition during childhood is due to bone formation processes being more active than resorption (163) and hence these processes are said to be uncoupled compared to in adulthood.

2.6.2 Biological markers of Bone Formation and Resorption in Young Children

Since bone formation and resorption are a coupled process (162), it is important to have biomakers to measure each of them. First, procollagen type I N-terminal propeptide (P1NP) is a sensitive marker of bone formation, which is a process that is highly active in young children (45). P1NP is a derivative of the most common type of collagen found in bone (type I collagen). Osteoblasts synthesize procollagen (collagen precursor) which contains N-terminus and C-terminus peptides that are cleaved by specific proteinases during the formation of collagen molecules (45). Though P1NP may be produced by other tissues (tendons, skin, fibrocartilage) these tissues account for a very small amount of P1NP in circulation. Second, osteocalcin is the

most abundant non-collagenous protein of bone matrix. It is produced by osteoblasts (vitamin K dependant synthesis) and is essential for the deposition of hydroxyapatite crystals into bone matrix (164). Since osteocalcin is released into circulation during bone resorption, it is considered a marker of bone turnover rather than bone formation (164). For bone resorption, the C-terminal telopeptide of type 1 collagen (CTX) is a sensitive marker as it is the section cleaved by osteoclasts during resorption (165) and thus is a marker of osteoclastic activity. Lastly, PTH can be a marker of resorptive activy, because sustained elevated PTH promotes calcium resorption from bones when serum calcium concentration is low (105).

2.6.3 Skeletal Function of Vitamin D

Vitamin D acts on 3 levels to maintain calcium homeostasis; in the duodenum to improve calcium absorption, on bone cells, osteoblasts and osteoclasts, to mobilize calcium and on renal cells to regulate calcium reabsorption (27, 28). The major types of bone cells, osteoblasts, osteoclasts and osteocytes are all able to metabolize 25(OH)D to $1,25(OH)_2D$ (166). CYP27B1 is strongly expressed in human osteoblasts and beyond being a precursor for $1,25(OH)_2D$, 25(OH)D stimulates osteoblast maturation and mineralization along with reducing cell proliferation. In osteoblasts and osteoclasts in response to elevated $1,25(OH)_2D$, fibroblast growth factor 23 (FGF23) is secreted and acts on the proximal tubule of the kidney to decrease the expression of a sodium-phosphate cotransporter NPT2 to decrease the reabsorption of phosphate in the kidney (167). In human peripheral blood mononuclear cells (pre-osteoclasts), CYP27B1 expression is related to the optimization of osteoclastogenesis in the presence of RANKL. Osteoclasts will have reduced resorbing activity compared to osteoclasts that went through maturation not in the presence of vitamin D metabolites (166). The expression of CYP27B1 in osteocytes has been associated with acquisition and accumulation of mature

osteocyte genes. The regulation of CYP27B1 in bone cells is relatively unknown as the production of this enzyme is not upregulated by the combination of low $1,25(OH)_2D$ and high PTH (166). Similar to renal CYP27B1 expression, CYP27B1 expression in bone decreases with age and inversely correlate with CYP24A1 expression (166). However, expression of CYP27B1 in osteoblasts may increase due to high circulating calcium concentrations, acting to increase bone matrix mineralization (168). Also, expression in osteoclasts and osteoblasts could have coordinated actions to regulate trabecular bone remodelling (169) and expression in chondrocytes is thought to be vital for normal bone growth (93).

2.6.4 Vitamin D Studies With Bone Outcomes in Young Children

An inverse relationship is seen between PTH and serum 25(OH)D concentrations in both children and adults (26). However, recent work in children and young adults (5-21 y, n=145) has shown that PTH, within the normal range, is positively associated ($p < 0.001$) with an increased rate of bone mineral accrual independent of 25(OH)D (170). This suggests that a combination of indicators rather than 25(OH)D alone, may be a more appropriate biomarker of bone growth and development. In healthy children and term infants, respectively, it was shown that increasing dietary milk intake (171) or consuming vitamin D supplements (172) suppresses CTX. Vitamin D supplemented milk intake was also positively associated with biomarkers of bone formation including osteocalcin and P1NP (172).

Vitamin D intakes in a representative cross-sectional cohort of 2-5 y old children (n=508) in Montreal showed positive associations with whole body and radial bone mineral density (BMD) (29). In a 2 y study in Chinese girls (n=757, 10 y), a vitamin D intervention of 200-320 IU/d (fortified milk) significantly increased whole body BMC and BMD (30). Though almost all of these girls were vitamin D deficient (mean: 17.7-20.1 ± 8.8 nmol/L), a Sri Lankan study of

children with a mean 25(OH)D of 72.0 ± 32.3 nmol/L (n=32, 3-5 y) also found that a 9 mo vitamin D intervention in fortified food (100 IU/d) significantly improved lumbar spine BMD (31). However, a 2011 meta-analysis found that vitamin D supplementation trials in children with BMC and BMD outcomes show inconsistent results, and as such, the precise effect of vitamin D status on bone growth and development are still unclear (32). Thus, the relationship of vitamin D and bone development needs to be investigated longitudinally in children.

2.7 MUSCLE PHYSIOLOGY AND THE FUNCTION OF VITAMIN D IN MUSCLE

2.7.1 Muscle Physiology

Extra skeletal functions of vitamin D are less understood but muscle has become a tissue of interest for vitamin D action as muscle expresses VDR and has complex inter-relationships with bone. In regards to muscle tissue, there are three major types, skeletal, cardiac and smooth muscle. Skeletal muscle is made of bundles of muscle fibers that are enclosed in layers of connective tissue called fascia. Multinucleated muscle fibers (myocytes), which are cylindrical in shape, are made when myoblasts fuse together (myogenesis) (173). Each myocyte is composed of more than a thousand myofibrils, which are the structures responsible for muscular contraction. Myofibrils are long chains of sarcomeres, attached by crossbridges, that are in turn composed of actin (thin filaments) and myosin (thick filaments). Myofibrils are also made of multiple other proteins including troponin and tropomyosin (regulatory proteins) (173). Sarcomeres are the functional units of the muscle that allows muscle contraction. Muscle contraction involves multiple steps given the name “the sliding filament theory of muscle contraction”. Contraction is caused by ATP binding to myosin resulting in ATP hydrolysis, movement of the myosin head and then a power stroke where myosin pushes the attached actin

filaments towards the center of the sarcomere (M line) (173). These events are initiated by action potentials that result in calcium signalling to activate muscular contraction.

Skeletal muscle generates energy from oxidative glycolysis or anaerobic glycolysis and can be categorized as slow twitch oxidative (type I), fast twitch oxidative-glycolytic (type IIa) or fast twitch glycolytic (type IIb). The speed (slow or fast) denotes how quickly the muscle can generate maximum tension and the speed of activity of the myosin ATPase (173). Type I fibers are capillary and mitochondrial dense, whereas type II have fewer mitochondria and capillaries resulting in paler coloured larger cells. Type IIa fibers can adapt to endurance aerobic training and become more oxidative instead of glycolytic.

Based on the WHO growth charts, for children 2-8 y, the average height velocity is 7 cm/y and weight velocity is 2 kg/y (174). In reference data from the US, though data only went as young as 5 y, lean mass accretion from ages 5-10 y was ~1.5 kg/y (or 125 g/mo) and did not differ between girls and boys until after 12 y (36). The rate of lean mass accretion means that changes in lean mass should be able to be measured over a shorter period than changes in BMC. Occurring around the ages of 6-8 y, a mid-childhood growth spurt occurs that results in an increased rate of height and weight gain. In regards to body fat, percent body fat usually reaches a maximum between 3-6 mo of age (29-32%), and gradually decreases throughout early childhood due to fat mass accretion being minimal (36). However, fat accretion was shown to become greater in girls than boys around 10 y (36).

2.7.2 Function of Vitamin D in Muscle

As shown in section 2.5, vitamin D is well known to improve calcium reabsorption, act on bone cells and regulate calcium reabsorption, however, because of the discovery of the VDR in skeletal muscle, vitamin D is understood to have both genomic and non-genomic functions in

muscle (175). Genomic actions of vitamin D are mediated through the nuclear VDR in muscle cells (34). There are 2 ways for vitamin D to bind to the nuclear VDR; first, $1,25(OH)_2D$ bound to DBP in circulation, gets transported to the nucleus by the intracellular binding protein and binds to VDR. Second, 25(OH)D in circulation gets transported into the myocyte and into the mitochondria where CYP27B1 converts it to $1,25(OH)_2D$, which is then transported to the nucleus and binds to VDR (175). Though there has been debate about the presence of CYP27B1 in skeletal muscle cells, recent studies in mice (46) and in C2C12 muscle cells (176) shows evidence of both their presence and their signalling activities.

VDR then becomes the heterodimer complex with the retinoid-X receptor (RXR) and this allows vitamin D response element (VDRE) related mRNA gene transcription and *de novo* protein synthesis (175). Importantly, this leads to an increased activation of calmodulin-dependant kinases (calmodulin is responsible for calcium binding), which was shown in cell line studies to increase the transcription activity of the VDR coactivator steroid receptor coactivator (SRC) (177). Thus, VDR-mediated transcription in myocytes can be synergistically improved from vitamin D induced calmodulin-dependant kinase activation. A second protein of importance is Insulin-like growth factor binding protein three (IGFBP3) and serum concentration of IGFBP3 has been shown to increase by as much as 50% in vitamin D interventions. This increase in IGFBP3 is thought to increase the half-life of the anabolic hormone IGF-1 and thus, increase the concentration of IGF-1 in circulation. IGF-1 is responsible for muscle protein synthesis through activating protein kinase B and the mammalian target of rapamycin signaling pathway, thus stimulating of cell growth and proliferation (178). This is supported by a study in mice with an over-expression of IGF-1, that had increased muscle cross sectional area and mass compared to wild type mice (179). The VDR may also have direct roles in muscle development by increasing

cellular differentiation and proliferation (mediated by cyclic AMP) as well as stimulating muscular contraction (regulating phosphate metabolism) (34).

The fast acting, non-genomic functions of vitamin D in skeletal muscle are mediated through $1,25(OH)_2D$ binding to the plasma membrane VDR (34). This then stimulates secondary messenger pathways that can become active within seconds (175). One important pathway in calcium regulation involves VDR increasing the activity of phospholipase C-g, which in turn stimulates downstream effects that mobilize Ca^{2+} from sarcoplasmic reticulum by stimulating calcium pumps in the sarcoplasmic reticulum. Because of this calcium mobilization, calmodulin and protein kinase C become active which act to stimulate the extracellular influx of Ca^{2+} and the activation of voltage-gated calcium channels (35). Thus, by modulating intracellular calcium concentrations, vitamin D effects muscular contraction and muscular function. Some of the fast responses to vitamin D in muscle are also due to the activation of the mitogen-activated protein kinase (MAPK) signaling pathways. Through the phosphorylation of other proteins, activated MAPKs can initiate cellular processes including proliferation and differentiation (35). Due to these above described functions of vitamin D, it primarily affects type II muscle fibers, increasing diameter and area. However, as recently noted in a review of vitamin D and muscle function, it is not clear if vitamin D causes type I fibers to convert to type II or if it increases production of new type II fibers (180).

2.7.3 Vitamin D Studies With Lean Mass Outcomes

Based on a 2010 meta-analysis of 17 RCT's in adults, vitamin D supplementation has a significant effect on global muscle strength (standard mean difference (SMD): 0.17, 95%CI 0.03,0.31) but did not effect muscle mass or muscle power in subsets of studies (181). From another subset analysis, when baseline 25(OH)D is >25 nmol/L, vitamin D_3 supplementation

does not seem to effect proximal lower limb strength (SMD 0.1, 95%CI −0.01,0.22) (181). More recent vitamin D supplementation trials in adults have shown significant increases in muscular strength (1000 IU/d) in women (182) and muscle mass in collegiate males (4000 IU/d) (183).

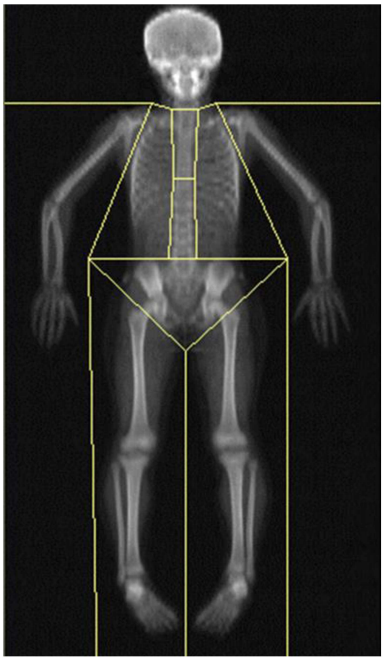
In healthy children, there are limited data on the relationship between vitamin D intake and muscle outcomes. Recently in 3 y old children in Montreal, a higher vitamin D status from infancy to 3 y was associated with a leaner body composition (37), however, with sufficient vitamin D status, vitamin D intakes > 400 IU/d did not improve lean mass accretion. In a cross-sectional analysis of 15 y old Chinese girls (n= 323), total lean body mass, but not fat mass, associated with 25(OH)D ($r= 0.446$, $p < 0.001$) (38). Also, hand grip strength was significantly greater in girls with 25(OH)D ≥ 50 nmol/L, compared to those with 25(OH)D < 50 nmol/L. Similarly, in girls 12-14 y in Britain with a median 25(OH)D of 21.3 nmol/L, vitamin D status had positive associations with jump velocity, jump height and power (184). Similar results were shown in the 1 y trial that followed, with the vitamin D intervention group (4 doses of 150,000 IU) having significantly greater jump power and velocity compared to control (185). With outcomes of lean mass accrual, a vitamin D supplementation RCT (200 IU/d or 2000 IU/d for 1 year) in vitamin D deficient pre-pubertal Lebanese girls (n=34) showed a significant greater increase ($p=0.04$) in percent lean mass compared to the control group but supplementing with 2000 IU/d did not confer additional benefit above 200 IU/d (ctrl: 10.7 ± 5.2, 200 IU/d: 16.8 ± 6.6, 2000 IU/d: 18.1 ± 6.7) (39). Thus, trials are needed looking at lean mass accretion in pre-pubertal male children and both male and female Canadian children.

2.8 MEASURING BONE MINERAL PARAMETERS AND BODY COMPOSITION

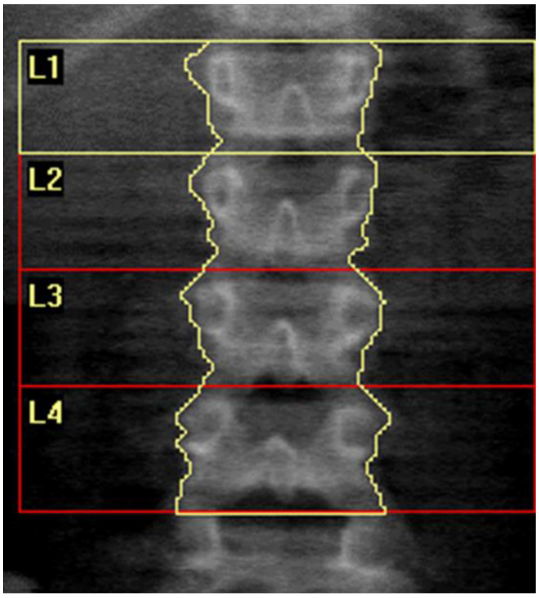
There are many methods to measure body composition as well as bone mineral content (BMC) and bone mineral density (BMD) including quantitative ultrasound, magnetic resonance

imaging, computed tomography and dual-energy X-ray absorptiometry (DXA). BMC is a measurement of the total amount of mineral present in the bone at a certain time point. Areal bone mineral density (aBMD) refers to the amount of mineral content per square centimeter of bone (186) and is commonly assessed as a predictor of osteoporosis. The quantitative assessment of BMD and BMC by DXA is the most widely validated technique, and the preferred method of analysis in children by the ISCD (187). Specifically, the posterior-anterior spine and whole body scans are preferred for measurement (**Figure 2.4**), whereas hip is not due to variations in skeletal development. Other reasons for DXA being commonly used in children is the low radiation dose (~3 mSv for the 3 minute whole-body scan), performance speed and accessibility (188). Though forearm scans are not as commonly used, because the forearm is the most common fracture site in children, these fast scans have the potential to be highly used in clinical assessments (189). Importantly, to measure changes in bone parameters, the ISCD states the minimum interval between scans should be 6-12 months (189). DXA is also a preferred method for quantitating body composition (adipose tissue and lean tissue) as it can perform whole body scans and generate regional body composition for specific areas of the body. Since it is a 2-dimensional scan, it cannot measure visceral body composition, however, values for trunk adiposity align well with those from computed tomography scans in women and children (36, 190, 191).

Figure 2.4 Whole body and lumbar spine dual-energy x-ray absorptiometry scans, with permission, from an 8 y old female.



Whole body



Lumbar spine

Since DXA gives assessments of density as areal density (g/cm^2), instruments able to measure volumetric density (g/cm^3) are also commonly used. Peripheral quantitative computed tomography (pQCT) is a semi-portable instrument able to give 3-dimensional computed tomography scans of the forearm or the lower leg (**Figure 2.5**). Due to its 3-dimensional nature, it can distinguish between cortical and trabecular bone compartments and give density and area measures of muscle and fat tissues. Density measures are not affected by the 2-dimensional bias of bone size (192). However, according to the ISCD, there is no preferred method of QCT and there are no standardized methods for pQCT, making it challenging to compare results among studies (193). Little pQCT data is available for children < 6 y, illustrated by the lack of precision data available (193) and the variation in methodology used previous to our work (**Table 2.4**). This may be somewhat due to a bias in pQCT measurement in children called the partial volume effect. Voxels (pixels) are the discrete unit of the scan volume because of the reconstruction of the scan data. Typical pQCT scanners can achieve voxel size of around 200 μm whilst high resolution pQCT (HRpQCT) can achieve voxel size of 82 μm (194). The voxel size of pQCT is relatively small compared to cortical thicknesses that have been measured in adults (over 2.5 mm) but is large compared to the trabecular thickness of young children, which is below 150 μm and the mean cortical thickness of 3-4 year olds is 1.2 mm (195). When the voxel size is relatively large compared to the thickness, errors due to the partial volume averaging effect may be introduced. The density value calculated for the voxel would be the mean of all tissues in the voxel meaning that the partial volume effect may cause trabeculae or cortical bone to appear less dense and thicker. This could mean, for example, that young children with thin cortical bone shell could be misdiagnosed with osteomalacia (196). It was stated by authors of a study on 3-4 year old children that because of the small cortical thickness, cortical density could not be

analyzed (197). Using HRpQCT would decrease this partial volume effect, however, instruments are more expensive and scans take longer resulting in a higher radiation exposure. The number of HRpQCT scanners worldwide (50), as published in 2014, is much lower less than pQCT scanners (600), meaning less data is available from them (193).

2.9 CONCLUSION

Vitamin D intake in the majority of young Canadian children does not meet the recommendations of the EAR because current fortified foods do not contain sufficient vitamin D. Though the year-round mean vitamin D status is well above 50 nmol/L in young Canadian children, winter decreases in vitamin D status may still pose a concern to health. This literature review highlighted that there is a lack of North American vitamin D food fortification trials in young children and thus, the relationship between vitamin D intake and status during winter still needs to be elucidated. Due to the absence of North American vitamin D trials in young children with functional outcomes of bone development or lean mass, it is also critical to understand the relationship of vitamin D and musculoskeletal health.

Figure 2.5 Peripheral quantitative computed tomography scan at the 66% lower leg in a 7 y old male, with permission.

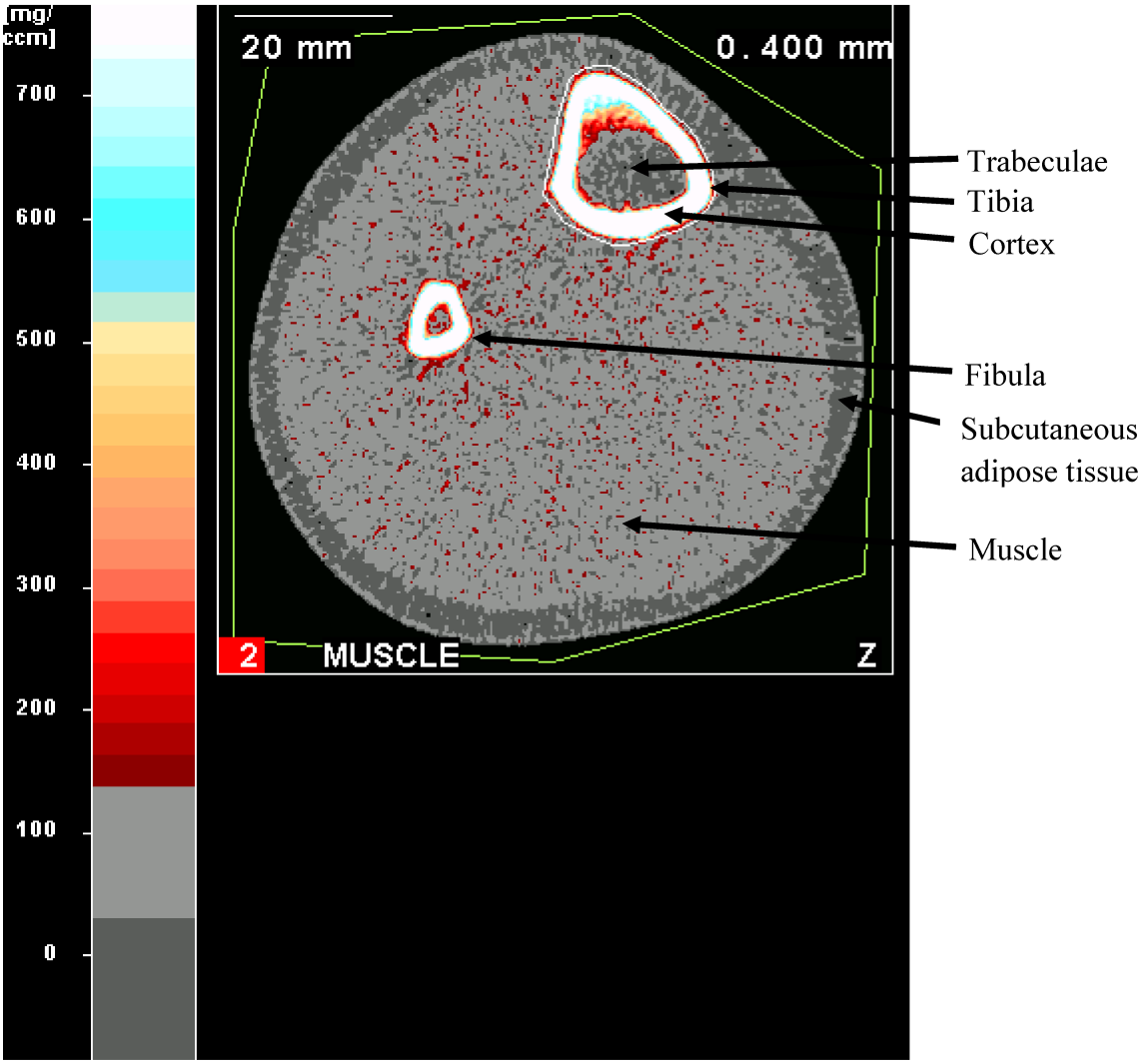


Table 2.4 Peripheral quantitative computed tomography methods used previously in young children

Authors	Slice placement and thickness	Voxel size (mm^2)	Speed (mm/s)	Population
Moon et al 2015 (UK) (198)	4%, 38% One 2 mm slice	0.5	30	n= 200, healthy 6-7 y
Leonard et al 2015 (USA) (199)	3%, 30, 38, 66% One 2.3 mm slice	0.4	25	n= 91 obese, 51 healthy 10-15 y
Moyer-Mileur et al 2008 (USA) (200)	4%, 66% One 2 mm slice	0.4	30	n= 416, healthy 5-18 y
Specker et al 2003 (USA) (201)	20% Two 2 mm slice	0.4	20	n= 239, healthy 3-5 y
Binkley et al 2000 (USA) (202)	20% Two 2 mm slice	0.4	20	n= 101, healthy 3-4 y

BRIDGE 1.

Since the current vitamin D fortification policy in Canada (mandatorily adding vitamin D to milk and margarine) (14-16) was set when intake recommendations were lower, it is unrealistic for young children in Canada to be able to reach the current EAR through currently available foods. Though yogurt and cheese are considered part of the milk and alternatives food group, they have < 30% of the vitamin D that fluid milk contains (8). This means that the maximum amount of vitamin D that can be consumed by meeting the milk and alternative food group recommendation is 200 IU/d. This is problematic because 60-85% of vitamin D intake for children comes from the milk and alternatives food group.

Average 25(OH)D concentration (> 50 nmol/L) of Canadian children is higher than expected based on intake, and vitamin D deficiency is uncommon. Based on nationally representative data (19) and cross-sectional studies (1, 2) the average winter 25(OH)D may only be 4-10 nmol/L. Based on these data, it is possible that children do not need to consume the EAR vitamin D intake to maintain vitamin D status at 40 nmol/L or 50 nmol/L, respectively year-round. Due to this and current vitamin D recommendations being based on studies in adolescents and adults, there is a need for vitamin D research in young children. Highlighting this need for research, no vitamin D fortification trials have been undertaken in young children in North America. The following study examined how 25(OH)D concentration in Montreal children 2-8 y, responded during winter to 12 wk of meeting the EAR (400 IU/d) or RDA (600 IU/d) for vitamin D through fortified cheese and yogurt. This pilot study gathered evidence so that a longer trial of similar design could be undertaken throughout winter investigating vitamin D and functional health outcomes.

CHAPTER 3: MANUSCRIPT 1

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Dietary vitamin D dose-response in healthy children 2 to 8 y of age: a 12 wk randomized controlled trial using fortified foods

Neil R. Brett, Paula Lavery, Sherry Agellon, Catherine A. Vanstone, Jonathon L. Maguire, Frank Rauch, Hope A. Weiler.

School of Human Nutrition, McGill University, Ste Anne de Bellevue, QC. (NB, PL, SA, CV, HW)

Applied Health Research Centre, Li Ka Shing Knowledge Institute of St. Michael's Hospital, Department of Pediatrics, University of Toronto, Toronto, ON. (JM)

Shriners Hospital for Children, Department of Pediatrics, McGill University, Montreal, QC. (FR)

PubMed indexing: Brett, Lavery, Agellon, Vanstone, Maguire, Rauch, Weiler

Disclaimers: JM and HW are members of the Dairy Farmers of Canada Expert Scientific Advisory Committee. All authors have no conflicts of interest.

ABSTRACT

Background: Vitamin D is fundamental for bone health. A high proportion of Canadian 2 to 8 y olds do not meet the Estimated Average Requirement (EAR) of 400 international units (IU)/day.

Objective: To determine whether vitamin D intakes consistent with the EAR or Recommended Dietary Allowance (RDA), through fortification of additional dairy products, would result in higher vitamin D status in young children.

Design: Participants 2 to 8 y (n=77; Montreal, Canada), were randomized to 1 of 3 dietary vitamin D targets (control, EAR: 400, or RDA: 600 IU/day) for 12 wk, January to April 2014. Anthropometry, demographics, dietary intakes, fasting serum parathyroid hormone (PTH), 25(OH)D (Liaison, Diasorin) and ionized calcium (iCa; Radiometer) were compared using mixed model analysis of variance (ANOVA).

Results: Participants were 5.1 ± 1.9 y (mean ± standard deviation), 54.5 % male with BMI Z-scores 0.50 ± 0.85. Compliance was 85% overall. No differences were observed in baseline dietary vitamin D intake or serum 25(OH)D. At 12 wk, the EAR and RDA groups had significantly higher vitamin D intakes (median (interquartile range), control: 227 (184-305), EAR: 410 (363-516), RDA: 554 (493-653) IU/d, $p < 0.05$) and serum 25(OH)D concentrations (control: 55.8 ± 12.3, EAR: 64.1 ± 10.0, RDA: 63.7 ± 12.4 nmol/L, $p < 0.05$) compared to control. Ninety-six percent of children in the EAR and RDA groups and 67% of the control group had 25(OH)D ≥ 50 nmol/L.

Conclusion: Increasing the vitamin D intakes of young children through fortification of alternative dairy products results in significantly higher serum concentrations of 25(OH)D and a significantly greater proportion of children with serum 25(OH)D above 50 nmol/L during periods of minimal ultra-violet beta radiation exposure.

3.1 INTRODUCTION

Vitamin D is a fundamental nutrient for bone mineral accrual (43). Since vitamin D cannot be endogenously synthesized year round above 40º N latitude due to limited ultraviolet beta (UVB) solar radiation from October through March (9), it is considered an essential nutrient in the diet (8, 43). Children age 2 to 8 y are recommended to consume 2 to 2.5 servings of milk and alternatives by Canada's Food Guide and MyPlate (10, 11). However, greater than 50% of children in Canada and the USA do not meet the Estimated Average Requirement (EAR) for vitamin D based on national surveillance surveys (12, 13). In cross sectional studies in young children, vitamin D status is significantly higher in the summer months compared with the winter months (1, 20, 21). To the best of our knowledge, no studies of young children in North America have investigated whether winter-time declines in vitamin D status are significant when followed prospectively. The amount of dietary vitamin D needed to sustain vitamin D status throughout winter, in young children, is unknown.

Vitamin D fortification policy in Canada includes adding vitamin D to milk and margarine and in the USA, to milk and some yogurt and cheese products as well as other foods (14-16). Average fluid milk intake of Canadian 4 to 8 y olds was 375-575 mL/d in 2004 (4). NHANES 1999-2002 data shows that preschool age children have a mean milk intake of 364 mL/d (203) and NHANES 2001-2004 data showed that 59.1 ± 3.17% of 2-3 y olds and 57.8 ± 2.14% of 4-8 y olds were meeting the recommended milk intake (204). Meeting the food group recommendation by consuming only fortified fluid milk provides half of the EAR of 400 IU/d and a third of the Recommended Dietary Allowance (RDA) of 600 IU/d of vitamin D. These values were set to align with serum 25-hydroxyvitaminD (25(OH)D) concentration of 40 nmol/L and 50 nmol/L, respectively (8). This means children are at risk of not meeting the Institute of

Medicine (IOM) population target of 40 nmol/L of 25(OH)D (14). By consuming yogurt or cheese instead of fluid milk, vitamin D intakes may be lower as they have < 30% of the vitamin D milk contains (8).

