MATERNAL VITAMIN D STATUS DURING PREGNANCYAS A PREDICTOR OF OFFSPRING BONE MASS AT THREE YEARS OF AGE

MATERNAL VITAMIN D STATUS DURING PREGNANCY AS A PREDICTOR OF OFFSPRING BONE MASS AT THREE YEARS OF AGE

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A Thesis
Submitted to the School of Graduate Studies
in Partial Fulfillment of the Requirements
for the Degree
Master of Science

McMaster University
Department of Medical Sciences

MASTER OF SCIENCE (2011) McMaster University

(Medical Sciences, Metabolism and Nutrition) Hamilton, Ontario

TITLE: Maternal vitamin D status during pregnancy as a predictor of offspring bone mass at three years of age

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NUMBER OF PAGES: 160

ABSTRACT

Emerging evidence indicates that *in utero* exposure to vitamin D metabolites may influence fetal and neonatal bone development due to their role in placental calcium transport and endochondral ossification of fetal bone. Bone accretion in prenatal and early postnatal life may impact peak bone mass achieved in early adolescence; and peak bone mass is a well-established predictor of osteoporosis risk in later life. **Hypothesis**: We hypothesized that offspring of mothers with higher serum vitamin D status (assessed by circulating 25-hydroxyvitamin D (25OHD)) during pregnancy will have higher whole body BMC z-score and bone size at 3 years of age, after adjustment for confounders. **Methods**: In a prospective, longitudinal study, 372 mothers with singleton birth were recruited during pregnancy, and maternal blood samples were obtained during the third trimester. Child bone outcome measures at 3 years of age included: whole body BMC, femoral and humeral lengths by DXA. We controlled for other relevant factors such as maternal nutrition, pre-pregnancy BMI, physical activity during pregnancy, maternal BMD, as well as the child's nutrition at 6 months and 3 years, and the child's physical activity. **Results & Discussion**: Maternal vitamin D status during pregnancy did not predict whole body BMC z-score of the child at 3 years of age. Over 92% of Canadian women in our sample were vitamin D sufficient with mean intakes of 435 IU/day from food and supplements and mean serum 25OHD of 111.2 nM. Further, data indicate a potential negative effect on offspring bone size at maternal serum 25OHD concentrations that exceed the upper limit suggested by the most recent DRI report (>125 nM), at which adverse health effects may occur. Our findings may differ from previous studies in the United Kingdom, India and Finland that found a positive relationship between maternal vitamin D status and child bone outcomes due to the high frequency of our mothers that had optimal vitamin D status. In addition, we adjusted for most of the key covariates that were not adjusted for in previous studies, which may contribute to the different findings compared to previous investigations.

ACKNOWLEDGEMENTS

It is my pleasure to thank the many people who made this project possible. I would first like to thank Dr. Stephanie Atkinson, my supervisor and encourager as I completed this project. I am thankful for having the opportunity to be a part of her research, and have learned from her enthusiasm for nutrition research. I am especially grateful for her dedication to helping me complete this project on time, and the many hours she poured into editing my thesis. I would also like to thank my supervisory committee members, Dr. Sarah McDonald and Dr. Christoph Fusch, for their regular constructive feedback that kept my project on track, and encouragement to think critically and carefully as a scientist.

I would like to thank the FAMILY study team as well, including Nora Abdallah, Aisha VanderLoo, Natalie Jakymyshyn, Sharon Kotlan, for making the FAMILY study visits run so consistently and smoothly, and for their friendship and positive attitudes no matter how early the visits were on a dreary winter days, or how late on Christmas Eve!

I am also greatly indebted to members of the Fusch lab, including Dr. Gerhard Fusch and Arum Choi, who kindly offered their expertise, evenings and weekends to help me complete the vitamin D analyses. Their attention to detail and work ethic are a great example that I will remember. I would also like to thank Susan Docherty-Skippen for her invaluable support in completing the vitamin D analyses, as well as her mentorship in training me to do clinical visits, and for taking genuine interest in my research project and willingness to sharing her research experiences with me.

A portion of my thesis project would have also not been possible without the aid of Dr. Troy Farncombe and Dr. Colin Webber in developing the MATLAB software to measure bone lengths, turning my simple idea into a reality. I am also thankful for their advice and guidance to my project.

I am also very grateful to the work-study students, Emily Roth, Jessica Graham, and Irena Doubelt, who have weathered the years with me by completing tons of diet record analyses and helping me put together a written protocol. I would also like to thank Kim Chau, for being a dependable friend and supporter, as well as for the laughter we've shared while in this lab.

I would like to thank Rachel Chu and Dr. Thabane for their statistical support, and for being excellent teachers to me throughout the process of the statistical analyses I did for my project.

I am also very grateful to the participants of my study, who made it to our clinical visits in rain or snow, and at times traveling far across Ontario.

My heartfelt thanks also go out to Mom and Dad, for their commitment to raising me with the best opportunities they could offer, as well as to Jon, for his unyielding and unconditional support at all times. None of this would have been possible without your love and encouragement – so thank you!!

LIST OF ABBREVIATIONS

1,25OHD – 1,25-hydroxyvitamin D, or calcitriol

25OHD – 25-hydroxyvitamin D

aBMD – Areal bone mineral density

BA – Bone area

BMC – Bone mineral content

BMD – Bone mineral density

BMI – Body mass index

CDC – Centre for Disease Control and Prevention

CHMS – Canadian Health Measures Survey

CSA – cross sectional area

CT – computed tomography

CV – coefficient of variation

DRI – dietary reference intake

DXA - dual-energy x-ray absorptiometry

FAMILY study - **F**amily **A**therosclerosis **M**onitoring **I**n Ear**LY** life

FDA – (US) food and drug administration

pQCT – peripheral quantitative computed tomography

FFQ – food frequency questionnaire

GH – growth hormone

HAES – Habitual activity estimation scale

HPLC – high performance liquid chromotography

ICTP – Serum carboxyterminal telopeptide

IGF-1 – insulin growth factor-1

IQR – inter-quartile range

IUGR – intra-uterine growth restriction

ISCD – International Society for Clinical Densitometry

LC-MS/MS – liquid chromatography mass spectrometry

MUMC - McMaster University Medical Center

NIST – National Institute for Standards and Technology

nM – nanomols per litre

PBM – peak bone mass

PCA – principal component analysis

PHRI – Population Health Research Institute

PTH – parathyroid hormone

RDA – recommended dietary allowance

RIA – radioimmunoassay

RXR – retinoid –X-receptor

SD – standard deviation

SGA – small for gestational age

SOP – standard of practice

SJH – St. Joseph's Hospital

SRM972 – standard reference material 972

TQD – tandem quadrulpole mass detector

TXR – thyroid hormone receptor

UPLC – ultra performance liquid chromatography

USDA – United States Department of Agriculture

vBMD – Volumetric bone mineral density

VDR – vitamin D receptor

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GENERAL INTRODUCTION

Chapter One

GENERAL INTRODUCTION

1.1 Rationale, objectives and hypothesis

Rationale

The past decade has seen a marked shift in our approach to understanding osteoporosis, with a growing realization that early life events play a key role in bone health. Studies have shown that early life bone accretion influences peak bone mass achieved in adolescence, and peak bone mass in turn, is a well-established predictor of fracture risk. Thus, while traditionally labelled as a geriatric disorder, osteoporosis is increasingly recognized as a disease with its roots in early life events and exposures[1,2]. In particular, there has been a growing interest in maternal nutrition during pregnancy as a predictor of offspring bone health. Prospective cohort studies have found that maternal nutrition predicts bone mass of offspring up to 16 years of age [3-5]. A nutrient key to skeletal health is vitamin D. The contribution of maternal vitamin D status during pregnancy to *in utero* bone development remains unclear. This is reflected in the lack of consensus in vitamin D recommendations for pregnant women: the current RDA for all women of child-bearing age is 600 IU/day[6], while the Canadian Pediatric Society recommends up to 2000 IU/day during pregnancy[7]. A number of maternal health outcomes drive the establishment of vitamin D requirements during pregnancy; however, in relation to optimizing offspring bone health, evidence has been sparse [8-10].

Across the globe, vitamin D deficiency is common in pregnant women[11], raising concerns of long-term implications for fetal bone health. For example, birth month, reflective of UV-B exposure in early life, is a predictor of adult BMD[12]. As well, maternal veiling during pregnancy,

which reduces the mother's UV-B exposure and dermal vitamin D synthesis, has a negative effect on the bone mass of male offspring [13]. On the other hand, higher maternal UV-B exposure during the third trimester is linked with higher bone mass in the offspring at 9.9 years of age [14], as is a higher pregnancy serum vitamin D status [15]. Nevertheless, a common criticism is that these cohort studies do not sufficiently account for confounders, such as lifestyle factors passed on from mother to child. In addition, higher maternal vitamin D status may simply be associated with a generally healthier profile, such as women who are physically active, less likely to be overweight, non-smokers, and who consume a healthy diet. Hence, this study aims to elucidate the contributions of other maternal and early postnatal factors in relation to vitamin D, to determine whether maternal vitamin D status is a significant determinant of bone health in the offspring. We also include a measure of the mother's bone mass to assess how her genetic contribution to the child's bone mass may interact with the aforementioned factors.

Another point of uncertainty in existing literature is whether maternal vitamin D status may also program a larger body size *in utero*[14,16-18] . This could also explain any effect on bone mass, since a larger body size potentially means having a larger skeleton with longer and/or thicker bones that contain higher mineral content. Of note, greater bone width renders the skeleton less fracture prone[19]. Since it is not possible to assess differences in bone size using direct outputs from DXA, for this study we have developed other means of measuring bone size and shape, using a combination of newly developed computer software and estimations of bone size via statistical tools. The results of this study will shed light on the potential lasting influence of maternal vitamin D status during pregnancy on offspring skeletal development.

Primary objective

To investigate whether higher maternal vitamin D status during pregnancy predicts higher offspring BMC z-score at three years of age after adjustment for confounders, which include:

A. Maternal
    1. Bone mass
    2. Smoking during pregnancy
    3. Vitamin D and calcium supplement use during pregnancy
    4. Nutrition (calcium, vitamin D, protein) during pregnancy
    5. Pre-pregnancy BMI
    6. Physical activity during pregnancy

B. Child
    1. Season of birth
    2. Vitamin D supplements at 6 months of age
    3. Nutrition (calcium, vitamin D, protein)
    4. Vitamin D and calcium supplements at 3 years of age
    5. Physical activity at 3 years of age

Secondary objective

To elucidate the effect of maternal vitamin D status during pregnancy on offspring bone size based on two parameters:

A. Whole body bone area

B. Long bone lengths (average femoral length and average humeral length)

Hypothesis

The over-arching hypothesis is that after adjustments for relevant modifiable confounders, maternal vitamin D status during pregnancy will remain a significant predictor of BMC z-score in the offspring, as well as bone size.

1.2 Childhood skeletal development

Bone accrual occurs at a rapid pace throughout childhood and adolescence, making these formative years of lifetime skeletal health. Approximately 30-40% of bone mass is gained during the peripubertal years in adolescence, and 80-90% of bone mass is accrued by age 20, with variations by skeletal site [20]. Consolidation of bone continues until late adolescence, when peak bone mass is achieved 6-10 years later [1,21]. Thus, the years of childhood and adolescence represent both a window of opportunity for optimizing skeletal health, as well as a time of vulnerability. Deficiencies in growth during this time may translate into suboptimal adult peak bone mass, which is a predictor of bone fragility and fracture risk later in life [1,19,22]

Indeed, emerging research has pointed to life *in utero* as an important determinant of skeletal health. Large volumes of calcium are transferred to the fetus during pregnancy, with approximately 30 g accumulated in the fetus for bone mineralization by the third trimester of pregnancy[23]. Slower rate of long bone growth between 19 and 34 weeks gestation has been linked to lower bone mineral content (BMC) and areal bone mineral density (aBMD) at 4 years of age [24]. A longitudinal study that followed children through puberty found strong correlations between pre- and post-puberty BMC at several skeletal sites at risk of fracture in later life[25]. A comparison of their BMC z-scores indicated that most children ranking below their peers remained so post-puberty. Hence, growth deficits *in utero* may persist late into adolescence, the years during which the majority of bone mass is acquired. As well, the rise of pediatric fractures and the plethora of pediatric illnesses and drugs that have long-term skeletal effects also emphasize the importance of optimizing skeletal health early on[21].Thus, a focus on optimizing prenatal factors for bone growth provide the foundation for lifetime bone health, and therefore may be an effective prophylactic strategy against fractures.

1.2.1 Measurement of bone by DXA

BMC is the total amount of mineral content present in the skeleton, and BMD is an expression of the mineral content per volumetric unit (cm^3) of bone. The two measures are related as follows:

$$\text{Volumetric BMD } (g/cm^3) = \text{BMC } (g)/\text{volume of bone } (cm^3)$$

This is also known as the true BMD, or the volumetric BMD. In contrast, the measure obtained in DXA is a two-dimensional measure, also known as areal BMD. This value is obtained by dividing BMC by the total area of bone (in cm^2), as follows:

$$\text{Areal BMD from DXA } (g/cm^2) = \text{BMC } (g)/\text{area of bone } (cm^2)$$

Since DXA is the most widely and commonly used for assessing whole body bone in the literature, we will refer to areal BMD as simply BMD from here on, and distinguish it from volumetric BMD measured by CT when necessary. BMD is useful for predicting fractures in adults[26], but there is some disagreement over the best measure of bone, BMC or BMD, for children. BMD by DXA is greatly affected by bone size[27,28]; children with smaller bones appear to have lower BMD, and in repeated longitudinal measures, increase in bone size due can be mistaken for increase in density. Comparisons between DXA and CT results indicate that BMC from DXA are the best parameter to use in children, because it is more sensitive to changes due to growth in the child than areal BMD[27]. Whole body BMC was also predictive of childhood fractures in an eight year prospective study[29], and is the recommended bone measure to report by

the ISCD[30]. Whole body BMC in children can be used interchangeably with the more general term, bone mass.

Recent literature has also raised the importance of excluding head BMC from measures of whole body BMC in children because algorithms for predicting BMC from age are more accurate when the head is excluded[31]. Other reasons for exclusion include that the head does not grow in proportion to rest of body, and that the skull's high density of minerals contributes "noise" to repeated measurements of whole body BMC[31]. Nevertheless, due to the lack of headless reference data for our age group, we were not able to report headless BMC values. Since we performed a single measure at one time point rather than repeated measures, we expected that the impact of skull density on our results would be low.

One last issue to consider is that a measure of the whole body BMC is affected by body size (i.e. height), and also does not give information on the dimensions and size (i.e. thickness) of bones. Lower whole body BMC could be reflective of shorter stature or narrower bones. Thus, when calculating z-scores for BMC, we used a calculator that accounted for height of the child at 3 years of age[32]. Measuring bone size in children poses a unique challenge; some studies have used pQCT to provide a measure of bone thickness in older children[33]. Multiple adjustment methods for estimating bone size in children have also been attempted in the literature; for example, height-adjusted BA has been used as an estimate of periosteal bone growth (related to bone width)[14], and estimations of humeral dimensions from a DXA scan have also been used[19]. In our study, we applied a novel way of calculating an index of bone size for children by combining both a whole body skeletal size measure (whole body BA) with specific dimensions (femoral and humeral lengths) to derive a measure reflective of overall bone size, using both

information directly available from a whole body DXA scan and newly developed computer software at McMaster University.

1.3 Vitamin D in pregnancy

1.3.1 Vitamin D status

Skin exposure to sunlight (UV-B rays, 290-315 nm) initiates the process leading to vitamin D_3 synthesis. Upon hydroxylation by 25-hydroxylase in the liver, vitamin D_3 is converted into 25-hydroxyvitamin D_3 ($25OHD_3$). This metabolite is further hydroxylated in the kidney by the enzyme 1-alpha-hydroxylase, forming the active hormone 1, 25 di-hydroxyvitamin D (also known as calcitriol)[34,35]. Another form of the prohormone is $25OHD_2$ synthesized from vitamin D_2. Since vitamin D_2 is the form found in vitamin D supplements in the U.S. only, we considered that only $25OHD_3$ was of relevance to our Canadian population, and from hereon will be referred to simply as 25OHD.

In the serum, the half-life of both vitamin D_3 and calcitriol is less than one day, and of little clinical relevance to measure[36]. In contrast, the half-life of the pro-hormone, 25OHD, is 15 days[36]. Hence, a measure serum 25OHD is used to assess an individual's adequacy in vitamin D. Importantly, a measure of serum 25OHD also reflects both dietary and skin sources of the vitamin. To date, this is the most commonly used and accepted method; circulating 25OHD is considered a measure of the individual's "vitamin D status" [6].

During pregnancy, a measure of maternal vitamin D status is also an indicator of the availability of calcitriol to the fetus since neither vitamin D nor calcitriol cross the placenta in appreciable amounts. The production of calcitriol by 1-alpha-hydroxylase in the placenta and fetal skeleton is contingent up on the availability of 25OHD from maternal serum[37,38], which

does cross the placenta[39] (Figure 1). Clinically, it has been observed that the mother and neonate's vitamin D status are closely linked[40-43].

Although measurement of serum 25OHD is the accepted gold standard to assess vitamin D status, there is controversy surrounding the cut-points for serum 25OHD to define deficiency or sufficiency. Some argue that sufficiency should be defined as a serum 25OHD above 75 nM, as there are studies showing that this corresponds to maximal benefit on calcium homeostasis[44-46], and whole body, spine, and hip BMD [46-48]. In contrast, the Dietary Reference Intake (DRI) report published in 2011 by the Food and Nutrition Board of the Institute of Medicine (IOM) states that the benefits associated with vitamin D status above 75 nM are not consistent throughout all of the relevant literature[6]. Thus, a cut-point of 50 nM was defined as a better reflection of sufficiency for bone health outcomes. A level lower than 30 nM is associated with rickets in children and osteomalacia in adults, and hence was accepted as the definition of deficiency. The report also states that among people with vitamin D status between 30 and 50 nM, some, but not all, may potentially be inadequate. A serum concentration greater than 125 nM suggests cause for concern, based on case studies of vitamin D toxicity. In concluding, the DRI report calls for greater consensus in scientific literature regarding cut-points for serum 25OHD[6].

Establishing proper serum cut-point values for pregnant women may be complicated by hemodilution[49] or changes in vitamin D metabolism during pregnancy[50,51]. Thus, it is uncertain whether the same ranges for non-pregnant adults should be applied; further research is needed to clarify this. Serum cut-points for sufficiency in pregnant women may also need to take into consideration offspring health outcomes, and particularly skeletal health. A lack of consensus on

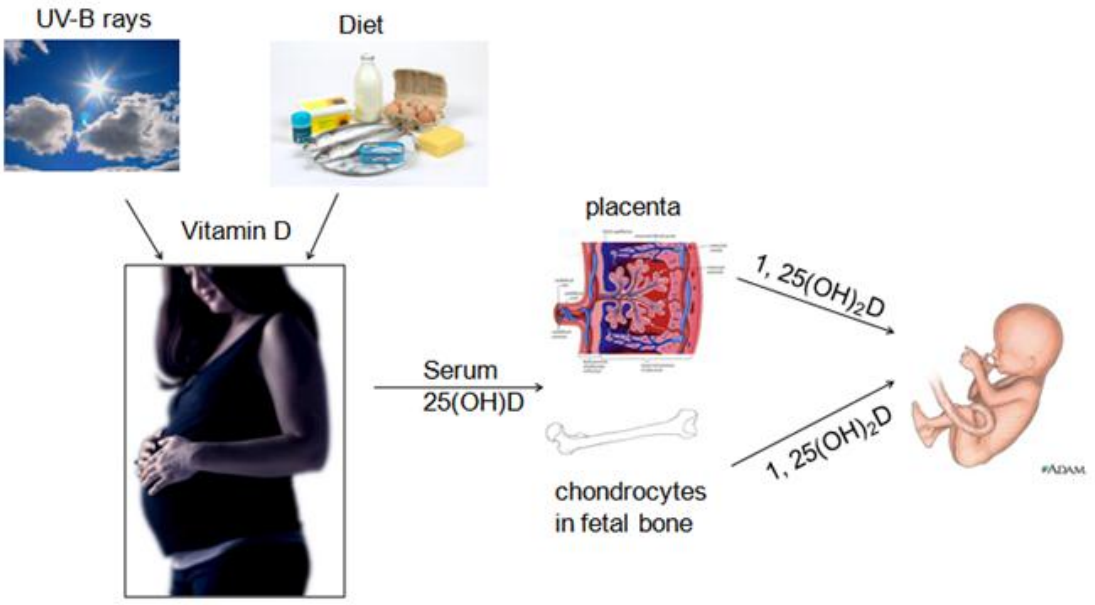

Figure 1 – Maternal serum 25OHD passes the placenta and supplies the fetus with the prohormone needed for conversion to the active hormone, calcitriol. (Figure created by Melody Ng, 2010)

these serum values also makes it difficult to evaluate studies on vitamin D and pregnancy, as each study varies in its choice of defining deficiency; comparisons must be made with caution.

1.3.2 *Vitamin D status in pregnancy*

Countries with long winters and relatively little sun exposure year-round are at risk of vitamin D insufficiency. In combination with recommendations to wear sunscreen to guard against skin cancer, as well as the scarcity of this nutrient in common foods, vitamin D status can dip below optimal levels in many populations. A study of Caucasian women living at a latitude of 54-55 degrees North found that 16% had <25 nM of 25OHD and 75% had sub-optimal vitamin D status (<50 nM/L)[52]. In countries with abundant sunlight, cultural clothing that covers skin entirely from sunlight such as veiling can inhibit sufficient vitamin D synthesis[13,53], increasing rates of deficiency among these women. In addition, skin tone also plays a role in vitamin D synthesis. Melanin inhibits the production of pre-vitamin D from 7-dehydrocholesterol. A study in Pittsburg, Pennsylvania, found that 74-95% of African American pregnant women compared to 46-62% of Caucasian women were insufficient in vitamin D, defined as <37.5 nM[54]. Lifestyle changes in developing countries have resulted in a shift to spending less time outdoors; the resulting lack of sun exposure could be a limiting factor in achieving vitamin D sufficiency.

Survey results from the Canadian Community Health Survey (in 2004) revealed that women of child-bearing age consume limited milk, one of the major sources of vitamin D in Canada[55]. Approximately 65% of women between 17-30 years old and 72% between 31-50 years old consumed less than the recommended servings of dairy[55]; however, there have been no population-based surveys of Canadian pregnant women, leaving a gap in the literature surrounding their dietary choices and vitamin D sufficiency. In other countries such as the UK,

UAE, Iran, India, New Zealand, and the Netherlands, maternal vitamin D deficiency (defined as <25 nM) during pregnancy and a high prevalence of mother and infant pairs deficient at birth is well documented[43]. Our study will provide data on the vitamin D status of Canadian pregnant women, which is not available in the current literature.

