BONE METABOLIC RESPONSE TO NUTRITION+EXERCISE INTERVENTION IN PREGNANCY

MATERNAL CALCIOTROPIC AND BONE BIOMARKER PROFILES IN RESPONSE TO NUTRITION+EXERCISE INTERVENTION IN A RANDOMIZED CONTROLLED TRIAL IN PREGNANCY

BY

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A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

McMaster University

Department of Medical Sciences

DOCTOR OF PHILOSOPHY (2019) McMaster University

(Medical Sciences, Metabolism and Nutrition) Hamilton, Ontario

TITLE: Maternal calciotropic and bone biomarker profiles in response to a Nutrition+Exercise intervention in a randomized controlled trial in pregnancy

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NUMBER OF PAGES: xviii, 192

LAY ABSTRACT

Background: Adaptations in maternal bone metabolism during pregnancy and the post-partum period provide the offspring with the nutrients needed to mineralize their bones. Maternal diet and hormonal changes influence these metabolic changes.

Method: In 203 women recruited to the Bone-Be Healthy in Pregnancy Study randomized controlled trial, we compared changes in bone metabolism during pregnancy and at six months post-partum between women randomized to an individualized and monitored Nutrition + Exercise intervention or usual care (control) throughout pregnancy.

Results: The intervention group consumed more protein and calcium, but had similar and adequate vitamin D status. At the end of pregnancy, women in the intervention group had less bone loss compared to the control group, but all measures were similar at 6 months post-partum.

Clinical significance: The nutrition and exercise intervention reduced maternal bone loss during pregnancy, and could be a feasible intervention to support bone health of pregnant women.

ABSTRACT

Background: Pregnancy induces transient bone mass loss. Dairy foods might promote bone health, yet few interventions have been conducted to optimize maternal bone health in the perinatal period.

Objectives: To conduct a Nutrition+Exercise randomized controlled trial (RCT) in pregnant women to assess the impact on maternal bone health by measures of calciotropic and bone biomarkers at the end of pregnancy and in the post-partum period.

Study design: In the Be Healthy in Pregnancy (BHIP) RCT, 203/241 women consented at randomization (12-17 weeks (wk) gestation) to the bone health sub-study and received either usual care or a Nutrition+Exercise intervention that provided an individualized high protein diet (50% as dairy products) and a walking program throughout pregnancy. Maternal characteristics and fasting blood samples were obtained at 12-17 wk and 36-38 wk gestation, and at six months post-partum. Vitamin D status from the BHIP participants was compared to the FAMILY birth cohort participants (assessed at 24-36 wk gestation) to assess changes over a ten-year span. The response of the calciotropic and bone biomarkers to the RCT intervention was assessed at the end of pregnancy and in the post-partum period.

Results: Adequate vitamin D status in pregnancy was observed in 322 participants from

the FAMILY and 191 from the BHIP study, impacted by season and supplement intake. For participants in the BHIP study, serum 1,25-dihydroxyvitamin D concentrations increased throughout pregnancy and were not associated with serum 25(OH)D. Participants from the intervention group had lower serum bone resorption marker CTX compared to control group, which was reflected in cord serum. No differences were observed with other bone biomarkers at the end of pregnancy or in the post-partum period.

Conclusion: Higher protein and calcium intake compared to the control group during pregnancy minimized bone resorption, thus protecting maternal bone health in the perinatal period.

ACKNOWLEDGMENTS

First, I would like to thank my supervisor, Dr. Stephanie Atkinson. I am grateful for the many opportunities and the support you have offered me over the last years. You are an exceptional role model as a female in academia. It has been inspiring and rewarding to be working under your mentorship through my PhD. I would also like to thank my committee members, Dr. David Meyre and Dr. M. Constantine Samaan, for sharing their expertise and providing constructive feedback on my projects and progress. Thank you to Dr. Gerhard Fusch and Ms Caroline Moore for numerous discussions and expertise in analyzing my data and pushing me to think critically. Thank you to the funding agencies that made this work possible and who awarded me scholarships to dedicate my time to my research.

Thank you to all the FAMILY and BHIP families who have shared such an important moment of their life with us. Getting to know so many women and meeting their children have definitely been my favorite part of this work!

Thank you to everyone who has worked as part of the FAMILY and BHIP teams. The amount of work that has been put in this research is tremendous and everyone had played such an important part. Thank you for sharing the ups and downs of science.

Thanks to Sarthak and James, for reminding me that life isn't just about this PhD.

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LIST OF ABBREVIATIONS

DOHaD	Developmental Origins of Health and Disease
DXA	dual-energy x-ray absorptiometry
$1,25(OH)_2D$	1,25dihydroxyvitamin D
25(OH)D	25-hydroxycholecalciferol
RCT	randomized controlled trial
PINP	serum procollagen type I N propeptide
CTX	carboxyl-terminal telopeptide of type I collagen
PTH	parathyroid hormone
PTHrP	parathyroid hormone related peptide
IGF-1	insulin-like growth factor-I
BMD	bone mineral density
BMI	body mass index
DRI	Dietary Reference Intakes
EAR	Estimated Average Requirement
RDA	Recommended Dietary Allowance
IOM	Institute of Medicine
LC-MS/MS	liquid chromatography-tandem mass spectrometry
BHIP study	Be Healthy in Pregnancy study
Bone-BHIP Study	Bone-Be Healthy in Pregnancy study

GWG	gestational weight gain
ELISA	enzyme-linked immunosorbent assay
NIST	National Institute of Standards and Technology
FAMILY study	Family Atherosclerosis Monitoring In Early Life study
FFQ	food frequency questionnaire
ANOVA	analysis of variance
CIs	confidence intervals
SD	standard deviation
UL	Tolerable Upper Intake Level
CHMS	Canadian Health Measures Survey
CV	coefficient of variation
DEQAS	Vitamin D External Quality Assessment Scheme

FORMAT AND ORGANIZATION OF THESIS

This thesis was prepared in the "sandwich format" as outlined by the School of Graduate Studies in the 'Guide for the Preparation of Doctoral Theses'.

This thesis is comprised of 4 original research papers (Chapters 2-5), preceded by a general introduction and followed by a general discussion/conclusion.

Two papers are published, one has been accepted for publication pending minor revisions, and one is ready for submission.

All papers have the candidate as first author.

CONTRIBUTIONS TO PAPERS WITH MULTIPLE AUTHORSHIP

CHAPTER 2

Publication

Perreault, M; Atkinson, SA; Mottola, MF; Phillips, SM ; Bracken, K; Hutton, EK; Xie, F; Meyre, D; Morassut, RE; Prapavessis, H ; Thabane, L. *Structured diet and exercise guidance in pregnancy to improve health in women and their offspring: study protocol for the Be Healthy in Pregnancy (BHIP) randomized controlled trial*. Trials 2018, *19*, 1–15.

Contributions

SAA conceived the study as the principal investigator. Co-investigators collaborated for the grant applications and implementation of the core study including facilitation of recruitment of study participants. At the McMaster University study site, **MP** conducted the core study visits with the participants, with the exception of the intervention so to remain blinded to the group allocation. MFM was responsible for recruitment and measurements at the Western University site. **MP** performed laboratory analysis for all the biomarkers (for McMaster University and Western University sites), in collaboration with the study coordinator and conducted the DXA scans at McMaster as well as overseeing the conduct and analysis of the DXA scans at Western. **MP** wrote the paper. SAA and MFM oversaw the data collection. All authors read and approved the final manuscript.

CHAPTER 3

Publication

Perreault M, Moore CJ, Fusch G, Teo KK, Atkinson SA. *Factors associated with serum 25-Hydroxyvitamin D concentration in two cohorts of pregnant women in Southern Ontario, Canada*. Nutrients. 2019;11:123.

Contributions

MP conducted research for the BHIP study. **MP** performed laboratory work for the samples collected as part of the BHIP study, in collaboration with GF and CJM. **MP** analyzed data and performed all statistical analysis for both FAMILY and BHIP data. MP wrote the paper. SAA conceptualized and oversaw both studies, obtained the grant support and had primary responsibility for the final content. SAA and MFM oversaw the data collection. All authors read and approved the final manuscript.

CHAPTER 4

Publication

Perreault M, Atkinson SA, Meyre D, Fusch G, Mottola M. *Summer season and recommended vitamin D intake support adequate vitamin D status throughout pregnancy in healthy Canadian women and their newborns.* Accepted for publication pending minor revisions in the Journal of Nutrition, September 23 2019.

Contributions

MP conducted research, and performed laboratory analysis of the vitamin D metabolites in collaboration with GF. **MP** analyzed the data and performed statistical analysis. **MP** wrote the manuscript. SAA designed the study, obtained the grant support and had primary responsibility for the final content. SAA and MFM oversaw the data collection. All authors read and approved the final manuscript.

CHAPTER 5

Publication

Perreault M, Atkinson SA, Mottola MF, and the BHIP study team. *Individualized high dairy protein + walking program supports bone health in pregnancy: a randomized controlled trial*. To be submitted to American Journal of Clinical Nutrition, Fall 2019.

Contributions

MP conducted the core study visits with the participants, with the exception of the intervention so to remain blinded to the group allocation. **MP** performed laboratory analysis for all the biomarkers and the DXA scans, in collaboration with the study coordinator. **MP** analyzed data and performed statistical analysis. **MP** wrote the paper. SAA designed the study, obtained the grant support and had primary responsibility for the final content. SAA and MFM oversaw the data collection. All authors read and approved the final manuscript.

CHAPTER 1

INTRODUCTION

1.1. Clinical problem

Osteoporosis can be studied under the lens of the developmental origins of health and disease (DOHaD) as it has been hypothesized to have its root in early life (1). From the maternal perspective, it is hypothesized that pregnancy and lactation themselves may contribute to maternal osteoporosis later in life (2–4).

Both pregnancy and lactation induce bone mineral mobilization to accommodate the increased nutrient demands for fetal and infant skeletal formation, leading to a deterioration of maternal bone status (reviewed in (5)). Yet, perinatal maternal bone metabolism adaptations are poorly characterized (reviewed in (6)).

Vitamin D deficiency is often claimed as a major problem in pregnant women globally leading to potential adverse risks to mother and offspring (reviewed in (7)). Despite such claims, few studies have assessed dietary intake and serum vitamin D metabolites using gold standard methods prospectively throughout pregnancy. Gaps in knowledge exist regarding the maternal determinants of vitamin D status at different trimesters during pregnancy, and how it impacts maternal bone health outcomes (reviewed in (8)).

Maternal habitual dietary intake throughout pregnancy and post-partum are not often well documented in studies looking at bone health (reviewed in (5)) despite the importance of nutrition on normal bone turnover. As well, the duration and extent of lactation is not

often described, which could encompass various breast-feeding practices that influence maternal bone health status in the post-partum.

Dairy foods might promote bone health during pregnancy, with potential sustained effects in the post-partum period (9), being rich in protein, calcium and other bone-nutrients, such as vitamin D (if fortified) (10). Very few structured and personalized lifestyle interventions have been tested in randomized controlled trials (RCTs) conducted during pregnancy with the goal to optimize maternal bone health and monitor the sustained impact in the post-partum period. Studies have either focused on simply providing vitamin D supplements (reviewed in (8)) or calcium supplements during pregnancy (11) or lactation (12), with inconclusive findings on maternal bone outcomes. To our knowledge, only one study assessed the impact of a maternal calcium supplementation with milk powder on maternal bone outcomes in a Chinese cohort (13), showing a positive benefit to bone mass density. No RCT has evaluated the impact of a Nutrition+Exercise intervention on maternal bone health while completing a global assessment of dietary habits and bone metabolism, including measures of serum bone turnover markers and vitamin D metabolites, throughout pregnancy and the post-partum period.

1.1.1. Objectives

To address these knowledge gaps, the specific objectives of my thesis are:

1) To conduct a RCT in pregnant women focused on bone health and to publish this as a

RCT protocol of the BHIP study;

2) To conduct a comparative analysis of vitamin D intake and status in two cohorts of pregnant women studied ten years apart in Southern Ontario;
3) To characterize the vitamin D metabolite profiles across pregnancy, and identify the factors associated with maternal serum 25(OH)D concentrations during pregnancy;
4) To determine the effect of a maternal Nutrition+Exercise intervention in comparison to the control (usual care) group during pregnancy on maternal bone biomarkers from early (12-17 weeks) to late (36-38 weeks) of pregnancy, at six months post-partum and in cord blood; and maternal bone mass at six months post-partum.

1.1.2. Hypotheses

For objective 2, I hypothesized that the primary source of dietary vitamin D intake would be milk, and that total consumption of vitamin D would be higher in the cohort recruited in 2012-2014 when compared to the cohort recruited in 2004-2009 due to recent media hype about vitamin D's role in health, leading to higher serum concentrations of 25(OH)D in the recent cohort compared to the older one;

For objective 3, I hypothesized that maternal dairy product consumption, especially milk, would be associated with vitamin D status during pregnancy, and that serum concentrations of 25(OH)D would remain stable, but that serum $1,25(OH)_2D$ concentrations would rise significantly by the end of pregnancy;

For objective 4, I hypothesized that maternal high protein dairy consumption combined with exercise in pregnancy would reduce bone turnover during pregnancy, but the effects would not be sustained at six months post-partum.

1.2. Pregnancy changes in bone metabolism

1.2.1. Pregnancy and the developmental origins of osteoporosis

The developmental origins of health and disease (DOHaD) paradigm postulates that environmental factors such as maternal nutrition and lifestyle choices during pregnancy and in early life can modify the offspring physiological processes, impacting risks of non-communicable disease later in life (1). Osteoporosis is described by a systemic impairment of bone mass and microarchitecture leading to elevated risks of fracture (14). Although osteoporosis is hypothesized to have environmental, genetic and biological components, it can also be studied under the lens of DOHaD, as it has been proposed to have its roots in early life (1). Adequate bone accretion *in utero* has been observed to be dependent on maternal nutritional status (1). Bone accretion of the fetus *in utero* and in early life can program for peak bone mass, as it has been shown that childhood bone mass tracks until puberty (15) when peak bone mass is achieved. Sub-optimal peak bone mass is a predictor of fracture risk in later life (1). Adequate nutrient transfer to the fetus is dependent on the mother's nutritional status and overall health.

Beyond the focus put on the offspring's health, the DOHaD hypothesis also implies that mothers' health needs to be nurtured to ensure a healthy pregnancy as well as successful future pregnancies (16). To that effect, the concept of maternal constraint suggests that mothers need to give relative priority to themselves over the fetus (16), and limit the fetal growth to ensure a successful delivery, and sustain maternal capacities for future pregnancies, at least in well-nourished and healthy women (16). Nutritional and

environmental signals are at play to restrict excessive fetal growth (16). Maternal constraint in the context of bone metabolism can impact the fetus' supply of nutrients, and ultimately its bone mass accretion. Signals involved in maternal constraint might impact systems regulating vitamin D and calcium homeostasis, as well as the placenta, which controls the synthesis and release of various hormones during pregnancy **(Figure 1)**. Optimal maternal health and adequate nutrition are key to balance maternal constraint processes in a way that preserve the mother's reproductive health but still support the optimal development and survival of the fetus.

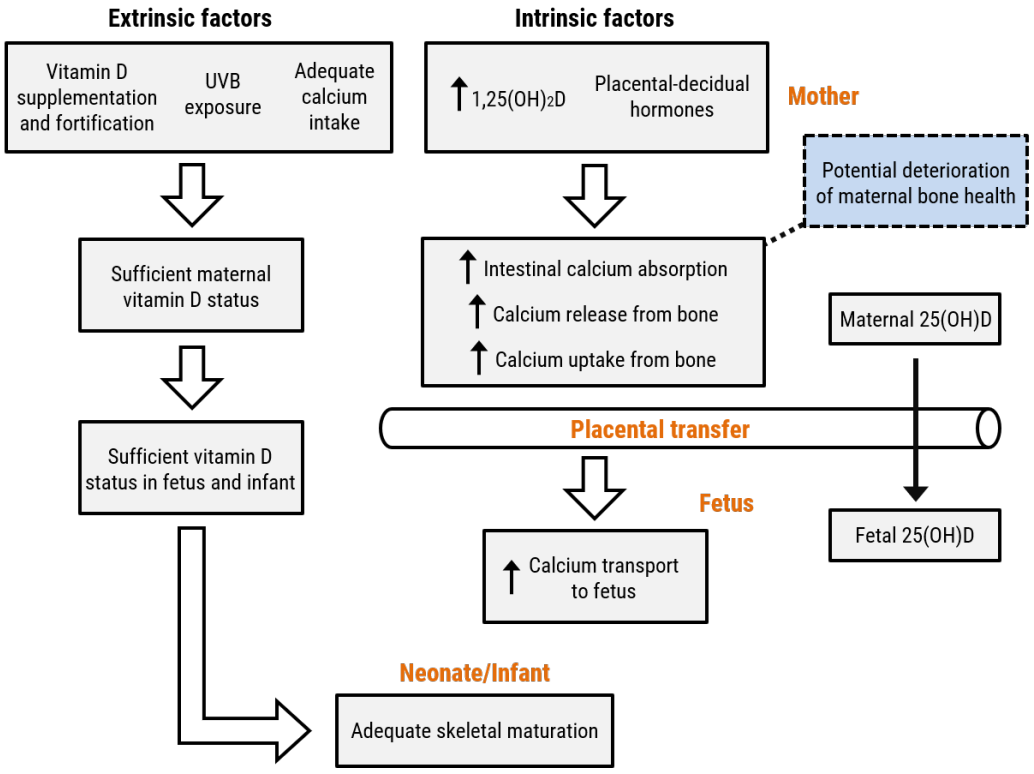

Figure 1: Maternal factors and physiological changes impacting calcium and vitamin D metabolism during pregnancy. Adapted from Fiscaletti M, Stewart P, Munns CF. The importance of vitamin D in maternal and child health: A global perspective. Public Health Rev. Public Health Reviews; 2017;38:1–17.

Pregnancy represents a period where the mother provides a substantial amount of calcium for transplacental transfer to the fetus (**Figure 2**, discussed in more details in subsequent sections), which places her at risk for demineralization of her own skeleton (reviewed in (6)). Several metabolic changes occur during pregnancy in order to support growth *in utero*. Although these changes aim to maximize bone health of the offspring, observational evidence suggested that it might be detrimental to the mother's own bone health (reviewed in (5)). Some evidence suggested that loss of maternal bone is transient (17), and the impact was modulated by the nutritional status of the mothers (18–21). Accordingly, pregnancy is hypothesized to be a risk factor for maternal osteoporosis (2).

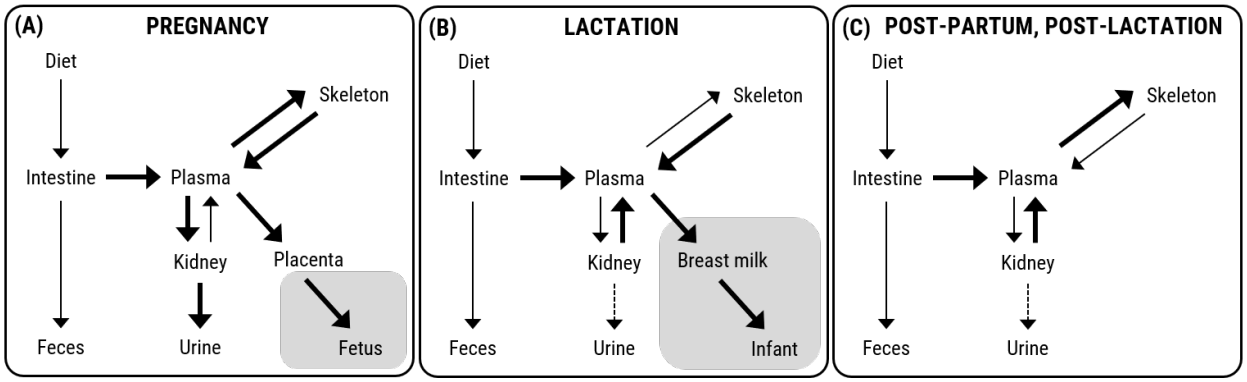

Figure 2: Schematic diagram of the calcium flux in non-pregnant, pregnant, and lactating women. Adapted from Olausson H, Goldberg GR, Ann Laskey M, Schoenmakers I, Jarjou LM, Prentice A. Calcium economy in human pregnancy and lactation. Nutr Res Rev. 2012;25:40–67.

Sub-optimal vitamin D status during pregnancy can impact the adaptations in calcium metabolism that typically occur in pregnancy such as increased intestinal absorption and increased renal retention (reviewed in (5)). Although a high calcium demand supports the

hypothesis that pregnancy and lactation are risk factors for osteoporosis (2–4), a recent systematic review of observational studies reported inconclusive results (22). Thus, a lack of consensus remains as to the metabolic adaptations associated with pregnancy, the magnitude of bone loss, and the lasting impact of pregnancy changes on bone health status in the post-partum period. A better understanding of bone metabolism changes associated with pregnancy is needed in order to intervene and limit maternal bone mass loss, and subsequently prevent osteoporosis later in life.

1.2.2. Changes in bone turnover by biomarkers

It is estimated that 30 grams of calcium are transferred to the fetus during pregnancy (5). Accordingly, maternal dietary calcium and vitamin D insufficiency, as well as inadequate metabolic adjustments can result in the decalcification of the mother's own skeleton to support fetal bone accrual.

Healthy bone is metabolically very active and constantly renewed through the action of bone-forming osteoblasts and bone-resorbing osteoclasts (23). The ongoing process of bone remodeling follows a time course including maturation and mineralization of the collagenous matrix (23). Bone is made of mineralized collagen fibrils, of which 90% is type I collagen. The remaining components include mineral crystals, such as the hydroxyapatite composed of calcium (23). Cross-links are formed between collagen helices in order to create strong collagen fibrils, ensuring strength to the skeleton.

Serum concentration of bone turnover markers reflect short-term changes in bone turnover, and reflects the rate of bone remodelling (24). Of all of the measurable serum and urinary bone markers, the International Osteoporosis Foundation and the International Federation of Clinical Chemistry and Laboratory Medicine (25) recommend measurement of serum procollagen type I N propeptide (PINP) as a marker of bone formation, and serum carboxyl-terminal telopeptide of type I collagen (CTX) as a marker of bone resorption. PINP is a precursor molecule to the formation of mature collagen that is highly specific to bone (26). CTX is a product of the collagen cleavage that occurs during resorption. The greatest contribution to circulating CTX is assumed to be from bone (26), so its specificity to bone is considered moderate to great. Measured together, serum PINP and CTX reflect bone turnover.

An overall bone mineral mobilization during pregnancy is evident from longitudinal studies in which concentrations of plasma bone turnover markers (20,27–35) or urinary markers (27–29,34) were higher during pregnancy when compared to pre-pregnancy. Specifically, elevated concentrations of the bone resorption marker CTX were detected as early as the first trimester in some (33,36) but not all studies (20,30,32). No significant increases in concentrations of the bone formation marker PINP were reported before the third trimester (29,30,32). Collectively, markers of bone resorption and formation reached their maximal concentrations in the last trimester of pregnancy, when fetal bone accrual peaks.

Of the 15 studies that have measured bone turnover throughout pregnancy, only two (30,32) measured simultaneously serum CTX and serum PINP as suggested by expert consensus (25). In these observational longitudinal studies representing 41 English pregnant women (30) and 49 German pregnant women (32), circulating concentrations of PINP and CTX in early pregnancy (e.g. 12 week gestation) were significantly lower compared to pre-pregnancy, but rose significantly by the end of pregnancy at 36 weeks gestation. This suggests that changes in bone resorption are coupled to changes in formation, supporting an overall increased bone turnover during pregnancy.

Among other studies on bone metabolism changes during pregnancy, some have measured either serum CTX (20,33,36) or serum PINP (29), limiting the interpretation of pregnancy's impact on bone turnover. Limitations of the published studies include sample size, which averaged 27 participants, and some with ten participants or less (21,27,28). Most studies took place in Europe (n=10), with three from the United Kingdom (27,29,30), concentrating the results on European descent women but not from North America. Only half of the studies (20,27,29–32,34,36) repeated measures of bone turnover markers throughout pregnancy, which is essential to have a rigorous assessment of pregnancy related changes with baseline values. Additionally, older studies have used suboptimal or outdated analytical methods, which are difficult to interpret within the current body of knowledge. For example, some studies have measured bone turnover markers in urine (27–29) which is not optimal due to an increase in glomerular filtration rate observed during pregnancy that can affect the interpretation of urinary measures (5).

Assays are now readily available to measure concentrations in serum or plasma, which is preferred (25). Even when measured in serum, attention to time of day is required for markers such as CTX, which must be measured in the morning after a fasted night in order to minimize variability as its metabolism is highly dependent on circadian rhythm and food intake (37). To gain a clear understanding of the pregnancy-induced bone metabolism changes, there is a need for high quality studies with large sample sizes, and repeated measures throughout pregnancy using gold standards techniques and the recommended bone turnover markers serum CTX and PINP.

1.2.3. Changes in calciotropic hormones

Changes in calciotropic hormones, including vitamin D metabolism occur during pregnancy to support adequate calcium transfer to the fetus. Sub-optimal maternal vitamin D status in pregnancy can limit fetal bone accrual, as fetal bone growth relies on both maternal optimal calcium intake and vitamin D status (38). Most evidence to date suggests that serum 25(OH)D concentrations, the indicator of vitamin D status (39), did not change throughout pregnancy (34,35,40). However, serum $1,25(OH)_2D$ concentrations, the active form of vitamin D, reached supra-physiologic concentrations by the end of the third trimester. Indeed, the conversion of 25(OH)D to $1,25(OH)_2D$ was greatly up-regulated compared to a non-pregnant state (41). The renal hormone 1-α-hydroxylase became uncoupled with its feedback system, and concentrations of serum $1,25(OH)_2D$ were reported to increase as a function of serum 25(OH)D availability (42,43). The rise in circulating $1,25(OH)_2D$ concentration occurred in the first trimester of

pregnancy (28,40), and continued to rise in late pregnancy to concentrations several fold higher than pre-pregnancy (28,40) and early pregnancy (20,21,28,34,40). Compared to early pregnancy, serum $1,25(OH)_2D$ increased by 50-100% in second trimester, and by another 100% in third trimester (44). Although that would be considered toxic concentrations in non-pregnant women, it did not lead to hypercalcemia in pregnant women (41). Rather, this rise in serum $1,25(OH)_2D$ concentrations is hypothesized to support increased maternal calcium absorption, ultimately supporting the calcium needs for fetal bone accretion (41).

While 1-α–hydroxylase is regulated via negative feedback by parathyroid hormone (PTH) in a non-pregnant state, it does not seem to be the case during pregnancy, as PTH and $1,25(OH)_2D$ circulating concentrations have not been found to correlate (reviewed in (6)). It is more probable that the rise in $1,25(OH)_2D$ is due to a PTH-independent mechanism (reviewed in (6)), and several other hormones were proposed such as parathyroid hormone-related protein (PTHrP), estradiol, prolactin, placental lactogen, and calcitonin, all of which increase throughout pregnancy and could have a central role in stimulating renal 1-α-hydroxylase (**Figure 1)** (41,42,45). It was also postulated that the increased production of maternal $1,25(OH)_2D$ early in pregnancy could be a compensatory mechanism to the increase in DBP that binds 25(OH)D, decreasing the free 25(OH)D (reviewed in (45)). Another contributor during pregnancy may also relate to the maternal decidua and fetal placental cells that exhibit action of 1-α–hydroxylase, being a major extra-renal site of conversion of 25(OH)D to its active form (reviewed in (45)). This is

further enhanced by the observed decreased CYP24A1 expression in the placenta in early gestation, leading to the prevention of the catabolism of $1,25(OH)_2D$ (reviewed in (45)). However, the majority of the rise in concentration of $1,25(OH)_2D$ during pregnancy seems to be attributed to the activity of 1-α–hydroxylase in the maternal kidneys as opposed to the placental and decidual cells. This is substantiated in animal studies of nephrectomized rats injected with 25(OH)D as well as observational studies involving pregnant women with non-functioning kidneys, where lower $1,25(OH)_2D$ levels compared to healthy pregnant state were observed in both cases (reviewed in (45)).

The growth factor insulin-like growth factor-I (IGF-1) is among the other regulators of changes in calcium metabolism during pregnancy as it is a bone anabolic hormone (5). Similar trends to CTX and PINP were observed for IGF-1 with elevated concentrations reported only at the end of pregnancy (21,29,34,46). Circulating concentrations of IGF-1 correlated with changes in bone turnover markers of resorption and formation during pregnancy (21,29,34), but no studies to date have measured serum IGF-1 concentrations in combination with serum CTX and PINP throughout pregnancy. It is apparent that IGF-1 plays an important role in the calcium metabolism adaptations seen in pregnancy, yet its specific role requires further investigation.

1.2.4. Changes in bone mineral

Mobilization of bone mineral was reported to occur during pregnancy (reviewed in (5)). Both a decline in bone mineral and no change from pre-pregnancy to early post-partum at

one or more skeletal sites were reported in a review including ten studies (reviewed in (5)). The response of bone to physiological and environmental stimuli differed between regions of the skeleton, so there is a need to assess different skeletal sites to have an overview of bone metabolism (reviewed in (5)). Both trabecular and cortical bone were affected by pregnancy-related changes, but in different magnitudes. Cortical bone is compact, while trabecular bone is spongy and reported as more metabolically active (47). As a result, trabecular bone was more rapidly remodeled compared to cortical bone (47). The axial skeleton, including the spine, is made of a greater ratio of trabecular-to-cortical bone compared to other skeletal sites. Accordingly, it is important to assess several skeletal sites when evaluating the effects of pregnancy on bone health (reviewed in (5)).

Dual-energy x-ray absorptiometry DXA is recognized as the gold standard method to measure whole body and site specific bone mineral density (BMD) (48). Out of the ten observational longitudinal studies that assessed the impact of pregnancy on maternal bone mineral, seven reported a significant decrease in bone mineral at one or more skeletal sites when measured by DXA from pre-conception to early post-pregnancy (29,31,49–53). Two studies reported non-significant decreases in bone mineral from pre- to post-partum (54,55), while one study did not see any changes due to pregnancy (40). Excluding implausible data (51), a significant average loss of -1.1% of bone mineral (range -1.7 to -0.5%) at the whole body, and an average loss of -2.7% (range -4.5 to -1.5%) of bone mineral at the spine was reported from pre-conception to up to six weeks post-partum. Yet, a lot remains unknown and limitations of published studies to date such

as small sample size and wide range of pre-pregnancy measurement (from 16 months to three months pre-conception) limits the generalizability of the results.

In assessing the variations in skeletal response to pregnancy between studies it is important to consider factors such as genetics, and body size in the perinatal period that might explain inter-individual variations. With regard to body size, pre-pregnancy body mass index (BMI), gestational weight gain, and body fat mass are all reported to impact bone mineral density changes during pregnancy (18,50). For example, women with a low pre-pregnancy BMI were reported to have greater decline in whole body bone mineral content than women of higher pre-pregnancy BMI, suggesting that higher body weight might be protective of bone mass due to increased mechanical loading (50). Fat mass gain, rather than total gestational weight gain, might play an important role in modulating bone metabolism changes during pregnancy. Greater maternal adiposity, but not gestational weight gain, was associated with an attenuated bone loss in pregnancy (18), supporting the protective effect of higher fat mass on maternal skeleton. With regards to genetics, a portion of the variance in bone mass was reported to be attributable to genetics (56). Recent genome-wide association studies (GWAS) revealed the importance of genetic determinants in various skeletal phenotypes in human, such as BMD (57). Strong genetic correlations are reported with BMD (58), emphasizing the importance of considering genetic risk factors in maternal bone health.

1.2.5. Maternal dietary calcium intake and vitamin D status

The extent to which bone metabolic changes during pregnancy are dependent on maternal intake and status of calcium and vitamin D remains unclear (reviewed in (5)). Changes in bone mineral by DXA during pregnancy were independent of dietary calcium in observational studies including women with adequate habitual calcium intake averaging the Dietary Reference Intakes (DRI; Estimated average requirement (EAR): 800 mg/day; Recommended dietary allowance (RDA): 1,000 mg/day (39)) across the United States (59), Denmark (53) and the United Kingdom (52,55). In contrast, changes in bone mineral throughout pregnancy in a cohort with low habitual calcium intake (e.g. 400-500 mg/day) seemed to be dependent on maternal calcium intake. Longitudinal studies using ultrasound reported that pregnant women consuming less than 568 mL of milk/day (representing an estimated 560 mg of calcium) in the United Kingdom (18) and less than 1000 mg/day of calcium in Spain (19) had an accentuated bone loss throughout pregnancy when compared to women with higher maternal calcium intake. Taken together, these studies suggest that effects on bone loss might be larger in women with low habitual intake (e.g. ≈500 mg/day) compared to women with high habitual intake. In a small cohort of Argentinian pregnant women (n=39), greater bone turnover by biomarkers, specifically serum CTX, was observed in women with low intakes of dietary calcium compared to those with adequate calcium intake (20). To further complicate the interpretation, it was suggested that pregnant women with low habitual calcium intake might have compensatory enhanced intestinal calcium absorption (21). Taken together, these results suggest that the bone metabolism changes in pregnancy might be dependent

on maternal calcium intake, and that bone loss might be greater in women with low habitual intake.

Currently a lack of consensus exists among recommendations as to the optimal maternal vitamin D intake and status during pregnancy (**Table 1**). Vitamin D status, as defined by circulating serum concentration of 25(OH)D (39), accounts for both endogenous and exogenous sources of vitamin D, including food, supplements, and sunshine exposure (42). Sub-optimal vitamin D status during pregnancy has the potential to exaggerate maternal skeletal response due to its role in calcium metabolism (5).

Table 1: National and International guidelines on thresholds for vitamin deficiency and dietary recommendations for vitamin D intake during pregnancy. Adapted from Curtis EM, Moon RJ, Harvey NC, Cooper C. Maternal vitamin D supplementation during pregnancy. Br Med Bull. 2018;1–21.

Guideline	**Countries covered by recommendation**	**Vitamin D status deficiency; 25(OH)D (nmol/l)**	**Vitamin D status insufficiency; 25(OH)D (nmol/l)**	**Vitamin D status sufficiency; 25(OH)D (nmol/l)**	**Dietary recommendation for vitamin D intake in pregnancy (IU/day)**
Institute of Medicine (IOM)	USA and Canada	< 30	30 – 50	≥ 50	600
Canadian Pediatric Society	Canada	< 25	25 - 75	75 - 225	No recommendation
Endocrine Society Clinical Practice Guidelines	Worldwide	< 50	50 – 75	≥ 75	600
Global Consensus Recommendations on Prevention and Management of Nutritional Risks	Worldwide	< 30	30 – 50	≥ 50	600

Using systematic reviews and meta-analysis as a basis, the Institute of Medicine (IOM; (39)) defined serum concentrations of 25(OH)D below 30 nmol/L to be deficient, 30-50 nmol/L to be inadequate, and above 50 nmol/L to be sufficient, based on bone health outcomes such as prevention of rickets and osteomalacia. Health Canada adopted the IOM guidelines (60), so the same guidance holds in Canada. In contrast, other organizations have suggested serum concentrations of 25(OH)D above 75 nmol/L for maximal health benefits, but most of these recommendations are based on expert consensus rather than systematic review of the literature (Osteoporosis Canada (61); the American Endocrine Society (62); and the Canadian Pediatric Society (63)). Little is known about the impact of maternal vitamin D status during pregnancy on bone health outcomes of the mother, as most studies focused on infant outcomes (42). One observational study of 304 British women (ethnicity unreported) showed that entering pregnancy during the winter accentuated the maternal bone loss observed during pregnancy compared to women entering pregnancy during the summer, suggesting an interaction between maternal bone metabolism adaptations and vitamin D status through sunshine exposure (18).