Vitamin D fortification regulations were set when recommended intakes were lower (14-16). Thus, the vitamin D EAR cannot be met by most children consuming currently available marketplace foods. The primary objective of this study was to determine whether vitamin D intakes consistent with the EAR or RDA, through fortification of additional dairy products, would result in higher vitamin D status in young children. The secondary objective was to confirm if vitamin D intakes reaching the RDA sustains a serum 25(OH)D concentration of 50 nmol/L in young children during winter and early spring months.

3.2 SUBJECTS AND METHODS

Subjects

Families and children were recruited from daycare centers within the greater Montreal region. Parents were provided a recruitment letter through registered daycares and contacted the research unit if interested. For those > 6 y, previous participants of a vitamin D study (1) were invited and media recruitment strategies were also used. Inclusion criteria were: healthy, pre-pubertal, regularly consume milk and milk products, within 2 BMI Z-scores from zero for age and sex based on World Health Organization (WHO) growth charts (174) and not taking any nutritional supplements. Exclusion criteria included chronic diseases or medications known to affect vitamin D, infections of the immune system, known anaemia, small size at birth or preterm birth <37 wk gestation. During screening, if children were taking nutritional supplements, the parents were asked to stop the supplement intake of their children for one month (2 half-lives of 25(OH)D) (205) prior to the study. Twelve 2-3 y olds and twenty two 4-8 y olds were taking

vitamin D supplements (68% took 200 IU or less per day, 29% took 400 IU/d and 1 child took 1000 IU/d). There were no differences in supplement intake among groups (**Table 3.1).**

Study Design

This was a 12 wk double blind randomized controlled trial, following the CONSORT statement, with recruitment from January 8th to February 16th, 2014. Children were randomly allocated 1:1:1 to 1 of 3 groups (**Figure 3.1**), in a double blind fashion stratified by 2 y blocks for age, using http://randomization.com. Group codes were randomly chosen by the safety officer for this trial to ensure blinding of researchers. All children consumed normally fortified fluid milk ad libitum. The control group consumed two 93 mL drinkable yogurts/ day and one 21 g piece of cheddar cheese with no added vitamin D in addition to their regular household meals. The two treatment groups consumed the same yogurt beverage and cheese products except with added vitamin D_3 to reach an estimated total dietary vitamin D intake consistent with the EAR (400 IU/d) and RDA (600 IU/d). It was expected that on average the control groups would consume 250 mL of milk/d (100 IU of vitamin D), and obtain 30 IU of vitamin D normally occurring in the yogurt beverage and 10-65 IU from other foods (fish/seafood, margarine or other products with vitamin D added) providing a total of 140-195 IU/d of vitamin D. The yogurt beverage in the EAR group contained a total of 42 IU/93 mL of vitamin D and in the RDA group contained a total of 125 IU/93 mL. Both groups consumed 200 IU/d through cheese (200 IU/21 g). The products were provided pre-coded by the companies and vitamin D content verified to be within ± 8% by Maxxam Analytique Inc., Saint Laurent, QC for the yogurt and within ± 5% by O'Neal Scientific Services Inc., St. Louis, MO for the cheese. Families were instructed to otherwise follow their normal lifestyle. Children and families were seen at baseline and at 12 wk.

At both visits fasting blood samples were obtained and anthropometric measures were taken along with surveys on demographics, sun exposure, physical activity and dietary intake.

Assessments

Blood Sampling, Vitamin D Status and Parathyroid Hormone (PTH)

Fasting venipuncture samples were taken (0700 h – 1100 h) to control for diurnal variation; parents were instructed that their child could not eat anything after midnight. At baseline and end of study, 2 mL of whole blood was separated to obtain serum for measurement of 25(OH)D and PTH and 0.1 mL of whole blood was immediately analyzed for ionized calcium.

Serum total 25(OH)D and intact 1-84 PTH were measured using an autoanalyzer (Liaison, Diasorin). The sensitivity of these assays was 10 nmol/L for 25(OH)D and 2.36 pg/mL for PTH. Using National Institute for Standards and Technology 25(OH)D standards 972a level 1 and 4, the inter- and intra-assay CVs were 2.1% and 2.2% for level 1 and 5.8% and 6.9% for level 4 with an accuracy of 96% or greater. The laboratory also maintains certification with the Vitamin D External Quality Assessment Scheme (DEQAS). PTH controls had inter- and intra-assay CV's of 3.6%, 2.6% for control 1 and 3.7%, 7.2% for control 2 with an accuracy of 95% or greater for control 1 and 2. Ionized calcium was measured immediately in whole blood (0.1 mL) as a safety measure using a portable blood gas unit (ABL80 FLEX, Radiometer Medical A/S, Copenhagen, Denmark).

Dietary Assessment and Compliance.

A 24-h food intake assessment (day prior to sampling) was used to assess energy and macronutrient intake. Having 3 days of intake data has been shown to be sufficient to estimate energy intake, however, more than 7 days are needed to estimate calcium intake (206).

Therefore, a validated 13-item semi-quantitative 1 month food frequency questionnaire (FFQ) was used specifically to estimate calcium and vitamin D (1). The FFQ had 13 questions asking about intake of milk and alternatives, vitamin D fortified juices, margarine and baby foods as well as fish and seafood. Both the FFQ and 24-h assessment were completed by the parents with the assistance of a registered dietitian at each study visit. Subsequent 24-h food intake assessments were completed over the telephone mid-way between study visits. Both the 24-h assessment and FFQ were repeated at the final visit. Nutrient intake was generated using Nutritionist Pro™ (Axxya Systems LLC, Stafford, TX, US) and the Canadian Nutrient File version 2010b. Data was also expressed as food group servings according to Canada's Food Guide (2). Compliance was reported by parents using a daily calendar check-sheet, keeping track of how many of the study products their child consumed each day. Parents were contacted over the telephone or by e-mail once per month to arrange delivery of fresh product and to encourage compliance. Calendar check-sheets were collected and verified with parents at 12 wks to add up the product intake of their child over the study period.

Demographics, Skin Pigmentation and UVB

At baseline, survey data was obtained for child sun exposure as well as self-reported income and ethnicity to facilitate understanding of the relationships among socio-demographics, skin pigmentation and baseline vitamin D status. In case of UVB exposure from travel to southern locations, data at baseline and 12 wk was collected regarding sun exposure during the previous 30 days as a percentage of body surface area (BSA) exposed (207), frequency of sunscreen use and total hours spent in direct sunlight per day. At each time-point the sun index was calculated for each child, by multiplying the % BSA exposed by the time spent outside. Skin type at baseline was established using a spectrophotometer (CM-700d/600d, Konica Minolta,

Ramsey, NJ, USA) by measuring pigmentation three times at the inner upper arm for constitutive pigmentation. Facultative pigmentation at the forehead, mid-forearm and lower leg was also measured thrice at each site to estimate recent exposure to ultraviolet beta radiation (UVB) (e.g. travel during December break or later during March break). The individual typological angle (ITA^{o}) was calculated using the L* and b* values with the equation from the Commission Internationale de l'Éclairage (208) and classified into 6 skin types based on Fitzpatrick descriptions. Differences between facultative and constitutive values assisted in qualitative assessment of previous UVB exposure (209, 210) at all visits.

Anthropometry

Height was measured to the nearest 0.1 cm using a wall mounted stadiometer (Seca 216, Seca Medical Scales and Measuring Systems, Hamburg, Germany). Body weight was measured to the nearest 0.5 kg using a balance-beam scale (Detecto, Webb, USA) with the child wearing light clothing and no shoes. From these values, BMI (kg/m^2) was calculated and then Z-scores for weight, height and BMI were calculated using the WHO 2007 growth standards/references for children under/over 5 y (WHO AnthroPlus, Geneva, Switzerland). At end-point, dual-energy x-ray absorptiometry (DXA) (Hologic Discovery 4500A QDR series with Apex V13.2 software, Bedford, Mass. USA) was used to scan the whole body to provide total and regional fat mass (kg and %). Children wore standardized clothing, light pants and t-shirt, and were scanned without sedation. Total body and regional fat mass were quantified since regional fat mass in adults and children inversely correlates to vitamin D status (211, 212). Regional adipose was estimated using sub-region analysis of the full torso as well as between the last floating rib and the iliac crests to reflect visceral and subcutaneous fat of the abdominal region. Even though DXA estimates of trunk fat do not distinguish subcutaneous from visceral fat, values align well with

those from computed tomography scans in women and children (36, 190, 191). The whole body scans take 3 minutes, resulting in a low dose radiation exposure (<3 μSV).

Ethics

This study was approved by the McGill University Faculty of Medicine Research Ethics Board in accordance with the Tri-Council policy on ethics (213), Temporary Marketing Authorization letters were obtained from Health Canada for both products (TM-13-0432 and TM-13-0433). All daycare centers agreed in writing to facilitate the study and all parents or legal guardians provided written informed consent prior to the study.

Statistical analyses

A sample size estimate of 20 per group was set to enable detection of clinically meaningful group differences of 20 ± 16 nmol/L of 25(OH)D, based on a representative sample of pre-school age children in Montreal (1) at a 5% significance level with 80% power. To account for a 5-10% drop out rate and the additional possibility of insufficient blood volume sampled we aimed to recruit 25 participants per group.

Intent-to-treat analyses were conducted using SAS (version 9.3, SAS Inst. Cary, North Carolina). All data entry was double audited and tested for normality using the Kolmogorov-Smirnov test and homogeneity of variance using the Bartlett test. For the primary analysis, mixed model ANOVA was used to analyse continuous data accounting for fixed effects (sex/ age strata, dietary group) and random effects (e.g. demographics etc.) with post-hoc testing where necessary (i.e. 3 age groups or interactions) using Bonferroni correction. Non-normal data were log transformed where applicable (e.g., 25(OH)D). At end point there were not enough children in each vitamin D status category for 30-39.9 and 40-49.9 nmol/L serum 25(OH)D concentration to enable reliable statistical analyses, thus these were combined to form a category of 30-49.9

nmol/L. Less than 5% of data was missing and imputation approaches were not sought. Compliance, based on daily check sheets was compared among groups using mixed model ANOVA. Chi-square or Fisher exact testing was used for proportions. Linear regression analysis was used to explore correlations between serum 25(OH)D concentration and predictor variables. The model was constructed based on inclusion of predictor variables vitamin D intake, age, BMI Z-score and sex; single variables were the added to the model that were significant or that improved the model ($R^2 > 2\%$). The models were checked for pre-specified interactions, and if interactions were present, interaction terms were included in the final model. Visual examination was used to test for normality of the residuals. Data are presented as mean (SD) or median (IQR) depending on normality for continuous data or as proportions for ordinal data (e.g. sex). A probability < 0.05 was accepted as significant.

3.3 RESULTS

Demographics

At baseline, children were 5.1 ± 1.9 y (range 1.9-8.7 y) with 54.5% (42/77) being male and 72.7% (56/77) white. The majority of children were born to mothers who were well educated with 77% achieving college or university education and household income was above $65,000 in 56% of families with 9% not disclosing income. No differences in these characteristics were observed among allocation groups. Physical activity was not different among groups at baseline. On weekdays and weekends, 91% and 82% of children were very active for ≥ 60 minutes.

At baseline, BMI-for-age Z-score was 0.51 ± 0.89 overall, with no differences between males and females, 0.48 ± 0.82 and 0.55 ± 0.97, respectively. Mean height-for-age Z-score was 0.10 ± 0.98 and weight-for-age Z-score, 0.43 ± 0.93. No differences in anthropometry were observed among groups (**Table 3.1).** Height velocity was 0.6 ± 0.3 cm/mo and weight velocity

was 0.2 ± 0.2 kg/mo over the study period with no differences among groups (**Supplemental Table 3.1**). At the end of the study, there were no significant differences among study groups in body fat percentage (range: 15.1- 39.7 %), however, female participants had significantly higher ($p < 0.05$) mean android and gynoid fat percentages (android M: 20.5 ± 6.1, F: 25.1 ± 5.5 %; gynoid M: 33.0 ± 4.8, F: 41.0 ± 8.0 %).

Seventy four (96.1%) children completed the study over 12.1 (range 10.9-13.9) wk. Twenty two participants had their 12 wk follow up in early May instead of April due to rescheduling. Over the course of the study, the median (interquartile range (IQR) duration of childhood illnesses (e.g. common colds) per group was, control: 4.0 (3.0-5.5), EAR:5.0 (2.9-9.3), RDA: 4.5 (3.0-6.0) days with no differences among groups. Mean compliance for the study yogurt and cheese products was 86 ± 20% and 84 ± 17%. For the first wk of the study it was 95 ± 12% and 91 ± 18% and dropped to 79 ± 28% and 75 ± 29% by the 12th wk. Compliance was significantly different ($p=0.04$) between 2-3 y olds and 4-8 y olds for cheese (76 ± 21% vs 88 ± 13%), but not different for yogurt (84 ± 19% vs 88 ± 19%). Overall the EAR group had significantly lower compliance ($p=0.02$) for both products (yogurt: 80 ± 22%, cheese: 79 ± 17%) than the control (yogurt: 89 ± 19%, cheese: 88 ± 13%) and RDA (yogurt: 89 ± 16%, cheese: 84 ± 19%) groups.

Dietary Characteristics

Consumption of study products during the trial did not cause a significant change in total energy intake (baseline: 1520 ± 423, 12 wk: 1538 ± 390 Kcal/day), protein intake (baseline: 64 ± 23, 12 wk: 67 ± 24 g/day), fat intake (baseline: 49 ± 19, 12 wk: 54 ±19 g/day) or carbohydrate intake (baseline: 212 ± 64, 12 wk: 201 ± 54 g/day).

The children in all groups, on average, met or exceeded the recommended servings of milk and alternatives based on Canada's Food Guide (**Figure 2, Supplemental Table 2)**. There were no differences in servings of total milk and alternatives consumed (2-3 y: 2.0 ± 1.4; 4-5 y: 2.6 ± 1.2; and 6-8 y: 2.3 ± 1.3 servings/d) among the 3 age strata. At baseline the median (IQR)) vitamin D intakes from the 30 day FFQ of 198 (155-291) IU/d were below the EAR with no differences among groups or between age strata (**Table 3.2)**. The vitamin D intake of the control group did not significantly change throughout the study. According to the 30 day FFQ, none of the control group reached 400 IU/d. The percent of children in the EAR and RDA groups reaching their targets at 12 wk was 60% and 42%, respectively, according to the 30 day FFQ (**Table 3.2).** If compliance had been 100%, the median (IQR) vitamin D intakes for the EAR and RDA groups would have been 458 (390-509) and 629 (578-698) IU/d. Total milk product intake did not differ over time or among groups (**Figure 3.2).** Calcium intake was similar across groups at baseline (Control: 1092 ± 367 mg/d, EAR: 1042 ± 429 mg/d, RDA: 1039 ± 418 mg/d) and did not change over the 12 wk, with 61% of all participants reaching the RDA at baseline and 64% reaching the RDA at 12 wk.

Sun Exposure

Fifty six percent (43/77) children had Fitzpatrick skin types I, II or III, 44% (34/77) had skin types IV, V or VI. Only 4% (3/74) of children travelled to warm countries during the study period and none of them had changes in ITA from baseline to follow-up. Accordingly, there was no significant tanning of skin from ultra violet beta solar radiation. The mean change in ITA over the study on the 3 tanning sites (forehead, lower leg and lower forearm) was 1.84 ± 3.75 and was not significantly different from zero or significantly different among groups. Participants who

had their 12 wk follow up in May (n=22) did not have a significantly different mean change in ITA (0.85 ± 2.81) from those with follow ups in April (2.28 ± 4.06).

Biochemical Assessments

In regards to our primary analysis, at baseline, 77% (59) of participants had serum 25(OH)D concentrations between 50-125 nmol/L, none had concentrations over 125 nmol/L and only 1 participant had a 25(OH)D concentration < 30 nmol/L (**Table 3.1**). By 12 wk, no children had serum 25(OH)D below 30 nmol/L, 8 children in the control group and 1 child in each of the EAR and RDA groups had serum 25(OH)D below 50 nmol/L. No child had serum 25(OH)D concentrations above 125 nmol/L.

At 12 wk, both the EAR and RDA allocation groups had significantly higher 25(OH)D concentration compared to control (**Figure 3.3A**) and had a significantly greater change over time in 25(OH)D concentration (**Figure 3.3B**). In all groups, change in vitamin D status was not different according to age groups (p=0.499). At baseline, all groups had similar proportions of children with 25(OH)D above the target consistent with the EAR (control: 96%, EAR: 93%, RDA: 100%) and the RDA of 50 nmol/L (control: 71%, EAR: 74%, RDA: 85%). By 12 wk, 96% of the EAR and RDA groups had 25(OH)D concentrations equal to or above 50 nmol/L, which was significantly different compared to the control group (67% with 25(OH)D ≥ 50 nmol/L). At 12 wk, 100% of the EAR group and 92% of the control group had 25(OH)D concentration above 40 nmol/L.

Based on regression analysis (R^2 0.73), 25(OH)D increased significantly ($p < 0.05$) from skin type category 1 (Fitzpatrick I-III) to category 2 (Fitzpatrick IV-VI) by 5.5 (95% CI 1.1 – 10.0) nmol/L, but did not vary significantly based on other variables in the model, BMI Z-score, total body fat percentage (total or android and gynoid), or number of days in the study. Using the

same model, for every 100 IU increase in vitamin D intake, serum 25(OH)D increased by 1.7 (95% CI 0.6 – 2.8) nmol/L.

Ionized calcium showed a group by time interaction (p=0.027) suggesting values declined in the control group by 12 wk (Baseline: 1.31 ± 0.05, 12 wk: 1.28 ± 0.06 mmol/L), but did not reach statistical significance (p=0.059) with no differences in the EAR or RDA groups. Average ionized calcium (1.30 ± 0.04 mmol/L) was within normal limits (1.15-1.38 mmol/L) in all groups; however one child in the EAR group had a low value (1.10 mmol/L) at 12 wk. The pattern in PTH was the opposite of that of 25(OH)D, although with no significant differences among groups (**Figure 3.3**) and an overall mean change in PTH from baseline to 12 wk of -0.5 ± 5.1 pg/mL. Time of blood draw in the morning did not have an effect on PTH concentration (p=0.37). One child in the EAR group (6.3 pg/mL) at baseline and 4 children in the EAR group and 2 children in the RDA group (7.4-8.8 pg/mL) at 12 wk had PTH values below the normal range (9-60 pg/mL).

3.4 DISCUSSION

The primary objective of this study was to establish whether vitamin D intakes consistent with the EAR or RDA, through fortification of additional dairy products, would result in higher vitamin D status compared to normal vitamin D intakes. While we observed a significant difference in dietary intake, not all children were able to meet the EAR and RDA targets. Since the EAR is set to be a sufficient for 50% of individuals, and 60% of our EAR group met this target, we would have expected more than half of the children in this group to maintain the 40 nmol/L of 25(OH)D whereas almost all (96%) children maintained this. For the RDA group, the design was intended to elevate usual intakes to meet the RDA over the course of the study, this did not appear to be achieved based on the 30 day FFQ. In the real life situation of our study,

children had a median (IQR) of 4.5 (3.0-6.0) days of illness which contributed to intakes below the RDA as captured in the FFQ. Nonetheless, all but 1 child was able to achieve and maintain a 25(OH)D of > 50 nmol/L. These results suggest that the baseline vitamin D status observed in our study was sufficient to protect against 25(OH)D falling below 40 nmol/L or that the EAR is higher than necessary for the needs of young children. Overall, the significantly increased vitamin D consumption in the fortified groups compared to the control group led to significantly higher serum 25(OH)D concentrations in the winter and early spring months.

Interestingly, the RDA intake group (64.1 ± 10.0 nmol/L) in our study did not have significantly higher serum 25(OH)D compared to the EAR group (63.7 ± 12.4 nmol/L). Both the EAR and RDA groups also had 96 % of children with 25(OH)D above 50 nmol/L. The EAR and RDA recommendations from the IOM are mostly based on randomized controlled trials in adults from northern Europe (8), where vitamin D status is usually lower than in North America. In fact, all 16 trials that were used in revising the recommendations were supplement based with no trials using fortified foods (214). A recent trial in Denmark involving both children and adults (4-60 y) consuming vitamin D fortified foods (~ 360 IU/d from fortified milk and bread), showed that while this intake was closer to the EAR it was enough to ensure serum 25(OH)D above 50 nmol/L in children (33). These results along with ours could suggest that vitamin D when consumed regularly from foods better supports vitamin D status than inferred from the data derived from pill supplements. Alternatively, both of these trials in children suggest that the EAR could be too high regardless of exogenous source of vitamin D.

Another reason for similar results among EAR and RDA groups could be that serum 25(OH)D does not represent tissue 25(OH)D uptake or that 12 wk may not be long enough for 25(OH)D in children to plateau. Little is known about the mobilisation of stored vitamin D (215)

and if it is more efficient during childhood (216). In rats, oral vitamin D_3 quickly increased (1-2 days) adipose vitamin D_3 stores and was very slowly released while the rats were in a state of energy balance (217). The authors suggest that this may act to partially buffer vitamin D status and that in theory mobilization of vitamin D stored in adipose tissue in children increases when the body requires it, as it may in late winter. This could explain why the control group did not show a significant decrease in serum 25(OH)D over 12 wk (-2.5 ± 6.5 nmol/L) with 92% maintaining concentrations above 40 nmol/L even though vitamin D intakes were just over half of the EAR.

Addition of vitamin D to foods as a means to support vitamin D status during times of reduced endogenous synthesis has been tested mostly in adults. A meta-analysis of trials in adult populations showed that for every 40 IU/d increase in vitamin D intake, there would be a resulting increase of 1.2 nmol/L (95% CI: 0.72, 1.68) (218). A trial in adults ≥ 50 y (n=56) in Ireland through 10 wk in winter showed a 2.5 nmol/L increase for every 100 IU increase in vitamin D intake (219). However, previous trials in children that ran September to April (33) and January to March (34) have shown that each 100 IU/d increase in vitamin D intake leads to a 7-10 nmol/L increase in serum 25(OH)D. In our study, based on linear regression analysis, each 100 IU/d increase in vitamin D intake through fortified foods, resulted in an average increase in 25(OH)D of 1.7 (95% CI 0.6 – 10.0) nmol/L. Our results may differ from previous work in children due to our study taking place over only 12 wk, beginning in the middle of winter. Longitudinal results from adults in Denmark (14) show a mean decrease of 25 nmol/L of 25(OH)D between October to December and 4 nmol/L between January to March, similar to our results in young children. A larger increase in 25(OH)D may have been observed had baseline vitamin D status been deficient, as illustrated in young children in Mongolia (16).

When 25(OH)D is low, it is common to observe elevated PTH (220). At baseline PTH (15.6 ± 4.8 pg/mL) values in our study were similar to that of 4 to 8 y old children after supplementation with 1000 IU/d of vitamin D_3 (13.3 ± 7.6 pg/mL) (22), which may explain why significant decreases in PTH concentration were not seen in the fortified groups in our study. The lower PTH is likely due to the very good calcium intakes in the present study. Ionized calcium, being similar among groups and unchanged over the 12 wk study period demonstrates the safety of fortifying dairy products with vitamin D in an effort to reach EAR and RDA targets.

To our knowledge, our study methodology presented a novel fortification model for children, by using 2 fortified dairy products to augment usual intakes. Increased daily intake of vitamin D was chosen because it is more efficient at maintaining or raising serum 25(OH)D compared with large monthly (221) or seasonal (222) intakes. Another strength of this design was that it allowed children to maintain day to day consumption of dairy products, with the study products replacing those normally consumed (**Figure 3.2, Supplementary Table 3.2**). Allowing normal food intake habits for children likely contributed to our high level of overall compliance (84-86%). Poor compliance has been observed when children have difficulty meeting food intake or nutrient intake goals (223). Though our compliance declined by the end of the study, a higher level of compliance may have been observed with the availability of multiple yogurt flavours and types of cheese since parents may not have to put as much pressure on children to eat the foods. In an American study (n= 27 children, mean age 4.0 ± 1.0 y), parents putting pressure on a child to eat a food, was shown to significantly decrease the intake of that food by 20% and have children vocalize 5 times as many negative comments about the food (224).

Limitations of our study include that the data are not representative of all young Canadian children since a large proportion of the parents had university education and households had a

median family income above average for Quebec and Canada (225). Also, it is possible that dietary vitamin D intakes were underestimated since 25(OH)D present in animal-based foods were not included in our total dietary intake calculation. Based on NHANES data (n=8579, 2007-2008), contributions from all other foods besides milk and alternatives is estimated to be 68-116 IU/d of vitamin D in the diets of people > 2 y (226). Lastly, based on previous research, it was anticipated that baseline 25(OH)D values may differ among white and non-white children (146), and that the effect of vitamin D food fortification on serum 25(OH)D would not differ among white and non-white children (227). We detected a significant difference baseline and 12 wk in serum 25(OH)D between Fitzpatrick skin types 1-III vs IV-VI but could not further analyse differences among all six skin types due to a limited sample size.

In conclusion, young children who consumed vitamin D fortified yogurt and cheese products had significantly higher vitamin D intakes and improved vitamin D status over the winter and spring period studied. These results show a need for future studies to confirm the vitamin D EAR of 400 IU/d for young children on the basis of 25(OH)D response and functional outcomes.

Table 3.1. Baseline Characteristics[1]

Parameter	Control	EAR	RDA
N	24	27	26
Age (y)	5.0 ± 1.8 Range: 2.0-8.0	4.9 ± 2.1 Range: 1.9-8.7	5.3 ± 2.0 Range: 2.1-8.3
Male	12 (50%)	15 (56%)	13 (50%)
Maternal Education Achieved, College or Higher	19 (79%)	20 (75%)	20 (77%)
Family Income: > $65,000[2] Not Disclosed	 14 (58%) 4 (17%)	 14 (52%) 0	 15 (58%) 1 (4%)
Weight Z-Score	0.49 ± 0.96	0.23 ± 0.78	0.55 ± 0.90
Height Z-Score	0.07 ± 0.88	-0.12 ± 1.05	0.27 ± 1.00
BMI Z-Score	0.63 ± 1.00	0.40 ± 0.58	0.54 ± 0.91
Vitamin D supplements: Prior use Dose (IU/supplement) Frequency (#/wk)	 12 (50%) 291 ± 115 5.0 ± 2.3	 11 (41%) 290 ± 104 4.9 ± 2.5	 11 (42%) 291 ± 243 4.5 ± 1.6
Serum 25(OH)D Category: < 30 nmol/L 30-39.9 nmol/L 40-49.9 nmol/L 50-124.9 nmol/L ≥ 125 nmol/L	 1 (4%) 0 6 (25%) 17 (71%) 0	 0 2 (7%) 5 (19%) 20 (74%) 0	 0 0 4 (15%) 22 (85%) 0

[1] Data are mean ± standard deviation or n (%)
[2] Canadian Dollars

Table 3.2. Vitamin D (IU/d) intakes based on a 30 day FFQ[1]

	Group	Fluid Milk	Yogurt[2]	Cheese[2]	Other[3]	Total	% Met EAR	% Met RDA
Baseline	Control n=24	161 (117-206)	17 (14-22)	2 (0-3)	76 (50-201)	207 (169-383)[a]	25	0
	EAR n=27	173 (106-232)	18 (13-28)	3 (2-4)	77 (53-105)	218 (145-294) [a]	8	0
	RDA n=26	120 (97-215)	12 (6-18)	2 (1-3)	72 (48-99)	166 (148-306) [a]	8	4
12 wk	Control n=24	132 (100-160)	40 (36-44)	1 (0-2)	64 (36-95)	227 (184-305) [a]	0	0
	EAR n=25	121 (66-206)	77 (69-85)	161 (141-213)	53 (28-81)	410 (363-516)[b]	60	4
	RDA n=25	112 (74-163)	223 (203-263)	167 (134-202)	67 (30-99)	554 (493-653)[c]	80	42

[1] Data are median (interquartile range).

[a.b.c] Values with different superscripts are significantly different ($p < 0.05$) from all other groups, using mixed model ANOVA with Bonferroni *post hoc* testing, accounting for age, sex, ethnicity, number of days between baseline and follow-up.

[2] Includes study products at 12 wk accounting for compliance.

[3] Other foods include margarine, eggs, fish.

Figure Legends

Figure 3.1. CONSORT diagram. This was an intent to treat analysis

Figure 3.2. Servings of milk and milk products per day based on a 30 day FFQ. Data are median (interquartile range). Serving sizes according to Canada's Food Guide: fluid milk (250 mL); yogurt (175 g or 200 mL); cheese (50 g). At 12 wk, yogurt and cheese include study yogurt (186 mL/d) and cheese (21 g/d) accounting for compliance.

Figure 3.3. Serum 25(OH)D concentration at baseline and 12 wk (panel A); and change in 25(OH)D concentration over time (panel B). Serum PTH concentrations at baseline and 12 wk (panel C); and change in PTH concentrations over time (panel D). *denotes significantly different ($p < 0.05$) from control group at 12 wk, ** denotes significantly different ($p < 0.05$) from control at 12 wk and all groups at baseline. Using an intent to treat analysis Mixed model ANOVA (adjusted for age, sex, ethnicity, baseline 25(OH)D, number of days between baseline and follow up) with Bonferroni *post hoc* testing. Serum 25(OH)D data was log transformed before analysis and are presented as unadjusted mean ± SD.

