LEPTIN IN PREGNANCY: ASSOCIATION WITH BONE HEALTH IN THE OFFSPRING

LEPTIN IN PREGNANCY: ASSOCIATION WITH BONE HEALTH IN THE OFFSPRING

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ABSTRACT

Leptin, an adipose derived hormone, has emerged as a regulator of bone metabolism. Recent findings support a role of leptin in the process of fetal bone remodeling during pregnancy; however, the link between maternal leptin during pregnancy and offspring bone status is undocumented. Evidence exists that the intrauterine environment plays a role in programming peak bone mass that is achieved in late adolescence and thus osteoporosis risk later in life. We investigated the association between maternal leptin during the third trimester and offspring bone mass at 3 years of age. **Method:** Based on a sub-sample of a prospective birth cohort study, we conducted analysis on 425 mothers from whom maternal blood samples in pregnancy were analyzed for leptin and 25-hydroxyvitamin D, and whole body bone mass by dual energy x-ray absorptiometry were available for both mother and child at 3 years. Data were collected for maternal pre-pregnancy body mass index (BMI), lifestyle, and nutrition during pregnancy, as well as the child's nutrition and physical activity at 3 years. **Results:** Women obese on entering pregnancy have a two-fold greater circulating leptin during pregnancy than women with normal weight BMIs. Maternal age and skinfold thickness were positively associated with maternal leptin status. However, maternal leptin status was not a significant predictor of offspring BMC z-score at 3 years of age, when adjusted for relevant maternal and child variables. Maternal vitamin D status was also not a predictor of offspring bone status. Rather the key predictors of child BMC z-score were maternal bone mineral density z-score, and child's weight and vitamin D intake at 3 years.

Conclusion: While maternal leptin status during pregnancy is highly variable among women of different BMI categories, in utero exposure to leptin is not a significant factor that influences child bone status at 3 years of age when adjusted for other relevant variables.

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Lastly, thank you, reader. If you are reading this line after the others, you have at least read one page of my thesis. Thank you.

*"Anybody who has been seriously engaged in scientific work of any kind realizes that over the entrance to the gates of the temple of science are written the words: '**Ye must have faith**.' It is a quality which the scientist cannot dispense with."*

-Max Planck

LIST OF ABBREVIATIONS

25OHD – 25-hydroxyvitamin D
aBMD – areal bone mineral density
AMDR – acceptable macronutrient distribution range
Arc - arcuate nucleus
BBB – blood brain barrier
BMC – bone mineral content
BMD – bone mineral density
BMI – body mass index (kg/m^2)
CART – cocaine and amphetamine regulated transcript
CHMS – Canadian Health Measures Survey
CT – computed tomography
CV – coefficient of variation
DRI – dietary reference intake
DXA – dual energy x-ray absorptiometry
EAR – estimated average requirements
ELISA – enzyme-linked immunosorbent assay
FAMILY study - **F**amily **A**therosclerosis **M**onitoring **I**n Ear**LY** life
FFQ – food frequency questionnaire
GWG – gestational weight gain
HAES – Habitual Activity Estimation Scale
HF – high fat
HPLC – high performance liquid chromatography
I.C.V. – intracerebroventricular
IOM – Institute of Medicine
MUMC – McMaster University Medical Center
NIST – National Institute for Standards and Technology
nM – nmol/L
PHRI – Population Health Research Institute
PMB – peak bone mass

PPWR – post partum weight retention
pQCT – peripheral quantitative computed tomography
Ob – obese/leptin gene
ob/ob – leptin deficient genotype
SD – standard deviation
SWS – Southampton Women's Survey
RDA – Recommended Daily Allowance
vBMD – volumetric bone mineral density
VMH – ventromedial hypothalamus

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GENERAL INTRODUCTION

Chapter 1: General Introduction

1.1 Rationale, objectives, and hypothesis

Maternal health status on entering pregnancy, and nutrition and lifestyle during pregnancy, are important determinants of health outcomes for both mother and child. For example, overweight and excess gestational weight gain puts mothers at risk of health problems such as diabetes, high blood pressure, and increases the degree of obesity after pregnancy. Consequently their babies are at risk of being born too large or developing related health problems. This represents a major health challenge since almost 50% of women are entering pregnancy overweight or obese.[1] Additionally, excess gestational weight gain during pregnancy occurs in 55-75% of such women.

Leptin, an adipose derived hormone, plays a key role in food intake and energy expenditure by acting on receptors in the hypothalamus signaling a feeling of satiety. As such, leptin circulates in proportion to body fat, particularly white adipose tissue. As circulating leptin rises during pregnancy, its role in fetal growth and development [2] is of interest, especially in women of varying adipose mass. Although a normal level of leptin is necessary for growth and development of the fetus, there may be a narrow optimal window of fetal exposure since neonates with both high circulating leptin at birth (offspring of gestational diabetic mother) and low leptin (offspring of thin mothers) are at higher risk of developing obesity and diabetes in later stage of life.[2] Recently, leptin was established as a regulator of bone metabolism.[3] Studies have proposed leptin as the possible mechanism for the observed inverse relationship between visceral fat and bone.[4]

Our study aimed to investigate leptin status in pregnancy and determine its effect on the bone status of offspring at three years.

Our primary study objective was to determine the effect of maternal leptin in pregnancy on bone status of their child at 3 years of age after adjusting for key maternal and child variables. Before addressing this, we needed to investigate leptin status in women during pregnancy to observe if differences exist between women related to pre-pregnancy BMI and gestational weight gain (GWG).

Our hypotheses were that:

#1 – Maternal leptin will be highest in women with high pre-pregnancy BMI and excess GWG.

#2 – Offspring of mothers with higher serum leptin concentration will have higher bone mineral density z-scores compared to those offspring of mothers with lower serum leptin concentration.

1.2 Leptin

Leptin, discovered in 1994 is a peptide hormone and the soluble product of the *ob* gene.[5,6] It is produced primarily by white adipose cells but also expressed by brown adipose tissue, placenta, ovaries, skeletal muscle, stomach, mammary epithelial cells, bone marrow, pituitary and liver.[5,7] Leptin receptors (Ob-R) belong to the cytokine family and are found throughout the body, indicating numerous roles for leptin.[7]

The name leptin comes from the Greek word "leptos" meaning thin. Mutated ob/ob mice (deficient in leptin) were found to overeat and be very obese. When leptin was injected intracerebroventricularly (i.c.v) into these mice they lost a significant amount of weight.[8,9] Thus, the main endocrine function of leptin is to maintain whole body energy homeostasis. It achieves this by acting as a signal via the ventromedial hypothalamus (VMH). Leptin inhibits the neuropeptide Y pathway, a potent feeding simulant and stimulates the synthesis of alpha melenocartin, a suppressant of food intake.[10] It also decreases energy storage and increases energy consumption in the periphery in order to maintain energy balance.[11]

Leptin is secreted in circadian fashion with nocturnal rise in both lean and obese patients. Under conditions of weight maintenance, leptin circulates in proportion to adipose tissue mass.[5] The mean serum leptin levels for a normal lean male (BMI 18-25 kg/m^2) is about 3.8 (1.8) ng/ml, while those for a normal BMI female is 7.4 (3.7) ng/ml. Serum leptin is approximately 2.5 times higher in women per unit BMI as compared to males.[12]

Unlike lean individuals, obesity is generally characterized by increased circulating free leptin and lower levels of Ob-Re (soluble leptin receptor).[13,14] This is positively correlated with BMI and fat mass.[8,15] An increasing body of research suggests central leptin insufficiency as the mechanism. Although leptin is deemed to act as a satiety signal, the high levels in obesity are associated with central leptin insensitivity and reduced transmission of leptin across the BBB. This results in a loss of signaling capacity to satiety centers.

1.2.1 Leptin and its link to bone

Bone is a dynamic and unique structure in that it has components able to both build and destroy itself. Both the osteoblast (bone forming cell) and the osteoclast (bone resorbing cell) work in tandem to (re)model bone throughout an individual's lifetime.

The groundwork of the skeletal system is developed as early as 10 weeks gestation and continues to build until reaching peak bone mass (PBM) in early adulthood. Bone accrual is rapid throughout childhood and adolescence. Approximately, 30-40% of bone mass is gained during the peripubertal years and 80-90% is accrued by age 20 with variations by skeletal sites.[16] Total skeletal mass peaks about 6 to 10 years later. Accumulating a high adult peak bone mass is thus beneficial in combating the risk of fragility fractures later in life. It is considered that increasing PBM by one standard deviation would reduce fracture risk considerably.[17,18]

The intrauterine environment may play an important role in contributing to a child's peak bone mass and risk of fracture. During pregnancy, large volumes of calcium are transferred to the fetus, with almost 30g of calcium accumulated by the fetus for bone mineralization,[19] 80% of which is in third trimester.[20] Key regulation in the womb environment, particularly in the second half of pregnancy, may contribute to the extent that the skeletal matrix is established; either optimizing or diminishing PBM later in life. In the Southampton Women's Survey (SWS) a higher velocity of fetal femur growth was strongly associated with greater skeletal size at 4 years.[21] Moreover, a 28-year longitudinal study observed that bone mineral content (BMC) z-score of children (mean age 9) correlated with BMC z-score in adulthood (mean age 37). Furthermore, 55% of the

children in the lowest quartile of BMC were still in the lowest quartile in adulthood.[22] Hence, growth deficits in utero may persist into adolescence, the period during which the majority of bone mass is acquired. Thus, a focus on optimizing prenatal factors for later bone health may be an effective strategy against later life skeletal risks.

Leptin and bone have been linked as obesity has been shown to have protective benefits on bone. In prepubertal children, fat mass was a positive independent determinant of bone mass and size and of increases in these parameters over 2 years.[23] Also, heavier post-menopausal women have fewer fractures than thinner women.[5] It is proposed that mechanical loading stimulates bone formation by decreasing apoptosis and increasing proliferation and differentiation of osteoblasts and osteocytes through the Wnt/β-catenin signaling pathway.[18,24]

Several lines of evidence suggest that obesity and bone metabolism are interrelated. First, leptin does not appear during evolution with any aspect of energy metabolism but instead is associated with bone (re)modeling.[15] Second, both osteoblasts and adipocytes are derived from a common mesenchymal cell[25] and factors that inhibit adipogenesis stimulate osteoblastic differentiation and vice versa. Third, decreased bone marrow osteoblastogenesis with aging is usually accompanied with increased marrow adipogenesis.[26] Lastly, chronic use of steroid hormones like glucocorticoids results in obesity accompanied by rapid bone loss.[27]

Confirming this bone-adipose link, a landmark study by Ducy[28] demonstrated that ob/ob mice deficient in leptin are not only massively obese, but also have high bone mass. When leptin was injected i.c.v. into the brain, it decreased bone mass to a normal level.[28]

This study, along with others has now established leptin as a bone regulator. Nonetheless, the exact influence of leptin on bone metabolism has not yet been clarified. In humans, the correlation between serum leptin and bone mineral density (BMD) have been positive, negative or neutral.[5] Currently, it has been established that there are two bone-controlling mechanisms, which may or may not work concurrently, the indirect central pathway and the direct peripheral pathway.

1.2.1.1 Central hypothesis of action

Leptin regulates bone remodeling indirectly via the hypothalamus and the sympathetic nervous system.[29] This central pathway was first elucidated by Ducy's groundbreaking discovery that leptin deficient ob/ob and leptin receptor deficient db/db mice had a high bone mass phenotype, which could be recovered by i.c.v injection of leptin.[28,30] This led researchers to believe that leptin is a central inhibitor of bone mass accrual.

The current mechanism of action is shown in figure 1, involving the hypothalamus and sympathetic nervous system. It was first theorized that leptin bound to its receptors in the ventromedial hypothalamus (VMH) and the arcuate (Arc) nucleus, but this notion was shattered when selective inactivation of leptin receptors in these two sites did not affect bone or appetite. Yadav and his colleagues demonstrated that brain stem serotonin favors bone mass accrual on binding to receptors on VMH neurons as well as appetite via receptors on the Arc neurons.[3] They also showed that leptin reduces serotonin synthesis and firing of serotonergic neurons.[3,31] Leptin secreted from the adipose tissue binds to the

Ob-Rb receptor in the raphe nucleus, where it signals through the JAK-STAT pathway. It inhibits Tph2 expression thus inhibiting the production of serotonin. This in turn inhibits appetite via the Arc nucleus, and bone mass accrual via the sympathetic nervous system.

Once leptin acts on serotonin production in the hypothalamus, there are two different central pathways that leptin affects in order to inhibit bone mass accrual; the sympathetic nervous system (SNS) and CART (cocaine- and amphetamine-regulated transcript). The first is the CART pathway, which is still under investigation.[32] It basically acts by inhibiting RAKL in the osteoblast. The second mode of action is via the sympathetic nervous system. Noradrenaline binds to Adrβ2 on the membrane of the osteoblast.[29,32] Inhibition of osteoblastic differentiation occurs via CREB and the clock gene of the osteoblast which exerts dominant influence.[33,34] Moreover, ATF4 stimulates RANKL to cause osteoblast differentiation.[35]

It has been proposed that blocking leptin signaling of the sympathetic nervous system could potentially be a therapy for osteoporosis. It can be inferred that since obese individuals are central leptin insensitive/insufficient, this central decrease in bone mass accrual does not occur to the same extent.

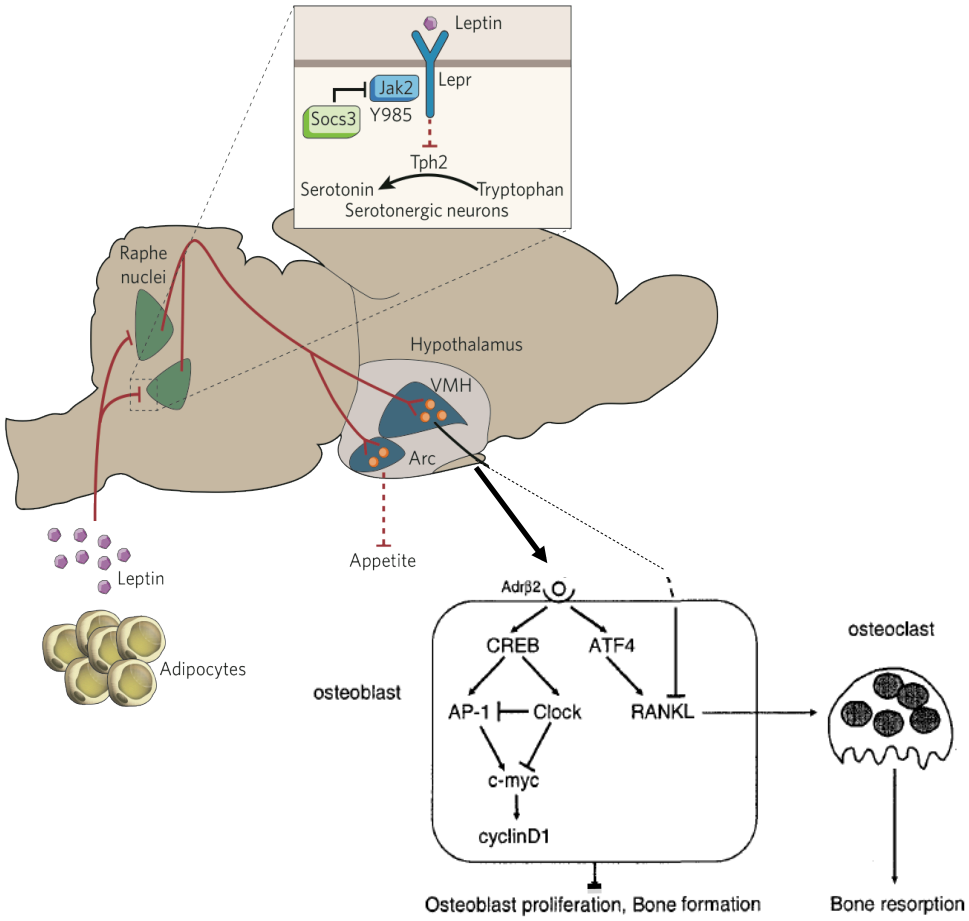

Figure 1. Mechanism of central action of leptin via the hypothalamus and sympathetic nervous system. [15,35]

1.2.1.2 Peripheral hypothesis of action

Traditionally, obesity has been linked with high bone mass, however the central pathway seems to contradict this observation. Moreover, other studies observed growth promoting effects of leptin on the skeleton of leptin-deficient ob/ob mice, as well as a protective effect of peripherally administered leptin in bone loss mouse models. [36] Since leptin originates in the periphery, scientists proposed a local effect of leptin on bone.