Several factors influence maternal vitamin D status during pregnancy (**Figure 3**). Pregnant women of higher pre-pregnancy BMI have lower serum 25(OH)D than women with lower pre-pregnancy BMI throughout pregnancy (64), and this might be due to the vitamin D being sequestered in the adipose tissue (65). Limited evidence exists on the impact of gestational weight gain and changes in body composition on serum 25(OH)D

concentrations in pregnant women. It is difficult to measure body composition changes in pregnancy due to the limitations of available methods. For example, DXA scanning is deemed unsafe during pregnancy due to x-ray exposure, while bioelectrical impedance analysis is safe but not validated in pregnancy (66).

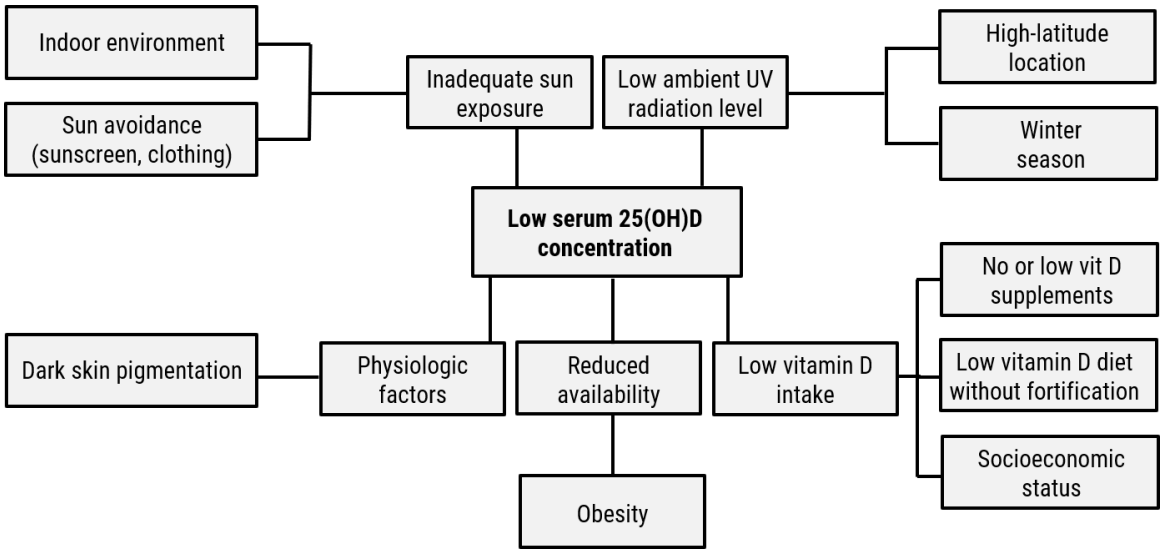

Figure 3: Factors affecting maternal vitamin D status during pregnancy. Adapted from Hossein-Nezhad A, Holick MF. Vitamin D for health: A global perspective. Mayo Clin Proc. 2013;88:720–55.

As endogenous vitamin D synthesis relies on ultraviolet radiation, many factors that affect sunlight exposure can also influence maternal vitamin D status. Cutaneous vitamin D production is influenced by skin pigmentation, sunscreen use, and clothing choices, where darker skin and the use of sunscreen and covering clothes lead to less production of pre-cholecalciferol (42). Other factors that affect cutaneous production of vitamin D include season, time of the day, latitude and altitude (42). Although the biological

evidence clearly supports the role of vitamin D status in maternal bone metabolism changes during pregnancy, the regulation of alterations in concentrations of 25(OH)D and $1,25(OH)_2D$ is unclear throughout pregnancy. Importantly, few studies measured circulating concentrations by the gold-standard method liquid chromatography-tandem mass spectrometry (LC-MS/MS) (40) while most are using assays (20,21,33–36) with important between-assay variability (67). LC-MS/MS is regarded as the gold standard to measure vitamin D metabolites due to the ability of this technique to separate and quantify various molecules with sensitivity and specificity: $25(OH)D_2$, $25(OH)D_3$, total 25(OH)D, and other metabolites such as $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$ (68).

Only two studies (34,40) measured both 25(OH)D and $1,25(OH)_2D$ in their cohort, and only Moller *et al.* (34) have done so in conjunction with measuring serum bone formation markers, but no serum bone resorption marker. A global assessment of vitamin D metabolites and other relevant serum bone biomarkers by gold standards methods is needed to understand the changes related to pregnancy.

1.2.6. Maternal skeletal benefits of interventions including a dietary or exercise component

Potential benefits of calcium supplementation alone on maternal bone turnover were demonstrated in some intervention studies. In a small randomized cross-over trial of 31 Mexican pregnant women with adequate habitual calcium intake, a ten day supplementation protocol with 1200 mg/day of calcium led to a significant decrease in

urinary NTX, a marker of bone resorption, compared to supplementation with a multivitamin without calcium (69). This contrasts with results from a randomized controlled trial of calcium supplementation including pregnant women with very low habitual calcium intake (350 mg/day) (11), and taken together suggests that compensatory mechanisms might enhance calcium absorption in women with very low daily calcium intake (reviewed in (5)).

The influence of vitamin D supplements in pregnancy was addressed in a Cochrane systematic review including four RCTs for a total of 414 women in which the women who received vitamin D supplements had higher circulating 25(OH)D concentrations at the end of pregnancy than those who received a placebo or no intervention (reviewed in (8)). Out of the trials included in this systematic review of vitamin D supplementation during pregnancy, none reported on maternal bone health, so it remains unclear if higher vitamin D status was linked to clinical benefits for the skeletal health of the mother during pregnancy.

Since the publication of this systematic review, several RCTs have been published. Most intervention studies included pregnant women with insufficient (70–72) or deficient (73) vitamin D status at enrolment. The geographical areas represented were East Asia (70,71) or the Middle East (72,73), and when assessed, their habitual calcium intake was low. Consistently, all studies reported that vitamin D supplementation during pregnancy led to a rise in maternal serum 25(OH)D concentrations at term whether provided as daily (73)

or a weekly (71,72) supplementation. High daily vitamin D supplementation was also efficient in raising maternal vitamin D status in pregnant women with adequate vitamin D status (74,75). Although these high supplement doses (i.e. up to 4,000 IU/day) were deemed safe (74,75), maternal bone health outcomes were not assessed in these trials.

Overall, results from clinical trials showed a clear benefit of vitamin D supplementation at improving maternal vitamin D status in pregnant women (especially if they were vitamin D deficient at baseline) and a potential benefit of calcium supplementation on bone metabolism. A combined calcium and vitamin D supplementation was effective at raising circulating 25(OH)D concentrations at the end of pregnancy but this was in pregnant adolescents with low habitual calcium intake but with adequate initial vitamin D status (76). Yet, it remains unclear if there were any maternal bone health benefits associated with such interventions, as none of the studies to date have measured the impact of such interventions on maternal bone metabolism markers in pregnancy (70–76).

Interventions supplementing food in conjunction with nutrient supplements (13) and promoting lifestyle changes such as physical activity (77) are promising avenues to maintain maternal bone health during pregnancy. In the only reported randomized controlled trial, 36 Chinese women with very low habitual calcium intake (480 mg/day) received either 350 mg/day of calcium (through milk powder, for a total daily intake of 900 mg), 950 mg/day of calcium (through milk powder and a calcium supplement, for a total daily intake of 1500 mg) or habitual diet from 20 weeks of pregnancy to the post-

partum (13). At the end of pregnancy, calcium supplementation led to a significant dose-dependent decrease in urine hydroxyproline, a marker of bone resorption, when compared to no supplementation.

Exercise might exert a positive impact on maternal bone health in pregnancy due to mechanical loading. Only one study (n=118) to our knowledge looked at the impact of physical activity in pregnancy on bone health, showing that Chinese women who engaged in physical activity during their pregnancy had preserved bone mineral density of the heel in early post-partum compared to women who were inactive (77).

Although the evidence to date is sparse, interventions that would combine active lifestyle and consumption of bone-related nutrient rich foods such as dairy products to support maternal bone health during pregnancy are promising. Large sample size and measurement of clinically relevant bone biomarkers and bone mineral density measures are needed.

1.2.7. Cord serum calciotropic hormones and bone biomarker concentrations in relation to maternal concentrations

Infant vitamin D status at birth is dependent on maternal serum concentrations of 25(OH)D during pregnancy (reviewed in (78)), and cord serum 25(OH)D concentrations strongly correlate with maternal concentrations at the end of pregnancy (79). While the optimal vitamin D status at birth is not defined, cord serum 25(OH)D concentration was

reported to average 60% of maternal concentrations at the end of pregnancy in a cohort of Caucasian women (N=107) from Denmark (80), thus suggesting that maternal adequate vitamin D status is required to ensure neonatal adequacy. In contrast, cord serum $1,25(OH)_2D$ concentrations did not correlate with maternal concentrations at the end of pregnancy, with cord concentrations averaging 52% of maternal concentrations (reviewed in (78)). No study to date has evaluated the cord serum concentrations of both 25(OH)D and $1,25(OH)_2D$ in relationship to maternal concentrations throughout pregnancy, using the gold standard LC-MS/MS method.

No reference for vitamin D adequacy exists for cord blood, with the exception that newborn serum 25(OH)D above 25-30 nmol/L will prevent nutritional rickets (reviewed in (78)). The current DRI for vitamin D for pregnant women (39) did not evaluate the intake in pregnancy that would ensure adequate vitamin D status for newborns. However, it is suggested that maternal intake greater than the current DRI (39) are required to ensure adequate maternal, fetal and neonatal bone health (74,81).

To our knowledge, measurement of serum bone turnover by the recommended CTX and PINP biomarkers (25) have never been completed in cord blood from healthy infants born at term, and limited evidence exists regarding bone turnover in cord serum. Only one study assessed the relationship between bone biomarkers in maternal and cord circulation in 41 dyads (82), measuring specifically serum CTX, and osteocalcin and bone-specific alkaline phosphatase as markers of bone formation. Significantly higher concentrations of

bone formation and bone resorption markers in cord blood compared to maternal concentrations suggested a higher fetal bone turnover state compared to the mother (82). Important to note, no correlation was noted for all bone biomarkers between cord serum and maternal serum concentrations, and fetal bone metabolism was suggested to be independent of the mother at term (82).

More evidence is needed to understand the relationship between maternal and fetal bone metabolism, and how the maternal adaptations might impact bone turnover status in the infant at birth.

1.2.8. Knowledge gaps

No consensus exists on the metabolic adaptations to pregnancy related to vitamin D and bone biomarkers, the magnitude of bone loss, nor the lasting impact of pregnancy changes in the post-partum period. In addition to being of small sample size, few studies measured simultaneously serum CTX and serum PINP as suggested by expert consensus. Only half of the studies repeated measures of bone turnover markers throughout pregnancy. To gain a clear understanding of the pregnancy-induced alterations in bone metabolism, there is a need for high quality studies with large sample sizes, and repeated measures throughout pregnancy using gold standards techniques and the recommended bone turnover markers serum CTX and PINP.

Knowledge is limited regarding the impact of maternal vitamin D status during pregnancy

on maternal bone health outcomes, as most studies focused on pregnancy and infant outcomes. How the concentrations of vitamin D metabolites, specifically 25(OH)D and $1,25(OH)_2D$, change throughout pregnancy and what systemic and lifestyle factors influence the changes remain unclear. Importantly, few studies measured circulating concentrations by the gold-standard liquid chromatography-tandem mass spectrometry (LC-MS/MS). A global assessment of vitamin D metabolites in addition to bone biomarkers by gold standard methods is needed to understand the changes related to pregnancy.

From a lifestyle perspective, it remains unclear the extent to which bone metabolic changes during pregnancy are dependent on maternal intake particularly of bone-dependent nutrients such as protein, calcium and vitamin D. Although the evidence to date is sparse, interventions that would combine active lifestyle and consumption of bone nutrient rich foods such as dairy products to support maternal bone health during pregnancy are promising. The use of dairy products rich in bone-health nutrients, rather than supplementation of single nutrients, might be more effective at preserving maternal bone health during pregnancy. Large sample size and measurement of clinically relevant bone biomarkers and bone mineral density measures are needed.

Data are sparse regarding the relationship between maternal and fetal bone metabolism. Serum vitamin D metabolites using gold standard techniques and the recommended bone turnover markers serum CTX and PINP measured in cord serum would enhance our

knowledge of fetal bone metabolism in relation to maternal factors influencing bone metabolism in pregnancy.

1.3. Post-partum changes in bone metabolism

1.3.1. Post-partum and the developmental origins of osteoporosis

During post-partum, maternal bone health is still precarious due to recovery of the pregnancy metabolic changes, with or without the additional demand of producing breast milk. Deleterious effects on the maternal skeleton appears extremely important during lactation, due to maternal demand to provide approximately 280-400 mg calcium per day through breast milk (reviewed in (6)). As a result, a temporary bone demineralization occurs with breast feeding with women losing three to ten percent of bone mineral density (reviewed in (6)) (**Figure 2**). The exacerbated bone loss associated with lactation might put the mother at higher risk of osteoporosis later in life (2–4).

1.3.2. Changes in bone turnover by biomarkers

High bone turnover in the post-partum period, particularly when women are lactating, was reported in longitudinal studies. A rise in serum bone formation and bone resorption markers was reported in the first weeks post-partum in lactating women when compared to values either in late pregnancy (21), or in pre-conception (27,35,40,83). In the following three to six months post-partum, concentrations of bone resorption markers were reported to return to lower concentrations, while bone formation markers remained

elevated (35,84). As a result, an uncoupling of bone turnover to favor bone resorption characterized the post-partum period. Lactation modulates the circulating concentrations of bone biomarkers in the post-partum period as higher concentrations of bone biomarkers are reported in post-partum lactating women when compared to post-partum women who are not lactating (85–87). Higher serum bone formation PINP and higher serum bone resorption CTX concentrations specifically were reported for the lactating women when compared to non-lactating women (88,89), yet lactation practices are not often well described in studies.

Few studies measured both biomarkers CTX and PINP in serum both in pregnancy and the post-partum to obtain a global assessment of bone turnover in the perinatal period. Of the studies that assessed women's bone health in pregnancy and post-partum with multiple bone biomarkers, the follow up in post-partum was short, often up to only two weeks post-partum (29,30). Such early post-partum measures of bone biomarkers might simply reflect pregnancy changes rather than post-partum changes, but it is difficult to determine due to the short length of follow-up in the post-partum period. Other limitations of the studies published to date include small sample size and selection of only one or two markers of bone turnover. Studies enrolling pregnant women early in pregnancy and following them well into post-partum would allow for a better understanding of changes associated with pregnancy and the post-partum.

1.3.3. Changes in calciotropic hormones

The interplay between calcium and vitamin D during the post-partum period was shown in a study of post-partum women using stable calcium isotopes (90), showing a positive impact of combined high intake of calcium and adequate vitamin D status on rates of bone calcium deposition. Serum $1,25(OH)_2D$ concentrations and dietary calcium intakes were among the factors that explained 99% of the variability in the rate of calcium deposition (90), thus suggesting an important role in maternal bone calcium metabolism during the post-partum-period.

During the post-partum period, serum 25(OH)D concentrations remained comparable to pregnancy, irrespective of lactation practices (including duration of lactation, and type such as 'exclusively breastfeeding', 'expressing breast milk' or 'not lactating') (34,40,88). In contrast, serum $1,25(OH)_2D$ concentrations declined compared to the elevated concentrations in pregnancy returning to pre-conception concentrations (11,21,91), and remained within the normal range irrespective of lactation (28,34,40,92). The decline in circulating concentrations of $1,25(OH)_2D$ in the post-partum might be due to the loss of pregnancy-specific placental hormones (41,42,45).

Bone metabolic changes during the post-partum, specifically during lactation, appeared to be independent of PTH and $1,25(OH)_2D$ metabolism, and might be mediated by low estrogen and the PTHrP produced by the breast tissue (reviewed in (6)). PTHrP

stimulated maternal bone resorption and maternal renal calcium reabsorption to ensure adequate calcium transfer to the infant (reviewed in (6)).

Serum concentrations of the bone anabolic hormone IGF-1 were reported to decline in early and late post-partum compared to late pregnancy concentrations (21,29) and appeared to be modulated by lactation practices (34). Overall, few studies assessed changes in concentrations of IGF-1 in the post-partum compared to pregnancy concentrations, while accounting for lactating practices.

1.3.4. Changes in bone mineral

Non-lactating post-partum women experienced either no change in bone mineral density (12,49,93–98) or an increase of up to two percent at the spine (99,100) and two percent at the whole body (17,93) by three to six months post-partum (**Figure 2**). It is difficult to determine if these increases in bone mineral are a catch-up recovery from the loss experienced during pregnancy or if it is a phenomenon specific to the post-partum.

Transient bone demineralization of the maternal skeleton appeared to be the primary metabolic adaptation to meet the calcium requirement of lactation. During lactation, women are reported to lose five to seven percent of bone mineral content at the lumbar spine (49,94,96,97) and about one percent at the whole body (93,101) over the period from early to six months post-partum (**Figure 2**). Women who lactate for longer periods had more pronounced bone mineral loss in the first six months post-partum compared to

women who lactate for shorter period of time (93,97). This may reflect differences in lactation practices such as breastfeeding versus expressing breast milk, frequency and duration of lactating sessions and the volume of breast milk produced (reviewed in (5)). Resumption of menses and its associated increase in estrogen is another factor that might explain differences in bone mass among women lactating for various length of time (reviewed in (6)). Supporting this hypothesis, maternal bone recovery was reported in women who stop lactating and presumably saw a return of their menses (84,100). Details pertaining to lactation practices and menstruation are not often reported in studies, and these two factors complicate the interpretation of the bone health changes observed in the post-partum period. Limitations of bone mineral assessment also include the lack of baseline data as methods such as DXA are not safe in pregnancy and it is difficult to recruit women pre-conception. Longitudinal studies assessing bone mineral at various skeletal sites in complement to measuring serum bone biomarkers are needed to gain knowledge of bone metabolism changes in the perinatal period.

1.3.5. Maternal dietary calcium intake and vitamin D status

The amount of maternal bone demineralization was not dependent on the maternal calcium intake during lactation, as both women with low habitual calcium intake (300 mg/day) (101–103) and women with high habitual calcium intake (950 mg/day) (91,99,100,104,105) experienced bone mineral loss during lactation. Since neither low nor high calcium intake minimized maternal bone loss in lactation (101), it suggests that bone demineralization is a physiological response independent of dietary intake.

In RCTs conducted during pregnancy and lactation the effects of calcium supplementation on bone mineral mobilization in the mother in the post-partum are inconsistent. Bone loss in lactation was not altered when women with low habitual calcium intake (300-500 mg/day) were supplemented with a daily dose of 1000 mg of calcium for six months (12) or 12 months post-partum (102). Thus, calcium supplementation itself might not be efficient at preserving bone health during post-partum, or starting the supplementation during the lactation period might be too late to see benefits on maternal bone health. It is also possible that women with low habitual calcium intake have beneficially adapted to their low calcium intake, and supplementing them with a high amount of calcium might be detrimental, as observed in a RCT cohort of women with low habitual calcium intake (350 mg/day) supplemented with 1500 mg/day of calcium during pregnancy (11). Supplementation did not alter bone mineral density at two weeks post-partum, but detrimental effects were observed at 12 months post-partum in women supplemented as they had greater bone loss and higher concentrations of bone turnover biomarkers than the control group (11). Taken together, supplementation might have a detrimental impact on maternal bone health by disrupting the adaptation to a low calcium intake.

Neither maternal vitamin D supplementation nor higher maternal serum 25(OH)D concentrations were beneficial to maternal bone health in lactation as noted in observational studies (106) and RCTs (107,108). Daily vitamin D supplementation with 6400 IU/day successfully raised maternal serum 25(OH)D concentrations (108), but it

remained unclear if any clinical benefits for the mother exist in the post-partum and lactation periods. Thus, to date, no evidence exists that the maternal requirement for vitamin D intake increases during lactation either to meet maternal needs (39) or to provide for transfer of calcium through breast milk to the infant.

Supplementation of vitamin D and calcium during pregnancy conferred a benefit to lactating teenagers at four months postpartum as demonstrated by higher lumbar spine BMD compared with placebo (76). However, results should be interpreted with caution as women in the intervention group significantly reduced their lactation frequency and had a higher rate of resumed menses when compared to women in the placebo group. Yet, the concept of combining vitamin D and calcium within a single supplement is interesting, as it potentially can increase the absorption of calcium but more evidence is needed.

1.3.6. Maternal skeletal benefits of intervention including a dietary or exercise component

To our knowledge, only one RCT employed food as part of their intervention (13) to assess its impact on maternal bone health in the post-partum. While providing milk powder in addition to calcium supplementation during pregnancy ((13) described in details in previous section), a dose-dependent increase in whole body and lumbar spine bone mineral density at six weeks post-partum was observed in the calcium supplementation compared to the control group. These results suggest that the positive impact of supplementation using food during pregnancy is sustained into early post-

partum. To our knowledge, no studies have supplemented mothers with whole food such as dairy products other than milk powder, and measured its impact on maternal bone health outcomes in post-partum.

Weight bearing exercise offers potential benefit to limit maternal bone loss in the post-partum. An exercise regimen including weight bearing aerobic exercise starting at one month post-partum in lactating women resulted in significantly less bone loss at five months post-partum when compared to no exercise (109). While the benefit of this exercise program was observed at the lumbar spine but not at the whole body (109), a similar intervention by the same group combined resistance training and energy restriction from one month post-partum to five months post-partum but failed to find a benefit to bone mass at either skeletal site when compared to no intervention (110). Both of these studies were of small sample size (20-31 participants), and had recruited overweight women (110) who were exclusively breastfeeding (109,110), thus limiting the generalizability of the results. In addition, although one study included a dietary component, it was centered around energy restriction only (110) and the authors suggested that the effects of a diet intervention designed to increase intake of protein, calcium and vitamin D in combination with an exercise intervention be assessed to determine the impact on maternal bone health in lactation.

1.3.7. Knowledge gaps

While many studies assessed maternal bone mineral in the post-partum, few measured both biomarkers CTX and PINP in serum during lactation. Measures of maternal bone resorption and formation both in pregnancy and the post-partum would provide a global assessment of bone turnover in the perinatal period. Such an approach addresses the current limitation of the studies published to date, which include the selection of only one or two markers of bone turnover. It would also address the current limitation of bone mineral assessment that cannot be performed during pregnancy, by providing a profile of bone biomarkers throughout pregnancy. In addition, a longer follow-up in the post-partum is also needed to delineate the impact of pregnancy versus the post-partum, specifically lactation, on maternal bone health.

Lactation practices are not often described in detail, and could encompass various lactation practices, which can differently affect maternal bone health status in the post-partum. Details should include frequency and intensity of breastfeeding and/or expressing breast milk.

Maternal dietary habits throughout pregnancy and the post-partum are not often well documented in the studies looking at bone health despite the importance of nutrition on normal bone turnover. As well, only one RCT employed food as part of their intervention showing promising impacts both at the end of pregnancy and in post-partum. The benefits of a diet intervention designed to increased intake of protein, calcium and vitamin D in

combination with an exercise program during pregnancy need to be evaluated to measure if benefits can be sustained in the post-partum period.

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CHAPTER 2

STRUCTURED DIET AND EXERCISE GUIDANCE IN PREGNANCY TO IMPROVE HEALTH IN WOMEN AND THEIR OFFSPRING: STUDY PROTOCOL FOR THE BE HEALTHY IN PREGNANCY (BHIP) RANDOMIZED CONTROLLED TRIAL

PREFACE TO CHAPTER 2

Transparent protocols are important to the good conduct and scientific validity of RCTs. Guidelines for RCT report, including protocol content, have been developed (e.g. Standard Protocol Items: Recommendations for Interventional Trials; SPIRIT guidelines), and such statements have been shown to improve report quality and their usefulness for the research community.

Our protocol outlines the rationale, primary and secondary outcomes, methods, and analysis plan of our RCT. A written protocol prior to data analysis and interpretation ensures internal validity and limits post-hoc revisions of study outcomes. We believe having it published in Trials will improve the usefulness for the research community of the RCT in pregnancy that we conducted. Our protocol includes the populated SPIRIT checklist and figure.

Authors' contributions: SAA conceived the study as the principal investigator. Co-investigators collaborated for the grant applications and implementation of the core study including facilitation of recruitment of study participants. At the McMaster University study site, **MP** conducted the core study visits with the participants, with the exception of the intervention so to remain blinded to the group allocation. MFM was responsible for recruitment and measurements at the Western University site. **MP** performed laboratory analysis for all the biomarkers (for McMaster University and Western University sites), in

collaboration with the study coordinator and conducting the DXA scans at McMaster and overseeing them at Western. **MP** wrote the paper. SAA and MFM oversaw the data collection. All authors read and approved the final manuscript.

Full citation: Perreault M, Atkinson S, Mottola M, Phillips S, Bracken K, Hutton E, et al. *Structured diet and exercise guidance in pregnancy to improve health in women and their offspring: study protocol for the Be Healthy in Pregnancy (BHIP) randomized controlled trial*. Trials. Trials; 2018;19(291):1–15.

Perreault *et al. Trials* (2018) 19:691
https://doi.org/10.1186/s13063-018-3065-x

Trials

STUDY PROTOCOL Open Access

Structured diet and exercise guidance in pregnancy to improve health in women and their offspring: study protocol for the *Be Healthy in Pregnancy (BHIP)* randomized controlled trial

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Abstract

Background: Evidence from epidemiological and animal studies support the concept of programming fetal, neonatal, and adult health in response to in utero exposures such as maternal obesity and lifestyle variables. Excess gestational weight gain (GWG), maternal physical activity, and sub-optimal and excess nutrition during pregnancy may program the offspring's risk of obesity. Maternal intake of dairy foods rich in high-quality proteins, calcium, and vitamin D may influence later bone health status. Current clinical practice guidelines for managing GWG are not founded on randomized trials and lack specific "active intervention ingredients." The Be Healthy in Pregnancy (BHIP) study is a randomized controlled trial (RCT) designed to test the effectiveness of a novel structured and monitored Nutrition + Exercise intervention in pregnant women of all pre-pregnancy weight categories (except extreme obesity), delivered through prenatal care in community settings (rather than in hospital settings), on the likelihood of women achieving recommended GWG and a benefit to bone status of offspring and mother at birth and six months postpartum.

Methods: The BHIP study is a two-site RCT that will recruit up to 242 participants aged > 18 years at 12–17 weeks of gestation. After baseline measures, participants are randomized to either a structured and monitored Nutrition + Exercise (intervention) or usual care (control) program for the duration of their pregnancy. The primary outcome of the study is the percent of women who achieve GWG within the Institute of Medicine (IOM) guidelines. The secondary outcomes include: (1) maternal bone status via blood bone biomarkers during pregnancy; (2) infant bone status in cord blood; (3) mother and infant bone status measured by dual-energy absorptiometry scanning (DXA scan) at six months postpartum; (4) other measures including maternal blood pressure, blood glucose and lipid profiles, % body fat, and postpartum weight retention; and (5) infant weight z-scores and fat mass at six months of age.

Discussion: If effective, this RCT will generate high-quality evidence to refine the nutrition guidelines during pregnancy to improve the likelihood of women achieving recommended GWG. It will also demonstrate the importance of early nutrition on bone health in the offspring.

Trial registration: ClinicalTrials.gov, NCT01689961 Registered on 21 September 2012.

Keywords: Nutrition, Exercise, Randomized controlled trial, Pregnancy, Bone, Infancy, Developmental origins of health and disease, Proteins, Dairy foods, Gestational weight gain

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Background

Consequences of excess gestational weight gain (GWG)

The documented adverse sequelae of excess GWG for both mother and child include undesirable pregnancy/birth outcomes and/or later health outcomes that are likely to impose a substantive downstream burden on healthcare costs [1]. The most common adverse effect of excessive GWG on maternal health is weight retention (five months and even up to three years postpartum) [2–4]. Mothers entering subsequent pregnancies at a higher weight are at greater risk for long-term obesity and future co-#morbidities such as diabetes and cardiovascular disease [5, 6]. The emerging evidence of developmental origins of health and disease implicates the impact of mothers' health during pregnancy on "programming" of the growing fetus in utero to develop adverse health outcomes [7, 8]. Maternal excess GWG has been reported as the strongest predictor of obesity in the offspring [9–11].

The use of low-fat dairy foods as the source of protein in pregnancy is proposed based on studies in animals [12] and humans [13–15] that provide support for the putative effects of components of dairy foods such as calcium and leucine as anti-adipogenic and pro-lipolytic agents. Consumption of high dairy protein may not only benefit GWG but also improve other maternal pregnancy outcomes such as dysglycemia, elevated blood pressure, blood lipid profiles, and risk of pre-eclampsia, the latter due to higher intake of calcium and vitamin D [16].

Guidelines for GWG exist and have been interpreted for practice in Canada [17, 18]. Until there is evidence of effective population level interventions to assist women in achieving healthy weights, programs aimed at interventions during pregnancy to improve weight management and pregnancy outcomes are a starting point. Pregnancy has been described as a "teachable moment" for weight control and obesity prevention in women [19]. The most convincing approach to achieve reduction in GWG is the combination of physical activity and diet counseling, preferably in combination with weight monitoring [1, 20, 21]. Yet current clinical practice guidelines for managing GWG are not founded on randomized trials and lack specific "active intervention ingredients" [21] that are proven effective in achieving target GWG.

Maternal programming of bone health

Findings from epidemiological and animal studies support a role for the programming of fetal, neonatal, and adult bone outcomes in response to exposures during pregnancy such as maternal nutrition and other lifestyle variables [22–25]. In three longitudinal observational studies, maternal intake of dairy foods during pregnancy was associated with higher bone mass in offspring aged 6–16 years [26–28]. In prospective cohort studies, maternal physical activity level has also been positively associated with infant bone mass and geometry at birth [29, 30]. Being born with optimal bone mass may have long-term benefits since childhood bone mass tracks until skeletal maturity, when peak bone mass is achieved [31]. Collectively, these findings suggest that bone mass during childhood and adolescence may be predictive of an individual's risk for osteoporosis, as peak bone mass is a strong predictor of osteoporotic fracture later in life [32].

To date, only one randomized controlled trial (RCT) in a small sample ($N = 36$) investigated the effect of dairy product supplementation on bone mineral density (BMD) and bone turn-over in pregnant women and these women entered pregnancy with a habitually low calcium intake [33]. The study showed a beneficial effect of increased milk consumption on maternal bone mass density at the spine and on suppression of bone resorption when measured at 6 ± 1 weeks postpartum. While promising, further studies are needed with larger sample sizes and evaluation of bone health outcomes in the offspring. In addition, no study to date has investigated the impact of dairy product supplementation during pregnancy on bone health outcomes of women after delivery.

Informed by our recent clinical and qualitative research, as well as existing systematic reviews, we have designed a RCT comprising several unique features with the objective of controlling GWG and optimizing bone health of mother and infant. We will test the effectiveness of a novel structured and monitored nutrition (high-protein dairy-based diet) along with an exercise (walking) intervention that is science-based, vetted for feasibility in pregnant women and care providers through focus groups, in pregnant women of most pre-pregnancy weight categories (except extreme obesity) in a community setting (as opposed to hospital-based).

Specific objectives and hypothesis

The primary research objective of the Be Healthy in Pregnancy (BHIP) study is to determine whether introducing a structured and monitored nutrition (high-dairy protein diet) and exercise (walking) program (intervention) in early pregnancy, compared to standard prenatal care (control), will increase the number of women attaining GWG (outcome) within the Institute of Medicine (IOM) recommendations for their pre-pregnancy body mass index (BMI) sub-category [17].

Secondary objectives include determination of the impact of a maternal high-dairy diet with exercise compared to standard care diet during pregnancy on: (1) bone status (e.g. blood biomarkers procollagen type I that contains N-terminal extensions (PINP), C-terminal telopeptide of type I collagen (CTX-I), insulin-like growth factor-1 (IGF-1), 25-hydroxyvitamin D (25(OH)D), and 1,25-dihydroxyvitamin D) in mothers

during pregnancy; (2) infant bone status at birth via cord blood; and (3) mother and infant bone status at six months postpartum by dual energy x-ray absorptiometry (DXA) scan.

The over-arching hypothesis is that maternal consumption of a high-dairy diet during pregnancy will have a positive impact on: (1) GWG; (2) bone health status of the mother during and after pregnancy; and (3) bone mass and bone size of the offspring, after adjustment for factors known to influence skeletal status.

Methods/design

Trial design

The BHIP study is a two-arm, two-site prospective RCT. This study is designed as a prospective superiority trial, with 1:1 allocation ratio to either the Intervention group (i.e. Nutrition + Exercise intervention: high-dairy nutrition intervention combined with structured exercise) or the Control group (i.e. usual care as per National Health Canada recommendations) during pregnancy. The study is open-label with blinded endpoints. The study protocol is described following the standard protocol items: recommendations for interventional trials (SPIRIT) guidelines [34]. See Additional file 1 for the SPIRIT checklist and Additional file 2 for the WHO trial registration data set.

The core BHIP trial follows women up to delivery as the primary outcome relates to GWG. The Bone-BHIP study is an extension of the core BHIP trial that continues a follow-up of the women and their offspring until six months after delivery, with the objective to assess maternal and infant bone status. This trial is registered at ClinicalTrial.gov (NCT01689961). The BHIP trial is conducted by collaborative research teams at McMaster University (Hamilton, ON) and Western University (London, ON) in Canada. The study takes place in academic hospitals and community healthcare clinics in London, Burlington, and Hamilton, Ontario, Canada. Recruitment is facilitated by healthcare professionals (e.g. family doctors and midwives) who ask their patients for consent to be contacted by the BHIP study team, as well as poster advertisements in midwifery, family practice, and ultrasound clinics, at community sites such as the YMCA, libraries, and coffee shops, and on Facebook or Kijiji.