1.3.3 Vitamin D recommendations for pregnant women

Currently, Health Canada recommends 600 IU/day of vitamin D for all individuals 1-70 years old, with no increase for pregnant or lactating women; this recommendation is based on the most recent DRI report[6]. These guidelines had recently been tripled from the previous recommendation of 200 IU/day[56], with the aim of promoting bone health across all age groups. Skeletal health was cited as the primary focus of the new DRIs over other outcomes such as cancer, heart disease, autoimmune diseases and diabetes. Nevertheless, the focus on bone alone did not make the task of establishing recommendations during pregnancy any less challenging. Few human studies have focused on skeletal outcomes of offspring later in life (four in total). Thus, in the DRIs, the bulk of evidence comes from studies in mice, rats, sheep and pigs, which suggest the development of the fetal skeleton is mainly independent of maternal vitamin D status. However, considering that nearly every other nutrient in the DRIs recognizes an increased demand during pregnancy, it seems counterintuitive to suggest that vitamin D is the exception without substantial human data.

1.4 Vitamin D's role in pregnancy and offspring skeletal outcomes

1.4.1 Vitamin D and calcium homeostasis during pregnancy

During pregnancy, the mother's need for calcium increases greatly in order to accommodate fetal skeleton mineralization. Nearly 30 grams of calcium is transferred across the

placenta for during the third trimester[23]. To meet this need, the mother's intestinal absorption of calcium doubles[57,58]. Whether this process is vitamin D dependent or not is debated. Most animal models support the latter[59,60]. Vitamin D-deficient pregnant rats and VDR-null mice experience an increase in intestinal calcium absorption during pregnancy, regardless of the absence of calcitriol or its receptor for action[61]. Animal studies suggest that other hormones such as prolactin[62] and placental lactogen may be responsible for stimulating this adaptation in the intestines[63], independent of calcitriol.

Studies comparing intestinal calcium absorption in vitamin D deficient versus vitamin D replete pregnant women are not available[64]. Among randomized controlled trials supplementing pregnant women with vitamin D, only two measured clinical outcomes[65,66]. Both of these studies were discussed in a recent Cochrane review, which concluded that there may be evidence for a reduction of neonatal hypocalcemia when mothers are supplemented with vitamin D[10]. Importantly, cord blood calcium concentration predicted bone mass at 9 years of age in one longitudinal study[15]. Calcitriol is also postulated to play a role in increasing the transcription of placental calcium transporters[67], which include TRPV6[68] and PMCA isoforms 1-4[69]. One study demonstrated that the mRNA expression of one of the transporter isoforms, PMCA3, predicts neonatal skeletal size, independent of several other maternal predictors[69], although the overarching relationship - vitamin D sufficiency *in utero* and offspring skeletal health, is not well understood.

1.4.2 Vitamin D and offspring skeletal outcomes

1.4.2.1 Vitamin D and fetal bone development

Calcitriol directly acts on fetal bone *in utero* by stimulating the proliferation and differentiation of mesenchymal cells into chondrocytes, forming the cartilage framework that

osteoblasts later invade in order to initiate mineralization. This process, called endochondral ossification, begins as early as 5 weeks gestation, and forms regions of the skeleton that are most vulnerable to osteoporotic fracture later in life[70]. Maternal vitamin D deficiency during pregnancy is reported to increase the risk of neonatal vitamin D deficiency[71], which is a risk factor for childhood rickets, a disease marked by "soft" bones due to under-mineralization, leading to a "bow-legged" phenotype[72]. Among Canadian infants diagnosed with rickets, nearly 80% of mothers refrained from consuming milk during pregnancy, and only 12.5% were taking vitamin D supplements[73]. Lower cord 25OHD is associated with altered bone turnover in the neonate indicated by measures of BALP (bone formation marker) and TRACP (bone resorption marker)[74].

1.4.2.2 Offspring bone mass

A study in pregnant women in Lebanon found that reduced maternal UV-B exposure during pregnancy predicted lower bone mass in offspring[13]. Similar findings were reported in the UK; mothers with lower UV-B exposure (assessed by using local meteorological data) in their third trimester give birth to children with lower bone mass at 9.9 years of age[14]. A cohort study also conducted in the UK measuring mothers' serum vitamin D status during pregnancy found that deficient mothers gave birth to children with significantly lower whole body BMC, as well as lower lumbar spine BMC[15].

While it has been demonstrated that correcting maternal vitamin D deficiency during pregnancy has a positive effect on offspring bone, it is uncertain whether increases beyond sufficiency have added benefit. In addition, interpretation of the above studies on bone mass in children as a function of maternal vitamin D status is hampered by a lack of detailed information

on other fetal and childhood exposures that may influence bone accrual. Thus, we have also collected data on key confounding variables to address this gap in knowledge.

1.4.2.3 Offspring bone size

There is evidence that vitamin D may affect skeletal development by influencing overall skeletal size. As early as 1936, Stearns et al. demonstrated that infants fed cod liver oil developed longer bones in comparison to infants without the supplement[75]. Directly supplementing infants with vitamin D during the first year of life also conferred a longer median body length[76]. There may be a similar effect in the fetus. Maternal vitamin D deficiency (serum 25OHD <28 nM) during late pregnancy is linked with reduced knee-heel length at birth, indicative of reduced intrauterine long bone growth[18]. A study by Mahon et al. observed that fetuses of vitamin D deficient mothers had a pattern of femoral growth that resembled childhood rickets, including an increased splaying index and metaphyseal cross sectional area[77] measured by 3D ultrasound at 19 weeks of gestation. There was no effect of maternal vitamin D status on fetal femur length, but a higher rate of femur longitudinal growth *in utero* was observed in mothers with higher vitamin D status. Lower dairy intake and UV-B exposure in the mother during pregnancy however, predicts a shorter fetal femur length in the third trimester[78] and birth length, respectively [14,16]. Indeed, seasonal fluctuations in birth length are widely reported in large prospective studies conducted in Denmark[79], Northern Ireland[80], Australia[16,81]. The seasons at which birth length peaks vary between countries however, and may be partially attributable to varied UV-B exposure among geographical regions. As a whole however, there is difficulty in relating these seasonal fluctuations to vitamin D. Some studies report shorter longitudinal measures in infants born in the winter and autumn, and the third trimester would have taken place during summer and spring, periods of greater UV-B exposure and presumably greater

dermal vitamin D synthesis in the mother during pregnancy[16]. In contrast, other studies have shown that higher maternal UV-B exposure during pregnancy positively correlates with the child's height at 1 and 9 years of age[14,16]. Consistent with this, animal studies show that the effect of vitamin D metabolites on longitudinal growth *in utero* may not be reversible even with sufficient postnatal supplementation with the vitamin[82]. Nevertheless, these studies generally show the effect of correcting deficiency in the mother, while it is uncertain whether additional increases in vitamin D status beyond the sufficient level confer additional increases in bone length.

There is also evidence that early life exposures may influence bone thickness[83]. Of clinical importance, bone thickness is a predictor of fracture risk both in children and later in adult life[19,84]. In a longitudinal study, maternal UV-B exposure during the third trimester positively predicted bone width in children at 9 years of age. However, to our knowledge, no study has yet to demonstrate the direct effect of maternal vitamin D status during pregnancy on offspring bone after accounting for relevant covariates. For this reason, in addition to traditional assessment of bone that focuses mainly on bone mass, we were interested in exploring any differences in bone size in children, using data we had already collected via DXA.

1.5 Other maternal predictors of offspring bone mass

In addition to vitamin D, there a number of other maternal predictors of offspring bone mass, including her genetic contribution to bone mass in the child, as well as her lifestyle factors during pregnancy. These variables are important to account for as potential covariates in elucidating the relationship between maternal vitamin D status during pregnancy and offspring bone mass.

1.5.1 Heritability of bone mass

Observational studies have noted that there is strong correlation between the BMC and BMD of parents and children[5], and the relationship between father and daughter skeletal size and BMC is particularly prominent[85]. Studies comparing BMD among twins (monozygotic and dizygotic), between twins, and non-twin siblings show that approximately three-fourths of the variance in peak bone mass is attributable to heredity[1]. This is a polygenic phenomenon, involving a number of genes, including those related to body size (genes of the growth hormone/IGF axis), reproductive sex steroid receptors, chromosome 11 (q12-13), the VDR gene, and genes encoding skeletally active cytokines[1]. For example, polymorphisms in the VDR gene may influence how efficiently an individual responds vitamin D and calcium intake.

Other anthropometric parameters of parents are predictive of offspring bone health status. Both the mother and father's own birth weight predicts neonatal bone mass, with stronger associations for paternal than maternal[86]. Taller mothers and fathers also have infants with higher spine and whole body BMC[86]. Since these studies report whole body BMC unadjusted for bone area or height in the offspring, it is possible that these associations are simply a result of passing on a larger body size, and hence a larger skeleton with more mineral content, to one's offspring.

1.5.2 Maternal smoking during pregnancy

In rats, there is a dose dependent relationship between maternal[74] smoking and a reduction in skeletal ossification centers in the fetus[87]. Women who smoke during pregnancy give birth to neonates with lower whole body BMC[83,86]. These effects persist into early childhood, with lower bone mass in the lumbar spine and femoral neck (skeletal sites prone to fracture later in life) observed in 8 year olds whose mothers smoked while they were pregnant[88].

Cigarette smoke affects fetal bone in a multifaceted way. Possible mechanisms include causing general growth retardation, increasing oxidative stress, decreased nutrient and oxygen delivery caused by reduced placental blood flow, and the direct toxic effects of cadmium[89,90]. Pregnant rats given a higher dose of cigarette smoke exhibit greater embryonic growth retardation[87,91]. Children of women who smoke during pregnancy have lower birth weight, shorter crown-heel lengths at birth, as well as higher bone resorption markers in cord blood[92 74]. Importantly, these children fail to catch up to their peers in height and weight at age 8[88]. Such general growth restriction also predicts a smaller skeleton with thinner bones; thus relatively, fracture risk is increased. Further, cadmium found in tobacco smoke affects the function of osteoblasts and placental calcium transport[86].

1.5.3 Maternal pre-pregnancy BMI

Neonatal whole body BMD and BMC are negatively correlated with decreasing maternal weight, BMI[93], and skinfold thicknesses[86], particularly in late pregnancy[83], which may suggest the effect of poor nutritional intake. However, the opposite trend has also been cited. Maternal obesity and gestational weight gain has an inverse relationship with cord blood concentrations of markers of bone formation (osteocalcin and bone specific alkaline phosphatase)[94]. Thus it is possible that maternal underweight and overweight both have negative consequences for offspring bone health. Two endocrine factors suggested to have a role in mediating the relationship between maternal fat mass and offspring bone include leptin[93] and estrogen[83].

1.5.4 Maternal physical activity during pregnancy

Lower frequency of maternal physical activity and a slower walking pace in late pregnancy predict greater whole body BMC and BMD in neonates[83,86]. In one study, slower walking pace also predicted greater bone width and thickness, suggesting that in addition to an

effect on volumetric mineralization, maternal physical activity also affects the geometric shape of bone in the offspring[83]. Importantly, bone geometry may predict fractures[19]. It is suggested that perhaps lower physical activity increases bone resorption in the mother, thus increasing the availability of minerals (calcium and phosphate) in the maternal circulation to transfer to the fetus[86].

1.5.6 Maternal nutrition during pregnancy

In addition to vitamin D, dietary protein and calcium are key nutrients to include in the maternal diet for optimal fetal bone development. Most clinical and animal studies suggest a positive association between protein intake during pregnancy and fetal bone health. Longitudinal cohort studies report that higher protein intake during the third trimester is associated with higher whole body BMD in the offspring[3,95]. Animal studies consistently show that depriving mothers of dietary protein during pregnancy has a notable effect on fetal bone, including delayed mesenchymal stem cell proliferation and differentiation in the skeleton[96], modified growth plate morphology, as well as differences in bone composition, length and mechanical strength[97,98].

Sufficient protein intake during pregnancy is crucial for several reasons. Firstly, a continuous supply of amino acids to the developing skeleton *in utero* is necessary because protein in the form of collagen is an essential constituent of bone. Secondly, dietary protein levels regulate calcium homeostasis. High protein intakes promote calcium absorption in the intestine[99], especially in low-calcium diets in postmenopausal women[100]. Other studies suggest that dietary protein high in sulfur amino acids increases the serum acidity, leading to dissolution of bone to provide calcium to act as a buffer[101,102]. Thus, during pregnancy, dietary protein intake may indirectly influence calcium availability to the fetus for bone mineralization. Finally, dietary protein also modulates serum IGF-1 levels. Low protein intake signals a state of starvation and

suppresses IGF-1 levels[103-105]. During pregnancy however, a spike in IGF-1 is important for facilitating placental nutrient transfer in guinea pigs, particularly by up regulating genes that encode amino acid transporters[106,107], suggesting that a protein deficient mother may induce a similar state of deficiency in the fetus with implications for bone. Indeed, mothers with lower protein intake in late pregnancy have lower cord serum IGF-1 concentrations[97,98 108], which is associated with low birth weight in offspring[109]. Birth weight is a predictor of adult bone mass[110]. Importantly, cord IGF-1 levels also positively correlate with the whole body bone mineral content of infants after adjusting for other independent predictors of bone mass[108,111,112].

The increased demand for calcium from the fetus during pregnancy necessitates sufficient calcium in the mother's diet[113]. A birth cohort study (n=216) found that adolescents whose mothers that had consumed more calcium and milk during pregnancy had higher lumbar spine BMD at age 16[4]; a similar finding was reported among 6 year olds (n=698) in rural India, which reported whole body BMD in addition to spine BMD[5]. Conversely, less than two dairy servings per day during pregnancy is associated with shorter femur length at 20-34 weeks gestation, which can be an indicator of fetal bone development[78].

1.5.5 Growth in early life

Birth weight is a well-established indicator of the quality of intra-uterine growth, which is often dependent on maternal nutrition. Both birth weight and growth rates in early postnatal life are predictors of long-term skeletal health[110]. Higher birth weight positively predicts whole body BMC and BMD in the infant[86,108,114]. A study of pubescent boys found that those born premature had lower proximal femur BMC, but it was appropriate for their overall smaller body size compared to term-born peers. Nevertheless, a measurement during puberty could potentially be masked by the accelerated bone growth that normally occurs in this stage. In adolescents

between 18 and 27 years old, those born prematurely showed deficits in skeletal density compared to their term-born peers, having lower BMD in areas of the skeleton prone to fracture such as the lumbar spine and femoral neck. Alarmingly, these associations persist even after adjustments for both shorter height and lower exercise intensity[115]. Hence, the skeletal effects of growth deficits *in utero* may indeed be permanent. Retrospective studies among older adults (65 years and older) find consistently that lower birth weight is associated with reduced whole body bone mass, and radial and tibial size and strength in both genders. Again, these relationships remained robust after adjustment for confounders[116,117].

Studies that measure growth rate find a similar link. A reduced growth rate in early life, indicated by several consecutive height and weight measurements during childhood, is a predictor of femoral neck BMC at 10 years of age[118], as well as a risk factor for hip fractures in adulthood[119]. Hence, it is vital to optimize growth in early life, since it establishes the foundation for a lifetime of good skeletal health.

1.6 Childhood predictors of offspring bone mass

A child's season of birth, as well as their nutrition and physical activity during early childhood contribute to their bone accrual, and are important to account for as potential covariates.

1.6.1 Season of birth

Neonatal bone mass varies according to month of birth, although the direction of the trend is at times inconsistent in the literature. In three separate studies conducted by Namgung et al.[91], American summer-born infants had significantly lower BMC compared to winter-born infants, suggesting that early pregnancy may be the critical period for the fetal skeleton to benefit

from exposure to vitamin D metabolites. Mothers of summer-born infants would have had lower UV-B exposure, and thus lower vitamin D status, during their first trimester. Opposite trends were observed in Korean infants; summer-born infants had higher BMC than winter-born infants[91]. Namgung et al.[91] attributed this to a lack of vitamin D supplementation in Korean women during pregnancy. This explanation falls short however, as it would instead suggest that Korean summer-born infants should have lower BMC than winter-born ones, if early pregnancy is indeed an important time for vitamin D exposure. Nevertheless, what is certain is that there is some persisting effect of birth season on bone accrual, as a retrospective study found that adult BMD varies according to the season in which they were born[14].

Seasonal variations in infant vitamin D status are consistently reported. In a cohort of 100 infants in southeastern United States, summer-born infants (April-October) had significantly higher umbilical cord 25(OH)D concentrations (28.3 nM higher) than winter-born infants (November-March), with a greater seasonality effect in Caucasian infants than African-Americans[120]. Thus, it is possible that seasonal differences in neonatal BMC are due to a combination of UV-B exposure in the mother and in early postnatal life.

1.6.2 Childhood nutrition

Dietary protein is essential to the developing skeleton. Protein in the form of collagen makes up approximately one third of total bone mass; both bone growth and turnover requires a steady supply of protein to replenish lost collagen. However, there is disagreement in the literature on whether high protein intake is indeed beneficial to bone. Some studies report that consumption of protein high in sulfur amino acids (mostly meat proteins) creates an acidic environment that disrupts the balance between osteoblastic and osteoclastic activity[103], as well as

increasing the need for calcium to act as a buffer[103]. Whether the source of this calcium is bone is still debated[99,121].

Most clinical studies thus far have focused on adult and elderly populations, several of which suggest that high dietary protein is linked to increased risk of hip fractures[122,123]. Studies on adolescents generally report the opposite effect. Dietary protein and diaphyseal bone strength, lumbar and femoral bone sites BMC and BMD were positively correlated in children and adolescents between 6 and 19 years old[124-126]. Consuming animal protein increases serum IGF-1[100,104,105,127,128] , and IGF-1 has an anabolic effect on bone by stimulating osteoblast activity, aiding in bone mineralization, and increasing calcium absorption[**103**]. In adult rats, protein insufficiency reduces IGF-1 levels and induces osteoporosis, resulting in lower femoral BMD and biomechanical resistance (bone strength)[104,105]. Consumption of milk protein increased IGF-1 concentrations and improved bone acquisition in adolescent girls (12.2 years old)[129]. Hoppe et al. [128] also observed positive correlations between dietary protein, IGF-1, and whole body BMC in 105 ten year old Danish boys.

Dietary calcium and vitamin D intake during childhood are also important determinants of bone health. From birth to adulthood, females and males gain approximately 875 g and 1175 g of calcium, respectively. These large gains in calcium are largely due to bone growth throughout childhood and adolescence. The skeleton is an enormous reserve of calcium, where 99% of the body's calcium is found[130]. Since few investigations of calcium requirements in young children exist, the most recent IOM DRI 2011 report recommendations of 700 mg/day for children between the ages of 1 and 3 was based on a single study. The EAR was estimated from a factorial analysis and knowledge of the average calcium retention level in children (142 mg/day), from which the RDA was then derived[6].

Most studies on calcium intake and bone have focused on children aged 6 years and older. During childhood, 25% of peak bone mass is acquired during a two-year period close to the time that peak height growth velocity is reached. Trials supplementing children between the ages 6 and 14 years old report significant gains in BMC and BMD, as well as increase in bone size[130], particularly in prepubertal children[131]. The magnitude of increase has been moderate, ranging from 2-5%. It is estimated that a 5% or (0.5 SD) increase in peak bone mass decreases fracture risk by 40%[132]. Few studies supplementing youth have followed them long term, and none have assessed peak bone mass, although one study found that the amount of milk consumed during childhood/adolescence predicts postmenopausal BMD[130].

Accompanying the high demands for calcium in a growing skeleton is the need for vitamin D to aid calcium absorption and bone accrual. Suboptimal intake of vitamin D during early years of childhood can cause delayed growth and bone abnormalities, thus increasing the risk of pediatric fractures[133] and preventing the accrual of the maximum amount of calcium that is genetically preprogrammed for the skeleton[72]. Although rickets was thought to be a disease of the past, it re-emerged in North America in the 1960s[134]. Among Canadian infants, there is a high prevalence of vitamin D deficiency, particularly those in Northern provinces and territories. Having dark skin and being breastfed without additional vitamin D supplementation is also a significant risk factor for deficiency[73,134]. Vitamin D supplementation during infancy is also associated with long term skeletal benefits. Prepubertal girls given a supplement during infancy had higher BMD at specific skeletal sites[135].

Thus, mindful of the contribution of the above nutrients to bone mass, we conducted careful assessment of dietary and supplement intake of these nutrients at 3 years of age using 3 day diet records.

1.6.3. Childhood physical activity

In the 1980s, Frost[136] first developed the mechanostat theory regarding bone, which purported that mechanical loading by muscle on bone regulated bone mass and geometry. Subsequently, studies have revealed that mechanical strain on bones is sensed primarily by an osteocyte network, and transmitted to effector cells (osteoclasts and osteoblasts)[136]. Osteocytes are repressed and osteoblasts are stimulated to differentiate, thus increasing bone remodeling and mineral accretion. In contrast, the absence of shear stress leads to apoptosis of the osteocytes and local resorption of bone is stimulated[136].

In children, physical activity is an important factor that influences bone accretion during growth[137,138]. Most studies report that running and jumping are beneficial to bone mass accrual[139]; in particular, one study found that the intensity of exercise was more predictive of femoral neck strength in 9 year old children than the duration[140]. A minority of others have argued however, that the benefits of physical activity on bone mass fail to compensate for the increased risk of fractures due to intense physical activity[22]. Since physical activity has some contribution to early life bone accrual, it was important to assess physical activity level in our study.