The peripheral action of leptin involves directly affecting cells localized in bone. This pathway appears to be osteogenic (promote bone formation). Burguera and colleagues proposed that the skeletal actions of peripheral leptin were anabolic whereas

its central actions were catabolic.[36] This was supported by Hamrick's observation that the ob/ob mouse does not have high bone mass globally as initially represented but rather exhibits a mosaic skeletal phenotype, where there is higher cancellous bone mass in certain regions like the lumbar, but lower cancellous bone mass in the distal femur. Moreover, there was lower cortical and total bone mass and decreased bone length.[37] Additionally, serum osteocalcin (a biochemical marker for bone formation) was increased in ob/ob mice after hypothalamic leptin therapy.[38]

Recently Turner et al, reevaluated this dual pathway and proposed that leptin acts primarily through its peripheral pathways to enhance bone growth and maturation by increasing osteoblast number and activity as well as osteoclast activity.[39] Turner used the same type of mice that Ducy had previously used, but found that ob/ob and db/db mice had lower bone formation rates as well as a lower value for osteoblast-lined bone perimeter. Subcutaneous replacement of leptin in these mice resulted in site-independent increase in bone formation and osteoblast-lined bone perimeter; suggesting that leptin increases osteoblast activity.[39]

The peripheral pathway of leptin action is still not clearly known, however scientists have some theories. Leptin is both expressed and secreted from osteoblasts.[40] Leptin Ob-Rb receptors are expressed in osteoblasts, osteoclasts and chondrocytes.[29] In vitro experiments have demonstrated that leptin promotes growth of primary osteoblasts and chondrocytes.[41] It is proposed that leptin promotes differentiation of bone marrow stromal cells to osteoblasts by stimulating production of IGF1.[39] IGF1 in turn stimulates the proliferation of osteoblast precursors, and further enhances bone formation through

suppression of osteoclast generation by causing osteoblastic stromal cells to make less RANKL (which stimulates osteoclast generation), and by stimulating the production of the osteoclast-suppressing osteoprotegerine.

Therefore, leptin's peripheral role in bone formation may help explain why obese individuals, who are often leptin resistant are protected from bone loss.[42]

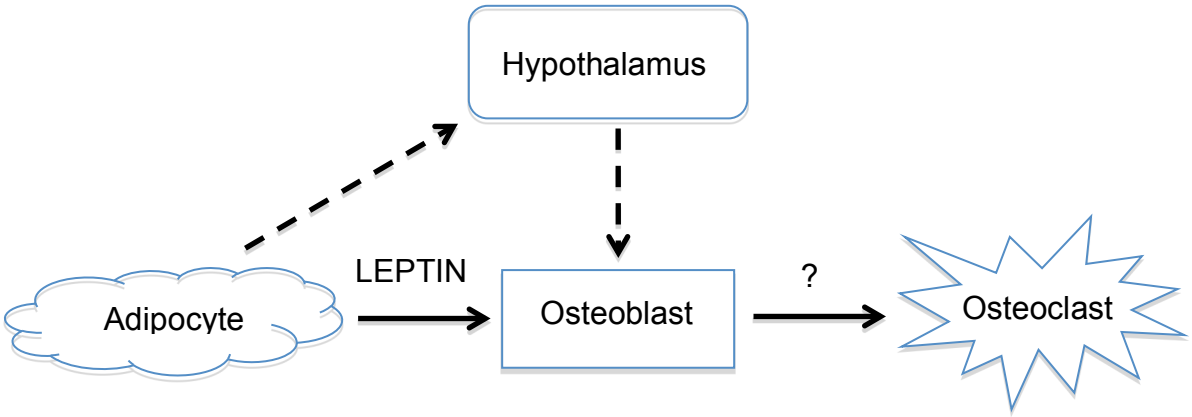

Figure 2: Proposed dual-pathway mechanism of leptin action. Solid lines indicate major route of action.[39]

1.2.2 Leptin in pregnancy

1.2.2.1 Role in mother

During pregnancy, leptin plays additional roles for both the mother and fetus. Leptin concentrations dramatically increase during pregnancy, beginning in early gestation and peaking in early third trimester.[43] As opposed to the concentrations in normal non-pregnant women described in section 1.2, serum concentration of leptin in pregnancy varies, with reported means of 23.29 (8.62) ng/ml for normal pre-pregnancy BMI women at 3rd trimester,[44] and 40.86 (2.81) ng/ml for mild pre-eclamptics.[45] These

high levels are maintained throughout the remainder of gestation and dramatically decline postpartum.[46] In rats, hyperleptinemia in maternal circulation during healthy pregnancy leads to central leptin resistance by down regulation of the Ob-Rb receptor in the VMH and increased circulating Ob-Re.[11] Normal weight pregnant women achieved circulating leptin similar to non-pregnant obese individuals. They also exhibit a form of central leptin resistance.

Leptin is an essential factor in regulating maternal and fetal energy balance. Since pregnancy requires a positive energy balance to provide for optimal fetal development, it is not likely that the increased leptin concentrations would function classically to reduce food intake as pregnancy progresses. This points to a role for leptin that is different than hypothalamic regulation of appetite suppression; pregnancy is another state, which alters leptin control. In the latter half of pregnancy, accretion of fat represents the main caloric demand the fetus has to be supported by adequate maternal nutrient supply. A rise in maternal leptin levels may enhance the mobilization of fat stores to increase availability and support trans-placental transfer of lipid substrates.[13] Additionally, it has been found that leptin is involved in macronutrient exchange, particularly amino acid transport,[47] as well as placental angiogenesis, trophoblast mitogenesis and immunomodulation.[47,48]

1.2.2.1.1 Association with gestational weight gain

Gaining weight in pregnancy is normal and recommended, however gaining excess gestational weight has been linked to adverse health outcomes for both mom and offspring, independent of pregravid weight, including large and small for gestational age

babies.[49,50] In 2009, the Institute of Medicine (IOM) reevaluated their recommendation that women should gain an appropriate amount of weight with respect to their entering BMI. Unfortunately, nearly 50% of women still gain more than the recommended amount in pregnancy and women who enter pregnancy with higher pre-pregnancy BMIs tended to gain the most gestational weight.[51] This excessive GWG has been associated with increased maternal postpartum weight retention.[50] New data suggest that the harms of excessive GWG extend well past birth into offspring adulthood, with an increased risk in greater BMI.[52]

To date, two studies have observed an association in maternal serum leptin with GWG in women of normal pre-pregnancy BMI.[53,54] Although the pregravid BMI of these women were normal, the high leptin concentration early in pregnancy may predict a greater gestational weight gain.[54]

Excessive GWG may increase risk of adverse effects, but maternal GWG is also essential to the fetus. Maternal tricep-skinfold thickness during late pregnancy has been shown to be positively related to BMC in neonates, suggesting the importance of maternal fat stores for skeletal development of the fetus.[55,56] Maintaining an appropriate GWG for pregravid BMI category is thus essential in optimizing skeletal development in children.

1.2.2.2 Role in placenta and fetus

The marked decline of serum leptin concentrations are in part attributed to the contribution of leptin secreted by the placenta. The placenta is an important source for leptin during pregnancy.

Leptin is expressed in the syncytiotrophoblast cells that are exposed to the maternal blood as well as the fetal vascular endothelial cells that are in direct contact with the fetal blood.[57] 95% of placental leptin is given to the mother's circulation, and the remaining 5% to the fetus.[58] However, the rate of leptin delivery to the fetus from the placenta is much higher than for other placental derived hormones like HCG.[59]

The leptin receptors located on the syncytiotrophoblast cells are accessible to maternal circulating leptin.[57] Both the maternally derived and placental leptin can bind to these placental leptin receptors. However, instead of stimulating JAK phosphorylation, these Ob-R receptors follow the mitogen activated protein kinase (MAPK) pathway, thereby increasing DNA synthesis in the placental cells,[60,61] as well as the effects previously stated in section 1.2.2.1. An overproduction of leptin by the placenta in pregnancy with diabetes mellitus or hypertension is associated with maternal hyperleptinemia.[13]

Leptin plays a role in fetal development, particularly in growth, hematopoiesis, lymphopoiesis and fetal bone remodeling. The fetal adipose tissue is also a source of leptin for the fetus. It produces leptin as early as 6-10 weeks of gestation.[62] Leptin controls fetal bone remodeling by enhancing the differentiation of human bone marrow stromal cells to osteoblasts and decreasing bone resorption.[32,63]

Studies of umbilical cord tissue have been helpful in understanding the influence of intrauterine leptin concentrations. Studies have demonstrated a positive association between infant size and cord leptin concentration. Recently, Brynhildsen and colleagues investigated the effects of GWG interventions on potentially normalizing maternal leptin,

and its effects on the offspring. They observed that the birth weight of children of pregravid obese women were significantly higher than the birth weight of children of normal weight mothers.[64] Moreover, irrespective of intervention, the cord blood concentration of leptin was significantly higher in children of overweight and obese mothers compared with normal weight mothers. The GWG intervention had no effect on cord blood leptin concentrations.[64] Another study found that umbilical cord leptin is predictive of fetal bone mass; positive association with vBMD, and BMC in 117 infants.[65] Therefore, leptin plays a pivotal role in the development of the placenta and the fetus, demonstrating a potential are for intrauterine modifications.

1.2.2.3 (Proposed) Role in offspring skeletal outcome

Based on the evidence reviewed above maternal leptin can bind to the placental leptin receptor and have an effect, or maternal leptin can cross the placenta.[66] Research in mice demonstrated that ossification centers in long bones of mice born to mothers treated with subcutaneous injection of leptin, grew more rapidly in length and cross-sectional area than mice of untreated mothers.[41] It would thus be of interest to observe if this link is valid in humans as well as the proper mode of action. If a direct link between leptin and intrauterine bone formation is clearly demonstrated, it may indicate that interventions for prevention of later bone risk should start in early life.

1.3 Assessment of bone outcomes in young children

1.3.1 Measurement of bone by dual energy x-ray absorptiometry

Dual energy x-ray absorptiometry (DXA) has become the Gold Standard in assessing bone mass in individuals of all ages. Bone mineral content (BMC) is the total amount of mineral content present in the skeleton, while bone mineral density (BMD) conveys the mineral content per volumetric unit (cm^2) of bone.

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

The volumetric BMD is known as the true BMD, as it is a three-dimensional measurement. It is usually obtained by computed tomography (CT). However, since DXA is measured in two dimensions, it provides an areal BMD (aBMD) rather than the true vBMD. With DXA, depth cannot be accounted for as it is in the same direction as the x-ray beam.[67] The aBMD 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 our machine of use, we will refer to areal BMD simply as BMD from here on, and distinguish it from vBMD measured by CT when necessary.

Although BMD is useful for predicting fractures in adults, there is disagreement over the best measure of bone for children. Due to the lack of the third dimension, DXA

derived BMD is greatly affected by bone size. This is seen in children with smaller bones who appear to have lower BMD. An increase in bone size can be mistaken for increase in density.[68] Comparisons between DXA and CT results indicate that BMC from DXA is the best variable to use in children, as it is more sensitive to changes due to growth in children than aBMD, and lacks areal density-related errors.[67] Furthermore, the International Society for Clinical Densitometry (ISCD) recommends using BMC to measure whole body bone status in children.[69]

For total body assessments, it has been suggested that head BMC from measures of whole body BMC in children because the algorithms for predicting BMC from age are more accurate when the head is excluded.[70] The skull is a dense mass of bone content, in which 80% of adult skull volume is achieved by 3 years of age. Eliminating the skull in whole body BMC measurements is better predicted by age.[70] Unfortunately, due to the lack of headless reference data for our age group, we did not report headless BMC values.

1.3.2 Variables influencing bone outcomes in children

1.3.2.1 Maternal predictors of offspring bone mass

A link between maternal factors during pregnancy and the outcome of bone mass in the offspring has been demonstrated. Maternal predictors of offspring bone mass include genetic contribution and lifestyle factors during pregnancy. These variables are important to account for as potential covariates in elucidating the relationship between maternal leptin status and offspring bone mass.

Observational studies have noted a strong correlation between parental and offspring BMC and BMD.[71,72] Evidence from twin and family studies suggest that the genetic contribution to BMD is between 60% and 90%.[73] Some skeletal sites are shown to have a higher degree of heritable contribution than others, with the greatest degree being the head.[74,75]

Other anthropometric parameters of parents are predictive of offspring bone health status. The birth weight of both parents and the height of the father are positively correlated with neonatal whole body BMC.[55] Moreover, ethnicity plays a role in bone health. Africans have a higher bone mass density than Caucasians of the same gender and age and East Asians have an even lower bone mass.[76]

Maternal smoking during pregnancy, has been linked to many adverse fetal outcomes,[77,78] one of which is the influence on the accrual of bone mass in utero. Two studies in the SWS showed that women who smoked during pregnancy had infants with a lower whole body BMC and BMD.[55,56] The effects persisted long-term with a deficit in offspring bone mass to age 8 years,[79,80] and lower height in females at 16 years.[80]

Potential mechanisms by which maternal smoking influences offspring skeleton include impaired placental function, reduction of utero-placental blood flow, and effect on fetal oxygen carrying capacity.[55] Additionally, cadmium, a heavy metal contaminant in tobacco smoke could affect fetal skeletal growth via its effects on osteoblast function and trophoblast calcium transport.[55]

The linkage of pre-pregnancy obesity and bone mass is conflicting, with studies showing both positive and negative relationships. In a multivariable regression analysis of

7121 children with a mean age of 9.9 years, maternal pre-pregnancy BMI was positively associated with offspring total bone less head (TBLH) BMC and BMD, and spine BMC and BMD. [81] In other maternal and gestational weight gain had an inverse relationship with cord blood concentrations of bone formation markers such as osteocalcin and bone specific alkaline phosphatase. [78] Thus, further investigation is needed into the link between maternal weight and offspring bone health, especially in terms of adiposity hormones such as leptin.

Physical exercise is generally promoted during pregnancy, although the effects on fetal outcome have yielded varying results depending on intensity, duration and type of exercise. Moderate exercise regimen has been found to promote healthy placental function, while vigorous exercise, particularly in the later stages of pregnancy has been correlated with adverse outcomes. [55,82] On the other hand, lack of exercise during pregnancy increases size of infant at birth and risk of obesity later in life.

The SWS observed that a lower walking speed in late pregnancy was a predictor of greater neonatal whole body bone are as well as BMC, in a sample of 841 mother-baby pairs. [56] This was a similar observation to a study done in 2001. [55] Furthermore, voluntary exercise in pregnant rats influenced fetal growth without causing maternal stress. Both resistance and cardio exercises increased the length of the fetus as well as placental weight. As expected, maternal weight gain in exercised pregnant rats was less than the controlled non-exercised rats. [83] Thus, maternal physical exercise is a potential confounding variable that must be considered.

Maternal nutrition plays an important factor in the growth and development of the fetus particularly with respect to bone status. The maternal diet during pregnancy, which is the main determinant of fetal nutrition, has been suggested to influence childhood bone mass.

Maternal calcium, vitamin D and protein have particularly been associated to outcomes in fetal bone health. A recent prospective study observed that higher first trimester intake of protein, calcium and phosphorus were associated with higher childhood bone mass, while higher carbohydrate intake was associated with lower bone mass.[84] The current Recommended Dietary Allowance (RDA) and upper intake levels for calcium and vitamin D established for pregnancy are the same for non-pregnant women of similar age. The calcium demand by the fetus, especially in the third trimester when fetal accrual of bone is at peak velocity, is accommodated by natural physiological responses that double the maternal intestinal absorption of calcium, as well as increase renal and bone calcium resorption.[85,86] Unfortunately, inadequate intakes of calcium and vitamin D during pregnancy were linked not only with excessive skeletal loss in the mother, but also impaired bone mineral accrual in the offspring well past infancy.[87]

Maternal vitamin D status is one variable that has been prominently linked to offspring bone outcome. Maternal 1,25-$(OH)_2$ vitamin D_3 (calcitriol) is the principle stimulus for calcium absorption,[19] while 25-hydroxyvitamin D (25 OHD) is the form measured in serum to determine vitamin D status. Low cord serum 25 OHD has been correlated with maternal vitamin deficiency [19] and was positively associated with child total body BMC.

Observational studies have demonstrated that offspring of vitamin D deficient women are born vitamin D deficient, have greater birth weight, [88] reduced intrauterine and fetal long-bone growth, [89] lower infant bone mass [88] and are at risk of neonatal rickets. [90] Moreover, this persists in childhood as studies have reported that children of mothers who were vitamin D deficient during pregnancy had significantly lower whole body BMC and lower lumbar spine BMC at 9 years of age. [86] Thus, sufficient maternal vitamin D intake throughout pregnancy is necessary to optimize bone status of offspring.