Study recruitment began in January 2013 and is expected to be completed by April 2018, reaching the a priori calculated sample size of 242 participants for our primary outcome. All participants who sign consent to contact forms receive a phone call by study personnel and are provided a general overview of the study and expectations using a scripted text. Figure 1 outlines the participant's timeline in the BHIP study. Participants are screened for eligibility during the telephone interview according to the criteria list below. Eligible women are enrolled in the study and sign informed consent by the end of the first trimester of pregnancy (i.e. 12–17 weeks of gestation).

Inclusion criteria

- Healthy pregnant women aged > 18 years
- Singleton pregnancy (either nulliparous or multiparous)
- Able to be randomized to group allocation by 17 weeks and six days of gestation
- Pre-pregnancy BMI < 40 kg/m^2
- Planning to deliver at a Hamilton, Burlington, or London regional hospital or by home birth and willing to attend research visits at either study site
- Approval of primary care provider to participate in exercise
- Able to provide signed informed consent

Exclusion criteria

- Not conversant in English
- Known contraindications to exercise as recommended by the Canadian clinical practice guidelines for pregnancy [35]
- Severe chronic gastrointestinal, heart, kidney, liver, or pancreatic diseases or conditions
- Refusal to consume dairy foods due to intolerance or dislike
- Pre-existing diabetes
- Currently smoking and will not discontinue smoking during the pregnancy
- Depression score > 12 on the validated Edinburgh Depression scale

Randomization: allocation and implementation

Block randomization is used with block sizes of two, four, and six selected at random, using an online Research Electronic Data Capture (REDCap) randomization service managed by an independent team in the Biostatistics Unit at St Joseph's Healthcare in Hamilton, ON, Canada. Randomization to the two study arms occurs in a 1:1 ratio and is stratified by study site and pre-pregnancy BMI category following IOM guidelines (underweight: BMI < 18.5, normal weight: BMI 18.5–24.9, overweight: BMI 25.0–29.9, and obese: BMI ≥ 30.0 kg/m^2). The eligibility of participants is confirmed at the first visit, when baseline data are also collected. The research assistant randomizes the participant at the second visit to the study center using an online third-party automatic randomization system. Once randomized, the research assistant consents the participant to the appropriate study arm at a time that is 14–17 weeks/6 days of

gestation. Assessments, regardless of the study arm, occur at < 18 weeks of gestation, 26–28 weeks of gestation, 36–38 weeks of gestation, birth, three months postpartum, and six months postpartum. The intervention arm consists of weekly or biweekly in-person visits (based on the participant's preferences) during pregnancy (i.e. from allocation to study group until 36–38 weeks of gestation). Baseline measures (i.e. before randomization), primary and secondary outcome measures, and other measures assessed during and after pregnancy are measured in all participants, regardless of their study group allocation.

The flow diagram (Fig. 2) will be included in future published results.

This study is open-label due to the nature of the intervention. The study research assistants (data collectors) administering the personalized Nutrition + Exercise intervention are not blinded to group allocation. In addition, the participants are not blinded to their own group allocation, in order to maximize adherence to the lifestyle treatment. Mothers randomized to the intervention and control groups are assessed at the study clinic on different days, to prevent interaction between participants of each study arm. The primary data outcome collector is blinded to group allocation and is conducting the study visits for all participants, regardless of their group allocation. To maximize the objectivity of findings, the primary outcome assessor and data analysts/statistician are blinded to the study allocation and are not collecting any study data.

Interventions

All participants receive usual care as delivered by their healthcare practitioner (family physician or midwife) during pregnancy. In addition, after enrolment in the RCT and regardless of their group allocation, all participants receive counseling by the study nutritionist on the latest recommendations by Health Canada as

		Enrollment		Allocation	Post-allocation		Follow-up		Close-out
	TIMEPOINT	12-17wk	17wk	<18wk	26-28wk	36-38wk	Birth record	3mo pp	6mo pp
Enrollment	Contact to consent obtained	X							
	Initial contact	X							
	Eligibility screen	X							
	Informed consent		X						
	Allocation			X					
	Informed consent to study arm			X					
Intervention	Nutrition + Exercise intervention			Weekly or Biweekly visits					
	Usual care (control)								
Assessments	***Baseline variables***								
	Pre-pregnancy BMI		X						
	Demographics		X						
	Family History		X						
	Outcomes variables								
	Maternal weight and fat mass			X	X	X	X	X	X
	Bone biomarkers mother			X		X			X
	Bone biomarkers infant						X Cord blood		
	Maternal and infant bone status by DXA								X
	Maternal metabolic status, blood pressure			X		X			X
	Pregnancy & birth outcomes						X		
	Infant growth and feeding habits						X	X	X
	Maternal dietary habits			X	X	X		X	X
	Physical activity level			X	X	X			X
	Quality of life			X	X	X			X
	Health care costs						X		
	Maternal and cord blood gene variants profiles					X	X Cord blood		

Fig. 1 Flow diagram

Table 1 BHIP study arms

Component	Control arm *Usual care* 12–17 to 36–38 weeks of gestation	Intervention arm *Nutrition + Exercise intervention* 12–17 to 36–38 weeks of gestation
Gestational weight gain (GWG)	Latest recommendation by Health Canada in terms of GWG [17] and the Pregnancy Weight Gain Calculator [44]	Latest recommendation by Health Canada in terms of GWG [17]
Nutrition during pregnancy	Latest recommendation by Health Canada in terms of nutrition during pregnancy [74] and Eating Well with Canada's Food Guide [46]	Latest recommendation by Health Canada in terms of nutrition during pregnancy [74] **Nutrition component of the intervention** 1. Individualized nutrition plan with a high protein content: ~ 25% of energy intake which is within the acceptable macronutrient distribution range a. Individualized to each mother's estimated energy requirement and calculated using the equation derived in the Dietary Reference Intake report for women [43] with energy intakes adjusted as recommended in the second and third trimesters [74] b. Provided by dairy foods: 4–5 servings of dairy food/d: fresh low-fat white milk and/or cottage cheese and/or low-fat Greek yogurt, as implemented in our previous study [38]
Exercise during pregnancy	Latest recommendation by Health Canada in terms of exercise during pregnancy [75]	Latest recommendation by Health Canada in terms of exercise during pregnancy [75] **Exercise component of the intervention** 1. Controlled walking program with the study nutritionist a. 25 min per session, 3–4 times per week, while increasing the time by 2 min/week to a maximum of 40 min maintained until delivery [39] 2. 10,000 steps per day a. Daily step counts and any other exercise tracked using a pedometer every day and an exercise log. Also used as a motivator and self-monitoring device which will also help improve compliance [39]
Wellbeing during pregnancy	**Nested qualitative study** 1. Focus group and information session given by a midwife in the third trimester of pregnancy a. Discussion on topics such as pain relief options during labor and breastfeeding techniques	

published online (https://www.canada.ca/en/health-canada/services/healthy-living/healthy-pregnancy.html). After delivery, both study groups receive usual care from their healthcare practitioners with no further intervention; the research team prospectively follows participants at three months and six months postpartum. Table 1 outlines the study arms.

Participants randomized to the personalized, monitored, and structured Nutrition + Exercise program group (intervention) begin early in the second trimester of their pregnancy until the end of pregnancy with counseling on a weekly or biweekly basis (Table 1 and Fig. 1). The nutrition component is an individualized nutrition plan tailored to each participant's energy requirements with a high protein content (25% protein energy) provided primarily by dairy foods. Dairy foods are accepted by women during pregnancy as a healthy choice as indicated from our pilot study [36] and in a recent birth cohort study in the same community in which women consumed an average of ≥ 3 servings of dairy foods per day [37]. Dairy foods are sources of high-quality proteins, calcium, and vitamin D in the case of milk as it is under mandatory fortification with vitamin D in Canada. The Nutrition + Exercise intervention is considered safe. First, the nutrient intake associated with the increased low-fat dairy consumption is less than the tolerable upper intake level for all nutrients as recommended per the Dietary Reference Intake by Health Canada. In addition, walking (the exercise component) is the easiest physical activity to undertake and implement in terms of goal-setting for step count and monitoring adherence using accelerometer-type devices as demonstrated in our previous studies [38–40]. Walking was the most practical exercise intervention of choice since women reduce moderate and vigorous physical activity during pregnancy yet maintain levels of walking [41]. Most importantly, these exercise guidelines are based on the Physical Activity Readiness Medical Examination (PARmed-X)

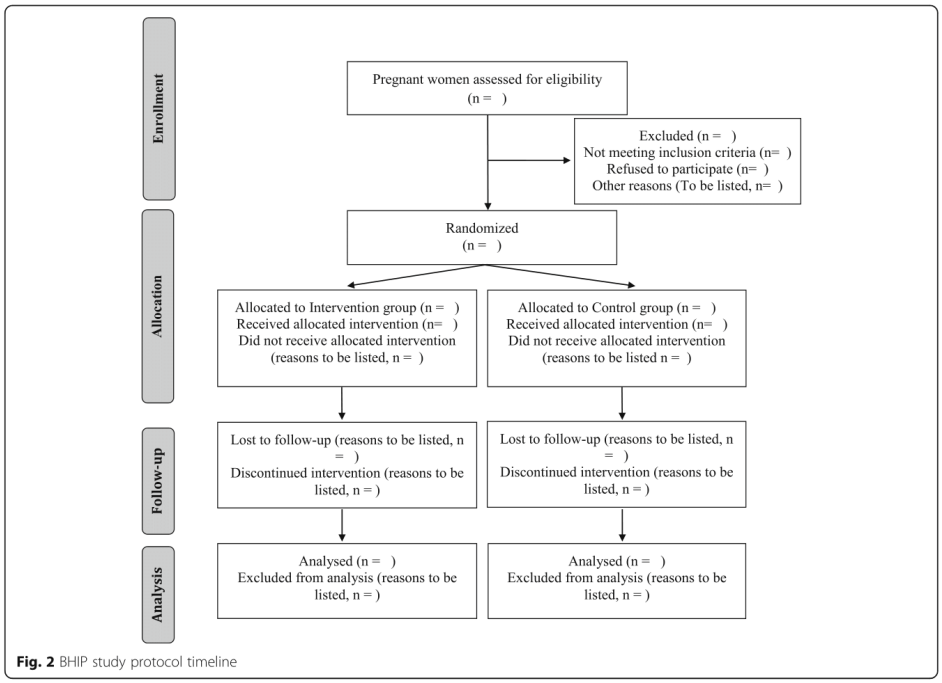

Fig. 2 BHIP study protocol timeline

for Pregnancy [35]. Data from the Nutrition and Exercise Lifestyle Intervention Program study [39] indicates that the goal of 10,000 daily steps is feasible under free-living conditions and has been effectively utilized in a number of trials [42].

A component of the intervention is discontinued if the medical condition of a participant changes and the intervention is modified if a participant requests a change due to personal reasons, such as food aversion or pain episode. The intervention is personalized for every woman, to ensure the goal of 25% energy from protein and 10,000 steps/day are achieved. Additionally, estimated energy requirements are calculated for each mother using the equation derived in the Dietary Reference Intake report for normal and overweight women [43] with energy intakes adjusted as recommended in the second and third trimesters [17]. The individualized nutrition plan is fashioned upon a standard three-day food intake record completed by the mothers that includes one weekend day as used previously in pregnant women [39]. The individualized nutrition plans and counseling are conducted by a study nutritionist providing the number of food servings to meet their estimated energy requirements. The nutrition plan is modified if GWG monitoring indicates weight loss or excess gain has occurred as recommended in the GWG guidelines [17, 44]. Adverse events related to the intervention are reported to our local ethics boards. Other non-intervention-related adverse events are noted in the participant's chart and monitored, in accordance with the N2 Network of Networks guidelines Canada [45].

Several strategies are used to improve adherence to the intervention. For the exercise component, participants wear and track their step counts on a weekly basis, increasing motivation and adherence to the treatment. In addition, they go for a walk with the research staff and have a personalized counseling session with the study nutritionist. For the nutrition component, participants are provided with low-fat milk, cottage cheese, and/or yogurt, as per their preference, to ensure they consume the recommended amount of dairy foods. Participants come to the study site at least every other week to receive their dairy foods and the study nutritionist shares strategies and recipes with the participants to ensure they consume the targeted amount of dairy food

servings. All these actions improved the adherence to the study protocol.

Participants randomized to the usual care group (control) receive usual prenatal care. Participants are given the most recent advice from Health Canada including Healthy Weight Gain During Pregnancy [17], the Pregnancy Weight Gain Calculator [44], and Eating Well with Canada's Food Guide [46]. Mothers are followed by their primary care provider who also receives the same Health Canada materials. After delivery, they are followed by their healthcare practitioners. In addition, the control participants are invited to participate in a focus group and to attend an information session led by a midwife; topics discussed include pain relief options during labor and breastfeeding techniques (Table 1).

Outcome measurements

Primary and secondary outcomes are summarized in Table 2 and described below.

- Primary outcome
  - GWG
- Secondary outcomes
  - Maternal and cord blood circulating bone markers: PINP, CTX-I, IGF-1, 25(OH)D, 1,25dihydroxyvitamin D
  - Maternal bone status at six months postpartum: whole-body bone mineral content (BMC), whole body BMD, lumbar spine BMD
  - Infant bone status at six months of age: whole body minus the head BMC
- Other outcomes
  - Maternal body weight and fat mass
  - Maternal metabolic status (including fasting glucose and lipid profiles, leptin and adiponectin)
  - Maternal blood pressure
  - Maternal pregnancy outcomes such as gestational diabetes and pre-eclampsia
  - Infant birth outcomes such as birth weight and body fat mass at six months
  - Safety outcomes
  - Quality of life using the EQ-5D questionnaire
  - Healthcare costs
  - Maternal and cord blood gene variant profiles

Data and biosample collection

The pre-pregnancy BMI is calculated using the measured weight at study entry minus the mother's self-reported GWG. Body weight is measured by trained research assistants using a body impedance Tanita Body Composition Analyzer BF-350 scale (Tanita, IL, USA) at 12–17 weeks of gestation, 26–28 weeks of gestation, 36–38 weeks of gestation, three months postpartum (self-reported weight only), and six months postpartum. GWG is calculated by subtracting the weight at the 36–38 weeks of gestation study visit by the pre-pregnancy weight. Adjustments such as rate of GWG will be made to account for gestational age at delivery, including pre-term births.

Fasted maternal blood samples for metabolic and bone health biomarkers are collected at 12–17 weeks of gestation, 36–38 weeks of gestation, and at six months postpartum; venous cord blood is collected at delivery. Serum is collected in serum-separating with gel (SST™) vacutainers and in spray-coated silica vacutainers; plasma is collected in sodium fluoride/ Na_2 ethylenediaminetetraacetic acid (Na2EDTA) vacutainers. Sample are centrifuged for 10 min at 3000 rpm at 4 °C; serum separator tubes are spun for an additional 5 min, aliquoted, and stored at − 80 °C. Samples are batched to include all samples from each mother for analysis in our laboratories when participants have completed the study. Fasting plasma glucose is determined using a hexokinase photometric assay (Architect kit, Abbott, Abbott Park, IL, USA). Serum triglycerides are analyzed using a glycerol phosphate oxidase photometric assay (Architect kit, Abbott, Abbott Park, IL, USA) completed by Hamilton Health Sciences Regional Laboratory Medicine Program. Serum leptin and insulin are analyzed using Luminex® human premixed multi-analyte enzyme-linked immunosorbent assay (ELISA) kit supplied by R&D Systems (Minneapolis, MN, USA). Serum adiponectin and C-reactive protein are analyzed by Luminex® premixed multi-analyte ELISA kit supplied by R&D Systems. Bone formation is assessed by measuring serum PINP by ELISA (Cloud Clone Corp., Houston, TX, USA). Bone resorption is assessed by measuring serum CTX-I by (Serum Crosslaps (CTX-1) ELISA, product code AC-02F1 Immunodiagnostic Systems, UK). Growth is assessed by measuring serum IGF-1 by ELISA (R&D Systems, Minneapolis, MN, USA). All standards and samples are analyzed in duplicate, following the manufacturer's instructions. A serum quality control is run in triplicate at the beginning and end of each ELISA plate to calculate inter- and intra-assay coefficients of variability. Serum vitamin D metabolites are measured by liquid chromatography tandem mass spectrometry (LC-MS/MS) using a Waters AQUITY Tandem Quadropole Detector coupled to an AQUITY UPLC system (Waters Corporation), after extraction that includes a saponification step as adapted from Hymoller [47]. Accuracy is determined using the certified Vitamin D analytes serum-based standard reference material (SRM 972a) from the US National Institute for Standards and Technology (NIST; National Bureau of Standards, Washington, DC, USA) [48]. Precision is measured using a serum quality control in all runs of samples

Table 2 Analysis plan: objectives, outcomes, hypotheses, and methods of analysis

Objective	Hypothesis	Outcome measure (type of outcome: B = binary or C = continuous)	Methods of analysis
1. Primary	An experimental combined Nutrition + Exercise intervention will increase the percentage of pregnant women who achieve GWG within current recommendations when compared with standard care provided in the primary care community setting	Proportion of women who are within the BMI appropriate GWG according to the IOM guideline for GWGs (B)	Logistic regression
2. Secondary	An experimental combined Nutrition + Exercise intervention will lead to better maternal and child bone health outcomes when compared to standard care	• *Maternal and cord blood circulating bone markers (C)* ○ Bone biomarkers: PINP, CTX-I, IGF-1, 25(OH)D, 1,25$(OH)_2$D • *Maternal bone status at 6 months postpartum (C)* ○ Whole body bone mineral content, whole body BMD, lumbar spine bone mineral density by DXA scan • *Maternal fat mass (C)* • *Maternal blood glucose, lipid profile, leptin, and adiponectin* • *Maternal blood pressure (C)* ○ Diastolic BP ○ Systolic BP • *Maternal pregnancy outcomes (B)* ○ Gestational diabetes ○ Pre-eclampsia • *Infant bone status at 6 months of age (C)* ○ Whole body minus the head bone mineral content by DXA scan • *Infant outcomes* ○ Birth weight z-score(C) ○ Body fat mass (B)	Regression analysis *We will use logistic regression for binary outcomes and linear regression for continuous outcomes
3. Subgroup analyses	The percentage of women within each of the normal, overweight, and obese BMI categories will be similar with respect to being with the IOM target GWG for each category	Proportion of women in each BMI category who reach appropriate GWG according to the IOM guideline for GWGs	Regression analysis including the interaction term of BMI group X Intervention group
4. Sensitivity analyses	Combined Nutrition + Exercise Intervention leads to a greater percentage of women who achieve GWG within current recommendations when compared to standard care	Primary outcome only	• Generalized estimating equations • Random-effects model

IMPORTANT REMARKS:
In all analyses, results will be expressed as difference or OR (95% CI) and associated *p* values, as appropriate
Bonferroni method will be used to adjust the overall level of significance for multiple secondary outcomes
We will examine residuals to assess model assumptions
The GEE [76] is a technique that allows to specify the correlation structure between patients within a site and this approach produces unbiased estimates under the assumption that missing observations will be missing at random. An amended approach of weighted GEE will be employed if missingness is found not to be at random [77]
*Infant growth outcomes at 6 months will be adjusted for feeding type (duration of breast feeding from birth to 6 months)

tested, allowing calculation of inter- and intra-assay coefficients of variability.

DNA extracted from maternal and cord blood plasma samples will be purified using the Chemagen 500 MSM I (PerkinElmer chemagen Technologie GmbH, Baesweiler, Germany) and genotyped using the Human Core Exome Bead Chip by Illumina. The chip is specifically designed to allow imputation of up to 18 million markers in multi-ethnic populations. Data cleaning and quality control of SNPs data are done according to the guidelines published by Winkler et al. [49].

Bone mass is measured by DXA scan using the research dedicated QDR®4500 series Hologic Inc. Discovery™ DXA machine (Waltham, MA, USA; Adult whole body software version 12.3.1 and Infant whole body software) at the McMaster University study site and the General Electric-Luna iDXA (Ames Medical enCORE, Version 14.1, Waukesha, WI, USA; CoreScan, GE) at the Western University study site. Daily quality control tests are conducted using an artificial L_{1-4} lumbar spine made from hydroxyapatite encased in epoxyresin. Weekly, a calibration test is performed using a step phantom

(composed of soft-tissue and lean-tissue equivalent materials); at this time, the uniformity test is also performed to evaluate the contribution of air molecules to the attenuation of the X-rays. The coefficient of variations for BMC and BMD were 0.65% and 0.38%, respectively (Hologic Inc., Bedford, MA, USA) in our past study [37]. Inter-site comparison of DXA measures is conducted by having two phantoms (i.e. Hologic lumbar spine phantom (#2603), BFP® phantom) tested at both study sites. This provides a cross-calibration standard and enables us to account for inter-site differences and to harmonize results. It also allows for verification of the instrument stability over the course of the study.

At six months postpartum, bone scans are performed on mothers and infants at six months by staff following a standard operating procedure. Mothers are dressed in regular clothes without any metal parts or in a light hospital gown, while infants are in a clean diaper only. The primary outcome assessor, who has expertise in analyzing DXA scans, reviews all scans from both study sites. This ensures consistency in analysis across all participants at both study sites. If objects are included in the scan such as a child's toy, a sub-regional analysis is done to isolate and subtract the object's contribution to overall results. If considerable movement artifacts are seen in infant scans, the well-captured limb is used as a surrogate for the one with movement [50]. Any scans with unsalvageable distortions due to movement are not included. Z-scores are calculated for all BMC and BMD results using data from an age- and sex-specific standard curve. Results for women are interpreted as z-scores in reference to an adult women population embedded in the Hologic software. Results for infants are expressed as whole body minus the head BMC and BMD. Data are interpreted as z-scores in reference to an infant population from in-house data collection on normal healthy term infants from birth to one year of age.

All participants receive instructions to complete a standard three-day food intake record at 12–17 weeks of gestation, 26–28 weeks of gestation, 36–38 weeks of gestation, and six months postpartum. The food record includes two weekdays and one weekend day, as used previously in pregnant women [39]. It captures food and beverage intake as well as drug and supplement (vitamin and mineral) intake. Trained assessors analyze all food records using standard operating procedure on the computer-based Nutritionist Pro Software (Axxya Systems, Stafford, TX, USA). Nutritionist Pro computes the average amount of nutrients consumed per day.

Physical activity level is assessed through wearing a BodyMedia Sensewear Pro II armband monitor device (BodyMedia, Pittsburgh, PA, USA) in all participants for the same three days (as food monitoring above) in their work/home environment. These small devices are comfortable and easily wearable on the arm [38]. The data collected for three days at each visit (12–17, 26–28, and 36–38 weeks of gestation and six months postpartum) includes daily steps, daily energy expenditure, and minutes of activity level in Metabolic Equivalent of Task (MET; very vigorous, vigorous, moderate, sedentary). The Sensewear unit does not provide feedback to the participant.

Pregnancy and infant outcomes are obtained from participant's medical records. Details are extracted about Cesarean section and/or vaginal delivery. For infant outcomes, data regarding gestational age, birth weight, birth length, birth head circumference, 1-min and 5-min Apgar scores, complications related to birth, and feeding practices at birth are extracted.

Infant weight and length at birth are recorded from medical charts and at three months of age as reported by the mother during the telephone interview. At the six-month visit, trained assessors measure infant weight by electronic scale (Medela BabyWeigh, McHenry, IL, USA), length using a measuring board for term infants (Pediatric Stadiometer, Ellard Instrumentation Ltd., Monroe, WA, USA), and head circumference using a constant-tension measuring tape (OHAUS, Dundas, ON, Canada), following standard operating procedures.

Infant feeding practices over the first three months are recorded by phone call and at six months in person using a standardized questionnaire administered by a trained assessor [51]. The questionnaire includes feeding history, duration and extent of breastfeeding, introduction of solid foods, and use of vitamins and supplements.

Study management and governance

This RCT is led by investigators from McMaster University (Department of Pediatrics, Department of Family Medicine, Department of Obstetrics and Gynecology, Department of Health Research Methods, Evidence and Impact, and School of Nursing) and Western University (School of Kinesiology) as well as practitioners from the City of Hamilton Public Health, all located in Ontario, Canada. The oversight of the study is guided by the steering committee that is composed of the lead investigators (SAA, SMP, MFM, LT, and EKH). The sponsors of the study are McMaster University and Western University. The sponsors are indemnified for any harms arising from trial participation and they approve protocol amendments when ethics approval has been obtained. Day-to-day running of the study is provided by the trial principal investigators, the study coordinators, and research assistants. The monitoring board consists of three well-established investigators from universities within Canada but outside McMaster and Western Universities.

All case report forms are anonymized using study identifiers and are stored in locked cabinets in a locked

office. An administrative assistant to the study team who is not involved in the study holds the key to participants' names. Research staff who are not involved in data collection enter data in a two-step process (entry and verification, following standard operating procedures) in our REDCap projects hosted at McMaster University [52]. REDCap is a secure, web-based application designed to support data capture for research studies, providing: (1) an intuitive interface for validated data entry; (2) audit trails for tracking data manipulation and export procedures; (3) automated export procedures for seamless data downloads to common statistical packages; and (4) procedures for importing data from external sources. Use of range check for data values promotes data quality. Double data entry is performed to ensure inter-study site reliability.

The research assistant monitors intervention adherence biweekly. For the nutrition portion, this includes ensuring dietary intake meets each participant's caloric needs and are within an acceptable macronutrient distribution range. In terms of the exercise portion, participants monitor and report biweekly their physical activity using a diary and by wearing a pedometer. Participants report any discomfort experienced (due to the natural course of pregnancy and/or the intervention) to the study coordinator who personalizes the intervention as needed.

Statistical methods

The analysis and reporting of the results will follow the SPIRIT statement for reporting RCTs [53]. The process of patient selection and flow throughout the study will be summarized using a flow-diagram (Fig. 2).

The results of patient baseline characteristics and outcome variables (both primary and secondary) will be summarized using descriptive summary measures: expressed as mean (standard deviation) or median (range) for continuous variables and n (%) for categorical variables. All statistical tests will be performed using two-sided tests at the 0.05 level of significance. Binary outcomes will be analyzed using logistic regression and continuous outcomes will be analyzed using linear regression. All analyses will account for stratification by including study site and participants' BMI category in the models. For all models, the results will be expressed as estimate of the difference for continuous outcomes (odds ratio [OR] or relative risk [RR] for binary outcomes), corresponding two-sided 95% confidence intervals (CI) and associated p values. P values will be reported to three decimal places with values < 0.001 reported as < 0.001. The Hosmer–Lemshow will be used to assess goodness-of-fit for logistic regression and explore the residuals to assess goodness-of-fit and model assumptions for linear regression.

An intention-to-treat principle will be adopted to analyze all outcomes. Multiple-imputation will also be used to handle missing data during data collection. Sensitivity analyses will be performed using some of the commonly used RCT patient-level methods (e.g. generalized estimating equations, the random-effect model which account for the potential correlation within sites, etc.) to assess the robustness of the results [54]. All the analyses will be done using SAS 9.2 (Cary, NC, USA) or SPSS 13 (Chicago, IL, USA). Table 2 provides a summary of the planned methods of analyses for each outcome and subgroup.

Power calculation

The sample size calculation for the core study is based on the test of the null hypothesis that the percentages of women with GWG within IOM guidelines in the two populations (intervention and control) are equal. To account for the uncertainty in these prior estimates, we calculated the sample size for different values of the percentage of women with weight within IOM guidelines in the control group in the range of 50–65%, with a risk difference of 15%, 20%, 25%, and 30%. While initially we targeted a total sample size of 350, the constraints of our funding timelines (which include a one-year CHIR grant extension) and a recruitment rate that was less than projected despite our best efforts, we revised our desired sample size to 242. Based on our a priori calculation, a sample size 111 per group corresponds to an absolute difference of 30% with our Nutrition + Exercise intervention, resulting in 30% of women in the treatment group having a GWG exceeding the IOM recommendations compared to 65% in the control group. With the revised sample size (i.e. assuming a 1:1 allocation ratio), the study will have power of 80% to yield a statistically significant result assuming a binomial distribution (using an intention-to-treat principle for the analysis) of the difference between percentages of women with GWG within IOM guidelines at alpha = 0.05. We hope to increase the sample size to 242 total participants in order to allow us to account for the two stratification variables – site (2 degrees of freedom) and baseline BMI strata (3 degrees of freedom).

The calculated sample size for the secondary outcome of maternal bone biomarker status is a total of 177 participants (allocation 1:1, $\alpha = 0.05$, $\beta = 20$ for two-group t-test), based on literature with similar outcomes [55, 56]. A sample size was also calculated for the secondary outcome of infant whole body minus the head BMD. The total is 240 participants (allocation 1:1, $\alpha = 0.05$, $\beta = 20$ for two-group t-test) based on the literature with similar primary outcomes in infant populations [57, 58]. For both sample sizes, a 15% attrition rate was calculated as a precaution in this type of clinical trial with follow-up.

Discussion

The BHIP study is novel as it proposes a feasible Nutrition + Exercise intervention that pregnant women could incorporate into their lifestyle and improve adherence to GWG recommendations. In addition, it will contribute to the knowledge of the perinatal developmental programming of body composition and skeletal phenotypes.

The BHIP RCT enrolls women in early pregnancy (12–17 weeks of gestation). Ideally, women should have access to general health interventions before pregnancy; however, currently pregnancy is often the first opportunity for health professionals to address issues beyond those directly related to the pregnancy state. Thus, one of the strengths of the BHIP study is that it starts in early pregnancy and monitors participants until six months postpartum. In addition, measures of health outcomes in both mother and child at various time points throughout pregnancy and postpartum allow for a comprehensive evaluation of the intervention effects. Lastly, one particular strength of the BHIP study is the individualized intervention and weekly monitoring. Evidence shows that the consumption of dairy foods (nutrition component) is well accepted during pregnancy [36], while walking (exercise component) is the best physical activity to promote adherence during pregnancy [38–40].

The primary outcome of GWG was selected because it is the key clinical problem we are addressing with our Nutrition + Exercise intervention. Pre-pregnancy obesity and excess GWG are the strongest maternal characteristics associated with offspring obesity [9, 11, 59] and childhood metabolic dysfunction [60]. Excess GWG in pregnancy is a major clinical challenge affecting Canadian women who enter pregnancy overweight and even women of normal pre-pregnancy weight [61, 62]. At the national level, a cross-sectional study on pregnancy experiences in Canada determined that over one-third of women entered pregnancy overweight or obese and nearly 60% experienced GWG greater than that recommended by the IOM recommendations for GWG [63]. The Alberta Pregnancy Outcomes and Nutrition prospective cohort study reported similar statistics with a greater number of overweight and obese women (80%) gaining excess weight during pregnancy [64]. Likewise, in the Hamilton region, data from the Family Atherosclerosis Monitoring In earLY life birth cohort demonstrated that > 50% of women entered pregnancy with a pre-pregnancy BMI of > 25.0 kg/m^2 and > 50% exceeded the IOM guidelines for GWG [65]. Evidence to date suggest that the most convincing approach to achieve appropriate GWG is an intervention combining physical activity and nutrition, in combination with weight monitoring [1, 20, 21].

The secondary outcome of bone status was selected due to the emerging evidence of risk of osteoporosis in later life being programmed from the womb [24]. Bone biomarkers were selected as outcomes following the International Osteoporosis Foundation and the International Federation of Clinical Chemistry and Laboratory Medicine recommendations, which is to measure a marker of bone formation and a marker of bone resorption to assess bone turnover [66]. Currently, the most sensitive markers recommended are subgroups of type I collagen representing the predominant component of the bone matrix wherein sub-products can be measured as markers of formation and resorption of the bone [66]. To support fetal bone growth, pregnant women undergo bone metabolic adaptations over the course of pregnancy, where bone resorption usually rises and formation declines [67]. These changes result in high bone turnover during pregnancy [68]. Maternal bone turnover peaks during the third trimester, with a marked increase in bone resorption observed at 38 weeks of gestation [69]. Based on this knowledge, two biomarkers of bone modelling are quantified in maternal blood at 36–38 weeks of gestation and in cord blood. The marker of bone formation is PINP and the marker of bone resorption is CTX-I. Blood samples are consistently collected in a fasted state and in the morning, as suggested by the National Bone Health Alliance [70]. In addition, vitamin D status is measured by serum 25(OH)D as it plays an important role in calcium homeostasis and bone metabolism [71] and is a reflection of dietary vitamin D intake and sun exposure. Both the frequency of milk consumption and sun exposure were shown to be positive predictors of serum 25(OH)D concentrations in the recent multivariate analysis of three cycles of the Canadian Community Health Measures surveys [72]. The active form of vitamin D, 1,25dihydroxyvitamin D, is also measured since it is known to be upregulated during pregnancy [73].

Bone mineral content was selected as an outcome because it is linked to the peak bone mass (i.e. highest bone mass accrued for an individual) and is predictive of osteoporotic fracture [32]. Furthermore, recent evidence suggests that childhood BMC persists until peak bone mass and could indicate individuals at risk of osteoporotic fracture [31].

The nature of the intervention brings a limitation; the research study personnel in direct contact with the participants are not blinded to the study allocation of each participant. To preserve the integrity of the study findings and the internal validity of the study, all other investigators and statisticians are blinded to the study allocation, including people collecting and performing the analysis of primary and secondary outcomes. Some sources of bias are inevitable in the context of the study: recruitment sites are mostly primary care clinics, with few participants recruited from advertisements in the community. Since women are excluded with limited comprehension of the English language, if documented

signs of depression or a pre-pregnancy BMI > 40 kg/m^2, the generalization of the study would exclude such groups. From previous studies completed in urban Southern Ontario [39, 65] we anticipate that our study population will be mostly Caucasian, holding a university degree, and with a medium to high socioeconomic status. This sample is representative of the populations in the cities were recruitment occurred as they are modern urban centers with universities, colleges, and major commerce. Thus, the results will be generalizable to populations of women living in many Canadian cities with academic centers and major commerce. In addition, our results will still be of high value for the general Canadian pregnant women population who are served through provincial public health units, as it will bring new knowledge to refine the Canada Prenatal Nutrition Program (CPNP, https://www.canada.ca/en/public-health/services/health-promotion/childhood-adolescence/programs-initiatives/canada-prenatal-nutrition-program-cpnp.html), which is currently offered through public health but not based on evidence.

The strengths of the design include that the Nutrition + Exercise intervention was vetted for feasibility in pregnant women and care providers through focus groups and was conducted in pregnant women of all pre-pregnancy BMI categories (except extreme obesity) and in a community setting (as opposed to hospital-based as in many reported studies).