Table 1: Summary of studies examining the relationship between maternal vitamin D status and/or UV-B exposure during pregnancy and bone health status in offspring

Reference	Study design	Population	Measurements	Findings/Conclusions
Javaid et al., 2006	Prospective cohort study	- n = 198 - Southampton, UK - 9 year old children - Insufficient (27.5-50 nM): 31% - Deficient (<27.5 nM): 18%	Baseline (34 weeks gestation): - Pre-pregnancy weight and body composition, nutrition, vitamin D status: RIA, UV-B exposure, smoking - Parental birth weight and anthropometrics - Cord blood Follow-up at 9 years: - SES, daily milk intake, and physical activity of mother and child - Child's anthropometry, and child whole body BMC, and lumbar spine BMC: DXA	- Lower maternal serum 25OHD predicted lower whole body BMC and BMD and lumbar spine BMC in children - maternal 25OHD did not predict body size (BA, height, birth length, weight, lean mass) - UV-B exposure and supplements predicted maternal serum 25OHD - Maternal 25OHD not associated with cord calcium or placental weight - Cord calcium levels predicted child's whole body BMC - Childhood milk intake and exercise did not predict whole body BMC *Conclusion:* Higher maternal 25OHD in the third trimester predicts higher bone mass in the child, but this is not mediated by an increase in placental calcium transport

Sayers and Tobias, 2009	Prospective cohort study	- n = 6995 - ALSPAC study in Bristol, UK - 9.9 year old children	Baseline (third trimester): - UV-B exposure - Maternal serum 25OHD in subgroup (n= 355) - Crown-heel length, birth weight Follow-up at 9.9 years: Child's whole body BMC, whole body BMD, area adjusted BMC, height adjusted BA, anthropometry	- 1 SD increase in UV-B exposure in mother → 0.5 SD (9.6 g) increase in BMC - Maternal UV-B exposure did not predict birth weight and fat mass - Maternal UV-B exposure has effect on periosteal bone growth (bone thickness), estimated by hBA; height accounted for 50% of this influence *Conclusion*: Maternal UV-B exposure predicted greater BMD and BMC but primarily because UV-B exposure also predicted a larger body size (birth length, height, weight and lean mass at 9 years)
Viljakainen et al., 2010	Prospective cohort study (self declared as "cross sectional with longitudinal follow up)	- n = 125 - Helsinki, Finland - 14 month old children	Baseline (1st trimester): - Maternal serum 25OHD and bone remodelling markers At birth: - Maternal serum 25OHD and bone remodelling markers - Cord blood - pQCT of tibia in child 14 months follow-up: - pQCT of tibia in child Adjustments made for: birth weight, maternal height, newborn	- Maternal 25OHD predicted higher tibia BMC at birth only - Maternal 25OHD predicted higher tibia CSA with no difference in BMD at 14 months of age - Fetal bone turnover differed between group with higher maternal 25OHD and lower *Conclusion*: Maternal 25OHD in first trimester affects bone mineral accrual in the fetus, and has long term effects on

			age at time of measurement	offspring bone size
McGrath et al., 2005	Cross sectional study	- n = 350, 171 (birth weight) - n = 1233 (other anthropometrics) - Queensland, Australia	Infant: - Birth date - Anthropometrics: birth weight, limb length, head size, skin fold thicknesses	- Birth weight was significantly higher in winter and spring (25 g difference between infants born in October compared to May), as were limb lengths - Winter and spring infants would presumably have had with lower prenatal vitamin D exposure (third trimesters during autumn and winter) *Conclusion:* Lower prenatal exposure to vitamin D may predict a larger body size; this effect may be due to role of calcitriol in regulating growth plate development in the proximal and distal end of long bones
Finch et al., 2010	Animal study (guinea pig model)	- Deficient/control sows (C/D) - Supplemented/placebo offspring (S/P); approximates dose of 400 IU in infant	Maternal: - Serum 25OHD at: conception, 3rd trimester, postpartum Offspring: - Serum 25OHD at: birth and 28 days - DXA - Biomechanical testing - Bone remodelling markers - pQCT of tibia and femur	- Less gestational weight gain in D sows - At birth and 28 days, offspring of the D sows had lower serum 25OHD, osteocalcin, body weight and length than those of C sows - D sow offspring also had lower whole body and total tibia BMC, CSA, and lower bone strength compared to offspring of the C sows, regardless of postnatal supplementation

				- Postnatal supplementation did not significantly improve offspring 25OHD, regardless of D or C mothers *Conclusion:* Maternal vitamin D status in pregnancy should be optimized, as supplementation in postnatal life alone may not reverse detrimental effects of deficiency in utero on bone mass and growth
Mahon et al, 2009	Prospective cohort study	- n= 424 - Southampton, UK	At 19 weeks gestation: - Maternal serum 25OHD - 3-D ultrasound measure of femur length and splaying index	- Lower maternal serum 25OHD predicted greater femoral metaphyseal cross-sectional area and higher femoral splaying index at 19 weeks of gestation - No effect on femur length *Conclusion:* Vitamin D sufficiency in early pregnancy is essential, as lower maternal 25OHD predicts fetal femoral geometry similar to rickets
Morley et al., 2005	Prospective cohort study	n = 374 Victoria, Australia	Baseline (<16 weeks and 28 weeks gestation): - Maternal serum 25OHD - Maternal PTH Birth follow-up: - Knee-heel length	- Gestation length was 0.7 weeks shorter in deficient mothers (<28 nM), as was knee-heel length - Other birth measures not affected - Maternal PTH positively predicted knee-heel length, birth weight, arm and calf

			- Birth weight - Upper-arm circumference - Calf circumference	circumferences (independent of 25OHD) *Conclusion:* Low maternal 25OHD during pregnancy may restrict longitudinal growth in fetus, possibly mediated by a decrease in gestational length
Mahomed and Gülmezoglu, 2010	Meta-analyses of randomized controlled trials supplementing pregnant mothers with vitamin D	- n = 232 (two trials) - Mallet et al., 1986 - Brooke et al., 1980	Electronic searches on MEDLINE and the Cochrane Controlled Trials Register, as well as review of 38 journals received via ZETOC Mallet et al. 1986 (n=77) - 1000 IU/day or single dose of 200 000 IU - Outcomes: birth weight, maternal and cord 25OHD, calcium levels Brooke et al. 1980 (n=126) - 1000 IU/day in third trimester - Outcomes: maternal weight gain, birth weight, neonatal hypocalcemia, craniotabes, 25OHD	- Higher pregnancy weight gain and lower number of low birth weight infants reported in Mallet et al. - Lower birth weight in supplemented group reported in Brooke et al. *Conclusion:* Insufficient evidence to evaluate effects of vitamin D supplementation in pregnancy, but supplementation in groups with high risk or in countries with long winters should be considered. Larger trials studying maternal and neonatal outcomes from supplementation needed.

CHAPTER 2

STUDY DESIGN AND METHODS

Chapter 2

STUDY DESIGN AND METHOD

2.1 Study Design

2.1.1 FAMILY study

This thesis project was an ancillary study of the FAMILY study[141], a longitudinal prospective birth cohort study exploring the early origins of obesity, diabetes, atherosclerosis and allergies at McMaster University. The FAMILY study aims to elucidate the relative contributions of maternal, infant, and childhood factors to the development of obesity and cardiovascular disease risk.

The participants for the study in this thesis project were a subset of the FAMILY study sample, which consisted of mothers recruited from prenatal ultrasound clinics at MUMC and SJH in Hamilton, as well as Joseph Brant Memorial Hospital in Burlington. Mothers were in the third trimester of pregnancy at the initial visit; blood samples were taken from the mother, and questionnaires on demographics, lifestyle and medical history were administered. Subsequently, a follow-up visit was conducted at birth, when the neonate's anthropometric measures were taken. When the child turned 6 months old, the family was contacted via telephone regarding the child's dietary practices including vitamin D supplementation. When the child turned 3 years old, the family attended a follow-up visit at which the child's diet and bone health status via DXA were assessed at MUMC. We also performed a DXA scan on the mother at this time.

All participants completed consent forms to participate in this ancillary study before measurements were taken. This study was approved by the Research Ethics Board of Hamilton Health Sciences and McMaster University.

2.1.2 Inclusion and exclusion criteria

We included only singleton children in this study. We excluded mothers from the study if they suffered from diseases that may affect bone health (i.e. malabsorption disorders including Celiac's disease, Irritable Bowel Syndrome, ulcerative colitis, or hypothyroidism, and hyperemesis), identified as treatment by steroid drugs (as self-reported); the same criteria were applied to the children at 3 years of age.

2.2 Maternal measurements during pregnancy

2.2.1 Vitamin D status

Maternal blood samples were collected at the initial visit during the third trimester of pregnancy were spun and were frozen at -80°C in the central research biobank at PHRI of McMaster University. When required, the samples were retrieved from storage and transported frozen to Dr. Atkinson's lab prior to being thawed and aliquoted for analysis of 25OHD in her lab, noting that serum 25OHD is unaffected up to four freeze-thaw cycles[142]. UPLC LC/MS-MS was used to quantify serum 25OHD. The protocol for sample preparation and the instrument parameters were based on the Waters Alliance protocol for HPLC LC/MS-MS adapted for use on a UPLC system. The method was validated for use on a Waters ACQUITY UPLC system and ACQUITY TQD (with Acquity UPLC BEH C18 column), using MassLynx 4.1 software in the Fusch laboratory[143]. The reported inter-assay CV for low samples was 13%, and for high samples was 7%, based on pooled blood samples with approximately 30-80 nM 25OHD, with or without spiking with 120 nM of 25OHD, respectively[143].

A calibration curve was run using standards prepared from a 25OHD sample obtained from Sigma (standard of concentration 1 mg/ml). The correlation coefficient of the curve was 0.995 (R-squared = 0.991). Low and high concentration control samples were run using samples from

ClinCheck (Recipe Chemicals, Germany), reference number 35082 from lot 015; the coefficients of variation for measurement of low and high $25OHD_3$were 11% and 10%, respectively. The accuracy of the method was confirmed by using the most recent certified NIST reference material for vitamin D analysis, SRM972, which contains a known quantity of 25OHD quantified by isotope dilution tandem mass spectrometry[144]. Our assay measured 117% and 114% of the certified concentration values for $25OHD_3$ of SRM972 Level 1 and SRM972 Level 3, respectively. Level 1 contained human serum, while Level 3 contained human serum spiked with additional $25OHD_2$. As discussed above, only $25OHD_3$, referred to simply as 25OHD, was of relevance to our Canadian population.

The volume of serum required per sample was 150 µL, and a total of 368 samples were processed. In each sample, a deuterated internal standard was included to account for potential losses in sample preparation. Vitamin D status was categorized as deficient, insufficient and optimal based on the most recent IOM guidelines in the DRI report released in December 2010; thus <30 nM was considered deficient, 30-50 nM potentially insufficient, and >50 nM optimal[6]. Values greater than 125 nM were considered excessive[6].

2.2.2 Anthropometric measures, lifestyle factors, and nutrition

At the initial visit, anthropometric measures of the mother were taken. Height was measured using a stadiometer measured to the nearest 0.1 m, and weight by an electronic scale measured to the nearest 0.1 kg[141]. At the 3 year visit, the mothers' bone mass was assessed by DXA. The mother's whole body BMD z-scores were calculated using reference data embedded in the Hologic software for analysis.

Demographics and lifestyle factors during pregnancy were assessed via questionnaires. Mothers were asked about their education, annual income, medical history, and smoking history (yes/no, and whether they had quit for pregnancy). Physical activity during pregnancy was assessed using a validated FAMILY questionnaire adapted from a previous study at PHRI; questions helped estimate both leisure and occupational activity. Leisure activities were divided into four categories: mainly sedentary (score = 0), mild exercise (score = 1), moderate exercise (score = 2) and strenuous exercise (score = 3). Occupational activities were divided into five categories: do not work or mainly sedentary (score =0), predominantly walking with no heavy lifting (score = 1), mainly walking and including climbing stairs, walking uphill or lifting (score = 2), heavy physical labour (score = 3). A total score between 0 and 6 was obtained for each mother. Hence, a total score of 0 was assigned to sedentary women, 1-2 for somewhat active, 3-4 for active, and 5-6 for very active.

Diet was assessed via an FFQ to capture the mother's vitamin D, calcium, and protein intake in the past year, which included the months of pregnancy. The FFQ contained 157 items, which were selected using the Study of Health Assessment and Risk in Ethnic groups (SHARE) database[145] in Hamilton. Each question in the FFQ asked about a specific food/drink item, its frequency of consumption (number of times per day, week, month, year, or never), and portion size consumed (small, medium, or large); photographs were provided to help participants estimate their portion sizes. These questions were followed by a section that asked participants to report any food/drink items consumed more than twice a month that was not included in a question in the FFQ. The next section consisted of 14 additional questions gathering information on cooking methods, as well as the consumption of nutritional supplements during pregnancy (i.e. prenatal vitamins, multivitamins). Data from the FFQ were computed by a registered

dietician at PHRI, using a calculator created in Excel 2007, which yielded the average nutrient intake per day.

2.3 Child measurements

2.3.1 Bone mass

BMC was assessed using a QDR® series Hologic Discovery^{TM} DXA machine. Software for whole body analysis (V12.3.1) provided by Hologic was used for analyzing the child and mother's scan. The DXA employs a fan-beam source of X-rays of two energies and measures differences in attenuation by the body to yield measures of body composition including body fat, lean mass, and bone mineral content. A multiple detector array captures the attenuated X-rays. Participants were asked to remove all metal before undergoing the scan, and were instructed to lie still on the DXA bed for 2-3 minutes in supine position while the scan was taken. For the children, the scan field was often shortened to 120-140 cm to accommodate their height and to minimize scan time. In those cases that metal or objects were included in the scan (i.e. toys to encourage the child to comply), sub-regional analysis was done to isolate and subtract the object's contribution to overall fat, lean or bone mass measurement. Movement artifacts (seen only in child scans) were dealt with in a similar fashion. If considerable movement was seen in one limb but not the other, the well-captured limb would be used as a surrogate for the one with movement. Any scans with unsalvageable distortions due to movement were discarded.

All scans were performed on the same machine by trained members of the research team, and analyzed immediately after the visit. In addition, all scans underwent final review and approval by Dr. Stephanie Atkinson, the principal investigator with expertise, to ensure consistency in analysis across all participant data. Daily quality control tests were performed using an artificial $L_{1\text{-}4}$ lumbar spine made from hydroxyapatite encased in epoxyresin. Weekly,

the step phantom (composed of soft and lean tissue equivalent materials) was performed to calibrate the machine; at this time, the uniformity test was also performed to evaluate the contribution of air molecules to the attenuation of the X-rays. The CVs for BMC, BMD and BA, respectively, were 0.651%, 0.376%, and 0.491% (Hologic Inc.; Bedford, MA).

2.3.2 BMC z-score calculation

For all BMC data, z-scores were calculated using data from an age and sex specific standard curve. The z-score is a standard score that reflects the number of standard deviations a particular data point is away from the mean. In clinical terms, this allows the comparison of an individual's score with peers. The z-score is computed in the following way:

$$\text{Z score} = (\text{individual data point}) - (\text{mean of the distribution}) / \text{distribution standard deviation}$$

In current scientific literature, bone mass data on children younger than 5 years of age is sparse, partly due to difficulties of conducting research in young children (i.e. non-compliance). The largest collection of bone mass measured in 3 year olds comes from the Baylor College of Medicine Body Composition Laboratory Z-score calculator, developed in the Children's Nutrition Research Centre (CNRC) in Texas[32,146]. The calculator utilized bone data from 84 children aged between 2 and 4 years of age to construct trends, which were used to derive z-scores (personal communication, Roman Shypailo, Biophysical Engineer, Baylor College of Medicine Children's Nutrition Research Center, Houston, August 11, 2011). We used this database to calculate z-scores for BMC of the children in our study.

Further, the ISCD has recommended height adjustment for pediatric DXA interpretation to account for high variability in growth among children[30]. There have also been suggestions that the effects of vitamin D on fetal bone may be partially mediated by effects on longitudinal growth[14]. In keeping with these suggestions, we also report BMC scores for children that are height-adjusted, by using an alternate form of the Baylor calculator that accounts for height (personal communication, Roman Shypailo, Biophysical Engineer, Baylor College of Medicine Children's Nutrition Research Center, Houston, February 17, 2011).

2.3.3 Bone size

We collaborated with Dr. Troy Farncombe (Nuclear Medicine, McMaster University) to develop a computer software in MATLAB that allowed measurement of femoral and humeral lengths from a subset of the child 3 year DXA scans (n=90). A procedure for correct and consistent placement of markers on the scan to measure length was developed and validated in a 4th year thesis project by Andrew Sripalan at McMaster University, which I supervised. The CV for measurements performed on nine different days were 1% and 2% respectively. Bone size was estimated using these measures in combination with whole body BA to derive a principal component to represent the underlying trait of bone size (further discussed below in statistical analysis section).

2.3.4 Anthropometric measures

Infant length was measured between 24 and 48 hours of age using an O-Leary Pediatric LengthBoard (Ellard Inc)[141]. The infant was placed on the board with his/her head against the stationary head piece, with the face towards the ceiling. Gentle pressure was applied to the infant's knees to extend the legs, and the foot piece was positioned firmly against the feet. The length measurement was then read through the magnifier and recorded to the nearest 0.1 cm.

Birth weight was obtained from chart review and recorded on the Birth Visit CRF (#3). Height at three years was measured using a stadiometer (Harpenden) fixed on the wall[141]. Weight was obtained using an electronic scale measuring in kg to 0.1 kg.

2.3.5 Lifestyle factors: diet and physical activity

To assess the child's diet, a 3-day diet record was mailed to participants, completed by parents, and returned to the investigators at the three-year visit. Clarifications of missing data in the diet records were made with the parents during the clinical visit. Parents were instructed to record all foods consumed by the child, including food type, brand, cooking methods and portion size for three days. The three days did not have to be consecutive, but needed to include two weekdays and one weekend day to account for different eating patterns on weekends.

The diet records were analyzed using Nutritionist Pro Software (Axxya Systems, Stafford, Texas), which contains a database with nutrient data for over 35 000 foods. Specific brand names, fast foods, and ethnic foods were available in the database. Food items based on the US Department of Agriculture (USDA) database were the preferred selection. Further, our analysis was also supplemented with the use of the online USDA reference standard (Release 22) established in 2009[147], which had a more up-to-date selection than the software. After entering all dietary data, Nutritionist Pro computed the average amount of vitamin D, calcium, protein, and total calories consumed per day. The intake of nutritional supplements at 3 years of age was obtained from a separate questionnaire and coded separately from the dietary data.

To ensure consistency in the analysis of the 3-day diet records, I developed a written standard of practice (SOP) protocol in collaboration with other staff (Appendix A); this protocol was followed by all research staff analyzing diet records. In addition, since more than one research staff analyzed the data, statistical analysis was performed to ensure that there were not

significant differences between staff; the analysis can also be found in the appendix (Appendix B).

The physical activity of the child was assessed using a modified HAES questionnaire[148] that collected information on the child's activity level (total hours per day the child spent being "active" and "very active") on one typical weekday and one typical Saturday.

2.4 Statistical analysis

A total of 372 mother and child pairs were included for analyses, with the primary criteria being the existence of a successful child DXA scan. Of this sample, 368 children had corresponding maternal blood samples from their third trimester, 247 had a corresponding maternal DXA scan, 208 children had complete 3-day diet records, and measures of bone length were conducted on 61 children DXA scans.

Statistical analysis was performed using SPSS (version 19.0; SPSS Inc., Chicago, IL). Descriptive statistics were computed by calculating the mean, standard deviation and median for all data; minimum and maximum values are also reported. All descriptive data is reported with the original dataset only, and not imputed data.

Child anthropometric and bone measures were stratified by gender, with two-tailed unpaired t-tests conducted to detect any gender differences. Predictors of maternal vitamin D status were assessed using simple linear regression. Both non-standardized (b) and standardized regression (β) coefficients are reported for all predictors; as well, a confidence level of $1 - \alpha$ where $\alpha = 0.05$ was chosen to construct the confidence intervals.

A priori, we intended to enter all maternal and child covariates along with maternal vitamin D status as predictors of child bone outcomes. However, many of the maternal and child variables had missing data of up to 10%. As a result, a single model containing all variables of

interest would decrease the sample size considerably. Thus, several multiple regression models were computed instead to examine the influence of maternal and child independent variables separately, as well as together.

Models showing the separate contributions of maternal and child variables are in Appendix C and Appendix D; their combined contributions, minus child diet (a missing rate of 44%), are displayed in Tables 15 and 16. Sensitivity analysis was conducted to examine the influence of imputing the mean for missing data (Appendix E); the impact was minimal, therefore the model with imputed data was chosen for further analysis.

2.4.1 Multiple regression of primary outcome of child BMC z-score: models 1a-6a

A total of six models were developed to explore the combined contribution of maternal and child lifestyle variables on the primary outcome of interest: the child's 3 year BMC z-score. One final model, model 5a, was adopted for reporting (models 1a-4a and model 6a are shown in Appendix F). Prior to developing the models, exploratory analysis was done by entering all variables backwards, in a stepwise fashion; predictors with significant colinearity (variance inflation factor greater than 10) were either excluded or re-defined (data not shown). Then, a full model was then run with all available variables (model 1a); next, only variables with $p<0.25$ and of strongest clinical significance were included in model 2a. Subsequently, model 3a and 4a were developed to test assumptions of linearity, equal variance, and normality. Independence of variables was inferred based on inclusion criteria of only one child from each family. Lastly, models 5a and 6a were developed to show the influence of outliers in the data. Non-standardized and standardized beta values are reported for all predictors; as well, a confidence level of $1 - \alpha$ where $\alpha = 0.05$ was chosen to construct the confidence intervals. Also, statistical significance was taken at p-values less than 0.05.

2.4.1 Multiple regression of secondary outcome of child bone size: models 1b-6b

To examine the secondary outcome of interest, bone size, a smaller subset of the sample was used (n=61). For each child, average femur length was calculated by averaging left and right femurs; the same was done to calculate average humeral lengths. Since whole body BA and bone lengths are likely to be co-linear due to the underlying shared factor of bone size, principal component analysis was performed on these outcome measures and one component was derived to obtain a single variable indicative of bone size. Subsequently, multiple regression was performed to examine the relationship between maternal and child lifestyle variables on the child's bone size (represented by the derived component). The same steps for developing models 1a-6a above were followed, and model 5b is reported (models 1b-4b and 6b can be found in Appendix G).

In all cases of multiple regression, when child or maternal vitamin D and calcium intake were entered, the quantity of each nutrient was standardized to the amount found in one cup of milk in Canada, in order to facilitate interpretation of results (i.e. to understand the effect of the amount of calcium in one cup of milk on the outcome of bone, instead of one mg of calcium). Thus, dietary vitamin D and calcium are expressed as "per 90 IU" and "per 300 mg", respectively, in the multiple regression results. Likewise, maternal serum 25OHD is expressed as "per 10 nM" in the multiple regression models, so that the reported non-standardized regression coefficient (b) represents the change in bone outcome associated with 10 nM change in serum 25OHD.

CHAPTER 3

RESULTS

Chapter 3

RESULTS

3.1 Study sample demographics

A total of 372 singleton children successfully completed a DXA scan at the 3 year visit; 5 scans were omitted from the statistical analysis owing to an invalid DXA scan due movement artefact or an unidentifiable object in the scan field that could not be eliminated in the analysis. Further, of this sample, 247 had corresponding maternal DXA scans; the remainder of mothers declined either because they were pregnant or for other reasons. Demographic characteristics of the mothers in this study are summarized in Table 1. Each variable had less than 5% of missing data due to lack of participant response; the total number of participants for which the data were available for each variable is indicated in the second column.

Approximately 86% of mothers reported to be of European descent, with the rest coming from a variety of ethnic backgrounds including: African, Asian, Hispanic, and Aboriginal. The average age of the mothers was 32.8 years. The majority of them (87%) had post-secondary education and 79% lived in a household earning more than $50 000 a year. The characteristics of this sub-population were comparable to the demographics of the entire FAMILY study sample, which consisted of 836 mothers[141].

Table 2 – Demographics of mothers recruited in pregnancy

Maternal characteristics	N	%	Mean (SD)
Ethnicity	372		-
European	319	85.8	
Other	53	14.2	
Age (yr)	372	-	32.8 (4.7)
Household Income (dollars)	359		-
<$29, 999	32	8.9	
$30-49,999	44	12.3	
>50,000	283	78.8	
Education (years)	372		-
< or = 13	49	13.2	
>13	323	86.8	

3.2 Maternal variables during pregnancy

3.2.1 Maternal lifestyle factors, nutrition and physical measures

Approximately half of the mothers were overweight (BMI >25), and on average, had whole body BMD one standard deviation above the age and sex matched reference population (Table 3). Fewer than 4% of mothers smoked during pregnancy and less than 5% of mothers reported consuming more than one drink per month during pregnancy (Table 4).