The high demand for calcium by the fetus during pregnancy necessitates sufficient calcium in the mother's diet. Maternal consumption of high amounts of calcium and milk during pregnancy were associated with BMC and higher lumbar-spine BMD in offspring at 6 [84] and 16 [91] years of age respectively.

Lastly, maternal protein intake during pregnancy has been implicated in impacting fetal bone development. A high protein intake during the third trimester was associated with higher whole body BMD in the offspring. [92] In rats, protein malnutrition regardless of exercise during both pregnancy and lactation promoted permanent damage to the bone structure of the offspring.[93]

In summary, an adequate maternal diet during pregnancy is not only beneficial for fetal bone development but also for the mothers as it prevents resorption from mother's bone if inadequate. Maternal dietary protein, calcium and vitamin D will be accounted for in our study as they may impact fetal skeletal outcomes.

1.3.2.2 Childhood predictors of bone mass

Since childhood is an important phase in bone mass accretion, adequate nutrition and physical activity is necessary to optimize an individual's peak bone mass accrual. These two factors were assessed in our study as possible co-confounders to child bone mass at 3 years of age.

Since childhood is the time of greater bone formation than bone absorption, attaining optimal peak bone mass is the key. Dietary calcium and vitamin D intakes during childhood are important determinants of bone health. From birth to adulthood, females and males gain approximately 875g and 1175g of calcium respectively. The skeleton is an enormous reserve of calcium, where 99% of the body's calcium resides. [94] The current RDA for calcium is 700 mg/day for children between the ages of 1 and 3.

Most studies investigating calcium's effect on bone health have focused on children 6 years and older as 25% of PBM is acquired during a two-year period close to the time that peak height growth velocity is reached. Cross sectional studies on children, adolescents and young women indicate that higher calcium intakes are associated with higher bone mass.[94]

In addition to the high demands for calcium is the need for vitamin D. As previously explained, vitamin D is necessary for calcium uptake, bone development and remodeling. The primary source of vitamin D is conversion in the skin via exposure to UVB radiation, however dietary intake is also necessary. The re-emergence of rickets in North America sparked further investigation into the status of vitamin D in children. Vitamin D deficiency is an increasing concern especially for individuals in northern

latitudes, those with dark skin, obese children, and breast-fed infants.[16] Vitamin D supplementation during infancy has been associated with long-term skeletal benefits. Moreover, in prepubertal children with low vitamin D concentrations, supplementation had greater effect on BMD in specific bone sites.[16] Due to the emphasis on vitamin D and bone health, many countries, including Canada and the United States, have fortified food with vitamin D precursors, including cow milk.

Dietary protein, including those from dairy products has the potential for both a beneficial and detrimental influence on bone accretion in children. Protein in the form of collagen makes up approximately one third of total bone mass and both bone growth and turnover requires a steady supply of protein to replenish lost collagen. It is especially important in young children, as protein requirement for deposition of bone is one sixth of the average total requirement for a 1 year old. Inadequate protein intake in undernourished children delays skeletal growth and reduce bone mass. Excess dietary protein, particularly animal protein, is associated with increased urinary calcium loss,[95] which poses risk of fracture.

Most clinical studies have focused on the adult and elderly populations. Increasing protein intake, especially in those who were inadequate has reduced hip fractures in adults and is positively associated with bone measures in premenopausal women and older men.[96] There are a few studies in children, adolescents and young adults that have observed positive relationships between protein intake and BMC, however most of these studies caution that the anabolic effect of dietary protein on bone only occurs with an adequate intake of potassium, magnesium and calcium.[96,97] Protein from dairy products

is one alternative that can counteract the calcium loss from increased protein intake as well as providing other necessary nutrients for bone health such as vitamin D.

Being mindful of the impact of these nutrients on childhood bone health, we conducted assessments of dietary and supplement intake of these nutrients at 3 years of age using three-day diet records.

Exercise, especially that which is weight bearing, has been deemed beneficial for bone mass development. Increasing bone strength has been hypothesized to enhance bone strength in adulthood. Evidence from animal and human studies suggest that bone is able to adapt to external mechanical strains associated with physical activity. [98] Studies have shown that an osteocyte network senses mechanical strain on bone and directs remodeling according to the type of strain. When shear stress is sensed, it induces repression of osteocytes and increases osteoblast differentiation. However, absence of any mechanical strain leads apoptosis of osteocytes, disrupting bone resorption and leading to severe osteoporosis.[99]

A recent longitudinal study by Scerpella and colleagues on female ex-gymnasts discovered that compared with non-gymnasts, ex-gymnasts had greater radius bone mass, size and aBMD. These skeletal benefits persisted more than 4 years after the gymnasts discontinued their sport. These girls were followed 4 years prior to menarche to 9 years after menarche. [100] This works supports the notion that exercise, particularly those that are intense and weight bearing, in childhood and early adolescence can result in sustained skeletal benefit into adulthood. Thus, since physical activity has some contribution to childhood bone accrual, it was important for us to assess this in our study.

CHAPTER 2
STUDY DESIGN AND METHODS

Chapter 2: Study Design and Methods

Section A: Method Optimization for Bone Measures by Dual Energy X-Ray Absorptiometry

A.2.1 Dual energy x-ray absorptiometry

Dual energy x-ray absorptiometry (DXA) is employed widely to measure body composition in research and may also be a clinically useful tool.[101] DXA has been validated across age groups from premature infants to seniors including both normal and overweight subjects, and against other body composition measures such as skinfold-thickness assessments, bioelectrical impedance analysis, computed tomography and magnetic resonance imaging.[102-104] DXA scans have the advantage of short scan time, low radiation dosage and the ability to provide direct measures of fat tissue, non-bone lean tissue and bone mineral content in an isolated region or the body as a whole.[105]

A.2.2 Validation of surrogate limb analysis

The application for DXA in infants and children is gradually growing in use to understand the impact of disease on bone health or nutritional impact on body composition.[67,106] A key limitation of DXA scanning in young children is the need to remain perfectly still during the entire scan albeit only about 3 minutes. Slight movement of the arms and legs when the scan arm passes, causes the image to be either blurred or

broken. The values for these images, if taken as is, are invalid thus causing loss of valuable data.

To our knowledge, no previous study has compared the substitution of surrogate limb measurements as estimates of complete original whole body scans using the Hologic DXA system. Reported validation of half-body scans in the obese population was determined to be closely comparable to whole body analysis; however, these were done using a Lunar DXA system and were performed on older and obese populations. [107-109] A similar validation has not been done in a very young population.

In order to examine an alternative method of DXA analysis whereby scans that showed flaws in limb measurements could still be used, we performed a comparison of surrogate limb scans versus whole body scans for measuring body composition in a sample of 3 year old children.

A.2.2.1 Study design

Subjects

Whole-body DXA scans were taken of 451 children aged 3 years as part of an ongoing birth cohort study at McMaster University. [110] For this validation study, scans were excluded if the child was reported as non-Caucasian, or the scan showed movement artifacts, overlap of body parts, or artifacts that could hinder correct analysis of limb sections (Figure 3). This provided 246 child scans (123 males and 123 females) of the quality shown in Figure 4 that were included in the statistical analysis for validation. Ethics approval for conduct of the DXA measures was obtained from the Research Ethics

Board of McMaster University and informed consent was obtained from the subject's guardian at recruitment.

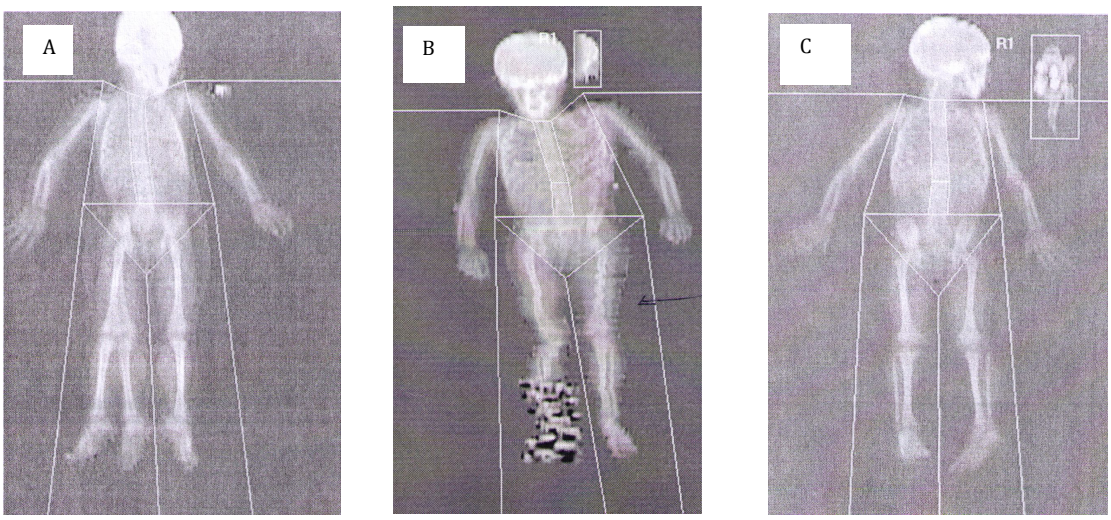

Figure 3. Excluded scans: A and B - due to movement artifact; C – due to artifact in limb sector.

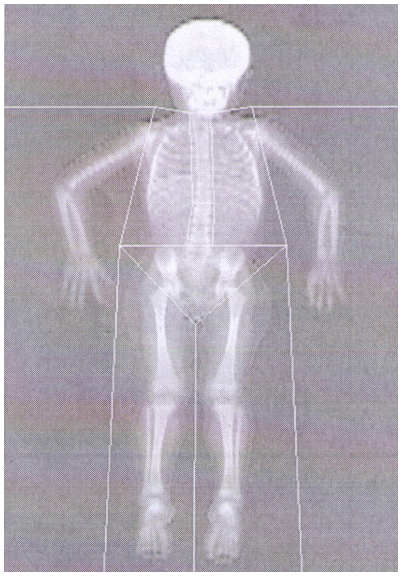

Figure 4. Example of a perfect scan that was included in study.

Instrumentation

DXA measurements were acquired with the Hologic Discovery QDR 4500 System (Hologic Inc., Waltham, MA). The Hologic device utilizes a constant x-ray source producing fan beam dual energy radiation with a very low effective dose equivalent of 5μSv. It takes approximately 3 minutes to run a whole-body scan. For this experiment all scans were performed using the auto whole body mode, which uses algorithms to improve the accuracy of edge detection of the bones in younger children and individuals with low BMD.[111]

Scan analysis was performed using the Hologic Discovery QDR Series Software V12.3 (Hologic Inc. Waltham, MA). This software allows for the adjustment of specific regions of interest. The body was divided into nine distinct regions for analysis, the head, spinal cord, left and right trunk, left and right arm, pelvis and left and right leg.

Each whole body scan was analyzed, and the data for each limb noted. By replacing the measurements of one scanned limb with those of the opposite limb we obtained an estimate value of the sum of the regional areas that was then compared to the whole body scan for fat, lean and bone mass, percent whole body fat and total mass.

Obtaining a good scan from a 3 year old

A few techniques were used to obtain a legible and valid scan of a 3 year old. Since mothers were also being scanned as part of the study, we scanned the mothers first in order to achieve greater cooperation of the child to be scanned. Watching the mother perform the scan first was a key model and stress reliever. The mother was not only able

to demonstrate the correct position to lie on the scan bed, but also dispelled any fears of the machine itself. It was also useful to show the child the mom's scan image on the computer as the DXA machine scanned the mother. Rocking horses or a comfortable seat for the child during the scan of the mother helped to put them at ease.

Once the child was on the DXA bed, staff spoke softly and calmly as well as used encouraging phrases such as "you are doing great." Visual aids, like pictures of popular cartoon characters on the ceiling or the scan arm aided to position a child's head to face up. Allowing the mother to hold the child's left hand during the first two thirds of the scan helped to keep the child comfortable. Toys aid in calming nerves, however, should be used as a last resort as it may interfere with the scan if the child refuses to be separated from the toy. Lastly, softly counting down from 10 during the last scan section, allowed for time perception.

Statistical analysis

Mean values for BMC, fat mass, percent body fat, lean tissue mass and total tissue mass were compared independently for each limb as well as surrogate estimates against whole body. All values are expressed as a mean $\pm$ standard deviation (SD) unless otherwise indicated. After finding the impact of the child's sex to be negligible, all data were pooled. Correlation analysis for upper and lower limb measurements as well as for whole body and surrogate estimates were calculated. Bland-Altman analysis was performed to test for the magnitude of agreement and bias in all parameters.[112] Statistical analysis was performed using GraphPad Prism 6 and SPSS version 16.0.

Section B: Longitudinal Study in Mother-Offspring Pairs

B.2.1 Study Design

B.2.1.1 FAMILY study

This project is an ancillary study of the **F**amily **A**therosclerosis **M**onitoring **I**n Ear**LY** life (FAMILY) study, 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 later in life.

The participants for this project were a subset of the FAMILY study sample, which consisted of mothers recruited from prenatal ultrasound clinics at McMaster University Medical Center (MUMC) and Saint Joseph's Hospital 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, food intake 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 3 years old, the family attended a follow-up visit at which the child's diet and bone health status via dual energy x-ray absorptiometry (DXA; Hologic Inc, Waltham, MA) were assessed at MUMC. A DXA scan on the mother was also performed at this time, analyzing both whole body and abdominal region.

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

B.2.1.2 Inclusion and exclusion criteria

We included only singleton children and their mothers in this study. As well, only mothers with available maternal serum sampled at third trimester of pregnancy were included.

B.2.2 Maternal measurements

B.2.2.1 Leptin status during pregnancy

Maternal blood samples were collected at the initial visit during the third trimester of pregnancy. They were spun and frozen at -80C in the central research biobank at PHRI of McMaster University. A small sample of each woman's serum was then transferred to Dr. Atkinson's lab and stored in the -80°C freezer.

Leptin was analyzed using a pre-coated human leptin enzyme-linked immunosorbent assay (ELIZA) kit (Lot E13-020, Ref. RD191001100) supplied by Biovendor (Asheville, NC). This kit was previously tested in studies with people from various age groups and BMI including an obese sample. Each kit included a 96 well plate. A fresh kit was used for each ELISA plate tested, with the accompanying Standards and QCs.

The volume of serum required per sample was 50 μL, and a total of 430 samples were processed. The protocol followed was based on those provided by BioVendor. The standards, quality controls and samples were incubated in microplate wells pre-coated with polyclonal anti-human leptin antibody. Both the high and low quality controls were human serum based, with inter-assay coefficient of variability (CV) of 6% and 4% respectively. After the incubation and wash, polyclonal antihuman leptin antibody conjugated with horseradish peroxidase (HRP) is added to the wells and incubated to bind to captured leptin. Once washed, the remaining HRP conjugate is allowed to react with the substrate solution. The reaction is stopped by addition of acidic solution and the absorbance of the resulting yellow product is measured. The Wallac 1420 Multilabel Counter (Perkin Elmer, Waltham, MA) was used to read the ELISA plate. The absorbance is proportional to the concentration of leptin. The concentration of unknown samples was determined by the Workout 2.0 software, using a standard curve constructed by plotting the absorbance values against the concentration of standards.

B.2.2.2 Vitamin D status during pregnancy

Maternal blood samples were collected as previously stated. Serum 25OHD analyzed using a UPLC LC/MS-MS (Waters ACQUITY UPLC) and the MassLynx 4.1 software in the Fusch laboratory, following the protocol outlined by Hymoller and Jensen.[113] This protocol enables the fat in serum to be saponified and provides a better homogenous concentration of vitamin D in the sample.[113]

The calibration curve was run using standards prepared form a 25OHD sample obtained from Sigma (standard of concentration 1mg/ml). Low and high quality control samples were run using samples from ClinCheck (Recipe Chemicals, Germany), reference numbers 35080 and 35081 from lot 015; the coefficients of variation for measurement of low and high $25OHD_3$ were 19% and 11% respectively. The accuracy of the method was confirmed using the most recent certified NIST reference material for vitamin D analysis, SRM972, which contains a known quantity of 25OHD quantified by isotope dilation tandem mass spectrometry.

The volume of serum required per sample was 150 μL, and a total of 467 samples were processed. In each sample, a deuterated internal standard was included to account for potential losses in sample preparation.