For the timely recruitment of study participants, challenges included prolonged ethics approval processes at secondary recruitment sites, reduced recruitment at community family practices due to influx of immigrants who were non-English speaking (thus not eligible), and competition with other studies sampling pregnant women. In light of these, we have taken measures to achieve the predetermined sample size based on a power analysis for the primary outcome so that the integrity of the study is preserved and the results are able to properly test the stated hypothesis and provide the needed new knowledge. To expand recruitment at McMaster University a new local study site was set up in a nearby city, Burlington, Ontario, with the collaboration of midwifery and obstetric clinics, as well as the local hospital. This partnership is very successful thus helping to achieve the recruitment goal. To date, retention after randomization to the study visit at 36–38 week of gestation when we assessed our primary outcome is 87%. To maximize retention of participants, we implemented a number of procedures such as study visit times at the convenience of the participants, including home visits. We also offered in-person, over the phone, and email correspondence to keep participants engaged. For participants in the intervention group, providing them with the dairy products (which are an integral part of the intervention) increases the likelihood of consuming the recommended amount. For the exercise component, participants receive a free pedometer as a self-motivation tool to reach their step count goals. Lastly, for both groups, participants are compensated on three occasions with $25 gift cards that they can redeem at a local grocery store.

If effective, this RCT will generate high-quality evidence to refine the nutrition guidelines during pregnancy to improve health of women during pregnancy and their offspring in early life, including acquisition of a strong skeleton.

Trial status

Active recruitment continues since a no-cost extension of one year from CIHR was obtained in December 2016, to allow the BHP study to achieve the target sample size.

Additional file

Additional file 1: SPIRIT checklist. (DOCX 76 kb)
Additional file 2: WHO Trial registration data set. (DOCX 18 kb)

Abbreviations

25(OH)D: 25-hydroxyvitamin D; AAFC: Agriculture and Agri-Food Canada; BHIP study: Be Healthy in Pregnancy study; BMC: Bone mineral content; BMD: Bone mineral density; BMI: Body mass index; CIHR: Canadian Institutes of Health Research; CTX-I: C-terminal telopeptide of type I collagen; DXA: Dual-energy X-ray absorptiometry scan; ELISA: Enzyme-linked immunosorbent assay; GWG: Gestational weight gain; IGF-1: Insulin-like growth factor-1; IOM: Institute of Medicine; LC-MS/MS: Liquid chromatography tandem mass spectrometry; MET: Metabolic Equivalent of Task; NIST: US National Institute for Standards and Technology; OR: Odds ratio; PARmed-X: Physical Activity Readiness Medical Examination for Pregnancy; PINP: Procollagen type I that contains N-terminal extensions; RCT: Randomized controlled trial; REDCap: Research Electronic Data Capture; RR: Relative risk; SPIRIT: Standard protocol items: recommendations for interventional trials; SST: Serum-separating vacutainers

Acknowledgements

We are grateful to partners in the community who assisted in recruitment and to all of the participants who enthusiastically volunteered for the study.

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Funding
This study is supported by CIHR (FRN 123347) and Dairy Research Cluster Initiative (Dairy Farmers of Canada and AAFC, the Dairy Network and the Canadian Dairy Commission) and in-kind provision of milk, yogurt and cottage cheese by GayLea Foods Coop & Ultima Foods, Canada. MP is supported by a doctoral Vanier CIHR Canada Graduate Scholarship and a Career Enhancement Program scholarship by the Canadian Child Health Clinician Scientist Program. REM is supported by a CIHR Master's Scholarship. DM is supported by a Canada Research Chair in Genetics of Obesity.

Availability of data and materials
The datasets during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authorship & Dissemination policy
As set out in the Collaborative Clinical Trial Agreement between McMaster University and Western University, under article 3, Intellectual Property and Publication, "*all rights, title and interest in and to any and all data arising out of the Study Data shall be the sole and exclusive property of McMaster University*." By agreement with Western University it is established that the first publication of the study will be made as a joint, multi-center publication of the analysis of the completed study of all participants involving all Investigators and Institutions. Authorship of the multi-center publication shall be in accordance with academic standards for authorship. If such a multi-center publication has not been submitted to an academic journal within 18 months after conclusion, abandonment, or termination of the study at all participating sites or if the principal investigator confirms in writing that there will be no multi-center study publication, the investigator may publish the information and/or results from the institution. As stated in the contract between Dr. Stephanie Atkinson and Dairy Farmers of Canada, "*Principal investigator reserves the right to publish the results of the study. Before publishing, however, the principal investigator will provide Dairy farmers of Canada with a copy of such proposed publications together with manuscripts related to the study, to be disclosed, for review and, if applicable, comments, which shall be provided within 30 days. The final decision concerning the content and journal of publication shall rest with the principal investigator, which shall nevertheless consider any comments and modifications proposed by Dairy Farmers of Canada, except where same are unreasonable or biased in the reasonable opinion of the principal i*nvestigator." Finally, the DSMB members will not be co-authors on future publications in order to avoid conflict of interest, but they will be acknowledged in all manuscripts for their work.

Authors' contributions
SAA conceived of the study and was the principal applicant for the grants awarded; SAA, SMP, MFM, KB, EKH, FX, and LT initiated the study design; SAA, MFM, KB, EKH, MP, HP, and LT helped with implementation; all members of the BHIP study team are investigators on the grant for the core BHIP study; SAA and DM initiated the design of the secondary objectives related to bone health outcomes and SAA, DM, MFM, and HP are grant holders for the Bone BHIP study; LT provided statistical expertise in clinical trial design; MP and REM conducted research pertaining to this manuscript; SAA and LT will be conducting the primary statistical analysis. MP, SAA and LT drafted the manuscript for the study protocol and other authors contributed to its revision. All authors contributed to refinement of the study protocol and approved the final manuscript.

Ethics approval and consent to participate
After independent, full, external peer review the study protocol and subsequent amendments have been approved by the Hamilton Integrated Research Ethics Board (REB Project no. 12–469, McMaster University and its associated sites, Hamilton, ON), the Joseph Brant Hospital Research Ethics Committee (Project titled "Be Healthy in Pregnancy (B-HIP): An RCT to study nutrition and exercise approaches for healthy pregnancy," Joseph Brant Hospital, Burlington, ON), and the Health Sciences Research Ethics Board (HSREB File no. 103272, Western University and its associated sites, London, ON). This study is being conducted in compliance with the protocol, Good Clinical Practice and the applicable regulatory requirements.

The Research Ethics boards have approved changes to the protocol since the inception of the clinical trial. All changes were communicated to relevant parties and modified on the trial registries. Of significance, the inclusion criteria was modified: increasing BMI from 30 to 40 kg/m^2 to capture a representative sample of the community who presents an important percentage of obese women; participants have to be randomized to group allocation before 17 weeks 6 days (initially set at 11 weeks 6 days) to maximize participant enrolment while ensuring an intervention period of at least 20 weeks. Further, two groups originally excluded were added to the inclusion criteria: women whose pregnancy is a result of in vitro fertilization and women currently breastfeeding from a previous pregnancy. Exclusion criteria were also modified to redefine exclusion of participants with a prenatal depression score > 12 (rather than the initial score of 10) on the validated Edinburgh Depression scale as that is indicative of severe depression and should be referred for treatment.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Received: 14 April 2018 Accepted: 21 November 2018
Published online: 19 December 2018

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CHAPTER 3

FACTORS ASSOCIATED WITH SERUM 25-HYDROXYVITAMIN D CONCENTRATION IN TWO COHORTS OF PREGNANT WOMEN IN SOUTHERN ONTARIO, CANADA

PREFACE TO CHAPTER 3

Maternal vitamin D status in pregnancy is critical to pregnancy health outcomes, the vitamin D status of the infant at birth, and may program bone development in childhood and later life. Many recent published papers cite a 'widespread prevalence of vitamin D deficiency in pregnancy'. Whether this applies to pregnant women in North America is unclear since no population-based data on vitamin D status in pregnancy exist in Canada or the United States to date. Further, use of supplements containing vitamin D has increased over the last decade among pregnant women in North America. Our paper addresses the issue of widespread vitamin D deficiency by examining vitamin D status in pregnant women from two studies conducted ten years apart in Southwestern Ontario, Canada. We employed gold-standard methodology to measure vitamin D status, and conducted a comprehensive assessment of vitamin D intake. Because of the extensive phenotypic data collected in the respected studies we were able to evaluate the factors associated with maternal vitamin D status. Our work addresses the misconception that pregnant women in Canada are vitamin D deficient and should receive high dose of supplements.

Authors' contributions: **MP** conducted research for the BHIP study. **MP** performed laboratory work for the samples collected as part of the BHIP study, in collaboration with GF and CJM. **MP** analyzed data and performed all statistical analysis for both FAMILY and BHIP data. MP wrote the paper. SAA conceptualized and oversaw both studies,

obtained the grant support and had primary responsibility for the final content. SAA and MFM oversaw the data collection. All authors read and approved the final manuscript.

Full citation: Perreault M, Moore CJ, Fusch G, Teo KK, Atkinson SA. *Factors associated with serum 25-Hydroxyvitamin D concentration in two cohorts of pregnant women in Southern Ontario, Canada*. Nutrients. 2019;11:123.

nutrients

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Article

Factors Associated with Serum 25-Hydroxyvitamin D Concentration in Two Cohorts of Pregnant Women in Southern Ontario, Canada

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Received: 29 November 2018; Accepted: 6 January 2019; Published: 9 January 2019

Abstract: Vitamin D deficiency in pregnancy is widely reported, but whether this applies in North America is unclear since no population-based surveys of vitamin D status in pregnancy exist in Canada or the United States. The objectives were to assess (i) the intake and sources of vitamin D, (ii) vitamin D status, and (iii) factors associated with serum 25-hydroxyvitamin D (25-OHD) concentration in two cohorts of pregnant women from Southern Ontario, Canada, studied over a span of 14 years. Maternal characteristics, physical measurements, fasting blood samples and nutrient intake were obtained at enrolment in 332 pregnant women from the Family Atherosclerosis Monitoring In early Life (FAMILY) study and 191 from the Be Healthy in Pregnancy (BHIP) study. Serum 25-OHD was measured by LC/MS-MS. The median (Q1, Q3) total vitamin D intake was 383 IU/day (327, 551) in the FAMILY study and 554 IU/day (437, 796) in the BHIP study. Supplemental vitamin D represented 64% of total intake in participants in FAMILY and 78% in BHIP. The mean (SD) serum 25-OHD was 76.5 (32.9) nmol/L in FAMILY and 79.7 (22.3) nmol/L in BHIP. Being of European descent and blood sampling in the summer season were significantly associated with a higher maternal serum 25-OHD concentration. In summary, health care practitioners should be aware that vitamin D status is sufficient in the majority of pregnant Canadian women of European ancestry, likely due to sun exposure.

Keywords: serum 25-OHD; pregnancy; developmental origins of health and disease; bone health

1. Introduction

Adequate maternal vitamin D status is critical to pregnancy health outcomes and the vitamin D status of the infant at birth, and may program for bone development in childhood and later life [1–3]. Vitamin D is essential for bone mineralization, proper bone accretion and growth of the fetus during pregnancy [4]. Systematic reviews of randomized studies identified the effects of maternal vitamin D supplementation on reducing low birth weight prevalence and improving infant growth, with some indications of its potential benefit on pregnancy complications such as pre-eclampsia [5–7]. Maternal vitamin D status in pregnancy has also been positively associated with bone health outcomes in infants [8], children [9,10] and adolescents [11].

Given the potential health benefits of vitamin D, supplementation has gained popularity in the general public over the last decade. The dose in common brands of prenatal multivitamins in Canada has increased from 300 to 600 IU/pill on average over the years, but doses of over-the-counter multi-nutrient supplements range from 200 to 1000 IU/tablet, and single vitamin D supplements are

available in doses of 1000–10,000 IU/tablet. As well, the consumption of vitamin D fortified products is gaining popularity in the market as pregnant women become aware of the possible benefits of vitamin D during and after pregnancy.

Despite the potential importance of maternal vitamin D status on the health outcomes of mother and child, no population-based data exist as pregnant women have not been sampled in the nutrition and health surveys in Canada or the United States to date. Claims of a "pandemic" or a high prevalence of vitamin D deficiency in pregnant and lactating women in Canada [12] are not founded on population-based surveys, but rather, cite literature primarily from Afro-American and Indigenous groups living in Canada. In a single US study [13], it was postulated that intakes of 4000 IU of vitamin D per day during pregnancy are required to optimize the production of 1,25-dihydroxyvitamin D (1,25-OH_2D) and cord blood 25-hydroxyvitamin D (25-OHD). However, no study to date has demonstrated that vitamin D intakes in pregnancy of >400 up to 4000 IU/day result in any functional benefits to mother or infant. Thus, in the recently revised Dietary Reference Intakes (DRI) [14], the Estimated Average Requirement (EAR) for vitamin D in pregnancy is the same as for non-pregnant women at 400 IU per day. This recommendation was confirmed by the Scientific Advisory Committee on Nutrition in the United Kingdom [15], as well as by the European Food Safety Authority in Europe [16].

The present study was undertaken with the objective to assess (i) the intake and sources of vitamin D, (ii) the vitamin D status, and (iii) the factors associated with maternal serum 25-OHD concentration as a measure of vitamin D status in two cohorts of pregnant women living in Southern Ontario, Canada, studied over a span of 14 years.

2. Materials and Methods

2.1. Study Design

Pregnant women enrolled in the FAMILY and BHIP studies were included, both of which were conducted in accordance with the Declaration of Helsinki. The Family Atherosclerosis Monitoring In early Life (FAMILY) study was a longitudinal, prospective birth cohort study designed to investigate the determinants of obesity, type 2 diabetes and cardiometabolic traits early in life [17]. A total of 857 pregnant women were recruited through three hospitals in Hamilton and Burlington, Ontario, Canada between the years of 2002–2009. For this ancillary study on factors associated with maternal serum 25-OHD concentration, separate ethics approval was granted for the assessment of vitamin D status and bone health in subjects of the FAMILY study by the Research Ethics Board at Hamilton Health Sciences/McMaster University (REB #02-060). Participants gave informed written consent for this sub-study. The Be Healthy in Pregnancy (BHIP) Study is an ongoing randomized controlled trial (RCT; Clinical Trials Ref: NCT01693510) [18] for which the primary research objective is to determine whether introducing a structured and monitored nutrition and exercise program in early pregnancy, compared to standard prenatal care, will increase the number of women attaining gestational weight gain within the Institute of Medicine (IOM), Health and Medicine Division recommendations for their pre-pregnancy body mass index (BMI) category [19]. The present analysis included data obtained at baseline prior to randomization. Between the years 2012–2018, 274 healthy pregnant women were recruited from health care clinics in Hamilton, Burlington and London, Ontario. Informed written consent was obtained upon study enrolment. Ethics approval was obtained from the Research Ethics Boards of Hamilton Health Sciences (REB Project#12-469), Western Ontario in London (HSREB 103272), and Joseph Brant Hospital in Burlington (JBH 000-018-14), all in Southern Ontario, Canada.

2.2. Maternal Data Collection

Maternal demographics, pregnancy history, fasting blood samples and physical measurements were obtained from each participant upon study entry. For the FAMILY study, the information and blood samples were obtained between 24 and 36 weeks of gestation, while for the BHIP study, they

were collected between 12 and 17 weeks of gestation. Maternal height and weight were measured, gestational weight gain was self-reported and pre-pregnancy BMI was calculated. Ethnicity, education level and annual household income were self-reported. Maternal health behaviours were self-reported using questionnaires. For the FAMILY study, participants completed a validated semi-quantitative multi-ethnic food frequency questionnaire (FFQ) including supplements [20,21] Nutrient composition was calculated as previously described [22], excluding records where the FFQ was <50% incomplete, or with implausible dietary intakes (<500 or >4500 kcal/day). For the BHIP study, participants completed diet records for three consecutive days (two weekdays and one weekend day) including both foods and supplements, as used previously in pregnant women [23]. Participants were asked to weigh the foods eaten when possible, using household measures such as cups/spoons when weight was not able to be determined. No diet records with implausible intakes were found in the BHIP study. Diet records were analyzed using Nutritionist Pro diet analysis software (Version 5.2, Axxya Systems, Stafford, TX, USA), and the Canadian Nutrient File (version 2015) to obtain daily intake of vitamin D. For the FAMILY study, dairy products were classified as low fat ($\leq$2% fat) or regular fat ($\geq$3.25% fat) dairy products. For the BHIP study, the categories were milk (low and regular fat combined), yogurt (low and regular fat combined), and other dairy products (i.e., regular fat sour cream, cream, cheese, and ice cream). In Canada, all milk and margarine products are fortified with vitamin D_3 by law. Yogurt and other dairy products are sometimes made from vitamin D_3 fortified milk, and this is noted on the label. Exercise was self-reported by participants in both studies. In the FAMILY study, participants completed a validated questionnaire [24], and were categorized as currently exercising or not. In the BHIP study, participants reported exercising or not at recruitment by completing the Physical Activity Readiness Medical Examination (PARmed-X) for Pregnancy [25].

2.3. Vitamin D Analysis

Serum 25-OHD (D_2 and D_3 isomers) was measured by ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) using the Waters application note 720002748 [26] with a modified sample preparation that included a saponification step [27]. Saponification prevents fat droplet formation in the supernatant after extraction, which can occur in plasma with a high triglyceride concentration. Saponification converts triacylglycerides into water-soluble fatty acid soaps. This is particularly important as circulating lipids can be elevated in pregnant women [28]. In short, 150 µL of serum and 10 µL of internal standard solution (800 nmol/L, 25-OHD_3-d_6; 99% pure; Medical Isotopes, Pelham, NH, USA) were vortexed and 50 µL of methanol (Fisher Scientific, Ottawa, Ontario, Canada), 100 µL of ascorbic acid (20% w/v, (>99.9% pure); Sigma Aldrich, Oakville, Canada) and 40 µL of potassium hydroxide (45% w/v; Fluka Analytical, Ronkonkoma, NY, USA) were added. Samples were placed in a 75 °C hot water bath for 20 minutes. After cooling down to room temperature, the mixture was extracted with 500 µL of heptane (Fluka Analytical, Ronkonkoma, NY, USA). The organic phase was evaporated under a gentle stream of nitrogen, and reconstituted in 75 µL of methanol (MS grade, Fisher Scientific, Ottawa, Ontario, Canada). The accuracy and precision of the LC-MS/MS method for measuring 25-OHD_2 and 25-OHD_3 was evaluated using National Institute of Standards and Technology (NIST) Standard Reference Material 972a (Bureau of Standards, Washington, DC, USA). 25-OHD_3/25-OHD_2 serum controls purchased from BioRad (Munich, Germany) served as a daily quality control. A Waters Acquity UPLC/TQD system was used with an Acquity UPLC BEH C18 column. The transitions m/z 401/383 for 25-OHD_3, 407/389 for 25-OHD_3-d_6, and 413.5/395.3 for 25-OHD_2 were used for quantification.

2.4. Statistical Analysis

Statistical analysis was performed using JMP®9.0 (Version 9.0.1, SAS Institute Inc., Cary, NC, USA) and GraphPad Prism (Version 7, La Jolla, CA, USA). Descriptive statistics were computed by calculating the means and standard deviations of normally distributed continuous data; medians and quartiles (Q1 and Q3) for non-normally distributed continuous data; and counts and percentages

for categorical data. *T*-tests were performed to compare groups and significance was established at $p < 0.05$. Analysis of variance (ANOVA) was performed to compare 25-OHD concentrations in women of different pre-pregnancy BMI categories. Significance was established at $p < 0.05$. Mean values are given as means ± standard deviations if not stated otherwise. To determine which factors were associated with maternal serum 25-OHD concentration, we conducted a multivariable linear regression analysis. The variables of interest included in our multivariable regression were decided a priori based on clinical rationale and evidence from the literature. The non-standardized regression coefficients and their corresponding 95% confidence intervals (CIs) and *p*-values for the multivariable analyses are presented.

3. Results

3.1. Demographics and Physical Characteristics

A total of 332 participants from the FAMILY study and 191 from the BHIP study were included in this report as they had available maternal serum samples analysed for 25-OHD. Participants of the FAMILY study were enrolled at a median of 28 weeks gestation, while the BHIP study participants were enrolled at a median of 13 weeks gestation. Half of the FAMILY participants (54%) were enrolled by 2006 and all by 2009, while most participants (96%) of the BHIP study were enrolled between 2013 and 2017. The mean (SD) age of the participants was significantly higher in the FAMILY study than in the BHIP study (32.5 (4.7) vs. 31.2 (3.9) years; $p = 0.001$). Pre-pregnancy BMI was not statistically different between studies (Table 1). According to the pre-pregnancy BMI data, about half of the participants had normal weight, while half were categorized as overweight or obese upon entering pregnancy (Table 1). Most participants were of European ancestry, had a tertiary level of education, and were currently exercising. Few participants in the FAMILY study and none in the BHIP smoked during pregnancy, the latter because it was an exclusion criterion.

Table 1. Demographic, lifestyle and physical characteristics of participants during pregnancy.

	FAMILY Study *N* = 332		**BHIP Study *N* = 191**	
Maternal Characteristics	***N***	**(%)**	***N***	**(%)**
Gestational age	24–36 weeks		12–17 weeks	
Pre-pregnancy BMI (kg/m^2)				
Underweight (<18.5)	4	1	3	1
Normal weight (18.5–24.9)	144	45	91	48
Overweight (25.0–29.9)	103	32	61	32
Obese (≥30)	69	22	36	19
Unknown	12	-	0	-
Ethnicity				
European descent	285	86	171	90
Other	47	14	20	10
Household income (CAD)				
<$50,000	68	21	15	8
$50,000–$99,999	138	43	91	48
≥$100,000	111	35	79	41
Unknown	15	1	6	3
Education (years)				
≤13	46	14	0	0
>13	286	86	191	100
Smoking status				
Smoked during pregnancy	10	3	0	0
Former smoker; quit before pregnancy	107	33	n/a	n/a
Never smoked	212	64	n/a	n/a
Unknown	3	-	n/a	n/a

Table 1. *Cont.*

	FAMILY Study *N* = 332		BHIP Study *N* = 191	
Maternal Characteristics	***N***	**(%)**	***N***	**(%)**
Gestational age	24–36 weeks		12–17 weeks	
Exercise at study entry				
Not currently exercising	49	15	42	22
Currently exercising	283	85	147	77
Missing data	0	-	2	1

Data not applicable (n/a) as smoking status was an exclusion criteria in the BHIP study, and such data was not collected.

3.2. Intake of Vitamin D in Both Studies: Trend Over 10 Years

The median (Q1, Q3) total vitamin D intake in the FAMILY study was 383 IU/day (327, 551) with the highest intake being 3050 IU/day (Figure 1). The median total vitamin D intake in the BHIP Study was 554 IU/day (437, 796) with the highest intake being 11,062 IU/day. Intakes of vitamin D met the EAR of 400 IU/day in 43% of participants in the FAMILY study and 80% in the BHIP study. Vitamin intake from food sources alone met the EAR in only 9% of participants in both the FAMILY and BHIP studies (Figure 1). No participants in the FAMILY study exceeded the Tolerable Upper Intake Level (UL) of 4000 IU/day, and only three participants in the BHIP (2%) exceeded the UL. Supplement intake represented 64% (289 IU/day) of total intake in the FAMILY study and 78% (629 IU/day) in the BHIP study. Supplements containing vitamin D, mostly prenatal multivitamins, were consumed by 87% of participants in the FAMILY study. The median (Q1, Q3) intake of vitamin D from multivitamins was 300 IU/day (300, 300) but ranged from 0 to 3000 IU/day. In the BHIP study, 92% of participants were consuming supplements containing vitamin D. The median (Q1, Q3) intake of vitamin D from multivitamins was 400 IU/day (400, 600), ranging from 0 to 11,000 IU/day for some participants.

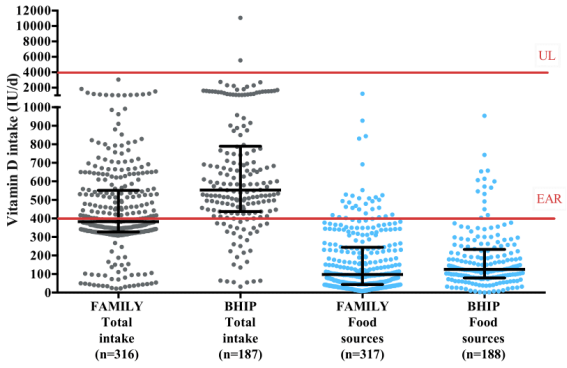

Figure 1. Maternal dietary intake of vitamin D of participants in the FAMILY and BHIP studies. Median and interquartile ranges are displayed for total intake and food sources contribution to total vitamin D. — Vitamin D recommendations for pregnancy: Estimated Average Requirement (EAR) = 400 IU/day; Tolerable Upper Intake Level (UL) = 4000 IU/day [14].

3.3. Maternal Serum 25-OHD Concentration during Pregnancy

The mean serum 25-OHD concentration was in the optimal range (serum 25-OHD 50–125 nmol/L [14]). The isomer 25-OHD_2 was detected in only 6% of participants in the BHIP study (data not available for the FAMILY study) at a concentration of 0.51 ± 2.89 nmol/L (mean ± SD); thus, the D2 isomer did not contribute significantly to the overall total serum 25-OHD. Accordingly,

the total circulating 25-OHD is a reflection of 25-OHD_3. Serum 25-OHD concentrations did not differ between women with different pre-pregnancy BMI values in either study (Table 2). The season that blood was drawn was significantly associated with 25-OHD concentrations in both studies, where blood samples collected in summer had higher serum 25-OHD than in winter ($p = 0.001$ in FAMILY and $p = 0.002$ in BHIP). The threshold representing a sufficient 25-OHD concentration of 50 nmol/L as set by the IOM [14] was met or exceeded by 77% of participants in the FAMILY study and 93% of participants in the BHIP study (Figure 2). The range of maternal 25-OHD concentrations was greater in the FAMILY study compared to in the BHIP study; 5% of participants in the FAMILY study had serum 25-OHD in the deficient range (<30 nmol/L) and 9% of participants exceeded 125 nmol/L, the level for excessive circulating 25-OHD. In the BHIP study, only 0.5% of participants were clinically deficient, while 3% surpassed the excessive threshold. No correlation was observed in the FAMILY study between maternal total vitamin D intake and maternal serum 25-OHD concentration ($R^2 = 0.01$, $p = 0.09$). In the BHIP study, a higher total intake of vitamin D was associated with higher serum 25-OHD ($R^2 = 0.11$, $p < 0.0001$). For BHIP, supplements contributed an important amount to the total vitamin D intake and contributed to a higher 25-OHD concentration (Figure 3).

Table 2. Maternal serum 25-OHD concentration by season of blood draw and pre-pregnancy body mass index (BMI) category.

	FAMILY Study			BHIP Study		
Category	Serum 25-OHD, nmol/L Mean (SD) (95% CI)	*N* (%)	*p*-value	Serum 25-OHD, nmol/L Mean (SD) (95% CI)	*N* (%)	*p*-value
All participants	76.5 (32.9) (72.9, 80.1)	332	-	79.7 (22.3) (76.5, 82.9)	191	-
Season of blood draw						
Summer (May–Oct.)	83.47 (34.3) (78.3, 88.7)	169 (51)	0.0001	84.9 (21.0) (80.9, 89.0)	106 (55)	0.0002
Winter (Nov–Apr.)	68.5 (29.3) (63.9, 73.1)	160 (48)		73.2 (22.2) (68.4, 78.0)	85 (45)	
Missing data	-	3 (1)		-	-	
Pre-pregnancy BMI (kg/m^2)						
Underweight (<18.5)	72.2 (44.3) (1.6, 142.8)	4 (1)	0.11	90.3 (15.1) (52.6, 127.9)	3 (1)	0.10
Normal (18.5–24.9)	79.5 (33.4) (74.0, 85.0)	144 (43)		82.2 (21.2) (77.8, 86.5)	93 (49)	
Overweight (25.0–29.9)	78.3 (33.7) (71.7, 84.9)	103 (31)		73.8 (20.5) (68.4, 79.2)	58 (31)	
Obese (≥30)	68.1 (30.0) (60.9, 75.3)	69 (21)		81.8 (26.6) (90.7, 73,0)	37 (19)	
Missing data	-	12 (4)		-	-	

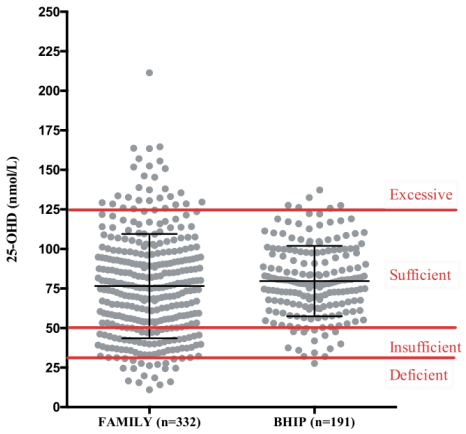

Figure 2. Maternal serum 25-OHD concentration (mean, SD) in participants from the FAMILY and BHIP studies in comparison to the recommendations by the Institute of Medicine [14]; — <30 nmol/L deficient, 30–50 nmol/L insufficient, ≥50 nmol/L sufficient, >125 nmol/L excessive.

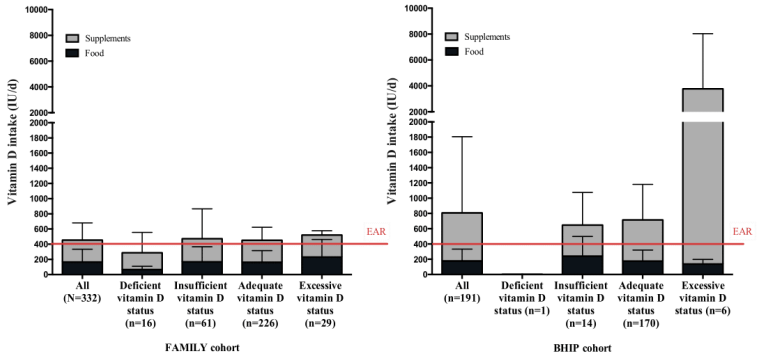

Figure 3. Total vitamin D intake in the FAMILY and BHIP studies, by maternal serum 25-OHD concentration. Mean and standard deviation are displayed. The Estimated Average Requirement (EAR) is indicated by — [14].

3.4. Factors Associated with Maternal Serum 25-OHD Concentrations in Pregnancy

For participants enrolled in the FAMILY study, a multivariable analysis demonstrated that a higher maternal 25-OHD concentration was significantly associated with being of European descent, having blood drawn in summer, and having a low pre-pregnancy BMI (Table 3).

For participants enrolled in the BHIP study, a multivariable analysis revealed that higher maternal 25-OHD concentrations were significantly associated with having blood drawn in summer and vitamin D intake from regular fat dairy products (i.e., sour cream, cream, ice cream, cheese) (Table 3).

Table 3. Multivariable analysis of factors associated with maternal serum 25-OHD concentrations during pregnancy in the FAMILY and BHIP studies.

Variables	FAMILY Study			BHIP Study		
	Estimated Coefficient	95% CI	*p*-Value	Estimated Coefficient	95% CI	*p*-Value
Ethnicity (European descent as reference)	**−5.85**	**−10.97, −0.72**	**0.025**	−5.91	−12.44, 0.61	0.075
Season of blood draw for baseline blood (Winter as reference)	**7.73**	**4.27, 11.18**	**<0.001**	**8.27**	**4.44, 12.09**	**<0.001**
Exercising at enrollment	0.53	−5.73, 4.66	0.840	3.08	−1.72, 7.88	0.206
Pre-pregnancy BMI	**−0.92**	**−1.52, −0.31**	**0.003**	−0.37	−1.19, 0.46	0.381
Total vitamin D intake	−0.01	−0.04, 0.02	0.469	−0.01	−0.04, 0.02	0.451
Vitamin D intake from supplement	0.01	−0.02, 0.05	0.422	0.02	−0.01, 0.05	0.174
Vitamin D intake from milk	1.03	−3.97, 6.02	0.687	0.03	−0.03, 0.10	0.272
Vitamin D intake from low fat dairy products	4.19	−0.43, 8.82	0.076	-	-	-
Vitamin D intake from regular fat dairy products (sour cream, cream, ice cream, cheese)	−2.11	−5.99, 1.77	0.285	**0.44**	**0.08, 0.81**	**0.017**

Bold format for significant results.

4. Discussion

The majority of healthy pregnant women in Southern Ontario in the last 10 years have had sufficient circulating 25-OHD both in early and late pregnancy using the reference cut-off values from the DRI report [14]. While risk for vitamin D deficiency may exist globally [29], this does not appear to apply to pregnant women living in Southern Ontario. Health care practitioners should be aware that in our community, <5% of pregnant women had serum 25-OHD <30 nmol/L, which is similar to what was found in the general Canadian population (4%, defined as <27.5 nmol/L) by the Canadian Health Measures Survey (CHMS) [30]. The overall circulating 25-OHD in the participants in the two combined pregnant cohorts was 77.7 nmol/L, a value slightly higher than the average serum 25-OHD of 69.5 nmol/L reported for females of child-bearing age (20–39 years old) in the CHMS [30], and which is significantly higher than observed in males in the same age category. The latter is likely attributable to higher vitamin supplement intake among females [30]. High intakes of supplements containing vitamin D were observed in our two cohorts of pregnant women where approximately 90% were taking prenatal supplements. Vitamin D supplementation is known to be associated with higher 25-OHD status, but the response can be highly heterogeneous among pregnant women [7,31], including those in our study. Despite the majority of participants taking prenatal supplements in both the FAMILY and BHIP studies, heterogeneity was indicated, in which prenatal supplement intake was not associated with 25-OHD status in FAMILY ($R^2 = 0.00$, $p = 0.58$) but a modest albeit significant association with 25-OHD status was observed in BHIP ($R^2 = 0.11$, $p < 0.0001$).

Although the average maternal intake of vitamin D from food did not reach the EAR of 400 IU/day and this was only weakly associated with 25-OHD concentration, the estimated total vitamin D intake from both food and supplements exceeded 400 IU/day in only 45% of participants in the FAMILY study but in 80% of the BHIP participants. Our results are in agreement with data reported from Canadian studies showing that the primary source of oral vitamin D (approximately 60% total intake) is supplements [32,33]. In the Canadian food chain, there are limited natural or fortified food sources of vitamin D. According to our data and those of other Canadian studies [32–37], intake of vitamin D supplements is common in pregnancy, and combined with sun exposure, the prevalence of inadequacy of vitamin D intake is low. As noted above, almost all participants in the two cohorts of pregnant women took prenatal supplements (containing between 200 and 600 IU of vitamin D), vitamin D supplements (up to 10,000 IU), or both. Although participants from the BHIP study consumed more vitamin D overall due to higher supplement intake, they had similar 25-OHD concentrations to participants in the FAMILY study. Participants in the FAMILY study presented a broader range of 25-OHD concentrations, likely due to the larger sample size of FAMILY as compared with the BHIP study, with participants at the extremes with either clinical deficiency or excessive 25-OHD concentration. These results also suggest that sun exposure plays an important role through cutaneous production of vitamin D, ensuring most participants achieved an adequate vitamin D status.