Figure 3.1

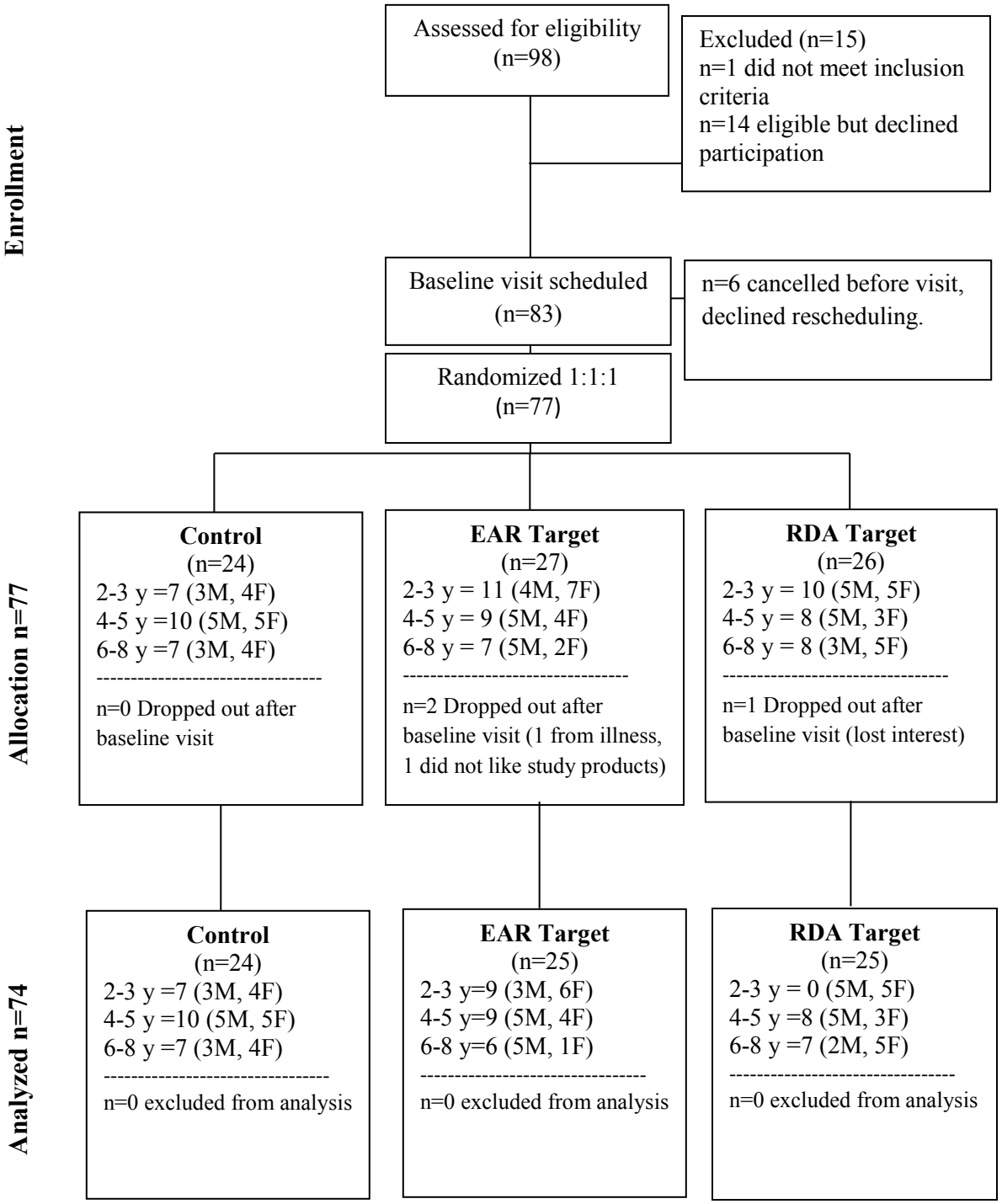

Enrollment
Assessed for eligibility (n=98)
Excluded (n=15)
n=1 did not meet inclusion criteria
n=14 eligible but declined participation
Baseline visit scheduled (n=83)
n=6 cancelled before visit, declined rescheduling.
Randomized 1:1:1 (n=77)
Allocation n=77
Control
(n=24)
2-3 y =7 (3M, 4F)
4-5 y =10 (5M, 5F)
6-8 y =7 (3M, 4F)
n=0 Dropped out after baseline visit
EAR Target
(n=27)
2-3 y = 11 (4M, 7F)
4-5 y = 9 (5M, 4F)
6-8 y = 7 (5M, 2F)
n=2 Dropped out after baseline visit (1 from illness, 1 did not like study products)
RDA Target
(n=26)
2-3 y = 10 (5M, 5F)
4-5 y = 8 (5M, 3F)
6-8 y = 8 (3M, 5F)
n=1 Dropped out after baseline visit (lost interest)
Analyzed n=74
Control
(n=24)
2-3 y =7 (3M, 4F)
4-5 y =10 (5M, 5F)
6-8 y =7 (3M, 4F)
n=0 excluded from analysis
EAR Target
(n=25)
2-3 y=9 (3M, 6F)
4-5 y=9 (5M, 4F)
6-8 y=6 (5M, 1F)
n=0 excluded from analysis
RDA Target
(n=25)
2-3 y = 0 (5M, 5F)
4-5 y =8 (5M, 3F)
6-8 y =7 (2M, 5F)
n=0 excluded from analysis

Figure 3.2

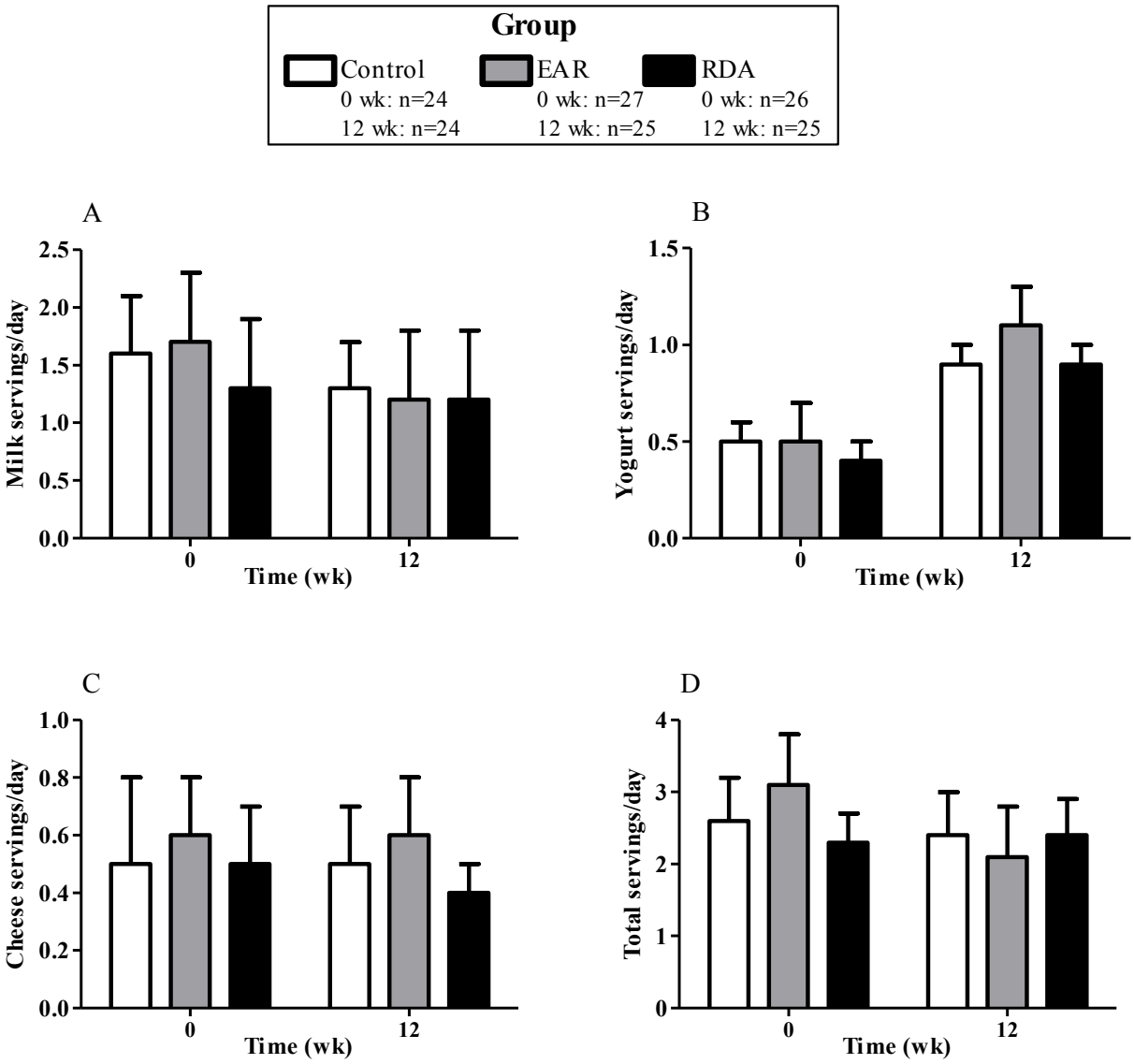
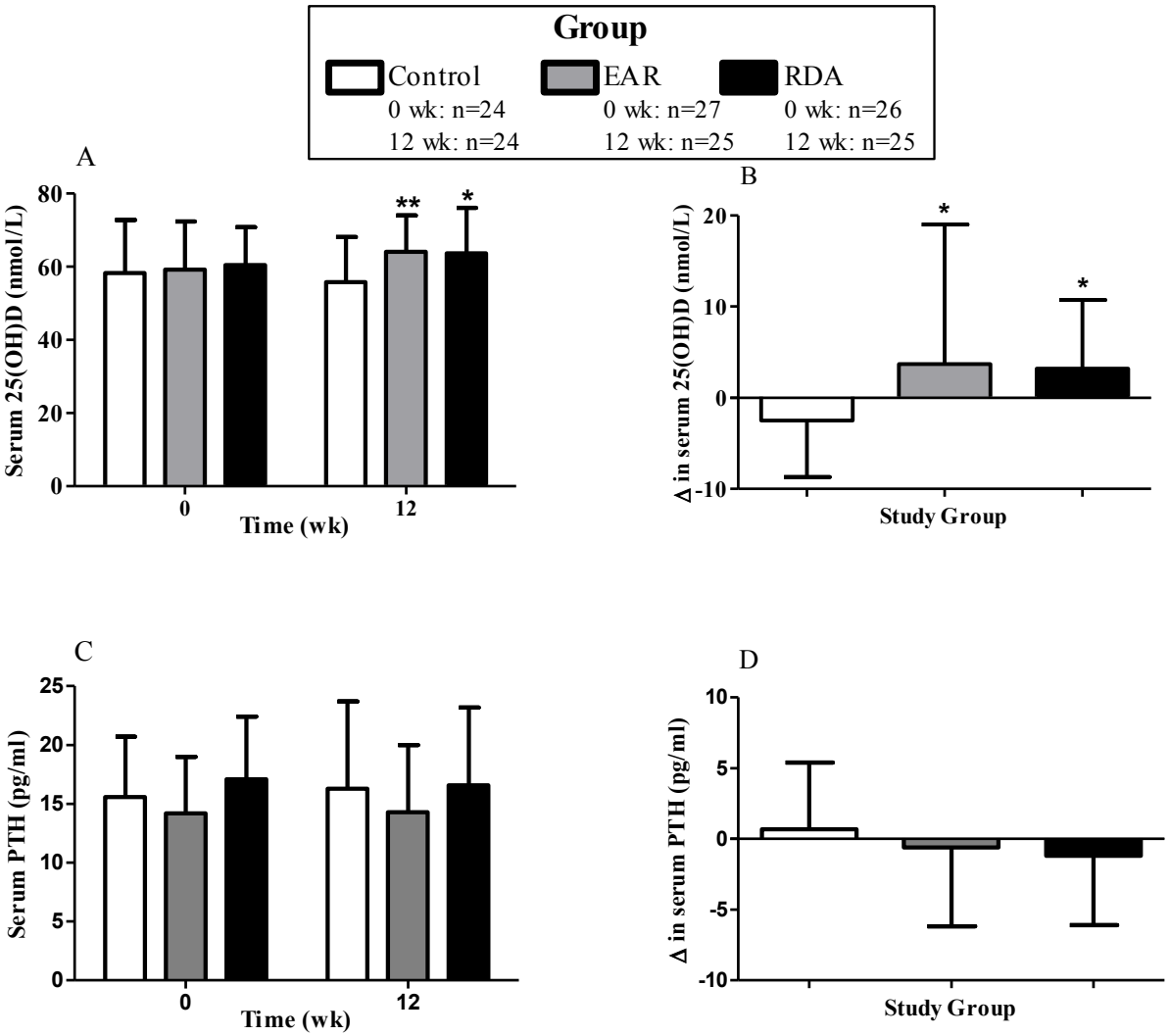

Group
Control
0 wk: n=24
12 wk: n=24
EAR
0 wk: n=27
12 wk: n=25
RDA
0 wk: n=26
12 wk: n=25
A
Milk servings/day
2.5
2.0
1.5
1.0
0.5
0.0
0
12
Time (wk)
B
Yogurt servings/day
1.5
1.0
0.5
0.0
0
12
Time (wk)
C
Cheese servings/day
1.0
0.8
0.6
0.4
0.2
0.0
0
12
Time (wk)
D
Total servings/day
4
3
2
1
0
0
12
Time (wk)

Figure 3.3


Group
Control
0 wk: n=24
12 wk: n=24
EAR
0 wk: n=27
12 wk: n=25
RDA
0 wk: n=26
12 wk: n=25
A
Serum 25(OH)D (nmol/L)
Time (wk)
B
Δ in serum 25(OH)D (nmol/L)
Study Group
C
Serum PTH (pg/ml)
Time (wk)
D
Δ in serum PTH (pg/ml)
Study Group

Supplementary Table 3.1 Anthropometry and growth over 12 wk[1]

	Group	Height (cm)	Height velocity (cm/mo)	Weight (kg)	Weight velocity (kg/mo)	Total body fat (%)
Baseline	Control	109.4 ± 14.2		20.2 ± 6.4		
	EAR	107.7 ± 17.1		19.3 ± 6.6		
	RDA	111.9 ± 13.9		20.9 ± 6.0		
12 wk	Control	111.1 ± 13.8	0.5 ± 0.2	20.6 ± 6.3	0.3 ± 0.2	26.0 ± 5.6
	EAR	108.9 ± 16.4	0.6 ± 0.3	21.2 ± 6.3	0.2 ± 0.3	26.1 ± 5.1
	RDA	113.5 ± 13.8	0.6 ± 0.3	21.6 ± 6.4	0.2 ± 0.2	26.2 ± 4.9

[1]Data are mean ± standard deviation

Supplemental Table 3.2 Total milk and milk product intake (mL/day) based on a 30 day FFQ[1]

	Group	Fluid Milk	Yogurt	Study Yogurt[2]	Cheese (g/day)	Study Cheese[2] (g/day)	Total servings/day[3]	≥ 2 servings/d (%)
Baseline	Control n=24	400 (275-525)	88 (70-105)	N/A	25 (10-40)	N/A	2.6 (2.3-3.6)	96
	EAR n=27	425 (275-575)	88 (52-122)	N/A	30 (20-40)	N/A	3.1 (2.3-3.8)	69
	RDA n=26	325 (175-225)	70 (70-105)	N/A	25 (15-35)	N/A	2.3 (1.6-3.4)	59
12 wk	Control n=24	325 (225-425)	18 (0-38)	160 (140-180)	10 (0-20)	15 (10-20)	2.4 (1.7-3.0)	60
	EAR n=25	300 (125-475)	38 (18-88)	160 (120-195)	15 (5-25)	15 (10-20)	2.1 (1.4-2.8)	64
	RDA n=25	300 (200-400)	18 (0-38)	120 (140-180)	5 (0-15)	15 (10-20)	2.4 (2.0-3.0)	80

[1] Data are median (interquartile range).
[2] Serving of study yogurt was 195 mL/day, serving of study cheese was 21 g/day. These columns are accounting for compliance
[3] Serving sizes according to Canada's Food Guide: Fluid milk (250 mL); Yogurt (175 g or 200 mL); Cheese (50 g).

BRIDGE 2.

In chapter 3, results of the 12 wk clinical trial showed that consuming 400 IU/d or 600 IU/d of vitamin D through fortified foods, had similar effects on 25(OH)D concentration and that both intervention groups had significantly increased 25(OH)D concentration compared to the control group. Since ~96% of children in the group consuming 400 IU/d maintaining 25(OH)D ≥ 50 nmol/L, it suggests that this intake fits the definition of the RDA. Meaning, it needed to be elucidated if a vitamin D intake of 400 IU/d, through fortified foods, would similarly effect 25(OH)D concentration across the entire winter period (6 mo). Since this level of vitamin D intake is more realistic to obtain through food sources compared to 600 IU/d, it is relevant to future updates of vitamin D food fortification policy in Canada.

As described in chapter 2, vitamin D has roles in both bone and muscle development. Cross sectional data from young children in Montreal showed positive associations between vitamin D status and muscle and bone outcomes (29, 37). However, since there are no vitamin D supplementation or fortification trials in North American young children investigating vitamin D related bone and muscle outcomes, it is not known how meeting vitamin D dietary intake guidelines impacts these functional outcomes longitudinally. Thus, chapter 4 also investigates functional outcomes associated with the vitamin D intake of 400 IU/d over 6 mo. This time frame was chosen as it covers the entire winter period of minimal UVB exposure and is the minimum amount of time needed to measure changes in bone mineral content (189).

CHAPTER 4: MANUSCRIPT 2

Vitamin D Status and Functional Health Outcomes in Children 2-8 y: A 6-month Vitamin D Randomized Controlled Trial

Neil R. Brett[1], Colleen A. Parks[1], Paula Lavery[1], Sherry Agellon[1], Catherine A. Vanstone[1], Martin Kaufmann[2], Glenville Jones[2], Jonathon L. Maguire[3], Frank Rauch[4], Hope A. Weiler[1].

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

[2] Department of Biomedical and Molecular Sciences, Queen's University, Kingston, ON.

[3] Applied Health Research Centre, Li Ka Shing Knowledge Institute of St. Michael's Hospital, Department of Pediatrics, University of Toronto, Toronto, ON.

[4] Shriners Hospital for Children, Department of Pediatrics, McGill University, Montreal, QC.

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ABSTRACT

Background: Canadian children do not meet the recommended dietary intake for vitamin D.

Objective: To test how much vitamin D from food is needed to maintain a healthy serum 25-hydroxyvitamin D_3 ($25(OH)D_3$) from fall to spring in young children and to examine musculoskeletal outcomes.

Design: Healthy children 2-8 y (n=51; Montreal, Canada) were randomized to 1 of 2 dietary vitamin D groups (control or intervention to reach 400 IU/d by using vitamin D fortified foods) for 6 mo, starting October 2014. At baseline, 3 and 6 mo, anthropometry, vitamin D metabolites (Liquid chromatography tandem mass spectrometry) and bone biomarkers (Liaison, Diasorin, IDS iSYS) were measured and physical activity and food intakes surveyed. At baseline and 6 mo, bone outcomes and body composition (dual-energy x-ray absorptiometry) were measured. Cross-sectional images of distal tibia geometry and muscle density were conducted using peripheral quantitative computed tomography scans at 6 mo.

Results: At baseline, participants were 5.2±1.9 y, 53% (27/51) of male sex and had BMI Z-scores of 0.65±0.12. There were no differences between groups in baseline serum $25(OH)D_3$ (66.4±13.6 nmol/L) or vitamin D intake (225±74 IU/d). Median (IQR) compliance was 96% (89-99) for yogurt and cheese 84% (71-97). At 3 mo, serum $25(OH)D_3$ was higher in the intervention group (p=0.001), but was not different between groups by 6 mo. Over 6 mo, 96% and 78% of all children maintained $25(OH)D_3$ ≥40 and 50 nmol/L respectively, whilst only 3 children maintained 25(OH)D3 ≥75 nmol/L. Lean mass accretion was higher in the intervention group (p=0.025); no differences in muscle density or bone outcomes were observed.

Conclusion: Consuming 400 IU/d of vitamin D from fall to spring did not maintain vitamin D stores or improve bone outcomes, but enhanced lean mass accretion in healthy young children.

4.1 INTRODUCTION

Vitamin D is important for bone growth and development in children (43) and cannot be synthesized from exposure of skin to sunlight at latitudes ≥ 40° N year round as solar ultra-violet beta (UVB) radiation is limited in winter months (9). Clinically, vitamin D status is assessed using serum 25-hydroxyvitamin D (25(OH)D) concentration, a composite reflection of total intake and synthesis. The Institute of Medicine (IOM) recommendations for vitamin D intake were set to meet the needs of the general population in the absence of UVB exposure. The Estimated Average Requirement (EAR: 400 IU/d), and Recommended Dietary Allowance (RDA: 600 IU/d) align with serum 25(OH)D concentrations of 40 nmol/L and 50 nmol/L, respectively (8). Given these recommendations, Canadian children, with an average intake of 244 IU of vitamin D/d, appear to be at risk of not meeting population targets for vitamin D status (14).

The IOM vitamin D recommendations for young children were based on results from supplementation trials in adults and older children (8). Since that time, vitamin D interventions in young children have been trialed during the winter months (40, 41, 202) and results suggest that the vitamin D EAR may be overestimated for young children. Furthermore, only 1 study had mean 25(OH)D concentrations equivalent to the mean 25(OH)D concentrations observed in national surveillance studies conducted in Canada and the US (children 3-11 y: 64-74 nmol/L) (6, 228). Thus, it remains questionable how much vitamin D is required to maintain healthy vitamin D status during seasonal UVB void periods. In addition, while there is a well-known positive impact of vitamin D on bone health in adults (8), data in young children are limited (31, 229) and it is possible that vitamin D interventions may only affect bone outcomes when baseline 25(OH)D concentration is <35 nmol/L (32). Data from children in Montreal (2-5 y)

showed vitamin D status ≥75 nmol/L positively related to bone mineral density (BMD) of the whole body and radius (29) and 25(OH)D was related to a leaner body phenotype (37). This agrees with a 1-y trial in vitamin D deficient pre-pubertal girls, where lean mass accretion was greater in those receiving a vitamin D supplement (39). Thus, the primary objective was to test how much vitamin D intake from food is required to maintain healthy vitamin D status from the beginning of the UVB-void period (end of October) to the end of the winter period (March). It was hypothesized that intake of 200 IU/d would result in serum 25(OH)D exceeding 40 nmol/L and that intakes reaching 400 IU/d would support serum concentrations of 75 nmol/L.

4.2 MATERIALS AND METHODS

Study Design

This was a 6 mo double blind randomized controlled trial in Montreal, Canada, following the CONSORT guidelines, with baseline assessments in October, 2014. Children were randomly allocated by family 1:1 and stratified by families with only young (2-4 y), only school age children (5-8 y), or families with both ages of children to double-blinded groups (**Figure 1**), using random numbers tables. Group codes were randomly assigned by a member of the research team with no direct contact with the participants. In addition to their regular food intake, the control group was instructed to consume 33 g/d of cheddar cheese or two 93 mL drinkable yogurts/d, neither with added vitamin D (expected vitamin D intake: 140-195 IU/d). To reach the 400 IU/d, the intervention group consumed the same yogurt and cheese products except with added vitamin D_3. The cheese contained 300 IU/33 g of vitamin D_3 and yogurt beverages contained 150 IU/93 mL. Children could consume the products at any time during the day so that the products would be incorporated into their normal eating habits. The products were provided pre-coded by the companies, with codes only disclosed after all data was analyzed. The vitamin

D content of each product was independently verified to be within ± 8% (Maxxam Analytique Inc., Saint Laurent, QC) for the yogurt and within ± 5% (O'Neal Scientific Services Inc., St. Louis, MO) for the cheese. Families were instructed to otherwise follow their normal lifestyle. Children were seen at baseline, 3 mo and 6 mo, where anthropometric measures were taken, fasting blood samples were obtained and surveys were completed on demographics, illnesses, sun exposure, physical activity and dietary intake. At baseline and 6 mo, body composition and bone geometry measures were taken.

Subjects

Recruitment was from August to October 2014 where children were recruited from daycare centers. Inclusion criteria were: 2 to 8 y of age, consumed milk products regularly, within ± 2 BMI Z-scores from 0 for sex and age based on World Health Organization (WHO) growth charts (174) or body fat percentage within normal ranges (230) and not taking supplements containing vitamin D. Exclusion criteria were chronic diseases or medications known to affect vitamin D, known anaemia, small size at birth or preterm birth <37 wk gestation.

Assessments

Blood Sampling, Vitamin D Status and Bone Biomarkers

Fasting venipuncture samples were taken between 0700 h – 1100 h to control for diurnal variation. Immediately, ionized calcium in whole blood (0.1 mL) was measured as a safety assessment using a blood gas unit (ABL80 FLEX, Radiometer Medical A/S, Copenhagen, Denmark), which had CV's of ≤ 5%. Two mL of whole blood was separated to obtain serum for measurement of 25(OH)D, PTH, osteocalcin, C-terminal telopeptide of type 1 collagen (CTX) and procollagen type 1 N-terminal propeptide (P1NP).

Samples were prepared for 25(OH)D assessment as previously described (231). Metabolites 25(OH)D_3, 24,25-dihydroxyvitamin D_3 (24,25$(OH)_2D_3$) and C3 epimer of 25-hydroxyvitamin D_3 (3-epi-25(OH)D_3) were quantified using ultra high performance liquid chromatography tandem mass spectrometry (Acquity UPLC with Xevo TQ-S mass spectrometer, Waters) at Queen's University, Canada (231). Lower limits of quantification for metabolites were 0.25-0.75 nmol/L. National Institute for Standards and Technology (NIST) 25(OH)D standards 972a level 1 and 4 had inter- and intra-assay CVs < 5% and accuracy ≥ 95%. Intact 1-84 PTH and osteocalcin were measured using chemiluminescent immunoassays on an auto-analyzer (Liaison, Diasorin). Sensitivity was 2.36 pg/mL for PTH and 3.0 ng/mL for osteocalcin. Controls for PTH and osteocalcin had inter- and intra-assay CV's of < 7% and accuracy ≥ 95%. CTX (45 µl) and P1NP (20 µl diluted 10x) were measured using chemiluminescent immunoassays at Shriners' Hospital for Children (Montreal, Canada) with an IDS-iSIS autoanalyzer. The CTX assay had sensitivity of 0.033 ng/mL (range: 0.033- 6.000 ng/mL) and P1NP a quantification limit <1.0 ng/mL (dynamic range: 2 to 230 ng/mL).

Dietary Assessment and Compliance

A validated 13-item semi-quantitative 30-day food frequency questionnaire (FFQ) was used to estimate vitamin D and calcium intake (1). A 24-h food intake assessment, documented the day prior to sampling, was used to assess macronutrient and energy intake. Though 3 days of 24-h intake assessments may be sufficient to measure energy and macronutrient intake, to represent usual intakes of calcium and vitamin D, a longer time period of assessment is needed (206). Thus, the 30-day FFQ was used to measure the intake of these micronutrients. Nutritionist Pro™ (Axxya Systems LLC, Stafford, TX, US) and the Canadian Nutrient File version 2010b were used to generate nutrient intakes. Parents used daily calendar check-sheets, collected and

verified every 3 mo, to record compliance. Compliance data was lacking for only 1 child who finished the trial.

Demographics, Physical Activity, Skin Pigmentation and UVB

At baseline, self-reported socio-demographic variables were surveyed. At all visits, data was collected on parent-reported physical activity for weekends and weekdays using the validated Habitual Activity Estimation Scale (HAES) questionnaire (232), sun exposure during the previous 30 days, frequency of sunscreen use and hours spent in direct sunlight/d based on the Canadian Health Measures Survey (233). Skin type was determined using a spectrophotometer to measure individual typological angel (ITA) (CM-700d/600d, Konica Minolta, Ramsey, NJ) and UVB exposure was qualitatively determined as previously described (234).

Anthropometry

Methods for measuring height, weight and BMI Z-scores were previously described (11). The International Society for Clinical Densitometry (ISCD) states that whole body and lumbar spine are the preferred dual-energy x-ray absorptiometry (DXA) measurement sites (235). In addition, forearm scans were performed since the forearm is the most common fracture site in children (236). For bone outcomes, DXA measures of whole body, lumbar vertebrae 1-4 (anterior-posterior) and distal forearm (non-dominant) were measured. Ultra-distal forearm data was reported as this region measures new bone close to the growth plate. Whole body scans were used to assess lean mass. All scans were performed using a Hologic 4500A (APEX software, v13.3:3, Bedford, MA) fan-beam clinical densitometer at baseline and 6 mo. Daily quality control measurements were obtained using a lumbar spine bone phantom (Hologic) with accuracy of ± 1.5% of the mean and CV's of 0.396%, 0.524%, 0.363% for BMD, bone mineral

content (BMC) and area, respectively. As per manufacturer recommendations, global standard deviations for the low air and high air measures of radiographic uniformity were always below 2.0.

Peripheral quantitative computed tomography (pQCT: XCT-2000; Stratec, Pforzheim, Germany) scans of the non-dominant tibia were performed by x-ray technicians at 6 mo to evaluate 3-D bone geometry and muscle parameters. There are no standard pQCT methods for children (237), so our methods were based on those used when producing normative data in children (200). Length of the tibia was measured between the superior margin of the medial condyle and the medial malleolus. A scout scan was performed to visualize the distal growth plate and the reference line placed at the most proximal line of the growth plate. The 4% and 66% sites, measured proximally from the distal end of the tibia, were each scanned with a single 2 mm slice, a voxel size of 0.4 mm^2 and speed of 30 mm/second.

Ethics

This study was approved by the McGill University Faculty of Medicine Research Ethics Board in accordance with the Tri-Council policy on ethics and the Declaration of Helsinki (213) and was registered at clinicaltrials.gov (NCT02387892). Temporary Marketing Authorization letters were obtained from Health Canada for the trial products (TM-14-0112 and TM-14-0113).

Statistical analyses

We aimed to recruit 25 children/group based on the 25(OH)D_3 power calculations with an expected group difference of 20 nmol/L and a SD of 16 nmol/L and accounting for a 5-10% drop out rate (11). Intent-to-treat analyses were conducted using SAS (version 9.3, SAS Inst. Cary, NC). All data entry was double audited and tested for normality using the Kolmogorov-Smirnov test and homogeneity of variance using the Bartlett test. A p-value of < 0.05 was categorized as

significant, after adjustment for multiple comparisons where applicable. A mixed model ANOVA was used to analyse continuous data accounting for fixed effects (group, sex and age) and random effects (e.g. within family, demographics, body composition) with *post-hoc* testing where necessary using Bonferroni correction. Non-normal data were log transformed where applicable (e.g., $25(OH)D_3$). There was less than a 5% dropout during the study period, so data imputation approaches were not sought. Fisher exact testing was used for differences in proportions. Log-log regression analysis was used to normalize fat mass and lean mass indices (238) since fat mass and lean mass have different relationships with height in children than weight does.

4.3 RESULTS

Demographics

No differences in baseline characteristics (**Table 4.1**) were observed among allocation groups. Physical activity was not different between groups, using HAES questionnaire activity categories, where 91% and 82% of control and intervention groups were very active for ≥ 60 minutes. Forty-nine (96.1%) children completed the study (**Figure 4.1**) over 25.3 ± 0.6 weeks. There were no differences in height, weight, height velocity or weight velocity between groups (Supplementary Table 1).