B.2.2.3 Anthropometric measures, lifestyle factors, and nutrition

At the initial visit, anthropometric measures of the mother were taken. Height was measured using a stadiometer, and weight by an electronic scale. Tricep and subscapular skinfold thickness was also measured by a trained individual. At the 3-year visit, the mothers body composition, total mass and bone mass was assessed by DXA. As described in section A.2.2.1, the Hologic Discovery QDR 4500 System (Hologic Inc., Waltham, MA) was the machine used. The mother's whole body BMD z-scores were calculated using the reference data embedded in the Hologic software for analysis.

Demographics and lifestyle factors during pregnancy were assessed via questionnaires. Mothers were asked about their medical history, smoking history,

education and annual income. Physical activity during pregnancy was assessed using a validated FAMILY questionnaire adapted from a previous study at PHRI; [114] the questions helped estimate both leisure and occupational activity. Leisure activities were divided into four categories, ranging from mainly sedentary (score = 0) to strenuous exercise (score = 3). Occupational activities were also divided into four categories, ranging from do not work or mainly sedentary (score = 0) to heavy physical labour (score = 3). A total score between 0 and 6 was obtained from each mother; 0 was assigned to a sedentary women, 1-2 for somewhat active, 3-4 for active, and 5-6 for very active.

To assess dietary intake in the past year, a food frequency questionnaire (FFQ) was completed by mothers. The FFQ contained 157 items, each of which asked for the frequency of consumption, and portion size consumed; photographs were provided as a visual aid to estimate sizes. Furthermore, a section of 14 questions pertained to cooking methods, as well as consumption of nutritional supplements during pregnancy such as prenatal vitamins and multivitamins. Data from the FFQ was computed by a registered dietician at PHRI, using a calculator created in Excel 2007, which yielded the average nutrient intake per day.

B.2.2.4 Gestational weight gain

During the initial visit, mothers were weighed and asked their current weight gain. The difference of these two measurements was used to obtain their pre-pregnancy weight. At the last visit before delivery, mothers were again weighed. The difference between this

last weight and their pre-pregnancy weight was calculated to provide the total gestational weight gained during pregnancy.

B.2.3 Child measurements

B.2.3.1 Z-score calculation for bone mineral content

BMC for children was assessed using a QDR 4500 Discovery Hologic DXA machine, the same machine noted in Section A. Analysis of BMC was just as noted in Section A. Movement artifacts were dealt with, using the alternative surrogate limb methodology which was evaluated in Section A. All scans were performed on the same machine by trained members of the research team, and were 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.

The numerical value of the BMC is meaningless if it is not compared to the appropriate normal controls. Therefore, the BMC is reported as a z-score, which is a standard deviation score in relation to a population of similarly aged children. A z-score of zero is equivalent to the mean, and a score of -1 and +1 are equivalent to values one standard deviation below and above the mean.[67] The largest normative data set for 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 in Texas. We used this calculator to compute z-scores for BMC of the children in our study.[115,116] This calculator had data for our age group specific for the

Hologic QDR 4500 measurements, as well as allowing us to account for gender, height and ethnicity.

B.2.3.2 Lifestyle factors – diet and physical activity

The child's diet was assessed using a 3-day diet record, which was mailed to participants, completed by parents and returned to the investigators during the three-year visit. Any clarifications in the diet record were made with the parents during the clinical visit. Parents were instructed to record all foods consumed by the child including the brand, cooking method, and portion size for three days. The three days needed to include two weekdays and one weekend to account for variable eating patterns during weekends.

The diet records were then analyzed by trained staff using Nutritionist Pro Software (Axxya Systems, Stafford, Texas), which contains a large database with nutrient data. Specific brand names, ethnic foods and fasts foods were available in the database. If a food was not listed in the database, the US Department of Agriculture's (USDA) online reference standard was used to supplement. Once the dietary data were entered, Nutritionist Pro computed the average amount of nutrients consumed per day. Average daily intakes for calcium, vitamin D, and protein were recorded for each child. To ensure consistency in the analysis of the 3-day diet records, all research staff followed a standard of practice protocol (SOP) developed by a previous Masters student. [117]

The physical activity of the child was assessed using a modified Habitual Activity Estimation Scale (HAES) questionnaire, [118] which collected information on the child's

activity level on a typical weekday and a typical Saturday. It recorded the total hours per day the child spent being "active" and "very active"

B.2.4 Statistical analysis

Statistical analysis was performed using STATA 13.0 (StataCorp LP, Texas). Descriptive statistics were computed by calculating the mean, standard deviation and median for all data; frequencies, minimum and maximum values are also reported.

Child anthropometric and bone measures were stratified by gender, with two-tailed unpaired t-tests conducted to detect any sex-based differences.

Univariate and multiple regression models were used to assess 1) the maternal predictors of leptin status in pregnancy 2) the significance of maternal leptin on offspring 3 year BMC z-score. Univariate regression was first done on key contributing variables. Only variables of $p < 0.2$ and of strongest clinical significance were included in the following multiple regression. Both the non-standardized (b) and standardized regression (β) coefficients are reported for all predictors. A confidence level of $1-\alpha$ where $\alpha = 0.05$ was chosen to construct the confidence intervals. Statistical significance for the multiple regression model, was taken at p-values less than 0.05.

CHAPTER 3
RESULTS OF VALIDATION STUDY OF SURROGATE LIMB ANALYSIS BY DUAL ENERGY X-RAY ABSORPTIOMETRY

Chapter 3: Results of Validation Study of Surrogate Limb Analysis by Dual Energy X-Ray Absorptiometry

3.1 Surrogate limb validation

The children had a mean age of 3.1 years, and were not statistically different in height or weight (Table 1). Estimates of whole body measurements using the surrogate limb values were significantly correlated with the original whole body measurements for all body composition variables. Both the right and left arm surrogate estimates for BMD correlated with whole body BMD ($R^2 = 0.998$, $p<0.001$), as did both leg surrogate estimates ($R^2= 0.996$, $p<0.001$). Right and left arm surrogate estimates for fat mass correlated with whole body fat mass ($R^2=0.997$, $p<0.001$), as did both leg surrogates ($R^2= 0.995$, $p<0.001$). Arm surrogate estimates for lean mass correlated with whole body lean mass ($R^2=0.998$, $p<0.001$), as did both leg surrogates ($R^2=0.997$, $p<0.001$). Total tissue mass for all surrogate measures correlated with whole body total tissue measures ($R^2=0.999$, $p< 0.001$). Lastly, surrogate arm measures for percent fat correlated with whole body percent fat ($R^2= 0.989$, $p<0.001$), as did both leg surrogate measurements ($R^2=0.986$, $p<0.001$). The mean differences between left and right limbs were small as noted (Table 2). For example, the mean difference between left and right arm for fat mass was only 0.006 kg (95% CI: -0.001, -0.0009) (Table 2).

The Bland-Altman analysis showed high levels of agreement between surrogate estimates and original total-body scans for all of the measurements assessed, with the

limits of agreement being small and closely comparable (Figure 3). For example, comparison of the surrogate leg estimate for fat mass with the whole body scan yielded a mean difference of only 0.014 kg with the limits of agreement of -0.158 and 0.129, and that for lean mass was only 0.031 kg with the limits of agreement of -0.177 and 0.114 (Figure 5). The estimates of total body BMC, fat mass, lean mass, total tissue mass or percent fat were not significantly different when the original analysis was compared with estimated measures calculated by substituting the values from the regional analysis of right or left limbs as surrogate (Table 3).

Table 1. Characteristics of study participants. – values expressed as mean (SD).

	All subjects (n=246)	**Male (n= 123)**	**Females (n= 123)**	**p-value**
Age (yr)	3.06 (0.1)	3.05 (0.1)	3.06 (0.1)	0.671
Height (cm)	95.4 (4.9)	95.8 (5.9)	95.0 (3.6)	0.182
Body mass (kg)	15.1 (1.7)	15.3 (1.7)	15.0 (1.8)	0.207

Table 2. Correlation analysis and mean difference between limbs for all comparisons noted.

Variables measured	**R^2**	**P value**	**Mean difference (SD)**	**95 % CI**
Left/Right Arm BMC	0.712	<0.001	0.001 (0.002)	-0.001, -0.0009
Left/Right Arm fat mass	0.724	<0.001	0.006 (0.056)	-0.001 0.013
Left/Right Arm lean mass	0.624	<0.001	-0.029 (0.063)	-0.037, -0.021
Left/Right Arm % fat	0.590	<0.001	1.788 (7.386)	0.860, 2.715
Left/Right Arm total tissue mass	0.888	<0.001	-0.024 (0.039)	-0.029, -0.019
Left/Right Leg BMC	0.879	<0.001	-0.002 (0.003)	-0.003, -0.002
Left/Right Leg fat mass	0.912	<0.001	-0.014 (0.073)	-0.024, -0.053
Left/Right Leg lean mass	0.902	<0.001	-0.031 (0.074)	-0.040, -0.022
Left/Right Leg % fat	0.850	<0.001	0.305 (3.162)	-0.092, 0.702
Left/Right Leg total tissue mass	0.966	<0.001	0.048 (0.063)	-0.056, -0.040

Table 3. Comparison of original whole body measure of bone, lean, fat and total tissue mass with estimated measures using values of regional analysis of right or left arm or right or left leg as substitutes (surrogate). Values are mean (SD).

	Whole Body (original)	**Right arm substitute**	**Left arm substitute**	**Right leg substitute**	**Left leg substitute**
BMC (kg)	0.52 (0.05)	0.52 (0.05)	0.52 (0.05)	0.52 (0.05)	0.52 (0.05)
Fat (kg)	4.49 (1.05)	4.48 (1.05)	4.49 (1.06)	4.50 (1.06)	4.47 (1.04)
Lean (kg)	10.10 (1.28)	10.13 (1.28)	10.07 (1.28)	10.13 (1.29)	10.07 (1.28)
Percent fat (%)	29.56 (5.31)	29.51 (5.31)	29.68 (5.37)	29.68 (5.33)	29.68 (5.29)
Total tissue mass (kg)	15.11 (1.74)	15.13 (1.74)	15.08 (1.74)	15.15 (1.75)	15.06 (1.73)

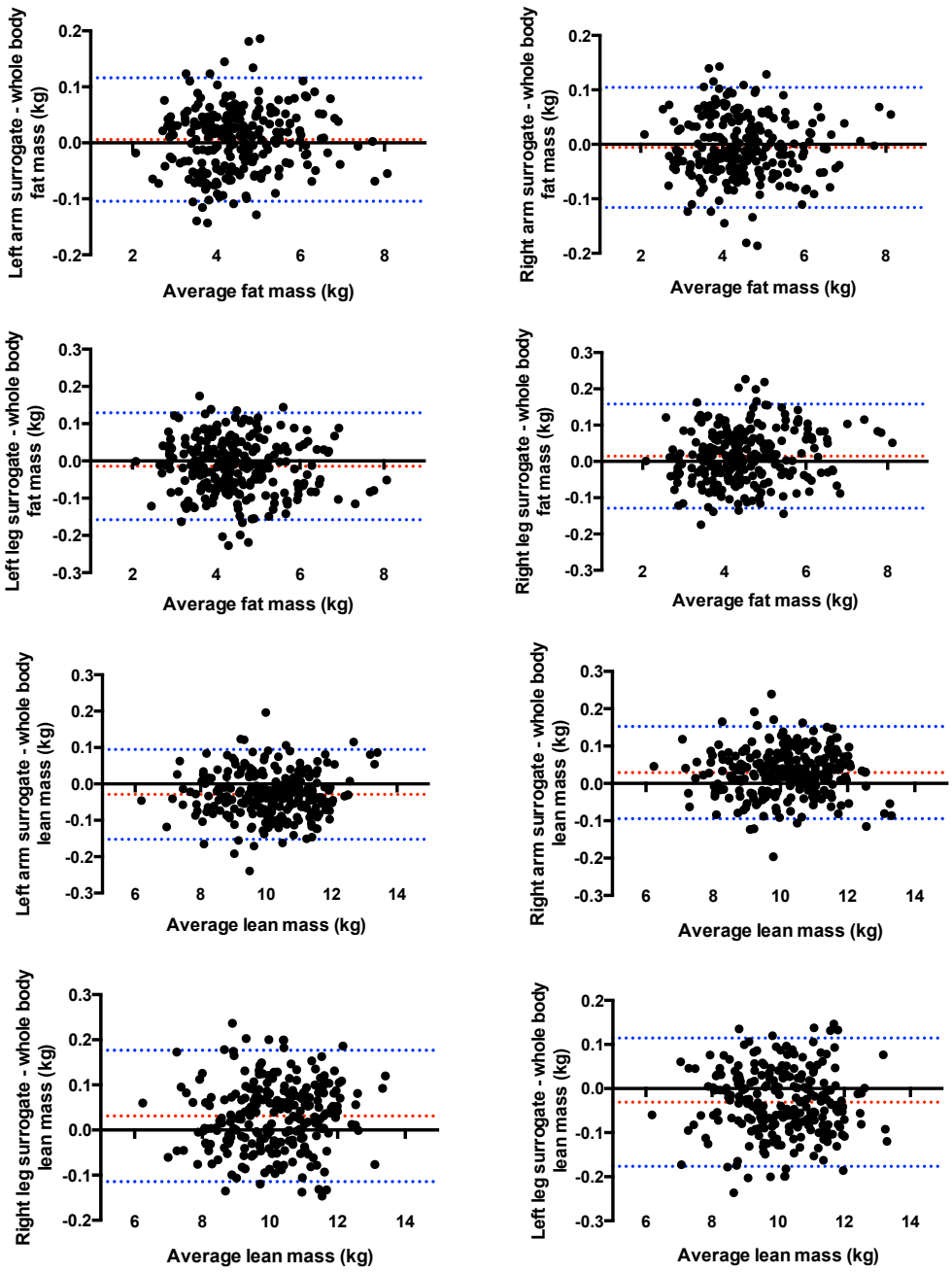

Figure 5. Bland-Altman plots comparing left and right limb surrogate scans to whole body scans for fat and lean mass. Each figure shows values for 246 subjects. Hashed lines represent mean difference between methods (red), and 95 % limits of agreement (blue).

CHAPTER 4
RESULTS OF LONGITUDINAL STUDY

Chapter 4: Results of Longitudinal Study

4.1 Study sample demographics

A total of 425 women had available serum samples that were tested for leptin and 25-OHD status. Of these, 357 singleton children successfully completed a valid DXA scan at the 3 year visit. Of these, 237 had corresponding maternal DXA scans. The remainder of mothers declined to have DXA scans, either because they were pregnant or for other reasons.

Demographic characteristics of the mothers are summarized in Table 4. Each variable had less than 5% missing data (except for gestational weight gain which had less than 10%). Approximately 90% of the mothers were of European descent, with the rest representing a variety of ethnic backgrounds including: African, Asian, Hispanic, and Aboriginal. The average age of mothers at recruitment was 32.7 years. The majority of the mothers were highly educated, with 87% having post-secondary education, 93% were married or living in a common law relationship and 63% lived in a household that earned more than $70 000 a year. The characteristics of this sample are comparable to the demographics of the entire FAMILY study sample, which consisted of 836 mothers.[110]

Table 4. Demographics of mothers recruited in pregnancy

Maternal characteristics	n	%	Mean (SD)
Ethnicity	425		
European	384	90.3	
Other	41	9.7	
Age (yr) at recruitment	425		32.7 (4.7)
Education	424		
≤ 13 yr	55	13.0	
> 13 yr	369	87.0	
Marital Status	425		
Married/Common Law	395	93.0	
Single/Divorced/Widow/ Separated	26	6.1	
Unknown	4	0.9	
Household Income	425		
≤ $49 999	83	19.5	
$50 K – $69 999	63	14.8	
$70 K – $99 999	117	27.5	
≥ $100 K	149	35.1	
Unknown	13	3.1	

4.2 Maternal variables during pregnancy

4.2.1 Maternal physical measures, lifestyle factors, and nutrition

Physical Measures: More than half of the mothers entered pregnancy overweight and obese (BMI >25) (Table 5). The skinfold thickness for both tricep and subscapular were greater in higher BMI categories ($p<0.001$ between groups for both tricep and subscapular) (Table 5). Across all BMI categories mean gestational weight gain was greater than the recommendation (IOM 2009) (Figure 6). Overall, mean BMC measured by DXA at 3 years post partum was 2.28 kg, while mean maternal BMD z-score was 0.92.

Lifestyle factors: Only about 3% of women smoked during pregnancy and less than 5% of mothers reported consuming more than one alcoholic beverage per month during their pregnancy. The majority (79%) of women were characterized as not being active or very active in the combined leisure and work activities during their pregnancy (Table 6).

Nutrition: Although the mean daily intake of vitamin D from food was below the EAR of 400 IU/day recommended for pregnant women, the majority of women (72%) took vitamin D supplements (Table 7). Mean total intake of vitamin D including supplements just barely met the EAR. Total vitamin D intake (mean food + supplement =394 IU/day) was below the EAR in 62% of mothers (Figure 7).

The mean daily intake of calcium from food exceeded the EAR of 800 mg. With addition of calcium intake from supplements consumed by 74% of mothers, average total

intake of calcium rose by 200 mg/day. With the addition of supplements, only 9% of mothers had intakes below the EAR for total daily intake of calcium (food + supplements), while 91% exceeded the EAR more than two fold, with a mean of 1753 mg/day (Figure 8).

The majority (79%) of mothers exceeded the EAR for protein, consuming greater than the recommended 0.88 g/kg/day (equivalent to 1.5 g/kg/day of protein) (Figure 9).

Sodium intake for mothers was much higher (mean = 2669 mg) than the AI of 1500 mg for pregnant women, with the highest intake almost 8 times the AI (11414 mg/day) (Table 7). The majority of the total daily energy came from carbohydrates (56%) and fat (29%) (Table 7). The mean percent energy of total calories from fat (29%), protein (17%) and carbohydrates (56%) were within the acceptable macronutrient distribution range (AMDR) for pregnant women (20- 35% for fat, 10-35% for protein and 45-65% for carbohydrates) (Table 7).

Table 5. Physical measures of mothers during pregnancy and at 3 years post-partum (For each maternal measure, values with unlike lower case letters are statistically different, p <0.001 by multiple means Tukey test)

Maternal measure	N	%	Mean (SD)	Median (min, max)
Height (m)	425		1.6 (0.1)	1.7 (1.4, 1.8)
Pre-pregnancy body mass index, kg/m^2	411			
<18.5	6	1.4	17.5 (0.9)[a]	17.8 (15.9,18.4)
18.5-24.9	191	46.5	22.4 (1.6)[b]	22.6 (18.8, 24.9)
25-29.9	117	28.5	27.1 (1.4)[c]	27.0 (25.0, 29.9)
> 30 kg	97	23.6	35.7 (5.0)[d]	34.4 (30.1, 54.7)
Tricep Skinfold Thickness (mm)	420		31.3 (11.8)	29.6 (7.8, 66.3)
By BMI: <18.5	5		20.3 (7.8)[a]	21.7 (11.7, 29.5)
18.5-24.9	189		24.7 (7.7)[a]	23.5 (7.8, 54.2)
25-29.9	115		31.8 (8.1)[b]	31.7 (9.0, 54.2)
≥ 30	97		44.1 (12.1)[c]	44.0 (20, 66.3)
Subscapular Skinfold Thickness (mm)	414		24.2 (9.4)	22.7 (7.5, 57.2)
By BMI: <18.5	5		15.7 (5.4)[a]	14.0 (10.0, 22)
18.5-24.9	188		18.8 (6.0)[a]	18.3 (7.5, 38.4)
25-29.9	112		25.0 (6.7)[b]	25.0 (13.0, 50.2)
≥ 30	95		34.8 (8.3)[c]	34.7 (16.7, 57.3)
Gestational Weight Gain	383		14.1 (5.3)	13.9 (0.38, 36.5)
BMC at 3 yr post-partum (kg)	302		2.28 (0.3)	2.25 (1.6, 3.9)
BMD z-score at 3 yr post-partum	302		0.92 (1.0)	0.9 (-1.5, 6)

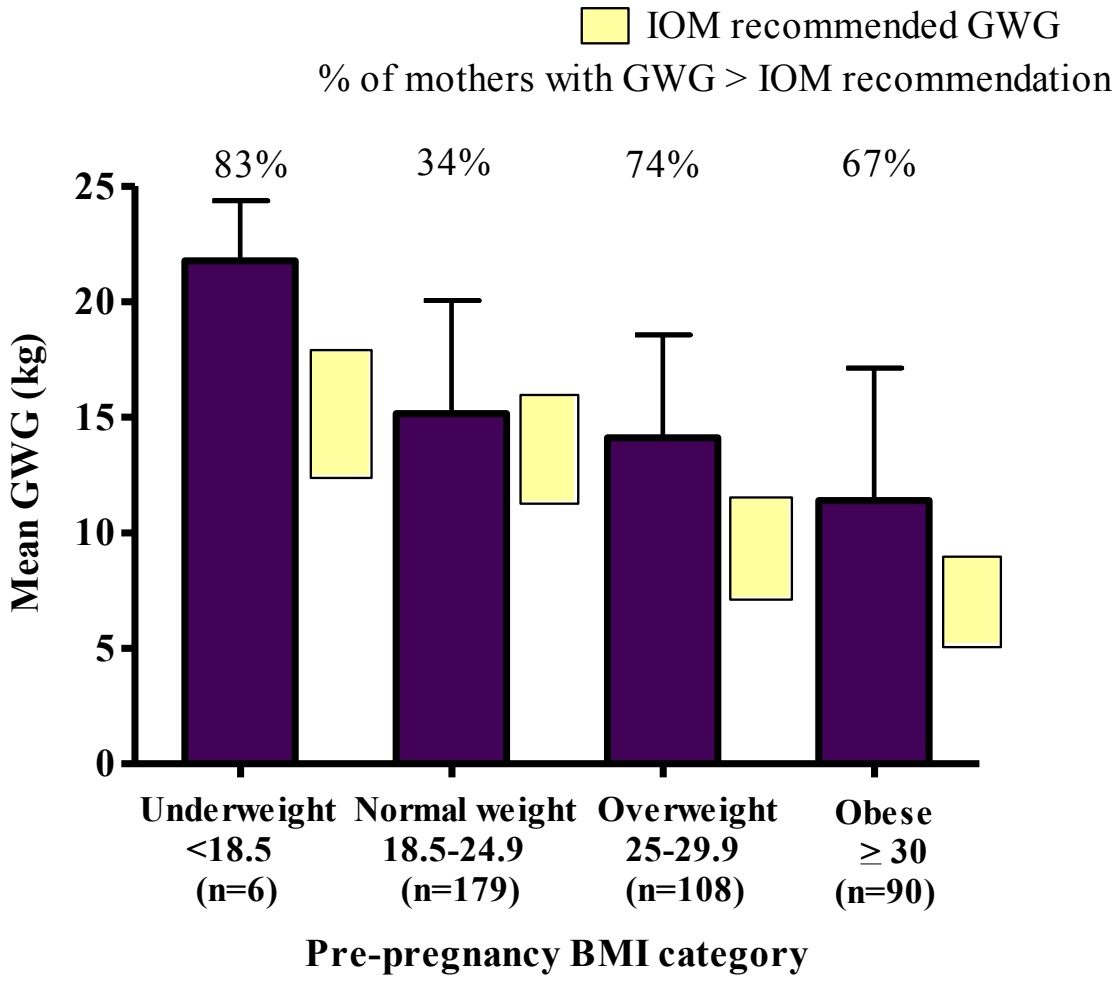

Figure 6. Average gestational weight gain of mothers by pre pregnancy BMI category, compared to IOM recommended total gestational weight gain during pregnancy for each BMI category.[49,133]

Table 6. Lifestyle factors of mothers during pregnancy

Maternal characteristics	n	%
Smoking Status	420	
Smoked during pregnancy	14	3.3
Formerly smoked but quit before pregnancy	131	31.2
Never smoked	275	65.5
Alcohol intake	420	
Never, <1 drink/month	400	95.2
>1 drink/month	20	4.8
Pregnancy physical activity (work + leisure)	423	
0 – sedentary	65	15.4
1 – somewhat active	128	30.2
2 – somewhat active	141	33.3
3 – active	60	14.2
≥4 – active, very active	29	6.9

Table 7. Maternal intake of specific nutrients and food groups during pregnancy

Maternal diet		n	%	Mean (SD)	Median (min, max)
Vitamin D	Supplement Use No use 200-400 IU >1000 IU	423 119 296 8	 28.1 70 1.9		
	From food (IU/day)	408		163 (172)	94 (5, 1219)
	Total intake, food + supplement (IU/day)	408		394 (245)	357 (5, 1844)
Calcium	From food (mg/day)	408		1457 (699)	1364 (250, 4345)
	From food (mg/day/1000 calories)	408		667 (214)	647 (183, 1506)
	From supplements (mg/day) None 200-300 mg 350-650 mg	423 109 292 22	 25.8 69.0 5.2		
	Total intake, food + supplement (mg/day)	408		1658 (714)	1565 (373, 4845)
Dietary protein	g/day	408		91 (32)	88 (19, 198)
	g/kg/day	398		1.3 (0.5)	1.2 (0.2, 3.4)
Energy intake	kcal/day	408		2172 (722)	2095 (631, 4448)
	kcal/kg/day	398		31 (12)	30 (8, 74)
Sodium Intake (mg/day)		408		2669 (1024)	2535 (701, 11413)
% Energy from fat		408		29.3 (4.6)	29.4 (18.1, 48.5)
% Energy from protein		408		16.8 (2.5)	16.8 (9.8, 23.9)
% Energy from carbohydrate		408		55.8 (6.1)	55.9 (34.8, 73.0)

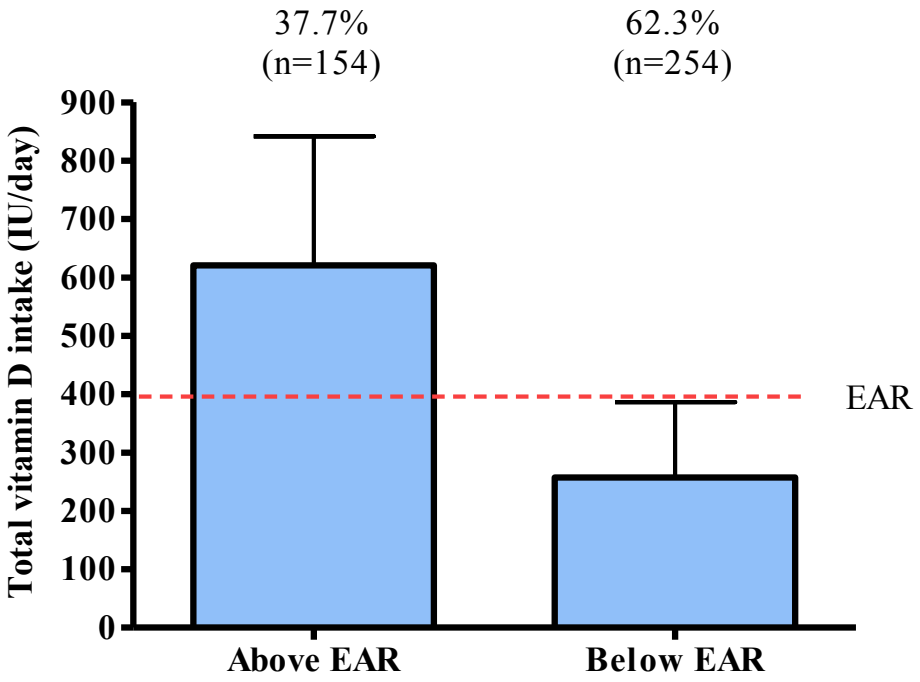

Figure 7. Mean maternal daily intake from food and supplements of vitamin D above and below EAR of 400 IU during pregnancy. Data are mean values and error bars as SD. Percentage of women in each category with intakes above or below EAR is indicated above bars.

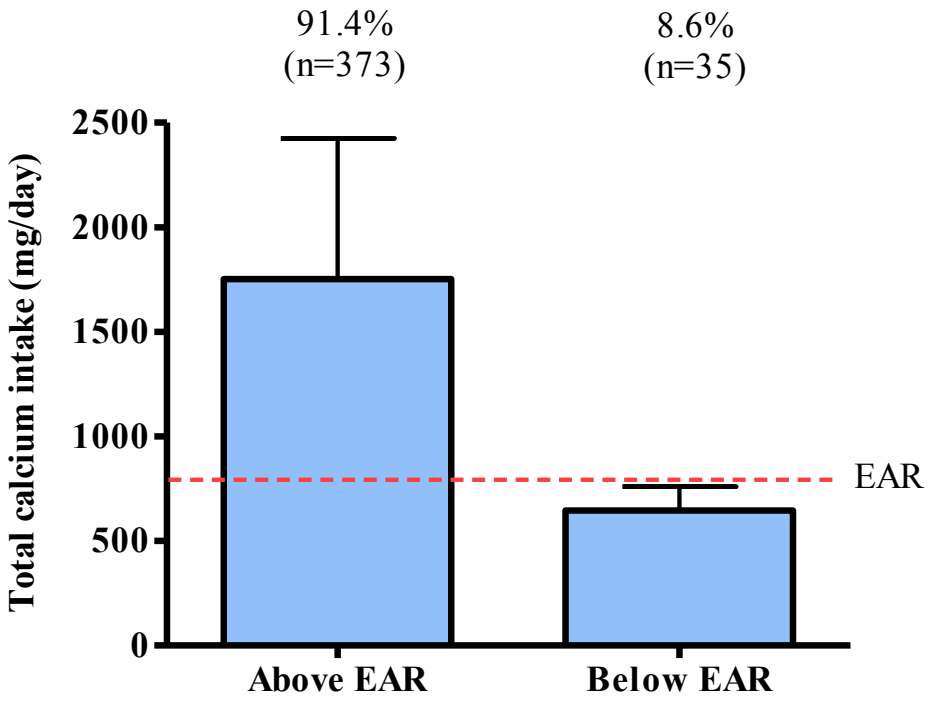

Figure 8. Mean maternal daily intake from food and supplement of calcium above and below EAR of 800 mg/day during pregnancy. Data are mean values and error bars are SD. Percentage of women in each category with intakes above or below EAR is indicated above bars.

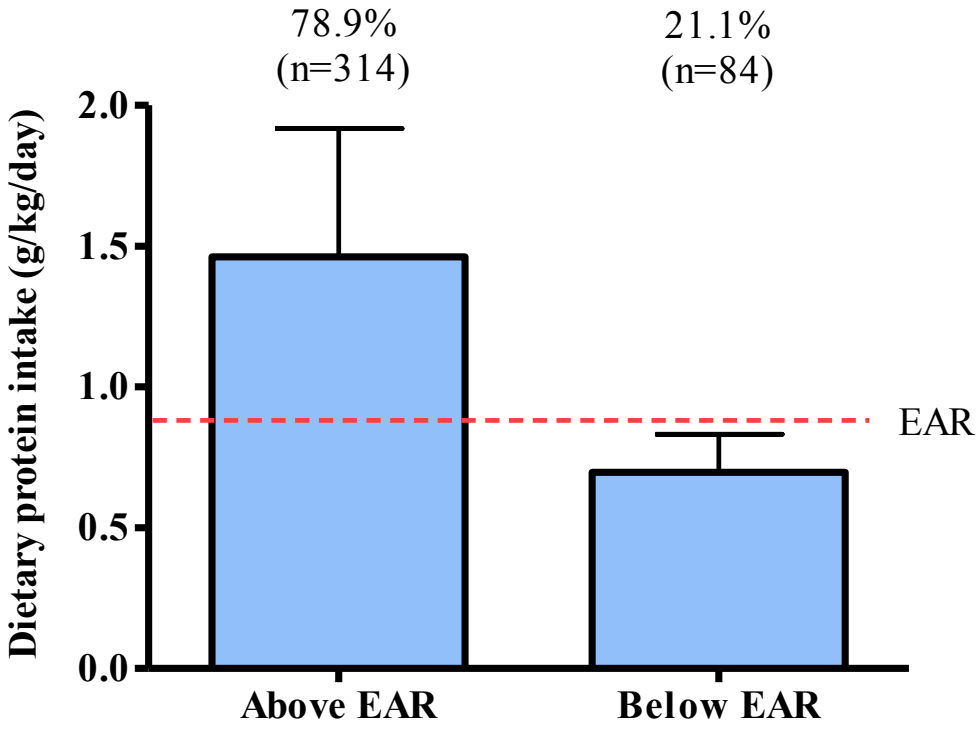

Figure 9. Mean maternal dietary intake of protein above and below EAR of 0.88 g/kg/day during pregnancy. Data are mean values and error bars are SD. Percentage of women in each category with intakes above or below EAR is indicated above bars.

4.2.2 Maternal serum measures for leptin and 25-hydroxyvitamin D

Circulating serum leptin in the third trimester of pregnancy was significantly higher in women with a pre-pregnancy BMI in the overweight and obese categories ($p<0.001$ by ANOVA) (Figure 10). The average leptin concentration for obese women was more than two fold that of the normal weight women (Figure 10).

For vitamin D status, the majority of women (70%) fell into the optimal range of 50-125 nmol/L (nM) defined as adequate for bone health by the IOM.[119,120] Vitamin D insufficiency was observed in only 21.7% (and only 4.8% had a value below 30 nmol/L, defined as true deficiency) while 7.9% of mothers would be classified as having excessive circulating vitamin D (Figure 11). The overall mean serum 25 OHD for mothers was 76.5 nmol/L. The mean maternal 25OHD assessed in the summer was significantly higher than that assessed in winter, but both were above 50 nM (Figure 12).

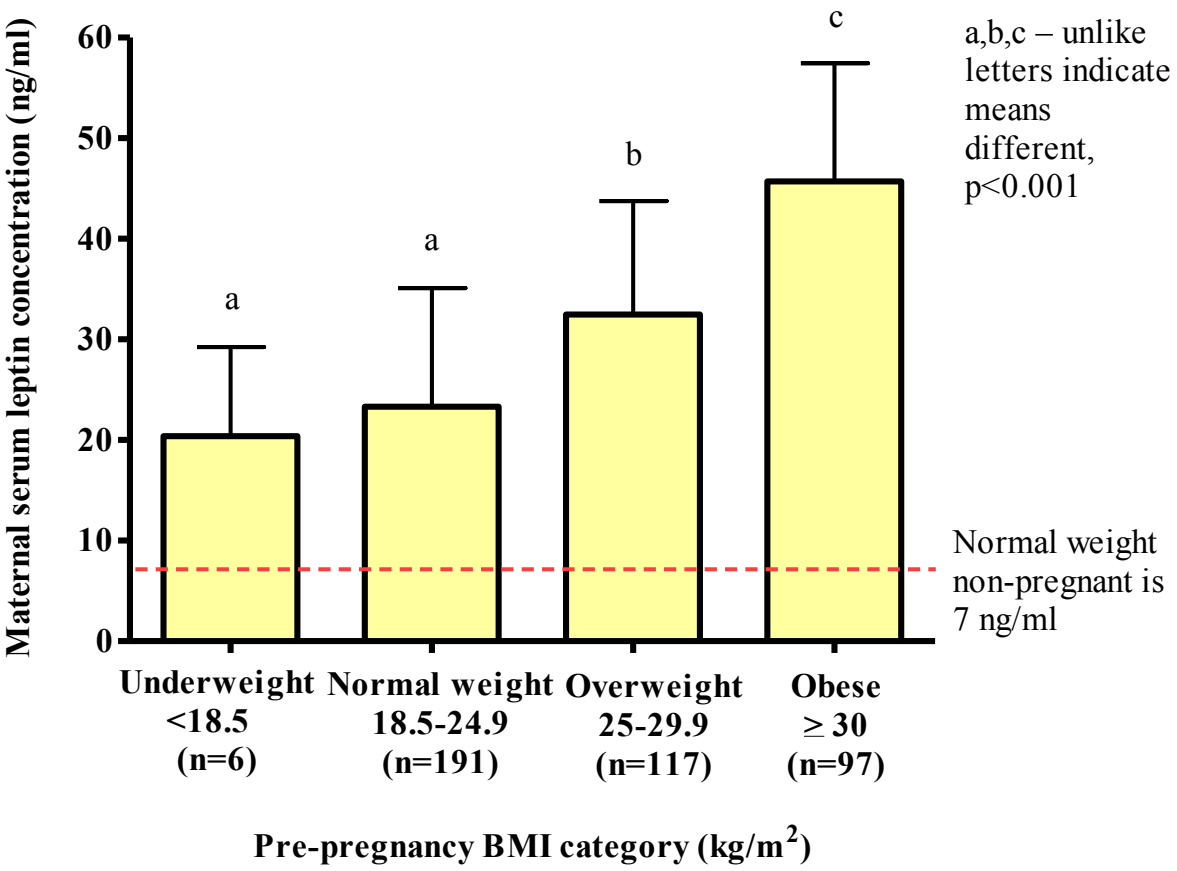

Figure 10. Circulating serum leptin at third trimester of pregnancy by BMI category. Data are mean values and error bars are SD. (p-value <0.001 by ANOVA).

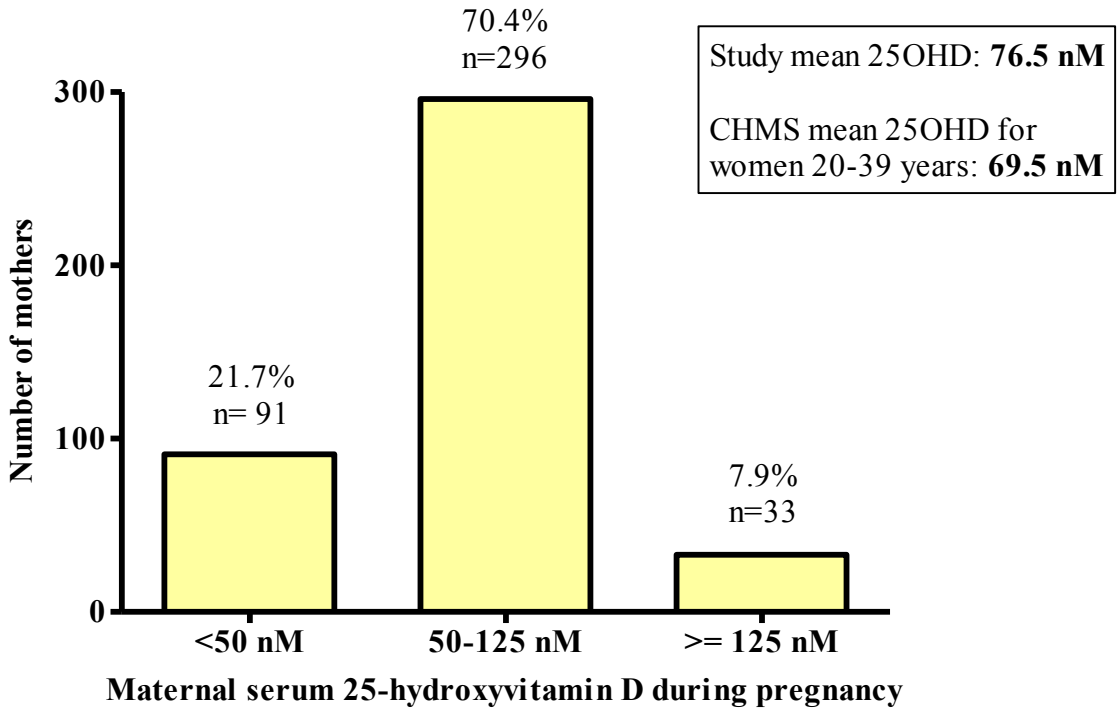

Figure 11. Frequency of distribution of mothers with serum 25 OHD <50 nM (sub-optimal), 50-125 nM (optimal) and > or equal to 125 nM (potential for toxicity) during pregnancy, defined according to DRI report, 2011. [120] CHMS – Canadian Community Health Survey.[128]

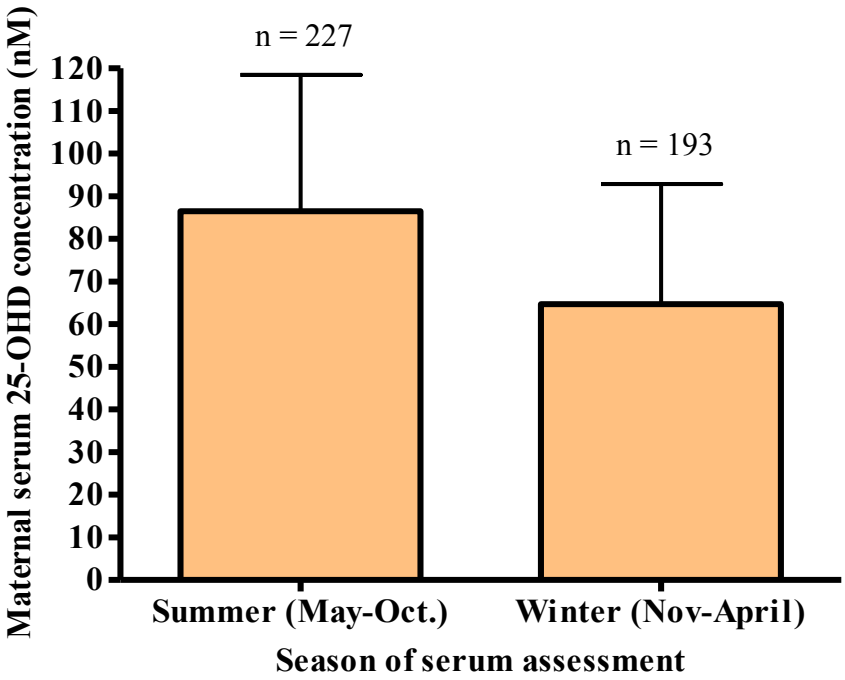

Figure 12. Circulating serum 25OHD at third trimester of pregnancy was significantly higher ($p<0.001$) when sampled in summer compared to winter months. Values are mean and error bars are SD.

4.3 Univariate and multiple regression analysis: maternal predictors of leptin status

Univariate regression analysis was performed for each of the independent values against leptin, with results listed in (Table 8). These included pre-pregnancy and during pregnancy variables such as pre-pregnancy BMI, maternal lifestyle, and dietary intakes during pregnancy. Of these, only variables indicating significant association, with maternal leptin status at p values less than 0.2, were placed into a multiple regression model (Table 9). Both the standardized (β) and the unstandardized (b) coefficients are displayed for comparison.

When maternal age at recruitment, ethnicity, pre-pregnancy BMI, physical activity score, total energy intake, percent energy from fat, and skinfold thickness were included, the multiple regression model was significant for predicting maternal serum leptin ($F_{12,376}$= 30.6, p<0.001, R-square = 0.478). Of the variables included in the model, higher maternal age was associated with lower leptin status, with each year older corresponding to a decrease of 0.25 ng/ml of leptin (Table 9). A pre-pregnancy BMI category of overweight and obese was associated with higher maternal leptin status. Similarly, the sum of the tricep and subscapular skinfold thickness was a positive predictor of leptin status (Table 9).

In this model, the predictor with the strongest effect on leptin status was maternal skinfold thickness, indicated by having the largest β among all the variables.

Table 8. Univariate analysis: maternal predictors of leptin ($p<0.2$ taken as statistically significant; * indicates statistically significant factors)

Maternal variables during pregnancy	n	b	95%CI		p-value
			Min	Max	
Age of recruitment	425	-0.29	-0.585	0.001	0.051*
Ethnicity	425				
Caucasian	384				
Non-Caucasian	41	-3.636	-8.346	1.074	0.130*
Pre- pregnancy BMI (kg/m^2)	411				
<18.5		-2.91	-12.372	6.552	0.546
18.5-24.9					
25.0- 29.9		9.17	6.491	11.849	0.000*
≥30		22.40	19.556	25.246	0.000*
Physical activity score	423				
0					
1		1.328	-3.018	5.673	0.548
2		-2.843	-7.121	1.435	0.192*
3		-4.167	-9.275	0.941	0.110*
≥4		-5.727	-12.099	0.645	0.078*
Maternal smoking	420				
Never					
Former		1.701	-1.353	4.755	0.274
Current		0.528	-7.354	8.409	0.895
Maternal alcohol consumption	420				
Never		-2.778	-9.366	3.810	0.408
>1/month					
Total energy intake	408	0.001	-0.000	0.003	0.138*
% Energy from fat	408	0.214	-0.093	0.521	0.172*
% Energy from carbohydrates	408	-0.148	-0.362	0.087	0.218
% Energy from protein	408	0.050	-0.526	0.625	0.865
Skinfold Thickness (sum of tricep + subscapular)	415	0.486	0.432	0.540	0.000*
Gestational Weight Gain	383	0.077	-0.203	0.357	0.590

Table 9. Multiple Regression analysis of maternal variables (R^2=0.478, $p < 0.001$) as predictors of maternal serum leptin (* indicates statistically significant factors)

Maternal variables during pregnancy	**n = 389**				
	β	**b**	**95% CI**		**p-value**
Age of recruitment	-0.08	-0.248	-0.475	-0.022	0.032*
Ethnicity					
Caucasian					
Non-Caucasian	-0.045	-2.273	-6.058	1.512	0.238
Pre- pregnancy BMI (kg/m^2)					
<18.5	-0.019	-2.788	-13.431	7.545	0.607
18.5-24.9					
25.0- 29.9	0.146	4.781	2.043	7.520	0.001*
≥30	0.273	9.506	5.645	13.367	0.000*
Physical activity score					
0					
1	0.048	1.553	-1.921	5.026	0.380
2	-0.005	-0.151	-3.563	3.260	0.930
3	-0.012	-0.505	-4.607	3.598	0.809
≥4	-0.008	-0.467	-5.361	4.427	0.851
Total energy intake	0.038	0.001	-0.001	0.002	0.312
% Energy from fat	0.015	0.047	-0.186	0.280	0.690
Skinfold Thickness (sum)	0.468	0.345	0.268	0.423	0.000*

4.4 Child physical measures, nutrition and physical activity measures

4.4.1 Child physical measures at birth and 3 years

Boys were significantly larger in body size than girls at birth, both with higher birth weight and birth length (Table 10). At 3 years of age, this trend continued, with boys displaying greater weight and height than girls (Table 11). The mean weight for both boys and girls was similar to the 50th percentile for 3 year olds (15 kg and 13.6 kg respectively). A comparison of z-scores for height and weight between boys and girls showed no significant difference, since each child was compared to an age and gender-matched reference data (Table 11).[121]

Table 10. Characteristics and anthropometric measures of children at birth – values expressed as mean (SD) (* indicates factors significant between genders)

Variable	Male		Female		p-value
	n	Mean (SD)	n	Mean (SD)	
Gestational age (wk)	177	39 (2)	177	39 (2)	0.528
Birth weight (g)	177	3531 (560)	178	3362 (572)	0.005*
Birth length	167	51 (2)	169	49 (3)	0.0002*

Table 11. Anthropometric measures of children at about 3 years of age (*indicates factors significant between genders)

Variable	Male (n=177)		Female (n=180)		p-value
	n	Mean (SD)	n	Mean (SD)	
Age (years)	177	3.06 (0.1)	180	3.07 (0.1)	0.690
Weight (kg)	177	15.12 (1.7)	178	14.61 (1.8)	0.007*
Weight z-score	177	0.32 (0.96)	178	0.24 (0.98)	0.429
Height (cm)	177	96.45 (3.9)	179	94.96 (3.7)	0.000*
Height z-score	177	0.17 (0.9)	176	0.21 (0.9)	0.712

4.4.2 Child nutrition and physical activity

Nutrition: Over 50% of the children in our sample were breastfed for greater than 6 months (Table 12). The majority of children (91%) were not given vitamin D supplements at 6 months of age (Table 12).

Among boys at 3 years of age, dietary daily vitamin D intake was less than the EAR of 400 IU (Table 13), with only about 10% of the boys exceeding this amount (Figure 13). Mean calcium intake exceeded the EAR of 500 mg, with 87% of boys consuming greater than this amount (Figure 14). The majority of boys did not consume supplemental vitamin D or calcium (Table 13). However, those boys who took supplements all exceeded the EAR for total daily intake for both calcium and vitamin D (Table 13). Mean intakes of protein exceeded the EAR with almost 100% consuming greater than 0.87 g/kg/day (Figure 15).

Among girls at 3 years of age, dietary daily vitamin D intake was considerably below the EAR of 400 IU (Table 13), with only about 6% of the girls exceeding this amount (Figure 13). Mean calcium intake exceeded the EAR of 500 mg with 91% of girls consuming greater than this amount (Figure 14). The majority of girls did not consume supplemental vitamin D or calcium at 3 years of age (Table 13). Of those girls who took supplements, the mean total daily intake did exceed the EAR for both calcium and vitamin D. All girls in the sample (100%) consumed greater than the EAR of 0.87 g/kg/day of protein (Figure 15).

The overall mean for both boys and girls for total energy intake (1399 kcal) was within the recommended intake for moderately active to active 3 year old children, of

1000 – 1400 kcal (based on EER from IOM 2002). About 43% of children consumed greater then 1400 kcal/day (Table 13).

Overall almost 45% of children consume less than 1 serving (1 cup) of milk per day. The mean milk consumed (1.3 servings) is less than the recommendation by Health Canada of 2 servings per day.

Physical Activity: Physical activity measured in daily hours of active and very active activity, were similar between weekday and weekend, and for both boys and girls (Table 14).

Table 12. Child nutrition at 6 months of age

	Male (n=177)	%	Female (180)	%
Breastfeeding duration				
>6 months	98	56.3	100	58.5
<6 months	64	36.8	61	35.7
Never	12	6.9	10	5.8
Vitamin D supplement use at 6 months				
No supplement	161	91.0	164	91.1
Reported supplement	16	9.0	16	8.9

Table 13. Child nutrition at 3 years of age for males and females

		Male (n=177)		Female (n= 180)	
		n	Mean (SD)	n	Mean (SD)
Vitamin D	From food IU/day	122	217 (127)	135	212 (111)
	Supplements None 400 IU/day	 152 (85.9%) 25 (14.1%)		 147 (81.7%) 33 (18.3%)	
	Total intake (food and supplement)	19	590 (122)	24	608 (117)
Calcium	From food mg/day	122	890 (374)	135	893 (324)
	Supplements None 150 mg/day	 155 (87.6%) 22 (12.4%)		 150 (83.3%) 30 (16.7%)	
	Total intake (food and supplement)	18	914 (342)	22	998(299)
Protein	g/day	122	55 (19)	135	57 (36)
	g/kg/day	122	4 (1)	135	4 (2)
Total energy intake	kcal/day	122	1407 (407)	135	1392 (365)
	kcal/kg/day	122	94 (25)	134	97 (25)

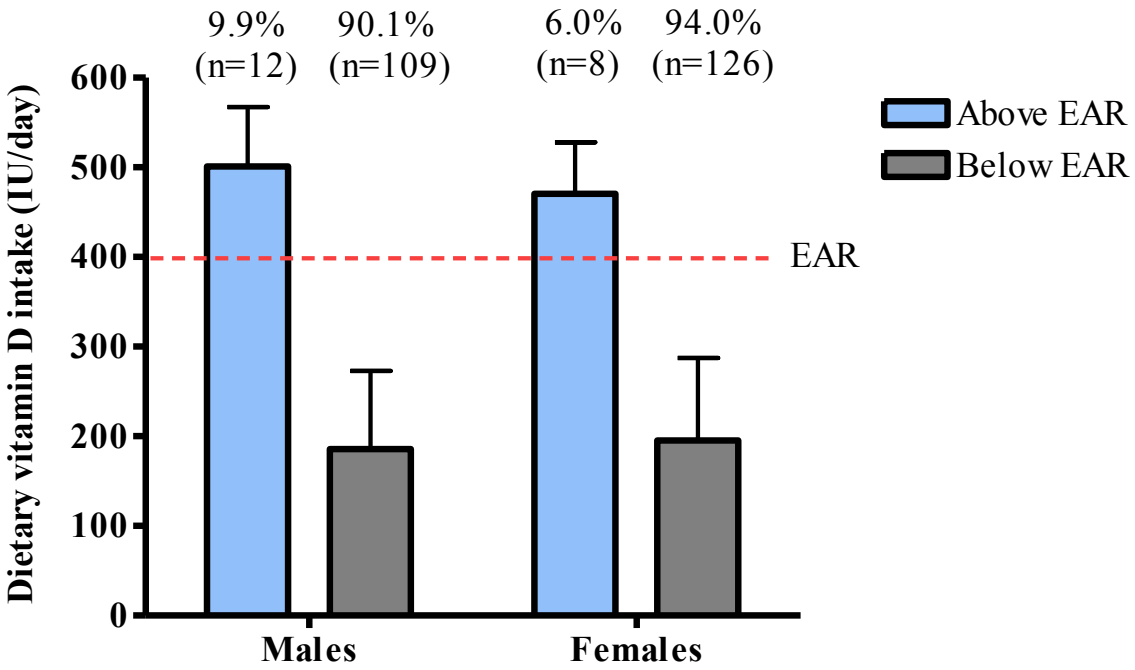

Figure 13. Mean child daily dietary intake of vitamin D above and below EAR of 400 IU/day at 3 years of age. Data are mean values and error bars as SD. Percentage of males or females in each category is indicated above bar.

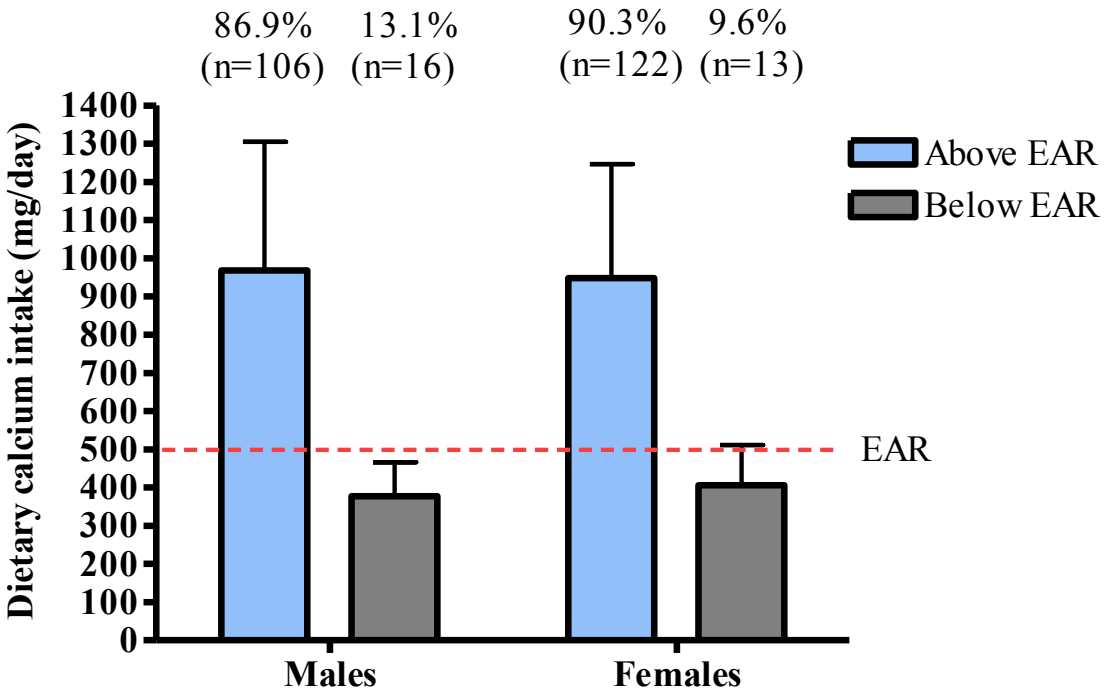

Figure 14. Mean child daily dietary intake of calcium above and below EAR of 500 mg/day at 3 years of age. Data are mean values and error bars as SD. Percentage of males or females in each category is indicated above bar.

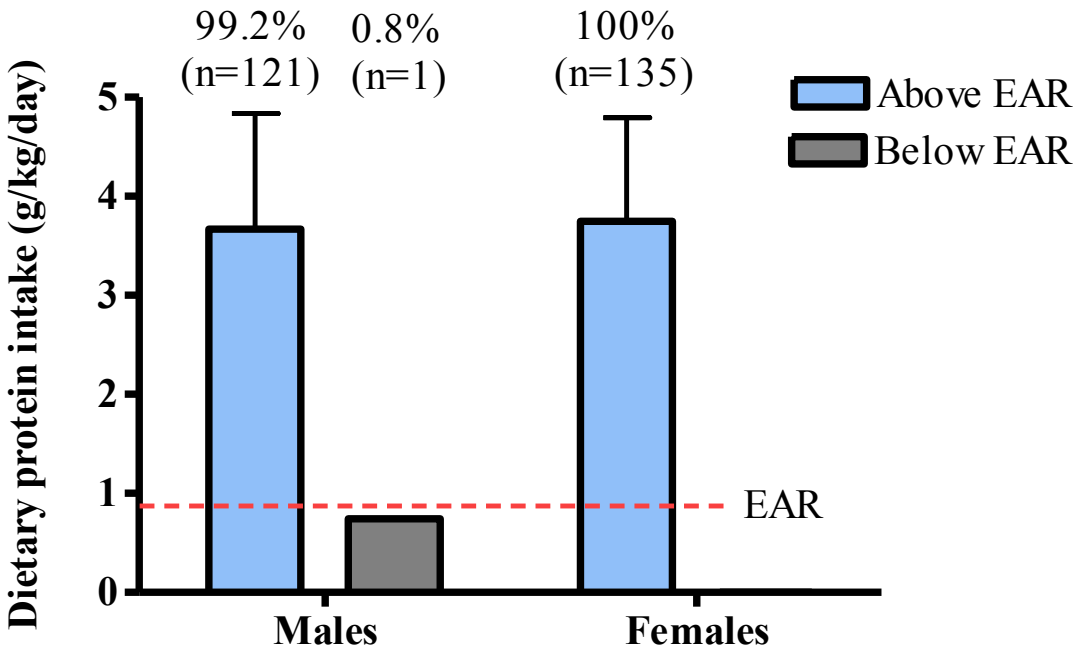

Figure 16. Mean child daily dietary intake of protein above and below EAR of 0.87g/kg/day at 3 years of age. Data are mean values and error bars as SD. Percentage of males or females in each category is indicated above bar.

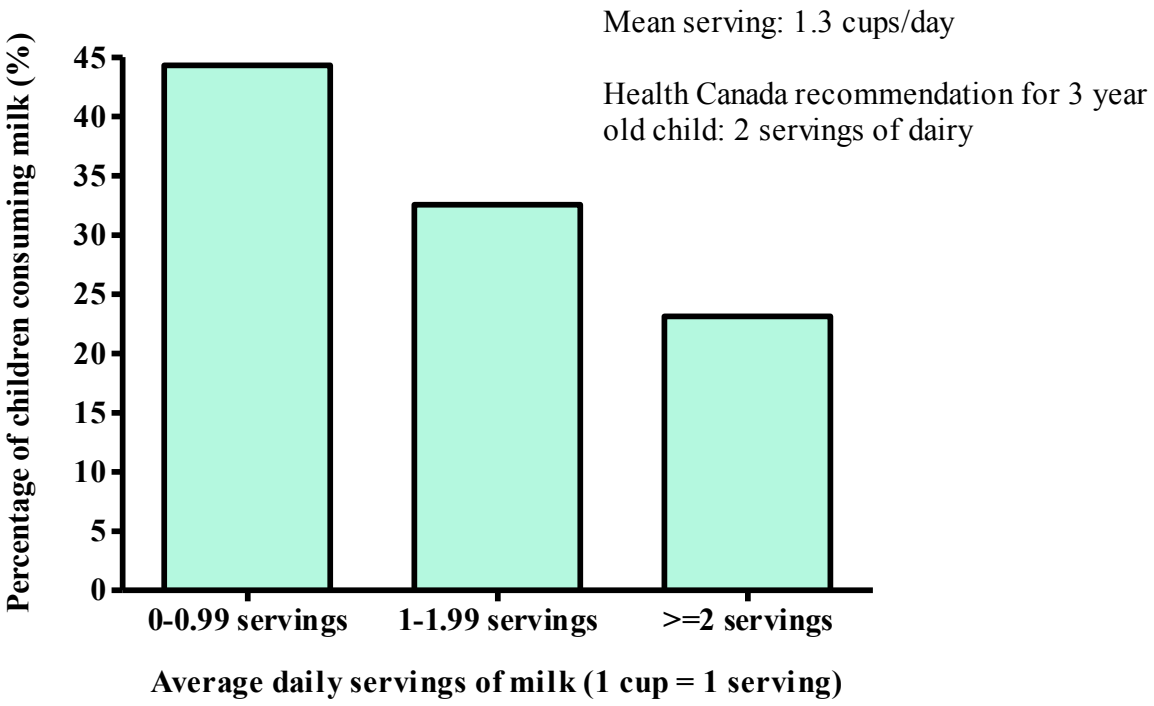

Figure 15. Percentage of 3 year old children consuming milk by average daily serving size category. 1 serving = 1 cup of milk (milk includes skim, 1%, 2% and homogenized).

Table 14. Child physical activity measured as active and very active at 3 years of age, assessed by modified HAES questionnaire.[118]

	Male		Female	
	n	**Mean (SD)**	**n**	**Mean (SD)**
Weekday physical activity (hours/day)	174	8.4 (2.0)	176	8.2 (1.9)
Weekend physical activity (hours/day)	169	8.7 (1.6)	171	8.8 (1.7)

4.4.3 Child bone outcomes at 3 years of age

For the DXA measurements absolute BMC and BMD values were significantly higher for boys than girls at 3 years of age (Table 15). BMC z-score corrected to age, gender and ethnicity matched reference data, which accounted for differences in child height, [116] were marginally negative compared to the reference for BMC for both boys and girls, while mean BMD z-scores were slightly positive (Table 15).

Table 15. Bone outcomes of children at 3 years of age (* indicates factors significant between genders)

Variable	Male (n=177)		Female (n=180)		p-value
	n	Mean (SD)	n	Mean (SD)	
BA (cm^2)	177	934.23 (50.2)	180	929.97 (56.6)	0.452
BMC (g)	177	532.61 (53.3)	180	510.99 (51.6)	0.001*
BMC z-score	177	-0.13 (1.5)	179	-0.24 (1.7)	0.538
BMD (g/cm^2)	177	0.57 (0.04)	180	0.55 (0.03)	0.000*
BMD z-score	177	0.764 (1)	180	0.839 (0.8)	0.439

4.5. Univariate and multiple regression analysis: predictors of child z-score at 3 years of age

A multiple regression model for predictors of child z-score at 3 years was developed to include maternal and child factors associated with child BMC z-score in the univariate analysis (p values <0.2) (Table 16). These included both maternal and child factors, which could possibly have an association with child BMC z-score.

Only variables indicating significant association ($p<0.2$) were placed into the multiple regression model. When maternal smoking, BMD z-score, and child weight at 3 years, total energy intake, calcium and vitamin D intake were included, this model was significant for predicting child BMC z-score ($R^2 = 0.272$, $F_{7,160}= 9.91$, $p<0.001$). Maternal leptin status in pregnancy was not a significant predictor of child BMC z-score, nor was maternal vitamin D status (Table 17).

The significant predictors of child BMC z-score were maternal BMD z-score, and child weight and dietary intake of vitamin D at 3 years (Table 17). For a unit increase in maternal BMD z-score there was associated a 0.339 unit higher child BMC z-score (Table 17). Each kilogram higher child weight was associated with a 0.416 higher BMC z-score. For dietary intake of vitamin D at 3 years, each 100 IU/day of intake of vitamin D was associated with a 0.3 higher BMC z-score.

In this model, the predictor with the strongest effect on child 3 year BMC z-score was the child's weight, indicated by having the largest β among all the variables.

Table 16. Univariate analysis for child 3 year BMC z-scores (adjusted for ethnicity and gender), ($p < 0.2$ taken as statistically significant; * indicates statistically significant factors)

Maternal and Child Variables	n	b	95% CI		p-value
Maternal leptin concentration during pregnancy (ng/ml)	356	0.001	-0.009	0.013	0.803
Maternal 25 OHD	352	0.001	-0.004	0.006	0.790
Maternal Protein (g/day)	341	0.001	-0.004	0.006	0.680
Maternal % energy from fat	341	-0.002	-0.039	0.035	0.913
Maternal vitamin D supplementation	354	-0.143	-0.576	0.289	0.515
Maternal dietary vitamin D intake	341	0.0006	-0.000	0.002	0.271
Maternal dietary calcium intake	341	0.0001	-0.000	0.000	0.712
Breastfeeding duration	345				
>6 months					
<6 months		0.168	-0.187	0.523	0.354
Never		0.310	-0.389	1.008	0.384
Pre- pregnancy BMI (kg/m^2)	343				
<18.5		0.650	-0.777	2.08	0.371
18.5-24.9					
25.0- 29.9		0.080	-0.319	0.478	0.694
$\geq$30		-0.008	-0.443	0.427	0.971
Maternal smoking	353				
Never					
Former		0.290	-0.072	0.653	0.116*
Current		0.305	0.584	1.193	0.500
Maternal alcohol consumption	352				
Never		0.408	-0.348	1.163	0.289
>1/month					
Maternal skinfold thickness (sum)	349	0.001	-0.008	0.01	0.843
Maternal BMD z-score	235	0.303	0.087	0.519	0.006*
Child birth length	336	0.028	-0.04	0.1	0.413

Child birth weight	355	0.000	-0.000	0.000	0.227
Steroid Use to 3 yr	356				
No entry					
Inhaled		0.353	-0.225	0.931	0.230
Oral		0.271	-0.844	1.387	0.633
Vitamin D Supplementation at 6 months	355	0.010	-0.438	0.458	0.965
Child Weight at 3 years	356	0.377	0.294	0.460	0.000*
Child Total Energy Intake at 3 years	257	0.0004	-0.000	0.001	0.112*
Child Ca^{2+} intake at 3 years	257	0.001	0.000	0.001	0.024*
Child Vitamin D intake at 3 years	257	0.003	0.001	0.004	0.001*
Child Vitamin D and Ca^{2+} supplementation	355	0.010	-0.438	0.458	0.965
Child Physical activity score					
Weekday	350	0.034	-0.051	0.120	0.431
Weekend	340	0.032	-0.068	0.132	0.533

Table 17. Multiple regression analysis of both maternal and child variables ($R^2 = 0.272$) as predictors of child z-score at 3 years of age (* indicates statistically significant factors)

Child Variables	**n= 168**				
	β	**b**	**95% CI**		**p-value**
Maternal smoking					
Never					
Former	-0.003	-0.011	-0.481	0.459	0.963
Current	0.027	0.237	-0.930	1.405	0.689
Maternal BMD z-score	0.197	0.339	0.111	0.566	0.004*
Child weight at 3 years of age	0.469	0.416	0.294	0.538	0.000*
Child total energy Intake at 3 years of age	0.004	0.000	-0.001	0.001	0.966
Child Ca^{2+} intake at 3 years of age	-0.035	-0.000	-0.001	0.001	0.755
Child vitamin D intake at 3 years of age	0.206	0.003	0.000	0.005	0.032*

CHAPTER 5

DISCUSSION AND FUTURE DIRECTION

Chapter 5: Discussion and Future Direction

Leptin is well documented to be associated with adiposity, but it is now emerging in a new role as a regulator of bone metabolism.[15] The importance of leptin during pregnancy for both the mother and fetus has been documented; however, no information existed to describe a possible link between maternal leptin status during pregnancy and offspring bone health. Evidence increasingly supports the concept that early life exposures, including exposures in utero, influence peak bone mass achieved in late adolescence and subsequently osteoporosis risk later in life. Whether exposure to varying maternal concentrations of leptin in utero influences programming of bone had not been previously explored.