The average serum 25-OHD in our participants was moderately higher than that reported since 2000 for Canadian pregnant women in other provinces. In the Vancouver area, pregnant women predominately of European descent ($N = 336$ at 20–35 weeks gestation) had a mean (95% CI) 25-OHD of 66.7 (64.2–69.1) nmol/L [34]. In a larger study in Québec City and Halifax ($N = 1635$ at 12–15 weeks gestation, primarily of European descent), the mean (SD) 25-OHD was 52.7 (16.9) nmol/L [38]. Further, in Edmonton and Calgary, Alberta ($N = 537$, primarily of European ancestry), the mean (SD) serum 25-OHD was 93.3 (25.6) nmol/L in the first trimester and 95.3 (25) nmol/L in the second trimester of pregnancy [36]. The 25-OHD concentration of women in our study is comparable to the first two studies but lower than reported by the APrON study in Alberta [36]. These discrepancies in 25-OHD concentration may be due to participants' exposure to sun, the nature of the study samples, where multiethnic participants have lower 25-OHD concentration [34], and because those with the highest socioeconomic status have the highest 25-OHD concentration [36]. In addition, the largest consumers of multivitamin supplements are found in Alberta, while the lowest consumers are in Quebec [30]. Discrepancies can also result from the methods used to measure serum 25-OHD—either

by ELISA [34,38] or LC-MS/MS [36]. In all cohorts, the prevalence of deficiency was very low (either defined as 25-OHD < 25 nmol/L [38] or < 30 nmol/L [34,36]); from < 1 to 7% [34,36,38], aligning with our observed prevalence deficiency (defined as 25-OHD < 30 nmol/L) of 5% in FAMILY and <1% BHIP studies. The prevalence of insufficient 25-OHD (< 50 nmol/L) in these cohorts was between 2% and 45% [34,36,38], similar to what we observed in the FAMILY study (18%) and BHIP study (7%).

Based on the contemporary studies noted above and using the IOM reference values, the majority of Canadian women have an adequate serum 25-OHD concentration in pregnancy, regardless of stage of pregnancy. However, controversy remains as to the 'optimal' 25-OHD concentration in pregnancy, since the clinical significance both for mother and infant of a 25-OHD above 50 nmol/L is still undefined [7,39–41]. Data from a recent systematic review suggests that pregnant women with bacterial vaginosis, gestational diabetes, pre-eclampsia and those with small for gestational age (SGA) babies have lower circulating 25-OHD than their healthy pregnant counterparts [42,43]. However, the value for what constitutes 'lower 25-OHD' was not defined in the review and their analysis included individual studies that used both 50 and 75 nmol/L as the cut-off for sufficiency. Conversely, a further systematic review of randomized controlled trials found no clear evidence for maternal benefits or reduced incidence of pre-term birth with supplementation of 25-OHD. In this review, only eight out of 43 trials were categorized as having an overall low risk of bias [39]. Many of the trials included in this review were small and of low quality; therefore, more research is needed before recommendations on optimal vitamin D status in pregnancy can be made. Further, a large prospective cohort study from New Zealand found that serum 25-OHD concentrations in pregnant women were not associated with pre-eclampsia, SGA babies or pre-term birth; however, of note, this population was largely 25-OHD replete [44].

Controversy also exists as to the optimal target for serum 25-OHD in pregnancy. A higher maternal 25-OHD concentration at delivery has been associated with infant cord blood 25-OHD status, and maternal use of vitamin D supplements was associated with higher odds of reaching sufficiency (defined as >75 nmol/L) for both mothers and infants [45]. However, uncertainty exists as to whether a higher maternal 25-OHD concentration (i.e., 25-OHD > 75 nmol/L) in pregnancy is linked with health benefits. It has been hypothesized that the optimal 25-OHD concentration would be the one leading to maximal conversion to the active form of vitamin D [46]. To that effect, data from one recent randomized trial were interpreted to indicate the total circulating 25-OHD must reach 100 nmol/L in order to optimize circulating 1,25-OH_2D in pregnancy [47]. In that case, only 21% of participants in our cohort would have met or exceeded this 25-OHD concentration. In contrast to benefits, high maternal, cord and infant 25-OHD concentrations may have disadvantageous effects on infant growth. In an RCT including 798 mother and infant dyads, mothers with pregnancy 25-OHD > 125 nmol/L had the smallest infants at 6 months [48]. Further, an evidence-based consensus statement determined that there is little evidence for any benefits of maternal vitamin D supplementation on early life anthropometry and growth in the offspring or on clinical benefits for the mother [49]. It was concluded that the cut-off for vitamin D sufficient status of 50 nmol/L, as suggested by the DRI, remains the accepted standard [49]. Based on our data, with the current level of vitamin D fortification in Canada the use of supplements might be essential for pregnant women to reach the EAR for vitamin D, but is not the most important factor in ensuring participants have adequate vitamin D status. Summer season, and by inference, amount of sun exposure, appeared to have the strongest impact on maternal serum 25-OHD concentration in our cohorts.

The factors that were significantly associated with maternal 25-OHD concentrations in this study are in agreement with reported maternal factors in pregnant women across Canada (Vancouver, Halifax and Québec City, Edmonton and Calgary). Summer season at time of blood draw [34,38] and being of European ancestry [34] were reported as significant factors associated with 25-OHD concentration in Canadian pregnant women living in Southern Ontario. While a pre-pregnancy BMI < 25 kg/m^2 was associated with a higher maternal 25-OHD concentration compared to pre-pregnancy BMI ≥ 35 kg/m^2 [38]; a relationship between pre-pregnancy BMI and maternal 25-OHD

was observed in the FAMILY study but not in the BHIP study. This may relate to the larger sample size in FAMILY. Consumption of milk, a mandatory vitamin D-fortified food in Canada, was surprisingly not associated with maternal 25-OHD concentrations in either study. Health Canada recently indicated plans to increase the amount of vitamin D for mandatory fortification of milk and margarine in an effort to help Canadians meet the DRIs [50]. Until that comes into effect by the end of 2022, it is likely that milk consumption alone will not be sufficient source of vitamin D intake for Canadian pregnant women.

Our study has several strengths including a detailed dietary intake including food and supplement sources and measurement of serum 25-OHD concentrations by the gold standard LC-MS/MS [51]. The generalizability of the findings may be limited due to the demographic homogeneity (primarily of European descent, highly educated women). Another limitation includes the reporting bias inherent to diet records, but our dietary assessment was combined with direct measurement of nutritional status, providing a better evaluation of nutritional adequacy. Lastly, the lack of quantitative measurement of sun exposure limits the interpretation of the results, as there might be differences between an individual's exposure due to variable time spent in outdoor activities, clothing coverage and use of sunscreen. Future steps include a prospective longitudinal assessment of participants in the BHIP study to investigate the association of maternal 25-OHD concentrations with pregnancy and neonatal health outcomes.

Author Contributions: Conceptualization, S.A.A.; Methodology, S.A.A.; Laboratory work, G.F., C.J.M.; Formal Analysis, M.P., S.A.A.; Investigation, S.A.A., M.P.; Data Curation, M.P., C.J.M., S.A.A.; Writing—Original Draft Preparation, M.P., G.F., S.A.A.; Writing—Review & Editing, M.P., G.F., S.A.A., C.J.M., K.K.T.; Visualization, M.P.; Supervision, S.A.A.; Project Administration, S.A.A.; Funding Acquisition, S.A.A.

Funding: The FAMILY study received separate funding from the Dairy Farmers of Canada to conduct the vitamin D analysis. The core FAMILY study was funded by the Canadian Institutes of Health Research (CIHR) and the Heart and Stroke Foundation. The BHIP study was funded by the CIHR; and the Dairy Cluster by Dairy Farmers of Canada, and Agriculture and Agri-food Canada (AAFC).

Acknowledgments: Authors would like to acknowledge all the families who participated in the FAMILY and BHIP study, as well as the community partners and collaborators involved in these studies. We are grateful for the contributions to data collection by the FAMILY Study team especially Nora Abdalla, and graduate students Melody Ng and Dilisha Rodrigopulle, and the BHIP study team, especially Michelle Mottola, PhD. The statistical consultation by Lehana Thabane, PhD is also gratefully acknowledged.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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CHAPTER 4

SUMMER SEASON AND RECOMMENDED VITAMIN D INTAKE SUPPORT ADEQUATE VITAMIN D STATUS THROUGHOUT PREGNANCY IN HEALTHY CANADIAN WOMEN AND THEIR NEWBORNS

PREFACE TO CHAPTER 4

Vitamin D deficiency in pregnancy has been reported as a prevalent public health problem globally, even among women in North America. To determine the extent of such deficiency in our province of Ontario in Canada, we studied vitamin D metabolite profiles across pregnancy and explored the determinants of vitamin D status (measured as serum 25(OH)D), as part of an assessment of bone health in pregnant women and their offspring in the BHIP RCT. Pregnant Canadian women provided fasting blood samples and detailed nutrient intake in early and late pregnancy as well as at delivery (cord blood) for a sub-sample. Vitamin D metabolites were quantitated by the gold standard method of LC-MS/MS, thereby providing reliable measures. Our findings contribute to the literature by providing longitudinal measures of vitamin D intake and metabolic profiles, which were assessed with consideration for relevant confounding variables in determining the factors of greatest influence on vitamin D status. Not one participant was vitamin D deficient at any time point. Further, in the adjusted multivariate analyses, maternal vitamin D status in early pregnancy was positively associated with summer season and supplement intake and in late pregnancy with summer season, non-milk dairy intake and supplement intake.

Our findings suggest that in Canadian women living in southern Canada, vitamin D deficiency is not an issue as the summer season and adequate vitamin D intake led to normal maternal vitamin D status throughout pregnancy, and cord vitamin D

concentrations. Thus, our findings counter the widely held view that even in North America vitamin D deficiency in pregnancy is highly prevalent. Based on our study, this might be leading to the use of high dose supplements of vitamin D that may also lead to adverse health outcomes. We conclude that statements about the prevalence of vitamin D deficiency in pregnancy was be made in the context of the country/region/specific population and then policy about vitamin D supplementation made within the context of the target population. Thus, our study provides a cautionary tale.

Authors' contributions: **MP** conducted research, and performed laboratory analysis of the vitamin D metabolites in collaboration with GF. **MP** analyzed the data and performed statistical analysis. **MP** wrote the manuscript. SAA designed the study, obtained the grant support and had primary responsibility for the final content. SAA and MFM oversaw the data collection. All authors read and approved the final manuscript.

Full citation: **Perreault M**, Atkinson SA, Meyre D, Fusch G, Mottola M. *Summer season and recommended vitamin D intake support adequate vitamin D status throughout pregnancy in healthy Canadian women and their newborns.*

Accepted for publication with minor revisions in the Journal of Nutrition as of September 23, 2019.

TITLE[1]:

Summer season and recommended vitamin D intake support adequate vitamin D status throughout pregnancy in healthy Canadian women and their newborns

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[1] Abbreviations list: serum 25-hydroxycholecalciferol (25(OH)D); 1,25-dihydroxycholecalciferol ($1,25(OH)_2D$); Institute of Medicine (IOM); liquid chromatography tandem mass spectrometry (LC-MS/MS); body mass index (BMI); National Institute of Standards and Technology (NIST); coefficient of variation (CV); Estimated Average Requirement (EAR); Tolerable Upper Intake Level (UL); Vitamin D External Quality Assessment Scheme (DEQAS). Financial disclosure: Supported by the Canadian Institutes of Health and Research (SAA), and Dairy Farmers of Canada and Agriculture and Agri-food Canada Dairy Research Cluster (SAA, DM); in-kind by GayLea Foods & Ultima Foods. MP is supported by CIHR-Vanier and Canadian Child Health Clinical Scientist Program doctoral awards. Conflict of interest disclosure: MP; no conflict of interest, SAA; no conflict of interest, DM; no conflict of interest, GF; no conflict of interest, MFM; no conflict of interest.

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Running title (50 or fewer characters and spaces): Serum vitamin D metabolites across pregnancy

Word count (introduction through discussion): 4035

Number of figures: 5

Number of tables: 3

Abstract page

Background: Vitamin D deficiency in pregnancy is reported as a prevalent public health problem.

Objectives: To evaluate in pregnant Canadian women: (i) vitamin D intake, (ii) maternal and cord serum 25-hydroxycholecalciferol (25(OH)D) and maternal 1,25-dihydroxycholecalciferol ($1,25(OH)_2D$), and (iii) factors associated with maternal serum 25(OH)D.

Methods: Women (n = 187; mean pre-pregnancy BMI 24.4 kg/m^2, mean age 31 yr) recruited to the Be Healthy in Pregnancy Study (NCT01693510) provided fasting blood samples and nutrient intake at 12-17 (early) and 36-38 (late) weeks of gestation, and cord blood. Vitamin D intakes (Nutritionist Pro™) and serum 25(OH)D and $1,25(OH)_2D$ concentrations (LC-MS/MS) were quantitated.

Results: Vitamin D intake was comparable in early and late pregnancy (median (Q1, Q3) = 586 (459, 859) vs 689 (544, 974 IU/day); p = 0.83), with 71% consumed as supplements. Serum 25(OH)D was significantly higher in late (mean ± SD) = 103.1 ± 29.3 nmol/L) compared to early pregnancy (82.5 ± 22.5 nmol/L); $p < 0.001$) and no vitamin D deficiency (< 30 nmol/L) occurred. Serum $1,25(OH)_2D$ concentrations were significantly higher in late (101.1 ± 26.9 pmol/L) compared to early pregnancy (82.2 ± 19.2) pmol/L, $p < 0.001$, n = 84). Cord serum 25(OH)D concentrations averaged 55% of

maternal concentrations. In adjusted multivariate analyses, maternal vitamin D status in early pregnancy was positively associated with summer season (13.07 (5.46; 20.69), $p < 0.001$) and supplement intake (est.β: 0.01, 95% CI(0.00; 0.01), $p < 0.001$); and in late pregnancy with summer season (24.4 (15.6; 33.2), $p < 0.001$), non-milk dairy intake (0.17 (0.02; 0.32), $p = 0.029$), and supplement intake (0.01 (0.00; 0.01), $p = 0.04$).

Conclusions: Summer season and recommended vitamin D intakes supported adequate vitamin D status throughout pregnancy and in cord blood at > 50 nmol/L in healthy Canadian pregnant women.

Keywords (5-10): serum 25(OH)D, serum $1,25(OH)_2D$, isomers, cord blood, human pregnancy, vitamin D intake, bone health.

Introduction

Globally, vitamin D deficiency in pregnancy is purported to be a prevalent public health problem (1–3). Yet, the importance of adequate maternal vitamin D status, as measured by serum 25-hydroxycholecalciferol (25(OH)D), to pregnancy and infant health outcomes has been highlighted by recent systematic reviews and meta-analyses (4–7). Despite this the optimal target for serum 25(OH)D in pregnancy remains undefined (8) as conflicting data exist as to the additional positive impact on maternal and neonatal outcomes of maternal 25(OH)D serum concentrations above the current threshold for adequacy set by the Institute of Medicine (IOM; ≥ 50 nmol/L, (9)) (4). Such diversity in observations may arise because of variations across protocols in the method employed to measure 25(OH)D concentration, timing of blood sampling across pregnancy or season (10). To our knowledge, no recent studies have profiled vitamin D metabolites using the gold standard method liquid chromatography tandem mass spectrometry (LC-MS/MS) (11) across uncomplicated pregnancy in women of European descent living in Canada to obtain a comprehensive view of the changes in metabolism inherent to pregnancy and lifestyle.

Infant vitamin D status at birth is controlled by maternal serum concentrations of 25(OH)D during pregnancy (12), yet no reference for vitamin D adequacy exists for cord blood, with the exception that newborn serum 25(OH)D above 25-30 nmol/L will prevent nutritional rickets (8). The current recommended intakes from the IOM for pregnant women of 600 IU vitamin D per day (9) did not evaluate the intake in pregnancy that would ensure adequate vitamin D status for newborns. As recently reviewed (12), debate

continues as reflected in papers that have challenged the IOM report indicating higher vitamin D intakes are required during pregnancy to ensure adequate maternal, fetal and neonatal health.

Trying to establish consensus on adequate vitamin D intake in pregnancy is complicated by the fact that maternal circulating vitamin D metabolites may vary by trimester due to modulation by normal physiological changes in pregnancy. Progressive gestational weight gain in the second half of pregnancy may reduce maternal circulating 25(OH)D as body adiposity has been associated with lower serum 25(OH)D concentrations due to sequestration in metabolic fat stores, at least in the non-pregnant state (13). Further, maternal serum 1,25-dihydroxycholecalciferol ($1,25(OH)_2D$) synthesis is up-regulated to facilitate increased intestinal calcium absorption and trans-placental transport to the fetus (14). This up-regulation is reported to be independent of calcium metabolism (15). The rise in $1,25(OH)_2D$ is hypothesized to be dependent on the availability of the substrate 25(OH)D, or due to other factors such as an increased renal and placental-decidual synthesis of $1,25(OH)_2D$ (12). Considering these pregnancy-induced adaptations, it is essential both in clinical care settings and in research settings to delineate trimester specific vitamin D status and how this is influenced by other maternal lifestyle factors. With such information women at risk of vitamin D deficiency/insufficiency can be targeted, and offered cost-efficient treatment or supplementation (16).

The objectives of this study were to determine in a cohort of pregnant women living in southern Canada the trajectory of vitamin D metabolites across pregnancy and the factors associated with observed changes by measuring: (i) intake and sources of vitamin D, (ii) maternal and cord 25(OH)D and maternal $1,25(OH)_2D$ for ergocalciferol (D_2) and cholecalciferol (D_3) isomers, and (iii) factors associated with maternal serum 25(OH)D at 12-17 weeks (early pregnancy) and 36-38 weeks of gestation (late pregnancy).

Methods

Study group

The data presented are based on 187 out of the 241 participants enrolled in The Be Healthy in Pregnancy (BHIP) Study, a randomized controlled trial (RCT; Clinical Trials Ref: NCT01693510). The full BHIP study protocol has been published (17). This observational sub-study of 187 participants represent those who had detailed dietary intake and blood samples available for both early and late pregnancy time points. A sub-sample of 41 participants provided cord blood.

Briefly, the study protocol involved healthy pregnant women recruited from health care clinics in Hamilton, Burlington and London, Ontario, Canada between 12 and 17 weeks of gestation according to the criteria as published (17). Ethics approval was obtained from the Research Ethics Boards of Hamilton Health Sciences (REB Project#12-469), Western Ontario in London (HSREB 103272), and Joseph Brant Hospital in Burlington (JBH 000-018-14), all located in Southern Ontario, Canada. The protocol complied with the

Helsinki Declaration.

Maternal data collection

Maternal demographics, pregnancy history, and physical measurements including skinfold thickness at four sites were obtained from each participant upon study entry as detailed in the research design paper (17). Fasted venous blood was collected at 12-17 weeks of gestation and at 36-38 weeks of gestation. Season of blood draw was classified as winter (November to April) or summer (May-October).

Dietary intake and primary food sources

Participants completed detailed diet records for three consecutive days, consisting of two week days and one weekend day, including both foods and supplements, as used previously in pregnant women (18). No diet records with implausible intakes were identified (e.g. <500 or >5000 kcal/d). Participants completed a diet record upon entry to the study (12-17 weeks gestation) and at the end of pregnancy (36-38 week gestation). Diet records were analyzed using Nutritionist Pro™ diet analysis software (Version 5.2, Axxya Systems, Stafford, TX, USA) and the Canadian Nutrient File (version 2015) to obtain daily intake of vitamin D. In addition, food and supplement sources of vitamin D were assessed manually. Food sources of vitamin D were grouped as milk (under mandatory fortification with vitamin D (100 IU per 250ml at the time of the study) in Canada (19)), non-milk dairy products (under voluntary fortification with vitamin D in Canada (19)), fortified food (e.g. fortified orange juice and breakfast cereals), animal

sources (e.g. fish, meat and eggs), plant sources (e.g. mushrooms), and 'others' when a dish combined more than one source of vitamin D.

Vitamin D status analysis

As previously reported (20), serum 25(OH)D concentrations (D_2 and D_3 isomers) were measured by ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) using the Waters application note 720002748 (21) with a modified sample preparation from Hymoller that included a saponification step (22). Accuracy and precision were determined by using human serum-based quality controls and standard reference materials (National Institute of Standards and Technology (NIST) Standard Reference Material 972a as previously described (20). Precision values for $25(OH)D_3$ were: intra-assay coefficient of variation (CV) 6.9 %, and inter-assay CV 8.2 % and the average mean bias was -1.3 %. For $25(OH)D_2$: intra-assay CV 10.0 %, and inter-assay CV 10.5 % and the average mean bias was 4.2 %. These values compare with previously established vitamin D standardization program performance criteria, namely CV $\leq$ 10 % and mean bias $\leq$ 5 %.

Serum $1,25(OH)_2D$ concentrations (D_2 and D_3 isomers) were measured by Quest Diagnostics Nichols Institute (CA, USA) using LC-MS/MS (AB Sciex LLC, MA, USA) in a sub-group of 81 individuals for whom sample was available. The Mass Spect Gold® (Golden West Biologicals, Inc.®, Temecula, CA, USA) ultra-sensitive human serum controls were used and the intra-assay CV% generated for samples was < 10 %. The

reporting range for this assay was 19 - 2400 pmol/L. Daily participant testing was performed with intra assay controls.

Statistical analysis

The observed changes in vitamin D intake throughout pregnancy are not attributable to the intervention (unpublished data) hence data were treated as observational, and all statistical models were adjusted for factors related to the study design of the RCT. This included the randomization stratification variables (i.e. study arm, study site and pre-pregnancy BMI). Statistical analysis was performed using JMP 9.0 (Version 9.0.1, SAS Institute Inc., Cary NC, USA) and GraphPad Prism (Version 7, La Jolla, CA). Descriptive statistics included the means and standard deviations of normally distributed continuous data; medians and quartiles (Q1 and Q3) for non-normally distributed continuous data; and counts and percentages for categorical data. Regression analyses were performed to compare values between time points and significance was established at $p < 0.05$. Linear regressions were used to assess the relationship between maternal and cord serum 25(OH)D concentrations. Mean values are given as mean ± standard deviation if not stated otherwise. To determine which factors were associated with maternal vitamin D status, we conducted multivariable linear regression analyses. The variables of interest included in our multivariable regressions were decided *a priori* based on clinical rationale and evidence from the literature. The non-standardized regression coefficients and their corresponding 95% confidence intervals (CIs) and p-values are presented**.**

Results

Demographics

A total of 187 participants were enrolled near the end of the first trimester of pregnancy (**Table 1**). The participants were mostly of European descent, and the ethnic composition of the women grouped under "Other" included Indigenous, Asian, Arab and South American ancestry. Participants had a mean pre-pregnancy BMI of 24.4 kg/m^2 (ranging from 17.4-39.6 kg/m^2), and university educated with a high percentage of high household income. About 50% of women entered pregnancy with a pre-pregnancy BMI classified as overweight or obese, and almost half of the participants were first time mothers. The participants excluded (*n*=54) in this report from the original data set of 241 participants had similar socio-demographics characteristics to the ones with a complete data set (*n*=187) (data not shown).

(TABLE 1 NEAR HERE)

Intake of total supplemental and dietary vitamin D during pregnancy

Total vitamin D intake was comparable in early and late pregnancy (**Figure 1**). Total intakes of vitamin D met the Estimated Average Requirement (EAR; (9)) of 400 IU/day in 93% of participants in early pregnancy and in 97% in late pregnancy. Vitamin D intake from food sources alone met the EAR in only 8% of participants in early pregnancy and 19 % in late pregnancy. Intake of vitamin D from food sources increased significantly ($p < 0.001$) from early (median 130 IU/d = 23% total intake) to late pregnancy (median 230

IU/d = 36%). Consumption of dairy products (i.e. milk and non-milk dairy products) represented the source of higher vitamin D intake from food (subsample *n*=129). Four participants exceeded the Tolerable Upper Intake Level (UL; (9)) intake of 4,000 IU/d in early pregnancy with the highest intake being 11,060 IU/d, and for two participants in late pregnancy with the highest intake being 11,050 IU/d. Exceeding the UL did not occur from food sources, but only when participants consumed single vitamin D supplements as part of their diet. Supplemental intake represented 77% (median 400 IU/d) of total vitamin D intake in early pregnancy, and 64% (median 400 IU/d) in late pregnancy. Consumption of supplements containing vitamin D was high primarily because 86% of participants took a daily prenatal multivitamin in early pregnancy and 81% in late pregnancy. An additional 14% of participants took a single vitamin D supplement in early pregnancy, and 16% in late pregnancy. The range of intake of vitamin D from supplements among participants spanned 0 to 10,000 IU/day.

(FIGURE 1 NEAR HERE)

Food sources of vitamin D intake during pregnancy

Vitamin D-fortified milk provided the greatest proportion of dietary vitamin D and rose significantly from early to late pregnancy (**Table 2**). The second most important source of dietary vitamin D was animal products (i.e. eggs, fish, meat) (**Table 2**).

(TABLE 2 NEAR HERE)

Maternal vitamin D status during pregnancy

The mean ± SD maternal serum 25(OH)D concentration (**Figure 2**) rose significantly in late compared to early pregnancy (103.1 ± 29.3 versus 82.5 ± 22.5 nmol/L), $p < 0.001$, $n = 187$) and was above the cut-off for vitamin D adequacy (≥ 50 nmol/L as set by the IOM (9) and recommended by a global consensus recommendation for prevention of nutrition rickets (23)) at both time points. The isomer 25-hydroxyergocalciferol ($25(OH)D_2$) was detected in minimal concentrations in only 14 participants in early pregnancy (0.51 ± 2.28 nmol/L) and 28 in late pregnancy (0.94 ± 2.56 nmol/L), thus did not contribute significantly to the total serum 25(OH)D concentration. A total of 93% of participants in early pregnancy, and 97% in late pregnancy met or exceed the threshold representing a sufficient 25(OH)D concentration of 50 nmol/L. No participants were vitamin D deficient (< 30 nmol/L) at either time point. In contrast, 4% of participants in early pregnancy and 22% in late pregnancy had serum 25(OH)D concentrations above the threshold of 125 nmol/L, which might be reason for concern as suggested by the IOM (9). Total vitamin D intake and circulating 25(OH)D concentration were weakly positively associated in early pregnancy ($r^2 = 0.10$, $p < 0.001$) and late pregnancy ($r^2 = 0.10$, $p < 0.01$). Most participants who consumed a dietary vitamin D below the EAR still had sufficient serum 25(OH)D concentrations throughout pregnancy (**Figure 2**). The small number of participants who consumed intakes (> 4000 IU/day) of vitamin D above the upper limit set by the IOM (9) tended to have serum 25(OH)D concentrations in early and late pregnancy that might be reason for concern at >125 nmol/L (**Figure 2**) as set by the IOM (9).

(FIGURE 2 NEAR HERE)

Maternal serum 1,25(OH)$_2$D concentrations

In a subsample of 84 participants, the mean ± SD serum 1,25(OH)$_2$D concentration was significantly higher in late pregnancy (101.1 ± 26.9 pmol/L) compared to early pregnancy (82.2 ± 19.2 pmol/L, $p < 0.001$) (**Figure 3**). The D_2 isomer was not detected in any serum sample at any time points. Thus, total 1,25(OH)$_2$D is a reflection of the D_3 isomer. No significant association was observed between 1,25(OH)$_2$D and serum 25(OH)D in early pregnancy. In late pregnancy, there was a significant weak positive association between 1,25(OH)$_2$D and serum 25(OH)D ($r^2 = 0.10$, $p = 0.04$). Participants with insufficient serum 25(OH)D (30-50 nmol/L) in late pregnancy had similar serum concentrations of 1,25(OH)$_2$D compared to those with values above 50 nmol/L. (**Figure 3**).

(FIGURE 3 NEAR HERE)

Factors associated with maternal vitamin D status in pregnancy

A multivariable analysis demonstrated that higher serum 25(OH)D concentration in early pregnancy was significantly associated with having blood drawn in the summer, and vitamin D intake from supplements (**Table 3**). In late pregnancy, higher maternal serum 25(OH)D concentration was significantly associated with blood drawn in the summer,

vitamin D intake from supplements and vitamin D intake from non-milk dairy products (**Table 3**).

(TABLE 3 NEAR HERE**)**

Cord serum 25(OH)D concentrations

Cord serum 25(OH)D concentrations at delivery (n = 41) were significantly associated with early and late pregnancy maternal serum concentrations (early pregnancy: r^2 = 0.25, $p < 0.001$; late pregnancy r^2 = 0.58, $p < 0.001$; **Figure 4**). The mean ± SD cord serum 25(OH)D concentration was 56.1 ± 23.6 nmol/L. Cord serum 25(OH)D concentrations averaged 55% of maternal 25(OH)D concentrations at the end of pregnancy (range 26-79%; **Figure 5**).

(FIGURES 4 and 5 NEAR HERE)

Discussion

Contrary to the reports of global widespread vitamin D deficiency in pregnant women including in North America (1–3), vitamin D deficiency defined as serum 25(OH)D < 30 nmol/L was not detected in this cohort of pregnant women living in southern Ontario, Canada, who were mainly of European descent taking a daily prenatal multivitamin. Further, maternal serum 25(OH)D concentrations actually increased significantly from early to late pregnancy by 25% and serum $1,25(OH)_2D$ concentrations by 23% when

measured by the gold standard LC-MS/MS, despite the two metabolites being only weakly correlated in late but not early pregnancy. These results are in line with those of a systematic review of 20 observational cross-sectional studies that found no association between serum 25(OH)D and $1,25(OH)_2D$ at the end of pregnancy (24). Our vitamin D metabolite profiles expand on this body of literature by having repeated measures in early to late pregnancy and support recent findings (8,12) suggesting that the concentration of serum 25(OH)D is not the driver of the observed rise in $1,25(OH)_2D$ from early to late pregnancy. Thus, our results challenge the hypothesis (15,25) of a direct positive relationship between the circulating substrate 25(OH)D and the active form $1,25(OH)_2D$ produced in pregnant women with adequate vitamin D status. Instead, it is postulated that other mechanisms are at play such as the increased production of $1,25(OH)_2D$ by the maternal kidneys independent of parathyroid hormone stimulation and/or the placental-decidual tissue through stimulation of Cyp27b1 by placental hormones leading to a higher conversion rate of $1,25(OH)_2D$ (15).

The finding that most women had adequate vitamin D status despite intakes both above and below the EAR is explained by the significant positive association of maternal serum 25(OH)D with summer season, thus contributing to cutaneous *de novo* synthesis of vitamin D. Women with excessive vitamin D intake due to high supplement intake had serum 25(OH)D concentrations above the 125 nmol/L concentrations that might be reason for concern as recommended by the IOM (9). Results from a systematic review and meta-analysis including 12 RCTs suggested that a vitamin D supplementation of

2,250 IU/d is required for pregnant women to achieve sufficient circulating 25(OH)D concentrations, in this case defined as 25(OH)D > 75 nmol/L (1). In contrast, a recent RCT from Ireland suggested a total daily maternal intake of 1150 IU/d of vitamin D is required to maintain maternal serum 25(OH)D concentrations >50 nmol/L (8). Our results do not support either of these conclusions as 63% of women in early and 86% of women in late pregnancy with mean intakes of 647 IU/D and 713 IU/D, respectively, had serum 25(OH)D > 75 nmol/L and 94% in early and 97% in late pregnancy had serum concentrations > 50 nmol/L. We hypothesize that the discrepancies between estimates of vitamin D intake are likely due to sunshine exposure and women's skin color, which directly influence vitamin D status as noted in a systematic review that concluded, "we could not exclude the influences from sun exposure, skin characteristics, dietary intake, and vitamin D intake because these confounding factors were not assessed in the available RCT data" (1). Such inconsistency in predictions of optimal vitamin D intake in pregnancy underlie the lack of consensus on recommended dietary intake as well as serum 25(OH)D concentrations for clinical benefits in pregnant women. In another approach, it was suggested that a serum 25(OH)D concentration of 100 nmol/L is needed for optimal conversion of 25(OH)D to the active form of vitamin D by the Cyp27b1 enzyme, maximising its potential health benefits (26,27). We did not see such a relationship in our study as we observed a weak association between serum 25(OH)D and $1,25(OH)_2D$ and only in late pregnancy.

The observed 23% rise in serum $1,25(OH)_2D$ concentration from early to late pregnancy is within the range of 10% to 54% reported by some (28–31) but not other (32) observational studies that have measured this metabolite across pregnancy However, our serum $1,25(OH)_2D$ concentrations in early and late pregnancy are lower than reported in these studies which ranged from 136.0 to 290.0 pmol/L in early pregnancy and from 212.0 to 371.8 pmol/L in late pregnancy (28–31). This is likely due to the fact that most studies used an immunoassay and not mass spectrometry (28–30), yielding potentially higher circulating concentrations. Indeed, as reported by the Vitamin D External Quality Assessment Scheme (DEQAS; (33)), $1,25(OH)_2D$ concentrations measured by LC/MS-MS tend to be lower than when measured by immunoassays. Unlike 25(OH)D, no reference material from the NIST (34) exists for $1,25(OH)_2D$ making it difficult to assess a 'true' value. As noted by the DEQAS committee (33), large variability exists among assays, highlighting the need for measuring $1,25(OH)_2D$ by the gold standard method LC-MS/MS. Our average mean bias for $25(OH)D_3$ using the NIST reference material measured by LC-MS/MS was -1.3%, which makes us confident in the method employed and in our results for $1,25(OH)_2D$.

In the Canadian context, detection of the D_2 isomer is not important as for both 25(OH)D and $1,25(OH)_2D$ the D2 isomer did not contribute to the overall status. This likely is a reflection of limited food sources of vitamin D_2 in the Canadian food chain. Our results align with those from another Canadian pregnancy cohort from northern Alberta where the reported $25(OH)D_2$ concentrations (median (Q1,Q3)) measured by LC-MS/MS were

2.9 (1.7, 4.4) nmol/L, contributing very little to the overall 25(OH)D status of 92.7 (79, 109.4) nmol/L in 537 pregnant women in their second trimester (35). Although in Canada most food sources and supplements on the market provide vitamin D_3, new plant-based and vegetarian/vegan products supplemented with vitamin D_2 are emerging and might contribute to serum 25(OH)D_2 concentrations in some consumers in the future. Season at time of blood draw was the factor most strongly associated with maternal 25(OH)D concentrations in early and late pregnancy, indicating the importance of sunshine exposure as reported by other Canadian (36,37) and non-Canadian studies (31,38,39). But winter season did not adversely impact maternal serum 25(OH)D concentrations across pregnancy, as values were above 50 nmol/L for most women all year round likely owing to the consistent consumption of supplements containing vitamin D even at the amount of 400 IU/day.

As was expected, cord serum 25(OH)D concentrations were significantly associated with maternal concentrations, and reflected general adequacy as indicated by concentrations above 30 nmol/L; only 6 samples were < 30 nmol/L, aligning with results from others in Canada (37,40,41). Newborn serum 25(OH)D concentrations were 55% of the maternal concentrations in late pregnancy, which is within the range of some non-Canadian reports (usually 50-80% (12)) and slightly lower than reported in Canadian cohorts (ranging between 72% (40) and 127% (37)). The lower percent of cord blood:maternal 25(OH)D we observed may reflect the high maternal vitamin D status in our cohort at the end of pregnancy (e.g. > 100 nmol/L) compared to other cohorts (37,40). A threshold of 25

nmol/L and 30 nmol/L has been suggested to prevent nutritional rickets in infants (8). To our knowledge, no evidence exists that cord blood 25(OH)D beyond 50 nmol/L confers any clinical benefit to the offspring. However, excessive circulating 25(OH)D concentration (> 125 nmol/L) in pregnancy was associated with infants that were smaller at 6 months of age compared to infants of women with maternal status < 125 nmol/L (42).

Strengths of the current study include the prospective and repeated assessment of vitamin D intake and status from early to late pregnancy and in cord blood. Vitamin D metabolites were measured using the gold standard method (43) and included both the D_2 and D_3 isomers of 25(OH)D as well as $1,25(OH)_2D$. Our study was seasonally balanced as we continuously recruited over 4 years, and included women with a wide range of pre-pregnancy BMI and body fat mass. Some limitations of our study also must be considered. The cohort of pregnant women was fairly homogenous, being primarily of European descent and highly educated. This might limit the generalizability of our findings to an ethnically diverse population as occurs in some areas of Canada. Also, the women were not vitamin D deficient and only a small percentage were vitamin D insufficient, so the results regarding factors associated with maternal status might not be extrapolated to pregnant women with low vitamin D status. Although we have recorded season, the lack of quantitative measurement of sun exposure limits the interpretation of the results, as there might be differences between an individual's exposure due to variable time spent doing outdoor activities, clothing coverage and use of sunscreen.

In conclusion, although both vitamin D metabolites increased throughout pregnancy, they were only weakly correlated in late pregnancy. This suggests that vitamin D status per se does not drive the rise in 1,25(OH)2D observed in pregnancy but rather other factors might be at play such as up-regulated synthesis in renal, placental or decidual tissue (12). In our population, being pregnant in the summer season and achieving currently recommended vitamin D intake supported adequate maternal vitamin D status throughout pregnancy, which was linked to cord vitamin D status > 50 nmol/L. Our results do not corroborate the reported widespread vitamin D deficiency in pregnant women (1,3) and highlight the importance of considering the context of the cohort or population under study when assessing vitamin D status, as factors such as sun exposure, ethnicity, and dietary intake influence maternal status (10). Global recommendations for vitamin D supplementation in pregnancy beyond regular prenatal multivitamin may lead to excessively high serum concentrations of 25(OH)D as we have observed. Based on emerging evidence, excessive maternal vitamin D status may also be associated with adverse outcomes (42). Future steps include investigating the association of maternal 25(OH)D concentrations with pregnancy and neonatal health outcomes, as part of the randomized controlled BHIP trial.

Sources of funding

Supported by Dairy Farmers of Canada (SAA), Canadian Institutes of Health and Research (SAA, MFM), and Dairy Farmers of Canada and Agriculture and Agri-food

Canada Dairy Research Cluster (SAA, DM, MFM); in-kind by GayLea Foods & Ultima Foods. MP is supported by CIHR-Vanier and Canadian Child Health Clinical Scientist Program career enhancement program awards. DM is supported by a Canada Research Chair.

Acknowledgments

Authors would like to acknowledge all the families who participated in the Be Healthy in Pregnancy study, as well as the community partners involved in this study. We are grateful for the contributions of the whole BHIP research team, particularly the study coordinators Ms Kristina Chandler and Ms Caroline Moore and research assistants who contributed to study management and data collection.

Author contributions

SAA designed the study and had continuous oversight. SAA and MFM oversaw the data collection and analysis. MP conducted research, analyzed the data and performed statistical analysis. MFM, DM and GF critically reviewed the manuscript. GF oversaw the laboratory analysis of the vitamin D metabolites. MP and SAA wrote the manuscript and had primary responsibility for the final content. All authors have read and approved the final manuscript.

Conflicts of interest: The authors declare no conflict of interest.

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Table 1. Demographic characteristics of pregnant women (*n* = 187) upon study entry (12-17 wk) and newborns (*n* = 41) at birth. Values are mean ± SD or (range) or frequency (percentage).

Maternal characteristics	*n* = 187
Gestational age (week)	13.3 ± 1.7
Age (year), mean (range)	31 (20-42)
Pre-pregnancy BMI (kg/m^2), N (%)	
Underweight <18.5	3 (1)
Normal weight 18.5-24.9	103 (55)
Overweight 25.0-29.9	50 (27)
Obese ≥30	31 (17)
Ethnicity, N (%)	
European ancestry	164 (88)
Other	23 (12)
Household income (CAD), N (%)	
<$ 75K/year	49 (26)
≥$ 75K/year	138 (74)
Education (highest degree), N (%)	
High school	3 (2)
College diploma	34 (18)
Bachelor's degree	62 (33)
Above bachelor's degree	88 (47)
Parity, N (%)	
0	89 (48)
1+	98 (52)
Newborn characteristics	*n* = 41
Gestational age (week)	39.4 ± 1.1

Table 2: Mean total, supplemental and dietary vitamin D intake, including food sources of vitamin D in pregnant women (n = 129) in early (12-17 wk) and late pregnancy (36-38 wk).

	Vitamin D sources (IU, % of total food intake)		**p-value between time points**
	Early pregnancy	**Late pregnancy**	
Milk	68 (39)	143 (54)	< 0.05
Non-milk dairy	12 (7)	27 (10)	< 0.05
Fortified food	31 (17)	21 (8)	0.22
Animal sources	52 (31)	55 (22)	0.80
Plant sources	1 (1)	1 (0)	0.68
Others	9 (5)	16 (6)	< 0.05
Total food sources of vitamin D (IU/d)	**174**	**263**	**< 0.05**
Supplemental sources of vitamin D (IU/d)	**400**	**400**	**0.38**
Total intake of vitamin D (IU/d)	**574**	**663**	**0.83**

Table 3: Multivariable analysis of factors associated with maternal serum 25(OH)D concentrations in pregnant women (*n* = 187) in early (12-17 wk) and late pregnancy (36-38 wk).

Variables	Early pregnancy			Late pregnancy		
	Estimated coefficient	95%CI	p-value	Estimated coefficient	95%CI	p-value
Season of blood draw (winter as reference)	13.07	5.46, 20.69	<0.001	24.35	15.55, 33.16	<0.001
Sum of skinfold	-0.14	-0.41, 0.13	0.312	0.06	-0.20, 0.32	0.654
Vitamin D intake from supplements	0.01	0.00, 0.01	<0.001	0.01	0.00, 0.01	0.001
Vitamin D intake from milk	0.01	-0.05, 0.07	0.779	0.01	-0.04, 0.05	0.843
Vitamin D intake from non-milk dairy products	0.03	-0.16, 0.23	0.734	0.17	0.02, 0.32	0.029

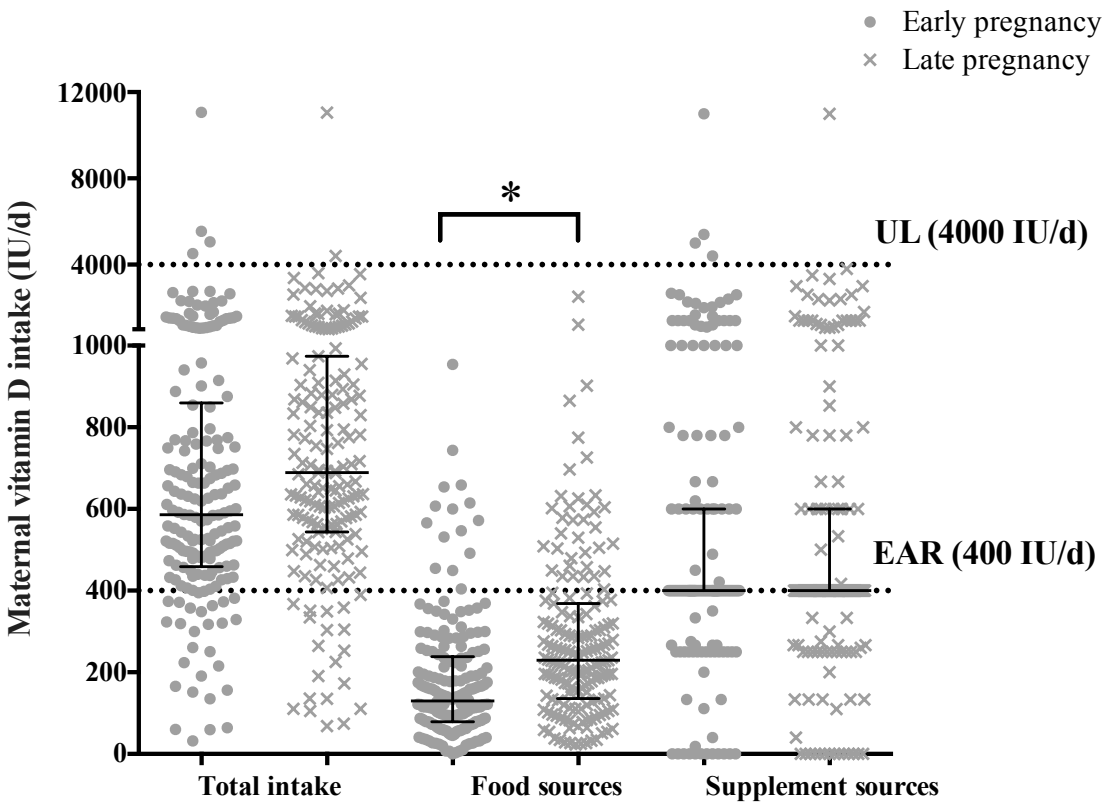

Figure 1. Maternal dietary intake of vitamin D, including food and supplement sources in pregnant women (*n* = 187) in early (12-17 wk) and late pregnancy (36-38 wk). Values are displayed as median and interquartile ranges.

Legend Figure 1: Dotted lines: Recommendations for pregnancy by the Institute of Medicine (9); * Different between early and late pregnancy, $p < 0.001$.

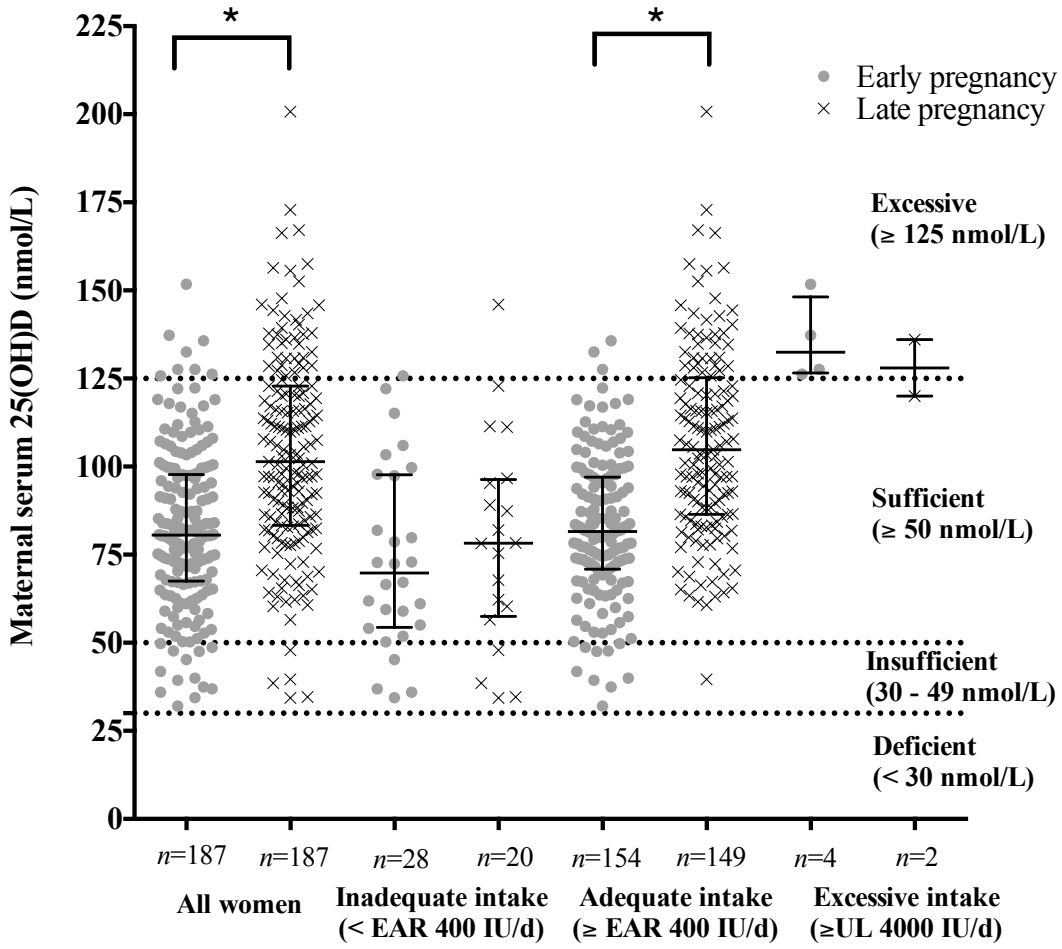

Figure 2: Maternal serum 25(OH)D concentration (mean ± SD) grouped by adequacy of vitamin D intake in pregnant women (*n* = 187) in early (12-17 wk) and late pregnancy (36-38 wk).

Legend Figure 2: Dotted lines: Recommendations for pregnancy by the Institute of Medicine (9); * Different between early and late pregnancy, $p < 0.001$.

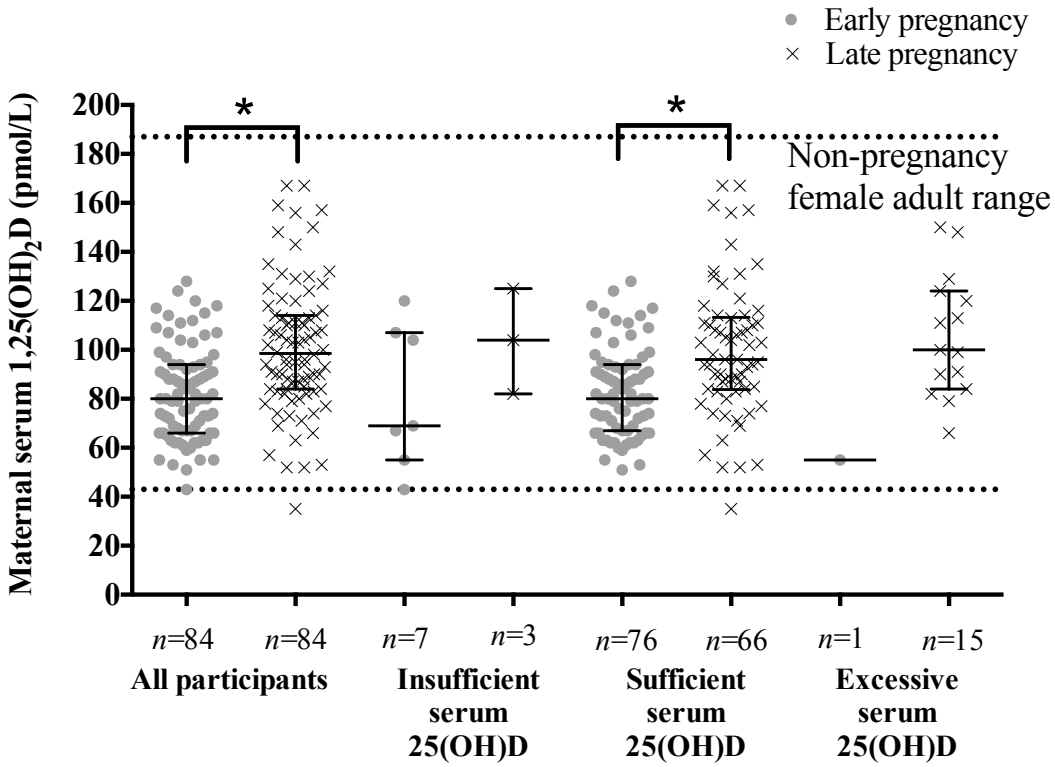

Figure 3: Maternal serum $1,25(OH)_2D$ concentrations (mean ± SD) grouped by maternal vitamin D status in pregnant women (n = 84) in early (12-17 wk) and late pregnancy (36-38 wk).

Legend figure 3: Recommendations for pregnancy by the Institute of Medicine (9): Insufficient serum 25(OH)D: 30-50 nmol/L; Sufficient serum 25(OH)D: ≥ 50 nmol/L; Excessive serum 25(OH)D: ≥ 125 nmol/L. Dotted lines: non-pregnancy female adult range values as reported by the Mayo Clinic Laboratories (44). * Different between early and late pregnancy, $p < 0.001$.

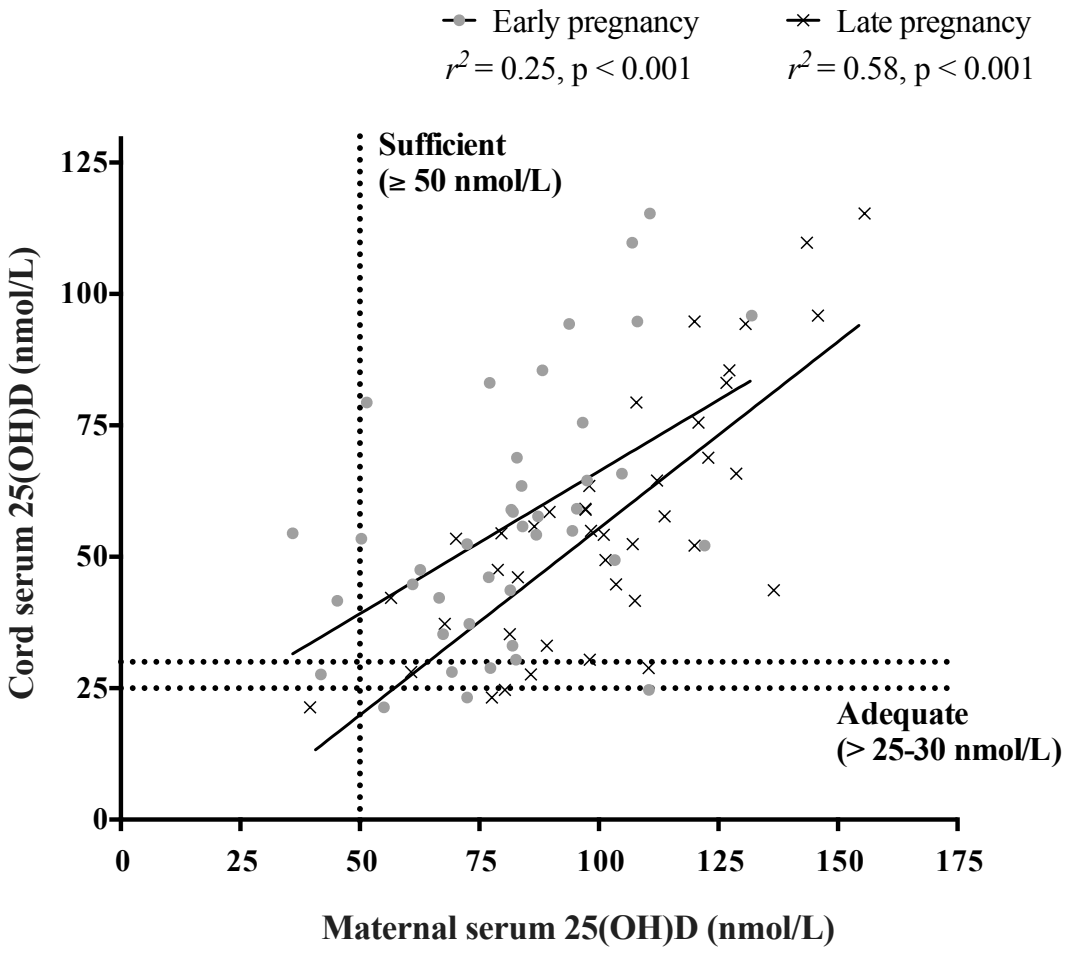

Figure 4: Relationship between maternal and cord serum 25(OH)D concentrations (individual values displayed; n = 41) in mother-infant dyads in early (12-17 wk) and late pregnancy (36-38 wk).

Legend Figure 4: Dotted lines: Recommendations for pregnancy by the Institute of Medicine (9). For cord serum, threshold of 25 nmol/L and 30nmol/L recommended to prevent nutritional rickets (8).

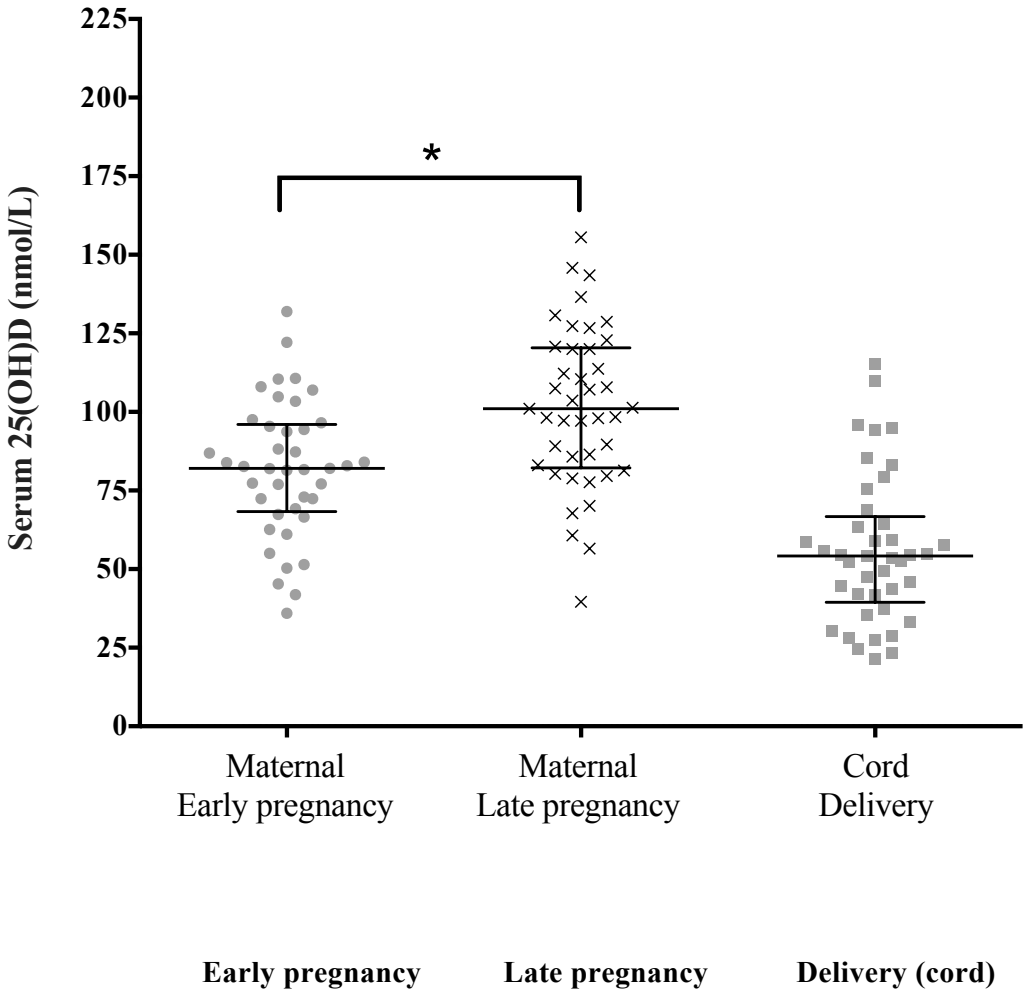

Figure 5: Maternal and cord serum 25(OH)D concentrations (mean ± SD) in mother-infant dyads (n = 41) in early (12-17 wk) and late pregnancy (36-38 wk), and at delivery.

Legend Figure 5: * Different between early and late pregnancy, $p < 0.001$.

CHAPTER 5

INDIVIDUALIZED HIGH DAIRY PROTEIN + WALKING PROGRAM SUPPORTS BONE HEALTH IN PREGNANCY: A RANDOMIZED CONTROLLED TRIAL

PREFACE TO CHAPTER 5

Evidence exists that pregnancy and lactation in the post-partum are associated with maternal bone mineral mobilization, which may be further compromised if dietary protein, calcium, vitamin D and physical activity are sub-optimal. To determine the impact of a Nutrition and Exercise intervention in pregnancy, we conducted a randomized controlled trial (RCT) in healthy pregnant women from Southern Ontario. We hypothesized that the high dairy protein diet + walking program would reduce maternal bone turnover during pregnancy, but that the benefits would not be sustained in the post-partum period.

Our findings contribute to the literature as most interventions to date focused on calcium or vitamin D supplements, with only one small RCT having used milk powder to supplement women with calcium. Our intervention is novel as it includes a practical nutrition component using dairy foods, in combination with an exercise component consisting of walking, a feasible activity for women throughout pregnancy, to maximize the potential impact on maternal bone health markers.

Our study contributes normative biochemical measures of bone status for women in the periconceptual period, as we globally evaluated maternal bone health by calciotropic and bone biomarkers throughout pregnancy and the post-partum, and complemented our

assessment by a maternal whole body and lumbar spine bone mineral density scan by dual energy absorptiometry at six months post-partum. We also measured bone biomarkers in cord blood, providing insights into how maternal bone status impacts fetal bone metabolism. We conducted detailed assessment of dietary practices and lactation practices throughout pregnancy and the post-partum period, as these factors are often overlooked despite their importance in modulating maternal bone metabolism in the periconceptual period.

The results demonstrated a lower serum concentration of the bone resorption marker CTX in women randomized to the intervention group when compared to the control group, and increasing concentrations of the bone formation marker PINP across pregnancy and the post-partum, suggesting that our intervention might preserve maternal bone health in pregnancy. Thus, our findings propose a feasible intervention that may ameliorate the transient bone loss reported to be associated with pregnancy.

Authors' contributions: **MP** conducted the core study visits with the participants, with the exception of the intervention so to remain blinded to the group allocation. **MP** performed laboratory analysis for all the biomarkers and the DXA scans, in collaboration with the study coordinator. **MP** analyzed data and performed statistical analysis. **MP** wrote the paper. SAA designed the study, obtained the grant support and had primary responsibility for the final content of the paper. SAA and MFM oversaw the data collection. All authors read and approved the final manuscript.

Full citation: **Perreault M**, Atkinson SA, Mottola MF, and the BHIP study team. *Individualized high dairy protein + walking program supports bone health in pregnancy: a randomized controlled trial*.

To be submitted to the American Journal of Clinical Nutrition, Fall 2019.

TITLE: Individualized high dairy protein + walking program supports bone health in pregnancy: a randomized controlled trial

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Sources of support: This study is supported by CIHR (FRN 123347) and Dairy Research Cluster Initiative (Dairy Farmers of Canada and AAFC, the Dairy Network and the Canadian Dairy Commission) and in-kind provision of milk by GayLea Foods Coop, ON, Canada, and yogurt and cottage cheese Ultima Foods, QC, Canada. MP is supported by a doctoral Vanier CIHR Canada Graduate Scholarship and a Career Enhancement Program scholarship by the Canadian Child Health Clinician Scientist Program.

Short running title: Dairy protein+walking RCT on maternal bone health

Abbreviation list: PINP **(**total procollagen type I N-terminal propeptide); CTX (C-terminal telopeptide of type I collagen); BMD (bone mineral density), DXA (dual energy absorptiometry); BHIP study (Be Health in Pregnancy study); EAR (Estimated average requirement); UL (Tolerable upper intake level);

Clinical Trial Registry: NCT01689961 (https://clinicaltrials.gov/ct2/show/NCT01689961?cond=NCT01689961&rank=1) Registered on 21 September 2012.

Abstract

Background: Pregnancy induces bone mineral mobilization, which may be further compromised if diet and physical activity are sub-optimal.

Objective: To determine the effects of a Nutrition+Exercise intervention during pregnancy on maternal calciotropic and bone biomarker profiles throughout pregnancy and the post-partum.

Design: In the Be Healthy in Pregnancy (BHIP) RCT, 203/241 women consented at randomization (12-17 weeks (wk) gestation) to the bone health sub-study and received either usual care (control) or a Nutrition+Exercise intervention that provided an individualized high protein diet (50% as dairy products) and a walking program (10,000 steps/day) throughout pregnancy. Serum total procollagen type I N-terminal propeptide (bone formation), C-terminal telopeptide of type I collagen (bone resorption), and insulin-like growth factor-1 were measured by ELISA, and vitamin D metabolites by ultra-performance liquid chromatography tandem mass spectrometry at early and late pregnancy, six months post-partum, and in cord blood. Maternal whole body and lumbar spine bone mineral density (BMD) were measured by dual energy absorptiometry scan at six months post-partum.

Results: A total of 187/203 participants completed all measures. Intakes of protein (median (Q1, Q3): 107 (87, 124) versus 86 (70, 105) g/day, $p < 0.0001$) and calcium (1762 (1375, 2052) versus 1208 (931, 1579) mg/d, $p < 0.0001$) were significantly higher in the intervention versus control group across second and third trimesters of pregnancy. Intervention compared to control participants had significantly lower maternal concentrations of CTX by the end of pregnancy (mean (SD): 0.78 (0.31) ng/mL versus 0.89 (0.33) ng/mL; $p = 0.038$; $n = 96$). Serum 25(OH)D status was > 50 nmol/L for 97 % of participants, and other bone markers were similar between groups. No differences in BMD z-score at both skeletal sites were observed between groups.

Conclusion: Higher maternal dietary protein and calcium intakes compared to usual care minimized bone resorption and maintained bone formation, thus conferring a protective effect on maternal bone health.

Keywords: serum PINP, serum CTX, serum IGF-1, pregnancy, post-partum, cord blood, bone health

Introduction

Maternal bone metabolism changes during pregnancy and the post-partum period to accommodate the increased nutrient demands for fetal and infant skeletal formation (1,2). The maternal bone mineral mobilization that occurs during pregnancy and lactation results in a transient bone loss (reviewed in (3)). In observational studies in pregnancy serum markers of bone resorption and bone formation increased from early to late pregnancy, peaking in the third trimester when fetal bone accrual is at its highest (4–7). A reduction of up to two percent of bone mineral density at one or more skeletal sites, as measured by dual-energy x-ray absorptiometry (DXA), was reported in a review of observational studies from pre-pregnancy to six weeks post-partum (3). During the post-partum period up to six months, mobilization of maternal skeleton occurs in association with lactation, leading to a transient loss of between five to seven percent of bone mineral density at the lumbar spine (8–11). To date, no recent studies have completed a comprehensive assessment of bone metabolism changes prospectively and repeatedly in the perinatal period, measuring serum bone biomarkers to complement measures of bone mineral content/density assessed by gold standard methods.

Although recent RCTs have employed single supplements of calcium (12,13) or vitamin D (14) to promote maternal bone health in pregnancy, there is scant knowledge of the influence of dairy foods on bone health in women in the periconceptual period. To our knowledge, only one small RCT including Chinese women (N=36) assessed the impact of a maternal calcium supplementation provided by milk powder on maternal bone health

outcomes, showing a positive benefit to bone mass density (15). Consumption of dairy products provides high quality dietary protein and calcium, as well as supporting calcium absorption due to the concomitant increase in vitamin D intake (if milk is fortified) and the whole food matrix (16). Dairy products are hypothesized to promote bone health during pregnancy, with potential sustained effects in the post-partum period (17).

Maternal habitual dietary intake throughout pregnancy and post-partum are often not well documented in studies looking at bone health (3) despite the importance of nutrition on normal bone turnover. As well, lactation is not often described and can encompass various lactation practices that may underlie the extent and duration of maternal bone loss in the post-partum period (18). Very few structured and personalized lifestyle interventions have been conducted during pregnancy with the goal to optimize maternal bone health and monitor the sustained impact in the post-partum period. It is unclear if optimized lifestyle, including nutrition and physical activity, can counterbalance the physiological changes associated with pregnancy that adversely influence bone health status. Whether the effect of an intervention during pregnancy is sustained in the post-partum period until cessation of lactation, has also not been explored. To address these gaps in knowledge we designed a structured lifestyle intervention that started early in pregnancy, using food rather than single nutrient supplementation, to measure the impact of optimized nutrition and exercise on maternal bone metabolism during and after pregnancy. Further, we measured the bone biomarker profiles in cord blood to assess the impact of the intervention on fetal bone metabolism. The objectives of this study were to

determine the effect of a maternal Nutrition+Exercise intervention in comparison to a control (usual care) group during pregnancy on i) maternal bone biomarkers from early (12-17 weeks) to late (36-38 wk) gestation, at six months post-partum, and in cord blood; and on ii) maternal bone mass at six months post-partum.

Subjects and Methods

Study design

Of the 241 participants enrolled and randomized in The Be Healthy in Pregnancy (BHIP) study, a randomized controlled trial (RCT; Clinical Trials Ref: NCT01693510), 203 participants consented to be included in the Bone-BHIP sub-study. The full BHIP study protocol, including details of this sub-study Bone-BHIP has been published (19).

Healthy pregnant women were recruited from health care clinics in Hamilton, Burlington and London, Ontario, Canada between 12 and 17 weeks gestation. Ethics approval was obtained from the Research Ethics Boards of Hamilton Health Sciences (REB Project#12-469), Western Ontario in London (HSREB 103272), and Joseph Brant Hospital in Burlington (JBH 000-018-14), all in Southern Ontario, Canada. The protocol complied with the Helsinki Declaration.

Experimental treatment

After baseline measures, participants were randomized to either a structured Nutrition+Exercise program (intervention) or to the control group (usual care) for the

duration of their pregnancy. Briefly, the intervention consisted of a monitored and personalized Nutrition and Exercise program starting at 12-17 wk gestation and continued until delivery. The nutrition component was an individualized nutrition plan tailored to each participant's energy requirements with a high protein content (target of 25% protein energy) provided primarily by low fat dairy foods (i.e. milk, Greek yogurt and cottage cheese). The exercise component was a monitored walking program, with the goal of 10,000 daily steps and 4 walks of 45 minutes each per week as previously shown to reduce the risks of excessive gestational weight gain, which was the primary outcome of the core BHIP study (20).

Maternal data collection

Maternal demographics, pregnancy history, and physical measurements including skinfold thickness, were obtained from each participant upon study entry and throughout pregnancy as published previously (19). Season at enrollment (12-17 wk gestation) was defined as summer (May-October) or winter (November to April). Fasted venous blood was collected in the morning at 12-17 wk and 36-38 wk gestation, and at six months post-partum. Cord venous blood was collected at delivery in a subgroup of 45 newborns when consent was obtained. Lactation practices were obtained through questionnaires at three months and six months post-partum and classified as 'Exclusively breastfeeding', 'Breastfeeding and expressing breast milk', 'Did breastfeed and/or expressed, but has stopped', or 'Never lactated'.