Although the median intake of vitamin D in mothers was below the EAR and only 9.8% of mothers exceeding the EAR of 400 IU recommended for pregnant women[6], over 83% of mothers took vitamin D supplements containing between 200 and 1000 IU per day during pregnancy (Table 5). Thus in total, the average total intake from both food and supplement exceeded the EAR of 400 IU. The median intake of calcium in mothers exceeded the EAR, with 89.4% of mothers consuming greater than the EAR of 800 mg. The median intake of protein in mothers exceeded the EAR, with 86.6% of mothers consuming greater than the EAR of 71 mg (Table 5).

Approximately 24% of mothers met Canada's Food Guide recommendation for daily servings of fruits and vegetables; 20% met the recommendation for daily servings of meat and alternatives, and 78% met the recommendation for servings of dairy (Table 5).

Among the mothers, 15.3% were sedentary during their pregnancy (total physical activity score 0), 64.2% were somewhat active (score 1-2), 19.3% were active (score 3-4) and 1.1% were very active (score 5-6) (Table 4).

Table 3 – Physical measures of mothers

Maternal measure	N	%	Mean (SD)	Median (min-max)
Self-reported pre-pregnancy weight (kg)	343	-	-	69.6 (45.6 – 119.3)
Height (m)	372	-	1.6 (0.07)	
Pre-pregnancy BMI	342		-	-
<18.4 kg/m^2	7	2.0%		
18.5-24.9 kg/m^2	155	45.3%		
25.0-29.9 kg/ m^2	100	29.2%		
>30 kg/ m^2	80	23.4%		
BMD z-score	247	-	0.90 (0.93)	-

Table 4 – Lifestyle factors of mothers during pregnancy

Maternal characteristics	N	%
Smoking Status	369	
Smoked during pregnancy	13	3.5
Formerly smoked but quit before pregnancy	114	30.9
Never smoked	242	65.6
Alcohol intake	368	
Never, or < 1 drink a month	350	95.1
Once a month	10	2.7
2-3 times a month	7	1.9
Once a week	0	0.0
2-3 times a week	1	0.27
4-6 times a week	0	0.0
Everyday	0	0.0
Pregnancy physical activity (work + leisure)	372	
0	57	15.3
1	112	30.1
2	127	34.1
3	53	14.2
4	19	5.1
5	4	1.1
6	0	0.0

Table 5 – Maternal intake of specific nutrients and food groups during pregnancy

Maternal diet		N	% of mothers	Mean (SD)	Median (min-max)
Vitamin D	From food (IU/day)	358	-	169 (171)	102 (4-1248)
	From supplement (IU/day)	373		-	-
	None	62	16.7		
	200-400 IU	302	81.2		
	>1000 IU	8	2.2		
	Total (IU/day)	366	-	434.6 (231.9)	378.5 (5.0-1860.0)
Calcium	From food (mg/day)	358	-	1680 (804)	1571 (423-5379)
	From food (mg/day/thousand calories)	358	-	587 (189)	558 (187-1448)
	From supplements (mg/day)	373	-	-	-
	None	57	15.3		
	200-300 mg	291	78.2		
	350-650 mg	24	6.5		
	Total (mg/day)	366	-	2185.5 (858.4)	2100.3 (425-5979)
Energy	kcal/day	358	-	2900 (1133)	2694 (961-7534)

intake	kcal/kg/day	334	-	42 (18)	38 (11-114)
Dietary protein	g/day	358	-	114 (44)	107 (29-320)
	g/kg/day	334	-	1.65 (0.71)	1.49 (0.28-5.0)
*Servings of dairy per day		368	-	3 (2)	3 (0-10)
*Servings of fruit per day		368	-	4 (2)	3 (0-16)
*Servings of vegetables per day		368	-	3 (2)	2 (0-10)
*Servings of meat per day		368	-	1 (1)	1 (0-10)

* Canada's Food Guide includes a recommendation to eat an extra two to three Food Guide Servings each day so that pregnant women will meet their energy needs[149]. Servings recommended by Canada's Food Guide for women of childbearing age are: Dairy = 3-4 ; Fruits and vegetables = 7-8 ; Meat and alternatives = 2

3.2.2 Primary predictor: maternal 25-hydroxyvitamin D status

The majority of mothers exceeded the recommended optimal serum vitamin D status in the DRI report[6], with 92.9% of mothers' serum 25OHD measuring greater than 50 nM (Figure 2). Several factors predicted maternal vitamin D status, including ethnicity, vitamin D intake from food, and BMI. Europeans had significantly higher vitamin D status than non-Europeans (an average of 14 nM greater), and both vitamin D intake from food and BMI positively predicted vitamin D status (Table 6). Mothers whose total vitamin D intake (from food and supplements) was above the RDA of 600 IU had significantly higher vitamin D status than those who were below (Figure 3).

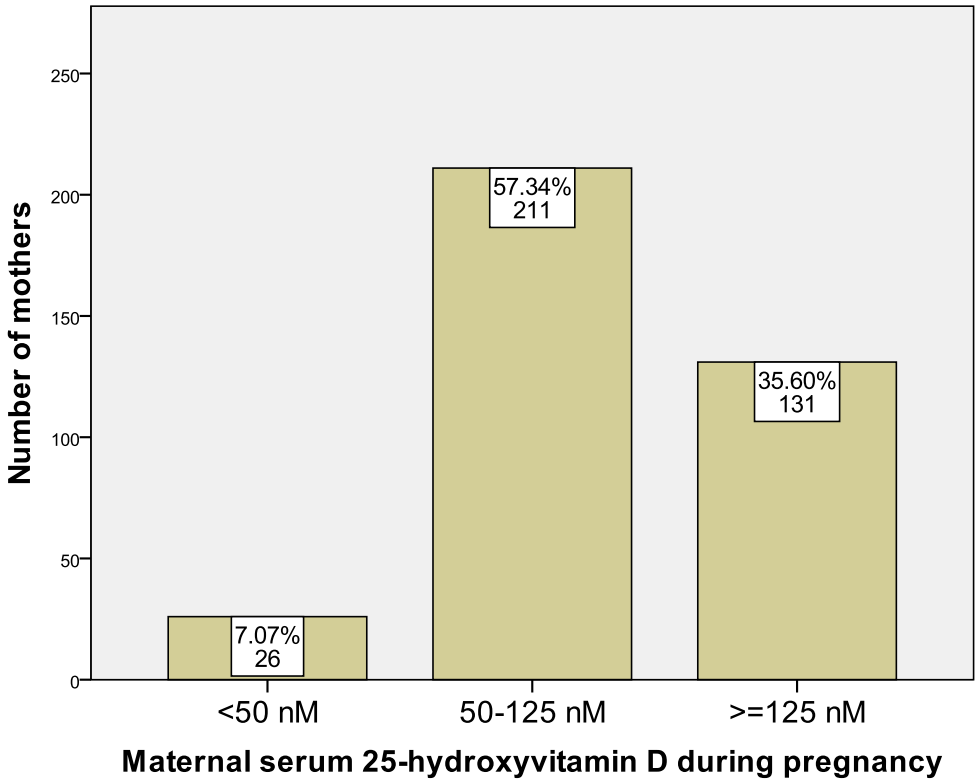

Figure 2 – Frequency of distribution of mothers with vitamin D status < 50 nM (sub-optimal), 50 nM – 125 nM (optimal) and > or equal to 125 nM (potential for toxicity) during pregnancy – defined according to DRI report, 2011

Table 6 - Predictors of maternal vitamin D status during pregnancy (* indicates statistically significant factors)

Maternal lifestyle factor	N	b	95% CI		r^2	p-value
Smoking	365					
Formerly smoked		2.110	-7.831	12.051	0.003	0.677
Currently smoking		13.073	-11.724	37.871		0.301
Physical activity (work + leisure)	313					
1		-4.823	-15.993	6.348	0.031	0.396
2		7.658	-6.656	21.972		0.293
3		21.844	0.542	43.146		0.044*
>4		32.131	-11.557	75.818		0.149
BMI	340	-0.834	-1.637	-0.031	0.012	0.042*
Food vitamin D intake	355	0.031	0.004	0.057	0.014	0.025*
Vitamin D supplement	372					
300 IU		10.852	-1.237	22.940	0.008	0.078
>1000 IU		10.046	-22.487	42.580		0.544
Age	368	0.782	-0.173	1.737	0.007	0.108
Ethnicity:						
Non-European	368	-14.214	-27.025	-1.402	0.013	0.030*

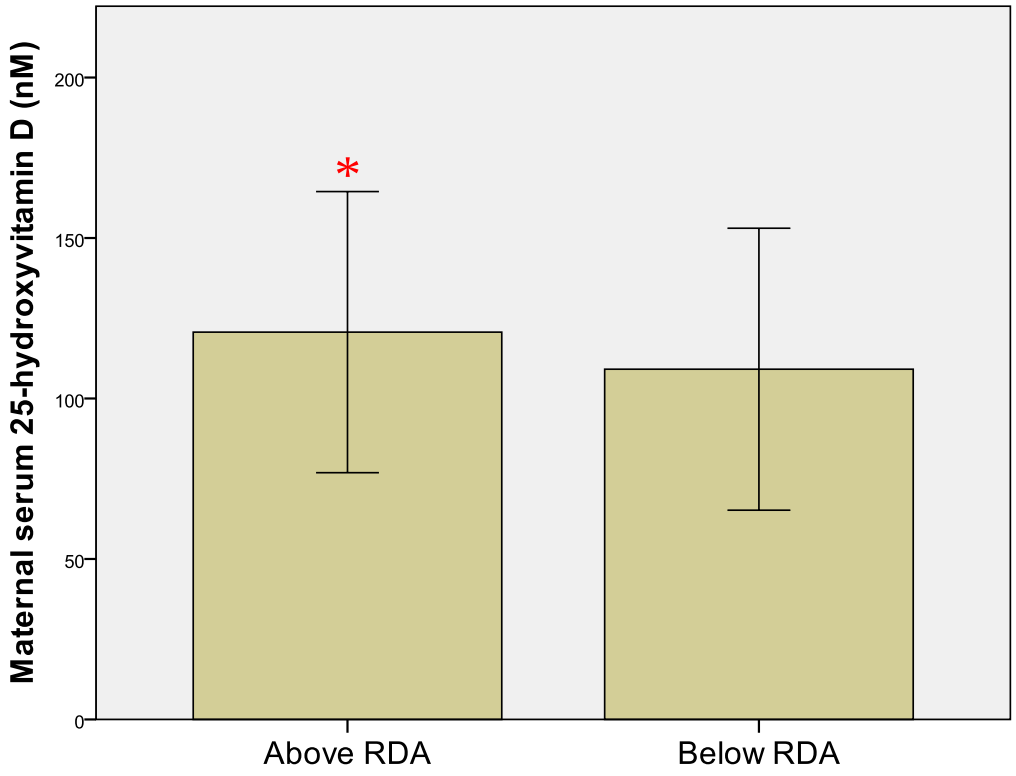

Figure 3 – Total maternal intake of vitamin D (food and supplements) above and below RDA of 600 IU as a predictor of maternal vitamin D status (nM) during pregnancy (*p=0.049). Data are mean values and error bars are SDs.

3.3 Child variables at birth, 6 months, and 3 years of age

3.3.1 Child nutrition and physical activity

Few children were exclusively breastfed for greater than 6 months, and the majority of children (91.5%) were not given vitamin D supplements at 6 months of age (Table 7).

Among boys, median daily vitamin D intake did not exceed the EAR, and only 5.3% of boys exceeded the EAR of 400 IU. Median calcium intake exceeded the EAR, with 87.4% of boys consuming greater than the EAR of 500 mg. Median protein intake also exceeded the EAR, with 100% of boys consuming greater than 0.87 g/kg of protein per day.

Among girls, median daily vitamin D intake did not exceed the EAR, and only 5.3% of girls exceeded the EAR of 400 IU. Median calcium intake exceeded the EAR, with 88.5 % of girls consuming greater than the EAR of 500 mg. Median protein intake also exceeded the EAR, with 100% of girls consuming greater than 0.87 g/kg of protein per day.

Physical activity was similar between weekday and weekends, and between boys and girls (Table 9).

Table 7 – Child nutrition at 6 months of age

	Male (n=188)	**%**	**Female (n=184)**	**%**
Exclusively breastfed for > 6 months	8	4.3%	5	2.7%
Supplement use at 6 months				
Vitamin D (400 IU)	16	8.5%	16	8.7%
None	172	91.5%	168	91.3%

Table 8 – Child nutrition at 3 years of age - values are expressed as mean (SD) and median (min-max)

		Male (n=188)			Female (n=184)		
		N	**Mean (SD)**	**Median (min-max)**	**N**	**Mean (SD)**	**Median (min-max)**
Total energy intake	Kcal/day	95	1386 (363)	1330 (739-3046)	113	1401 (382)	1342 (741-3062)
	Kcal/kg/day	95	93 (23)	90 (47-179)	113	98 (26)	95 (47-207)
Protein	g/day	95	56 (18)	54 (21-131)	113	56 (16)	55 (30-116)
	g/kg/day	95	4 (1)	4 (1-8)	113	4 (1)	4 (2-8)
Calcium	From food (mg/day)	95	862 (368)	765 (190-2432)	113	885 (338)	842 (107-1915)
	From food (mg/day/ thousand calories)	95	635 (184)	627 (131-1132)	113	657 (198)	649 (81-1337)
	Supplements: None	164 (87.2%)	-	-	153 (83.2%)	-	-

	150 mg/day	24 (12.8%)			31 (17.0%)		
Vitamin D	From food (IU/day)	95	275 (190)	185 (45-790)	113	271 (190)	168 (6-553)
	Supplements: None 400 IU/day	 160 (85.1%) 28 (14.9%)	-	-	 150 (81.5%) 34 (18.5%)	-	-

Table 9 – Child physical activity at 3 years of age by maternal report, assessed by modified HAES questionnaire and defined as average hours per day of being somewhat active to very active – values are expressed as mean (SD)

	Male (n=188)			Female (n=184)		
	N	**Mean (SD)**	**Median (min-max)**	**N**	**Mean (SD)**	**Median (min-max)**
Weekday physical (hours/day)	183	8.5 (1.8)	8.7 (12.8 – 11.2)	182	8.5 (1.8)	8.4 (4.7 – 12.5)
Weekend physical (hours/day)	183	8.5 (1.7)	8.9 (0 – 11.6)	182	8.8 (1.7)	8.8 (4.8-15.0)

3.3.2 Child anthropometrics and season of birth

Boys were consistently larger in body size than girls at birth, with higher birth weight and birth length (Table 10). A comparison of birth length z-scores between boys and girls showed no difference, since each child was compared to an age and gender-matched reference data[150]. Boys were also consistently larger than girls at 3 years of age, indicated by higher weight and height (Table 11). Once again, a comparison of height and weight z-scores between boys and girls showed no difference, since each child was compared to an age and gender-matched reference data[150]. There was an approximately even split between winter and summer born infants. Winter was designated as November – March, and summer as April – October (Table 10).

Table 10 – Characteristics and anthropometric measures of children at birth and 6 months – values are expressed mean (SD) (* indicates statistically significant factors)

Variable	Male (n=188)			Female (n= 184)			p-value
	N	Mean (SD)	Median (min-max)	N	Mean (SD)	Median (min-max)	
Gestational age (weeks)	188	39 (2)	39 (29-42)	184	39 (2)	40 (31-41)	-
Season of birth Winter Summer	188 82 (43.6%) 106 (56.4%)	-	-	184 76 (41.3%) 108 (58.7%)	-	-	-
Birth weight	188	3507 (593)	-	183	3352 (576)	-	0.011*
Birth length z-score	174	0.12 (1)	-	174	0.06 (1)	-	0.620
Birth length	174	50.4 (2.5)	-	174	49.5 (2.7)	-	0.001*

Table 11 – Anthropometric measures of children at 3 years of age (* indicates statistically significant factors)

	N	Male (n=188)	N	Female (n=184)	p-value
Age (years)	188	3.06 (0.17)	184	3.06 (0.15)	0.97
Weight z-score	188	0.4 (0.9)	183	0.3 (0.9)	0.516
Weight (kg)	188	15.1 (1.7)	183	14.6 (1.8)	0.003*
Height z-score	188	0.3(1)	183	0.2(1)	0.387
Height (cm)	188	96.5 (4.0)	183	95.1 (4.1)	0.001*

3.4 Child bone outcomes at 3 years of age

3.4.1 Primary outcome: child BMC z-score

DXA measurements showed higher BMC (Figure 4) in boys compared to girls, but no difference was observed in BMD. For both boys and girls, BMC was on average, below mean of the reference population, while BMD was above (indicated by z-scores in Table 12). The primary outcome of interest, child BMC z-score, was calculated by comparing BMC data to age and gender matched reference data that accounted for differences in child height at 3 years of age[32].

3.4.2 Secondary outcome: child bone size

Boys had higher whole body BA compared to girls. There were no gender differences in average femoral and humeral lengths, and no gender differences in bone size factor derived from principal component analysis of whole body BA and bone lengths (Figure 5). The secondary outcome of interest, bone size, was derived by principal component analysis of the femoral and humeral lengths and whole body BA; one component was extracted, reflective of the shared underlying trait of bone size. From this analysis, a standardized score was generated for each child, with a mean of 0, standard deviation of 1, median of -0.052, minimum value of -2.376 and maximum value of 2.027. Scores stratified by gender are shown in Table 12.

Table 12 – Bone outcomes of children at 3 years of age (* indicates statistically significant factors)

Bone outcome	Male (n=188)			Female (n=184)			P-value
	N	Mean (SD)	Median (min-max)	N	Mean (SD)	Median (min-max)	
BMC (g)	185	379 (59)	377 (244-351)	182	355 (60)	352 (236-576)	0.000*
BA	185	530 (73)	522 (367-761)	182	513 (83)	506 (343-870)	0.000*
BMD (g/cm^2)	185	0.72 (0.04)	0.71 (0.62-0.85)	182	0.69 (0.04)	0.69 (0.56-0.91)	0.339
BMC (g) z-score	185	-0.13 (1.4)	-	182	-0.33 (1.8)	-	0.217
BMD (g/cm^2) z-score	185	0.74 (0.95)	-	182	0.80 (0.88)	-	0.579
Average femoral length (cm)	62	21.5 (1.2)	21.6 (18-25)	61	21.6 (1.1)	21.7 (19-24)	0.282
Average humeral length (cm)	53	15.3 (0.92)	15.4 (13-17)	40	15.2 (0.89)	15.2 (13-17)	0.100
Bone size score	51	0.106 (0.985)	0.055 (-2.376-2.027)	39	-0.139 (1.015)	-0.124 (-2.191-1.657)	0.131

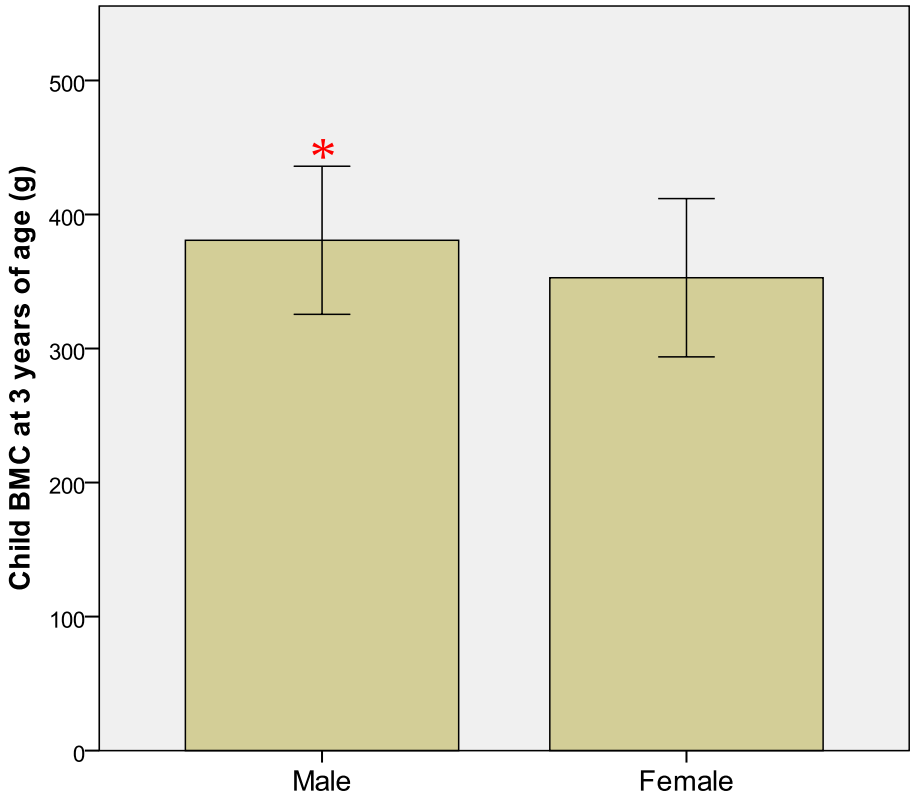

Figure 4 – Whole body BMC of boys and girls at 3 years of age assessed by dual-energy x-ray absorptiometry scans (* p<0.0001); data are mean values and error bars are SDs.

Table 13 – Principal component analysis of long bone lengths and whole body BA – one component was extracted, representative of shared underlying trait of bone size

Factor	Component
	1
Average femoral length	0.904
Average humeral length	0.854
Whole body BA	0.771
Variance explained	71%

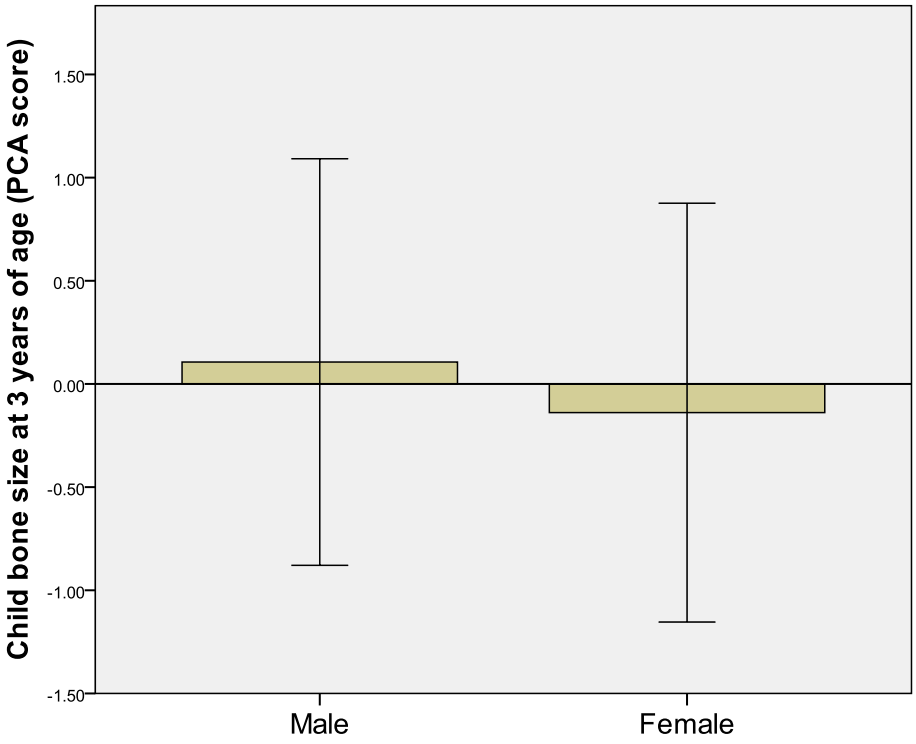

Figure 5 – Bone size score of boys and girls at 3 years of age derived by PCA of whole body BA and femoral and humeral lengths; data are mean values and error bars are SDs.