In this study of 425 Canadian mothers and their children, we demonstrated that leptin status varies in mothers during pregnancy as a function of their pre-pregnancy BMI category. However, maternal leptin status in pregnancy was not a significant predictor of whole body bone status of their offspring at age three years. This relationship was determined after adjusting for several potential confounding factors, which were not included in previous studies that investigated the impact of factors in the maternal uterine environment and early life factors on offspring bone health. These included variables that were found to be significantly associated with child BMC such as maternal BMD, child vitamin D intake and child's weight at three years of age.

5.1 Maternal leptin status

In the 425 women studied, 52% entered pregnancy overweight or obese. Average leptin concentration for obese women was more than two fold (mean=45.7 ng/ml) that of the normal weight women (mean=23.3 ng/ml). To our knowledge these are the first data to describe leptin status during pregnancy for different BMI categories, as previous reports were for women of normal weight [44] or with pre-eclampsia. [45] Thus, our data adds to current literature on the profile of leptin during pregnancy as a function of BMI status.

Since leptin circulates in proportion to body fat, and people with a higher BMI generally have higher circulating leptin and are leptin insensitive, we expected that pregnancy would be similar but with higher concentrations of leptin due to the addition of placental sources and other secretors of leptin. A previous study documented mean serum leptin for a non-pregnant normal BMI female to be 7.4 (3.7) ng/ml and approximately 2.5 times higher per unit BMI. [12] Our results show greater than 3 times this concentration in pregnant women of similar BMI (Figure 10). Although BMI in the overweight and obese categories was significantly positively associated with leptin it was not a highly linear relationship (R^2= 0.42, p<0.001). Leptin concentrations varied even within each BMI group; some women with low BMI had serum leptin expected of higher BMI and vice versa. BMI is a crude estimate of body composition, and since leptin circulates in proportion to fat, it was not surprising that the maternal skinfold thickness taken at the same time as the blood draw for leptin, had the strongest association with leptin status, further affirming that leptin circulates in proportion to fat mass even during pregnancy.

During pregnancy, excess GWG occurred in all BMI categories, particularly in the overweight and obese (74% and 67% of mothers, respectively) (Figure 6). This is of concern as excess GWG has been linked with adverse health outcomes for both the mother as well as the offspring, in addition to increasing the risk of maternal post-partum weight retention (PPWR). Unlike previous studies we did not observe a relationship between circulating maternal leptin and total gestational weight gained during pregnancy. [53,54] The one study that found a significant association between leptin status and GWG throughout each trimester of pregnancy was based only on 52 women all of whom had a normal pre-pregnancy BMI. [53] The different finding in our study may be in part due to our wide range of BMI and larger sample size, which is more indicative of the range of BMIs observed in the general Canadian population.

One other interesting predictor of maternal leptin status was maternal age at recruitment. A higher maternal age was associated with lower leptin status, with each increase in age, corresponding to a decrease of 0.248 ng/ml of leptin (Table 9); however, there was no correlation between age and BMI status (R^2 =0.003), so this relationship warrants further investigation.

In summary, our findings indicate that maternal weight is an important predictor of leptin status in pregnancy. Given the association of leptin and bone, it was then important to examine whether exposure of the fetus to variable maternal leptin in utero has a programming effect on fetal skeleton that can be detected at 3 years of age.

5.2 Predictors of child bone at 3 years of age

Maternal leptin status, although variable across BMI groups during pregnancy, was not associated with child BMC z-score at 3 years after adjustment for key covariates. Thus, we refuted our hypothesis that higher leptin status would predict a positive influence on bone health in the offspring. To our knowledge, this is the first study to investigate a possible relationship between maternal leptin during pregnancy and offspring bone outcome at 3 years. Prior studies have investigated maternal leptin and its relation to offspring adiposity and anthropometric measures particularly in infants [2,64] but have not examined the association with offspring bone health. We adjusted for many key variables, both maternal and child, including dietary intakes, physical exercise, and maternal BMD (Tables 16 and 17) that were not included as covariates in previous studies when investigating determinants of bone status in infants and children. [65,86,122,123] In an animal study by Bertoni and colleagues, [41] ossification centers in long bones of mice born to mothers treated with subcutaneous leptin were longer and with larger cross-sectional area than offspring of untreated mothers. The offspring were newborn pups, and thus they did not have to factor in the many covariates included in our analysis due to the age of our subjects. Perhaps the relationship between maternal leptin and offspring bone mass is more pronounced in infancy rather than childhood.

The only studies linking maternal leptin status to offspring bone have used cord serum leptin, as a predictor of DXA derived fetal bone mass, instead of maternal serum leptin during pregnancy. A study in 117 neonates found that cord blood leptin was associated with higher estimated vBMD and BMC as analyzed by DXA (scans taken

within 2 weeks of birth).[65] Since cord blood leptin is higher in offspring of overweight and obese women,[64] perhaps cord leptin is more strongly associated than maternal leptin. Since current research is limited to fetal bone outcomes, further investigation on later offspring bone health in relation to fetal exposure to maternal leptin should be investigated.

Our study found that at three years of age, the child's lifestyle was more predictive of bone status than maternal factors. We found that child weight had the strongest association with child BMC z-score adjusted for other variables. Traditionally, higher weight has been linked to greater BMC particularly in adolescent children and adults.[23,24,124,125] Higher weight has been linked to stimulation of osteoblast differentiation.[24] Since most studies on weight and bone health have focused on older children with obesity and adults, it is surprising that this association is seen in young children. It would be of interest to identify if the architecture and composition of bone is similar for children who are lighter versus those who are heavier.

An additional predictive factor of child bone status was child vitamin D intake. This is not surprising as vitamin D is necessary for calcium uptake, bone mineral accretion and remodeling. Child BMC z-scores were slightly negative, but fell within one standard deviation of reference values. Only about 10% of children exceeded the EAR for vitamin D intake (Figure 13), and only a fraction (12%) consumed vitamin D supplements. The low dietary intake of vitamin D may in part be explained by the low intake of milk, a good source of vitamin D due to fortification (1 cup of milk contains 105 IU of vitamin D).[126] Almost 45% of children had less than 1 serving of milk per day,

with an overall mean intake of 1.3 servings/ per day (Figure 16). This is well below the recommendation of 2 servings per day by Health Canada. However, vitamin D intake is not a good predictor of vitamin D status, rather serum 25 OHD is better. An important source of 25 OHD is synthesis in the skin in response to exposure to ultraviolet B; hence adequate vitamin D status observed in 78% of our mothers, despite marginally adequate intakes, may relate to the contribution of endogenous vitamin D synthesis to the overall circulating pool of 25 OHD. We cannot presume the same UV exposure in the children since it is increasingly common for young children to be protected from the sun, with shaded clothing and high UV blocking sunscreen. These protective measures may prevent children from obtaining adequate vitamin D from UV rays, making dietary intake of vitamin D increasingly important. Future studies should include analysis of the child's serum 25 OHD concentrations to gain a better understating of child vitamin D status. Our findings, however, still stress the importance of an adequate dietary vitamin D intake in early childhood as it sets the stage for optimal PBM.

Unlike many previous studies [19,86] we did not establish an association between maternal vitamin D deficiency and lower BMC of offspring. This may be explained by the low percentage of women who were truly deficient in serum 25 OHD (only 4.8% had lower than 30 nM) whereas 18% of mothers were determined to have vitamin D deficiency in previous studies. [86] Our results do support the recent findings of a large prospective cohort (n=3960 mother-offspring pairs) [123] that investigated BMC of offspring at age 9-10 years in relation to maternal vitamin D status during pregnancy. Similar to our study, a low percentage of women had vitamin D deficiency (6% with 25

OHD <27.5 nM), and they attributed the overall adequacy of maternal vitamin D status to the lack of association with child bone status. As in our study, the recent cohort study[123] also used an HPLC LC-MS method to assess for 25OHD as opposed to a radioimmunoassay used by previous studies [19,86] that did find associations between maternal vitamin D status and child bone outcomes. HPLC and LC-MS have increasingly become accepted as the more accurate methods to measure serum 25 OHD. [127] The low percentage of maternal vitamin D deficiency we observed is similar to that found in the Canadian general population (4% defined as <27.5 nM by the Canadian Health Measures Survey (CHMS)). [128] Despite the sub-optimal intakes of dietary vitamin D in mothers during pregnancy (62% <EAR), the majority of women (78%) had 25 OHD concentrations that were optimal (>50 nM). Mean maternal serum 25 OHD for our study was 76.51 nM, which is slightly higher than the average for Canadian women of childbearing years of 69.5 nM. [128] The observed adequate vitamin D status may be in part due to vitamin D supplement use as well as endogenous vitamin D production through exposure to ultra-violet radiation. Furthermore, the season in which serum samples were assessed may have contributed to the 25 OHD concentration, as those taken during the summer had significantly higher concentrations (mean=86.5 nM) than those taken during the winter (mean=64.7 nM), although both groups had mean concentrations above the optimal of 50 nM (Figure 12).

Maternal bone status measured as BMD z-score was positively correlated with child 3 year BMC z-score, indicating a significant genetic component to child bone status. This is consistent with other studies that have implicated the importance of genetics to

bone health.[71,72,74] To our knowledge, this is the first study that has controlled for maternal bone status when examining the relationship between maternal BMD and offspring BMC. Most studies that have described the genetic inheritance of bone have focused on twin studies[73] or on genes linked with bone development.[75] The SWS observed a positive association between paternal BMD and infant female offspring BMC.[72] However, they did not relate the offspring BMC to maternal BMD since at the time the investigators did not obtain measurements of bone composition for mothers.

5.3 Strengths and limitations of the study

There were several notable strengths in this study. First, this was a longitudinal birth cohort study with detailed assessment of a variety of covariates that were often omitted by past studies including maternal diet and supplement use, physical activity, BMI, and BMD. As well, our study included measures of multiple early life variables as well as the child's diet and lifestyle factors at three years that could potentially impact child skeletal development. Secondly, we had a high retention rate from the original cohort for the three year follow up. Lastly, we were able to include previously unusable DXA scans of children with movement artifacts, due to our novel validation technique. We discovered that substitution of the surrogate limb value provides a valid and reliable prediction of whole body percent fat, fat mass, lean mass, BMC, and total tissue mass in young children in cases where movement or other artifact eliminates the inclusion of a single limb when using the Hologic Discovery DXA scanner. By employing the surrogate

limb substitution method, we were able to include 20% more scans that were previously discarded due to movement artifacts.

Our study also had some limitations. One major limitation was the lack of third trimester serum samples for all moms who had three year follow up visits. Almost 118 (22% of total who attended the three year follow up visit) moms were missing serum samples and could not be included. Since this was one of our primary inclusion criteria, it greatly reduced the available sample size for this analysis. Additionally, the fairly high frequency of missing data reduced our evaluable sample size for multiple regression models. This included missing maternal food frequency data as well as child three year diet records, a problem of compliance of subjects with completing diet records. Missing DXA scans from moms due to pregnancy, and children due to fear of machine, further reduced available bone values, which could have been included. As a complete dataset is required for every participant in order to run an analysis, this reduced our observation size.

Volunteer bias limited our ability to generalize the results to a larger population outside our local area. Our study tended to attract individuals with higher education, higher income, and in general healthier lifestyle choices. This may in part be due limitation of recruitment to mothers interested in research and attending specific hospitals (such as a hospital associated with an academic institution). Additionally, the over-representation of middle-age, Caucasian women in our study sample suggests that this may not be representative of younger women or ethnic minorities in Canada.

Lastly, we could only make assessments for whole body bone mass and not bone quality, as DXA is not able to distinguish between cortical and trabecular bone the way a peripheral quantitative computed tomography (pQCT) scan could.

5.4 Contributions to existing literature and future directions

This study provides novel and additional information that contributes to existing scientific literature on maternal-child associations related to bone health in the offspring. Most importantly, in the Canadian context, we have elucidated key factors, from both mother and child that contribute to child bone status at three years of age.

Our study also provided information on dietary and lifestyle practices of pregnant women of which little is published for Canadian women as a pregnant group was not sampled in the Canadian Community Health nor Measures surveys. Based on our data, a large (>50%) of women enter pregnancy overweight or obese and this impacts on their leptin status during third trimester of pregnancy. The observed negative relationship between maternal age and leptin concentration warrants further investigation to determine whether there exists an age dependency for maternal leptin during pregnancy. Our data on maternal use of prenatal supplements and dietary intake represents information about the dietary habits of women during pregnancy in today's society. We observed that despite marginal dietary intake of Vitamin D, the majority of women had sufficient 25 OHD status. We predict that if 25 OHD status were analyzed in the child at 3 years of age, similar results may not occur.

The use of surrogate limb estimates provides a novel and useful tool to increase scan usability when dealing with movement and artifacts, in scans of young children using the Hologic DXA machine. Previous studies have used similar techniques but were analyzed for obese participants and on a Lunar machine that has half scan analysis incorporated into the software.[107-109] Additionally, to our knowledge, we have produced one of the largest existing sets of data on whole body bone mass in three-year-old children. A previous study of bone status in children and youth included only 84 children to provide reference data for three-year-old children.[115,116]

Future studies should investigate cord leptin status of offspring and observe if it is indicative of child BMC z-scores at 3 years. Previous studies in neonates have observed positive associations between cord leptin and adiposity, [43,64,129] as well as BMC. [65] Some studies suggest that cord leptin is more in line with fetal BMC than maternal leptin. [65] It would be of interest to observe if this impact is translated at 3 years.

It would also be of interest to assess the association between serum leptin during pregnancy with infant infant birth weight and adipocity up to 3 years. Recent studies have demonstrated an association between maternal mid-pregnancy leptin and infant birth weight, particularly in obese and overweight women. [130,131]

Lastly, future studies should investigate further into the role of maternal diet and nutrition (such as maternal fat intake), in association to leptin and bone status of the offspring. A recent animal study by Devlin and colleagues [132] found that female pups of high-fat (HF) moms had higher leptin compared to those of normal fed moms, while males of HF moms had lower leptin. At 14 weeks, both males and females of HF fed

moms had higher trabecular and cortical bone area.[132] Perhaps a similar effect would be observed in humans, as maternal diet may potentially alter the metabolism and skeletal acquisition of offspring.

In summary, our data demonstrates that leptin varies considerably during pregnancy and although general assumptions can be made regarding leptin status and pre-pregnancy BMI, the proportion of body fat accumulated during pregnancy is more relevant. We also demonstrated that a number of maternal and child lifestyle factors are important predictors of the child's bone mass in early childhood. Our sample consisted mostly of older, well-educated and financially secure mothers; over half entered pregnancy overweight and most exceeded their recommended GWG. Our findings contribute to existing literature by illustrating that although associations between the maternal intrauterine environment and offspring bone health may be present at birth; it may not be sustained later in life. Our data also suggests that nutrition and weight should be monitored in both mother and child as these variables can affect bone health later in life

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