Dietary intake and physical activity

At 12-17 wk, 26-28 wk and 36-38 wk gestation, and at six months post-partum participants completed three-day diet records over two week days and one weekend day that included both foods and supplements. Study nutritionists analyzed diet records using Nutritionist Pro™ diet analysis software (Version 5.2, Axxya Systems, Stafford, TX, USA) and the Canadian Nutrient File (version 2015) to obtain daily intake of nutrients. At the same time points, steps counts were measured using the SenseWear® armband tri-axis accelerometer (Model MF-SW; BodyMedia® Inc., Pittsburgh PA).

Bone turnover by bone biomarkers and vitamin D metabolites

Serum bone formation marker total procollagen type I N-terminal propeptide (PINP) and resorption marker C-terminal telopeptide of type I collagen (CTX) were assessed by enzyme-linked immunosorbent assays (ELISA) following manufacturer's instructions (P1NP, Cloud Clone Corp, Houston, USA; CTX, IDS Serum Crosslaps, UK). Insulin-like growth factor-1 (IGF-1) was assessed by ELISA following manufacturer's instructions (R&D Systems, Minneapolis, USA). Intra-assay and inter-assay coefficient of variation were the following: PINP: 16.9%, 16.45%; CTX; 1.9%, 13.3%; IGF-1: 5.0%, 17.0%.

Serum 25(OH)D concentrations were measured by ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) using the Waters application note 720002748 (21) with a modified sample preparation from Hymoller that included a saponification step (22) as published previously by our team (23). Serum

$1,25(OH)_2D$ concentrations were measured by Quest Diagnostics Nichols Institute (CA, USA) using LC-MS/MS (AB Sciex LLC, MA, USA) as previously published by our team (24).

Bone mass by dual-energy absorptiometry (DXA) scan

Maternal whole body bone mineral density (BMD) and lumbar spine BMD were measured by dual-energy absorptiometry (DXA) scan at six months post-partum in supine position (QDR®4500 series Hologic Inc. Discovery™ DXA machine, Waltham, MA, USA; Adult whole body software version 12.3.1 at the Hamilton study site; General Electric-Luna iDXA, Ames Medical enCORE, Version 14.1, Waukesha, WI, USA; CoreScan, GE at the London study site). The CV for BMD was 0.35%. If considerable movement was seen in one limb but not the other, the well-captured limb was used as a surrogate, as validated by our group (25). Any scans with unsalvageable distortions due to movement were discarded. Maternal DXA scan whole body BMD and lumbar spine BMD results are reported as z-scores using the software database as reference population.

Statistical analysis

Statistical analysis was performed using JMP 9.0 (Version 9.0.1, SAS Institute Inc., Cary NC, USA) and GraphPad Prism (Version 7, La Jolla, CA). Descriptive statistics included the means and standard deviations of normally distributed continuous data; medians and quartiles (Q1 and Q3) for non-normally distributed continuous data; and counts and percentages for categorical data. Mean values are given as mean and standard deviation if

not stated otherwise. Data were treated with a per protocol approach, including complete cases for the primary outcomes. Changes in calciotropic hormones and bone biomarkers throughout pregnancy and the post-partum period are reported based on one-way ANOVA. Differences between groups are reported based on regression analysis adjusted for randomization stratification variables (i.e. study site, pre-pregnancy BMI), maternal baseline status for each analyte, season at enrollment, maternal sum of skinfold, and lactation practices for outcomes measured in the post-partum. For regression analysis, the assumptions were assessed for all linear outcomes. For those that violated the normality assumption, other transformations (i.e. log, ln, square root, cube root) were explored to find a more appropriate model. However, in some cases, normality could not be achieved and has been stated in the results section. In such cases, interpretations of results should be done with caution. The non-standardized regression coefficients and their corresponding 95% confidence intervals (CIs) and p-values for the regression analyses are presented.

Results

Demographics and baseline data

Figure 1 represents the flow diagram of the Bone-BHIP study. A total of 18 participants were lost to follow-up at the end of pregnancy and did not provide primary outcomes. Hence the final sample size is 187 participants. No differences in demographic characteristics were observed between the participants from this sub-study when

compared to the whole BHIP study sample (data not shown). No differences in birth outcomes (i.e. gestational age, birth weight, birth length and birth APGAR scores) were observed between infants for whom cord blood was provided when compared to all infants of participants in the Bone-BHIP study. Reasons for loss to follow-up for 18 participants consented to the Bone-BHIP study are detailed in Figure 1. Participants were mostly of European descent, holding a university degree and with a moderate-to-high household income (**Table 1**). About half of the participants entered pregnancy with a pre-pregnancy body mass index (BMI) classified as overweight or obese, ranging from 17.4 - 39.6 kg/m^2.

(TABLE 1 NEAR HERE)

Dietary intake response to the intervention

While protein intakes were similar between groups prior to randomization (**Table 2**), participants in the intervention group consumed a significantly higher amount of total protein, and protein from dairy products, when compared to participants in the control group at mid-pregnancy and end of pregnancy (**Table 2**). At six months post-partum, the participants from the intervention group continued to consume significantly more protein from dairy foods compared to the control group (**Table 2**) but total protein intakes were similar between groups. Maternal dietary intakes of calcium pre-randomization were similar between groups, and met recommended intakes (> Estimated average requirement (EAR) 800 mg/d) in 85-88 % of participants (**Table 2**). Participants in the intervention

group consumed significantly higher amounts of calcium at mid-pregnancy and at the end of pregnancy compared to participants in the control group, but this was not sustained at six months post-partum (**Table 2**). Vitamin D intake was comparable between groups at all time points, and met recommendations (> EAR = 400 IU/d) in 84-85 % of participants at baseline at enrolment, 89-96 % at mid-pregnancy, and 87-92 % at the end of pregnancy (**Table 2**). Vitamin D intake decreased at six months post-partum compared to pregnancy, and was adequate for only 54-59 % of participants (**Table 2**).

(TABLE 2 NEAR HERE)

Bone biomarker response to the intervention

All maternal bone biomarker concentrations were similar between groups at baseline (**Table 3**). At the end of pregnancy participants in the intervention group had significantly lower mean serum concentrations of the bone resorption marker CTX when compared to the control group (**Table 3**). No significant differences in serum concentrations of the other bone biomarkers were observed between treatment groups at the end of pregnancy, nor at six months post-partum (**Table 3**).

(TABLE 3 NEAR HERE)

Bone biomarkers changes throughout pregnancy and the post-partum period

Serum concentrations of PINP rose significantly from early pregnancy to the post-partum for participants in the intervention group only (**Table 3;** ANOVA F-value, p-value: Intervention 4.08, 0.018; Control 2.77, 0.065). Serum concentrations of CTX changed significantly across pregnancy and the post-partum period in both groups (**Table 3;** ANOVA F-value, p-value: Intervention 57.9, <0.0001; Control 68.1, <0.0001). Serum concentrations of IGF-I changed significantly over pregnancy and the post-partum period for both groups (**Table 3;** ANOVA F-value, p-value: Intervention 130.1, <0.0001; Control 135.8, <0.0001). No participant had vitamin D deficiency (serum 25(OH)D < 30 nmol/L) at baseline and adequate vitamin D status was maintained throughout pregnancy and the post-partum as previously published (24). Serum 25(OH)D concentrations changed significantly over the pregnancy and post-partum period in both groups (ANOVA F-value, p-value: Intervention 29.08, <0.0001; Control 27.16, <0.0001) and remained above 50 nmol/L at all time points (**Table 3).**. Serum concentrations of $1,25(OH)_2D$ significantly changed throughout the pregnancy and the post-partum period in both groups (**Table 3;** ANOVA F-value, p-value: Intervention 66.24, <0.0001; Control 57.76, <0.0001).

Cord serum bone biomarker response to the intervention

Cord blood concentrations of bone resorption marker CTX were significantly lower in participants in the intervention group compared to the control group (**Table 4).** Serum concentrations of other biomarkers were similar between groups (**Table 4**).

(TABLE 4 NEAR HERE)

Response of maternal bone mass at six months post-partum to the intervention

In a sub-group of participants with successful DXA scans at six months post-partum (n=142), mean maternal whole body BMD z-score and lumbar spine BMD z-score were similar between groups (**Table 5**).

(TABLE 5 NEAR HERE)

Discussion

A personalized and monitored nutrition and exercise intervention during pregnancy that optimized dietary intakes of essential bone nutrients, particularly protein and calcium, led to lower maternal bone resorption at the end of pregnancy compared to usual care with self-selected diets. Further, serum PINP concentrations rose significantly across pregnancy and the post-partum in participants in the intervention group but remained stable in the control group. Serum concentrations of the growth marker IGF-1 rose similarly across pregnancy and the post-partum in both groups, consistent with results from a controlled cohort study of women followed from pre-conception to the post-partum (4). Collectively, bone turnover appears to favour formation in response to the intervention without excessive IGF-1 present, suggesting that the intervention conferred a protective effect on maternal bone health, and could represent a feasible intervention to

attenuate transient skeletal loss associated with pregnancy.

From our analysis, the impact of the nutrition versus the exercise component of the intervention is somewhat difficult to decipher. However, as observed in the core study (manuscript in preparation), exercise measured as step counts by accelerometry did not differ between intervention and control groups neither at baseline nor at the end of pregnancy. Daily steps counts ranged from 4800 to 6300 steps/d for participants in both groups throughout pregnancy, and equated to a 'low active' activity level (20). Thus, the BHIP RCT intervention could be considered primarily a dietary intervention, as the exercise intervention component did not lead to increased daily steps counts at any time point during pregnancy. We can surmise that the higher protein and calcium intakes as observed in the intervention group, supported by adequate vitamin D status, played the major role in suppressing bone resorption and promoting bone formation. Our results align with the only other RCT using dairy products to promote bone health in pregnancy (15). Calcium supplementation through milk powder in 36 Chinese pregnant women led to a significant dose-dependent reduction in urinary bone resorption marker at the end of pregnancy, when compared to no supplementation (15). Our results contribute to the limited evidence to date, and suggest that dairy products might confer an advantage over single nutrient supplementation during pregnancy on maternal bone health outcomes.

The difference in serum CTX between groups was not sustained at six months post-partum, likely reflecting the fact that higher dietary intakes of calcium and total protein

provided by the intervention were not maintained. Participants were instructed to follow the Nutrition+Exercise intervention until delivery, after which participants in the intervention group somewhat reduced their intake of dairy foods so that dietary intake of total protein and calcium was similar between the former treatment groups. No differences were observed between groups in serum concentrations of any of the other bone biomarkers, not surprisingly as these measures reflect short-term changes (26) and respond to dietary changes (27).

Our regression analysis models for biomarker outcomes were carefully controlled for factors beyond dietary intake that might influence circulating bone biomarkers including maternal body adiposity and lactation practices. For example, serum IGF-I concentrations are significantly lower in women who lactate compared to women not lactating (4). However, the high rate of breastfeeding and/or expressing breast milk in our cohort (83% of participants lactating at six months post-partum) made it difficult to further separate the women into categories to assess gradations of breastfeeding exclusivity and duration on maternal bone health. Maternal weight and pre-pregnancy BMI are other potential modulators of bone health status changes during pregnancy. Women with a low pre-pregnancy BMI were reported to have greater decline in whole body bone mineral content than women of higher pre-pregnancy BMI, suggesting that higher body weight might be protective of bone mass due to increased mechanical loading (28). The strength of our study is that we adjusted for maternal adiposity measured quantitatively by the sum of skinfolds at four sites at the end of pregnancy, maternal pre-pregnancy BMI (as

per study randomization stratification) as well as season at enrolment and lactation practices for the post-partum measures. Such factors are reported to modulate bone biomarker concentrations (28–31), yet have never been measured collectively in a study reporting on bone changes in pregnancy.

Vitamin D status was not likely a mitigating factor related to bone resorption as no clinical vitamin D deficiency was detected in any participant. As we reported previously (24), serum concentrations of both 25(OH)D and 1,25 $(OH)_2D$ rose at the end of pregnancy in both groups, irrespective of the intervention. This is reasonable given the similar intakes of vitamin D in early and late pregnancy for participants in both groups. Serum concentrations of 50-75 nmol/L appear to be sufficient to maintain bone homeostasis, and this observation does not support the hypothesis that serum 25(OH)D concentrations of at least 100 nmol/L are required to maximize 1,25$(OH)_2D$ conversion (14) and maintain bone health in pregnancy among women with adequate nutritional status.

The response of maternal serum bone biomarkers to the Nutrition+Exercise intervention was mirrored in cord serum, and contributed to the current knowledge as we are one of the few studies that reported on both serum vitamin D metabolites and serum bone biomarkers in mother-infant dyads. All mean values for cord concentrations of vitamin D metabolites and bone biomarkers were lower than maternal concentrations at the end of pregnancy, with the exception of PINP, which averaged four times (288%) higher than

the maternal concentration in late pregnancy, in 83% of the sample. In our cohort, the between-subject variability observed in cord serum concentrations of PINP cannot be explained by methodological issues, and it is unclear why a sub-group had up to nine fold lower concentrations (range 12.7 to 30.8 ng/mL, n=6) when most cord blood concentrations (range 107.1 to 199.9 ng/mL, n=30) surpassed maternal concentrations at the end of pregnancy. The high serum PINP cord concentrations might be explained by the bone formation that characterizes fetal skeletal bone accretion in the third trimester (reviewed by (32)), which happens irrespective of the mother's own bone turnover. Rather than reflecting maternal concentrations, cord serum PINP concentrations might reflect fetal somatic growth (reviewed in (33)). The role of PINP in somatic growth during early life was reported in a cohort of 690 preterm and term infants (34). Lower concentrations of cord PINP were reported with advancing gestational age, and cord PINP was lower in cord serum of infants born small-for gestational age when compared to those born average-for-gestational age (34). Our data revealed a promising area of research, as future studies including a larger sample size of newborns and cord blood samples could elucidate the relationship between maternal perinatal bone metabolism changes and the programming impact on the fetus and newborn.

For IGF-1, maternal consumption of milk and dairy products is hypothesized to raise maternal IGF-1concentrations, with mixed evidence suggesting both positive (reviewed in (35)) and negative impacts (reviewed by (36)) on fetal and neonatal growth. The systematic review of eight observational studies (N=104,485 participants) that addressed

the impact of maternal milk consumption on neonatal outcomes (35) concluded that evidence is limited, but pointed to a positive benefit of a maternal moderate milk consumption compared to minimal milk intake in pregnancy on fetal growth and infant birth weight. The BHIP study results suggest that increased maternal protein intake, due to higher consumption of dairy products including milk, is safe for mothers and the fetus/newborn as the intervention was not associated with either higher maternal serum concentrations of IGF-1 or cord serum IGF-1 concentrations. This aligns with evidence from twin pairs showing that cord concentrations of IGF-1 might be predominantly attributable to genetics, not to environmental factors such as maternal diet (37).

The lack of difference in maternal BMD between treatment groups at six months post-partum is understandable given that we could not adjust for baseline measures. What is important to note is that mean values of whole body BMD z-scores, as well as lumbar spine BMD z-scores, of participants in both groups indicate that they are within the expected range for sex and age (38). This reflects the overall appropriate nutrition, adequate vitamin D status, and general good health of the women in the BHIP study.

This study has several strengths, the most important one being a randomized intervention using food as part of a lifestyle intervention rather than individual nutrient supplements as used in most previous interventions trials in pregnancy (12,13,39,40). Also, the percentage of participants lost-to-follow-up post-randomization for the primary outcomes at the end of pregnancy was low (8%), and there was a high retention (80%) of

participants through to the last study visit at six months post-partum. Additionally, other factors known to impact bone metabolism (41) such as dietary intake including food and supplements were measured at several time points throughout pregnancy and the post-partum, and lactation practices were assessed at two time points. A full panel of serum bone biomarkers provided a global assessment of changes in maternal bone metabolism in the perinatal period, including the bone turnover markers suggested by the International Osteoporosis Foundation and the International Federation of Clinical Chemistry and Laboratory Medicine (27). Some limitations included the lack of baseline assessment before pregnancy due to the difficulty of recruiting women pre-conception, and the homogenous study cohort that might limit the generalizability of the results. Lastly, although bone density was measured by DXA in post-partum there was no baseline measure; further, it remains difficult to delineate the effect of pregnancy versus lactation on maternal bone mass. This caveat was addressed by measuring serum bone biomarkers at six months post-partum, as they reflects short-term changes (26), likely attributable to the post-partum changes in maternal diet and metabolism.

Future steps include evaluating the programming impact of the intervention on neonatal and offspring health outcomes, as well as studying the impact of maternal and cord blood gene variants profiles on bone health outcomes.

Acknowledgments

Authors would like to acknowledge all the families who participated in the Be Healthy in Pregnancy study, as well as the community partners involved in this study. We are grateful for the contributions of research coordinators Kristina Chandler and Caroline Moore, and students and assistants who contributed to data collection. We thank Dr. Lehana Thabane and Dr. Thuvaraha Vanniyasingam for statistical advice.

Sources of funding

Supported by Dairy Farmers of Canada (SAA), Canadian Institutes of Health and Research (SAA), and Dairy Farmers of Canada and Agriculture and Agri-food Canada Dairy Research Cluster (SAA); in-kind by GayLea Foods, ON, Canada & Ultima Foods, QC, Canada. MP is supported by CIHR-Vanier and Canadian Child Health Clinical Scientist Program doctoral awards.

Author contributions

SAA designed research; SAA, MFM and MP conducted research; MP analyzed data and performed statistical analysis; MP and SAA wrote paper; SAA had primary responsibility for final content.

Conflicts of interest: The authors declare no conflict of interest.

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Table 1: Demographic characteristics by study group (n=187).

Variables	Intervention (n=91)	Control (n=96)
Pre-randomization characteristics		
Maternal age (y); mean (SD)	32 (4)	31 (4)
Gestational age at randomization (wk); mean (SD)	13.8 (2)	13.6 (2)
Tertiary education; n (%)	88 (96)	92 (93)
Pre-pregnancy BMI (kg/m^2); mean (SD)	25.7 (4.5)	25.3 (4.6)
Pre-pregnancy BMI category; n (%) Underweight (<18.5 kg/m^2) Normal weight (18.5-24.9 kg/m^2) Overweight (25.0-29.9 kg/m^2) Obese (≥30 kg/m^2)	 2 (2) 47 (51) 26 (29) 16 (18)	 1 (1) 56 (58) 24 (25) 15 (16)
Ethnicity; n (%) European descent Mixed/Other Unknown	 80 (88) 11 (12) 0 (0)	 84 (88) 11 (11) 1 (1)
Total family income (CAN); n (%) <$45,000 $45,000-$74,999 >$75,000 Unknown	 4 (4) 12 (13) 73 (81) 2 (2)	 8 (8) 16 (17) 65 (68) 7 (7)
Married/living with significant other; n (%)	89 (98)	89 (93)
Nulliparous; n (%)	43 (47)	46 (48)
Study site; n (%) London Hamilton	 28 (31) 63 (69)	 32 (33) 64 (67)
Birth characteristics		
Gestational age (weeks); mean (SD)	39.4 (1.4)	39.5 (1.1)
Post-partum characteristics		
Lactation practices; n (%) Exclusively breastfeeding Breastfeeding and expressing Did breastfeed and/or expressed, but stopped Never lactated	 41 (54) 27 (35) 5 (7) 3 (4)	 43 (50) 33 (38) 3 (4) 7 (8)

Table 2: Dietary intake and step counts at baseline (12-17 wk), mid pregnancy (26-28 wk), end of pregnancy (36-38 wk), and at six months post-partum by study groups.

Variable	Intervention (N=91)			Control (N=96)			Mean difference (95% CI)*	P
	Median (Q1, Q3)	EAR N (%)	> UL N (%)	Median (Q1, Q3)	EAR N (%)	> UL N (%)		
Early-pregnancy (12-17 wk)								
Total protein (g/day) (g/kg/day)	87 (67, 99) 1.20 (0.92, 1.45)	86 (95)	n/a	88 (70, 103) 1.25 (0.97, 1.49)	93 (97)	n/a	-1.44 (-4.47, 1.57)	0.345
Protein from dairy products (g/day)	15 (11, 25)	n/a	n/a	16 (11, 26)	n/a	n/a	-0.04 (-0.21, 0.14)	0.691
Calcium (mg/day)	1164 (972, 1462)	80 (88)	2 (2)	1152 (936, 1535)	82 (85)	1 (1)	0.01 (-0.04, 0.05)	0.831
Vitamin D (IU/day)	585 (460, 766)	77 (85)	2 (2)	585 (458, 865)	81 (84)	2 (2)	-54.58 (-180.85, 71.68)	0.395
Step counts (steps/day)	6288 (5153, 8668)	n/a	n/a	6147 (4684, 7618)	n/a	n/a	0.04 (-0.01, 0.10)	0.130
Mid-pregnancy (26-28 wk)								
Total protein (g/day) (g/kg/day)	**111 (88, 128) 1.48 (1.15, 1.71)**	**86 (95)**	**n/a**	**87 (69, 103) 1.10 (0.97, 1.36)**	**84 (87)**	**n/a**	**0.54 (0.36, 0.71)**	**< 0.0001**
Protein from dairy products (g/day)	**38 (27, 53)**	**n/a**	**n/a**	**18 (11, 28)**	**n/a**	**n/a**	**10.05 (7.98, 12.12)**	**< 0.0001**
Calcium (mg/day)	**1797 (1366, 2051)**	**90 (99)**	**12 (14)**	**1204 (930, 1612)**	**86 (90)**	**0 (0)**	**0.17 (0.13, 0.22)**	**< 0.0001**
Vitamin D (IU/day)	739 (582, 1074)	87 (96)	0 (0)	599 (463, 918)	85 (89)	1 (1)	61.63 (-9.80, 133.07)	0.090
Step counts (steps/day)	6432 (4731, 8940)	n/a	n/a	5551 (4180, 7861)	n/a	n/a	110.40 (-221.75, 442.56)	0.513
End of pregnancy (36-38 wk)								
Total protein (g/day) (g/kg/day)	**103 (85, 119) 1.25 (1.08, 1.50)**	**82 (90)**	**n/a**	**85 (70, 106) 1.06 (0.87, 1.25)**	**72 (75)**	**n/a**	**9.70 (6.53, 12.87)**	**< 0.0001**
Protein from dairy products (g/day)	**35 (25, 48)**	**n/a**	**n/a**	**16 (11, 24)**	**n/a**	**n/a**	**10.16 (8.33, 11.99)**	**< 0.0001**
Calcium (mg/day)	**1727 (1384, 2053)**	**90 (99)**	**6 (7)**	**1212 (931, 1545)**	**84 (88)**	**0 (0)**	**0.17 (0.13, 0.22)**	**< 0.0001**
Vitamin D (IU/day)	734 (606, 1004)	84 (92)	1 (1)	615 (500, 893)	83 (87)	1 (1)	64.72 (-29.62, 159.07)	0.178
Step counts (steps/day)	4805 (3404, 6953)	n/a	n/a	5068 (3755, 6109)	n/a	n/a	-185.30 (-514.39, 143.78)	0.268
Six months post-partum								
Total protein (g/day) (g/kg/day)	88 (71, 103) 1.25 (1.08, 1.50)	61 (67)	n/a	84 (69, 102) 1.06 (0.87, 1.25)	66 (69)	n/a	1.51 (-1.82, 4.84)	0.371
Protein from dairy products (g/day)	**17 (9, 24)**	**n/a**	**n/a**	**13 (5, 21)**	**n/a**	**n/a**	**1.98 (0.29, 3.67)**	**0.022**
Calcium (mg/day)	1007 (777, 1426)	66 (72)	1 (1)	909 (728, 1227)	63 (66)	0 (0)	0.06 (-0.00, 0.12)	0.052
Vitamin D (IU/day)	450 (173, 927)	49 (54)	1 (1)	472 (197, 921)	57 (59)	1 (1)	0.01 (-0.39, 0.41)	0.956
Step counts (steps/day)	6133 (4420, 8207)	n/a	n/a	6200 (4541, 7708)	n/a	n/a	-117.72 (-543.14, 307.69)	0.585

Dietary reference intakes (42,43):
Total protein recommendations for women in pregnancy: Estimated Average Requirement (EAR)=0.66 g/kg body weight/day (first half of pregnancy) and 0.88g/kg body weight/day (second half of pregnancy); Tolerable Upper Intake Level (UL) = n/a;
Total protein recommendations for lactating women: EAR = 1.05 g/kg body weight/day.
Calcium recommendations for women in pregnancy and lactation: EAR=800 mg/day; UL=2,500 mg/day.
Vitamin D recommendations for women in pregnancy and lactation: EAR=400 IU/day; UL=4,000 IU/day.

Regression analysis with control group set as the reference group. Bold results indicate significant results ($p < 0.05$).
*estimates are based on model adjusted for study site, pre-pregnancy BMI, and baseline values at 12-17 wk pregnancy.
*Early pregnancy models: Total protein: not transformed, Protein from dairy products: square root transformed; Calcium: log transformed; Vitamin D: normality could not be achieved and interpretations of results should be done with caution; Step counts: log transformed.
*Mid pregnancy models: Total protein: square root transformed, Protein from dairy products: not transformed; Calcium: log transformed; Vitamin D: normality could not be achieved and interpretations of results should be done with caution; Step counts: not transformed.
*End of pregnancy models: Total protein: not transformed, Protein from dairy products: not transformed; Calcium: log transformed; Vitamin D: normality could not be achieved and interpretations of results should be done with caution; Step counts: not transformed.
*Six months post-partum models: Total protein: not transformed, Protein from dairy products: normality could not be achieved and interpretations of results should be done with caution; Calcium: log transformed; Vitamin D: cube root transformed; Step counts: normality could not be achieved and interpretations of results should be done with caution.

Table 3: Serum bone biomarker concentrations at baseline (12-17 wk), at the end of pregnancy (36-38 wk), and at six months post-partum.

Bone biomarkers	Intervention (N=91) mean (SD)	Control (N=96) mean (SD)	Mean difference (95% CI)*	*P*
Early pregnancy (12-17 wk)				
PINP (ng/mL)	32.58 (22.96)	36.95 (26.96)	-1.86 (-5.00, 1.28)	0.244
CTX (ng/mL)	0.42 (0.19)	0.46 (0.21)	-0.02 (-0.05, 0.01)	0.200
IGF-1 (ng/mL)	164.00 (62.74)	176.08 (58.85)	-0.04 (-0.09, 0,01)	0.069
25(OH)D (nmol/L)	80.80 (23.36)	82.10 (21.32)	-0.31 (-3.12, 2.50)	0.829
$1,25(OH)_2D$ (pmol/L)	83.78 (19.78)	80.39 (18.56)	1.20 (-3.08, 5.48)	0.577
End of pregnancy (36-38 wk)				
PINP (ng/mL)	37.91 (19.78)	42.57 (24.81)	-0.85 (-3.38, 1.68)	0.506
CTX (ng/mL)	**0.78 (0.31)**	**0.89 (0.33)**	**-0.02 (-0.044, -0.00)**	**0.034**
IGF-1 (ng/mL)	326.93 (128.35)	342.36 (132.95)	-0.01 (-0.05, 0.03)	0.571
25(OH)D (nmol/L)	104.00 (27.54)	101.38 (29.44)	1.78 (-1.28, 4.85)	0.253
$1,25(OH)_2D$ (pmol/L)	98.30 (27.20)	104.08 (26.61)	-4.27 (-10.11, 1.57)	0.149
Six months post-partum				
PINP (ng/mL)	44.57 (27.87)	48.06 (27.71)	1.05 (-2.14, 4.24)	0.514
CTX (ng/mL)	0.91 (0.38)	0.94 (0.37)	-0.01 (-0.07, 0.06)	0.967
IGF-1 (ng/mL)	138.45 (40.41)	141.95 (44.48)	-1.67 (-7.46, 4.11)	0.568
25(OH)D (nmol/L)	75.85 (28.80)	74.99 (22.97)	-0.95 (-3.98, 2.08)	0.538
$1,25(OH)_2D$ (pmol/L)	48.37 (10.32)	51.35 (15.41)	-0.03 (-0.09, 0.03)	0.347

Regression analysis with control group set as the reference group. Bold results indicate significant results ($p < 0.05$).

*estimates are based on model adjusted for study site, pre-pregnancy BMI, baseline values (except for outcomes in early pregnancy), season at enrollment, sum of skinfold, and lactation practices for the six-month post-partum time points.

*Early pregnancy models: PINP: normality could not be achieved and interpretations of results should be done with caution; CTX: not transformed, IGF-1: log transformed, 25(OH)D: not transformed, $1,25(OH)_2D$: not transformed.

*End of pregnancy models: PINP: normality could not be achieved and interpretations of results should be done with caution; CTX: square root transformed, IGF-1: log transformed, 25(OH)D: not transformed, $1,25(OH)_2D$: normality could not be achieved and interpretations of results should be done with caution.

*Six months post-partum models: PINP: normality could not be achieved and interpretations of results should be done with caution; CTX: log transformed, IGF-1: not transformed, 25(OH)D: not transformed, $1,25(OH)_2D$: log transformed.

Table 4: Cord serum bone biomarkers concentrations at delivery (n=53).

Variable	Intervention (N=31) mean (SD)	Control (N=22) mean (SD)	Mean difference (95% CI)*	*P*
PINP (ng/mL)	134.81 (48.49)	126.80 (68.69)	-1.22 (-15.18, 12.74)	0.857
CTX (ng/mL)	**0.58 (0.13)**	**0.69 (0.18)**	**-0.06 (-0.12, -0.01)**	**0.025**
IGF-1 (ng/mL)	80.71 (35.03)	75.98 (32.53)	0.05 (-0.11, 0.20)	0.545
25(OH)D (nmol/L)	57.07 (24.54)	56.91 (20.74)	2.17 (-4.44, 8.79)	0.509
$1,25(OH)_2D$ (pmol/L)	47.82 (12.10)	36.60 (9.90)	3.50 (-1.76, 8.76)	0.180

Regression analysis with control group set as the reference group. Bold results indicate significant results ($p < 0.05$).

*estimates are based on model adjusted for study site, pre-pregnancy BMI, maternal baseline values, season at enrollment, and maternal sum of skinfold at the end of pregnancy.

*PINP: not transformed; CTX: not transformed; IGF-1: log transformed, 25(OH)D: not transformed, $1,25(OH)_2D$: not transformed.

Table 5: Maternal bone mineral density z-score at six months post-partum (n=142).

Variable	Intervention (N=70) mean (SD)	Control (N=72) mean (SD)	Mean difference (95% CI)*	*P*
Whole body bone mineral density z-score	0.67 (0.92)	0.60 (0.91)	0.04 (-0.11, 0.20)	0.573
Lumbar spine bone mineral density z-score	-0.34 (1.14)	-0.41 (0.98)	0.04 (-0.16, 0.23)	0.704

Regression analysis with control group set as the reference group.

*estimates are based on model adjusted for study site, pre-pregnancy BMI, season at enrollment, sum of maternal skinfold at six months post-partum, and lactation practices in the first six months post-partum.

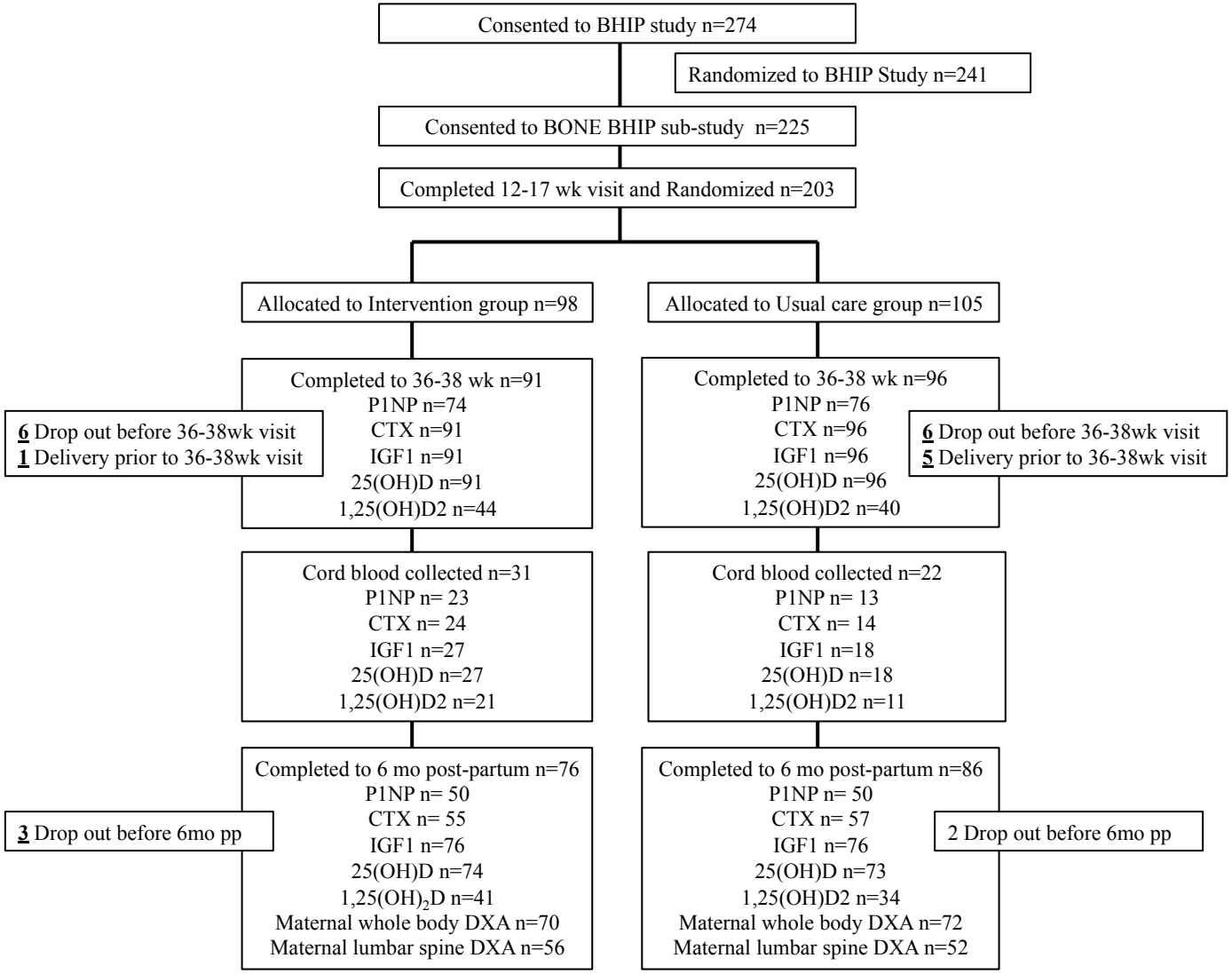

Figure 1: Flow chart

CHAPTER 6

INTEGRATIVE DISCUSSION

The research comprising this thesis enriches current knowledge of the dynamics of perinatal maternal bone metabolism, the contributors to maternal vitamin D status in pregnancy, and provides a rigorous global assessment of co-variables that impact maternal bone health in pregnancy and the post-partum period. A diagrammatic synthesis of the findings is presented in Figure 1. The knowledge gained from the Bone-BHIP RCT design using a whole food diet in combination with exercise, and the longitudinal prospective assessment of maternal and fetal outcomes, advocates for a practical and feasible approach to optimize bone health during pregnancy for women in the community.