3.5 Multiple regression analyses

Both non-standardized (b) and standardized (β) regression coefficients are reported for all models. The b tells us that the increase in one unit of the predictor (in the units it was entered into the regression model as) is associated with b amount of change in the outcome (in the outcome's original units), with all other predictors in the model held constant. The β is obtained when all variables are standardized to having a mean of zero and a standard deviation of one. Thus, the β tells us that an increase in one standard deviation of the predictor is associated with β number of standard deviations of change in the outcome. The b is usually preferred for interpretation of the contribution of a predictor because of its simplicity – interpretation is based on the original units of both predictor and outcome. However, the β is useful for comparing the *relative* contributions of the predictors to the outcome, as all the predictors are standardized to the same scale.

3.5.1 Child diet as a predictor of bone variables

For the primary outcome, using the enter method and including total energy intake, vitamin D, calcium and protein intake of the child at 3 years as independent variables, the model that emerged was not significant for predicting BMC z-score ($F_{4,203}$ = 1.637, p=0.166), R-square = 0.013 (Table 14). As well, none of the individual nutrients predicted BMC z-score. For the secondary outcome, again using the enter method with the same independent variables for predicting child bone size, the model that emerged was not significant ($F_{4,76}$ = 1.437, p=0.230), R-square = 0.070. However, the child's total energy intake predicted bone size, while vitamin D, calcium and protein intake did not (Table 14). An increase of one kilocalorie per day in the child's diet was associated with an increase of 0.001 in their bone size score (b=0.001) and an

increase in one standard deviation of kilocalorie per day was associated with an increase of 0.404 standard deviations in their bone size score (β=0.404).

Table 14 - Child diet at 3 years as a predictor of child BMC z-score (R-square = 0.013) and bone size (R-square = 0.070) (* indicates statistically significant factors)

Primary Outcome: child BMC z-score (n = 204)					
Predictor	**β**	**b**	**95% CI**		**p-value**
Vitamin D (per 90 IU/day)	0.135	0.170	-0.090	0.430	0.198
Calcium (per 300 mg/day)	-0.002	-0.003	-0.346	0.340	0.988
Total energy (kcal/day)	0.002	0.000008483	-0.001	0.001	0.987
Protein intake (g/day)	0.062	0.006	-0.019	0.030	0.683
Secondary Outcome: child bone size (n = 77)					
Predictor	**β**	**b**	**95% CI**		**p-value**
Vitamin D (per 90 IU/day)	-0.082	-0.068	-0.321	0.186	0.596
Calcium (per 300 mg/day)	-0.072	-0.062	-0.391	0.267	0.707
Total energy (kcal/day)	0.404	0.001	0.0	0.002	0.036*
Protein intake (g/day)	-0.168	-0.010	-0.035	0.015	0.417

3.5.2. Maternal and child variables as predictors of the primary outcome: BMC z-score

Using the enter method and including all collected maternal and child independent variables, a significant model emerged ($F_{23,180}$ = 2.095, p=0.004), R-square = 0.211 (model 5, Table 15 and 16), suggesting that 21.1% of variation in the outcome of child BMC z-score could be accounted for by the set of predictors entered into the model. In this model, the predictor with the strongest effect on child BMC z-score at 3 years was maternal dietary vitamin D intake during pregnancy, indicated by having the largest β value among all predictors (excluding the interaction term, which indicates the modulatory effect that one predictor has on the other, explained further below).

Maternal vitamin D status was not a significant predictor of child BMC z-score in this model (Table 15). However, maternal vitamin D and calcium intake from food negatively predicted child BMC z-score, while supplements had no effect. An additional 90 IU of vitamin D in the maternal diet during pregnancy (the amount of vitamin D found in a cup of milk) was associated with a 0.818 decrease in the child's BMC z-score, or a decrease of 0.818 standard deviations in the child's whole body BMC. An additional 300 mg of calcium in the maternal diet during pregnancy (the amount of calcium found in a cup of milk) was associated with a 0.379 decrease in the child's BMC z-score, or a decrease of 0.379 standard deviations in the child's whole body BMC (Table 15). A positive interaction was observed between maternal dietary vitamin D and calcium intake, indicating that the increase in one nutrient increased the effect that the other had on the outcome of child BMC z-score.

Maternal dietary protein intake positively predicted child BMC z-score; an additional gram of protein in the maternal diet during pregnancy was associated with a 0.028 increase in the child's BMC z-score, or an increase of 0.028 standard deviations in the child's whole body

BMC. In addition, the mother's own BMD z-score was positively associated with the child's BMC z-score, while maternal BMI and physical activity did not predict child BMC z-score (Table 15). Among the child lifestyle factors included, physical activity on weekdays positively predicted BMC z-score such that an additional hour of activity per day predicted an increase of 0.702 standard deviations in the child's whole body BMC. For birth length, one standard deviation higher than age and gender matched peers was associated with 0.461 standard deviation higher child whole body BMC. Winter born infants had a 0.504 standard deviation higher whole body BMC than summer born infants.

Table 15 – Multiple regression analysis of maternal variables (R-square = 0.211) as predictors of child's 3 year BMC-z-score (* indicates statistically significant factors)

Maternal variable during pregnancy		Model 5a; n = 181				
		β	**b**	**95% CI**		**p-value**
Vitamin D	Serum 25OHD (per 10 nM)	-0.033	-0.012	-0.067	0.042	0.654
	From food (per 90 IU)	-0.923	-0.828	-1.47	-0.187	0.012*
Calcium	From food (per 300 mg)	-0.599	-0.379	-0.611	-0.148	0.001*
Interaction between vitamin D & calcium from food		1.834	0.169	0.055	0.283	0.004*
Vitamin D & calcium supplements		0.135	0.631	-0.048	1.309	0.068
Dietary protein (g/day)		0.800	0.028	0.005	0.052	0.019*
Maternal BMD z-score		0.169	0.293	0.055	0.53	0.016*
Maternal BMI		0.024	0.007	-0.34	0.048	0.730
Maternal physical activity score						
1		0.015	0.049	-0.448	0.547	0.845
2		0.024	0.101	-0.538	0.741	0.755
3		-0.111	-0.723	-1.675	0.229	0.136
>4		-0.089	-1.437	-3.67	0.769	0.206

Table 16 - Multiple regression analysis of child variables (R-square = 0.211) as predictors of child's 3 year BMC-z-score (* indicates statistically significant factors)

Child variable	Model 5a; n=181				
	β	b	95% CI		p-value
Winter birth	0.157	0.504	0.052	0.957	0.029*
Weekday Physical activity at 3 years of age	-0.692	0.702	0.016	1.388	0.045*
Weekend physical activity at 3 years of age	0.323	-0.271	-0.835	0.294	0.346
Birth weight	-0.194	-0.563	-1.166	0.04	0.067
Birth length z-score	0.235	0.461	0.061	0.861	0.024*
Vitamin D & calcium supplement	-0.075	-0.311	-0.875	0.254	0.278

3.5.3 Maternal and child variables as predictors of the secondary outcome: child bone size

Using the enter method of including all collected maternal and child independent variables, a significant model emerged ($F_{22,60}$ = 5.018, p=0.000), R-square = 0.648 (model 5, Table 17 and Table 18), indicating that the predictors entered into this model could account for 64.8% of the variation in the outcome of child bone size. Maternal vitamin D status negatively predicted bone size after adjustment for maternal and child covariates. An additional 10 nM of serum 25OHD in the mother during pregnancy was associated with a 0.062 decrease in the child's bone size score. Maternal height positively predicted bone size, such than an additional centimetre in maternal height predicted a 0.0698 increase in the child's bone size score. As well, maternal BMD z-score negatively predicted bone size, with an additional increase in one standard deviation of BMD in the mother associated with a 0.323 decrease in the child's bone size score.

Higher maternal pre-pregnancy BMI predicted lower bone size, with each additional increase in one unit of BMI associated with a 0.366 decrease in the child's bone size score. When BMI was entered into the model as a squared term to test the assumption of linearity however, a significant positive relationship was observed between maternal $(BMI)^2$ and child bone size. In taking both the original and squared term non-standardized regression coefficients (b) into account, it can be demonstrated that the net effect of BMI on child bone size would be negative at values of BMI less than 52.3 kg/m2 (Appendix J). Given that only one mother in our study exceeded a BMI of 52.3 kg/m^2, the effect of BMI on bone size in this sample and model is negative, with a decreasing negative effect as BMI increases.

Maternal BMD z-score was negatively associated with bone size, such that each additional increase in one standard deviation of maternal BMD predicted a 0.323 decrease in

child bone size score. Children of non-European mothers had larger bones (b = 0.401) (Table 17). Maternal education negatively predicted child bone size, but when entered as a squared term, positively predicted bone size. In taking both the original and squared term non-standardized regression coefficients (b) into account, it can be demonstrated that the net effect of maternal education on child bone size would be positive but only at years of education greater than 39.5 (Appendix K). As the maximum number of years of education acquired by the mothers in our study was 31 years, the effect of maternal education on bone size in this sample and model is negative, with a decreasing negative effect as years of education increase.

Of the child variables, only birth length z-score positively predicted bone size, with both the original and squared term predicting bone size (Table 18); hence the contribution of birth length z-score can be expressed as the combination of two regression coefficients, $Y = 0.515x + 0.160x$ [with other covariates held constant], where x = birth length z-score.

Table 17 - Multiple regression analysis of maternal variables (R-square = 0.648) as predictors of child 3 year bone size (* indicates statistically significant factors)

Maternal variable during pregnancy		Model 5b; n = 61				
		β	**b**	**95% CI**		**p-value**
Vitamin D	Serum 25OHD (per 10 nM)	-0.241	-0.062	-0.111	-0.013	0.015*
	From food (per 90 IU)	-0.448	-0.265	-0.58	0.050	0.098
Calcium	From food (per 300 mg)	-0.027	-0.010	-0.169	0.149	0.901
Interaction between vitamin D & calcium from food		0.499	0.032	-0.011	0.075	0.139
Vitamin D & calcium supplements		0.080	0.221	-0.278	0.72	0.379
Dietary protein (mg/day)		0.067	0.001	-0.007	0.010	0.732
Maternal height (cm)		0.005	0.0698	0.040	0.099	0.000*
Maternal BMD z-score		-0.299	-0.323	-0.534	-0.111	0.003*
Maternal BMI		-1.819	-0.366	-0.713	-0.018	0.040*
Maternal BMI (squared)		1.847	0.007	0.000	0.013	0.036*
Maternal ethnicity: non-European		0.159	0.401	-0.084	0.104	0.104
Maternal education (years)		-2.187	-0.632	-2.187	-3.250	0.002*
Maternal education (years) (squared)		1.987	0.016	0.005	0.026	0.005*

Table 18 - Multiple regression analysis of child variables (R-square = 0.648) as predictors of child 3 year bone size (* indicates statistically significant factors)

Child variable	Model 5b; n = 61				
	β	b	95% CI		p-value
Winter birth	0.117	0.160	-0.189	0.508	0.363
Birth weight	-0.141	-0.251	-0.705	0.203	0.274
Birth length z-score	0.485	0.515	0.202	0.829	0.002
Birth length z-score (squared)	0.391	0.160	0.008	0.313	0.039*
Weekday physical activity score at 3 years of age	-0.082	-0.036	-0.149	0.077	0.527
Weekend physical activity score at 3 years of age	-0.018	-0.007	-0.112	0.097	0.887
Vitamin D & calcium supplement at 3 years of age	0.055	0.105	-0.385	0.595	0.671

CHAPTER 4

DISCUSSION AND FUTURE DIRECTION

Chapter Four

DISCUSSION AND FUTURE DIRECTION

Evidence increasingly supports the concept that early life exposures, including prenatal and early postnatal exposures, influence peak bone mass achieved in late adolescence and consequently, osteoporosis risk in later life. Vitamin D deficiency during pregnancy has been documented in many populations, and there are concerns that this may be associated with long-term bone health outcomes in the child[151].The relevance of such observations to Canadian women is uncertain as no population-based surveys of vitamin D status in pregnant women are available, and considerable controversy exists over the optimal vitamin D intake for pregnant women. In this study of 372 pairs of Canadian mothers and their children, we demonstrated that vitamin D deficiency among Canadian women represented in our sample is rare, and that maternal vitamin D status did not predict whole body bone status of the child at age three years. Further, there may even be a negative effect on offspring bone size at maternal serum 25OHD concentrations that exceed the upper limit of the range above which adverse health effects may occur as suggested in the most recent DRI report[6]. We refuted our hypothesis that higher maternal vitamin D status predicts higher child BMC z-score and bone size. Our findings may differ from previous studies in the United Kingdom[14,15], India[5] and Finland[74] that found a positive relationship between maternal vitamin D status and child bone mass and bone size due to the high frequency of our mothers that had optimal vitamin D status. In addition, we adjusted for most of the key variables that independently predict offspring bone mass that were not included as co-variables in previous studies [5,14,15,74,152]. Such adjustments for confounding variables may contribute to our different findings compared to previous investigations.

4.1 Maternal vitamin D status and bone mass in the child

Our study is the first to assess vitamin D status in a large number of Canadian pregnant women. The prevalence of vitamin D deficiency in our sample (defined as serum 25OHD <30 nM) was 1.4%, which is not dissimilar from what was found in the general Canadian population (4%, defined as <27.5 nM) by the CHMS[153]. In the CHMS, the average vitamin D status of females of child-bearing age (20-39 years old) was 69.5 nM, a value significantly higher than observed in males in the same age category, likely attributable to higher vitamin supplement intake among females[153]. Since our study focused on pregnant women, 83.4% of whom were taking prenatal supplements, it is perhaps not surprising that the average vitamin D status was higher (111.2 (44.1) nM) than in the general population of Canadian women. Among our mothers, serum 25OHD in 94.9% exceeded the 40 nM cut-off used to establish the EAR in the DRI report and 92.9% exceeded 50 nM, the value used to define the RDA[6] (Figure 1). What was of some concern was that 35.6- 17.7% of mothers, respectively, exceeded the cut-off range for the upper limit of intake defined as 125-150 nM, "a concentration which is at the high end of the range of serum levels associated with nadir risk of outcomes such as all-cause mortality"[6]. Although average maternal intake of vitamin D from food did not reach the EAR of 400 IU /day and only weakly predicted vitamin D status, estimated total vitamin D intake from both food and supplements exceeded 400 IU/day in 42.7% of mothers, indicating that maternal need for vitamin D was probably primarily met through supplements. As noted above, 83.4% of mothers in our study took either prenatal supplements (containing between 200 and 400 IU of vitamin D) or vitamin D supplements (up to 1000 IU), or both.

The adequate vitamin D status in the mothers studied may explain the lack of association between maternal vitamin D status and the child's BMC z-score after adjustment for covariates. Since 94.9% of mothers in our study exceeded the cut-off for serum 25OHD of 40 nM (and

92.9% >50 nM), the low prevalence of maternal vitamin D deficiency would limit our ability to detect any potential differences in offspring BMC. In support of this proposition, in the study that established a link between maternal vitamin D status in pregnancy and lower bone mass in the child at 9 years[15], almost half of mothers had sub-optimal vitamin D status (<50 nM). Similarly, Sayers et al.[14] found that maternal vitamin D status was associated with UV-B exposure, which in turn was predictive of the child's bone mass (both whole body BMC and BMD) at 9.9 years of age; however, in their population of 355 mothers, the average serum 25OHD at 36.3 weeks gestation was 53.3 (31.5) nM, which is considerably lower than the average of our mothers 111.2 (44.1) nM). A comparison of dietary and supplement data would have revealed the reasons for these differences; however, neither reported study[14,15] documented maternal vitamin D intake from food, and only Javaid et al[15] reported on supplement use. While 59% of the mothers used supplements of any kind, only 15% took vitamin D supplements[15], compared with 83.4% of our mothers taking supplements containing vitamin D. Thus, our findings indicate that optimizing maternal vitamin D status using readily available vitamin D supplements in our sample of Canadian pregnant women may be all that is needed to benefit offspring bone mass.

4.2 Maternal vitamin D status and bone size of the offspring

A novel way of calculating an index of bone size for children was applied in our study by combining both a whole body skeletal size measure (whole body BA) with specific dimensions (femoral and humeral lengths) to derive a measure reflective of overall bone size, using both information directly available from a whole body DXA scan and a newly developed computer software at McMaster University to estimate bone length from the DXA scan image. Using this approach we observed that maternal vitamin D status negatively influenced bone size (b = -

0.062, β = -0.241) at 3 years of age. This is the first report of such an association between maternal vitamin D status and bone size of the child. In a previous study, higher maternal vitamin D status during pregnancy was predictive of greater tibia BMC and cross-sectional area (CSA) in newborn infants as measured by pQCT, after adjustments for birth weight, maternal height and newborn age at time of measure[74]. Also, neonates born to vitamin D deficient mothers had shorter knee-heel length, which is reflective of shorter long bone length[18].

In interpreting the observations on bone size, key differences between the reported studies compared to our observations should be considered. Firstly, in the Viljakainen et al. study[74], their measure of maternal vitamin D status represented an average of measures during the first trimester and post-partum; this average better predicted newborn bone CSA than a single measure at each time point alone. We had only one measure of maternal vitamin D status from the third trimester. The development of a cartilage framework, which precedes the formation of the fetal skeleton, begins as early as 5 weeks gestation[70,154]. While the third trimester is the period when the majority of fetal bone mineralization occurs, bone size and shape is primarily determined by this cartilage framework[70]. Thus, it is possible that our single measurement lacked the same sensitivity that including a measure from an earlier time point in pregnancy may have offered. In contrast, Morley et al.[18], demonstrated a positive association between knee-heel length and maternal 25OHD in the third trimester only, and not earlier. Since our derived factor for bone size incorporates a direct measure of bone length and only an estimate of bone width offered by whole body BA (rather than a direct measure pQCT would offer), this may suggest that elements of bone dimensions – thickness and length – are determined during different periods *in utero*, with thickness being more sensitive to sufficient 25OHD exposure in early pregnancy and length being more responsive in late pregnancy.

A second issue to consider is differences in the maternal 25OHD status between studies. Of the 99 mothers in the Viljakainen, et al. study[74], 70.7% had vitamin D status less than 50 nM, with an average value of 44.8 (11.9) nM. As well, Morley et al.[18] found no association between maternal vitamin D status and knee-heel length at serum 25OHD concentrations above sufficiency defined as 30-40 nM. In contrast, about 36% of mothers had serum 25OHD above 125 nM in our study. Hence, our findings may be reflective of a negative *in utero* effect on offspring bone size at high serum values of 25OHD.

The notion of a negative effect of higher maternal 25OHD status on bone size is supported by observations of significantly shorter limb lengths in infants born in summer and autumn[16]. In this scenario, the third trimester of pregnancy occurred during spring and summer, periods of presumably higher maternal UV-B exposure and cutaneous vitamin D synthesis. In guinea pigs, a model validated for research on neonatal bone development[155], lower whole body BA and BMC (but not BMD, suggesting an effect on skeletal size)[82], was observed in offspring of vitamin D deficient mothers[156]. This finding is not surprising considering that the comparison was between a highly deficient and a sufficient group; thus any positive effect may be attributed more to correcting a deficiency than conferring additional benefit by higher doses. In contrast, in another guinea pig study that administered high doses of vitamin D to sows, offspring of the high dose group had narrower growth plates than those born to vitamin D deficient sows[156]. Histomorphometry on the bones revealed that the vitamin D deficient group in comparison to the high dose vitamin D group had greater expansion of the hypertrophic chondrocyte area of the proximal tibia, leading to widening of the growth plate[156]. The growth plate is located between the metaphysis (shaft of the bone) and the epiphysis (end of the bone)[157], and proliferation of the growth plate is responsible for long bone lengthening. Hence, this could explain the seasonal

pattern of limb lengths, as well as our finding that lower vitamin D status predicts larger bone size, keeping in mind that we had incorporated long bone lengths into our derivation of bone size factor from principal component analysis. The remaining interpretive challenge is how to understand the guinea pig dosages in the context of human research. There are currently no guidelines in the literature outlining what vitamin D intake or 25OHD serum values may be considered the equivalent of deficient, sufficient or excessive in guinea pigs compared to humans. Although the FDA Center for Drug Evaluation and Research Guide for Industry provides conversion factors to translate doses of drugs given to guinea pigs to a human equivalent doses, based on the assumption of a 1:1 scale of doses between species when body area is normalized[158], it is uncertain whether the same conversion would apply to nutrients.

In addition to acting directly on bone, vitamin D also induces gene transcription via the VDR by forming a heterodimer with the RXR[159]. One of the effects that vitamin D can have at the cellular level via the VDR is a potent anti-proliferative effect, causing cells to exit the cell cycle and even induce apoptosis[160,161]. The potency of the cellular effects of vitamin D depends on a competitive binding process that occurs between the TXR and VDR; at higher concentrations of $1,25OH_2D$, the VDR can displace the TXR, and vice versa[162]. Since the VDR is also found on the placental decidua and is suggested to have some role in regulating placental function and fetal development[163], it is possible that high serum concentrations of 25OHD may also influence fetal skeletal development indirectly via genomic effects.

4.3 Maternal demographics and vitamin D status

Based on our sample of Canadian pregnant women who were predominantly of European origin, concerns for vitamin D deficiency are likely unwarranted. Further, additional vitamin D

intake in this population is also unlikely to result in additional benefit to offspring bone, and may even have negative effects, as discussed above. While reports of vitamin D deficiency are high around the world, a closer look at these studies indicates that those at greatest risk reside in developing countries. Hypovitaminosis D is much more common in women of reproductive age and/or pregnant women in countries in Asia with religious and cultural practices dictating certain clothing, such as veiling[13,53,164]). Skin pigmentation is also a significant factor[11,165,166]. Studies in developed countries also find a negative impact of melanin on cutaneous vitamin D synthesis; in Pittsburg, Pennsylvania, only 5% of Caucasian pregnant women were deficient (defined as <37.5 nM) compared with 29.2% of African American women[54]. Similarly, in London, UK, Indian Asian, Middle Eastern and African American women all had higher rates of vitamin D insufficiency (defined as <50 nM) during pregnancy than Caucasian women[167]. Since over 85% of the mothers in our study were of European descent, this population generally has less difficulty in achieving optimal vitamin D status because of sun exposure and more efficient conversion of 7-dehydrocholesterol to vitamin D in white compared to pigmented skin.

One further caveat to extrapolating our study's results to the general Canadian population is that the composition of our sample was highly homogenous. With almost 80% of mothers in this sample residing in a household with a yearly income greater than $50,000, and 87% of mothers with post-secondary education, this sample represents middle to upper SES in Canada, including individuals with healthier lifestyle choices and habits. In fact, because 96.5% of mothers did not smoking during pregnancy and 95.1% had less than one drink per month during pregnancy, we were unable to include these factors as covariates. Associations between educational attainment, social status and a healthier dietary pattern, as well as lower likelihood of smoking, have been observed in other studies of pregnant women[3]. Consequently, we are one of

the first studies to document the postulated relationship between maternal vitamin D status and offspring bone mass in an affluent population, demonstrating a very different biology than may be expected in another sector of society, or in a developing country.