Dietary Characteristics and Sun Exposure

Median (IQR) compliance for the study yogurt and cheese was 96% (89-99) and 84% (71-97) with no differences between groups. Mean milk and alternatives intake (baseline: 2.8 ± 0.7, 3 mo: 2.7 ± 1.0, 6 mo: 3.2 ± 1.4 servings/day) exceeded Canada's Food Guide recommendations (2 servings/d). At baseline, vitamin D intakes were not different between groups and no children had intakes ≥ 400 IU/d (**Table 4.2**). The vitamin D intake of the control

group did not change throughout the study, whereas at 3 and 6 mo, the intervention group significantly differed from control (**Table 4.2**). Calcium intake was not different between groups at any time-point (**Table 4.2**). Baseline median (IQR) energy intake (control: 1670 (1416-2020) Kcal/d, intervention: 1420 (1302-1682) Kcal/d) and protein intake (control: 77 (66-84) g/d, intervention: 69 (45-76) g/d) did not differ between groups or change among time-points.

Sixty-seven percent (34/51) of the children had Fitzpatrick skin types I, II or III and 33% (17/51) had skin types IV, V or VI. Six percent (3/51) of children travelled to southern latitudes during the study period and none of them presented with significant tanning of skin as measured by changes in ITA at the forearm, forehead and lower leg (average of 3 sites Δ ITA: 5.6 ± 4.6^{o}).

Biochemical Assessments

At baseline, serum $25(OH)D_3$ ranged between 40-125 nmol/L (**Table 4.1**), with mean concentrations of 66.4 ± 13.6 nmol/L overall. Regarding our primary outcome, the control group had a decrease in $25(OH)D_3$ ($p = 0.001$) from 0-3 mo, whilst the intervention group decreased ($p = 0.001$) from 3-6 mo (**Figure 4.2A**). This resulted in serum $25(OH)D_3$ being lower in the control group than the intervention group at 3 mo ($p = 0.001$), but not 6 mo. Compliance did not significantly influence serum $25(OH)D_3$ and baseline $25(OH)D_3$ was not predictive of the 6 mo change in $25(OH)D_3$. No child at any time point had $25(OH)D_3$ concentrations over 125 nmol/L and only 1 child (4.3%), in the control group, had $25(OH)D_3 < 40$ nmol/L (39.4 nmol/L) at 6 mo. The proportion of children with $25(OH)D_3 \geq 75$ nmol/L was not different between groups at any time (baseline: control: 7/25 (28%), intervention: 6/26 (23%), 3 mo: control: 4/24 (16%), intervention: 6/26 (23%), 6 mo: control: 1/23 (4%), intervention: 2/26 (8%)), but significantly decreased only in the control group over 6 mo ($p=0.049$). Similarly, the proportion of children with $25(OH)D_3 < 40$ nmol/L or ≥ 50 nmol/L was not different between groups at any time, but

significantly decreased in the control group at 3 mo (**Table 4.2**). Serum $24,25(OH)_2D_3$ decreased (-1.0 ± 1.3, p = 0.001), over time (**Figure 4.2B**). The $25(OH)D_3$:$24,25(OH)_2D_3$ ratio (range of ratio: 10.1-25.0) and 3-epi-$25(OH)D_3$ did not change over time or differ between groups (**Figure 4.2A**).

Average ionized calcium (1.30 ± 0.04 mmol/L) was within normal limits (1.15-1.38 mmol/L) and did not vary by time or treatment group. PTH, P1NP, osteocalcin and CTX concentrations did not differ over time or between groups (**Figure 4.2C, D, E, F**). Concentrations of biomarkers did not vary based on the time of blood draw (0700 to 1100 h).

Body Composition Assessments

Fat mass index (control: 4.85 ± 1.31 $kg/m^{1.9}$, intervention: 4.56 ± 1.43 $kg/m^{1.9}$) as well as fat mass of the whole body, appendicular regions as a total and legs alone (**Table 4.3**) were not different between groups or over time. Lean mass index (control: 11.29 ± 1.07 $kg/m^{2.5}$, intervention: 10.85 ± 0.76 $kg/m^{2.5}$) did not differ between groups or over time, however, the percent increase in whole body, appendicular and legs alone lean mass was significantly greater over 6 mo in the intervention group (**Table 4.3**). Also, the 6 mo absolute change in lean mass for the whole body (control: 0.31 ± 0.67 kg intervention: 0.98 ± 0.59 kg, p = 0.038) and the appendicular skeleton (control: 0.35 ± 0.71 kg intervention: 0.67 ± 0.58 kg, p = 0.034) were significantly greater in the intervention group. The absolute change in lean mass of the legs was not different between groups (control: 0.30 ± 0.40 kg intervention: 0.51 ± 0.23 kg, p = 0.10). Lean mass outcomes were not related to physical activity. Lower leg muscle density and cross sectional area were not different between groups at 6 mo (**Table 4.4**).

Bone Assessments

BMD Z-scores (whole body Z-score: control: 1.46 ± 1.36, intervention: 0.90 ± 0.91, lumbar spine Z-score: control: 0.54 ± 0.90, intervention: 0.18 ± 0.85) were not different between groups and did not change over the 6 mo. Radius (33%) BMD Z-Scores (range: -1.2 to 1.9) were not available for all ages. The groups did not significantly differ in BMC accretion rates for whole body, lumbar spine and ultra-distal forearm over time (**Table 4.3**). All of the pQCT derived bone outcomes (**Table 4.4**) were not different between groups.

4.4 DISCUSSION

The IOM recommendations for vitamin D were set based on evidence from adolescents and adults (8), highlighting the need for randomized controlled trials in young children. Addressing this gap, the present study suggests that 400 IU/d of vitamin D maintains serum 25(OH)D ≥ 40 nmol/L in 100%, ≥ 50 nmol/L in 85% and ≥ 75 nmol/L in 8% of young children during UVB void periods in Canada. Despite interim benefits at 3 mo, the intervention did not maintain vitamin D status over 6 mo compared to the control group. It is possible that had we used greater amounts of vitamin D that the intervention group would have had higher status; however, that was not suitable for a fortified food-based trial as such foods would not realistically be approved for the Canadian food market (14). Within the secondary outcomes, greater lean mass accretion was observed in the intervention group and implies that meeting 400 IU/d for vitamin D may be beneficial for physical development beyond bone health. Lean mass may be an important consideration when setting future vitamin D recommendations.

The intervention in this study was designed to achieve dietary intakes of 400 IU/d of vitamin D, consistent with the EAR and below the RDA (600 IU/d). Though the intervention failed to support the hypothesized 25(OH)D_3 (75 nmol/L), it did maintain 25(OH)D_3 above 40

nmol/L in all participants with 85 to 88% of values ≥ 50 nmol/L, which is more similar to that anticipated when achieving the RDA. The baseline serum $25(OH)D_3$ of ~70 nmol/L in our study is similar to national surveillance data (3-5 y: 74 nmol/L; 6-11 y: 67 nmol/L) (6). In contrast, trials of children 4-8 y in Denmark (40) and 8-14 y in Pittsburgh (41) suggest that 780 IU/d and 1500 IU/d respectively are needed for 97.5% of children in winter to maintain 25(OH)D ≥ 50 nmol/L. Both trials had lower baseline 25(OH)D (Danish children: 56.7 ± 12.3 nmol/L, Pittsburgh children: 50 ± 7.7 nmol/L), meaning that almost half of the children had to first increase their 25(OH)D concentration to 50 nmol/L before maintaining it. Though designed to influence national policy, these studies did not reflect national data (US NHANES 2001-2006, children <11 y: geometric means 64-69 nmol/L (228), Danish children 4-17 y: fall 25(OH)D: 72.8 nmol/L, IQR 64.0-88.9 nmol/L) (23). In addition, the higher intakes needed among Pittsburgh children may be due to overweight and obese status since median BMI (20.6, IQR 17.8-23.3) was at the 80-85th percentile (174) whilst Danish and Montreal children had average BMI Z-scores of 0.08 ± 0.83 kg/m^2 and 0.67 ± 0.94 kg/m^2 respectively. These points highlight that extrapolation of data from studies where baseline 25(OH)D or BMI are not reflective of a general population needs careful consideration before applying it to dietary recommendations.

Multiple pediatric trials have shown that adiposity negatively associates with serum 25(OH)D (143, 239, 240). Vitamin D can be absorbed into fat cells instead of being quickly hydroxylated to 25(OH)D since serum 25(OH)D > 15 nmol/L associates with saturation of liver 25-hydroxylase (CYP2R1) (133). Fat mass did not differ between groups in our study and thus sequestration of vitamin D (241) may not have been a factor behind the serum $25(OH)D_3$ concentrations observed at 3 or 6 mo. However, since $25(OH)D_3$ declined over the study and fat mass was relatively stable, it is possible that adipose stores of vitamin D were not sufficient or

that needs for tissue expansion were increased, both of which are consistent with a significant decline in serum $24,25(OH)_2D_3$ after 3 mo and greater lean mass accretion in the intervention group. It has been shown that increased vitamin D intake could be taken-up by multiple other body tissues (242), though there is little known about vitamin D tissue distribution in children.

Early work on 25(OH)D tissue distribution (243, 244) demonstrated 25-66% was found in muscle tissue. A 2009 review (133) stated that in an average adult female, ~ 20% of 25(OH)D is in muscle tissue (133), suggesting that muscle consumes a significant proportion of 25(OH)D. Calcitriol is implicated in regulating the expression of transcription factors within the myocyte, important for muscle development (176) as well as increasing serum concentrations of insulin-like growth factor binding protein 3 (IGFBP3) (245) and activation of calmodulin-dependant kinases (177). Increased IGFBP3 has been suggested to increase the half-life of insulin-like growth factor 1 resulting in an increased concentration in circulation and an increase in downstream protein synthesis (245). Calmodulin-dependant kinases were shown to enhance vitamin D receptor-mediated transcription activity and thus may have a synergistic effect with vitamin D on vitamin D receptor-mediated transcription (177). In our trial, the biggest % difference in lean mass accretion between groups was in the legs, similar to results in postmenopausal women (182). With physical activity in our trial not relating to lean mass, these results suggest that vitamin D interventions may have a larger effect on fast twitch muscle fibres (246). Our results also agree with previous trials in Chinese girls (15 y) (38) and pre-pubertal Lebanese girls (10-13 y) (39) that showed significant associations between lean mass and vitamin D intake. In the trial in Lebanese girls, when compared with the control group, supplementing with 200 IU/d of vitamin D significantly increased lean mass accretion but not vitamin D status, suggesting that differences in vitamin D intake and not status led to changes in

lean mass (39). However, a recent study (37), showed that if serum 25(OH)D was ≥ 75 nmol/L, vitamin D intakes > 400 IU/d did not improve accretion. This underscores the importance of using the serum 25(OH)D concentration measurement when examining the interrelationships with lean mass accretion.

The serum 25(OH)D concentration recommended by the IOM for bone health is 50-125 nmol/L (8). All DXA-based BMD Z-scores were within a healthy range, agreeing with a 2012 meta-analysis (32) showing that vitamin D supplementation in children with serum 25(OH)D > 35 nmol/L did not affect hip or lumbar spine BMD. Interestingly, a 1 y trial in Finnish girls (n=228, mean age: 11.4 ± 0.4) showed that vitamin D supplementation elevated total hip BMD without significantly affecting serum 25(OH)D (baseline: 46.3 ± 17.4 nmol/L) (247). This highlights that associations between BMD and vitamin D status need careful examination. With no differences between groups for bone accretion or density outcomes, it is not surprising that in our study we did not find between group differences for bone biomarkers. Importantly though, bone health biomarkers can rapidly respond to changes in nutritional status, as P1NP was shown to significantly increase over a time frame as short as 4 weeks due to zinc supplementation (248). Thus, if we would have seen significant between-group differences in bone accretion in our 6 mo trial, we would have expected significant increases in markers of bone formation.

Significant strengths of our study include a high compliance rate, and a 6 mo UVB void period with study visits every 3 mo, giving us the ability to track seasonal changes in serum 25(OH)D. By assessing other vitamin D metabolites, we could see whether vitamin D intake related to the conversion of $25(OH)D_3$ to $24,25(OH)_2D_3$. A limitation of our trial was that few children started with $25(OH)D_3$ ≥75 nmol/L, meaning we were not able to test if this status could be maintained or affected functional outcomes. Also, by focusing on the population target for

vitamin D intake (400 IU/d) (8), we were not able to test if higher amounts would better support serum 25(OH)D_3 concentration or other functional outcomes. It is possible that either a longer time-frame would be needed to investigate bone mineral accretion or that a greater sample size would be required to investigate this outcome, especially given the healthy vitamin D status throughout the study. However, as stated in our methods, DXA has an accuracy of ± 1.5% and CV's for BMD and BMC being < 0.55%. Thus, the 5-10% changes of BMC (shown in table 3) and over 6 mo, are large enough to be outside the range of instrumental error, meaning that 6 mo was long enough to measure change. Since pQCT scans were only performed at 6 mo, we were not able to look at changes in bone or muscle outcomes measured by pQCT. Also, with scans at 4% and 66% sites of the lower leg, we were not able to assess bone outcomes at predominantly cortical sites. Lastly, we did not look at whether muscle strength of the children was impacted by vitamin D intake as has been shown previously (38, 249).

In conclusion, 96% of all children maintained 25(OH)D_3 ≥ 40 nmol/L and 70% of the control group (~ 200 IU/d of vitamin D) maintained 25(OH)D_3 ≥ 50 nmol/L (set to align with bone health) whereas 85% of the intervention group (~ 400 IU/d of vitamin D) achieved this healthy target. By 6 mo, only 2 children achieved the 75 nmol/L target in the intervention group because serum 25(OH)D_3 concentrations in both groups declined over the study, illustrating that vitamin D stores are utilized during the UVB void period, but not depleted. The increased vitamin D intake of the intervention group did not lead to improved bone health outcomes, but may explain the higher lean mass accretion in the intervention group compared to the control group. These results show a need for future vitamin D food fortification trials to examine the relationships among vitamin D intake, serum 25(OH)D and lean mass outcomes in children. Such trials will further inform dietary recommendations and food fortification policies.

Table 4.1 Baseline characteristics of participants

Parameter	Control	Intervention	p-value[3]
n	25	26	
Age (y)	5.4 ± 2.0 Range: 1.9-8.6	5.0 ± 1.8 Range: 2.0-8.4	0.752
Male	15 (56%)	12 (50%)	0.300
Ethnicity, White[1]	13 (52%)	18 (69%)	0.264
Maternal Education Achieved, College or Higher	20 (80%)	18 (69%)	0.172
Family Income:			
> $65,000[2]	18 (72%)	12 (50%)	0.065
Not Disclosed	0	1 (4%)	
Weight Z-Score	0.75 ± 0.87	0.64 ± 1.09	0.874
Height Z-Score	0.31 ± 0.90	0.44 ± 1.10	0.413
BMI Z-Score	0.81 ± 0.88	0.55 ± 0.98	0.275
Serum 25(OH)D_3			
< 30 nmol/L	0	0	
30-39.9 nmol/L	0	0	
40-49.9 nmol/L	2 (8%)	2 (8%)	0.313
50-124.9 nmol/L	23 (92%)	24 (92%)	
≥ 125 nmol/L	0	0	

Data are unadjusted mean ± SD or n (%).
[1] Non-white= Hispanic, Black, Asian
[2] Canadian Dollars.
[3] Testing for differences between groups, using a mixed model ANOVA or Fisher's exact test.

Table 4.2. Intakes of calcium (mg/d), vitamin D (IU/d), energy (kcal/d) and serum 25(OH)D_3 concentration (nmol/L) across the 6 month study

	Time-point	Control[3]	Intervention[4]
Calcium[1]	Baseline	909 ± 223	946 ± 351
	% meeting EAR	64%^{ab}	73%^{ab}
	3 mo	842 ± 262	869 ± 363
	% meeting EAR	48%^{a}	62%^{ab}
	6 mo	1066 ± 360	1034 ± 391
	% meeting EAR	87%^{b}	77%^{b}
Vitamin D[1]	Baseline	202 ± 76^{a}	248 ± 73^{a}
	% meeting 400 IU/d	0%^{a}	0%^{a}
	3 mo	239 ± 117^{a}	466 ± 95^{b}
	% meeting 400 IU/d	12%^{a}	81%^{b}
	6 mo	241 ± 124^{a}	486 ± 90^{b}
	% meeting 400 IU/d	12%^{a}	81%^{b}
25(OH)D_3	Baseline	67.5 ± 15.1^{a}	65.3 ± 12.2^{a}
	% meeting 50 nmol/L	92%^{a}	92%^{a}
	3 mo	58.3 ± 15.3^{b}	64.7 ± 12.2^{a}
	% meeting 50 nmol/L	67%^{b}	88%^{ab}
	6 mo	56.6 ± 13.9^{b}	58.4 ± 8.7^{b}
	% meeting 50 nmol/L	70%^{ab}	85%^{ab}
Energy[2]	Baseline	1670 (1416-2020) ^{ab}	1420 (1302-1682) ^{ab}
	3 mo	1527 (1273-1821) ^{ab}	1540 (1345-1799) ^{ab}
	6 mo	1583 (1160-1878) ^{ab}	1503 (1362-1647) ^{ab}

Data are unadjusted mean ± SD or median (IQR).
[1] From a validated 30-day food frequency questionnaire. [2] From 24 h intake assessments.
[3] Control group: baseline: n=25, 3 mo n=23, 6 mo n=23, [4] Intervention group: baseline: n=26, 3 mo n=26, 6 mo n=26.

[a,b] Different superscripts denote significant differences between groups and over time (p = 0.001-0.042). Fisher's exact test was used to test for between group differences in the proportion meeting recommendations. A mixed model ANOVA with Bonferroni correction, adjusted for age, sex, ethnicity, BMI Z-score, family cluster, length of study and baseline serum $25(OH)D_3$ was used to test for between group differences in continuous variables.

Table 4.3 Parameters of bone and body composition from DXA scans in children 2 to 8 y across the 6 month study period

	Control			Intervention		
	Baseline	6 mo	% Change	Baseline	6 mo	% Change
Whole Body BMD (g/cm^2)	0.696 ± 0.093	0.711 ± 0.093	2.7 (1.9-3.5)	0.691 ± 0.118	0.704 ± 0.119	2.9 (1.9-3.9)
Whole Body BMC (g)	805.78 ± 202.83	865.73 ± 223.90	6.6 (5.4-7.8)	731.13 ± 185.93	769.03 ± 198.55	5.3 (3.6-6.6)
Lumbar Spine BMD (g/cm^2)	0.519 ± 0.072	0.523 ± 0.076	1.7 (-0.8-4.2)	0.502 ± 0.073	0.513 ± 0.073	3.9 (2.3-5.5)
Lumbar Spine BMC (g)	16.68 ± 4.48	18.40 ± 5.29	9.4 (-0.5-15.5)	15.18 ± 4.18	16.11 ± 3.79	7.9 (4.3-12.1)
U-D Forearm BMD (g/cm^2)[1]	0.267 ± 0.06	0.288 ± 0.07	7.4 (1.2-13.6)	0.238 ± 0.04	0.245 ± 0.03	3.0 (1.3-4.9)
U-D Forearm BMC (g)[1]	0.93 ± 0.3	1.03 ± 0.3	10.1 (1.3-18.9)	0.77 ± 0.2	0.82 ± 0.2	6.1 (1.6-12.0)
Whole Body Lean Mass (kg)	15.33 ± 4.89	15.64 ± 4.89	5.9 (4.4-7.4)[a]	14.18 ± 4.11	15.16 ± 4.25	8.4 (6.3-10.5)[b]
Appendicular Lean Mass (kg)	6.76 ± 2.96	7.11 ± 2.75	7.7 (5.1-11.3)[a]	5.92 ± 2.26	6.59 ± 2.33	13.3 (8.9-17.1)[b]
Legs Lean Mass (kg)	4.56 ± 1.96	4.86 ± 2.05	8.9 (4.1-12.7)[a]	3.86 ± 1.65	4.37 ± 1.72	15.7 (11.7-19.7)[b]
Whole Body Fat Mass (kg)	6.10 ± 2.37	6.23 ± 2.56	6.0 (1.4-10.6)	5.34 ± 2.10	5.48 ± 2.20	2.6 (-1.0-6.2)
Appendicular Fat Mass (kg)	3.44 ± 1.46	3.75 ± 1.76	4.5 (-1.4-10.4)	2.81 ± 1.09	2.89 ± 1.19	-0.5 (-10.4-9.7)
Legs Fat Mass (kg)	2.70 ± 1.10	2.79 ± 1.20	5.0 (-1.3-11.3)	2.34 ± 0.86	2.35 ± 0.95	-0.6 (-6.6-7.3)

Data are unadjusted mean ± SD or mean (95%CI) (Control: baseline: n=24, 6 mo: n= 22 (1 child without scan at each time-point due to lack of co-operation), Intervention: baseline: n=26, 6 mo: n=25 1 child without scan at 6 mo due to lack of co-operation).

[1] U-D: Ultra-distal non-dominant forearm.

[a,b] Denotes significant differences between groups for % change in lean mass (total p= 0.038, appendicular p= 0.045, legs p= 0.025), using a mixed model ANOVA adjusted for age, sex, ethnicity, height velocity, family cluster, length of study and baseline serum 25(OH)D.

Table 4.4 Bone and muscle parameters from peripheral quantitative computed tomography scans of the lower leg in children 2-8 y after 6 months of study

	Control	Intervention
Tibia Geometry		
4% Trabecular Density (mg/cm^3)	179.3 ± 32.6	187.1 ± 25.2
4% Trabecular CSA (mm^2)[1]	189.5 ± 35.0	179.8 ± 50.2
66% Cortical Density (mg/cm^3)	986.8 ± 68.1	994.0 ± 47.3
66% Cortical CSA (mm^2)[1]	114.7 ± 39.4	116.5 ± 42.5
66% Cortical Thickness (mm)	0.47 ± 0.23	0.41 ± 0.13
Muscle Parameters		
66% Muscle Density (mg/cm^3)	56.4 ± 15.9	51.6 ± 11.1
66% Muscle CSA (mm^2)[1]	2696.1 ± 758.8	2465.0 ± 550.0

Data are unadjusted mean ± SD. No differences between groups ($p > 0.050$) for outcomes, using a mixed model ANOVA, adjusted for age, sex, ethnicity and family cluster. Control: n=22, Intervention: n=25 (1 child without scans for each group due to lack of co-operation).
[1] CSA: Cross sectional area.

Supplementary Table 4.1. Anthropometric measurements and growth of children over the 6 mo study

	Group	Height (cm)	Height velocity (cm/mo)	Weight (kg)	Weight velocity (kg/mo)
Baseline	Control	112.5 ± 15.0	N/A	22.1 ± 7.3	N/A
	Intervention	111.0 ± 13.5	N/A	20.3 ± 5.1	N/A
6 mo	Control	115.2 ± 14.4	0.5 ± 0.2	22.9 ± 7.2	0.3 ± 0.1
	Intervention	114.2 ± 14.2	0.5 ± 0.1	21.8 ± 6.1	0.2 ± 0.2

Data are mean ± SD. No significant differences between groups, using a mixed model ANOVA, adjusted for age, sex, ethnicity, family cluster and length of study.

Figure 4.1 CONSORT diagram

Figure 4.2 Serum $25(OH)D_3$ and 3-epi-$25(OH)D_3$ (panel A), $24,25(OH)_2D_3$ (panel B), parathyroid hormone (PTH) (panel C), procollagen type 1 N-terminal propeptide (P1NP) (panel D) osteocalcin (panel E) and C-terminal telopeptide of type 1 collagen (CTX) (panel F) concentrations at baseline, 3 and 6 months (mean ± SD); * Denotes significant differences between groups (p=0.001) at that time point. For $25(OH)D_3$ and $24,25(OH)_2D_3$, both groups significantly decreased (p=0.001) from baseline to 6 mo using a mixed model ANOVA adjusted for age, sex, ethnicity, BMI Z-score, family cluster, length of study and baseline serum $25(OH)D_3$.

Figure 4.1

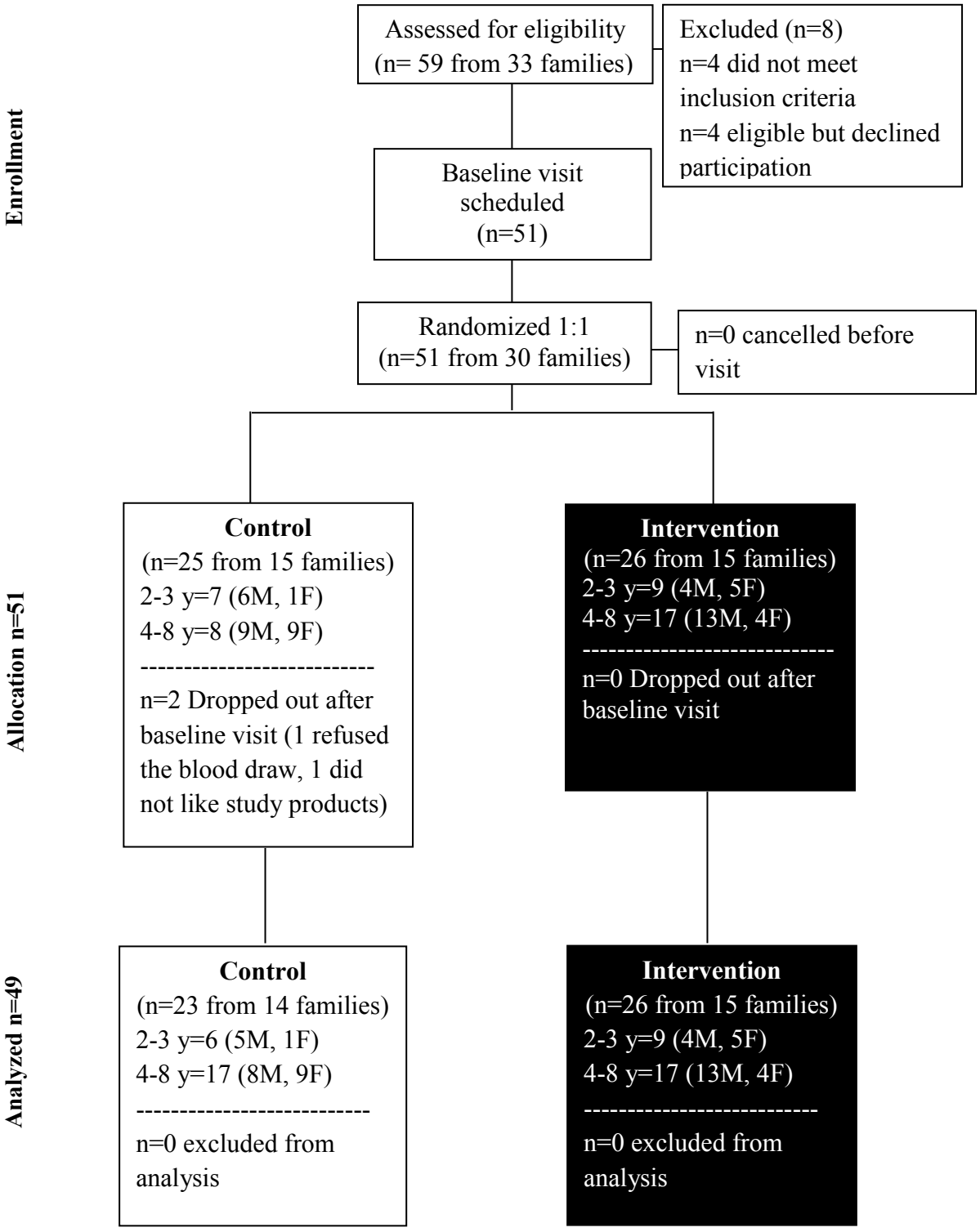
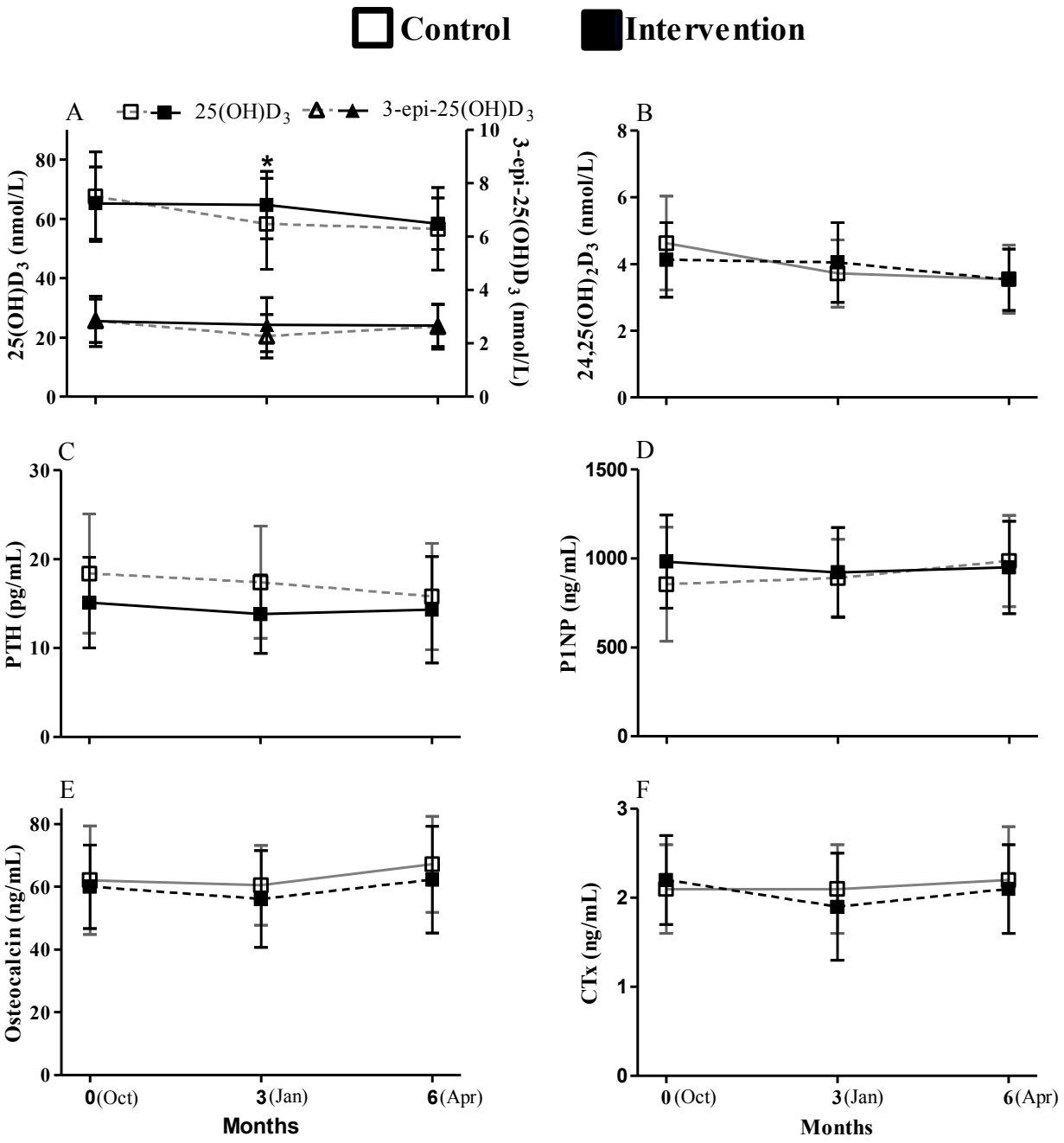

Enrollment
Assessed for eligibility
(n= 59 from 33 families)
Excluded (n=8)
n=4 did not meet inclusion criteria
n=4 eligible but declined participation
Baseline visit scheduled
(n=51)
Randomized 1:1
(n=51 from 30 families)
n=0 cancelled before visit
Allocation n=51
Control
(n=25 from 15 families)
2-3 y=7 (6M, 1F)
4-8 y=8 (9M, 9F)
n=2 Dropped out after baseline visit (1 refused the blood draw, 1 did not like study products)
Intervention
(n=26 from 15 families)
2-3 y=9 (4M, 5F)
4-8 y=17 (13M, 4F)
n=0 Dropped out after baseline visit
Analyzed n=49
Control
(n=23 from 14 families)
2-3 y=6 (5M, 1F)
4-8 y=17 (8M, 9F)
n=0 excluded from analysis
Intervention
(n=26 from 15 families)
2-3 y=9 (4M, 5F)
4-8 y=17 (13M, 4F)
n=0 excluded from analysis

Figure 4.2


Control
Intervention
A
25(OH)D3
3-epi-25(OH)D3
25(OH)D3 (nmol/L)
3-epi-25(OH)D3 (nmol/L)
*
B
24,25(OH)2D3 (nmol/L)
C
PTH (pg/mL)
D
P1NP (ng/mL)
E
Osteocalcin (ng/mL)
F
CTx (ng/mL)
0 (Oct)
3 (Jan)
6 (Apr)
Months

BRIDGE 3.