The contribution of this thesis to current literature and considerations for future research are presented in the subsequent sections.

6.1. Perinatal maternal bone metabolism: contribution to the literature

The Bone-BHIP study addressed current knowledge gaps by providing a prospective, longitudinal assessment of perinatal bone metabolism changes, as measured by serum biomarkers and BMD assessment in a cohort powered to detect statistical changes. Our study is the first one to measure a panel of clinically relevant serum bone biomarkers including PINP, CTX, and IGF-1, as well as 25(OH)D and $1,25(OH)_2D$, important vitamin D metabolites that are physiologically regulated during pregnancy (Chapters 4 and 5). A major finding of our study is that the high dairy food diet (intervention) compared to self-selected diets (control) supported lower bone resorption and a greater

rise in bone formation in the intervention compared to the control group (**Figure 1**). Importantly, analyses of the primary biomarker and BMD outcomes were adjusted for key confounding variables including season at enrollment, quantitatively measured changes in maternal body adiposity throughout pregnancy, and lactation practices during the first six months of post-partum, thus addressing common limitations identified in the current literature (Chapter 1).

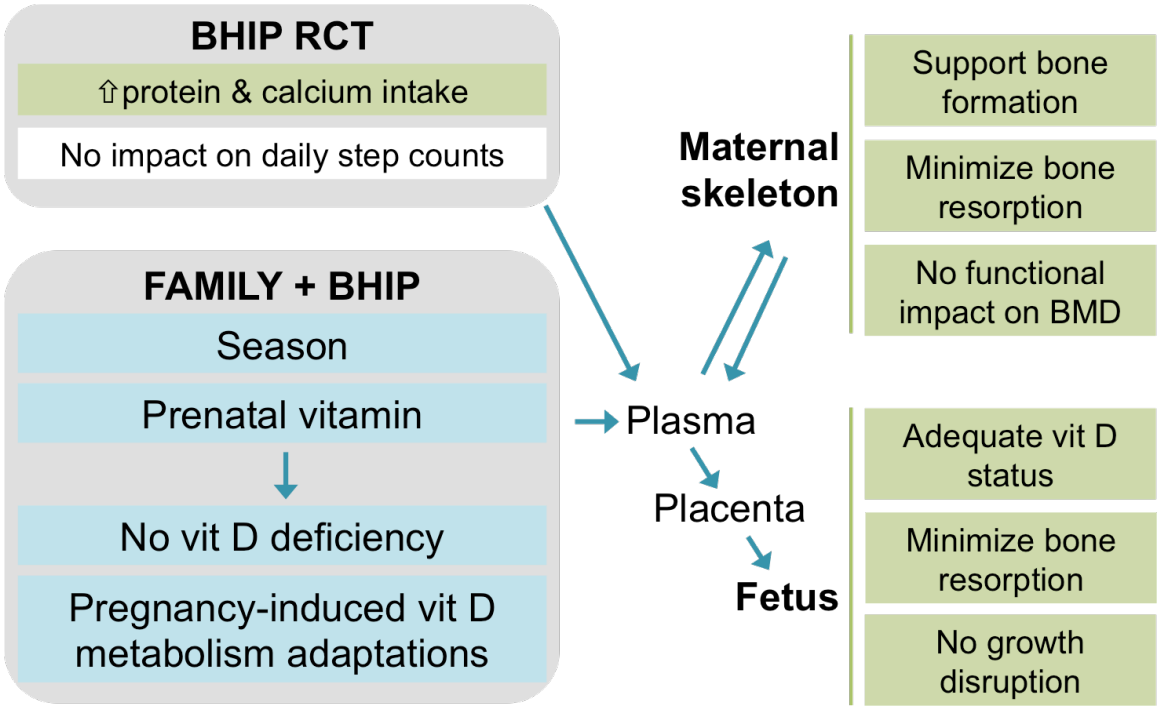

Figure 1: Overall impact of the BHIP intervention on maternal and fetal bone metabolism – a summary of the reported research.

A major finding of our study is that vitamin D status was likely not a contributing factor to the bone metabolism changes observed in pregnancy and the post-partum period, as no clinical vitamin D deficiency occurred in the Bone-BHIP cohort and 94-97% women had serum 25(OH)D > 50 nmol/L and 63-86% > 75 nmol/L in early and late pregnancy. This observation is important as it challenges the widespread information conveyed in the literature (1–3) and media (4) that promotes vitamin D supplementation during pregnancy, and which categorically states that vitamin D deficiency among pregnant women is widespread in North America and indeed globally. Such claims about vitamin D deficiency in pregnancy might be misleading for some pregnant women and the rationale behind the use of high dose supplements (up to 10,000 IU/day) of vitamin D that we observed in the Bone-BHIP study (Chapter 3). Further, there is no apparent biochemical or clinical benefit to consuming a vitamin D beyond the recommended RDA or supplementation with more than the recommended prenatal multivitamin. Our study validates the current EAR/RDA for vitamin D in pregnancy and provides evidence that the majority of women in the two cohorts studied follow the recommended practice of prenatal multivitamin supplementation in pregnancy which ensures an intake of 400 IU/day or more of vitamin D.

In addition to measuring key recommended serum bone formation (PINP) and resorption (CTX) markers, a strength of our study was to have concurrently measured serum IGF-1. Our observations of a rise in maternal serum IGF-1 concentrations at the end of pregnancy, followed by a significant decline in the post-partum, align with previous

reports (5). As this pattern occurred in both treatment groups of the Bone-BHIP study, it cannot be attributed to dairy intake specifically. In pregnancy, IGF-1 is produced primarily by the mother under the regulation of growth hormone until the ≈20th week of gestation, after which IGF-1 production is modulated by the human placental growth hormone rather than the growth hormone, leading to the maximal peak concentration in maternal circulation in the third trimester (reviewed by (6)). A rise in serum IGF-1 concentrations is hypothesized to be a key signal for osteoblastic stimulation, subsequently leading to bone formation, while the role of IGF-1 on osteoclasts is less clear (reviewed by (8)). In the Bone-BHIP study, serum maternal concentrations of IGF-1 in late pregnancy were significantly positively associated with maternal serum concentrations of both bone formation marker PINP and bone resorption marker CTX. These results suggest a role of IGF-1 in both formation and resorption during pregnancy. Having measured only two time points, it is difficult to speculate if IGF-1 is the trigger for any of the changes observed in the serum concentrations of bone biomarkers, but our overall results corroborate the pattern seen in other pregnancy cohorts (5,9), and support a role for IGF-1 in the regulation of maternal bone turnover during pregnancy. Our results contribute to the current knowledge, as we are one of the few studies that reported on the combination of serum bone biomarkers PINP, CTX, and IGF-1, as well as vitamin D metabolites, in a cohort prospectively followed from early pregnancy to the post-partum period.

The relationship between maternal and cord serum bone biomarkers is another knowledge gap that our study addressed. Firstly, this study contributes normative data on bone biomarkers in cord blood from healthy infants born at term, since to our knowledge, measurement of cord serum CTX and PINP together have never been completed (**Figure 1**). Further, our data contribute to the growing body of evidence suggesting that maternal metabolic status influences fetal metabolism *in utero*, which is suggested to impact on the programming of optimal bone health in offspring (reviewed by (10)). With the exception of PINP, all cord blood concentrations of bone biomarkers were significantly correlated with maternal concentrations in late pregnancy. The lower maternal CTX concentrations in participants from the intervention group were mirrored in cord blood (Chapter 5), suggesting that the bone preservation effect of the Nutrition+Exercise intervention might also have benefited the fetus in support of its fetal skeletal development.

The bone formation marker PINP was the only marker with a cord serum concentration higher than maternal circulating concentrations at the end of pregnancy. Such results might be explained by the important bone formation characterizing skeletal bone accretion (reviewed by (11)) that happens irrespective of the mother's own bone turnover. It is also hypothesized that cord serum PINP concentrations reflect fetal somatic growth, and accordingly cord concentrations are higher than in adult PINP circulating concentrations, which is directly reflective of bone turnover (reviewed in (12)). The role of PINP in somatic growth during early life was reported in a cohort of 690 preterm and term infants (13). Lower concentrations of cord PINP were reported with advancing

gestational age, and cord PINP was lower in cord serum of infants born small-for gestational age when compared to those born average-for-gestational age (13). Our data revealed a promising area of research, as future studies including a larger sample size of newborns and cord blood samples could elucidate the relationship between maternal perinatal bone metabolism changes and the programming impact on the fetus and newborn.

Cord serum concentrations of IGF-1 were positively associated with maternal concentrations in early and late pregnancy in participants of both groups in the Bone-BHIP study (Chapter 5). While protein intake during infancy is reported as positively associated with serum IGF-1 (14), our results suggest that the maternal Nutrition+Exercise intervention during pregnancy did not modulate cord serum IGF-1 concentrations. Indeed, higher maternal protein intake in the intervention compared to control group was not associated with higher cord serum IGF-1 concentrations. Our results align with recent evidence indicating that IGF-1 does not mediate the relationship observed between maternal protein intake, and offspring growth and body composition (15–17). The role of maternal protein intake in programming neonatal body composition is reported in observational cohorts where higher protein intake was associated with lower abdominal adiposity (18), and with lower birth- and length-for-gestational-age (16). Future studies should assess the programming potential of maternal protein intake on offspring bone health and body composition.

We completed our extensive assessment of perinatal maternal metabolic changes by measuring maternal bone mineral density (BMD) assessed by DXA, a clinically relevant measure of osteoporosis later in life. Whole body BMD z-scores, as well as lumbar spine BMD z-scores of participants in both groups, were within the expected range for a sex and age matched reference population (19), which reflects their overall adequate health and dietary intake, and adequate vitamin D status (Chapter 5). The absence of a BMD baseline measure is a limitation to our study, as DXA scanning could not be performed during pregnancy for safety reasons. As a result, we cannot assess a potential impact of the intervention on a change in BMD throughout pregnancy and the post-partum period. Addressing a current gap in knowledge, our analysis was adjusted for multiple variables reported to impact bone metabolism both in pregnancy such as season of enrollment and body adiposity (20,21), and in addition the lactation practices in the post-partum period (22). To our knowledge, this is the first report of such a combination of co-variables being measured within a pregnancy cohort and adjusted for in the analysis.

In summary, the Bone-BHIP study contributes to the current literature by being the first study to provide a high quality global assessment of bone metabolism performed from early pregnancy to six months post-partum, including a detailed evaluation of maternal dietary habits, measures of body adiposity, and description of lactation practices. No Canadian population-based normative data exist as pregnant women have not been reported to be sampled in the nutrition and health surveys in Canada to date. Our results provide data for healthy Canadian women, at least those of European descent living in

Southern Ontario, that can be interpreted as normative data in pregnancy and in the post-partum period (Chapter 5). Overall, results from the Bone-BHIP study improve our knowledge of the perinatal maternal bone metabolism changes, and how it relates to fetal adaptations in healthy pregnancy.

6.2. Maternal vitamin D status and contributing factors: contribution to the literature

Our longitudinal Bone-BHIP study contributes new knowledge through a detailed analysis of vitamin D metabolism in pregnancy (Chapter 4), and a historical comparative analysis with the FAMILY study completed 10 years ago (Chapter 3). Novel insights were gained into the dynamics of vitamin D metabolism that occur across pregnancy, the factors associated with status, specifics about dietary sources and a perspective on the importance of geography and demographics on evaluating the need for vitamin D supplementation at the level of clinical and public health practice. Taken together such information provides normative data measured by gold standard methodologies, for both pregnancy and the post-partum period in a large number of participants.

Since maternal vitamin D deficiency was rare in the FAMILY cohort and non-existent in the Bone-BHIP cohort of women studied at various trimesters during pregnancy and in the post-partum period, our results contradict the widely held conception by the general public and health care practitioners that vitamin D deficiency is prevalent (1,4,23,24), and therefore pregnant women should take a high dose vitamin D supplement. Based on the

Bone-BHIP and FAMILY birth cohorts studied 10 years apart (Chapter 3), it is clear that vitamin D deficiency is not a concern in pregnant women of European ancestry in Southern Ontario over the last decade or more. What is important to note is that participants in the Bone-BHIP study consumed more vitamin D than the participants of the FAMILY study, and a larger percentage of the Bone-BHIP cohort met the EAR as hypothesized. Despite the lower intake of vitamin D, the FAMILY participants had similar vitamin D status to those in the Bone-BHIP study.

This thesis provides new insights into the factors associated with maternal vitamin D status in pregnancy. Summer season was a significant factor influencing vitamin D status in both cohorts, and thus refutes the idea that in Canada, sunshine exposure plays a limited role to maintain adequate vitamin D status (25), which has important repercussions for general public health recommendations for pregnant women. Indeed, season, as a proxy of sunshine exposure, in combination with adequate vitamin D intake from food and a modest amount as a supplement, is sufficient for maintaining adequate serum 25(OH)D concentrations in most women of European ancestry throughout pregnancy (Chapter 4). Contrary to our hypothesis, the provision of vitamin D fortified dairy products in the Bone-BHIP intervention did not significantly increase intake of vitamin D compared to the control group, with the intake remaining adequate and stable throughout pregnancy for all participants (Chapter 5). Among the low-fat dairy products provided to participants in the intervention group (e.g. milk, Greek yogurt or cottage cheese), only milk is under mandatory vitamin D fortification in Canada (100 IU vitamin

D per 250ml (26)), while the other products are under voluntary fortification at the discretion of the company (26). Milk consumption rose throughout pregnancy in the intervention group, as did the amount of vitamin D from milk from early to late pregnancy. Yet, overall vitamin D intake (84 - 96% > EAR) of participants did not significantly change due to 75% of intake being derived from supplements, which remained stable throughout pregnancy (Chapter 4). Prenatal multivitamins were the predominant source of supplemental vitamin D, and were a factor associated with maternal serum 25(OH)D concentrations throughout pregnancy (Chapter 4). Most often, prenatal supplements, which are routinely recommended for women of child-bearing age as part of Health Canada recommendations (27), provide 400 to 600 IU/d if taken daily, which ensure women meet the EAR (28). Measurement of the D2 and D3 isomers of serum vitamin D metabolites, which is only possible using LC-MS/MS, revealed low amounts of vitamin D2 contributed through food or supplements. This was anticipated as most fortification of vitamin D in Canada is with D3 (in contrast to D2 used predominantly in the USA). However, with the rising availability of vegetarian and vegan vitamin D supplements (likely containing D2) on the market, measurement of the D2 isomer will be essential. Taken together, our results support the importance of the public health recommendation to take a daily prenatal supplement containing vitamin D during pregnancy (29) (Chapters 3 and 4), but not the use of single high dose vitamin D supplements in addition to the prenatal supplement. As mentioned previously, the role of season and thereby sunshine exposure is not to be disregarded in Canada as a contributor to adequate vitamin D status in pregnant women of European descent.

Our results of the maternal factors associated with vitamin D status align with those reported in the MAVIDOS RCT including 829 pregnant women in the United Kingdom (30), where season and adequate vitamin D supplementation were also important factors associated with sufficient maternal serum 25(OH)D concentrations. In contrast to the MAVIDOS RCT (30), where gestational weight gain as a proxy of maternal body fat was a predictor of vitamin D status, our results did not support maternal body fat measured by the sum of skinfold thickness at four sites as being a significant contributing factor to maternal serum 25(OH)D concentrations. This can be explained by our measure being a more quantitative measure of fat mass than total body weight or gestational weight gain, specifically during pregnancy. Accordingly, our data provide evidence for refinement of public health recommendations in Southern Ontario, where pregnant women of European descent should focus on adequate and safe sunshine exposure, and taking a daily prenatal multivitamin containing vitamin D to ensure an adequate vitamin D status throughout pregnancy.

Our Bone-BHIP study is unique in that we are the first Canadian cohort to measure serum 25(OH)D and 1,25$(OH)_2$D by LC-MS/MS concentrations prospectively throughout pregnancy and the post-partum period. To our knowledge, only one other study, conducted in Brazil, measured vitamin D both metabolites by LC-MS/MS throughout pregnancy (31), but this represents a population who is different in ethnicity and skin tone, factors known to be important in vitamin D metabolism (32). As hypothesized,

vitamin D status remained stable throughout pregnancy and the post-partum period, while serum $1,25(OH)_2D$ concentrations significantly increased. Only a weak correlation was observed between maternal serum 25(OH)D and $1,25(OH)_2D$ in late pregnancy. This observation refutes the hypothesis put forward by Hollis *et al.* (33) that a serum 25(OH)D concentration > 100 nmol/L is required to maximize its conversion into the active form $1,25(OH)_2D$ in pregnancy. We propose that evidence to date does not support additional clinical benefits of serum 25(OH)D concentrations above 50-75nmol/L. Participants in the Bone-BHIP study had adequate vitamin D status, which supported their bone health as assessed by serum bone biomarkers and BMD by DXA (Chapter 4). Knowing that the immunoassay kits often employed to measure 25(OH)D can yield falsely higher or lower circulating concentrations compared to values obtained by LC-MS/MS (34,35), our data from pregnant women in the FAMILY and Bone-BHIP studies (Chapters 3 and 4) provide valuable data that can be interpreted as normative for Canadian pregnant women of European descent. Such data are scarce, primarily because the Canadian Community Health Survey (CCHS) and Canadian Health Measures Survey (CHMS) have not to date specifically reported data for pregnant women, thus our findings provide valuable insights into key factors that influence bone health status for Canadian women in the periconceptual period.

Adequate maternal vitamin D status in late pregnancy, such as observed for the majority of women in the Bone-BHIP study, has potential benefits for fetal bone health as it is the third trimester where fetal bone accrual is maximal (reviewed by (11)). Fetal

concentrations of 25(OH)D rely on maternal stores (reviewed by (36)), and as expected, cord serum 25(OH)D concentrations were significantly associated with maternal serum concentrations in the total group (Chapter 4). Beyond cord blood status, the association of maternal vitamin D status in pregnancy and anthropometric measures of the offspring has been explored in a 2019 systematic review and meta-analysis based on 54 observational studies (37). Vitamin D deficient mothers (25(OH)D < 30 nmol/L) gave birth to newborns with lower birth weight and head circumference compared to mothers with serum 25(OH)D > 30 nmol/L. However, no significant differences in weight, length and head circumference at birth were observed between newborns born from vitamin D insufficient mothers (25(OH)D 30-50 nmol/L) and vitamin D adequate mothers (25(OH)D > 50 nmol/L). High heterogeneity and the small numbers of studies also limit the interpretation of the results from this recent systematic review and meta-analysis. The Bone-BHIP study lays the foundation for future steps assessing the determinants of offspring bone health outcomes, as these are often not reported in studies and systematic reviews (37).

In addition to concerns about vitamin D deficiency, emerging evidence indicates that excessive circulating 25(OH)D concentrations (> 125 nmol/L as per the IOM (28)) in pregnancy may be detrimental to the infant's growth. In a recent RCT of 798 mothers from Finland, pregnant women with serum 25(OH)D > 125 nmol/L had infants that were smaller at six months of age compared to infants of women with maternal status < 125 nmol/L (38). Although not many women had excessive vitamin D status in the Bone-

BHIP study, the use of single dose vitamin D supplement in pregnancy is concerning. Future studies need to characterize the risks and clinical benefits of excessive intake, and excessive vitamin D status on maternal, fetal and infant outcomes.

Future steps in the Bone-BHIP study include evaluation of the impact of the maternal intervention on birth outcomes, and infant bone health outcomes at six months of age. Based on current knowledge, adverse neonatal outcomes are most likely to happen when mothers are clinically vitamin D deficient, and especially when combined with low calcium intake (39). No convincing evidence exists to date to support any clinical benefit for offspring of a maternal vitamin D status higher than the adequate threshold of 50 nmol/L set by the IOM (28), such as observed in participants from the Bone-BHIP study. That was highlighted through the MAVIDOS RCT including 665 infants (40) born from mothers randomized to receive vitamin D supplementation or a placebo throughout pregnancy. No difference in whole body bone mineral content was observed between groups, suggesting that vitamin D might not be the sole determinant of offspring bone mass. Within the Bone-BHIP cohort, we can assess the impact of the maternal intervention on infant BMD at six months of age as measured by DXA (Chapter 2). The Nutrition+Exercise intervention provided higher maternal protein and calcium intake to women who had adequate vitamin D status, and it might positively impact offspring bone health in a similar way that it supported maternal bone health in pregnancy. The Bone-BHIP study lays the foundation for future steps assessing determinants of offspring bone health outcomes.

Other future directions in the Bone-BHIP study include exploring SNPs of genes involved in the vitamin D pathway and how different genotypes might impact maternal vitamin D status, and ultimately modulate maternal and offspring bone health. For example, a meta-analysis revealed single nucleotide polymorphisms (SNPs) in the vitamin D receptor (VDR) gene may alter vitamin D metabolism and impact fetal development and pregnancy outcomes (41). Recent evidence identified the maternal vitamin D receptor gene as an important genetic predictor of a higher concentration of vitamin D during pregnancy, but with potential negative impact on birth outcomes such as lower birth weight and early delivery (42). As it is often the case, no bone health outcomes were reported in that study (42). Other candidate genes of interest include the CYP2R1 genes coding for an enzyme involved in the vitamin D pathway, ultimately modulating serum 25(OH)D concentrations (43). As previously mentioned, the impact of maternal vitamin D status on offspring skeletal development is still unclear and under debate, and the role of genetics need to be studied to better understand these relationships. The data collected as part of Bone-BHIP study will help address these knowledge gaps.

6.3. RCT using whole food and exercise during pregnancy: contribution to the literature

The Bone-BHIP study is the first RCT to measure the impact of a lifestyle intervention centered on whole food dairy products and exercise on maternal bone health in the periconceptual period (**Figure 1**). The Nutrition and Exercise intervention tested in the Bone-BHIP RCT protected maternal bone status during pregnancy by minimizing bone resorption and maintaining bone formation and growth (Chapter 5). Overall, the intervention contributed to supporting maternal bone homeostasis during pregnancy.

The novelty of the design of the Bone-BHIP study resides in employing dairy foods rather than a single nutrient supplementation such as calcium (44,45) or vitamin D (46,47) as done in most studies to date. Indeed, the protective effect of the intervention on maternal bone health may be attributed to the intervention providing dairy products, which are well known to support bone health (48). Dairy products provide essential vitamins and minerals such as calcium and vitamin D (if fortified), as well as high-quality proteins that might be difficult to obtain with other foods (reviewed by (48)). For example, exclusion of dairy products from the diet caused aberrations in bone metabolism as measured by serum bone biomarkers in an adult vegan population (49), suggesting a unique biological role of dairy foods in supporting bone health. Data from the Bone-BHIP study are consistent with existing literature showing the benefits of dairy products on overall bone health. Given that bone metabolism is disrupted in the perinatal period,

our data promotes whole dairy food products as an accessible and affordable solution for women to maintain bone health during pregnancy.

Provision of dairy food led to significantly higher dietary protein intakes over the second and third trimesters of pregnancy and likely contributed to the effectiveness of the intervention. Although current recommended protein and essential amino acid requirements are higher in the pregnant than non-pregnant state (50), recent studies using the indicator amino acid oxidation method have challenged these Dietary Reference Intakes (DRI). The recent study suggested that protein requirements in pregnancy should be about 1.2 g/kg body weight/day in early pregnancy (~16 wk gestation) and 1.5 g/kg body weight/day during late pregnancy (~36 wk gestation), substantially higher amounts than the current DRI of 0.66 g/kg body weight/day (first half of pregnancy) and 0.88 g/kg body weight/day (second half of pregnancy) (51). Although higher than the current recommendations, these suggested intakes still fall within the DRI Acceptable Macronutrient Distribution Range for protein to provide 15-25% of total energy (50). The Bone-BHIP study was designed to provide 25% of each participants calculated energy needs as protein. Measured intakes of the participants in the intervention group yielded average protein intakes of 1.5 g/kg body weight/day in the second trimester (26-28 wk gestation) and 1.3 g/kg body weight/day in the third trimester (36-38 wk gestation), which is more in line with the new suggested amounts (51) than the DRIs (50) (Chapter 5). Proteins are the major components of bone tissue, and an adequate amount of amino acids are needed to support continuous bone remodeling (reviewed by (52)). Our results

support the hypothesis that women could benefit from higher protein intake during pregnancy to maintain their bone health, and potentially support fetal skeletal growth. The Bone-BHIP intervention puts these more recent estimates of protein requirements into context and shows it is feasible to adopt this protein distribution throughout pregnancy, with significant benefits to maternal bone health.

Another explanation for the benefits associated with the Nutrition+Exercise intervention is the significantly higher calcium intake provided by inclusion of more dairy products (Chapter 5). The Bone-BHIP intervention aimed to provide half of the calculated protein intake from dairy products. Typically, this represented four to six servings of dairy products per day (based on Canada's Food Guide 2007 (53), in effect at the time of study inception). This nutrition intervention ensured that participants' calcium intake surpassed the estimated average requirements (EAR) for pregnant women of 800 mg/d (28) in 99% of participants at mid- and end of pregnancy (Chapter 5). In comparison, the control group consumed their habitual intake, which was above the EAR in 88% of participants at mid- and early pregnancy. On average, participants in the intervention group consumed 500 mg/day more calcium than the control group, which is statistically and clinically significant, supporting its role in the observed protection effect on maternal bone of the Nutrition+Exercise intervention at the end of pregnancy. Moreover, this additional calcium intake might have supported fetal growth. Indeed, an estimated 300 to 350 mg of calcium per day is delivered through the placenta from the 35th week gestation onwards (reviewed by (11)). Although the current DRI for calcium intake during pregnancy

assumes compensatory maternal mechanisms, specifically enhanced intestinal absorption mediated by the up-regulation of $1,25(OH)_2D$ to meet the fetal calcium needs, our observations suggest that higher calcium intake is beneficial for the mother's bone health by reducing bone resorption and maintaining bone formation. As we have not measured intestinal calcium absorption, we can only speculate that a calcium intake higher than the current DRI can minimize the body's reliance on the other compensatory mechanism of bone resorption to meet fetal calcium needs. The higher total daily calcium intakes in participants in the intervention group are attributable to higher food calcium intake, as supplement intake was stable. Results from the Bone-BHIP study support the hypothesis that dairy foods provide a unique benefit, partly explained by higher calcium intake, on maternal bone health during pregnancy.

Another strength of the Bone-BHIP study and its contribution to current knowledge was the attention put towards improving compliance and measuring adherence to the two components of the intervention. Adherence to the nutrition component of the intervention was maximized by providing participants with dairy products and recipe ideas during their bi-weekly personalized nutrition counseling session (Chapter 2). This strategy also ensured participants consumed the targeted amount of dairy food. For the exercise component, having participants track their step counts with a pedometer and record their daily counts maximized adherence (Chapter 2). We planned *a priori* to measure adherence to both the diet and exercise interventions (Chapter 2). In general, participants complied with the nutrition component, but not with the exercise component.

Unexpectedly, women in the intervention group did not significantly increase their daily step counts over the pregnancy period (Chapter 5). The exercise component of the intervention focused on steps counts and daily walking. Current recommendations by the Society of Obstetricians and Gynaecologists of Canada, and the Canadian Society for Exercise Physiology (54) encourage women to practice exercise throughout pregnancy for health benefits beyond bone health, and walking is among the recommended physical activity women can undertake. The goal of 10,000 steps is a reasonable estimate of daily activity that would likely confer health benefits to most adults (55), while being safe and accessible to women throughout pregnancy irrespective of their baseline physical activity level (56). Yet, we observed low adherence to this component of the intervention, where participants in the intervention consistently did not reach the target of 10,000 steps/day, but instead counts were similar to the control group (mean daily steps in second trimester = 5992, third trimester = 4937). Assessing other forms of exercise that participants engaged in during pregnancy might decipher subgroups of women who might have benefited from the positive impact of exercise on bone health, notably when associated with a mechanical load (57). The next steps are to assess all forms of self-reported exercise captured by the Bone-BHIP Study exercise questionnaire that participants completed prospectively throughout pregnancy, and corroborate these results with the accelerometry data. This global assessment of their physical activity practices will allow evaluation of the effects of walking, as well as other bouts of exercise with detailed intensity, duration and frequency on maternal bone health in the periconceptual period.

Compliance with the nutrition component appeared greater than with the exercise component, with the caveat that nutrition was predominantly self-reported, while steps counts were objectively measured by accelerometry at each trimester. Indeed, participants verbally reported their food intake during interviews with study nutritionists and completed three-day food logs at each trimester (Chapter 2). The bias in under-reporting dietary intake is documented in adults (58,59), and the reliability limitations of food records are reported in the pregnant population (systematically reviewed in (60)). Yet, energy intakes were similar between groups during pregnancy, suggesting that results can be confidently interpreted even if reporting bias might have occurred. Even with the possibility of underreported food intake, the intervention successfully provided more servings of dairy products, proteins and calcium when compared to baseline. The next step is to employ metabolomics techniques to develop serum metabolomic biomarkers of dairy protein intake, and overcome the limitations associated with self-reported food intake. Metabolomic profiling of participants enrolled in clinical trial is a promising avenue to identify robust markers of dietary composition, and ultimately measure individual adherence to a given intervention (61). In the context of the Bone-BHIP study, a serum biomarker would allow for a true measure of adherence to the intervention, and would strengthen the approach to measure the intervention impact on maternal bone outcomes.

The effect of the Nutrition+Exercise intervention was not sustained in the post-partum period, as observed by similar concentrations of bone biomarkers between groups. This

aligns with what we hypothesized, and is not surprising since participants in the intervention group did not integrate the dietary changes into their lifestyle in the post-partum period (Chapter 5). As a result, participants in the intervention group returned to their baseline dietary intake of total protein and calcium, which seemed to have been the key changes explaining the benefits observed at the end of pregnancy. Although their protein intake from dairy products remained statistically higher than participants in the control group, the difference was not clinically significant (17g versus 14g in the control group). No differences were observed between groups in serum concentrations of bone biomarkers, not surprisingly as these measures reflect short-term changes (62) and respond to dietary changes (63).

No differences were observed between groups on the BMD measured at six months post-partum. In addition to participants going back to their usual dietary intake, another explanation for the lack of impact of the Nutrition+Exercise intervention is the overall low adherence to the exercise component of the intervention during pregnancy (Chapter 5). When exercise is practiced outside, sunshine exposure can support adequate vitamin D status in pregnant women, as reported in a cohort of pregnant women enrolled in an exercise program during pregnancy (64). Additionally, weight-bearing exercise might exert a positive impact on maternal BMD. The positive impact of weight-bearing exercise on BMD in non-pregnant pre-menopausal women was reported by some (57) but not all studies (65). Given the limitation of a one-time DXA measure post-partum we could not adjust for baseline BMD measured due to the study design and enrolment happening in

pregnancy. Ideally, future studies would enroll participants pre-conception and obtain a baseline BMD measure.

The Bone-BHIP study addresses an important knowledge gap by following participants for six months post-partum, thus delineating the effect of pregnancy versus the post-partum period, and how important factors such as lactation practices and maternal adiposity changes might impact perinatal maternal bone metabolism adaptations. This allowed us to carefully adjust our statistical analysis for outcomes at six months post-partum such as duration and intensity of lactation, maternal adiposity and season at enrollment. These covariates have been reported in the literature to impact maternal bone health metabolism (20,21,30), but have never been studied synergistically within a cohort at repeated time points throughout pregnancy and the post-partum.

As alluded to specifically regarding the vitamin D pathway, genetics is emerging as an important modulator of skeletal phenotypes. The future step of the Bone-BHIP study is to investigate the importance of genetics in modulating perinatal changes, and how the intervention might interact with genes to impact bone metabolism outcomes. Observational studies (66,67) and genome-wide association studies (GWAS) have revealed the importance of genetic determinants in various skeletal phenotypes in human, including BMD (reviewed by (68)). Up to 50-80% of the variance in BMD is attributable to genetics (66). Within the Bone-BHIP study, we can investigate the interactions between genes associated with bone health, and high dairy diet supplementation on bone

status in mothers in the periconceptual period. Although we have a modest sample size to perform a gene x intervention interaction analysis, doing so could identify subgroups of women who might have responded better to the Nutrition+Exercise intervention than others, based on SNPs of relevant genes. Identifying 'good' and 'poor' responders to lifestyle interventions is of public health interest within the context of osteoporosis prevention, and the Bone-BHIP study could represent an opportunity to elucidate questions about interaction of genes and environmental factors such as diet and exercise on maternal and offspring bone metabolism.

6.4. Concluding statement

The Nutrition+Exercise intervention in the Bone-BHIP study reduced maternal bone loss during pregnancy compared to the control group. Our RCT is novel as it was based on a lifestyle intervention, using whole dairy foods and a feasible walking program for women throughout pregnancy, and thus represents a realistic intervention to support maternal bone health in a community setting (**Figure 1**).

Our results provide normative biochemical measures of bone metabolism for women of childbearing age as we completed a global assessment of bone biomarkers at three time points throughout pregnancy and the post-partum, including cord blood analysis. Our results provide valuable data for healthy Canadian women throughout pregnancy and the post-partum period, at least those living in South Western Ontario as pregnant women are

not sampled by the national nutrition and health surveys in Canada.

The serum calciotropic hormones were measured using the gold standard analytical method (69), and we selected clinically relevant serum bone biomarkers as per recommendation from an international expert consensus (63). We completed our bone metabolism assessment by performing maternal DXA scanning of two skeletal sites at six months post-partum, the gold standard method to assess BMD (70). Our study is of high quality having prospective repeated measures, detailed assessment of maternal dietary habits including supplement intake, and lactation practices described in the post-partum period. As a result, the Bone-BHIP study addresses many of the knowledge gaps identified (Chapter 1) in the field of maternal bone health in the periconceptual period. Our study is clinically relevant as our comprehensive assessment of the determinants of maternal vitamin D status revealed that contrary to popular belief, the majority of healthy pregnant women of European descent are not vitamin D deficient in Southern Ontario, due to season and prenatal supplement intake. Thus, public health messages can be derived from our data.

Future steps could include studying the gene x intervention interaction in the individual response to the Nutrition+Exercise intervention. Detailing participants exercise profile beyond daily step counts could also provide knowledge of the potential impact of exercise on maternal bone health outcomes. Lastly, measuring offspring bone health could provide insight on the potential programming effect of maternal lifestyle and

maternal vitamin D status on early life skeletal growth.

6.5. References

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