Another noteworthy observation in our study is that there were a number of maternal characteristics that predicted maternal vitamin D status. Mothers with a higher BMI had lower vitamin D status, which may be due to sequestering of lipid-soluble vitamin D compounds in adipose[71,168], enhanced production of $1,25OH_2D$ leading to negative feedback on 25OHD synthesis[169], avoidance of sun exposure, and lower vitamin D intake in women with a higher BMI[170,171]. Being of European descent predicted a serum 25OHD status approximately 14 nM higher than in non-Europeans, which is consistent with previous research[54,153].

4.4 Maternal lifestyle factors as predictors of child bone outcomes

Maternal education was negatively predictive of offspring bone size, which may be understood in light of the dietary patterns observed to be associated with individuals with higher educational attainment and SES[3], as discussed above. Although maternal serum 25OHD status was not a significant predictor of child BMC z-score at 3 years, maternal intake of calcium and vitamin D during pregnancy was predictive of child bone measures. In both models predicting child BMC z-score and bone size, when maternal vitamin D and calcium intake from food were entered as an interaction term, a positive interaction was observed, indicating that an increase in one nutrient increased the effect of the other on the outcome of child bone measures. This was expected, given the role of vitamin D in promoting calcium absorption in the gut[172]. When considered as independent terms in our multiple regression model, both maternal dietary vitamin D and calcium intake negatively predicted BMC z-score in the child, and dietary vitamin D also predicted lower bone size. While the associations with dietary vitamin D may be understood in

light of the discussion above on serum 25OHD, the finding with calcium intake is initially counter-intuitive. Other studies have reported positive associations between maternal calcium intake during pregnancy and child bone mass at ages 6-10 years old[5,173]. It must be noted that these observations were made in women whose average dietary calcium intake was as low as 952 mg (SD 275)[173] and 268 mg/day (IQR 208-332)[5], the latter being a study on women in rural India. Studies reporting maternal calcium intakes more comparable to those found in our study conclude that it is more the overall dietary pattern that predicts offspring bone mass[3], and that any benefits from calcium are likely site specific, targeting mainly the lumbar spine[4]. High average dietary calcium during pregnancy (greater than 1200 mg/day), such as those observed in our study, have not been associated with offspring bone mass[95] or are even weakly negatively associated with bone mass[4].

Very recent evidence suggests that maternal age impacts offspring bone mass, and that there may be an interaction between maternal age and dietary calcium intake during pregnancy. In a sample of mothers with an average age of 29.5 (SD 4.8) years, higher maternal age predicted lower bone mass in male offspring at the age of 18.9 (0.6) years, after adjustments for maternal physical activity, smoking, and calcium intake during pregnancy, as well as infant anthropometric measures[174]. Further, maternal dietary calcium had different effects on maternal bone resorption during pregnancy depending on the mother's age[175]. In mothers younger than 34 years old, higher calcium intake from food was associated with reduced maternal bone resorption; however, beyond this age, there was an increase in maternal bone resorption[175]. The level of bone resorption during pregnancy influences the amount of calcium available in the maternal circulation, and plays an important role in maintaining sufficient mineral supply to the developing fetal skeleton[57,58,176]. Since the mothers in our study were almost evenly divided

above and below this age threshold (approximately 60% less than 34 years old), this may have influenced our finding of a weak, negative correlation between maternal dietary calcium intake and offspring bone mass.

Existing literature consistently reports that maternal milk intake during pregnancy is predictive of higher offspring birth weight, which is a predictor of bone mass in later life[177,178], and offspring bone mass itself [4,5]. However, most studies indicate that this is not fully explained by considering either vitamin D or calcium alone[4,5,178], and instead suggest that milk proteins may be contributing to the observed benefit on offspring bone[5,178]. Positive associations between maternal total protein intake with offspring bone mass have also been reported widely in literature[3,4,95]. This is consistent with our finding that maternal total protein intake positively predicted offspring BMC z-score. Suggested reasons for the association between maternal protein intake and child bone include the provision of amino acids for collagen formation in the fetal skeleton and the increase of IGF-1 associated with higher protein intake[103-105], since cord IGF-1 levels positively correlate with infant bone mass, after adjustment for other independent predictors of bone mass[108,111,112]. In addition, the acid-ash hypothesis of osteoporosis suggests that high intakes of animal protein lead to mobilization of calcium from bone to act as a buffer against increased acidity from sulfur-containing functional groups on amino acids[103]. This would lead to increased serum calcium, and in the case of pregnancy, an increase of available calcium to the fetus.

4.5 Maternal BMI as a determinant of child bone outcomes

Maternal pre-pregnancy BMI did not predict offspring BMC z-score at 3 years of age. Past studies report that maternal fat stores and pre-pregnancy BMI positively predicted offspring bone mass at birth[86] and 9.9 years old[179], respectively. Interestingly, correlations with paternal

BMI were equally strong[179], suggesting that a shared environment is more likely to explain the association with maternal BMI. Further, the child's own BMI may be a confounder because individuals with higher BMI have higher bone mass due to increased mechanical loading from greater body weight[180], and parental BMI may predict the child's BMI[181]. Considering that nutrition and physical activity have an important role in determining BMI[182], it is perhaps not surprising that the association between maternal pre-pregnancy BMI and bone mass of the 3 year old child was found to be less prominent in our study than the relationship with a 9.9 year old's bone mass[179], because an older child would have been sharing the same lifestyle factors as the parent(s) for much longer.

The negative correlations we observed between maternal BMI and bone size may also be due to different reasons. Approximately 30% of the mothers in our study were overweight, and 23% were obese. Leptin, which is produced by adipose tissue, regulates placental function and influences the intra-uterine environment [183]. One study demonstrated that umbilical venous leptin negatively correlated with male offspring corticol bone size[184] (female offspring were not studied), while another study did not[93]. Offspring of pregnant rats injected with leptin during pregnancy have reduced cortical bone dimensions as well[185]. To our knowledge, few other studies have examined the impact of maternal leptin on offspring bone. Clearly, the role for leptin in fetal and neonatal bone development, particularly in overweight and obese populations, should be investigated in future studies.

4.6 Maternal bone mass as a determinant of child bone outcomes

Our observation of a positive correlation between maternal and child bone mass (standardized regression coefficient of 0.169) suggests a definite genetic component of bone mass, which is consistent with other studies[1,5,85,186]. The association between maternal height and

child bone size however, was much stronger (standardized regression coefficient of 0.512). As our index of bone size partially reflects longitudinal size and there is a genetic component to height[187], this association is intuitive. The stronger association between maternal and child linear dimensions in contrast to bone mass is likely attributable to the pattern of skeletal development in childhood. The skeleton first grows longitudinally, and subsequently mineralization occurs; thus there is a transient decrease in bone density[119]. Thus, it is possible that at age 3 years, the children are further ahead in attaining their genetic potential in longitudinal growth than in bone mineralization.

The negative associations that we observed between maternal BMD z-score and child bone size, however, were unexpected and difficult to reconcile at first. Careful consideration of the bone measurements provided by DXA helped shed light on this. BMD as measured by a DXA scan is obtained by dividing grams of BMC by BA (g/cm^2) thus yielding areal BMD; it is actually not an estimate of true volumetric BMD (g/cm^3). This would mean that BMD is inversely correlated with BA. Considering that our index of child bone size includes a measure of whole body BA, a negative correlation between maternal BMD and child bone size then, may in reality reflect a positive correlation between maternal skeletal size (BA) and child skeletal size.

4.7 Child variables as determinants of bone outcomes

In comparing our 3 year old children to the reference population available, we found that BMC z-score was on average, lower, while BMD z-score was higher, indicating a higher density of minerals per cm^2 of the skeleton, but a lower total amount of mineral content. This observation suggests that children in our sample have smaller, denser bones compared to the reference, which may be explained by differences in ethnic composition. Our sample consisted of mainly Caucasian children, while the reference population consisted of two-thirds African American and Mexican American, and only one-third Caucasian[146]. As this was the only reference data available that included representation of 3 year old children we had no option but to use it. Comparisons between African American and Caucasian children aged between 6-16 years old show that African American children have consistently higher BMC and BMD at all skeletal sites[188]. Other physical measures of the children in our study indicated that they were close to the general population, as indicated in the reference data from CDC[150], in weight and height at 3 years of age.

Gender differences in bone measurements were small at 3 years of age, and primarily reflected differences in skeletal size rather than density, as both BMC and BA were greater in boys, but not BMD. This is consistent with previous studies that observed a faster increase in BMC in males during development, which accounts for higher peak bone mass and skeletal size in males compared to females; the increase in BMD is similar between males and females across all skeletal sites[189]. Also consistent with a study by Clark et al.[19], we saw no gender differences in long bone length, but higher BA in prepubescent boys, suggesting that differences in bone size and periosteal diameter of bones are may not be solely driven by sex hormones.

The child's diet at 3 years of age was not predictive of the child's BMC z-score, whether alone in a model or with maternal factors. Season of birth however, had a significant effect; children born in the winter had significantly higher BMC z-scores than summer-born children. Candidate seasonal effects include variations in UV-B exposure between winter in summer, as well as temperature differences. A previous study reported that maternal UV-B exposure during the third trimester of pregnancy positively predicted offspring BMC at 9.9 years of age, and maternal vitamin D status was suggested to be the explanation for this[14]. Since maternal vitamin D status had no observable effect on the child's BMC z-score in our study, we explored other possible factors to explain the seasonal difference. Birth weight, a predictor of adult bone mass, also fluctuates with season of birth, with lowest birth weights found in spring and summer births[16,190,191]. It has been suggested that colder temperatures during pregnancy induce vasoconstriction in the placenta, affecting delivery of nutrients to the fetus[190]. Another possible explanation is the child's own vitamin D status in early life[76]. At birth, cord blood 25OHD is strongly and positively associated with maternal 25OHD, but consistently lower than maternal concentration[192-194]. Maintenance of infant serum 25OHD is dependent on several factors. The level of vitamin D in human breast milk is very low, and not reflective of the mother's own vitamin D status[64,195,196]; hence, the high frequency of vitamin D sufficiency that we observed among mothers would confer no benefit to the infant that they are breastfeeding. This leaves the infant largely reliant on sun exposure for sufficient dermal vitamin D synthesis and supplementation. We observed that most children in our study were breastfed at some point, but few had vitamin D supplements; thus, sun exposure in early life may have been an important determinant of infant vitamin D status.

4.8 Strengths and weaknesses of the study

As a whole, there were several noteworthy strengths in this study. First, this was a longitudinal birth cohort study with detailed assessment of a wide variety of covariates often omitted by past studies including maternal diet and supplement use, physical activity (both occupational and leisure), BMI, BMD, as well as child lifestyle factors permitted greater control over important variables, as well as the examination of additional correlations. The use of validated questionnaires and trained researchers ensured accurate and consistent measures. Loss to follow-up at three years of the original cohort was less than 10%. Secondly, the use of several novel techniques in our study, including a computer software for measuring bone length, as well as the new gold standard for measuring 25OHD (UPLC-MS/MS), also allowed us to examine our research question with greater clarity.

One limitation in our study includes the fairly high frequency of missing data, which reduced our evaluable sample size for multiple regression models. For example, our attempts to perform DXA scans on the 3 year old children were sometimes unsuccessful because some of the children were frightened by the machine. Other times, a DXA scan of the mother was not possible because she was pregnant. As a complete dataset is required for every participant in order to enter them into the analysis, this reduced our sample size.

Also, volunteer bias limited our ability to generalize the results to larger populations outside of our catchment area. By limiting the selection of participants to mothers interested in research and attending specific hospitals (for example, a hospital associated with an academic institution) within one area, our study tended to attract individuals with higher educational attainment, higher income, and in general, healthier lifestyle choices. Further, the overrepresentation of middle-age, Caucasian women in our study sample suggest that our sample may not be representative of ethnic minorities or younger women in Canada.

We were also not able to account for the father's genetic contribution of bone mass by acquiring a paternal DXA scan, which is shown to also strongly predict the child's bone mass[85]. Lastly, we could only make observations on the children's whole body bone mass, and not on bone quality, as DXA is not able to distinguish between corticol and trabecular bone the way a pQCT scan could.

4.9 Contribution to existing literature and future directions

This study contributes new information on the bone health status of young children. To our knowledge, we have produced one of the largest existing dataset on whole body bone mass in 3 year old children. In a previous study of bone status in children and youth, only 84 children were included to provide reference data for 3 year old children[32,146]. Our data on the dietary practices of 3 year old children also contributes to a gap in literature. As well, our data on maternal use of prenatal and vitamin D supplements and dietary intake (a population not sampled in the CCHS or CCMS) provides information about the dietary habits of women during pregnancy in today's society where vitamin D has been promoted by the popular press; what we may be observing is in fact an over-consumption of vitamin D in women represented in our sample.

Future studies are necessary to examine whether the negative effects of higher maternal vitamin D status on offspring bone size that we observed are long term. There is also evidence that the VDR genotype of the infant modulates the effect of maternal vitamin D status on birth weight, a predictor of adult bone mass[197]; future studies including a genetic measure may shed light on this further. In addition, with increasing rates of obesity in Canada[198], it is also important to determine the effect of maternal leptin on offspring bone development *in utero,* as our study suggests that there may be a detrimental effect. Additional measurements including infant

vitamin D status may help explain the seasonal differences we observed in the child's BMC z-score.

In summary, our study demonstrates that a number of maternal and child lifestyle factors are important predictors of the child's bone mass in early childhood; higher maternal vitamin D status in particular, was a negative predictor of bone size. Our sample consisted mostly of financially secure and well-educated mothers; over half of mothers were overweight, and we found generally adequate levels of nutritional intake and widespread vitamin and mineral supplement use. Thus, our findings contribute to existing literature by illustrating that there may be a very different biology in relating maternal factors to offspring bone health when considering women at the upper end of the spectrum of nutritional status than is found in populations with lower intakes, such as in developing countries. In particular, our data suggest that nutrition during pregnancy should be monitored with caution, especially at high intakes, as there could potentially be negative effects on offspring bone health.

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Appendix A: Standard Operating Procedure for analyzing 3-day diet records

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Data Collection & Diet Record Review

- All 3-day diet records (refer to Appendix D) are to be prospectively completed by the subject (i.e. prior to study visit) so that an accurate record of dietary information is recorded at the time of food intake. If, however, a subject has not completed the 3-day diet record prior to the study visit, they will be asked to complete it and return by mail.
- All diet records are to be reviewed thoroughly, with the subject (at the time of the study visit) to clarify any incomplete information and/or inconsistencies regarding food type, product brand, cooking method and quantity consumed. *Table 1* lists common oversights the researcher should clarify with the subject.
- Each page of the diet and nutrient supplement records **MUST** be labeled (at the time of study visit) with the subject ID# and the visit # on the top of each page (e.g. #101 V1).

Table 1: Common Oversights & Clarification Choices

Type	Common Oversights	Points to Clarify with Subject DURING Diet Record Review
Food type	Not Specific	Questions to ask subject: Rather than provide 'leading' questions (i.e. was the bread whole wheat bread), ask the subject to describe the food item listed on the diet record in more detail – i.e. what type of bread did you eat? Examples: Milk→ 1%? 2%? homo? skim? soy? Bread →white? enriched white? whole wheat? 12 grain? Ranch Salad Dressing → regular? low fat? light? Apple → skin on? skin off? Chicken → breast? leg? skin on? Ground Beef → regular? lean? extra lean? Ham → deli cut? cooked ham roast?
Product Brand	Missing	Questions to ask subject: Do you remember the name brand of the product? Can you describe the package label? Regular or light?
Cooking Method	Missing or Not Specific	Questions to ask subject: What cooking method was used? Any additional ingredients used as part of the cooking process? Examples: - Chicken → Fried in Oil? Breaded? Oven Roasted? - Mashed Potatoes → Boiled and mashed with milk? Instant with butter?
Quantity Consumed	Missing or Not Specific	Questions to ask subject: Unless quantities are recorded (by volume, weight or standard serving quantities i.e. 1 granola bar, 1 cup of cereal/pasta, 3 ounces of meat – not 'granola bar', '1 *bowl* of cereal', '1 *slice* of meat') on the diet record, always ask subjects to estimate the quantity of food consumed by comparing the amount eaten to the size of 'standard objects' such as the following: - 3 ounces of cooked meat = the size of a deck of cards or a bar of soap - 1 cup of cereal or mixed vegetables/fruit, cooked pasta/rice = the size of a baseball - 2 tablespoons = the size of a ping pong ball - 1 teaspoon = the size of a woman's thumb (from top of thumb to the first joint) - 1 ½ ounces of cheese = 6 stacked dice
Combination Foods	Missing or Not Specific	Questions to ask subject: - What food items are in the combination food? Examples: - Chicken Stir-fry → 3 ounces of roasted chicken breast, ¼ cup broccoli sautéed, ¼ cup red peppers sautéed, ¼ cup mushrooms sautéed, ¼ cup onions sautéed, ¼ cup carrots sautéed, 2 tbsp olive oil

Food Item Entry into *Nutritionist Pro* Diet Record (refer to Figure 1):

Personnel not familiar with the Nutritionist Pro software must complete the Nutritionist Pro training tutorial prior to diet record analysis.

1. After subject client file has been created in *Nutritionist Pro*, begin the process of entering the food items recorded in the diet record for analysis,
    - Label the diet record as: Family 3YR# _ _ - _ _ _ _ - _ _
2. Select **Add Food** in the subject's diet record menu template
    - Right click in the menu template and select add food OR
    - Click the Add Food icon (half-eaten apple) in the toolbar on the top of the page
3. Search the *Nutritionist Pro* database for the food item
    - In the food item search box, enter food item as recorded on the subject's diet record (include brand if available).
    - Browse the search results for the specific food item (brand included) entered from the 'USDA Standard Reference Database'
    - If a 'USDA Standard Reference Database' entry for the specific food item is not available in the *Nutritionist Pro* database, browse the search results for the specific food item (brand included) entered under the 'Brand' Reference Database.
    - If the specific food item (brand included) that is recorded on the diet record is **NOT** found in the in the 'USDA Standard Reference' or the 'Brand' Reference databases, a new food item must be created within the *Nutritionist Pro Database*.
4. Search the Internet for nutrition information on the specific food item (brand included) and input a new food item into the Nutritionist Pro Database.

    Note: For instructions on how to input a new food item into the Nutritionist Pro Database, refer to Appendix C.1: Entering a New Food item by Brand.
    - Hierarchy of internet resources:
        - USDA Website
            - The USDA "What's In The Foods You Eat" Search Tool is available at: http://199.133.10.140/codesearchwebapp/%28m12asau2bp5clino5ycg3a45%29/codesearch.aspx
                - *See appendix C.2 for a screenshot of the USDA website*
            - Search for the specific food item (brand included) on the USDA website and enter the nutrient information provided into the *Nutritionist Pro database*.

- Company Website
    - If nutrition information for the specific brand is **NOT** provided by the USDA website, a new food item must be created by inputting the nutrition information found on the 'Nutrition Facts' label provided by the company manufacturing the food item.
    - Company websites can generally be located by searching the company/manufacturer's name in Google.
      NOTE: Ensure that you are using the Canadian website
    - Browse the company website for product nutrition information and enter the nutrition information provided for the specific food item into the *Nutritionist Pro* database.
- Daily Plate
    - If nutrition information for the specific brand of food is **NOT** provided by the USDA website or the manufacturer's website, search for nutrition information for the specific food item (brand included) on the DAILY PLATE
    - The Daily Plate website is available at:
      http://www.livestrong.com/thedailyplate/
        - *See appendix C.2 for a screenshot of the Daily Plate website.*
    - Enter the name of the specific food item (brand included) in the search box and select *Food & Fitness* from the drop down box
    - From the search results, select the most appropriate option available for the specific food item and enter the nutrition information into the *Nutritionist Pro* database.

5. Search *Nutritionist Pro* database for comparable food item.
    - If nutrition information is **NOT** provided by the USDA website and the label provided by the company manufacturing the food item is **NOT** available on the manufacturer's website or the DAILY PLATE, refer back to the Nutritionist Pro database and select a comparable food item.
    - Food selection choices should be made from the 'USDA Standard Reference Database' or from the 'Brand' Reference Database.
    - (*Helpful hint: select smooth peanut butter from the 'USDA Standard Reference Database' if nutrition information on KRAFT smooth peanut butter is unavailable).*

6. Search USDA website for comparable food item and input a new food item into the *Nutritionist Pro Database.*
    - If there is no comparable food item in the 'Nutritionist Pro Database', search on the USDA website for the general food item. The USDA website contains averages and general entries of various food items.

Note: When multiple comparable food items are available, choose the food item that is average in caloric value among the choices—i.e. not the extremes.

Trouble Shooting

- If quantity is not recorded on the diet record, then a standard portion size is always used as the default quantity. Refer to the FAMILY Toddler and Pre-School (age 3-5) Food Frequency Questionnaire. As a secondary resource, refer to the USDA website. This website provides standard information on portion sizes for the most common food items.
- When the brand of the product is not specified, either pick the brand they had on other days OR if non-existent, pick USDA
    - Always pick the "most common" option that is comparable to the food item recorded on the diet record
    - Ex. if the child had Neilson milk on day 1, they most likely had Neilson milk on day 2, even though it isn't recorded.
    - When the product details are not specified (i.e. **1%** milk, **whole wheat** bread), either pick the type they had on other days OR if-non-existent:
        - Ex. 1 If percentage of milk not specified anywhere on the diet record, select 2% milk (most common percentage drank by children)
        - Ex. 2 If bread type is not specified
            - If they routinely have whole wheat products i.e. whole wheat pasta, select whole wheat bread as this would be reflective of their usual food habits
            - If there is no discernable pattern in their food choices, input ½ slices of white bread and ½ slice of whole what bread, to get an average.

Exporting Diet Record to Shared Drive from *Nutritionist Pro*:

- After the entire diet record has been inputted into *Nutritionist Pro*, begin the process of exporting the diet record to the shared drive.