The results of the 6 mo trial in Chapter 4 demonstrated that an intervention of 400 IU/d of vitamin D maintained 25(OH)D for 3 mo, but not the entire winter. There were no differences between groups for bone health outcomes. Though lean mass accretion was significantly greater in the intervention group, by the end of winter, 25(OH)D concentration did not differ between the intervention and control groups.

Studies in Denmark (40) and Boston, USA (41) recently investigated the dose of vitamin D needed for 97.5% of children age 4-10 y to have 25(OH)D concentrations of ≥ 50 nmol/L by the end of winter. Their results showed 800 IU/d and 1500 IU/d, respectively were needed to reach and maintain this vitamin D status, whilst in contrast to this, the 12 wk trial in chapter 3 found 400 IU/d was needed to maintain 25(OH)D concentrations of ≥ 50 nmol/L. Therefore, further clarification of the relationship between vitamin D intake and status was needed. Adding to this, there has not been a meta-analysis looking at how vitamin D intake relates to status in children. It was timely to undertake a meta-analysis due to the relatively recent increase in vitamin D research, with the same number of trials in children over the last 5 years as published in all previous years. Thus, chapter 5 is a meta-analysis exploring how vitamin D interventions in randomized controlled trials effects vitamin D status in children. A 2012 meta-analysis in adults was done with a similar objective, however they only included food fortification trials (218). By including trials using methods of food fortification, supplementation and bolus injection, as well as across the age range of 2-18 y, it was possible to investigate how intake method and age along with other factors including location and baseline 25(OH)D concentration affect the relationship of vitamin D intake and status.

CHAPTER 5: MANUSCRIPT 3

The effect of vitamin D supplementation, food fortification or bolus injection on vitamin D status in children 2-18 years: a meta-analysis

Neil R. Brett, Nathalie Gharibeh, Hope A. Weiler.

School of Human Nutrition, McGill University, Ste Anne de Bellevue, QC.

Abstract

Background: Meta-analyses on the effect of vitamin D intake on vitamin D status specifically in children are lacking.

Objective: This meta-analysis investigated the effect of vitamin D interventions on vitamin D status, in children 2-18 years of age.

Design: Following PRISMA guidelines, literature searches were conducted up to December 2016. Randomized placebo-controlled vitamin D interventions in healthy children 2-18 y were included. A random-effects model was used with I2 to assess heterogeneity.

Results: We included 26 trials (5146 children) with interventions (n=9 fortified foods, n=15 supplements, n=2 bolus injections) from 100-4000 IU/d of vitamin D over 4 wk to 2 y. The 25(OH)D weighted mean difference (23.5 nmol/L (95% CI 20.7-26.3 nmol/L) I2=99.9%) resulted in a 1.0 nmol/L increase/100 IU/d of vitamin D. The 25(OH)D increase/100 IU/d of vitamin D was greater in trials with mean baseline 25(OH)D <30 nmol/L, using fortified foods and with baseline vitamin D intakes < 100 IU/d.

Conclusion: The 25(OH)D response to vitamin D intake appears to differ based on baseline status and intakes and delivery mode.

5.1 INTRODUCTION

Vitamin D is an essential nutrient for musculoskeletal growth and development in children (43). When living at ≥ 40° latitude, vitamin D cannot be synthesized year-round from exposing skin to sunlight (9), and thus, must be obtained from the diet. The harmonized Canadian and US vitamin D recommendations from the Institute of Medicine (IOM) include an Estimated Average Requirement (EAR, 400 IU/d) and a Recommended Dietary Allowance (RDA, 600 IU/d) for children 1-18 y (215) set to align with serum 25-hydroxyvitamin D (25(OH)D) concentrations of 40 nmol/L and 50 nmol/L, respectively. However, since these recommendations were only based on research in adults and teenagers, there is a need for vitamin D research spanning the full range of childhood. Recent results in the literature are also highly varied (400-1500 IU/d) regarding the amount of vitamin D needed for children to regarding the amount of vitamin D needed to reach the definition of the RDA (97.5% of children maintaining a status of ≥50 nmol/L throughout winter) (40, 41, 234, 250). Thus, a meta-analysis dedicated to exploring vitamin D dose-response patterns in children is needed and timely.

A 2012 meta-analysis of food fortification trials in adults showed a significant effect of vitamin D fortification (251) and a linear dose response of 3 nmol/L per 100 IU of vitamin D intake. Having baseline 25(OH)D <50 nmol/L compared to ≥ 50 nmol/L, and study location being ≥ 40° N latitude resulted in significantly greater dose responses to vitamin D intake. However, since this meta-analysis did not test for a non-linear relationship between vitamin D intake and status it is unclear if the effect of vitamin D intake on status is linear (41, 251), or non-linear as suggested recently in trials of healthy adults (252) and children (40, 250). It is thought however, that children with deficient (<30 nmol/L) baseline 25(OH)D concentrations may have a greater dose response to vitamin D intake than those with 25(OH)D ≥ 30 nmol/L

(222). Since Canada and the US (15, 253), the UK and some countries in Europe (254, 255) fortify some foods with vitamin D, it is important to explore if the vitamin D dose response in children is greater from food fortification versus supplementation and bolus injection. Thus, the primary objective of this meta-analysis was to investigate the mean effect of vitamin D interventions on vitamin D status, in children 2-18 years of age. The secondary objectives were to explore if the effect of vitamin D intake on status is linear and if factors including age, sex, latitude, baseline vitamin D status, measurement method and administration method have a significant role in the relationship between exogenous intakes and status.

5.2 METHODS

Data Searches and Study Selection

Using PRISMA guidelines (256), literature searches without language restrictions, were performed in Ovid MEDLINE, PubMed, Embase, and Cochrane Central Register of Controlled Trials were conducted through December 2016 (**Supplemental Table 5.1**). The search strategy was developed in MEDLINE and was adapted, as appropriate, for use in the other databases. Eligible studies were randomized controlled trials (RCTs), in children 2-18 y that compared either vitamin D supplements, bolus injections or fortified foods with placebo supplements, injections or unfortified foods and reported the 25(OH)D concentrations in all study groups. This age range was selected as it aligns with current dietary guidelines in Canada and the US (10, 11). Vitamin D status did not need to be the primary outcome for studies to be included. Though we were only including studies in healthy children, study populations with vitamin D deficiency were included if children were otherwise healthy. Studies were excluded if populations were predominantly obese, had chronic diseases or disease states influencing vitamin D metabolism. Corresponding authors were contacted for additional information if needed. If mean (SD)

vitamin D status was not able to be obtained, studies were excluded as analyses would not be possible

Two authors independently initially screened search results by titles, keywords and abstracts. Potentially relevant records were then screened independently with the use of the full text reports. Discrepancies were resolved through consensus of the reviewers.

Data Synthesis and Quality Assessment

Vitamin D intakes were converted to and reported as daily intakes in IU/d (μg x 40= IU). Vitamin D status was reported as serum 25(OH)D concentration in nmol/L (ng/mL x 2.497= nmol/L). An absolute change in 25(OH)D concentration was calculated from baseline and endpoint data when necessary. The SE of the absolute change and the conversion of 95% CI to SD were calculated as previously described (251). If a study had multiple intervention groups, the results of the intervention groups were combined and averaged, as per PRISMA guidelines (256). The Cochrane qualitative bias tool was used to qualitatively assess bias (257) of included trials and the Jadad 5-point scale was used to assess evidence strength with a score of 5 denoting trials with the lowest risk of bias (258).

Data Analysis

Using linear regression analysis, the effect of vitamin D intake on the change of serum 25(OH)D in control groups was explored in trials conducted in conditions of minimal UVB exposure. The treatment effects were shown as the mean difference (95% CI) in the change of 25(OH)D concentration between intervention and control groups. This mean difference was then converted to the mean difference per 100 IU of vitamin D intake per day. The Chi-square (Q) and I^2 statistics were used to assess heterogeneity (259) where an I^2 <25% is considered low heterogeneity and an I^2 >75% is considered high heterogeneity. Due to high heterogeneity among studies, the meta-analysis used a random effects model (260) to calculate the overall weighted

mean difference. Pre-specified subgroup analyses were carried out for age (2-8, 9-18 y), baseline 25(OH)D (<30, 30-49.9, ≥50 nmol/L), latitude (≥40^{o} N or S, <40^{o} N or S), method of 25(OH)D measurement (immunoassays, competitive protein binding assays, high performance liquid chromatography) and vitamin D administration method (daily supplements, fortified foods or bolus injections). Differences between subgroups were analyzed using a mixed model ANOVA in SAS (version 9.3, SAS Inst. Cary, North Carolina). For the effect of vitamin D intake on status, dose response curves were explored using both linear and non-linear regression. The non-linear regression was a quadratic-plateau model as assessed by segmental polynomial (knot) regression (SAS PROC NLIN). For linear regression models, condition indices and variance inflation factors were used to test for collinearity whilst plots of the distribution of residuals and Cook's D values tested for normality of the residuals. A probability < 0.05, after adjustment for multiple comparisons where applicable, was accepted as significant.

5.3 RESULTS

Literature Search

Searches of Ovid MEDLINE, PubMed, Embase, and Cochrane Central Register of Controlled Trials initially identified 2472 papers. After the removal of duplicates and an initial screen of titles and abstracts, there were 40 potentially relevant papers (**Supplemental Figure 5.1**). After careful examination, 14 articles were excluded for various reasons (**Supplemental Figure 5.1**). The summary of RCT's included in this meta-analysis are shown in **Table 5.1**. Vitamin D interventions and 25(OH)D concentrations are shown in **Table 5.2**.

Characteristics of Studies

From the 26 RCTs (n=5146 children) included in this analysis, 18 were published in the last 5 years (2012-2016). Though the mean of the vitamin D interventions was 778 IU/d, the

interventions were highly variable, ranging from 100-4000 IU/d vitamin D equivalents. The vitamin D delivery vehicle also varied with 9 trials that used fortified foods whilst 15 used supplements and 2 used bolus injections. The length (mean: 6.9 mo, range: 4 wk-24 mo) and location (20 of 26 trials were in locations ≥40^{o} N or S) of the interventions were also highly variable; only 1 trial was conducted in the southern hemisphere. The age range across trials was 2-18 y by meta-analysis design and though 24 (92%) of the individual trials had a within-trial spread of age of ≤ 6 years, 1 trial had an age spread of 3-17 y (**Table 5.1**). There were 3 trials only in boys and 4 trials only in girls (**Table 5.1**).

As shown in **Table 5.1** and **Table 5.2**, baseline dietary vitamin D intake was measured in 13 trials (range of mean/median intakes: 36-216 IU/d) and mean baseline 25(OH)D concentration was <30 nmol/L in 7 studies, 30-49.9 nmol/L in 10 studies and was >50 nmol/L in 9 studies. One trial (31) had mean baseline 25(OH)D >75 nmol/L, however this was only in the control group (103.4 ± 26 nmol/L), which significantly differed from the intervention group (72.0 ± 32.3 nmol/L). Studies used a range of methods to analyze 25(OH)D concentration (**Table 5.1**), 12 used either high performance liquid chromatography (HPLC) or HPLC tandem mass spectrometry (LCMS) whilst 14 used immunoassays or competitive protein binding assays.

Quality of Studies

Based on the Cochrane tool for assessing bias, study designs were qualitatively high, (**Supplemental Table 5.2 and Supplemental Figure 5.1**) and 96% had Jadad scores ≥4 (**Table 5.2**). Using the Cochrane tool for assessing bias criteria, 8-92% of studies had low risk of bias for the 7 categories of bias (**Supplemental Figure 5.1**). Though dropout percentage was reported in 17/26 trials (range: 1-22%), 2/26 studies only used data from completers (41, 261). One study

only reported on those with compliance > 80% (247), 13/26 trials did not report compliance and 10/26 trials had average compliance ≥ 80%.

Additional issues of study quality include that the study with the largest dose response of 25(OH)D to vitamin D intake (31.4 ± 6.5 nmol/L per 100 IU of vitamin D/d) was the only study with 25(OH)D differences between groups at end-point (31) and that 5 trials did not report season during which the intervention took place (**Table 5.1**). In 3 studies (262-264), the data for 25(OH)D concentration was highly skewed at either baseline or endpoint due to some children with very high 25(OH)D concentrations.

Intervention Efficacy and Subgroup Analyses

Out of the 11 trials that had conditions of minimal UVB exposure (conducted only during winter months in locations >40° N latitude), the control groups had an average monthly 25(OH)D decrease of 2.8 ± 1.9 nmol/L. Six of these trials gave dietary vitamin D intake information and those with intakes ~ 100 IU/d (n=3) had greater ($p<0.01$) average monthly changes in the control group (-4.2 ± 0.9 nmol/L) compared to those with intakes ~200 IU/d (n=3) (-1.9 ± 1.0 nmol/L).

All studies showed significant effects of vitamin D intervention on increasing serum 25(OH)D concentration (**Figure 5.1**). The 25(OH)D weighted mean difference from the random effects analysis was 23.5 nmol/L (95% CI 20.7-26.3 nmol/L). Due to the large variation in study designs, heterogeneity was high (I^2=99.9%). Using linear regression (**Figure 5.2A**), the mean effect of the treatment increases by 1.0 nmol/L for every 100 IU/d of intake, with a Y-intercept of 15.4 nmol/L (R^2=0.25). However, when the data was transformed to the mean change in 25(OH)D for every 100 IU/d of vitamin D intake (**Figure 5.2B**), a non-linear relationship emerged as shown by the segmented-plateau quadratic regression model. Because of this non-

linear relationship, the vitamin D dose was accounted for in all subgroup analyses described below.

First, the 25(OH)D increase per 100 IU/d of vitamin D in trials using fortified food (n=9) (9.3 nmol/L, 95% CI 6.7-11.9 nmol/L, I^2=99.9%) was greater than daily supplements (n=15) (3.0 nmol/L, 95% CI 2.1-3.9 nmol/L I^2=56%) (p=0.001) and bolus injections (n=2) (2.3 nmol/L, 95% CI 0.9-3.9 nmol/L I^2=0%) (p=0.02). Studies with baseline 25(OH)D concentration < 30 nmol/L (n=7) (5.0 nmol/L, 95% CI 3.2-6.6 nmol/L I^2= 98%) had greater increases in 25(OH)D per 100 IU/d than studies with baseline 25(OH)D ≥ 30 nmol/L (n=19) (3.0 nmol/L, 95% CI 1.9-4.9 nmol/L I^2= 8%) (p=0.01). Studies with baseline vitamin D intakes ≤ 100 IU/d had greater 25(OH)D increases per 100 IU/d (n=5) (5.8 nmol/L, 95% CI 4.1-7.4 nmol/L I^2=98%) compared to studies with intakes > 100 IU/d (n=5) (2.6 nmol/L, 95% CI 1.3-3.8 nmol/L I^2=84%) (p=0.01). Increases in 25(OH)D per 100 IU/d of vitamin D were significantly less (p<0.05) in studies that used chemiluminescent immunoassays (CLIA) (n=5) (1.5 nmol/L (95% CI 0.9-2.2 nmol/L, I^2=98%) compared with LCMS (n=5) and competitive protein binding assay (CPBA) (n=4) techniques (LCMS: 7.6 nmol/L (95% CI 7.4-7.8 nmol/L), CPBA: 6.1 nmol/L (95% CI 5.5-6.6 nmol/L) (I^2: 95-98%). Latitude, age, sex and study length did not significantly affect the change in 25(OH)D per 100 IU/d of vitamin D (all I^2 statistics > 90%).

Out of the 11 trials that had conditions of minimal UVB exposure (conducted only during winter months in locations >40° N latitude), the control groups had an average monthly 25(OH)D decrease of 2.8 ± 1.9 nmol/L. Six of these trials gave dietary vitamin D intake information and those with intakes ~ 100 IU/d (n=3) had greater (p<0.01) average monthly changes in the control group (-4.2 ± 0.9 nmol/L) compared to those with intakes ~200 IU/d (n=3) (-1.9 ± 1.0 nmol/L).

When baseline vitamin D status was <50 nmol/L, fortified food intervention groups (n=4) had increases in 25(OH)D of 4.2-10.8 nmol/L per 100 IU/d of vitamin D whilst supplement and bolus injection intervention groups (n=10) had increases of 1.1-5.6 nmol/L per 100 IU/d of vitamin D intake. Across all studies with baseline status <30 nmol/L (n=7), 30-49.9 nmol/L (n=7) and ≥50 nmol/L (n=12), changes in vitamin D status in the intervention groups per 100 IU/d of vitamin D intake were 1.1-10.8 nmol/L, 1.1-5.6 nmol/L and -1.6-24.3 nmol/L respectively. There were 4 trials that roughly spanned the UVB void period [7,23,29,49], and the one trial [49] that had a decrease in vitamin D status (baseline 25(OH)D: 74.9±17.4 nmol/L, change: -5.1±17.2 nmol/L) in the intervention group over the study also had the lowest dose of vitamin D (320 IU/d).

5.4 DISCUSSION

With no previous meta-analyses in children evaluating vitamin D intake and status, this study fills an important knowledge gap by showing that vitamin D interventions, from 26 randomized placebo-controlled trials in children (2-18 y), increased 25(OH)D concentration in a dose dependant manner. However, due to the high level of heterogeneity among studies (99.9%), the overall results should be used cautiously. Heterogeneity was expected to be high due to the age range, range of the dose of vitamin D interventions, baseline vitamin D status, location and length of study. Although subgroup analyses were undertaken in efforts to decrease heterogeneity, heterogeneity remained high in all subgroups except for those taking vitamin D supplements or bolus injections. The effect of the intervention per 100 IU/d of vitamin D was greater in subgroups with baseline 25(OH)D <30 nmol/L, baseline vitamin D intake < 100 IU/d, using fortified foods and for which LCMS or CPBA were used to measure 25(OH)D concentration (compared only to CLIA).

The overall results of this meta-analysis are similar to a 2012 vitamin D fortified foods meta-analysis in adults that had an overall mean difference of 19.4 nmol/L (95% CI 13.9-24.9 nmol/L, I^2=89%) (251). However, the dose response in adults of 3.0 nmol/L for every 100 IU/d of vitamin D was greater than the 1.0 nmol/L dose response seen in children. The dose response may have been lower than in adults because of growth in children, with muscle, fat, bone and other tissues consuming vitamin D (133), or because of the non-linear dose response observed in children. By using non-linear regression, similar to that of previous trials (40, 250) it was possible to account for vitamin D dose in subgroup analyses to help reduce type 1 statistical errors.

Because of 11 trials being conducted during periods of minimal UVB exposure, it was possible to investigate how vitamin D status changed in the control groups during winter. With 6 of these trials including data on vitamin D intake, this analysis showed that studies with vitamin D intakes of ~ 100 IU/d, similar to that of many European countries (265), had significantly greater monthly decreases in 25(OH)D (-4.2 ± 0.9 nmol/L) compared to those studies with intakes ~ 200 IU/d (-1.4 ± 0.6 nmol/L), similar to those of Canada (266) and the US (13). The results of this analysis highlight that a small increase of vitamin D intake (~100 IU/d), equivalent to consuming a 250 ml serving of vitamin D fortified milk in Canada and the US, may be protective against the more drastic winter decreases in 25(OH)D seen in European countries.

Strengths of this study were inclusion of only randomized placebo-controlled trials and studies using supplements, fortified foods or bolus injections of vitamin D, resulting in a robust sample of 26 trials. Though not all studies had vitamin D status as the primary outcome, extracting this data did not prove challenging. Since only randomized controlled trials were included, study quality was generally high and most data is recent with 16/27 (60%) trials having

been published within the last 5 years (2012-2016). However, as shown in **Supplemental Figure 5.1**, though the majority of studies had a low risk of bias in 6 of the 7 categories, over 50% had an unsure risk of bias in free of source of funding and 19% had a high risk of bias for selective reporting. Short statements should be added to funding information describing the role (if any) of funding organizations for added transparency. The selective reporting, including the 3 studies (41, 247, 261) that either only included completers or those with compliance > 80%, as well as those with high drop-outs percentages (15-22%) (24, 262, 267) may result in an overestimation of the effect of the intervention.

There were important variables that were not consistently reported. Vitamin D intake and skin colour/ethnicity were both missing in 15/26 (58%) of studies, limiting the possible analyses including dietary vitamin D intake or ethnicity. The season of the intervention was missing in 4 trials, however, since the trial locations were 7° N to 33° N latitude, some endogenous vitamin D production from UVB exposure would have been possible year-round. In addition, although some trials recorded data on winter vacations to southern locations, only 6 trials (30, 41, 234, 250, 264, 268, 269) measured UVB exposure and methods varied from skin colour to erythmal dose measurements. Nine trials did not include information on independent measurement of vitamin D concentration in study products and the possibility of high or low vitamin D content of supplements or foods could have added to the high heterogeneity among studies. Moreover, only one study was conducted in the southern hemisphere. Lastly, there was large variability in analytical methods used to measure vitamin D status (**Table 5.1**). As shown by our results and previous work from the vitamin D external quality assessment scheme (DEQAS) (270, 271), it is possible that CLIA underestimated 25(OH)D concentrations, or concentration changes, compared to liquid chromatography measurement techniques. Data from DEQAS also illustrates

that variation among laboratories can be high using the same 25(OH)D measurement technique (272), highlighting the need for thorough quality assessment, including the publication of assay accuracy and precision.

As more Northern countries implement vitamin D food fortification programs, results of this meta-analysis show fortification can be beneficial to pediatric populations since food fortification may be more efficient at raising or maintaining 25(OH)D concentrations compared to supplementation. In addition, since the minority of the population uses vitamin D supplements regularly (12, 273, 274) and vitamin D intake from fatty fish may be inconsistent or non-existent, vitamin D food fortification efforts are warranted. The study by Madsen et al. (23) suggests that over the entire UVB void period in children with sufficient vitamin D status (74.9±17.4 nmol/L), 400 IU/d of vitamin D, mostly from fortified foods, exceeded the definition of the EAR, but may not be enough to reach the definition of the RDA. With multiple studies of populations at locations <40^{o} N or S, having deficient or insufficient baseline 25(OH)D (**Table 5.2**) vitamin D food fortification may be important for countries regardless of latitude.

Since this meta-analysis showed similar response to vitamin D intervention regardless of latitude or age, setting of global EAR and RDA values for children may be feasible. Because baseline 25(OH)D may have a strong influence on the derived EAR and RDA values (40, 41, 234, 250), for countries to formulate vitamin D recommendations, studies need to reflect national vitamin D status. Future studies should span the winter period of minimal UVB exposure, where applicable, and should have 25(OH)D measurements at baseline, midpoint and endpoint, to best track changes in 25(OH)D concentration.

Table 5.1 Summary of 26 randomized placebo-controlled trials in healthy children with vitamin D supplement, injection or fortified food interventions

Reference	Location, Ethnicity or skin colour	Age (y), n (% male)	Duration, season	Baseline vitamin D intake (IU/d) (median (IQR) or mean (95%CI) or ±SD)	25(OH)D analysis method[1]
Abrams, 2013 (275)	Houston, USA (30° N), N.R.	4-8, 64 (NR)	8 wk, N.R.	216±80	CLIA
Ala-Houhala, 1988 (276)	Finland (60 º N), N.R.	8-10, 51 (45%)	13 mo, baseline January	N.R.	CPBA
Al-Shaar, 2014 (264)	Beirut, Lebanon (34º N), N.R.	10-17, 336 (51%)	1 y, baseline December to June	N.R.	CPBA
Andersen, 2008 (268)	Copenhagen, Denmark (55º N), Pakistani origin	10-14, 26 all female	1 y, baseline January to November	80 (IQR 52-96)	HPLC
Brett, 2016 (234)	Montreal, Canada (45.5º N), 73% white	2-8, 77 (52%)	12 wk, January to April	198 (IQR 155-291)	CLIA
Carnes, 2012 (269)	Southern Australia (42 º S), N.R.	15-17, 22 (45%)	1 y, baseline August to December	N.R.	RIA
Du, 2004 (30)	Beijing, China (40° N), N.R.	10, 757 all female	2 y, baseline in April	36±24	CPBA
Dubnov-Raz, 2015 (277)	Israel (31-32º N), N.R.	12-18, 53 (62%)	12 wk, November to January	N.R.	RIA
Duhamel, 2000 (278)	Rouen, France (49° N), N.R.	10-15, 68 (NR)	9 mo, October to June	N.R.	HPLC
Economos, 2014 (262)	Boston, USA (42º N), 44% black	6-10 176 (62%)	12 wk, January to June	N.R.	HPLC
Guillemant, 2001 (261)	Gouvieux, France (49° N), N.R.	13-16, 54 all male	6 mo, October to March	N.R.	CPBA
Hettiarachchi, 2010 (31)	Galle, Sri Lanka (7º N), N.R.	3-5, 60 (48%)	9 mo, N.R.	N.R.	RIA
Hower, 2013 (24)	Dusseldorf, Germany (51° N), 98% light-skinned	2-6, 80 (56%)	8 mo November to July	median 76 IU	CLIA

Khadgawat, 2013 (279)	Delhi, India (29° N), N.R.	10-14, 713 (42%)	12 wk, N.R.	N.R.	CLIA
Lewis, 2013 (280)	Athens, USA (34º N), West Lafayette and Indianapolis USA (40° N), 51% black	9-13, 323 (50%)	12 wk, October to December	N.R.	RIA
Maalouf, 2008 (263)	Beirut, Lebanon (34º N), N.R.	10-17, 340 (51%)	1 y, baseline N.R.	N.R.	RIA
Madsen, 2013 (23)	Gladsaxe, Denmark (56º N), N.R.	4-17, 321 (48%)	6 mo, September to March	88 (IQR 60-116)	LCMS
Molgaard, 2010 (281)	Denmark Copenhagen and Frederiksberg (55° N), White	11-12, 225 all female	1 y, baseline November to the following December	104 ±56	HPLC
Mortensen, 2016 (40)	Denmark (55º N), white	4-8, 130 (52%)	20 wk, October to March	80 (95% CI 60-108)	LCMS
Neyestani, 2013 (282)	Tehran, Iran (34 º N), N.R.	9-12, 410 (68%)	3 mo, November to March	N.R.	HPLC
Rajakumar, 2016 (41)	Pittsburgh, USA (40º N), 54% black	8-14, 96 (52%)	2 mo, October to April	189 (IQR 130-392)	LCMS
Rich-Edwards, 2011 (283)	Mongolia (48 º N), N.R.	9-11, 579 (36%)	1.6 mo, January to March	N.R.	LCMS
Smith, 2016 (250)	United Kingdom (51º N), white	14-18, 110 (43%)	20 wk, October to March	160± 96	LCMS
Tavakoli, 2016 (267)	Birjand, Iran (33° N), N.R.	10-14, 47 (49%)	1 mo, N.R.	N.R.	CLIA
Viljakainen, 2006 (247)	Helsinki, Finland (60° N), N.R.	10-12, 212 all female	1 y, baseline September to March	200±108	HPLC
Ward, 2011 (284)	Manchester, United Kingdom (53° N), N.R.	12-14, 73 all female	1 y, baseline December	N.R.	HPLC

[1] LCMS: high performance liquid chromatography tandem mass spectrometry, CLIA: chemiluminescent immunoassay, CPBA: competitive protein binding assay, RIA: Radioimmunoassay, HPLC: high performance liquid chromatography. NR: Not reported.

Table 5.2 Description of intervention and 25(OH)D concentrations in 26 randomized placebo-controlled trials in healthy children.

	Study design		Control Group			Intervention Group			
Reference	**Vitamin D vehicle**	**Vitamin D added (IU/d)**	**Baseline 25(OH)D (nmol/L)**	**Endpoint 25(OH)D (nmol/L)**	**25(OH)D change (nmol/L)**	**Baseline 25(OH)D (nmol/L)**	**Endpoint 25(OH)D (nmol/L)**	**25(OH)D change (nmol/L)**	**Jadad score**
Abrams, 2013 (275)	Tablets	1000	69.0±18.3	74.8±31.0	5.8±27.0[2]	69.2±18.5	90.0±25.8	21.8±23.0[2]	5
Ala-Houhala, 1988 (276)	Tablet	400	46.0±15.5	43.3±19.5	-2.7±18.9[2]	49.3±19.0	71.3±23.8	22.0±26.8[2]	4
Al-Shaar, 2014 (264)	Tablets: Boys	200 2000	41.2±14.8	43.5±16.3	2.3±23.1[2]	41.2±18.0 40.8±17.8	50.5±17.0 87.3±23.5	9.3±17.5[2] 46.5±21.5[2]	4
	Girls	200 2000	35.5±18.8	39.3±21.0	3.8±15.6[2]	34.8±23.3 35.3±16.8	42.8±15.5 94.8±78.0	8.0±20.5[2] 59.5±71.1[2]	
Andersen, 2008 (268)	Tablets	400 800	12.9±11.0	9.5±5.7	-3.4±10.0[2]	18.8±13.7 11.6±8.4	43.9±22.8 38.2±21.8	25.1±5.0[2] 26.6±5.0[2]	4
Brett, 2016 (234)	Yogurt/ cheese	250 450	58.3±14.5	55.8±12.3	-2.5±6.2	59.2±13.2 60.4±10.5	64.1±10.0 63.7±12.4	3.7±15.3 3.2±7.3	5
Carnes 2012 (269)	Tablet	820 1640	43.4±5.9	35.8±NR	-7.6±14.3[2]	39.4±9.8 37.9±9.1	41.1±NR 63.0±NR	1.5±9.8 25.1±9.1	4
Du, 2004 (30)	Fluid milk	Mean intake: 133	19.1±7.4	19.4±10.2	0.3±9.1[2]	20.6±8.8	47.6±23.4	27.0±20.1[2]	4
Dubnov-Raz, 2015 (277)	Liquid supplement	2000	62.0±11.5	51.3±10.5	-10.7±12.2[2]	61.0±12.3	74.0±16.3	13.0±14.6[2]	5
Duhamel, 2000 (278)	Injection[1]	830	49.0±25.8	41.5±18.8	-7.5±23.1[2]	45.0±21.5	55.8±18.3	10.8±20.0[2]	4
Economos, 2014 (262)	Orange juice	200 200	64.3±20.9	80.8±30.5	16.5±26.5	64.2±92.8 75.6±27.2	92.8±36.0 95.9±27.7	28.6±24.6 20.4±25.2	4
Guillemant, 2001 (261)	Liquid supplement[1]	1660	61.0±15.5	20.2±0.5	-40.8±15.2[2]	53.7±12.2	55.2±11.5	2.5±11.9[2]	4
Hettiarachchi, 2010 (31)	Fortified cereal based food	100	103.4±26.4	96.3±36.9	-7.1±7.3	72.0±32.3	96.3±27.5	24.3±6.5	2

Hower, 2013 (24)	Fluid milk	100/ 250 ml (median intake 284)	53.0±19.8	68.0±12.7	15.0±17.1[2]	54.5±19.3	72.8±15.0	18.3±17.0[2]	5
Khadgawat, 2013 (279)	Fluid milk	600 1000	29.4±13.0	27.0±13.0	-2.4±13.0	28.5±13.0 29.8±14.0	57.3±13.0 69.3 ±21.3	28.8±13.0[2] 39.5±18.8[2]	4
Lewis, 2013 (280)	Tablets	400 1000 2000 4000	71.5±18.6	61.4±NR	-10.1±2.9	71.4±19.5 71.1±19.7 65.8±7.3 70.0±17.5	76.9±NR 91.4±NR 103.4±NR 146.1±NR	5.5±2.6 20.3±2.6 37.6±2.7 76.1±3.0	5
Maalouf, 2008 (263)	Tablet: Boys	200 2000	40.0±15.0	42.5±15.0	2.5±15.0[2]	40.0±17.5 40.0±17.5	50.0±17.5 87.5±22.5	10.0±17.5[2] 47.5±16.4[2]	4
	Girls	200 2000	35.0±17.5	40.0±20.0	5.0±15.0[2]	35.0±17.5 32.5±20.0	35.0±15.0 95.0±77.5	0.0±20.4[2] 62.5±70.4[2]	
Madsen, 2013 (23)	Milk/bread	320	74.6±17.5	46.3±19.9	-28.3±17.5[2]	74.9±17.4	69.8±17.0	-5.1±17.2[2]	5
Molgaard 2010 (281)	Tablet	400 800	43.4±17.1	39.7±17.7	-3.1±7.3	41.9±17.6 44.4±16.6	52.9±16.3 57.9±14.3	11.0±10.3 13.0±10.8	4
Mortensen, 2016 (40)	Tablet	400 800	55.2±10.8	31.1±7.5	-24.1±1.2	56.9 ±12.7 58.1±13.5	61.8±10.6 75.8±11.5	4.9±1.3 17.7±1.8	5
Neyestani 2013 (282)	Fluid milk	200 200	25.3±1.3	23.9±1.1	-1.4±1.1[2]	24.9±1.2 24.9±1.4	34.4±1.5 33.5±1.8	9.5±1.4[2] 8.6±1.6	5
Rajakumar, 2016 (41)	Tablet	1000	47.3±18.3	42.8±17.5	-4.5±17.9[2]	52.0 ±19.8	65.3 ±20.8	13.3±20.3[2]	4
Rich-Edwards 2011 (283)	Fluid milk Fluid milk Tablet Tablet	300 300 300 300	20.0±10.0	20.0±10.0	0.0±10.0[2]	20.0±10.0 25.0±13.0 18.0±8.0 20.0±10.0	50.0±15.0 73.0±25.0 53.0±15.0 30.0±10.0	30.0±13.2[2] 48.0±21.7[2] 35.0±13.0[2] 10.0±10.0[2]	4
Smith, 2016 (250)	Tablet	400 800	46.8±11.4	30.7±8.6	-16.3±8.7	49.2±12.0 51.7±13.4	56.6±12.4 63.9±10.6	6.7±9.9 12.1±10.6	5

Tavakoli, 2016 (267)	Tablets	1000	24.3 ±13.8	23.2±12.5	-1.1±4.4	18.2±12.5	28.8±14.6	9.9±5.4	5
Viljakainen, 2006 (247)	Tablets	200 400	47.8±18.2	42.7±NR	-5.0±12.3	46.3±17.4 46.7±16.2	51.7±NR 58.8±NR	5.4±15.3 12.1±15.5	5
Ward, 2011 (284)	Injections[1]	1640	17.9±7.4	15.7±6.6	-0.8±3.4	18.1 ±8.0	56.0±8.9	37.9±4.5	4

[1] Bolus injections or supplements were converted to vitamin D intake/day.
[2] Calculated from baseline and endpoint data.
NR: Not reported.

Supplemental Table 5.1 Online keyword title and abstract search terms

(Teen* OR Pediatric* OR Paediatric* OR Adolescent* OR Child* OR Toddler* OR Girls OR Boys OR Youth)

AND

(25(OH)D OR Vitamin D* OR 25-hydroxy-vitamin D* OR 25OHD OR Serum 25-hydroxy-vitamin D* OR Cholecalciferol OR 25-OH vitamin D* OR 25-hydroxycholecalciferol OR Serum 25OHD)

AND

(Randomized* Controlled trial OR RCT OR randomized*)

Supplemental Table 5.2 Methodological quality of studies included in this meta-analysis using the Cochrane bias assessment tool.

	Bias Criteria[1]						
	1	2	3	4	5	6	7
Abrams, 2013 (275)	+	?	+	?	+	−	+
Ala-Houhala, 1988 (276)	?	?	+	?	+	−	+
Al-Shaar, 2014 (264)	+	+	+	?	+	−	?
Andersen, 2008 (268)	+	+	+	?	+	+	+
Brett, 2016 (234)	+	+	+	+	+	+	+
Carnes, 2012 (269)	?	?	+	?	?	+	+
Du, 2004 (30)	+	+	+	?	+	−	?
Dubnov-Raz, 2015 (277)	+	+	+	?	+	+	+
Duhamel, 2000 (278)	+	?	?	?	?	+	?
Economos, 2014 (262)	+	+	+	?	+	+	?
Guillemant, 2001 (261)	+	+	?	?	?	+	?
Hettiarachchi, 2010 (31)	?	?	+	?	?	+	+
Hower, 2013 (24)	+	+	+	?	+	+	?
Khadgawat, 2013 (279)	+	?	+	?	+	+	?
Lewis, 2013 (280)	+	+	+	?	+	+	+
Maalouf, 2008 (263)	?	?	+	?	?	+	?
Madsen, 2013 (23)	+	+	+	+	+	+	?
Molgaard, 2010 (281)	+	+	+	?	+	+	+
Mortensen, 2016 (40)	+	+	+	?	+	+	+
Neyestani, 2013 (282)	+	+	+	?	+	+	+
Rajakumar, 2016 (41)	?	?	+	?	+	−	+
Rich-Edwards, 2011 (283)	+	+	+	?	+	+	?
Smith, 2016 (250)	+	+	+	?	+	+	+
Tavakoli, 2016 (267)	?	?	+	?	+	+	?
Viljakainen, 2006 (247)	+	+	+	?	+	+	?
Ward, 2011 (284)	+	+	+	?	+	+	+

[1] Columns: 1: random sequence generation, 2: allocation concealment, 3: blinding of participants and personnel, 4: blinding of outcome assessment, 5: incomplete outcome data, 6: selective reporting, 7: free of source of funding.

Supplemental Table 5.3 Registered placebo-controlled vitamin D trials in healthy children that were not published as of December, 2016.

Principal Investigator	Trial registration[1], location	Age (y), n	Duration	Intervention, IU/d
Weiler, Hope	NCT02387892, Montreal, Canada	2-8, 51	6 mo, October to April	Fortified yogurt/cheese, 300
Winzenberg, Tania.	ACTRN12613000700730, Tasmania, Australia	15-17, 28	1 y, Rolling recruitment	Supplements, 1667
Loeb, Mark	NCT01705314, Vietnam	3-17, 1300	8 mo, Rolling recruitment	Supplements, 2000
Reyes, Maria	NCT02046577, Santiago, Chile	1.5-3, 276	6 mo, Baseline February	Supplements, 800, 1600
Ohland, Inger	NCT01741324, Sweden	5-7, 220	3 mo, Baseline November	Fortified milk, 400, 1000

[1] NCT: Clinicaltrials.gov, ACTRN: Australian New Zealand Clinical Trials Registry.

Figure Legends

Figure 5.1 The mean (95% CI) change in 25(OH)D concentration (nmol/L) from vitamin D supplementation, food fortification or injection (n=26). Using a random effects model, the overall weighted mean difference was estimated, with an I^2 statistic of 99.9%.

Figure 5.2 The 25(OH)D (nmol/L) dose response to vitamin D supplementation, fortification or injection interventions was estimated using linear regression, (Panel A) and the dose response of 25(OH)D per 100 IU/d of vitamin D was estimated using a quadratic-plateau model as assessed by segmental polynomial (knot) regression (Panel B).

Figure 5.1

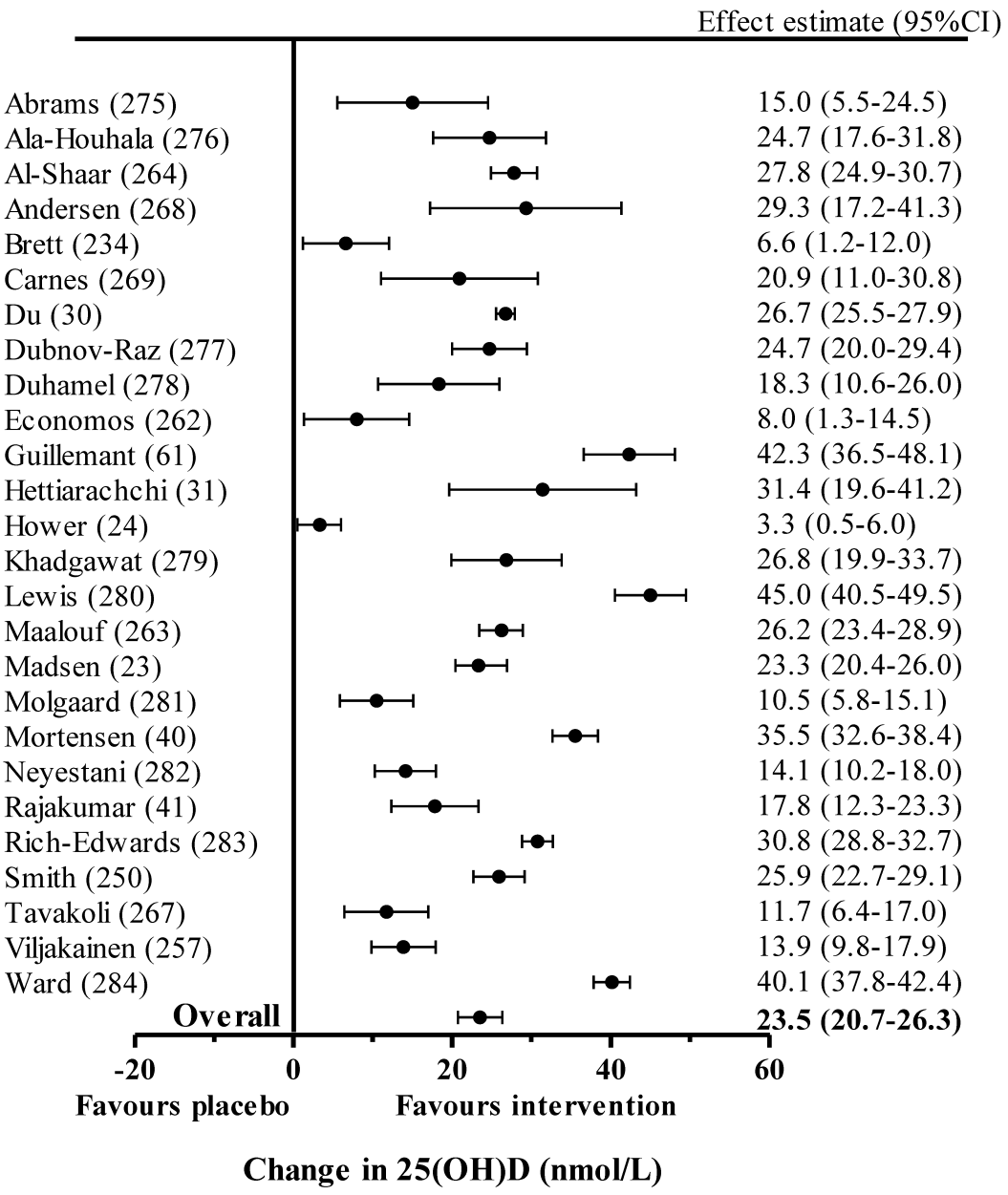
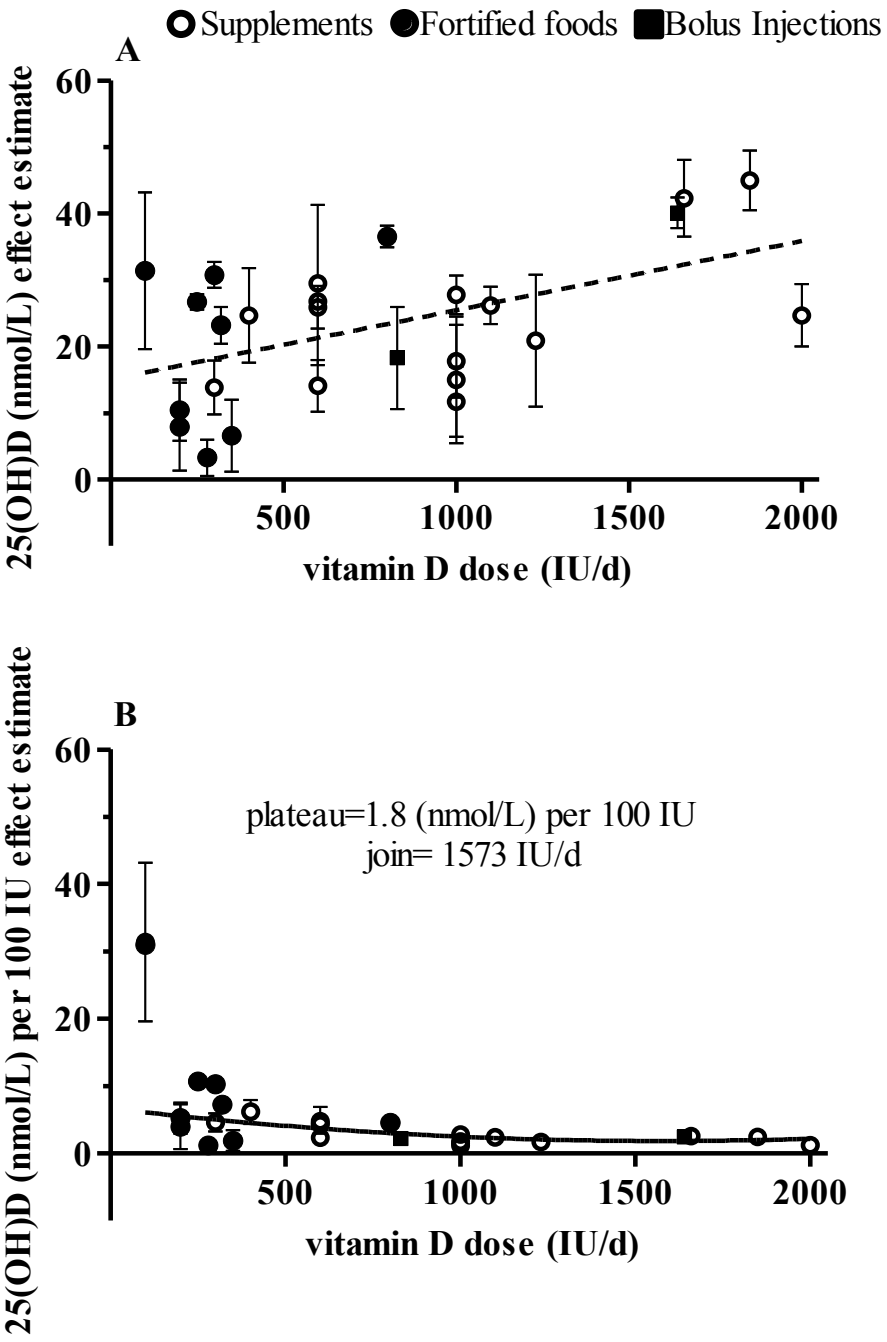
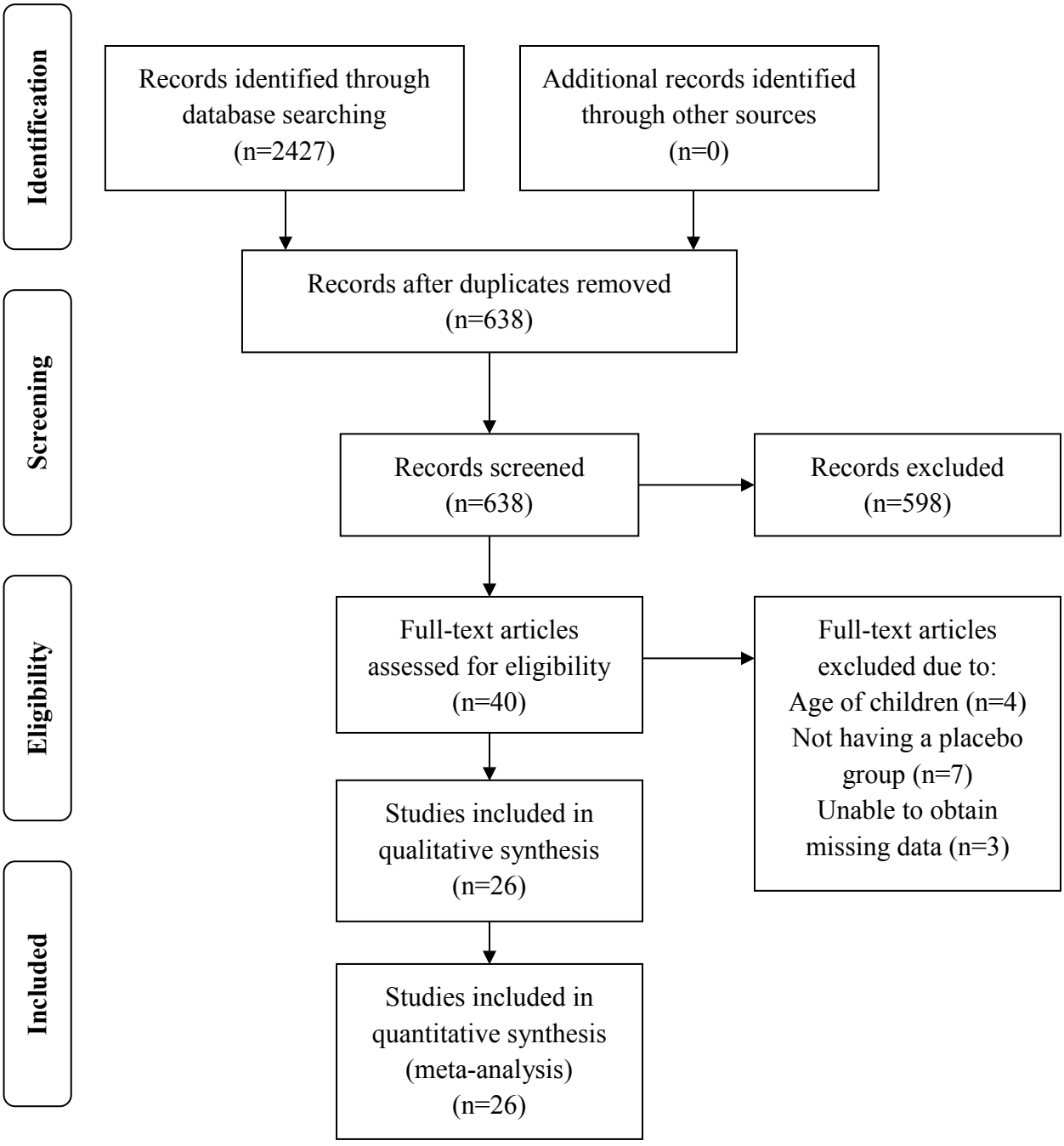

Effect estimate (95%CI)
Abrams (275) 15.0 (5.5-24.5)
Ala-Houhala (276) 24.7 (17.6-31.8)
Al-Shaar (264) 27.8 (24.9-30.7)
Andersen (268) 29.3 (17.2-41.3)
Brett (234) 6.6 (1.2-12.0)
Carnes (269) 20.9 (11.0-30.8)
Du (30) 26.7 (25.5-27.9)
Dubnov-Raz (277) 24.7 (20.0-29.4)
Duhamel (278) 18.3 (10.6-26.0)
Economos (262) 8.0 (1.3-14.5)
Guillemant (61) 42.3 (36.5-48.1)
Hettiarachchi (31) 31.4 (19.6-41.2)
Hower (24) 3.3 (0.5-6.0)
Khadgawat (279) 26.8 (19.9-33.7)
Lewis (280) 45.0 (40.5-49.5)
Maalouf (263) 26.2 (23.4-28.9)
Madsen (23) 23.3 (20.4-26.0)
Molgaard (281) 10.5 (5.8-15.1)
Mortensen (40) 35.5 (32.6-38.4)
Neyestani (282) 14.1 (10.2-18.0)
Rajakumar (41) 17.8 (12.3-23.3)
Rich-Edwards (283) 30.8 (28.8-32.7)
Smith (250) 25.9 (22.7-29.1)
Tavakoli (267) 11.7 (6.4-17.0)
Viljakainen (257) 13.9 (9.8-17.9)
Ward (284) 40.1 (37.8-42.4)
Overall 23.5 (20.7-26.3)
-20
0
20
40
60
Favours placebo
Favours intervention
Change in 25(OH)D (nmol/L)

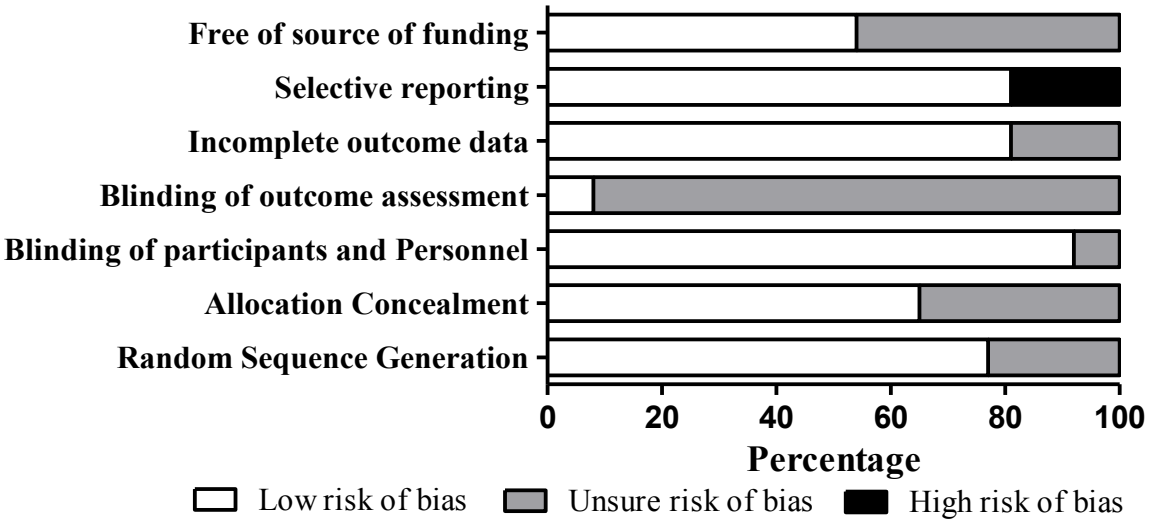
Figure 5.2


Supplements
Fortified foods
Bolus Injections
A
25(OH)D (nmol/L) effect estimate
60
40
20
0
500
1000
1500
2000
vitamin D dose (IU/d)
B
25(OH)D (nmol/L) per 100 IU effect estimate
plateau=1.8 (nmol/L) per 100 IU
join= 1573 IU/d
60
40
20
0
500
1000
1500
2000
vitamin D dose (IU/d)

Supplemental Figure 5.1 PRISMA flow diagram



Supplemental Figure 5.2 Percentage of studies in methodological quality categories of low, unsure and high risk of bias assessed using the Cochrane bias assessment tool. Details for each study are shown in **Supplementary Table 2**.



CHAPTER 6: DISCUSSION

6.1 MAIN OUTCOMES AND HYPOTHESES

The overall goal of this thesis was to investigate how the vitamin D status of children 2-8 y was affected by consuming foods fortified with vitamin D so that their intake would meet the recommendations. Two sequential randomized controlled trials and an independent meta-analysis were designed to investigate the relationship between vitamin D intake and status as well as how vitamin D intakes affect musculoskeletal health outcomes. With no previous vitamin D fortified food trials in North America in young children, the 12 wk trial (study 1) fills an important research gap. The 6 mo trial in chapter 4 adds to this work by investigating similar vitamin D outcomes over a longer time-frame and adds to the very limited paediatric literature on how vitamin D intakes/status affect bone and muscle health outcomes. Study 3 (meta-analysis) adds a significant contribution to knowledge and has public health implications by examining the relationship of vitamin D intake and status, in trials of children 2-18 y from around the world.

Study 1 found that having vitamin D intakes consistent with the EAR or RDA resulted in significantly higher 25(OH)D concentration compared to control, though the differences among intervention and control groups was not as large as hypothesized. Study 2 showed that a vitamin D intake consistent with the EAR maintained 25(OH)D for 3 mo, but not for the entire winter, and 25(OH)D in the intervention group was not different from control at the end-point of the study. Thus, the alternative hypothesis was rejected and null (H_0) of no change was accepted. However, when looking at the secondary objectives of functional outcomes, it was found that lean mass accretion was significantly greater in the intervention group over the 6 mo study. No differences were found between groups for bone biomarkers or bone health or for bone mineral

accretion. Study 3 found that vitamin D interventions had a significant positive effect on 25(OH)D and subgroup analysis found that the significantly larger effects were found in subgroups consuming fortified foods, having baseline 25(OH)D of < 30 nmol/L and using LC-MS/MS to measure 25(OH)D.

It is clear from this work that vitamin D recommendations for healthy young children of 400 or 600 IU/d are adequate to achieve and maintain a vitamin D status above deficiency, and thus, prevention of vitamin D dependant rickets is likely achieved. In the absence of ultraviolet B radiation (UVB), vitamin D intakes of 400 IU/d seem to exceed the definition of the EAR (50% of the population with 25(OH)D ≥ 40 nmol/L), however, the vitamin D intake needed for 97.5% of the population to have 25(OH)D ≥ 50 nmol/L (definition of the RDA) remains unclear and vitamin D stores may not be adequately reflected by 25(OH)D concentration. With lean mass accretion being greater in the intervention than the control group in the 6 mo trial, even though vitamin D status did not differ at 6 mo, it is possible that 25(OH)D alone is not the best marker of vitamin D status in young children. Since most children in both groups maintained 25(OH)D ≥ 50 nmol/L, but none maintained status ≥ 75 nmol/L, it is unclear if 25(OH)D concentration above either of these thresholds would improve bone health outcomes compared to below these thresholds. Longer vitamin D interventions than 6 mo are needed to examine bone health outcomes and future investigations of the inter-relationship between muscle and bone development in young children would be beneficial.

6.2 VITAMIN D AND MUSCULOSKELETAL HEALTH

As described in chapter 2, vitamin D has many potential roles in muscle and bone. By further discussing these roles in this section, I am able to also delve into the complex relationships of musculoskeletal interactions, and speculate on important interactions to be

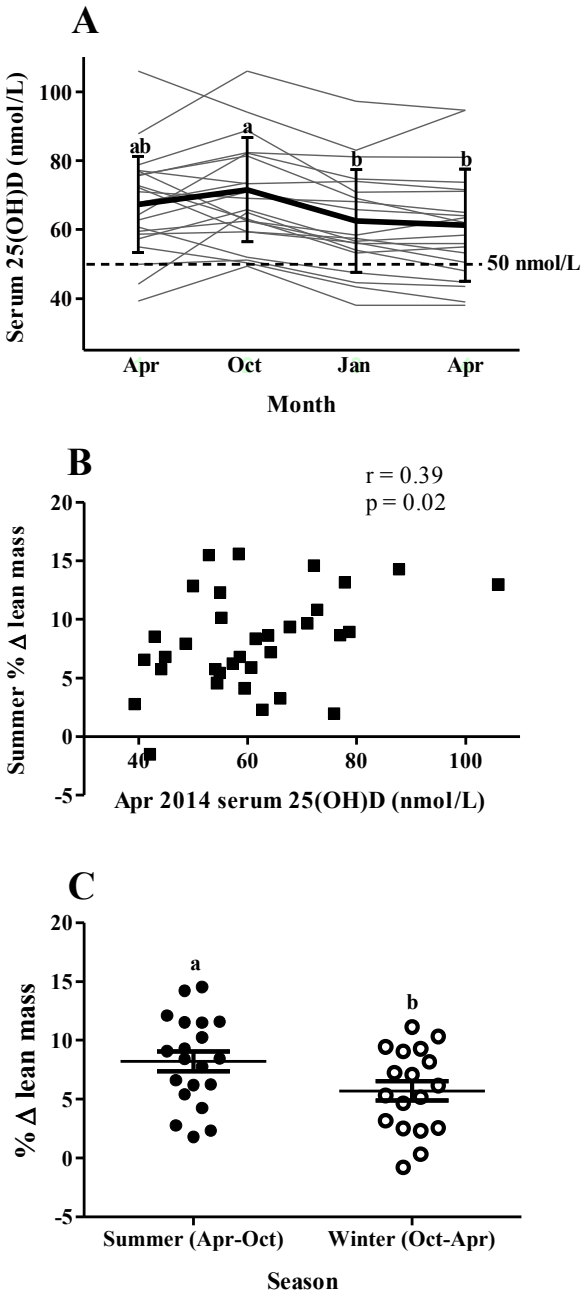
further investigated in future trials. First, in study 2, lean mass accretion was significantly increased in the intervention group even though 25(OH)D concentration at endpoint did not differ between groups. As explained in study 2, the gained lean mass may have been responsible for vitamin D status not differing between groups because lean mass consumes a significant portion of vitamin D with 25-66% of 25(OH)D in the body found in muscle tissue of humans (243) and rats (244). Chapter 2 describes mechanisms of how vitamin D could increase muscle mass including that vitamin D can induce VDR in a dose dependant manner (285) and increase protein synthesis and mRNA expression of VDR in myocytes (286). Whilst increased activation of calmodulin-dependant kinases can increase calcium transport and VDR-mediated protein transcription (177), increased serum IGFBP3 concentration can stimulating cell growth and proliferation by increasing the concentration of IGF-1 in circulation (178). Also as described in chapter 2, improvements in lean mass accrual could, in addition, be due to fast responses to vitamin D in muscle through the vitamin D induced tyrosine phosphorylation (activation) of MAPK signaling pathways. This results in significantly improvements of myotubular cross-sectional area (176).

As expected, when the majority of participants are vitamin D deficient and have low vitamin D intakes, like in studies of Chinese girls 15 y (38) and pre-pubertal Lebanese girls (10-13 y) (39), there were significant associations between lean mass and vitamin D intake. In our 6 mo trial, vitamin D status was maintained ≥ 50 nmol/L in > 70% of all participants, but differences in lean mass accretion were still seen. Because of muscle consuming a significant portion of vitamin D intake, lean mass may be an important consideration when setting future vitamin D recommendations. However, it is unclear what 25(OH)D threshold should be used or even if a vitamin D threshold should be used to define sufficiency for muscle outcomes. In

support of a higher threshold, in children 3 y, having 25(OH)D ≥ 75 nmol/L was associated with a leaner body phenotype (37). Interestingly using data from children who were in study 1 and 2 and consumed their normal vitamin D intake, lean mass accretion was significantly greater in 2-8 y old children during summer compared to winter (**Figure 6.1**) when adjusted for physical activity, age, sex, ethnicity, height velocity and BMI Z-score. In these 21 children consuming their normal dietary vitamin D intakes, vitamin D status was highest during the summer (Panel A) and vitamin D status at baseline positively associated with lean mass accretion ($r=0.37$, $p=0.02$). However, due to the variability in this association and to the factors explained above, it is possible that both vitamin D intake (including endogenous synthesis) and vitamin D status explain the increased lean mass accretion in summer compared to winter.

Muscular strength was an outcome not explored in the RCTs of this thesis; this could be interesting because in children it may also be related to vitamin D. The mechanism is probably due to the fast acting non-genomic vitamin D pathway in muscle that is able to increase contraction of the sarcomere through the influx of calcium from both the sarcoplasmic reticulum and the extracellular compartment (35). Beyond trials of vitamin D and muscular strength mentioned in chapter 2, in publications from the last 12 mo, vitamin D status was positively associated with muscular strength and jump height in Italian children (287), muscle power in Indian children 5 y (288) and in children 5-19 y (289). Longitudinal studies are still needed to investigate these outcomes in Canadian children.

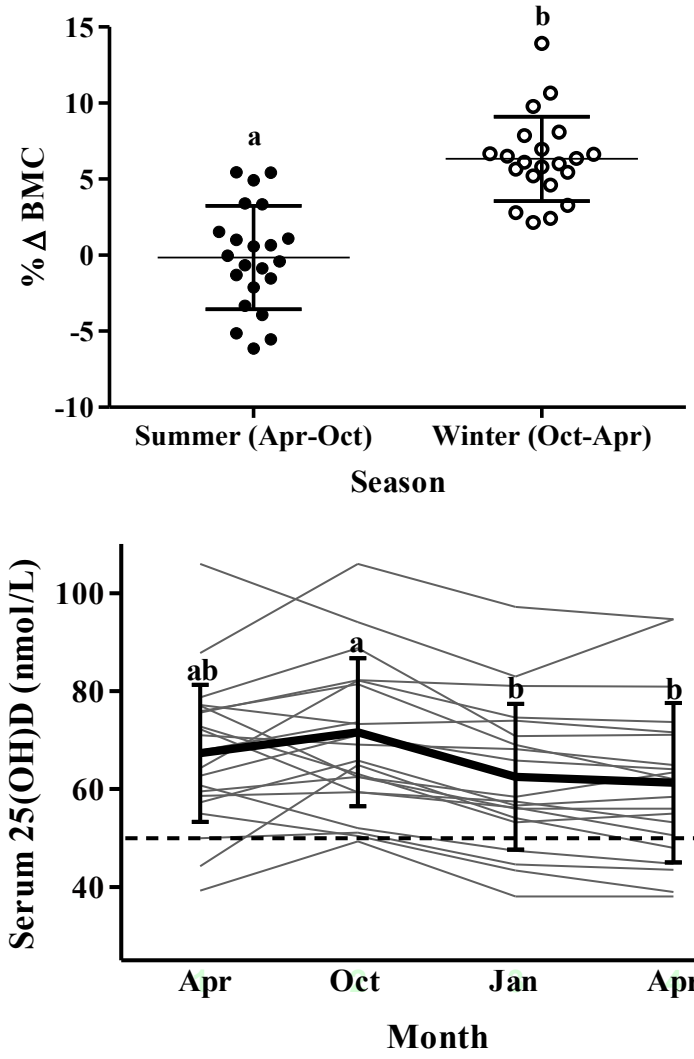
Figure 6.1 In healthy 2-8 y old children consuming their normal vitamin D intake from April 2014 to April 2015, 25(OH)D at all 4 time-points (Panel A). Spearman correlations between summer % Δ in lean mass and baseline 25(OH)D (Panel B). Lean mass % Δ (Panel C) during summer and winter in the subgroup. [a,b] Different superscripts depict significant differences ($p < 0.05$) using a mixed model ANOVA adjusted for physical activity, age, sex, ethnicity, height velocity and BMI Z-score. Panel A, C: Apr, Oct, Jan n=21, Apr 2015 n=19. Panel B: n=36 (3 children did not have DXA). Data are mean (SD) as a group and individual participant data over time.



It is well recognized that vitamin D is important for bone health and development. The various roles of 25(OH)D in bone health are described in chapter 2. Vitamin D interventions of 2 y and 9 mo, respectively, showed positive effects on whole body BMC and BMD (30) and lumbar spine BMD (31) in young children. However, a 2011 meta-analysis found that with vitamin D status > 35 nmol/L vitamin D supplementation trials in children with BMC and BMD outcomes did not show significant results (32). Agreeing with this, study 2 in this thesis did not see differences between groups for any of the bone health outcomes. The International Society for Clinical Densitometry states that 6-12 mo is the minimum time to examine changes in DXA bone parameters in children (189), meaning that the 6 mo length of our study may have not been long enough. However, from 2-8 y old children consuming their usual vitamin D intake from April 2014-2015, bone mineral accretion was higher in winter than summer, when adjusted for physical activity, age, sex, ethnicity, height velocity and BMI Z-score (**Figure 6.2**). Interestingly, for every 5% increase in summer lean mass accrual, there were 2.6% and 5.5% increases in whole body and lumbar spine bone mineral accrual during the following winter.

Interactions between muscle and bone are complex but it is well known that muscle loading induces biomechanical signals for bone growth and remodeling, commonly referred to as the mechanostat theory (290). It is possible that physical activity of children from my trials has an important role in the lean mass and bone relationships described above. But, it is challenging to quantify the effect of physical activity on bone in our trials because almost all children were meeting the daily physical activity recommendations (≥ 60 min/d). Also, it is possible that there is inaccuracy in our physical activity data because activity was measured using surveys completed by parents about their child's activity throughout days at school or daycare.

Figure 6.2. Bone mineral (BMC) % Δ (Panel A) in summer and winter and 25(OH)D at all 4 time points (Panel B) in children 2-8 y consuming their normal vitamin D intake. [a,b] Different superscripts depict significant differences ($p < 0.05$), using a mixed model ANOVA adjusted for physical activity, age, sex, ethnicity, height velocity and BMI Z-score. Apr 2014, Oct 2014 and Jan 2015: n=21, Apr 2015: n=19. Data are mean (SD) as a group and individual participant data over time.

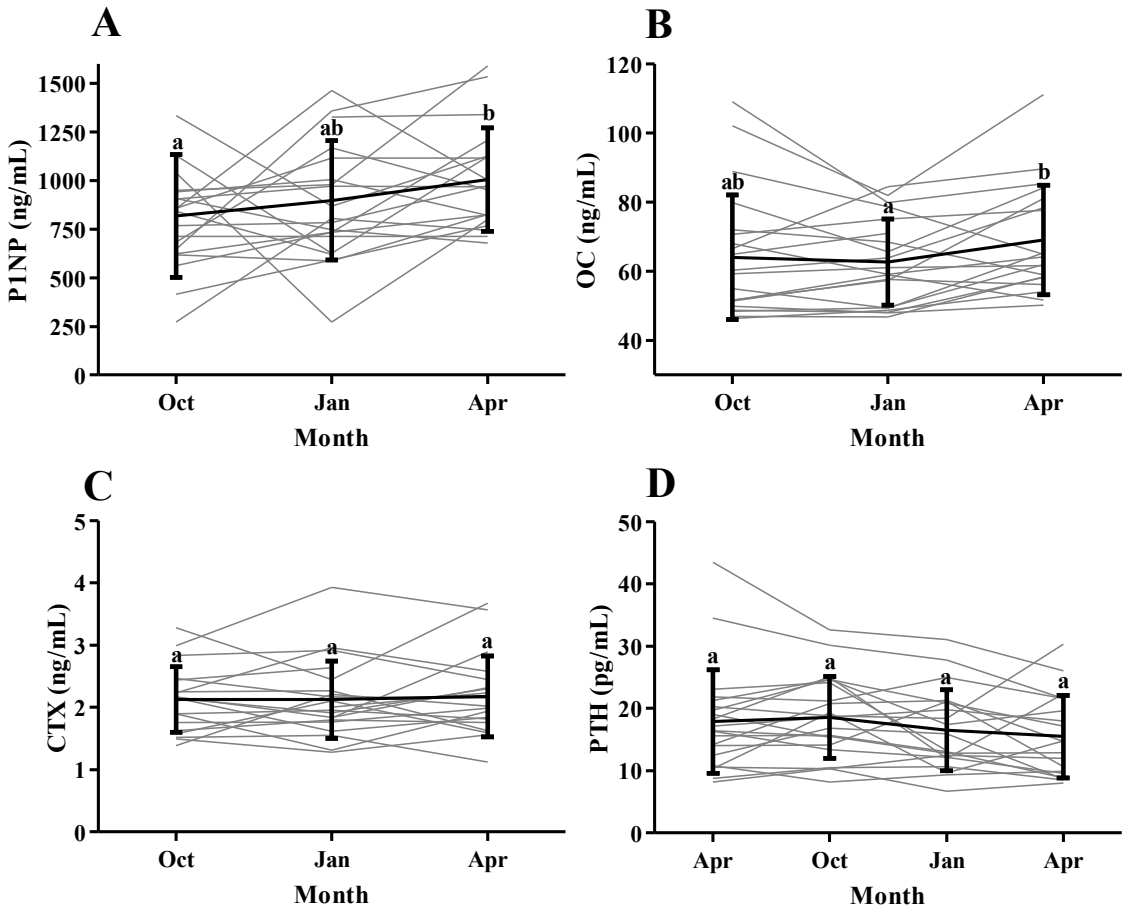


A 2007 meta-analysis on the effect of exercise on bone outcomes (measured by dual energy x-ray absorptiometry) in children showed that bone mineral accrual increases significantly in pre-pubertal children (0.9-4.9%) due to 6 mo exercise interventions and that this effect was greater than in pubertal children (0.3-1.9%) (291). Agreeing with these results, a 2014 systematic review of trials using 3-dimensional imaging techniques showed that weight bearing exercise interventions increased cortical thickness and cross-sectional area by 3-4% (292). A more recent 4 y exercise intervention in healthy children (baseline age: 6-9 y), showed that girls had significant increases in mid-tibia cortical area (5.0% (95% CI, 0.2% to 1.9%)) and girls and boys both had significant increases in cortical thickness (girls: 7.5% (95% CI, 2.4% to 12.6%), boys: 5.2% (95% CI, 0.4% to 10.0%)) (293). In comparison to the 2011 meta-analysis of vitamin D and bone health outcomes (i.e., no significant effect on bone parameters when status > 35 nmol/L) (32), these results from exercise interventions suggest that exercise may play a more important role than vitamin D for maximizing bone mineral accrual in healthy children.

It is also evident that additional endocrine and paracrine crosstalk is present between muscle and bone, including the many myokines have been proposed to effect bone including myostatin, interleukin-6 (IL-6), and fibroblast growth factor-2. Irisin, a recently discovered exercise induced myokine may also have a positive effect on bone by increasing cortical bone mass (294, 295), but more work is needed to properly elucidate its function. Beyond this, bone secretes a range of osteokines including FGF-23 (290). Bone also secretes osteocalcin that stimulates insulin secretion as well as insulin sensitivity and increased energy expenditure in muscle tissue (296). This means that the significantly increased osteocalcin concentration shown in **Figure 6.3** may be acting on muscle metabolism. Also, increased osteocalcin and P1NP, without changes in CTX and PTH supports that bone formation can be increased without

affecting resorptive activity. Lastly, IGF-1 is considered to have an integral role in cortical bone development and in muscle-bone crosstalk since both tissues can secrete IGF-1, resulting in autocrine and paracrine actions (297). In adolescent boys and girls, plasma IGF-1 was positively associated with bone mineral accrual (298) and in children 8-11 y was positively associated with fat free mass index (a measure of the composite amount of bone and muscle for a given height) (298). Plasma IGF-1 was also associated with bone mineral accrual and bone alkaline phosphatase in children 4-8 y (298) and late-pubertal girls (299), respectively. It was recently stated by Breen, et al. (298) that using DXA measures of bone mineral would result in not being able to measure the effect of plasma IGF-1 on bone because IGF-1 promotes periosteal mineral deposition, not detectable by DXA. In the 12 wk and 6 mo trials shown in chapter 3 and 4, it is possible that IGF-1 production in the control group is the reason there were no differences in bone outcomes between groups. Milk intake has been consistently linked to linear growth in healthy children, possibly partially due to the high tryptophan content (twice as high as meat) (300). Tryptophan acts to induce the IGF-1-mTORC1 (mammalian target of rapamycin complex 1) pathway, increasing cell growth and proliferation and thus increasing endochondral ossification (298). Studies in Danish children 2-5 y (301) and 8 y (302) both showed that milk consumption was associated with increased serum IGF-1 concentration. Because of the design of our studies, control and intervention groups were both consuming milk products and had average milk and alternatives intakes > 2 servings/d. This highlights a possible need for a vitamin D supplement intervention in Canadian children to help clarify if milk product intake is confounding the vitamin D and bone relationship.

Figure 6.3 Osteocalcin, type 1 procollagen N-terminal propepetide (P1NP) and c-terminal telopeptide of type 1 collagen (CTX) at 6, 9 and 12 mo (Panels A-C) and Parathyroid hormone (PTH) and 25(OH)D at all 4 time points (Panel C) in children 2-8 consuming their normal vitamin D intake. [a,b] Different superscripts depict significant differences ($p < 0.05$), using a mixed model ANOVA, with post hoc Bonferroni correction. Apr 2014, Oct 2014 and Jan 2015: n=21, Apr 2015: n=19. Data are mean (SD) as a group and individual participant data over time.



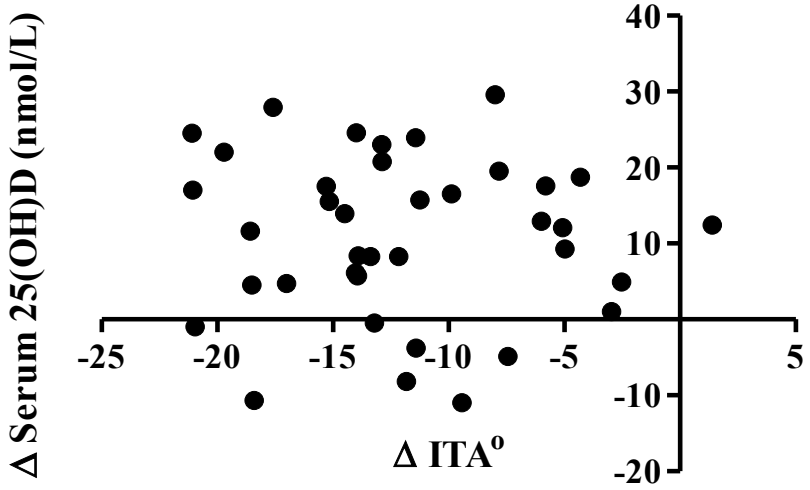
Because early childhood is still a period of rapid development, it is possible that epigenetic regulation (gene methylation) of genes, induced by vitamin D deficiency, could impact muscle and bone. CYP27B1, CYP24A1, CYP2R1 and importantly, VDR can be repressed through DNA methylation. The VDR interacting repressor (VDIR) can act as a co-repressor when VDIR contains histone deacetylase instead of histone acetyltransferase (303). This step is followed by DNA methylation through a DNA methyltransferase at CpG sites in both the promoter and exon regions of the CYP27B1 gene and this methylation contributes to VDR repression. Several genes responsible for muscle and bone development can be also be methylated and repressed including IGFBP3, bone morphogenic protein 2 and receptor activator of NF-Kappa B ligand (bone growth and development) (304-306). Studies in young children are needed to investigate these epigenetic effects related to vitamin D.

6.3 FACTORS AFFECTING VITAMIN D STATUS AND PUBLIC HEALTH IMPLICATIONS

There are numerous factors that may affect the relationships of vitamin D intake and status shown in chapters 3-5. By discussing important factors related to vitamin D intake or status, this section allows an in-depth discussion of vitamin D recommendations for children and highlights areas of further research needed. First, within studies 1 and 2, there was not only variation in vitamin D status, but also in the response to the intervention. As described in chapter 2, a number of trials (125, 128, 129), as well as a meta-analysis (127), have shown that single nucleotide polymorphisms of DBP, CYP2R1 and CYP24A1 most likely significantly effect vitamin D status. Canadian data is now needed to gain a more detailed understanding of how genetic variation within the Canadian population is affecting the relationship of vitamin D intake and status.

Second, the large decrease of 25(OH)D (~30 nmol/L) in the only previous trial with fortified foods over the entire winter, could partially be due to low vitamin D intakes in control groups (< 100 IU/d) (23). Though the higher mean vitamin D intakes (> 200 IU/d) in study 1 and 2 of this thesis may have been protective against the winter 25(OH)D decrease, other factors probably contribute as well. The fortified breads in the Danish study had 30% less vitamin D on average than the target whilst our 12 wk and 6 mo trials had vitamin D content of the fortified foods independently verified to be within ± 8% of the target. Also, children from our 12 wk and 6 mo trials may have a greater storage depot of vitamin D in adipose tissue that can be potentially mobilized. In Montreal, there is an average of 44% of daylight hours with bright sunshine (2000 total daylight hours/y) (307), however, in Denmark only 36% of daylight hours have bright sunshine (1600 total daylight hours/y). Montreal is also 10 degrees latitude farther south than Gladsaxe, Denmark, meaning that endogenous UVB synthesis could happen for a greater proportion of the year. Thus, having a greater potential for vitamin D endogenous synthesis and storage, children in Montreal may be partially protected from 25(OH)D winter decreases. Importantly however, stores of vitamin D may not be adequately reflected by serum 25(OH)D concentration since vitamin D storage in adipose tissue can happen within 24 hours (135) proportionally to the 25(OH)D concentration in circulation. This is illustrated by data from children 2-8 y showing vitamin D status during summer was not significantly related to tanning of skin (**Figure 6.4**).

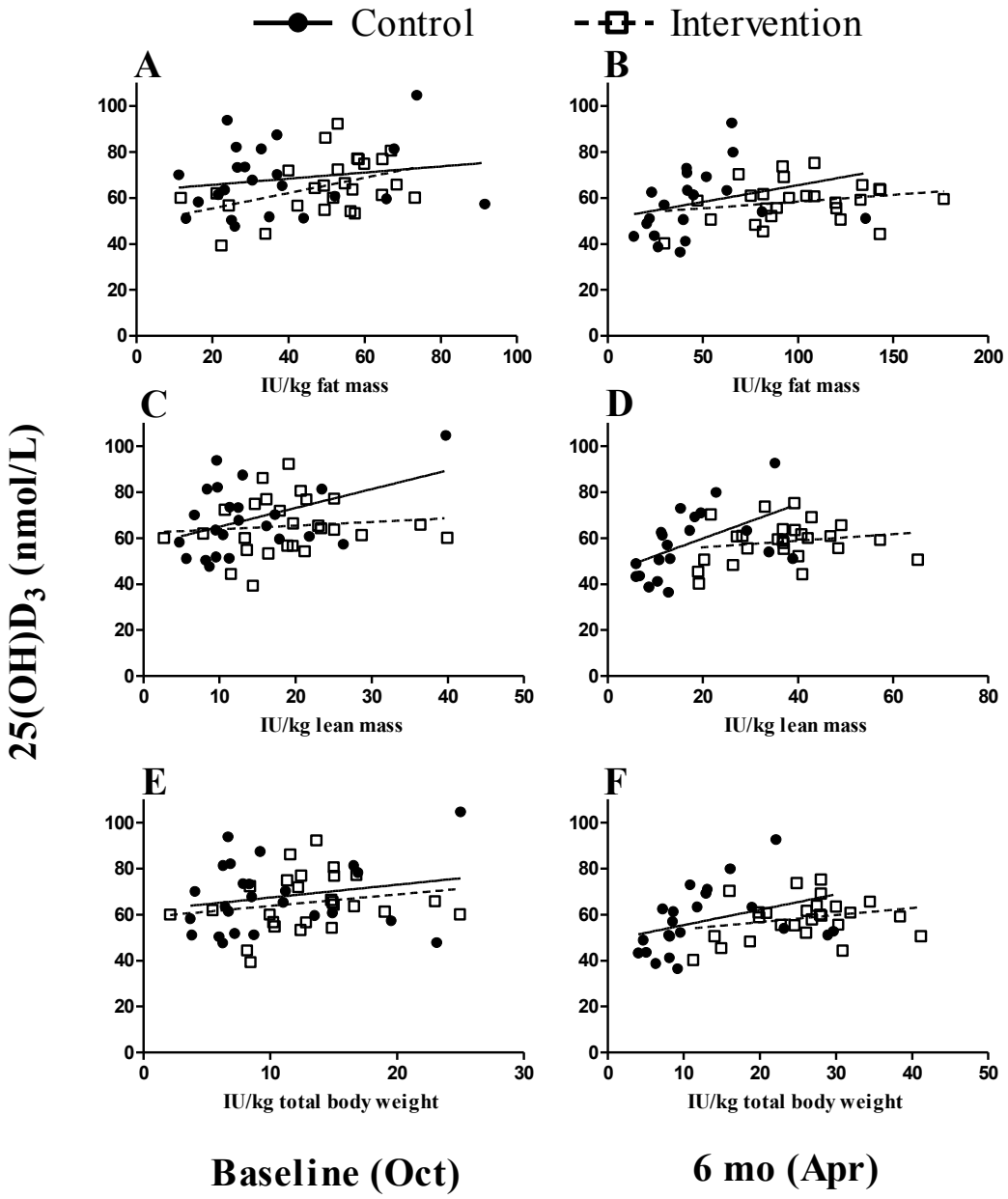
Figure 6.4 The April to October change in 25(OH)D and individual typological angle (ITA) for children 2-8 consuming their normal vitamin D intake (n=38). The more negative the change in ITA, the greater the tanning of the skin. Linear regression uncorrected for body composition and corrected for body composition showed the summer Δ ITA was not a significant predictor of Δ 25(OH)D ($p > 0.05$).



It is however, unclear how fluctuations of adiposity can affect mobilization of vitamin D from adipose tissue. Though as shown in **Figure 6.5**, adiposity did not have a linear relationship with vitamin D status in children from our trials, a 2007 study showed loss of adiposity over 1 y in obese children (mean age 12.1 ± 2.4 y) resulted in a 12 nmol/L increase in 25(OH)D concentration, compared to children who did not lose weight (308). Lastly, because our 6 mo trial showed that the C3 alpha epimer of 25(OH)D was at low concentrations in almost all participants and that 24,25$(OH)_2$D did not further help to explain individual differences in 25$(OH)D_3$, dietary vitamin D must have been stored. Overall, the above results show that our trials may not be generalizable to obese children. It is also apparent that research is needed to further elucidate how adiposity effects vitamin D storage and the magnitude to which adipose stores of vitamin D can protect from winter decreases in 25(OH)D concentration.

Though age was not a factor in the effect of vitamin D interventions in chapter 5, possibly due to the high heterogeneity of studies, the sister trials of children 4-8 y (40) and 14-18 y (250) found intakes of 800 IU/d and > 1200 IU/d were needed for the respective ages to have 97% of participants with 25(OH)D > 50 nmol/L. It is possible differences between age groups of Canadian children may be seen, however, these doses are not applicable due to the average vitamin D intakes, statuses and possibly sun exposure being different in Canadian children. **Figure 6.5** shows that the control group from the 6 mo trial had significant correlations of vitamin D intake/kg lean mass with serum 25(OH)D at both baseline and 6 mo. Correlations of vitamin D intake/kg of fat mass, lean mass and total body weight are not correlated with vitamin D status in the intervention group at any time-point, agreeing with the vitamin D meta-analysis of chapter 5 showing that age is not a significant factor influencing the relationship of vitamin D intake and status.

Figure 6.5 Vitamin D intake per kg fat mass (Panels A, B), lean mass (Panels C, D) and total body weight (Panels E, F) compared to serum 25(OH)D_3 concentration in children 2-8 y in 6 mo trial Oct (control: n=25, intervention: n=26) and Apr (control: n=22 (1 DXA scan missing), intervention: n=26). All spearman correlations were not significant ($R^2 < 0.10$, $p > 0.05$) except for the control group in Panels A, C and D (R^2= 0.18-0.26, $p < 0.05$).



When thinking about vitamin D recommendations for children, the European Union in 2016, came to the conclusion that there was insufficient evidence to derive a vitamin D EAR and RDA (309). As shown in the meta-analysis of chapter 5, the vitamin D fortification and supplementation studies had large variation in regard to the intervention effect on 25(OH)D, thus possibly agreeing with the above point. Heterogeneity was due to multiple factors including the large age range (2-18 y), location and length of interventions, dose of vitamin D, baseline vitamin D status, vitamin D intake of the control group and method of measuring 25(OH)D. This makes it significantly more challenging trying to derive vitamin D intake recommendations from this data. Complicating this, trials with vitamin D status below that of the respective national population status may have overestimated vitamin D intake requirements. In adults, the DRI for vitamin D were set with goals of preventing osteoporosis and adequacy of bone health outcomes (16). In this manner, it would seem that vitamin D intakes of 400-600 IU/d may not be needed in children to support bone health, based on our 12 wk and 6 mo trials as well as others (32). However, with a spark of controversy, The Endocrine Society rebuked the IOM recommendations by setting a 25(OH)D target of 75 nmol/L (33). More evidence is needed before using this vitamin D status cut-off in children, because there are very few trials in children maintaining ≥ 75 nmol/L, as illustrated in chapter 5. Multiple factors for these recommendations were considered, including maximizing calcium absorption, avoiding vitamin D deficiency and a plateau of PTH.

The consumption of vitamin D fortified foods in Canada, and average vitamin D status > 60 nmol/L (7), would most likely result in lower vitamin D doses to meet recommendations. The 96% of children in both intervention groups of our 12 wk trial maintaining 25(OH)D ≥ 50 nmol/L (matching the criteria for the RDA), differs from the 85% of children in the intervention

group of the 6 mo trial who maintaining 25(OH)D ≥ 50 nmol/L from October to April. Due to the design of our 6 mo trial only having 1 intervention group (400 IU/d), it is possible that the RDA intake of 600 IU/d would have maintained 25(OH)D concentration ≥ 50 nmol/L over the winter. However, results of the 12 wk and 6 mo trials do both agree that 400 IU/d exceeds the definition associated with the EAR (50% of children maintaining 25(OH)D concentration ≥ 40 nmol/L). Overall, the results of these two trials show that a study spanning the UVB void period using an intervention of 600 IU/d for young children in Canada, could be further informative towards public policy. Results from a further 600 IU/d vitamin D intervention study may be applicable to children outside of Canada as well because the 2016 European Union AI for vitamin D (600 IU/d), is the same for children as the IOM RDA.

Results from chapter 5 show that fortified foods are a more efficacious vehicle for vitamin D interventions than supplements. Because supplements are only used by the minority of children and have a wide range of doses, food fortification strategies should continue to be used and investigated. This meta-analysis also showed that there was variation in results between 25(OH)D measurement techniques. Chemiluminescent and radioimmune assays may overestimate or underestimate vitamin D status (272) and lack the specificity to measure different vitamin D metabolites. To help in setting future recommendations by decreasing heterogeneity, using LC-MS/MS techniques should be encouraged. The effect on vitamin D status may not differ in children based on location, sex or if vitamin D status is between 30-49.9 nmol/L versus ≥ 50 nmol/L. Thus, this thesis provides detailed evidence for policy makers in Canada and abroad when setting vitamin D recommendations for young children.

6.4 STRENGTHS AND LIMITATIONS

Findings from this thesis provide a thorough evaluation of the vitamin D intake recommendations and, more broadly, the relationship between vitamin D intake and status. To quantify vitamin D intake, the 12 wk and 6 mo trials not only used 24 h recalls but also utilized a validated 30 day FFQ for vitamin D intake (310). This FFQ associated strongly with observed vitamin D intakes of children and is able to display longer term vitamin D intake that is not possible for 24 h recalls without multiple day diet records (206). In both trials, the vitamin D intake of children, when not consuming the intervention products, was similar to the national average of vitamin D intake for children 4-8 y (244 ± 16 IU/d) (12). Using fortified foods that were popular with young children, and were appropriately sized was another strength of these trials. These products were independently tested by third parties for vitamin D content, which was within 10% of goal content for all products. By having companies provide products unlabelled and pre-coded with intervention group codes, it allowed all research staff to stay blinded to the randomization. Though compliance was high, it was measured by parent reported calendar check-sheets (**Appendix 1**), which is a weaker method of compliance measurement than the labour-intensive method of participant observation. After the 12 wk trial, parents were given surveys asking about how products could be improved for children (**Appendix 1**) and the main weakness revealed was that without multiple flavours of products, it was more likely for children to get flavour fatigue, and have decreased compliance. Products were shipped in batches to participants, which only presented challenges when families did not have sufficient fridge space for 2-4 wk worth of products.

Studies 1 and 2 were both double blinded randomized placebo-controlled trials (RCTs). This type of study design is seen as the gold standard and is thus most effective for informing

guidelines and public policies (311). Both trials were designed, carried out and reported following the CONSORT statement guidelines. The CONSORT statement is meant to reduce the risk of bias of reporting results from RCTs (internal validity) as well as improve the broader applicability of a trials results (external validity) (311). In line with this, studies 1 and 2 stated methods of randomization and allocation, blinding, analysis and funding. An intent to treat (ITT) analysis was used for both studies (analysis based on intervention allocation) to reduce the reporting bias (312). Data analysis of main outcomes was done blinded, to further reduce bias. For both studies, there were no differences among groups for baseline characteristics, showing that the randomization of participants was successful. By assessing many factors including physical activity, demographics and sun exposure it was possible to account for these variables in statistical analyses if needed. Using parent reported illness questionnaires **(Appendix 1),** we could measure illnesses of children and if they were taking medications that may compromise vitamin D status. By using a blood gas analyzer to immediately test for ionized calcium, and by acquiring complete blood cell counts at all time points of trials, we had safety measures for vitamin D status and for other health outcomes (hemoglobin, hematocrit, white blood cell counts). In study 1, although body composition outcomes were not investigated, body composition was assessed so that it could possibly be accounted for in statistical analyses.

A strength of the design of the 6 mo study was having a mid-point visit to better understand the changes of 25(OH)D over winter. This revealed that study 1 and 2 had consistent results, with significant 25(OH)D differences among groups at 12 wk. Average vitamin D status of the participants in both studies was similar to that of national data and the winter decrease of status in the 6 mo control group was similar to the expectation of 10-15 nmol/L (7). Though the 6 mo trial used LC-MS/MS to measure 25(OH)D, the 12 wk trial used chemiluminescent assays

on the Liaison autoanalyzer. The 6 mo trial also measured 25(OH)D using both Liaison assays and LC-MS/MS because the Liaison allowed analyses of samples soon after visits and was able to be used as a safety measure for vitamin D deficiency. LC-MS/MS methodology is recognized as having high precision and accuracy, whereas Liaison assays on average will under-report 25(OH)D by ~10 nmol/L according to DEQAS (272). Another strength of LC-MS/MS is the ability for this method to distinguish between vitamin D metabolites and quantitate metabolites that usually have low concentration like 24,25$(OH)_2$D (271). Interestingly, in our 6 mo trial, there were no significant differences between average concentrations from LC-MS/MS vitamin D status measurement (both as 25$(OH)D_3$ and total 25(OH)D) and total 25(OH)D concentrations from Liaison measurements (**Figure 6.6**). Though this may be expected when using stringent quality control methods, Bland-Altman plots of agreement show a large range of differences (~ ± 20 nmol/L) between LC-MS/MS and Liaison measurement of serum samples (**Figure 6.7**). These results suggest that continuing to use LC-MS/MS methods for vitamin D status measurement in future studies would be advisable.

Besides measuring vitamin D status, both studies measured PTH and the 6 mo trial also measured other biomarkers of bone metabolism, allowing an understanding of how bone is developing beyond what is shown in the bone imaging. By using DXA at baseline and 6 mo in study 2, it was possible to investigate whole body bone mineral and lean mass accrual as well as bone mineral accrual at lumbar spine and forearm sites. These scans were low radiation and relatively quick to complete with participants.

Figure 6.6 Comparison of the mean total 25(OH)D measured by Liaison with 25(OH)D_3 (Panel A) and total 25(OH)D (Panel B) measured by LC-MS/MS in children 2-8 y. Different superscripts depict significant differences between time-points using a mixed model ANOVA with post hoc Bonferroni correction. No differences between measurement methods. Oct: n=51, Jan: n=49, Apr: n=49.

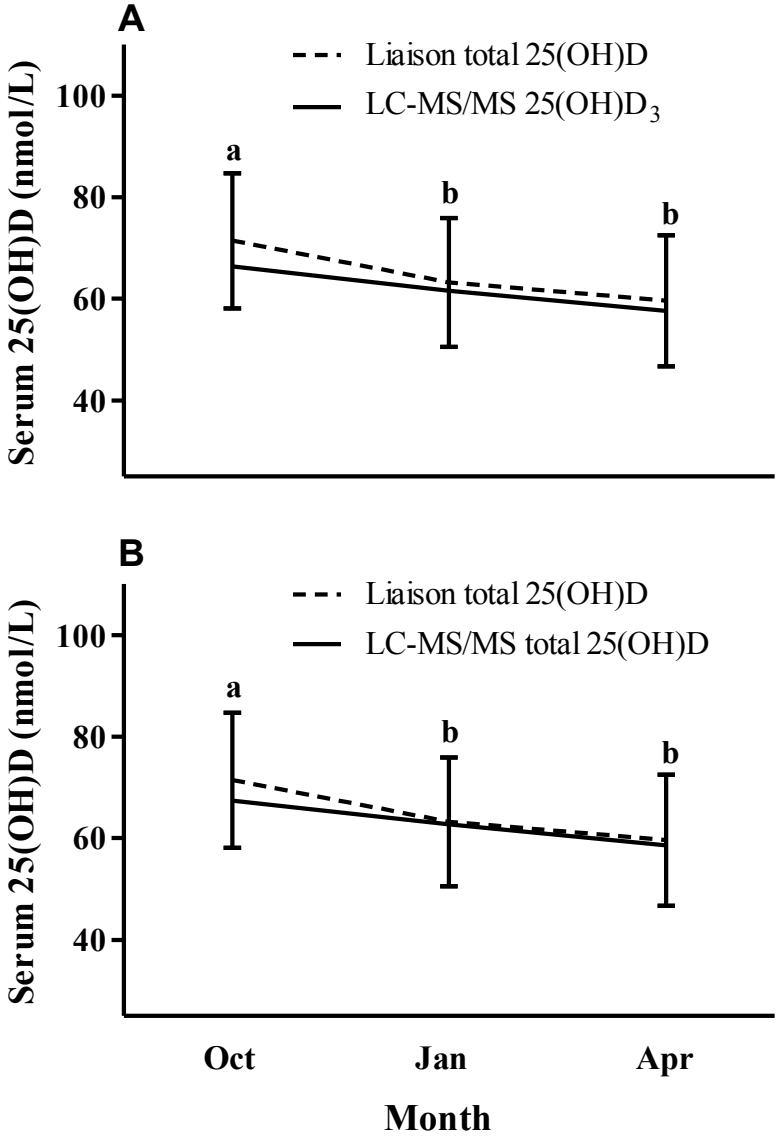
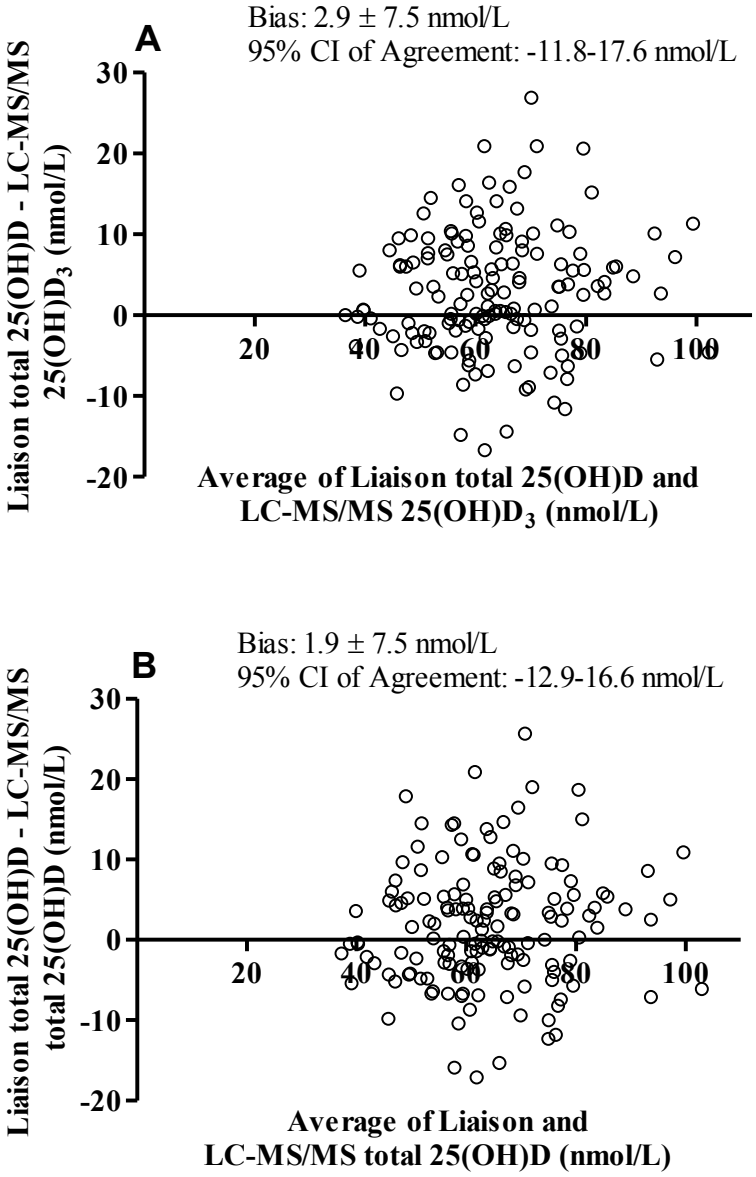


Figure 6.7 Bland-Altman plots of agreement between total 25(OH)D measured by Liaison and 25(OH)D_3 (Panel A) and total 25(OH)D (Panel B) measured by LC-MS/MS at all 3 time-points (Oct: n=51, Jan: n=49, Apr: n=49) in children 2-8 y.



Using 3-dimensional CT scans to compare with DXA, further strengthened these studies. However, a weakness of our design, was only using acquiring pQCT scans at the endpoint of study 2. Because of participant burden, radiation dose and lack of standardized methodology, it was not feasible to complete pQCT scans at baseline and endpoint. Since pQCT scans could be acquired in children as young as 2 y, these methods (described in study 2) could be utilized in further longitudinal research. However, the challenge of using pQCT longitudinally is that by only measuring a slice of growing bone, you will not be measuring the same exact location over time, making interpretation of results more challenging. Our methods were also designed based on the only normative pQCT data in young children (200), making them more broadly applicable. Lastly, our trials also lacked the measurement of muscle strength, meaning it is unknown if the lean mass accretion of the intervention group of study 2 also led to increases in strength. Future work could incorporate force plates to measure jumping force and velocity, methods that have been previously used in children (184).

Following the CONSORT statement guidelines for reporting clinical trials (311) in our 12 wk and 6 mo studies minimized biases to do with all aspects of the studies. Following the PRISMA guidelines (256) standardized reporting and analyses used and ensured the literature search was thorough and systematic. Only including placebo-controlled RCTs strengthened the design of the analysis and decreased bias. The effect of vitamin D intake on status, showed the importance of accounting for baseline status and subgroup analyses elucidated factors significantly effecting the vitamin D intake and status relationship. Importantly, by using trials from around the world, it was shown that the vitamin D intake of the control groups had a linear relationship with the change in 25(OH)D, with low vitamin D intakes (≤ 100 IU/d) having significantly greater decreases in 25(OH)D during winter. Lastly, by qualitatively and

quantitatively assessing bias, this meta-analysis highlighted specific areas of bias that were common among trials.

For study 1 and 2, sample size estimates were based on the primary objective of vitamin D status. Though statistical differences were shown among groups in study 1 and at the 3 mo time-point of study 2, a larger sample size would have provided more confidence. This is because the differences in 25(OH)D concentration between control and intervention groups was not as large as anticipated (hypothesis: > 20 nmol/L) based on previous food fortification trials (23, 25). However, the standard deviation of 16 nmol/L based on previous data in Montreal preschool age children was either like those in study 1 and 2, or higher than the standard deviations found in studies 1 and 2. In study 1, the sample size of 25/group allowed the detecting a 55% difference in the change of 25(OH)D between intervention and control groups. Thus, a larger sample size would be needed to detect smaller differences; although a smaller difference would not be meaningful. In study 2, the 25(OH)D difference between groups was 7.4 nmol/L at 3 mo and 1.8 nmol/L at 6 mo (SD:~ 15 nmol/L), meaning at 3 mo this allowed detecting a 38% difference between groups and a 7% difference at 6 mo. A sample size of 73 participants per group would be needed for a power of 80% at 3 mo and the 1.8 nmol/L difference at 6 mo is too small to realistically expect to measure differences between groups. Though between group differences in lean mass accretion were measured in study 2, it is unclear if this analysis was underpowered to measure differences in bone accretion. For whole body, appendicular and legs only lean mass accretion, the power for detecting differences between groups was 42%, 55% and 76%, respectively, meaning that the largest sample size would be needed when looking at whole body lean mass accretion to have a power of 80%.

6.5 FUTURE DIRECTIONS

This thesis has provided an advanced understanding of the relationship between vitamin D intakes and status in healthy young children, yet many new questions have arisen because of this work. Future vitamin D interventions in young children in Canada should test the vitamin D RDA over the entire winter as well as testing the recommended intakes year round. Having a population that is representative of a city, like the previous cross-sectional study in Montreal (1) would give added strength and broader applicability. Standardized methods of measuring 25(OH)D and other vitamin D metabolites, likely using LC-MS/MS techniques, would increase the comparability between trials. Vitamin D intervention trials in Canadian obese children need to be undertaken as obese children, may require different vitamin D recommendations. Vitamin D trials in other countries testing intake recommendations need to use populations that reflect national 25(OH)D data. With national data in mind, Canadian vitamin D recommendations would be strengthened by having better representative 25(OH)D data across the age range of young children (in summer and winter). To better understand vitamin D metabolism in children, stable isotopes of 25(OH)D (313) to distinguish endogenous production from exogenous intake or biomarkers of enzyme activity (314) could be used in conjunction with interventions. Lastly, by quantifying how phenotypes of the vitamin D binding protein affect 25(OH)D binding (85), or other enzymes involved in vitamin D metabolism affect 25(OH)D concentration (315), the relationship between vitamin D intake and status could be better understood.

Future trials should also explore how to improve vitamin D intakes in children using existing foods. The majority of vitamin D intake for children in Canada comes from the milk and alternatives food group, and more than one third of children 2-8 y do not meet daily intake recommendations for milk and alternatives (12). To improve milk and alternative intake there are

multiple knowledge gaps that need to be addressed. First, parental intake of milk and alternatives may be predictive of the intake of their children, though it has only been reported in American adolescents (316). Also from this American research, serving children milk and alternatives at meals was shown increases total intake of this food group (316). Second, research is lacking when it comes to the level of parental knowledge about serving sizes and recommended intakes for their children. Interestingly, both the perceived healthiness of foods and the perceived importance of nutrients may have a significant influence over what parents provide their children (317, 318), however, research in the Canadian population is lacking. Lastly, it is possible that there are multiple barriers to the consumption of milk and alternatives, including lack of time to prepare food, lack of money to purchase food, children disliking the taste of milk products or preferring sugar sweetened beverages to milk (319, 320).

Study 2 made it obvious that a longer trial in Canadian children is needed investigating vitamin D and bone health outcomes. Biomarkers of bone metabolism should continue to be included in longer studies, to increase the understanding of the bone and vitamin D relationship. Portable bone densitometers may make it possible to investigate longitudinal bone health outcomes in subgroups of the population not near urban centers (321), however they have not been validated longitudinally for changes in bone density in young children. More work is necessary to elucidate if forearm DXA scans in children have sufficient agreeability with lumbar spine or whole body scans to be used clinically. Longitudinal analysis in Canadian young children is also needed using 3-dimensional imaging like pQCT. Study 2 illustrated that standardized pQCT methodology is also needed for children to increase comparability between studies and replicability. A systematic review could be done to illustrate and compare published pQCT methodology in children. Since CHMS will soon begin to use DXA and pQCT as part of

its national data collection, results from this forthcoming work could help inform further bone health research in children.

When studying how vitamin D intake affects muscle development in children, measures of muscle strength are needed to compliment DXA or pQCT measures of lean mass or muscle. Longer trials in children could elucidate if lean mass accretion rates increase enough during summer, due to endogenous vitamin D synthesis, to make up for the significant differences that study 2 showed in winter. Epigenetic studies in young children are needed to elucidate if sufficient vitamin D status results in a leaner body phenotype. Included in this, measurement of IGF-1 and IGFBP3 would help to further understand the role that IGF-1 has in the function of vitamin D in muscle. Future studies also need to investigate muscle and bone interactions over periods of time longer than 6 mo since it is possible that initial improvements in lean mass accretion could lead to improvements in bone mineral accretion (322). Studies should also investigate whether having serum 25(OH)D $\geq$ 75 nmol/L is beneficial to functional outcomes, as was suggested cross-sectionally in young Montreal children (29). Future follow-ups of the groups of children who participated in study 2 could enable the study of possible long term benefits of an increased vitamin D intake.

6.6 CONCLUSIONS

In conclusion, this thesis has shown that vitamin D intakes of the EAR or RDA can significantly improve vitamin D status over part of winter. However, over the entire 6 mo winter period, an EAR equivalent vitamin D intake did not improve status or bone health outcomes but did significantly improve lean mass accretion. How vitamin D intake affects status in children significantly differs based on baseline 25(OH)D, vitamin D intake of the control group, method of vitamin D intervention and method of measuring 25(OH)D. This body of work significantly

contributes to the knowledge of the adequacy of vitamin D recommendations for children and the relationship between vitamin D intake and functional outcomes. It will be an integral part of future updates to vitamin D recommendations for children and food fortification guidelines. Although intake recommendations may not change in the future, vitamin D status outcomes beyond circulating 25(OH)D could be important to consider as part of these recommendations. Future longer-term investigations of vitamin D related functional health outcomes will help confirm our findings and bring a stronger understanding to this field of research.

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APPENDIX 1. PERTINENT QUESTIONNAIRES AND FORMS

Illness Form For 12 wk And 6 mo RCTs.

Participant ID: HW-14-01-P2-	**Randomization Code:**
YOUR CHILD'S INFORMATION	
Date of illness: Start: DD / MM / YYYY End: DD / MM / YYYY	**Diagnosis (type of illness):**
Temperature: _____ °C / °F	**Person completing questionnaire:** Mother □1 Father □2

Mark this line with an 'X'

Worst possible health l----------l **Best possible health**

Check off all boxes that apply to this illness	No Problem1	Minor problem2	Moderate problem3	Major problem4	Don't know or Not applicable5
1. Poor appetite					
2. Not sleeping well					
3. Irritable, cranky, fussy					
4. Feels unwell					
5. Low energy tired					
6. Not playing well					
7. Crying more than usual					
8. Needing extra care					
9. Clinginess					
10. Headache					
11. Sore Throat					
12. Muscle aches or pains					
13. Fever					
14. Cough					
15. Nasal congestion, runny nose					
16. Vomiting					
17. Diarrhea					
18. Not interested in what's going on					
19. Unable to get out of bed					

20. Needing medical attention	Yes □1 No □2
21. Unable to attend school, daycare	Yes □1 No □2 If yes, how many days missed? _____
22. Need to take medication	Yes □1 No □2 If yes, which one? _____
23. Were other family members ill?	Yes □1 No □2 If yes, who? Mother □1 Father □2 Sibling □3 Other □4
24. Did the ill family member miss work, school or daycare?	Yes □1 No □2 If yes, how many days missed? _____

Illness Form Supplementary Questionnaire For The 6 mo Trial

Q.1 Did your child get a flu shot this winter?

- Yes ☐
- No ☐
- Decline to answer ☐
- Don't know ☐

Is this the same as previous years? _____

Q.2 Over the past 6 months did your child get any other shots or vaccinations?

- Yes ☐
- No ☐
- Decline to answer ☐
- Don't know ☐

If yes, which ones? _____

Q.2 Over the past 6 months did your child start any new medications?

- Yes ☐
- No ☐
- Decline to answer ☐
- Don't know ☐

If yes, which ones? _____

Q.3 How many days of school/daycare did your child miss due to illness over the past 6 months?

- 0 days ☐
- 1-3 days ☐
- 4-7 days ☐
- 1-2 weeks ☐
- > 2 weeks ☐
- Decline to answer ☐
- Don't know ☐

Q.4 In total, how many days of work did your family members miss due to illness of your child over the past 6 months?

- 0 days ☐
- 1-3 days ☐
- 4-7 days ☐
- 1-2 weeks ☐
- > 2 weeks ☐
- Decline to answer ☐
- Don't know ☐

Q.5 Is your child's health about the same as previous years during October-April?

- Sick more often than previous years ☐
- The same as previous years ☐
- Sick less often than previous years ☐
- Decline to answer ☐
- Don't know ☐
- Comments: _____

Q.6 Compared to other children their age that you know, how has your child's health been?

- Sick more often than other children □
- The same as others □
- Sick less often than others □
- Decline to answer □
- Don't know □

Comments: _____

Exit Survey About Fortified Yogurt and Cheese Products For Parents of Participants In The 12 Wk Trial

Thank you for participating in the D-Kids Study. Since you and your child have graduated from the study we would like to ask for advice from you that will help us to create new programs to help children as they grow.

Place a checkmark for the answer you feel is best for you and your family.

		Strongly disagree						**Strongly agree**
	Question	**1**	**2**	**3**	**4**	**5**	**6**	**7**
1.	I would prefer vitamin D be in foods rather than having to give a supplement							
2.	Overall my child liked the drinkable yogurt							
3.	Overall my child liked the cheddar cheese							
4.	a) The size of the drinkable yogurts was appropriate							
	b) If not appropriate, what size would be better?							
5.	Consuming 2 drinkable yogurts each day was easy for my child							
6.	a) The size of the cheese pieces was appropriate							
	b) If not appropriate, what size would be better?							
7.	Consuming the entire piece of cheese each day was easy for my child							
8.	It would have been helpful to have a sheet with tips of different							

	ways to consume the cheese and yogurt							
9.	Having two or more flavours of yogurt would have been preferable to 1 flavour							
10.	Having a different type of cheese besides mild cheddar would have been preferable							
11.	During the study do you think your child typically met Canada's Food Guide for milk and milk products							

12. Besides strawberry, what other flavour of yogurt would your child have liked?

_____

13. Besides mild cheddar, what other type of cheese would your child have liked?

_____

14. What do you think would be the best way to advertise/reach families for studies in the future?

_____

15. Would you be interested in having us call you in the fall with information about Phase 2 of the D-Kids Study? (check one box)

☐ Yes ☐ No

16. What was the most difficult aspect of this study if any?

_____

Do you have any other comments or suggestions?

30 Day Food Frequency Questionnaire

State how often (if ever) your child ate the following vitamin D-containing foods during the last month, and then indicate the number and average portion size.

Food Item	Frequency and # of servings: Never	Monthly	Weekly	Daily	Check Serving Size: (mark one only)
EXAMPLE: Milk for drinking (incl. Choc milk/hot cocoa with milk)			**10**		☒ 125 ml (.5 cup) ☐ 250 ml (1 cup) ☐ 375 ml (1.5 cup)
Milk for drinking (incl. Choc milk/hot cocoa with milk)					☐ 125 ml (.5 cup) ☐ 250 ml (1 cup) ☐ 375 ml (1.5 cup)
Milk on cereal or in soups					☐ 60 ml (.25 cup) ☐ 125 ml (.5 cup) ☐ 250 ml (1 cup)
Baby food cereal a) made with milk Quantity milk_____ b) made with water					☐ 60 ml (.25 cup) ☐ 125 ml (.5 cup) ☐ 250 ml (1 cup) ☐ 60 ml (.25 cup) ☐ 125 ml (.5 cup) ☐ 250 ml (1 cup)
Soy or rice beverage or orange juice with added calcium and vitamin D					☐ 125 ml (.5 cup) ☐ 250 ml (1 cup) ☐ 375 ml (1.5 cup) Soy or rice beverage ☐ orange juice ☐
Eggs and egg dishes (including yolk) (ex. Fried, hard boiled, omelettes, quiche).					☐ 1 (large) ☐ 1 (medium) ☐ 1 (small)
Fish: Including salmon (canned & fresh), mackerel, herring, oysters or tuna (fresh) Specify Types:					☐ 75 g (2 ½ oz) ☐ 150 g (5 oz) ☐ 225 g (7 ½ oz)
Fish: Including fish sticks, shrimp, sole/flounder or tuna (canned) Specify Types:					

Margarine Brand:_____					☐ 5 ml (1 tsp) ☐ 15 ml (1 tbsp) ☐ 45 ml (3 tbsp)
Yogurt Specify brands:					☐ 60 ml (.25 cup) ☐ 125 ml (.5 cup) ☐ 250 ml (1 cup) ☐ 30 g (1 oz) ☐ 60 g (2 oz) ☐ 90g (3 oz)
Cheeses (Including cheddar, mozzarella, Kraft Singles®, Cheez Wizz®, parmesan, gouda, edam, brie, havarti, feta, blue, and chèver)* Specify brands:					☐ 60 ml (.25 cup) ☐ 125 ml (.5 cup) ☐ 250 ml (1 cup) ☐ 30 g (1 oz) ☐ 60 g (2 oz) ☐ 90g (3 oz)
Ice cream and frozen desserts (including sundae, ice cream cone, Fudgesicle®) Specify brands:					☐ 60 ml (.25 cup) ☐ 125 ml (.5 cup) ☐ 250 ml (1 cup)
For breastfeeding and formula fed children enter daily total # of 1 oz feeds and frequency below. For weaned children enter "0".					
Breast milk					☐ 30 ml (1 oz) OR ☐ # feed/day
Infant formula Specify brand (if not mixed according to manufactures instructions please describe):					☐ 30 ml (1 oz) OR ☐ # feed/day
Additional sources of vitamin D					
Cod or Halibut Liver Oil					☐ 15 ml (1 tbsp) ☐ 30 ml (2 tbsp) ☐ 45 ml (3 tbsp)
Vitamin D or multivitamin supplement					☐ 200 IU ☐ 400 IU ☐ 800 IU Specify brands:
*excluding cream cheese and cottage cheese.					

Sun Exposure Questionnaire Over The Previous 30 Days

Q.01 Has your child spent time outdoors?

Yes □1 Between 10:00 and 16:00? Y □ N □

No □2

Q.02 Was his/her skin exposed to direct sunlight?

Yes □1 Between 10:00 and 16:00? Y □ N □

No □2

Q.03 Did you use sunscreen on your child?

Yes □1 *Brand and SPF:*

_____

No □2

Sometimes □3 _____*% of the time*

Q.04 On average, how many minutes per day was your child exposed to direct sunlight?

5 min or less □1 16 to 30 min □3

5 to 15 min □2 31 to 60 min □4

More than 1 hour □5

Q.05 Which parts of the skin were typically exposed?

Face □1 Neck □4 Hands □7 Feet □10

Shoulders □2 Upper Arms □5 Thighs □8 Chest □11

Legs □3 Forearms □6 Lower legs □9 Back □12

Q.06 Did your child wear a hat / sunhat?

Yes □1

No □2

Sometimes □3 _____*% of the time*

✈Q.07 Did your child travel to a warm sunny southern location over the past 30 days?

Yes □1 Where?

_____

No □2

✈Q.08 How many days did your child spend there?

_____

✈Q.09 How many hours per day was your child exposed to direct sunlight?

_____

✈Q.10 Which parts of the skin were exposed to the sunlight?

	Face	□1	Neck	□4	Hands	□7	Feet □10
	Shoulders	□2	Upper Arms	□5	Thighs	□8	Chest □11
	Legs	□3	Forearms	□6	Lower legs	□9	Back □12

✈Q.11 Did your child wear a sunhat?

Yes	□1	
No	□2	
Sometimes	□3	_____*% of the time*

✈Q.12 Did you use sunscreen on your child?

Yes	□1	*Brand and SPF:* _____
No	□2	
Sometimes	□3	_____*% of the time*

Compliance Check-sheet Calendar (January 2014)

Sun			Mon			Tue			Wed			Thu			Fri			Sat		
									1			2			3			4		
									Y	Y	C	Y	Y	C	Y	Y	C	Y	Y	C
5			6			7			8			9			10			11		
Y	Y	C	Y	Y	C	Y	Y	C	Y	Y	C	Y	Y	C	Y	Y	C	Y	Y	C
12			13			14			15			16			17			18		
Y	Y	C	Y	Y	C	Y	Y	C	Y	Y	C	Y	Y	C	Y	Y	C	Y	Y	C
19			20			21			22			23			24			25		
Y	Y	C	Y	Y	C	Y	Y	C	Y	Y	C	Y	Y	C	Y	Y	C	Y	Y	C
26			27			28			29			30			31					
Y	Y	C	Y	Y	C	Y	Y	C	Y	Y	C	Y	Y	C	Y	Y	C			