1. Save completed diet record in Nutritionist Pro
   - Diet records should be saved as FAMILY 3YR# (subject's client number)
2. Select **file** and then **print**. Check off **nutrient analysis** and then select **preview** to view a breakdown of the child's daily intake.
   - Check for any extreme values (*i.e. 500% daily intake of saturated fats*). This process will help to catch any errors made while inputting the diet record.
   - If any value appears abnormal based on the child's intake, review the data inputted into Nutritionist Pro.
   - Ensure that the correct serving sizes were inputted and look for food items that are irregularly high or low in the nutrient in question.
3. Close the preview window. Check off **extract file** and then click **extract** (ensure nutrient analysis is still selected).
4. Save the export in the **Family Diet Record Exports** folder.
5. Open **Excel**. Go to **My Documents**. Select the **Family Study Diet Record Exports** folder.
6. Select your subject ID #. Click YES to the ERROR Message and CLOSE for the next message.
7. Delete the following rows IN THIS ORDER: 9, 17, 18. These are rows that correspond to empty cells in both of the large columns.
8. Copy the first large column on the left. Open a new excel document and paste the column in it by clicking **paste special**, and selecting **transpose**.
   - This will convert the large, vertical column into a horizontal row
9. Repeat step 8 for the large column on the far right. **Transpose** the column into the new excel document in the next available cell (directly beside left column that was previously transposed).
   - From the two, large vertical columns in the original extracted excel file, you should now have one, long horizontal row of data.
10. Click on the **Citrix** icon located on the desktop. Log in to access the shared drive
    - **Username**: ngmel
    - **Password:** vitDbmc3y
11. Select the **Windows Explorer** icon at the bottom, right hand side of the page, and then select the **Peds Nutrition on 'ipans01\peds$'(Z:)** icon
12. Open the **FAMILY Diet Records** folder, click on the **3 Year folder**, and then select the appropriate excel file that corresponds to your diet record:

- **Priority 1 mother child DXA pair diet records, log and nutrient file**
- **Priority 2 child only list, log and nutrient file**
- **Priority 3 mother only list, log and nutrient file**

13. Copy and paste the transposed data into the nutrient file tab and complete the chart under the log tab.
14. Save the excel file.
15. Open the Nutritionist Pro Training Log file
    - Record your name, the subject I.D.# and the date that the diet record was completed.
16. Save the excel file. Log out of the shared drive.

Diet Record Analysis: Standardization

- Personnel will be considered 'qualified' to analyze diet records using *Nutritionist Pro* only after successfully completing an independent analysis of 10 diet records previously analyzed by the Study Coordinator. As per the INTERMAP UK Standardized Coding of Diet Records Study (Conway *et. al, 2004)*, a successful completion of the independent diet record analysis will be defined as achieving no more than 6% error rate (per diet record) as expressed as a percentage of the total number of coding differences from the original diet record analysis.
- Ongoing verification that standardized food item selection using *Nutritionist Pro* database for diet record analysis will occur. For every 30 diet records analyzed, 3 random records will be reanalyzed by the Study Coordinator to ensure that no more than 6% error rate (per diet record) exists. If discrepancies are discovered during the verification process, the Study Coordinator will investigate the issue and report the outcome to the Principal Investigator in order to determine if further system analysis and/or personnel training is required.
- Once subject diet records are analyzed, all data files **MUST** be saved and backed-up by external hard-drive and network drive as directed by the Study Coordinator. Individual data files will be labeled according to study subject ID #, visit # and diet day, e.g. FAMILY Study Diet Record V1. (Note: subject # is already recorded when identification # is entered into the client record file) .

Entering a New Food Item by Brand

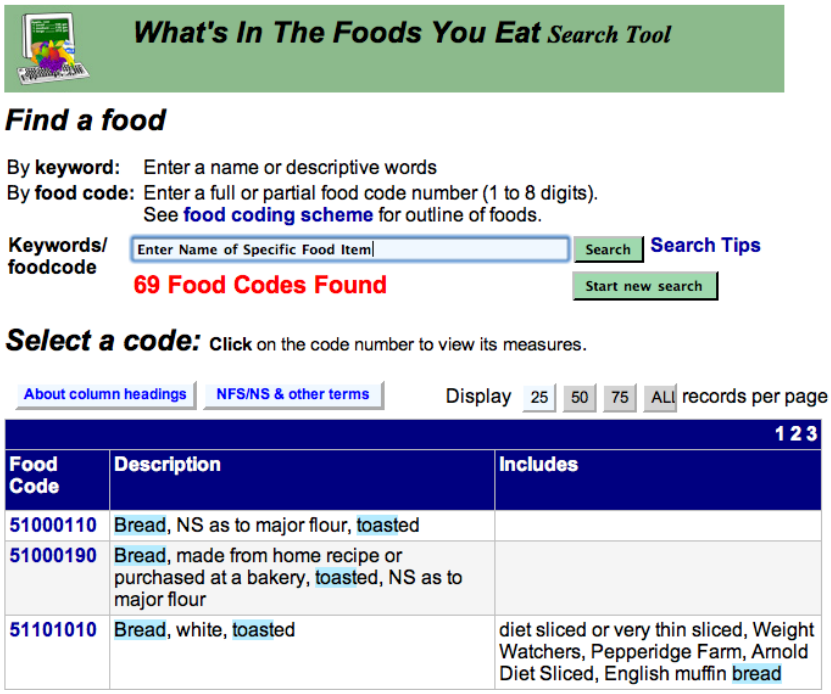
- If a specific food item brand is recorded on the diet record but **NOT** found in the *Nutrionist Pro* database, a new food item must be created within the *Nutrionist Pro* database.
- This is done by entering the nutrient information found on the 'Nutrition Facts' label provided by the company manufacturing the food item. *(Helpful hint: use the internet and search by manufacturer's name → i.e. 'Tim Horton's Ice Cappuccino – search internet for Tim Horton's website)*

1. In an open **Nutritionist Pro** menu template select **File → New → Food → Basic Food**
2. Input the food items name in the **Name (New Food)** text box along the top of the page
   - The food items BRAND should be inputted in ALL CAPS
   - Include as much information as possible in the name (i.e. low sodium, no sugar added, light, etc.)
   - Ex. KRAFT Light Smooth Peanut Butter (25% less fat)
3. Under the **General Tab:**
   i. Click the **Reference** drop down list and select Brand
      - ALL food items inputted manually will be listed under the **Brand** reference category
   ii. Input the **Serving amount** provided on the 'Nutrition Facts' label
      - Ensure that you select the appropriate unit of measurement from the serving amount drop box (i.e. cups, mL, grams, etc.)
      - A **Gram weight** value must ALWAYS be entered when inputting a new food item
        - If the **gram weight** for the specific food item is not available in the nutrition fact label, search for a comparable food item on the USDA website or daily plate website.
        - Liquids are an exception:
        - Ex. Creating new food item for ALLEN's Apple Juice
          - Gram weights are typically not provided for liquids
          - The serving amount on the ALLEN'S Apple Juice 'Nutrition Facts' label is 1 cup (250 mL)
          - Input 1 gram as the weight value for all liquids; there is no meaning to reporting grams of liquid.
        - *NOTE: if the gram weight and another unit of measurement is provided on the 'Nutrition Facts' label, be sure to incorporate both values into the new food item instead of using only the gram weight as the serving amount. This will make it easier for people in the future to input this food item.*

- *Ex. The 'Nutrition Facts' label for smooth peanut butter tells us that 2 tablespoons is equal to 32 grams*
- *Input 2 tablespoons as the **Serving amount** and 32 grams as the **Gram weight***
- *This makes the food item more applicable and provides more options for selecting the serving amount when inputting the food item in the future.*
- *Ex. Recorded on diet record that child had 0.25 cups of smooth peanut butter*
  - *If 32 grams were inputted as the **serving amount** and the **gram weight** for smooth peanut butter, cups would not be available as a serving amount option for this food item.*

4. Click the Nutrients Tab
   - Input the values provided on the 'Nutrient Facts' label
   - NOTE:
     - For vitamins, ALWAYS input using international units (IU) UNLESS a different unit is provided on the 'Nutrient facts' label
     - I.e. Nutritional information provided by the USDA website gives the value of vitamins in mcg
5. Save the New Food Item
   - While the New Food Item is still open, click the Save icon in the toolbar

USDA Website:



What's In The Foods You Eat *Search Tool*

Find a food

By **keyword:** Enter a name or descriptive words
By **food code:** Enter a full or partial food code number (1 to 8 digits).
See **food coding scheme** for outline of foods.

Keywords/ foodcode Enter Name of Specific Food Item | Search | Search Tips

69 Food Codes Found | Start new search

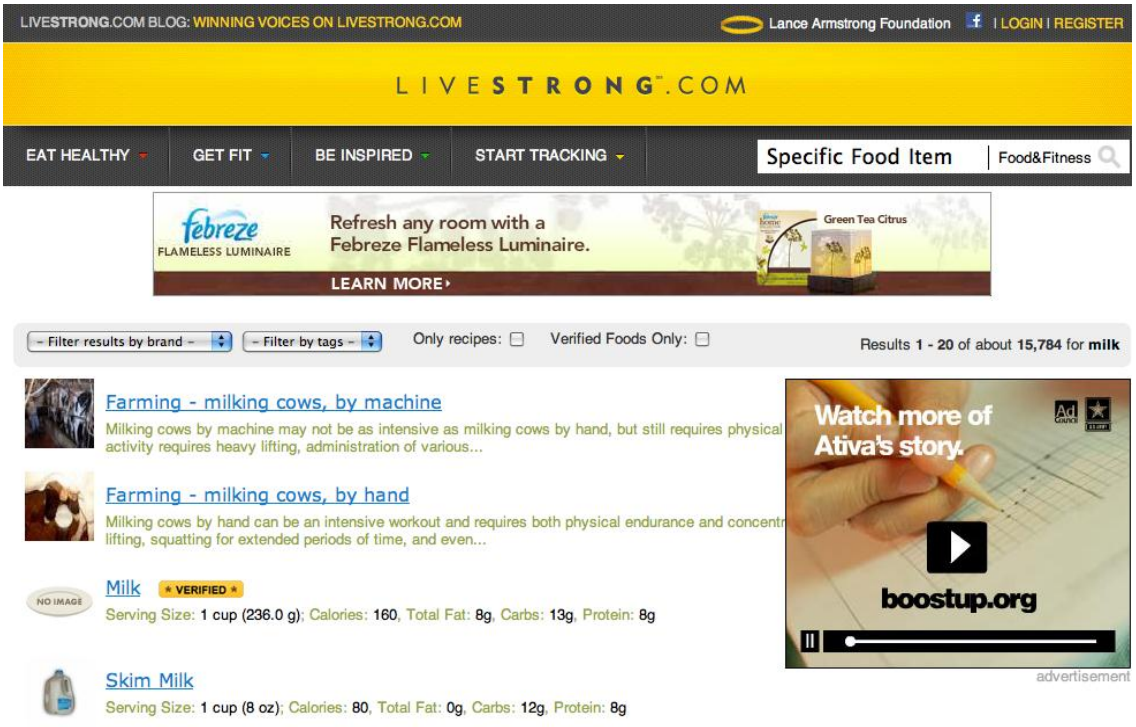
Select a code: **Click** on the code number to view its measures.

About column headings | NFS/NS & other terms | Display 25 50 75 ALL records per page

1 2 3

Food Code	Description	Includes
51000110	Bread, NS as to major flour, toasted	
51000190	Bread, made from home recipe or purchased at a bakery, toasted, NS as to major flour	
51101010	Bread, white, toasted	diet sliced or very thin sliced, Weight Watchers, Pepperidge Farm, Arnold Diet Sliced, English muffin bread

Daily Plate Website:



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My signature indicates I have read and understood the procedures described, and they have been reviewed with me by my trainer.

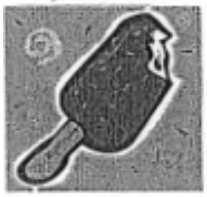
_____	_____	_____
Trainee Name (print)	Signature	Date
_____	_____	_____
Trainer Name (print)	Signature	Date

McMaster University **3 Day Diet Record**
DECCODE

HOW TO FILL OUT THIS DIET RECORD

We would like to know about your usual eating habits OVER THE FOLLOWING WEEK. Complete the diet record with as much detailed information as possible. Please indicate the day of the week and the date on each sheet in the area indicated.

1. Fill out a sheet for each day of the three (3) required; 2 weekdays and 1 weekend day.



2. For each meal/snack indicate the place (e.g. Home or McDonald's) and the time the food was eaten.

3. In the "**Food**" column give the general food name (e.g. tuna, bread).

4. In the "**Description of Food or Drink, Brand Name**" column provide a description of the type of food (e.g. bread - white) and/or preparation (e.g. tuna – baked). If the item is made up of several food items, such as a sandwich, record each item in the sandwich on a separate line. If applicable, provide a brand name for the food consumed (e.g. Oreo).

5. In the "**Amount Consumed**" column provide exact weights determined from the package labeling. Volumes may also be reported. Please use standard measurement units (e.g. grams, cups, tsp, ounces) or dimensions. Please do not use vague descriptions, such as a chunk or a serving.

6. Include all vitamin and/or mineral supplements, as well as medications taken on the separate sheet provided.

7. Please do not write in the last three columns on the far right.

Place Eaten	Time Served	Food	Description of Food or Drink	Amount Consumed
Home	12:30	tuna	Canned in water, Clover Leaf	25 grams
Home	5:45	Salad	Lettuce	¼ cup
			Celery	2 tbsp
			Tomato	¼ of small
			Cucumber, with skin	4 slices
			Italian dressing, light, Kraft	2 tbsp
McDonald's	8:00	Ice cream	Oreo McFlurry	Small

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DECCODE Study
Participant's Center No.: _____

3 Day Diet Record
Recruitment No.: _____

Initials: ___ ___
F M

Day of the Week: _____
Date: _____

Place Eaten	Time Served	Food	Description of Food or Drink Brand Name (If Applicable)	Amount Consumed	For Investigators Use Only		
					D.M. Code	Food Code	Amount Code

Appendix B: Comparison of 3-day diet record analysis by three different research staff

Descriptives

		N	Mean	Std. Deviation	Std. Error
total protein intake	1.00	23	53.2804	15.93664	3.32302
	2.00	23	53.6019	18.07812	3.76955
	3.00	23	57.8020	15.61573	3.25610
	Total	69	54.8948	16.46520	1.98218
total calcium intake	1.00	23	863.2607	331.07615	69.03415
	2.00	23	844.9680	404.94996	84.43790
	3.00	23	838.4457	251.58814	52.45975
	Total	69	848.8915	330.31285	39.76499
total vitamin D intake	1.00	23	237.2220	162.30668	33.84328
	2.00	23	255.2923	149.78234	31.23178
	3.00	23	288.1006	151.70012	31.63166
	Total	69	260.2050	153.87193	18.52400
total caloric intake	1.00	23	1331.9493	340.69390	71.03959
	2.00	23	1385.6275	383.13051	79.88823
	3.00	23	1462.7759	279.45021	58.26940
	Total	69	1393.4509	336.50284	40.51018

Figure 6 – Descriptives of dietary analysis performed by three different research staff (Research staff #1, 2, and 3)

Test of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
total protein intake	1.084	2	66	.344
total calcium intake	1.061	2	66	.352
total vitamin D intake	.086	2	66	.917
total caloric intake	.982	2	66	.380

Figure 7 – Levene's test reveals homogeneity of variances of the dependent variable (protein, calcium, vitamin D, total caloric intake) across groups

ANOVA

		F	Sig.
total protein intake	Between Groups	.533	.590
total calcium intake	Between Groups	.034	.967
total vitamin D intake	Between Groups	.639	.531
total caloric intake	Between Groups	.875	.422

Figure 8 – Comparison of means for each nutrient revealed no significant differences between dietary analyses done by research staff # 1, 2, and 3.

Appendix C: Child factors alone as predictors of BMC z-score

N = 181; R-square = 0.073

Predictor	b	SE	β	p-value
Gestational age	-.116	.092	-.121	.212
Birth length z-score	.290	.199	.175	.146
Birth weight	-.108	.379	-.036	.777
Child energy intake (kcal/day) at 3 years ofa ge	.000	.001	-.031	.818
Child protein intake (g/day)	.002	.014	.022	.880
Child vitamin D intake (per 90 IU) at 3 years of age	.171	.143	.132	.233
Child calcium intake (per 300 mg) at 3 years of age	.043	.188	.031	.818
Season of birth	.425	.244	.132	.083
Child use of vitamin D and calcium supplements at 3 years of age	-.024	.318	-.005	.941
Exclusively breastfed for > 6 months	.368	.649	.043	.571
Vitamin D supplements at 6 months	.240	.396	.044	.546
Child weekday physical activity at 3 years of age	.100	.077	.108	.195
Child weekend physical activity at 3 years of age	-.060	.075	-.066	.424

Appendix D: Maternal factors alone as predictors of BMC z-score

N = 323; R-square = 0.011

	B	Std. Error	Beta		
Maternal 25OHD (per 10 nM)	-.004	.020	-.012	-.215	.830
Maternal age (years)	-.003	.019	-.008	-.130	.896
Maternal BMI (kg/m2)	-.002	.015	-.006	-.114	.910
Maternal calcium intake (per 300 mg)	-.079	.071	-.129	-1.121	.263
Maternal education (years)	.036	.029	.071	1.232	.219
Maternal vitamin D intake (per 90 IU)	.066	.057	.080	1.153	.250
Maternal protein intake (g)	.003	.004	.069	.674	.501
Maternal use of vitamin D and calcium supplements	.124	.244	.028	.507	.612

Appendix E: Sensitivity Analysis

	Without imputation				With imputation			
Predictor	**b**	**CI**		**p-value**	**b**	**CI**		**p-value**
Maternal 25OHD (per 10 nM)	-.006	-.065	.053	.840	-.013	-.068	.042	.644
Maternal age (years)	-.011	-.068	.045	.693	-.015	-.066	.037	.571
Maternal height (m)	-1.319	-5.210	2.571	.504	.011	-3.438	3.460	.995
Maternal BMI (kg/cm2)	-.008	-.054	.038	.736	-.002	-.044	.040	.926
Maternal calcium intake (per 300 mg)	-.205	-.414	.003	.054	-.134	-.318	.050	.153
Maternal education (years)	-.012	-.098	.074	.776	-.010	-.086	.065	.790
Maternal vitamin D intake (per 90 IU)	.204	.035	.374	.019	.160	.003	.317	.046
Maternal protein intake (g/day)	.010	.000	.021	.057	.009	.000	.018	.059
Birth length z-score	.498	.001	.994	.049	.242	-.161	.645	.238
Birth weight	-.700	-1.472	.071	.075	-.305	-.943	.333	.347
Gestational age	.365	.074	.657	.014	.067	-.096	.230	.419
Maternal BMD z-score	-.582	-1.235	.071	.080	.333	.074	.591	.012
Child vitamin D & calcium supplement use at 3 years of age	.303	-.453	1.059	.430	-.337	-.917	.243	.253
Maternal ethnicity	-.017	-.584	.551	.954	.026	-.651	.704	.939
Maternal physical activity score 1	.126	-.609	.861	.735	.154	-.363	.672	.557
Maternal physical activity score 2	-.903	-1.967	.161	.096	-.001	-.661	.660	.999
Maternal physical activity score 3	-1.320	-3.672	1.031	.269	-.651	-1.626	.325	.190
Maternal physical activity score 4+	.747	-.475	1.969	.229	-1.476	-3.797	.845	.211
Exclusively breastfed at 6 months	.478	-.052	1.007	.077	.402	-.727	1.532	.483
Season of birth	-.031	-.236	.174	.765	.375	-.093	.843	.116
Maternal calcium and vitamin D supplements	.653	-.117	1.423	.096	.429	-.249	1.108	.213

Supplement use at 6 months	.537	-.447	1.520	.283	.660	-.271	1.592	.164
Child weekday physical activity at 3 years of age	.059	-.102	.220	.472	.091	-.052	.233	.210
Child weekend physical activity at 3 years of age	.017	-.146	.179	.840	-.003	-.148	.142	.968

Appendix F: Models 1a-4a and 6a for predicting primary outcome of child BMC z-score

Model 1a: N = 185; R-square = 0.144

Predictor	Beta	SE	CI		p-value
Maternal 25OHD (per 10 nM)	-.013	.028	-.068	.042	.644
Maternal age (years)	-.015	.026	-.066	.037	.571
Maternal height (m)	.011	1.748	-3.438	3.460	.995
Maternal BMI (kg/cm2)	-.002	.021	-.044	.040	.926
Maternal calcium intake (per 300mg)	-.134	.093	-.318	.050	.153
Maternal education (years)	-.010	.038	-.086	.065	.790
Maternal vitamin D intake (per 90 IU)	.160	.079	.003	.317	.046
Maternal protein intake (g/day)	.009	.005	.000	.018	.059
Birth length z-score	.242	.204	-.161	.645	.238
Birth weight (g)	-.305	.323	-.943	.333	.347
Gestational Age	.067	.083	-.096	.230	.419
Mom BMD Z-score	.333	.131	.074	.591	.012
Child vitamin D and calcium supplements at 3 yrs	-.337	.294	-.917	.243	.253
Maternal ethnicity	.026	.343	-.651	.704	.939
Maternal physical activity score 1	.154	.262	-.363	.672	.557
Maternal physical activity score 2	-.001	.335	-.661	.660	.999
Maternal physical activity score 3	-.651	.494	-1.626	.325	.190

Maternal physical activity score 4+	-1.476	1.176	-3.797	.845	.211
Exclusively breastfed for 6 months	.402	.573	-.727	1.532	.483
Season of birth	.375	.237	-.093	.843	.116
Maternal vitamin D and calcium supplement use	.429	.344	-.249	1.108	.213
vitamin D supplement at 6 months	.660	.472	-.271	1.592	.164
Child weekday physical activity at 3 years of age	.091	.072	-.052	.233	.210
Child weekend physical activity at 3 years of age	-.003	.074	-.148	.142	.968

Model 2a: N = 191; R-square = 0.135

Variables with p-value < 0.25 or of clinical importance from model 1 were kept for model 2.

Predictor	**Beta**	**SE**	**CI**		**p-value**
Maternal 25OHD (per 10 nM)	-.017	.027	-.069	.036	.534
Maternal BMI (kg/cm2)	-.002	.020	-.042	.037	.902
Maternal calcium intake (per 300mg)	-.129	.092	-.310	.051	.159
Maternal vitamin D intake (per 90 IU)	.163	.077	.011	.314	.035
Maternal protein intake (g/day)	.009	.005	-.001	.018	.064
Birth length z-score	.215	.196	-.170	.601	.272
Birth weight (g)	-.162	.282	-.717	.394	.566
Mom BMD Z-score	.302	.123	.060	.545	.015
Child vitamin D and calcium supplements at 3 yrs	-.339	.287	-.906	.227	.239

Maternal physical activity score 1	.124	.254	-.377	.625	.626
Maternal physical activity score 2	-.056	.324	-.695	.582	.862
Maternal physical activity score 3	-.665	.487	-1.627	.296	.174
Maternal physical activity score 4+	-1.469	1.159	-3.756	.818	.207
Season of birth	.319	.230	-.135	.774	.168
Maternal vitamin D and calcium supplement use	.407	.337	-.258	1.072	.228
vitamin D supplement at 6 months	.739	.459	-.167	1.645	.109
Child weekday physical activity at 3 years of age	.090	.070	-.048	.227	.201
Child weekend physical activity at 3 years of age	-.007	.072	-.148	.135	.928

Model 3a: N = 185; R-square = 0.165
Model 2 plus one interaction formed model 3.

Predictor	**Beta**	**SE**	**CI**		**p-value**
Maternal 25OHD (per 10 nM)	-.020	.026	-.072	.032	.452
Maternal BMI (kg/cm2)	-.001	.020	-.042	.039	.945
Maternal calcium intake (per 300mg)	-.241	.106	-.450	-.032	.024
Maternal vitamin D intake (per 90 IU)	-.144	.185	-.509	.221	.437
Maternal protein intake (g/day)	.010	.005	.001	.019	.027
Birth length z-score	.419	.204	.016	.822	.042
Birth weight (g)	-.524	.307	-1.130	.083	.090
Mom BMD Z-score	.308	.122	.068	.549	.012
Child vitamin D and calcium supplements at 3 yrs	-.310	.290	-.883	.263	.287

Maternal physical activity score 1	.070	.254	-.431	.571	.783
Maternal physical activity score 2	.161	.328	-.486	.808	.625
Maternal physical activity score 3	-.752	.482	-1.703	.199	.120
Maternal physical activity score 4+	-1.226	1.145	-3.485	1.034	.286
Season of birth	.454	.232	-.004	.912	.052
Maternal vitamin D and calcium supplement use	.655	.346	-.029	1.338	.060
vitamin D supplement at 6 months	.795	.455	-.103	1.693	.082
Child weekday physical activity at 3 years of age	.104	.070	-.033	.242	.136
Child weekend physical activity at 3 years of age	-.023	.072	-.165	.119	.752
Interaction - maternal vitamin D and calcium intake	.042	.023	-.003	.087	.066

Model 4a: N = 174; R-square = 0.227

Model 3 plus each continuous variable entered as a squared term formed model 4. Squared terms with p-value < 0.1 were kept for subsequent models.

Predictor	**Beta**	**SE**	**CI**		**p-value**
Maternal 25OHD (per 10 nM)	-.009	.121	-.249	.231	.941
Maternal BMI (kg/cm2)	.040	.173	-.302	.381	.819
Maternal calcium intake (per 300mg)	-.736	.268	-1.265	-.207	.007
Maternal vitamin D intake (per 90 IU)	-.921	.405	-1.721	-.122	.024
Maternal protein intake (g/day)	.039	.014	.010	.067	.008
Birth length z-score	.465	.207	.057	.874	.026

Birth weight (g)	-.218	1.697	-3.567	3.132	.898
Mom BMD Z-score	.334	.241	-.141	.809	.167
Child vitamin D and calcium supplements at 3 yrs	-.332	.294	-.912	.248	.260
Maternal physical activity score 1	.044	.256	-.460	.549	.862
Maternal physical activity score 2	.130	.330	-.521	.780	.695
Maternal physical activity score 3	-.705	.492	-1.676	.266	.154
Maternal physical activity score 4+	-1.356	1.163	-3.651	.939	.245
Season of birth	.499	.235	.036	.963	.035
Maternal vitamin D and calcium supplement use	.656	.352	-.039	1.350	.064
vitamin D supplement at 6 months	.830	.458	-.073	1.734	.071
Child weekday physical activity at 3 years of age	.695	.355	-.005	1.395	.052
Child weekend physical activity at 3 years of age	-.365	.296	-.950	.221	.220
Interaction - maternal vitamin D and calcium intake	.170	.059	.052	.287	.005
Maternal 25OHD (per 10 nM) - squared	.000	.005	-.010	.010	.985
Maternal BMI (kg/cm2) - squared	-.001	.003	-.006	.005	.854
Maternal calcium intake (per 300mg) - squared	.024	.016	-.008	.057	.139
Maternal protein intake (g/day) - squared	.000	.000	.000	.000	.041
Birth length z-score - squared	-.104	.138	-.376	.169	.453
Birth weight (g) - squared	-.043	.243	-.522	.436	.860
MomBMDZscore - squared	-.031	.114	-.256	.194	.786
Child weekday physical activity at 3 years of age - squared	-.037	.021	-.078	.005	.085

Child weekend physical activity at 3 years of age - squared	.023	.017	-.012	.057	.196
Maternal vitamin D intake (per 90 IU) - squared	.037	.049	-.059	.133	.447
Interaction - maternal vitamin D and calcium intake - squared	-.001	.000	-.002	.000	.029

Model 6a: N = 174; R-square = 0.214

Model 5 minus outliers (determined by box-plot of standardized residuals) formed model 6.

Predictor	**Beta**	**SE**	**CI**		**p-value**
Maternal 25OHD (per 10 nM)	-.006	.024	-.054	.041	.790
Maternal BMI (kg/cm2)	.000	.018	-.037	.036	.997
Maternal calcium intake (per 300mg)	-.299	.107	-.511	-.087	.006
Maternal vitamin D intake (per 90 IU)	-.607	.290	-1.179	-.035	.038
Maternal protein intake (g/day)	.025	.011	.004	.046	.019
Birth length z-score	.390	.181	.033	.747	.032
Birth weight (g)	-.262	.272	-.800	.275	.337
Mom BMD Z-score	.225	.106	.016	.434	.035
Child vitamin D and calcium supplements at 3 yrs	-.122	.256	-.627	.384	.635
Maternal physical activity score 1	.189	.226	-.256	.634	.404
Maternal physical activity score 2	.171	.289	-.400	.742	.555

Maternal physical activity score 3	-.591	.424	-1.428	.246	.165
Maternal physical activity score 4+	-1.617	.989	-3.570	.335	.104
Season of birth	.352	.205	-.053	.757	.088
Maternal vitamin D and calcium supplement use	.431	.310	-.181	1.044	.166
vitamin D supplement at 6 months	.727	.393	-.049	1.504	.066
Child weekday physical activity at 3 years of age	.672	.308	.065	1.279	.030
Child weekend physical activity at 3 years of age	-.227	.250	-.721	.268	.367
Interaction - maternal vitamin D and calcium intake	.120	.052	.019	.222	.021
Maternal protein intake (g/day) - squared	.000	.000	.000	.000	.090
Child weekday physical activity at 3 years of age - squared	-.032	.018	-.068	.005	.086
Child weekend physical activity at 3 years of age - squared	.013	.015	-.017	.042	.393
Interaction - maternal vitamin D and calcium intake_squared	.000	.000	-.001	.000	.140

Appendix G: Models 1b-4b and 6b for predicting primary outcome of child bone size

Model 1b: N = 52, R-square = 0.534

Predictor	B	SE	CI		p-value
Maternal vitamin D status (per 10 nM)	-.029	.031	.031	.031	.358
Maternal age (years)	.039	.024	.024	.024	.120
Maternal height (m)	5.451	1.732	1.732	1.732	.003
Maternal BMI (kg/m2)	.000	.022	.022	.022	.993
Maternal calcium intake (per 300 mg)	-.017	.097	.097	.097	.858
Maternal education (years)	-.088	.032	.032	.032	.009
Maternal vitamin D intake (per 90 IU)	-.030	.075	.075	.075	.696
Maternal protein intake (g)	.004	.005	.005	.005	.497
Birth length z-score	.468	.204	.204	.204	.026
Birth weight	.126	.315	.315	.315	.691
Gestational age	-.056	.075	.075	.075	.465
Mom BMD Z-score	-.262	.140	.140	.140	.066
Child use of vitamin D and calcium supplements at 3 years of age	-.228	.282	.282	.282	.423
Maternal ethnicity	.613	.288	.288	.288	.038
Maternal physical activity score 1	.203	.229	.229	.229	.378

Maternal physical activity score 2	.378	.303	.303	.303	.219
Maternal physical activity score 4+	.226	.609	.609	.609	.712
Breastfed exclusively for >6 months	.067	.434	.434	.434	.878
Season of birth	.027	.240	.240	.240	.911
Maternal vitamin D and calcium supplement use	.191	.308	.308	.308	.537
Vitamin D supplement at 6 months	.411	.410	.410	.410	.321
Child weekday physical activity at 3 years of age	-.057	.077	.077	.077	.462
Child weekend physical activity at 3 years of age	.026	.066	.066	.066	.702

Model 2b: N = 65, R-square = 0.514

	B	**SE**	**CI**		**p-value**
Maternal vitamin D status (per 10 nM)	-.034	.026	-.086	.018	.194
Maternal age (years)	.031	.020	-.008	.071	.117
Maternal height (m)	5.694	1.514	2.671	8.717	.000
Maternal BMI (kg/m2)	-.004	.019	-.042	.034	.842
Maternal calcium intake (per 300 mg)	.064	.084	-.103	.230	.449
Maternal education (years)	-.073	.028	-.130	-.017	.012

Maternal vitamin D intake (per 90 IU)	-.030	.068	-.165	.106	.663
Maternal protein intake (g)	.000	.005	-.009	.010	.918
Birth length z-score	.387	.173	.042	.733	.029
Birth weight	.046	.242	-.437	.528	.851
Mom BMD Z-score	-.280	.117	-.512	-.047	.019
Child use of vitamin D and calcium supplements at 3 years of age	-.164	.250	-.664	.336	.514
Maternal ethnicity	.576	.245	.088	1.065	.022
Season of birth	.002	.188	-.373	.378	.990
Maternal vitamin D and calcium supplement use	.146	.259	-.371	.663	.575
Vitamin D supplement at 6 months	.427	.361	-.293	1.148	.241
Child weekday physical activity at 3 years of age	-.082	.061	-.205	.040	.186
Child weekend physical activity at 3 years of age	.051	.057	-.063	.164	.376

Model 3b: N = 64; R-square = 0.551
Model 2 plus one interaction formed model 3.

	B	**SE**	**CI**		**p-value**
Maternal vitamin D status (per 10 nM)	-.050	.026	-.103	.003	.062
Maternal age (years)	.037	.020	-.004	.077	.073
Maternal height (m)	7.052	1.612	3.831	10.272	.000
Maternal BMI (kg/m2)	.005	.019	-.032	.043	.774

Maternal calcium intake (per 300 mg)	.010	.087	-.164	.184	.909
Maternal education (years)	-.082	.029	-.140	-.024	.006
Maternal vitamin D intake (per 90 IU)	-.259	.173	-.604	.086	.139
Maternal protein intake (g)	.000	.005	-.010	.009	.920
Birth length z-score	.419	.170	.080	.758	.016
Birth weight	-.102	.245	-.591	.386	.677
Mom BMD Z-score	-.307	.116	-.539	-.076	.010
Child use of vitamin D and calcium supplements at 3 years of age	.000	.261	-.522	.523	.999
Maternal ethnicity	.628	.254	.121	1.136	.016
Season of birth	.012	.184	-.356	.379	.949
Maternal vitamin D and calcium supplement use	.166	.270	-.374	.705	.542
Vitamin D supplement at 6 months	.521	.360	-.198	1.239	.153
Child weekday physical activity at 3 years of age	-.075	.061	-.197	.048	.227
Child weekend physical activity at 3 years of age	.027	.057	-.086	.140	.633
Interaction - maternal vitamin D and calcium intake	.036	.023	-.011	.082	.132

Model 4b: N = 50, R-square = 0.691
Model 3 plus each continuous variable entered as a squared term formed model 4. Squared terms with p-value < 0.1 were kept for subsequent models.

	B	SE	CI		p-value
Maternal vitamin D status (per 10 nM)	-.031	.120	-.272	.210	.795
Maternal age (years)	.005	.199	-.394	.404	.981
Maternal height (m)	36.506	57.093	-78.226	151.238	.526
Maternal BMI (kg/m2)	-.404	.199	-.803	-.005	.047
Maternal calcium intake (per 300 mg)	-.390	.246	-.885	.105	.120
Maternal education (years)	-.684	.230	-1.146	-.222	.005
Maternal vitamin D intake (per 90 IU)	-1.073	.551	-2.180	.035	.057
Maternal protein intake (g)	.017	.014	-.012	.046	.255
Birth length z-score	.546	.185	.175	.917	.005
Birth weight	-1.470	1.278	-4.038	1.098	.255
Mom BMD Z-score	-.291	.207	-.707	.124	.165
Child use of vitamin D and calcium supplements at 3 years of age	.007	.276	-.547	.560	.980
Maternal ethnicity	.505	.291	-.080	1.091	.089
Season of birth	.076	.211	-.347	.500	.719
Maternal vitamin D and calcium supplement use	.153	.292	-.433	.739	.602
Vitamin D supplement at 6 months	.284	.359	-.437	1.006	.432
Child weekday physical activity at 3 years of age	.028	.368	-.711	.768	.939

Child weekend physical activity at 3 years of age	-.122	.187	-.498	.254	.516
Interaction - maternal vitamin D and calcium intake	.166	.096	-.026	.359	.089
Maternal vitamin D status (per 10 nM) - squared	-.001	.005	-.011	.009	.839
Maternal age (years) - squared	.000	.003	-.006	.007	.917
Maternal height (m) - s quared	-9.268	17.474	-44.384	25.848	.598
Maternal BMI (kg/m2) - squared	.007	.003	.000	.014	.046
Maternal calcium intake (per 300 mg) - squared	.018	.013	-.008	.043	.178
Maternal education (years) - squared	.017	.006	.004	.030	.010
Maternal vitamin D intake (per 90 IU) - squared	.070	.072	-.074	.214	.333
Maternal protein intake (g) - squared	.000	.000	.000	.000	.476
Birth length z-score - squared	.170	.099	-.030	.369	.094
Birth weight - squared	.199	.193	-.189	.586	.308
Mom BMD Z-score - squared	-.003	.107	-.218	.212	.974
Child weekday physical activity at 3 years of age - squared	-.004	.022	-.048	.040	.855

Child weekend physical activity at 3 years of age - squared	.010	.013	-.016	.036	.442
Interaction - maternal vitamin D and calcium intake	-.002	.001	-.004	.001	.206

Model 6b: N = 57, R-square = 0.727

Model 5 minus outliers (determined by box-plot of standardized residuals) formed model 6.

Predictor	**B**	**SE**	**CI**		**p-value**
Maternal vitamin D status (per 10 nM)	-.070	.021	-.113	-.027	.002
Maternal age (years)	.030	.016	-.002	.062	.068
Maternal height (m)	6.829	1.317	4.191	9.467	.000
Maternal BMI (kg/m2)	-.242	.151	-.545	.061	.115
Maternal calcium intake (per 300 mg)	.002	.069	-.136	.141	.972
Maternal education (years)	-.705	.168	-1.042	-.369	.000
Maternal vitamin D intake (per 90 IU)	-.313	.136	-.584	-.041	.025
Maternal protein intake (g)	.000	.004	-.007	.007	.987
Birth length z-score	.496	.136	.224	.769	.001
Birth weight	-.167	.195	-.559	.225	.396
Mom BMD Z-score	-.277	.092	-.461	-.092	.004
Child use of vitamin D and calcium supplements at 3 years of age	.158	.211	-.265	.582	.458
Maternal ethnicity	.480	.207	.066	.894	.024

Season of birth	.148	.151	-.156	.451	.334
Maternal vitamin D and calcium supplement use	.350	.221	-.092	.793	.118
Vitamin D supplement at 6 months	.476	.284	-.092	1.045	.099
Child weekday physical activity at 3 years of age	-.055	.050	-.154	.044	.272
Child weekend physical activity at 3 years of age	.016	.045	-.074	.105	.729
Interaction - maternal vitamin D and calcium intake	.039	.019	.002	.076	.042
Maternal BMI - squared	.005	.003	-.001	.010	.092
Maternal education (years) - squared	.018	.005	.008	.027	.000
Birth length z-score - squared	.120	.065	-.011	.251	.071

Appendix H: Coding for maternal vitamin D and calcium supplements during pregnancy used at FAMILY study at PHRI

Table 1 – Questions from FAMILY study initial maternal questionnaire used to obtain data on maternal supplement use

Plate number	Question
5	29
57	Question for "Vitamins/supplements", including multivitamins plus iron or multivitamins plus minerals
58	Question for "Other vitamins"
59	Question for calcium only

Table 1 – Codes assigned by FAMILY study for supplements reported by mothers

Supplement	Code	Dosage
Vitamin D	28 28.1 28.2	Unknown dose Low dose (200-400 IU) High dose (1000 IU)
Prenatal vitamins	29	Low dose vitamin D (200-400 IU) Low dose calcium (600 mg or less)
Calcium	37 37.1 37.2	Unknown dose Low dose (600 mg or less) High dose (1000 mg or more)

Table 3 – Brands of supplements containing vitamin D taken by FAMILY study mothers

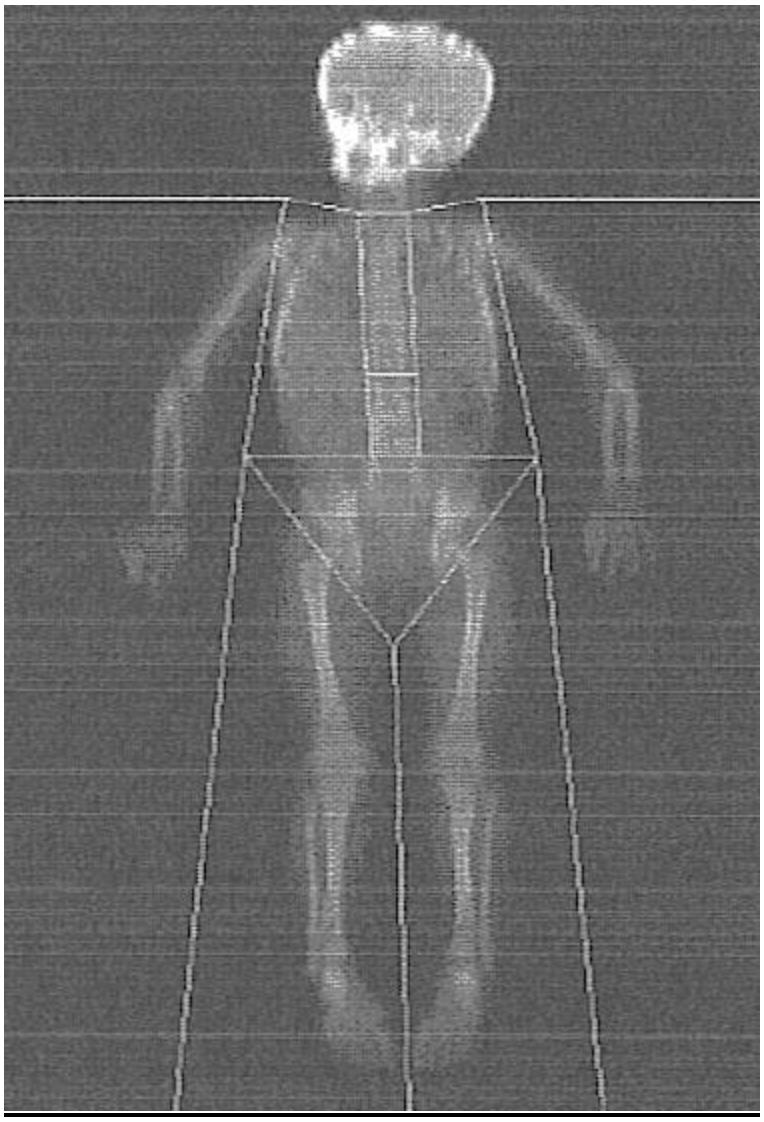
Supplement	Dose
Prenatal vitamins • Materna • Equate • Maternity supplement • Pregnancy vitamin	400 IU
Vitamin D D3 Cholecalciferol D3 Vitamin D drops	1000 IU
Calcium *with* vitamin D	400 IU
Multivitamins • Kirkland • Exact	400 IU

• Nature's Harmony • Genestra • Quest • GNC • Orifer F	

Table 4 – Brands of supplements containing calcium taken by FAMILY study mothers

Supplement	Dose
Prenatal vitamins • Materna • Equate • Maternity supplement • Pregnancy vitamin	250 mg
Multivitamin • Kirkland • Exact • Nature's Harmony • Genestra • Quest • GNC • Orifer F	100 mg
Calcium only	500 mg or less 1,000 mg 1,500 mg 2,000 mg or more

Appendix I: DXA scan of child at 3 years of age



Appendix J: interpreting the effect of maternal BMI on child bone size

Non-standardized regression coefficient (b) for BMI: -0.366; for BMI^2: +0.007

$$Y = -0.366x + 0.007x^2 \ldots \text{[other covariates held constant]}$$

Where
Y = net effect of maternal BMI on child bone size
X = maternal BMI

The b for BMI is much greater than the b for BMI^2 suggesting that at large enough values of BMI, there may be a net positive effect on the outcome. To test this:

Solve for X when Y > 0

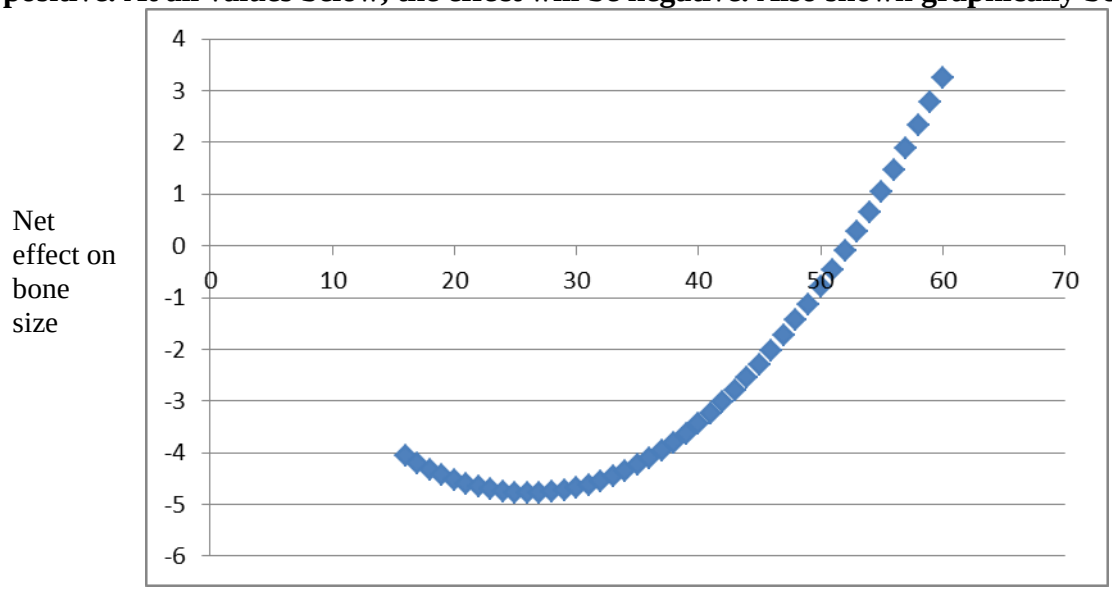
STEP 1: $0 < -0.366x + 0.007x^2$

STEP 2: $0.366\ x < 0.007x^2$

STEP 3: $0.366 < 0.007x$

STEP 4: $52.3 < x$

Therefore, at values above 52.3, the effect of maternal BMI on child bone size will become positive. At all values below, the effect will be negative. Also shown graphically below:



Appendix K: interpreting the effect of maternal education on child bone size

Non-standardized regression coefficient (b) for maternal education: -0.632; for (maternal education)^2: +0.016

$$Y = -0.632x + 0.016x^2 \ldots \text{[other covariates held constant]}$$

Where
Y = net effect of maternal education on child bone size
X = maternal education

The b for education is much greater than the b for education^2 suggesting that at large enough values of education, there may be a net positive effect on the outcome. To test this:

Solve for X when Y > 0

STEP 1: $0 < -0.632x + 0.016x^2$

STEP 2: $0.632x < 0.016x^2$

STEP 3: $0.632 < 0.0016x$

STEP 4: $39.5 < x$

Therefore, at values above 39.5, the effect of maternal education on child bone size will become positive. At all values below, the effect will be negative. Also shown graphically below: