THESIS

RELATIONSHIPS AMONG POSTPARTUM MATERNAL BODY COMPOSITION, BREASTFEEDING, DOCOSAHEXAENOIC ACID (DHA) STATUS, AND PHYSICAL ACTIVTY

Submitted by

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ABSTRACT

RELATIONSHIPS AMONG POSTPARTUM MATERNAL
BODY COMPOSITION, BREASTFEEDING,
DOCOSAHEXAENOIC ACID (DHA) STATUS, AND PHYSICAL ACTIVTY

BACKGROUND: The time periods of pregnancy and lactation are recognized as times of changes in maternal weight and high bone turnover and theoretically can be transitional time periods regarding female body composition. We aim to inquiry associations between postpartum body composition, breastfeeding, maternal docosahexaenoic acid (DHA) status and intake, and physical activity. METHODS: 27 women 37.3 ± 17.3 months postpartum participated. Blood samples were assessed for DHA. DEXA analyses provided body composition data. Pearson's correlations and linear regression models tested for significance. RESULTS: Total MET hours per week significantly positively correlated with whole body BMD and lumbar BMC. Both physical activity and RBC DHA explained significant amounts of variance within lumbar and pelvic BMC. CONCLUSIONS: Associations between exercise and bone mineralization within the postpartum period were further elucidated, though the role of DHA is still unclear.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

LITERATURE REVIEW

A. HEALTH ISSUES AMONGST WOMEN OF CHILDBEARING AGE

The population of the United States (US) faces a number of health concerns. Amongst these are issues facing women of childbearing age. Using data collected from the 2011-2012 National Health and Nutrition Examination Survey (NHANES), it is estimated that approximately one-third of all US adult women (36.1%, adjusted for age) are obese with 31.8% of women aged 20-39 being classified as obese [1]. Additionally, 58.5% of US women within this age group classify as either obese or overweight [1]. Overweight and obesity are defined as a body mass index (BMI) of 25.0 – 29.9 kg/m^2 or ≥30 kg/m^2 ($kilograms/meters^2$), respectively, by the National Heart, Blood, and Lung Institute (NHBLI) and are associated with increased adiposity [2]. Dating from 1960 to present day, obesity among women in the United States has increased by 20.8 percentage points (Figure 1) [3]. Obesity has been recently estimated to increase the risk of all-cause mortality by 18% (95% CI, 12-25%) compared to maintenance of a healthy weight (BMI between 18.5 and 24.9 kg/m^2) [2]. It is well established that overweight and obesity are related to hypertension, type 2 diabetes mellitus (T2DM), coronary heart disease (CHD), stroke, gallbladder disease, osteoarthritis, sleep apnea, respiratory problems, and some cancers such as endometrial and breast cancer [4]. Additionally, obesity has been associated with psychological disorders (specifically depression) and, amongst women specifically, menstrual cycle irregularities and a variety of complications during pregnancy [4].

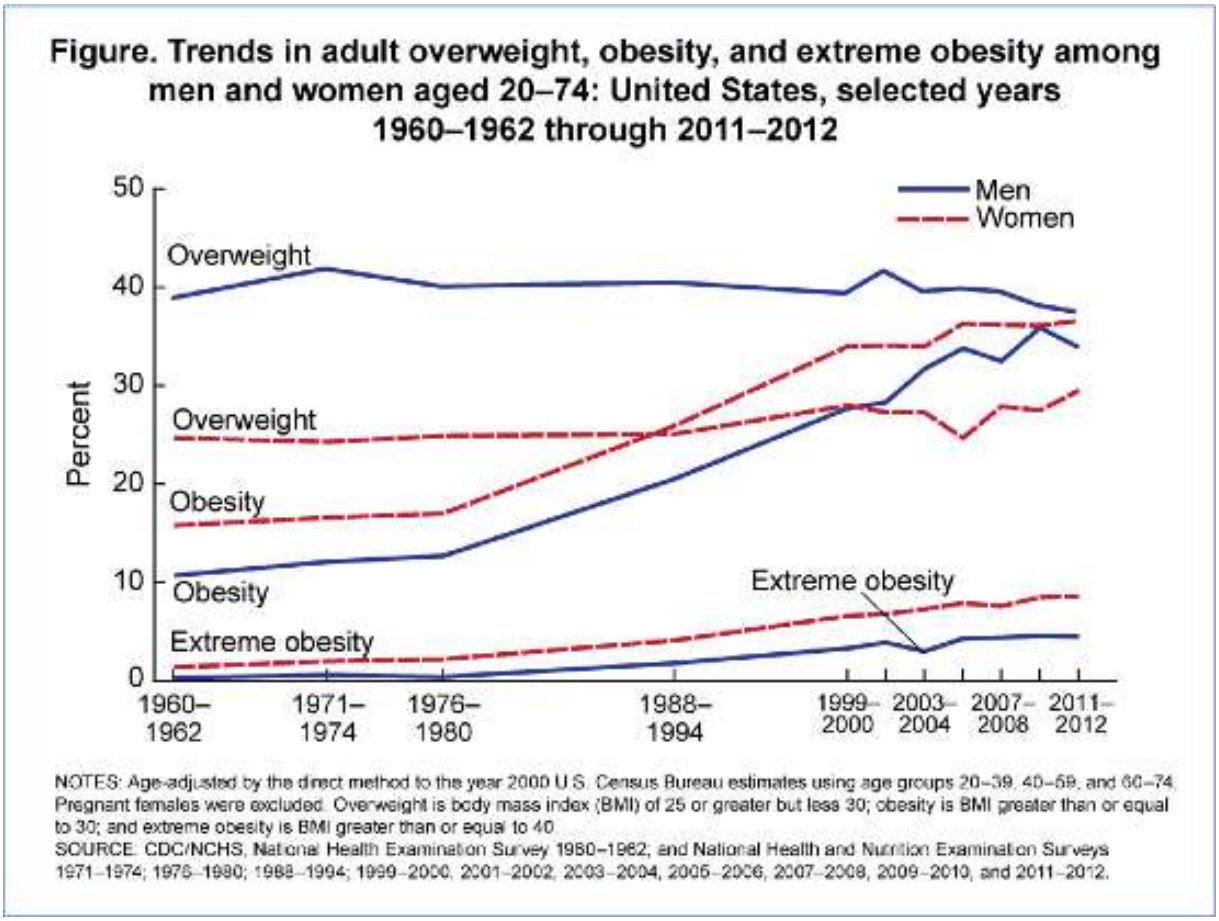

Figure 1: Changes in overweight and obesity prevalence since 1960.

Fryar et al., 2014

Chu et al., when pooling data collected from 26 US states and New York City, estimated that in the United States, one in five women who delivered live births were obese during pregnancy [5]. Within two subgroups, African-American women and women whose delivery was paid for by Medicaid, one in three pregnant women were obese [5]. Unique risk factors are associated with increased adiposity in the time periods surrounding pregnancy as both conditions increase morbidity for mother and child [6]. During pregnancy, elevated BMI increases the risk for gestational diabetes, preeclampsia, maternal thrombosis (during both the antenatal and postnatal periods), postnatal hemorrhage, macrosomia, fetal shoulder dystocia, fetal death, as well as childhood obesity [5,6].

Additionally, as BMI increases, a corresponding increase in the numbers of cesarean deliveries has been observed in many studies; such deliveries can harbor additional complications, including an increase in maternal blood loss during delivery, an increased incidence of postoperative wound infections, and endometritis [6].

Pregnancy itself can be a transitional period into overweight or obesity, placing women at risk for subsequent poor health outcomes and future pregnancy complications. It is important to consider that recent literature has shown between 15 and 20% of women will retain ≥ 5kg (10.4 lbs) of their gestational weight gain at 12 months postpartum [7]. In addition to failure to lose gestational weight in a reasonable timeframe, the risk of excessive weight retention (or transition to overweight or obesity) after pregnancy is also associated with the degree of obesity present prior to pregnancy as well as gestational weight gain above the recommended levels [7]. Evidence shows that women who fail to lose weight postpartum have a greater risk of long-term obesity [8]. Within the intra- and peripartum periods, maternal overweight and obesity increases the risk of development of T2DM, dyslipidemia, and cardiovascular disease (CVD) later in life [8]. A recent prospective study found that women who gained excessive amounts of weight during pregnancy, when compared to women who gained recommended amounts of weight during pregnancy as defined by the Institute of Medicine 2009 Guidelines (Figure 2), were 47% (95% CI, 11-94%) more likely to develop diabetes when controlling for maternal age, parity, smoking, race, ethnicity, TV watching, and exercise [9, 10]. Much research has been conducted assessing the risk for transition to overweight and obesity postpartum. In addition to the proposed risk for increased fat mass postpartum, it has also been postulated

that pregnancy as well as lactation could be transitional periods regarding a woman's bone mineralization and thus possible risk for osteopenia and osteoporosis later in life [11].

Prepregnancy Weight Category	Body Mass Index*	Recommended Range of Total Weight (lb)	Recommended Rates of Weight Gain† in the Second and Third Trimesters (lb) (Mean Range [lb/wk])
Underweight	Less than 18.5	28–40	1 (1–1.3)
Normal Weight	18.5–24.9	25–35	1 (0.8–1)
Overweight	25–29.9	15–25	0.6 (0.5–0.7)
Obese (includes all classes)	30 and greater	11–20	0.5 (0.4–0.6)

*Body mass index is calculated as weight in kilograms divided by height in meters squared or as weight in pounds multiplied by 703 divided by height in inches.

†Calculations assume a 1.1–4.4 lb weight gain in the first trimester.

Modified from Institute of Medicine (US). Weight gain during pregnancy: reexamining the guidelines. Washington, DC. National Academies Press; 2009. 2009 National Academy of Sciences.

Figure 2: Institute of Medicine (IOM) 2009 guidelines for pregnancy weight gain.
American College of Obstetricians and Gynecologists, 2013

B. PREGNANCY, LACTATION, AND BONE TURNOVER

In pregnancy and lactation both, the maternal environment adapts to meet the calcium demands of the developing fetus. How this specifically affects maternal bone mineralization is not entirely understood. It is known that during pregnancy, 2-3% of maternal calcium is transferred to the fetus with most of this occurring during the second and third trimesters [11]. In the final trimester, as calcium transfer increases to between 110-120 mg/d, there is a possibility of net maternal decalcification despite upregulation of renal calcium retention and intestinal calcium absorption [12]. Mechanisms underlying these phenomena are still being elucidated. Vitamin D has shown to possibly play a role in upregulating maternal calcium absorption, while parathyroid hormone (PTH) related peptide (PTHrP) along with local changes at the bone level involving the receptor activator

of nuclear factor kappa B ligand (RANKL) and osteoprotegerin (OPG) have been implicated in increasing bone resorption, allowing maternal calcium stores to supply calcium to the fetus as shown in Figure 3.

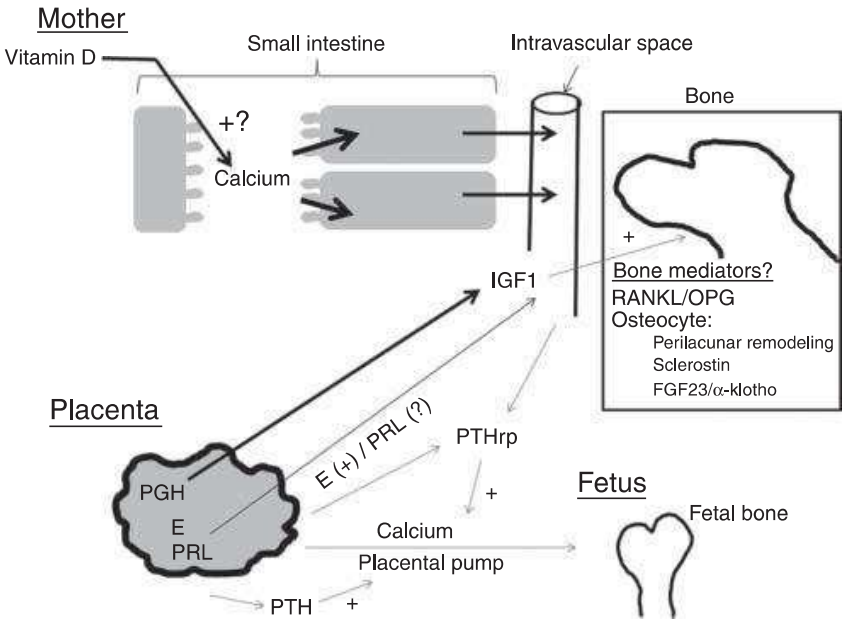

Figure 3: Mechanisms underlying maternal bone turnover during pregnancy.
E: estrogen, IGF-1: insulin-like growth factor 1, PGH: placental growth hormone, OPG: osteoprotegerin, PRL: prolactin, PTH: parathyroid hormone, PTHrp: parathyroid hormone related peptide, RANKL: receptor activator of nuclear factor kappa B ligand
Sanz-Salvador et al., 2015

It is also documented that during lactation, 300-400mg of calcium daily are transferred from the mother to the infant, with most of this coming from maternal bone stores [11]. During a period of 6 months of breastfeeding, women are estimated to lose between 5-10% of their total bone mass. Mechanisms behind this have been poorly understood, however PTHrP is likely involved in stimulating bone turnover [11,12]. Much of the proposed bone loss has been documented to occur at sites of cortical bone such as

the lumbar spine and femoral neck [11]. Prolactin has shown to be an additional stimulator of bone resorption [11].

Because of the possibility of net loss of bone mineralization during these time periods, it has been hypothesized that pregnancy and lactation, specifically when considering factors such as multifetal pregnancies, parity, closely spaced pregnancies, discrepancies in environmental factors such as maternal diet and calcium intake, as well as duration and lifetime history of breastfeeding, could be considered risk factors for subsequent development of post-menopausal osteoporosis if appropriate measures are not taken to reduce bone loss [11]. However, most longitudinal studies suggest that within one year of weaning a child, maternal bone calcification returns to baseline [12]. Still, aforementioned confounding factors in addition to the role of pre- and postpartum physical activity levels complicate much of the present research. Physical activity across the lifespan has associated with greater bone mineralization. However, the effect of physical activity during periods of pregnancy and lactation on future risk for poor mineralization is unclear. Dietary factors in pregnancy and lactation and their relationship with future bone mineralization is also presently poorly elucidated. Postulated has been a role for proteins that are pro-inflammatory in promoting poor bone mass [13]. In osteoporosis, we know there is an inflammatory state akin to that seen with obesity. It is also known that pregnancy is a pro-inflammatory period, thus the possibility that dietary interventions could help to reduce risk has proven to be of interest.

C. STRATEGIES FOR REDUCING RISK

Presently in the United States, greater than 40% of pregnant women gain more weight than recommended during their pregnancy and an estimated 18% begin pregnancy

obese [14,15]. Strategies for the prevention of obesity and associated complications can therefore be conducted either during pregnancy or during the interpartum period. A recent meta-analysis evaluated the effectiveness of maternal dietary and physical activity interventions during pregnancy at reducing gestational weight gain. After reviewing 30 randomized studies reporting intervention to promote recommended weight gain, women participating in an intervention were found to have gained 0.97 kg less than non-intervention counterparts [16]. Women who participated specifically in a trial using a dietary intervention gained on average 3.36kg less than non-intervention counterparts [16]. Typical dietary interventions were often as simple as promotion of a balanced diet consisting of carbohydrates, fats, and protein while maintaining a food diary. Standard physical activity interventions included promotion of light-intensity resistance training, walking for 30 minutes, or weight bearing exercises [16]. Dietary interventions were also found to reduce incidence of preeclampsia (RR 0.67, 95% CI 0.53 to 0.85; p = 0.0009), gestational hypertension (RR 0.30, 95% CI 0.10 to 0.88; p = 0.03), and preterm births (RR 0.68, 95% CI 0.48 to 0.96; p = 0.03) [16]. Often, such interventions incorporate behavioral change theories such as the transtheoretical model to assess self-efficacy of individuals and readiness to adapt [17]. Low-intensity interventions, including measures such as counseling women on recommended gestational weight gain at the first prenatal visit, increasing weight monitoring, and discussing basic nutrition messages at each prenatal visit such as limiting consumption of sugary beverages have been proposed as basic, but needed additions to the present standard care [18].

Decreasing the degree of weight retention, particularly in between pregnancies, is important as there is an association with increased weight between pregnancies and long-

term obesity [7]. Postpartum, breastfeeding has shown in some studies to assist in weight loss for women just having given birth. This effect is often attributed to the increased caloric expenditure required for lactation or metabolic changes that are favorable to weight loss [19]. At present, some 40.7% of US women breastfeed their children through the first 3 months of life and 18.8% breastfeed through the first 6 months of their child's life [20]. In a recent study, exclusive breastfeeding for 3 months led to a weight loss of 3.2 lbs greater than that seen in a matched cohort of women not breastfeeding exclusively for any duration of time [14]. Additionally, breastfeeding appeared to increase the chances of re-attaining pre-pregnancy weight by 6.1% (95% CI: 1.0, 11.1) [14]. Given this, there is a clear documented relationship between breastfeeding and post-partum body weight changes; however, there has been little inquiry into the relationship between breastfeeding, postpartum weight loss, physical activity, and nutrient intakes.

Dietary interventions can be an important strategy for health promotion. Stendell-Hollis and colleagues provided groups of breastfeeding women one of two diets postpartum, a Mediterranean diet or a diet based on the USDA My Pyramid for Pregnancy and Breastfeeding guidelines, and found that both groups of women had decreased TNF-α levels at 4 months postpartum compared to baseline measures taken around the time of delivery, however there were no between group differences [7]. Dietary interventions such as supplementation of specific nutrients like docosahexaenoic acid (DHA) have also been conducted in pregnancy, but the majority of studies have focused on neonatal health and few have looked at long term maternal outcomes.

D. DOCOSAHEXAENOIC ACID (DHA), PREGNANCY, AND THE BASIS FOR INTEREST

The very long-chain omega-3 (n-3) fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have been shown to play many physiological roles, including crucial roles in the maintenance of pregnancy and fetal cognitive development as well as inflammation, bone turnover, and possibly accumulation and storage of body fat [21]. Given its molecular composition, DHA is important to the membrane composition of many highly specialized cells such as those of the central nervous system [22]. During pregnancy and lactation, DHA is of importance as it is transferred to child across the placenta and through breast milk [22]. Fetal DHA plasma phospholipids are roughly 300-fold greater than those found in maternal blood [22]. High concentrations of DHA in retinal tissue and brain grey matter suggest it is crucial to proper retinal and neural development [22]. Additionally, low consumption of fish, a primary source of DHA, is associated with risk for preterm delivery; however, supplementation of DHA at levels of 600 mg/d decreases risk for early preterm birth (<34 weeks gestation) and very low birth weight, a risk factor for infant mortality and has been associated with T2DM later in life [23,24]. Cortical neuroplasticity, or the ability for neuronal cells to change over time, has associated with preterm birth, again indicating a possible link between DHA availability to the fetus or neonate and neural development [25]. Preterm supplementation of long chain polyunsaturated fatty acids (LCPUFAs) like DHA have shown beneficial effects on child neural development primarily when the n-6:n-3 is kept near 1:1 or 2:1 [26].

Inquiries into the effects of long chain n-3 fatty acids on health date back to the 1980s. These fatty acids proved to be of interest given their molecular composition, as the high degree of unsaturation meant that incorporation into cell membranes could lead to

changes in the fluidity of the cell membranes [27]. More recently, DHA through mechanisms not completely elucidated may play a role in they biosynthesis of extracellular signaling molecules such as prostaglandins (PGs) [21]. DHA has shown to reduce synthesis of metabolites derived from arachidonic acid (AA) as DHA intake, via incorporation into cell membranes, inhibits cyclooxygenase activity by displacing AA from the phospholipid bilayer [21].

DHA may affect bone turnover via fatty acid derivatives such as prostaglandins and may involve affects on the receptor activator of NF-κB (RANK) found on osteoclasts, responsible for bone resorption. DHA, likely through its displacement of AA from cell membranes and later conversion to anti-inflammatory mediators, reduces the availability of AA for prostaglandin-2 (PGE_2) synthesis [28]. PGE_2 stimulates both RANK and RANK ligand (RANKL) upregulation, and thus bone resorption. DHA, through displacement, may diminish RANKL production and has been implicated in limiting osteoclast maturation while upregulating osteoblast maturation [29]. Supporting this, animal studies have found DHA supplementation to correlate with greater osteoblast density, less osteoclast activity, and greater bone mass [29].

A graphic for proposed mechanisms of action for DHA within the context of bone health is described in Figure 4. In the small intestine, DHA has shown in animal studies to possibly upregulate calcium absorption acting on calcium ATP transferase proteins (Ca^{2+} ATPase) [28]. Within bone, DHA may act in two different ways to promote bone mineralization. One is to inhibit pre-osteoclast (pre-OC) cell advancement to mature osteoclasts (mature OC). This likely occurs in part through reduction of AA within osteoblast (OB) cell membranes [28]. Additionally, DHA through poorly understood

mechanisms might also promote differentiation of mesenchymal stem cells (MSC) to osteoblasts, thus maintaining levels of active bone forming cells [28].

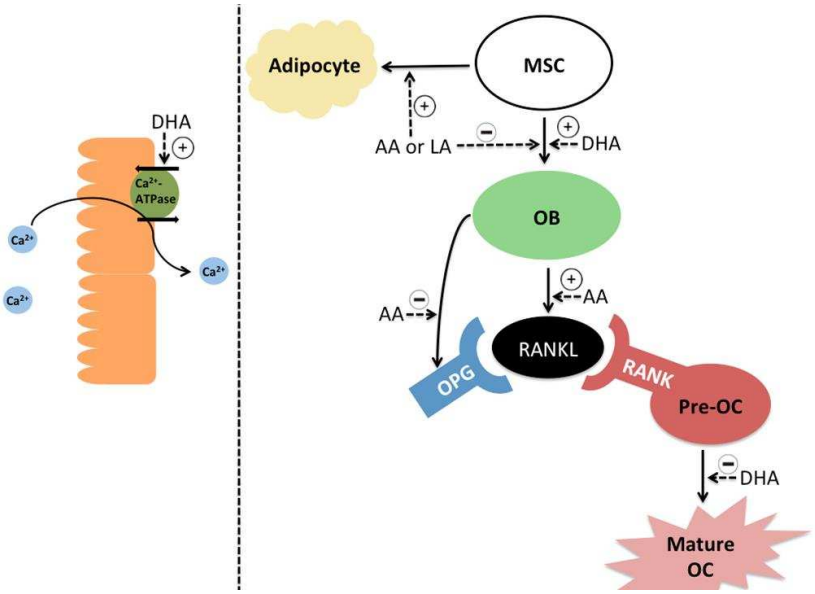

Figure 4: Possible mechanisms of action for DHA in bone health
AA: arachidonic acid, Ca^{2+} ATPase: calcium ATP transferase, DHA: docosahexaenoic acid, LA: linoleic acid, MSC: mesenchymal stem cell, OB: osteoblast, OC: osteoclast, OPG: osteoprotegerin, RANK: receptor activator of nuclear factor kappa B, RANKL: receptor activator of nuclear factor kappa B ligand

Lau et al., 2013

DHA has additionally been described as a precursor for D-series resolvins and protectins, lipid mediators that act to resolve the inflammatory process [30]. Resolvins D1 and D2 have shown to increase adiponectin concentrations and decrease concentrations of leptin, promoting a phenotype associated with a healthy BMI [30]. Additionally, dietary DHA has also been described as a possible appetite suppressor through actions that promote the release of the anorexogenic protein pro-opiomelanocortin (POMC) from neurons of the hypothalamus [31].

There is also evidence for DHA acting as a ligand for both intracellular and cell surface receptors [27]. DHA has been demonstrated to bind the transcription factor PPARγ, expressed in adipose tissue as demonstrated in Figure 5 [27]. The PPARγ receptor, once having bound its ligand, may interact with the transcription factor NF-κB or it may dimerize with an activated retinoic-X-receptor, altering gene transcription within the cell [32]. The latter action appears to be involved in promoting production of proteins related to a healthier phenotype, including adiponectin and adipose differentiation related protein (ADRP) which appear to promote greater fatty acid oxidation and increased energy expenditure by way of protein uncoupling [32].

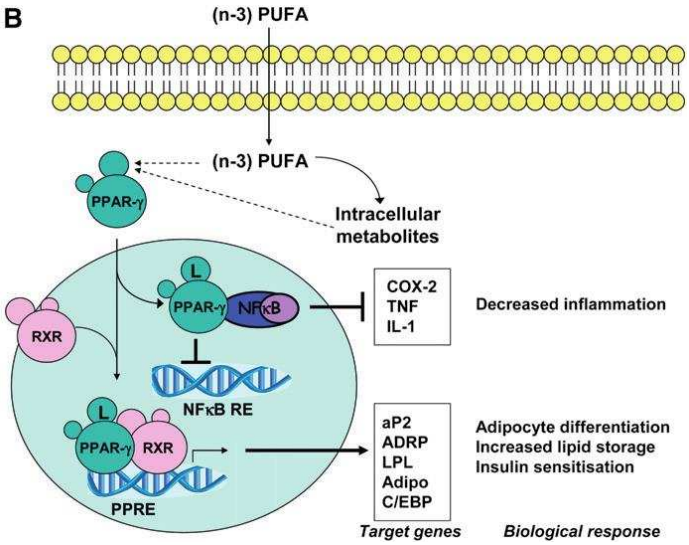

Figure 5: Activation of PPARγ by DHA
Adipo: adiponectin, ADRP: adipose differentiation related protein, ap2: adipocyte protein 2, C/EBP: CCAAT/enhancer-binding proteins, COX: cyclooxygenase, IL-1: interleukin 1, LPL: lipoprotein lipase, NF-KB: nuclear factor kappa B, PPAR: peroxisome proliferator-activated receptor, PUFA: polyunsaturated fatty acid, RE: response element, RXR: retinoic acid receptor, TNF: tumor necrosis factor

Calder, 2012

E. DHA AND AFFECTS ON BODY COMPOSITION

Relatively few well-controlled studies have assessed the impact of omega-3 fatty acids on body composition in humans and markers of a healthy phenotype, however animal research provides evidence suggesting omega-3 fatty acids can play a role in reducing body fat and improve bone mineralization. Additionally, even fewer studies have been conducted in pregnant cohorts. The results of those studies that have been conducted in humans appear to be inconsistent.

In a double-blinded, randomized controlled trial of overweight and obese men and women, Gammelmark and others showed omega-3 fatty acids to increase serum adiponectin levels [33]. Overweight and obese participants (n= 49, mean BMI = 30.2 kg/m^2) were randomly assigned to one of two groups, receiving supplements containing either 2g/d olive oil (control) or 2g/d (640 mg EPA, 480 mg DHA) of fish oil for 6 weeks. Serum concentrations of adiponectin increased significantly ($p = 0.04$) from baseline concentrations after 6 weeks of supplementation with fish oil when compared to the control, however no significant changes in other markers of inflammation (i.e. TNF-α, IL-6, CRP) were observed [33]. In the marine omega-3 supplementation group, an inverse correlation was observed between serum adiponectin and anthropometric measures of BMI and waist circumference. Additionally, serum adiponectin concentrations were independently associated with fish oil supplementation; BMI, waist circumference, low-density lipoprotein (LDL-C), total cholesterol, and circulating triacylglyerols did not significantly differ between baseline and post-intervention [33].

Using a randomized, double-blinded, 2-way parallel study design, Harden and colleagues assigned female subjects (n=40, BMI = 30.4 ±3.7) to one of two groups,

supplementing either a DHA emulsion (2.8g/d) or an oleic acid (OA) emulsion (unspecified amount per day) taken as 6 mL doses twice daily [34]. Energy intake, calculated from self-reported 3-day food diaries provided at baseline and after 12 weeks of intervention, proved to be significantly reduced ($p = 0.02$) in the DHA group compared to those taking OA supplements after intervention [34]. Additionally, in both groups reductions in BMI (Δ from baseline = -0.8 kg/m^2 OA group, -1.3 kg/m^2 DHA group) and body fat percentage (Δ from baseline = -5.6% OA group, -5.9% DHA group) were observed, however statistical significance between groups was not found [34]. Overall body weight was also reduced after DHA provision (Δ from baseline = -2.9 kgs) and OA supplementation (Δ from baseline = -1.4 kgs), however statistical significance was not achieved ($p= 0.89$) [34].

Kabir organized a double-blind, parallel designed study in which female participants (n=27) previously diagnosed with type 2 diabetes mellitus were randomly assigned to supplement either 3g/d fish oil (1.08g EPA, 0.78g DHA) or a paraffin oil placebo for 2 months [35]. In addition to supplementation, participants were advised to consume their recommended diet (total kcals distributed as 55% CHOs, 15% protein, and 30% fat) more strictly, to maintain a consistent dietary intake for the duration of the study, and were asked to complete 7 day food diaries both pre- and post-intervention to assess compliance to the recommendations. To assess changes to body composition, dual-energy X-ray absorptiometry (DEXA) scans and computerized tomography (CT) scans were performed. Additionally, fasting blood samples were taken to measure plasma adipokines (leptin, adiponectin, IL-6, and TNF-α) and plasminogen activator-inhibitor 1 (PAI-1). Fat biopsies were also taken to determine changes in gene expression and adipocyte size [35]. Body weight did not change, however supplementation with fish oil proved to significantly

decrease total percent body fat ($p = 0.02$) and adipocyte diameter ($p = 0.002$) when comparing groups, controlling for baseline values [35]. Adiponectin proved to negatively correlate with atherogenic index values ($r = -0.44$, $p = 0.015$), however no significant relationships were detected regarding the remainder of plasma biomarkers [35].

F. SUMMARY AND PURPOSE

Pregnancy and lactation have been postulated as possible transitional periods regarding female health, specifically regarding risk for weight retention and poor bone mineralization. These time periods may be appropriate times for intervention. Breastfeeding has been indicated in promoting postpartum weight loss. Routine physical activity and DHA intake have been implicated in promoting bone health. In reviewing recent literature, few inquiries appear to have been made attempting to establish the relationships between breastfeeding, physical activity, measures of body composition, and DHA status and daily intakes.

Therefore, the purpose of this study was to examine aforementioned relationships in a group of women 2 to 3 years postpartum. Hypotheses were: (1) physical activity status would be positively associated with measures of bone mineralization, while inversely associated with measures of adiposity; (2) duration of breastfeeding would be positively correlated with increased bone mineralization and inversely associated with adiposity, independent of physical activity status; and (3) circulating DHA concentrations would be inversely associated with adiposity and positively associated with bone mineralization in the inter-partum period, and would further strengthen the relationship between physical activity and a healthy body composition.

CHAPTER II

INTRODUCTION

The time periods of pregnancy and lactation are regarded as possible transitional periods regarding female health. An estimated 15 to 20% of recently pregnant women will retain ≥ 5kg (10.4 lbs) of their gestational weight gain at 12 months postpartum [7]. Additionally, these time periods are times of well-documented, high rates of bone turnover. Thus, these periods in life may be appropriate times for nutritional intervention to attenuate the possibility of transition toward poor health [11]. Postpartum breastfeeding is associated with a reduction in gestational weight retention. Additionally, physical activity is known to positively affect bone mineralization. However few inquiries into the relationships between breastfeeding, physical activity, and body composition postpartum have been made. Additionally, no studies to our knowledge have examined how dietary intake of docosahexaenoic acid (DHA), may contribute to explaining any associations that exist between body composition, breastfeeding duration, and bone density in the inter-partum period. In animal studies, DHA has shown to possibly increase calcium absorption within the small intestine and to limit osteoclastic maturation via arachidonic acid (AA) displacement within osteoblastic cell membranes, reducing the availability of AA for production of osteoclast stimulating metabolites such as prostaglandin-2 (PGE_2) [29]. The implication is that DHA may positively affect bone mineralization.

Therefore, the purpose of this study was to examine possible associations between postpartum body composition, levels of physical activity, breastfeeding duration, dietary DHA intake, and circulating levels of DHA at 2 to 3 years postpartum. Additionally

examined were if possible relationships were further associated with DHA status. Hypotheses were: (1) physical activity status would be positively associated with measures of bone mineralization, while inversely associated with measures of adiposity; (2) duration of breastfeeding would be positively correlated with increased bone mineralization and inversely associated with adiposity, independent of physical activity status; and (3) circulating DHA concentrations would be inversely associated with adiposity and positively associated with bone mineralization in the inter-partum period, and would further strengthen the relationship between physical activity and a healthy body composition.

CHAPTER III

METHODS AND PROCEDURES

A. Recruitment of Participants

The Colorado State University Internal Review Board Human Subjects Committee approved the study protocol before study initiation. The current project was a follow-up study to the Omega Smart Baby (OSB) study, a double-blind randomized placebo controlled trial in which women were provided either placebo or fish oil supplementation beginning in the final trimester of pregnancy and continuing to 3 months postpartum. Outcomes such as gestation length, infant neurological development, and concentrations of DHA within breastmilk were measured. All 116 women who participated in the previous OSB project were contacted regarding enrollment in the follow-up study. Contact with these mothers was maintained from conclusion of the OSB project and all contact information has been kept on file with Colorado State University (CSU). A research associate working with CSU conducted first contact with eligible participants. During initial contact, individuals were asked of their interest in participation in a follow-up study and their current pregnancy status. Women expressing interest in participating in the follow-up study, who self-reported not being pregnant during the previous 24 months, were contacted by a graduate research assistant to further discuss details of the present study. Twenty-one women contacted provided verbal intent to participate in the present study. Women enrolled were those who met the aforementioned criteria and were exempt from any exclusionary criteria. Exclusionary criteria, in addition to not being pregnant during the previous 24 months, were diagnosis of type 2 diabetes mellitus, hyperparathyroidism, celiac disease,

osteopenia, osteoporosis, rheumatoid arthritis, or use of medications that may alter bone density. Upon agreement to participate in the study, participants scheduled a time to meet with the graduate research assistant at the Human Performance and Clinical Research Laboratory (HPCRL) on the CSU campus.

An additional 6 parous women, matched for age, were recruited from the community for enrollment to act as a comparison group. Those eligible for participation were women aged 18-40, without pregnancy during the previous 36 months and did not self-report medical diagnoses that would exclude them from the study. Possible participants were recruited from places frequented by mothers of young children, i.e. child care centers, local health clubs that offer child care services, and CSU's Early Childhood Center (ECC). Flyers for the project were distributed at many childcare centers and health clubs across the Fort Collins, Colorado community. The flyer (appendix A) provided a brief synopsis of the project, what would be needed of the participants, and contact information for the graduate research assistant. Upon contact with interested individuals, confirmation of eligibility and details of the study were discussed. Six individuals met all inclusionary criteria, provided consent to participate, and scheduled to meet for an assessment at the HPCRL.

B. Procedures

Once enrolled, participants met with trained university staff at the HPCRL on the main CSU campus at scheduled times for assessments ranging between 30 and 40 minutes. Participants were then asked to provide written consent (appendices B and C) for participation in the research project. Two consent forms existed, and provision depended on whether or not the participant had previously participated in the OSB project. After

provision of consent, individuals completed a series of questionnaires. These included a demographics questionnaire that also asked about history of breastfeeding (appendix D), a form asking about any pre-existing conditions which may affect bone health (appendix E), a previously validated food frequency questionnaire (FFQ) designed to assess intake of foods containing DHA (appendix F), and a physical activity questionnaire (PAQ) (appendix G) designed by the European Prospective Investigation into Cancer (EPIC) Study – Norfolk research team, also previously validated [36].

After completion of all assessment forms, a trained graduate research assistant obtained a 7 mL blood sample for laboratory analysis from each participant. Blood collection was conducted using BD Vacutainer blood collection tubes with disodium ethylenediamin tetraacetic acid (EDTA) for preservation. Samples were stored on ice for approximately 30 minutes before separation for final storage. Whole body DEXA body composition scans using Hologic Discovery W (Hologic, Inc.) device with software version 13.4:7 were conducted.

Before conducting DEXA analyses, anthropometric measures of height and weight were taken and data were input into the DEXA software. Upon completion of the assessment, participants were given a copy of their DEXA results along with an information sheet (appendix H) intended to assist individuals interpret the results of the analysis. This was viewed by the research team as an incentive to the participants for participation in the study. Completed questionnaires and DEXA results were kept on file in the Department of Food Science and Human Nutrition.

C. Laboratory Analyses

Plasma and red blood cell (RBC) phospholipid fatty acid (FA) composition assessment was obtained for all subjects at assessment. Whole blood samples were separated via centrifugation at 1000g x 10 minutes directly after participant assessment and RBCs and plasma were aliquoted and stored at -80°C for later analysis. Plasma phospholipids were extracted in methanol and directly methylated with sodium methoxide (25% w/v) using the method of Glaser, Demmelmair and Koletzko [37]. RBC samples were treated with a 2:1 cholorform to methanol mixture, centrifuged, and the liquid phase was removed and evaporated under an oxygen-free nitrogen stream to concentrate the total lipid fraction. The phospholipid portions were methylated through addition of boron trifluoride in methanol (~14% BF3) and heated to 100°C to form fatty acid methyl esters (FAMEs). FAMEs were extracted in hexanes and separated using microcapillary gas liquid chromatography with flame ionization detection. Individual FAMEs were identified by comparison of retention times with known FAME standards. FA composition was expressed as percentages of total fatty acids.

D. Statistical Analyses

Statistical analyses were conducted using Statistical Package for the Social Sciences (SPSS) Version 22. Bivariate and partial correlations were conducted among measures of body composition, circulating DHA concentrations, self-reported dietary intake of DHA, breastfeeding duration, weight gain during most recent pregnancy, and total metabolic equivalent of task (MET) hours per week as calculated from a self-reported assessment of physical activity. Linear regression analyses were conducted to determine the best predictive models for measures of body composition which proved to be interest through

prior analyses. Due to many comparisons with a small number of participants, associations were determined significant at $p < 0.025$.

CHAPTER IV

RESULTS

There were 27 participants who completed measures for this study. All 27 women provided blood samples, completed DEXA body composition analyses, and completed the food frequency, breastfeeding, and pre-existing conditions questionnaires. Of the participants, 24 provided a completed physical activity questionnaire. The majority identified as white (n=25) while one individual identified as African-American and one identified as Hispanic. Thirteen participants indicated taking a multivitamin, including calcium and vitamin D, while 2 indicated taking an oral contraceptive; neither influenced statistical results. One individual indicated diagnosis of celiac disease and 4 indicated a diagnosis of asthma. Descriptive statistics are shown in Table 1. Participants were generally of a healthy BMI, were 3 years postpartum, and breastfed for just over 1 year.

Table 1: Description of study sample.

	N	Minimum	Maximum	Mean	Std. Deviation
Age (years)	27	25.0	42.0	35.2	3.7
Height in meters (m)	27	1.56	1.73	1.66	0.05
Weight in kilograms (kg)	27	54.1	81.8	67.8	8.6
Body Mass Index (kg/m^2)	27	18.7	32.0	24.6	3.5
Time Postpartum (months)	27	22.0	116.0	37.3	17.3
Pregnancy Weight Gain (kg)	21	6.8	20.5	14.2	3.8
Time breastfeeding (months)	21	0.5	40.0	16.4	9.6
Time since cessation of breastfeeding (months)	21	0.0	36.0	15.6	10.1

Correlations among bone mineralization and self-reported data regarding recent pregnancy and breastfeeding were conducted. Measures of bone mineralization were not significantly related to time removed from pregnancy, gestational weight gain, recent breastfeeding duration, or time since cessation of breastfeeding. Additionally, relationships between measures of adiposity, BMI, and weight and the provided pregnancy and breastfeeding data were also conducted. Again, no measures of percent body fat, BMI, or weight significantly correlated with time since pregnancy, gestational weight gain, recent breastfeeding duration, or time since cessation of breastfeeding.

All individuals completed an FFQ asking about intake of foods and supplements as sources of DHA. Participants also completed a previously validated physical activity questionnaire, from which total metabolic equivalent of task (MET) hours per week were evaluated. Average daily intake of DHA was 193.4 ± 157.4 mg/d. Reported DHA intake significantly correlated with RBC DHA concentrations ($r = 0.526$, $p = 0.005$), though not plasma DHA concentrations. There proved no relationship between MET hours per week and DHA status or intake.

Dietary DHA intake, total MET hours per week, as well as plasma and RBC DHA status were assessed for relationships with measures of body composition. Pearson correlation coefficients are shown in table 2. Neither dietary intake of DHA nor plasma DHA status significantly associated with measures of body composition. Both lumbar and pelvic BMC (bone mineral content) significantly inversely correlated with RBC DHA status ($p = 0.023$ and $p = 0.017$, respectively). Total MET hours per week correlated significantly with lumbar BMC ($p = 0.017$), whole body bone mineral density (BMD) ($p = 0.018$), and T-score ($p = 0.017$) as postulated, though no measures of percent body fat, weight, or BMI.

Table 2: Pearson correlation coefficients for physical activity, DHA status, and body composition amongst women 2-3 years postpartum (n = 27).

	MET Hours Per Week	RBC DHA
Lumbar BMC	0.484*	-0.436*
Pelvic BMC	0.415	-0.454*
Whole body BMD	0.479*	-0.245
T Score	0.481*	-0.236

* - achieved significance at $p < 0.025$
** - achieved significance at $p < 0.01$
BMC: bone mineral content, BMD: bone mineral density, MET: metabolic equivalents of task, RBC: red blood cell, DHA: docosahexaenoic acid

Partial correlations controlling for possible confounding factors were run to assess the strength of observed correlations. Controlling for age, BMI, and RBC DHA status, MET hours per week was no longer correlated with lumbar BMC, whole body BMD, and T score. Controlling for age, BMI, and MET hours per week, RBC DHA was inversely associated with lumbar ($p = 0.007$, $r = -0.570$) and pelvic ($p = 0.003$, $r = -0.608$) BMC.

Linear regressions were run to explore the best models for explaining variance within variables of interest. For lumbar BMC, the best predictive model incorporated MET hours per week and RBC DHA status, explaining 40.3% of the variance within the variable ($p = 0.004$). Pelvic BMC was best explained again by MET hours per week and RBC DHA, explaining 42.3% of the variance ($p = 0.003$). β-weights and levels of significance for the aforementioned variables are described in tables 3 and 4. Whole body BMD and T score were only significantly explained by MET hours per week at 22.9% and 23.1% of variance, respectively ($p = 0.018$ and $p = 0.017$).

Table 3: β-weights and individual levels of significance for included variables in linear regression model for dependent variable lumbar bone mineral content (BMC).

	Standardized β	Significance
(Constant)	--	0.000
RBC DHA	-0.413	0.024
MET Hours Per Week	0.447	0.015

Table 4: β-weights and individual levels of significance for included variables in linear regression model for dependent variable pelvic bone mineral content (BMC).

	Standardized β	Significance
(Constant)	--	0.000
RBC DHA	-0.503	0.007
MET Hours Per Week	0.370	0.037

CHAPTER V

DISCUSSION

A. DISCUSSION OF FINDINGS

This cross-sectional pilot study of women recently pregnant was undertaken to examine the possible associations among postpartum body composition, breastfeeding, physical activity, and DHA status. Positive relationships were found between habitual physical activity and lumbar bone mineral content (BMC), whole body bone mineral density (BMD), and T score, though there were no relationships with BMI, weight, or measures of adiposity. It is well understood that bone mineralization is typically higher in exercising compared to non-exercising individuals [38]. Also, prospective studies of humans as well as animal studies indicate a role for physical activity in maintaining bone health [38]. Within the postpartum period, exercise appears to attenuate the loss of bone mineralization. Lovelady et al., assigning women to either an exercise or control group at 4-16 weeks postpartum, observed a protective effect on lumbar BMD related to exercise [39]. In fact, much of the body of similar literature has been conducted close to the time of delivery. Participants in this study were on average 37.3 months postpartum, and findings add to previous research that the benefits of exercise continue to be observed as time moves forward. When controlling for age, BMI, and DHA status, aforementioned relationships failed to remain significant. This could imply that DHA, along with age and BMI, accounts for some of the positive impact on bone, however bivariate correlations between DHA and measures of bone mineralization as well as results from the linear regression models indicate an inverse relationship between DHA and bone health. These

same regression models continued to indicate a positive relationship between habitual physical activity and bone mineralization.

No significant associations were found between breastfeeding duration, months since cessation of breastfeeding, pregnancy weight gain, or months postpartum and body composition. This may be a reflection of the study timing, seeing individuals between 2 and 3 years postpartum and on average 16 months after having last breastfed. We know from prospective studies that within 6-12 months post-weaning many mothers return to baseline levels of bone mineralization [12]. Prolactin levels, which are high during lactation, support calcium release from maternal bone stores [39]. However, as time since delivery advances, the eventual return of menses and simultaneous increase in estrogen levels decrease bone resorption [39]. Still, previous studies have shown relationships between bone metabolism and breastfeeding. Yeo et al. observed in a study of Korean women that longer duration of breastfeeding resulted in lower total femoral, femoral neck, and lumbar spine BMD [40]. Hopkinson et al., in a study of lactating and non-lactating women observed that at 24 months postpartum duration of breastfeeding (specifically breastfeeding for greater than 9 months) was inversely related to net regain of whole body BMC and, regarding thoracic spine BMC specifically, significant differences between lactating and non-lactating women existed out to 12 months postpartum [41]. When discussing specifically gestational weight gain and its relationship with bone mineralization, few studies have made inquiry. Widen et al. showed that patterns in gestational weight gain correlated with differences in percent fat mass and total body water, but did not inquire into bone mineralization [42].

Finally, there were inversely significant associations between circulating levels of DHA and both lumbar and pelvic BMC. Mechanistically, basis for a role for DHA is provided by many animal and cell-culture models, however these and human models have yet to consistently show a role for DHA. Provided the mechanisms behind bone resorption, DHA and it's action as an inhibitor of AA bioavailability could indicate a plausible role in limiting bone resorption, as indicated here when controlling for DHA, assessing the correlations observed between physical activity and bone mineralization. However, there is also indication that in addition to DHA levels, another important factor is the availability of antioxidant nutrients. Izquierdo and colleagues proved that high levels of DHA lead to peroxidation risks, and the deleterious results of high DHA levels, including damage to cartilaginous structures and bone formation, could be the result of free radicals [43]. Administration of α-tocopherol reduced these risks [43].

B. STRENGTHS AND LIMITATIONS

The results of this study are affected by both strengths and limitations. In our study, we regard the DHA FFQ and the PAQ given to our participants at assessment as strengths. Both questionnaires have been previously validated and again in this study, the responses provided to us on the DHA FFQ are strongly correlated with RBC DHA providing further confidence in the measure. Aside from being previously validated, the PAQ provided was a 12-page assessment, asking for a thorough account of one's habitual daily physical activity and therefore our calculations of total MET hours per week for participants is presumed to be well representative.

Another strength of our study is the use of DEXA analysis for analyses of body composition. Kuriyan et al. in a study assessing the use of DEXA, air displacement

plethysmography (ADP), bio-electrical impedance, and a 4-skinfold technique to measure percent body fat, using a 4-compartment (4C) model as reference, found DEXA to be the only analysis to not underestimate percent body fat [44]. Compared to other measures of body composition, DEXA has a unique ease of use and allows simultaneous insight into both whole body and regional measurements. DEXA has additionally been shown to be superior compared to other body fat measures [45].

One limitation to the study is the number of participants. In our original grant proposal, we wrote that our power analysis indicated the need for 36 participants recruited from our previous cohort. Recruiting 36 participants from our cohort of previously studied women proved difficult, as many had moved from the surrounding area and were unavailable to meet with a member of the research team while others were simply not interested. It is possible that the low number of participants in this study has impacted the significant observations seen.

Another limitation of our study is the cross-sectional nature of the project. In our project, we have something of a snapshot into the lives of our participants. In their study to assess relationships between bone mineralization and lactation, Hopkinson et al. followed participants beginning shortly after delivery to 24 months postpartum collecting data at 3-month intervals. Were our study to have taken on a prospective design, we may have gained greater insight into the associations between our variables in question while also possibly addressing our low number of participants through collection of more data at multiple time points.

A final limitation to our project is that all but two participants identified as white, as one identified as African-American and one identified as Hispanic. Per 2010 U.S. census

data, the Fort Collins, Colorado community from which participants were largely recruited, is 89.0% white and therefore participants for this study were recruited from a largely homogenous population. Extrapolation of the results obtained in this study to the national population would thus be difficult given the ethnic disparity that exists between the demographics of the local community and the nation at large in addition to the aforementioned limitations.

CHAPTER VI

CONCLUSIONS AND RECOMMENDATIONS

This study of women roughly 3 years postpartum has provided additional insight into the relationships between body composition, physical activity and DHA status. By showing associations between variables such as physical activity postpartum and bone mineralization, we have provided additional evidence to support the present body of literature. However, any future studies should take account of study limitations and make changes accordingly. Future inquiries may wish to employ a prospective design if working with human subjects, as was our case. Such a design would alleviate the fact that we have large gaps of time for which we have no data, but instead have data from a single time point from which we have attempted to arrive at significant associations.

Finally, in our research we found recruitment of individuals, specifically new individuals from the community, to be difficult. Though we used an incentive-based technique for recruitment and maintained relationships with previous study participants, giving us access to the appropriate population of individuals, our numbers remained low. We would recommend to other researchers the use of special events, such as asking recruiters to present the project idea in a more formal manner, presenting the pertinent information regarding the project to a larger audience at once.

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APPENDIX A

RECRUITMENT FLYER

Participants Needed For Nutrition Study

Colorado State University (CSU) is looking for mothers, whose youngest children are 3-4 years of age, to participate in a research study regarding omega-3 DHA as it relates to body composition and bone density.

Testing will take place at the Human Performance Laboratory near CSU's Moby Arena and will take no more than 30 minutes. Free parking will be provided. You will also receive a copy and explanation of your results.

Participants will be asked to:

1) Give a small blood sample (equivalent to about 2 teaspoons)
2) Complete a short medical questionnaire and a diet questionnaire
3) Have height and weight measured when receiving a **free** DEXA body fat and bone density test.

For information please contact Wesley Pendleton, Graduate Research Assistant, Dept. of Food Science and Human Nutrition: phone 970-237-9442 or email wesley.pendleton@colostate.edu

Note: you are **not** eligible for this study and should not participate if you are not 18-40 years of age, are now pregnant, breastfeeding, or have been pregnant in the last 3 years or if you have any condition such as diabetes, hyperparathyroidism, rheumatoid arthritis, celiac disease or use certain medications which affect bone density or have been diagnosed with osteopenia or osteoporosis.

Concerns about human subjects protection can be addressed to: Janell Barker, Research Integrity and Compliance Review Office 321 General Services Building, Colorado State University, Phone: 970-491-1655

APPENDIX B

CONSENT FORM FOR PARTICIPANTS OF THE OSB PROJECT

Consent to Serve as a Subject in Human Research
(For Participants in the Omega Smart Baby Project)

Title of Project: Omega Smart Baby Project: Follow Up Study of Moms Treated with DHA During Pregnancy

Principal Investigators: Mary Harris, PhD, RD
Mary.Harris@ColoState.EDU
(970) 491-7462

Co-Investigators: Christopher Bell, PhD
Kimberly Cox-York, PhD

WHO IS DOING THE STUDY? Colorado State University is conducting this research.. The study is being funded by the USDA Agricultural Experiment Station.

WHAT IS THE PURPOSE OF THIS STUDY?
The primary purpose of the study is to see if DHA taken during pregnancy and lactation improves your body composition (body fat and bone density) 18 months after you gave birth. There is a growing body of research that shows that DHA may be involved in burning body fat and also in promoting bone density. Since women lose bone and increase body fat during pregnancy, we are testing to see if taking DHA during pregnancy and breastfeeding may help to restore pre-pregnancy weight and body composition.

WHY AM I BEING ASKED TO PARTICIPATE IN THIS RESEARCH? You are being asked to participate in this follow-up study because you were part of the Omega Smart Baby Project and may or may not have received a supplement of the essential nutrient (an omega-3 fatty acid called DHA, or docosahexaenoic acid) during pregnancy and for the first 3 months of breastfeeding.

WHAT WILL I BE ASKED TO DO? If you agree to be in the study, you will come into the Human Performance laboratory at Colorado State University to have a DEXA bone density and body composition test. At that same time, we will ask you to provide a small follow-up blood sample (10 ml or about 2 teaspoons) for analysis of your DHA stores and blood markers of inflammation and body fat metabolism and to fill out a one-page food intake and supplement form. We will measure and record your height and body weight.

What is a DEXA?DEXA is an FDA approved device for the measurement of bone density. Dual X-ray absorptiometry (DEXA) is the preferred technique for measuring bone density. The DEXA scanner produces 2 X-ray beams, each with different energy levels. One beam is high energy while the other is low energy. The amount of X-rays that pass through the bone is measured for each beam. This will vary depending on the thickness of the bone. The DEXA test is relatively easy to perform, you simply lay flat on a platform for about 20 minutes as the scanner moves over you. The amount of x-ray exposure is very low.

Dose from the DEXA measurement is well below natural background radiation levels. For example, total average x-ray exposure with the DEXA is around 0.2 microSv (units used to describe radiation) compared to dental bitewing xray 60 microSv and 5000 times lower than the recommended daily limit of 1000 micro Sv/day.

WHERE WILL THE STUDY TAKE PLACE?All testing will take place in the Human Performance Laboratory in Moby Gym at Colorado State University.

HOW LONG WILL THE STUDY LAST? The study will take about 30 minutes of your time.

WHAT ARE THE POSSIBLE RISKS AND DISCOMFORTS? It is not possible to identify all potential risks in a research procedure, but the researchers have taken reasonable precautions to minimize any known and potential, but unknown, risks**.** There are no anticipated physical risks for participating in the DEXA test other than a very small amount of radiation (equal to about 15 minutes outside on a sunny day). This is a commonly used clinical test to measure bone density and risk for osteoporosis in women. The risks of blood drawing are: a possible hematoma (bruise) at the site where blood was drawn and the remote risk of infection. There are no other known or anticipated risks associated with the study other than those addressed above.The results of the study will be published BUT information will be combined with that of other people taking part in the study and you will NOT be identified by name in any written or oral communication.

WHAT ARE THE BENEFITS OF PARTICIPATION IN THE STUDY?You will receive the results of your free DEXA scan (both your body fat composition and your bone density compared to expected values for your age). It is anticipated that this study will contribute understanding of the role of omega-3 DHA status in regulating body weight and bone density.

WILL I RECEIVE ANY COMPENSATION FOR TAKING PART IN THIS STUDY? You will not receive any compensation other than the results of your DEXA.

WHAT WILL IT COST ME TO PARTICIPATE? There will be no costs to you. You will receive a prepaid permit for parking on campus.

DO I HAVE TO TAKE PART IN THE STUDY?/ CAN MY TAKING PART IN THE STUDY END EARLY? Your participation in this research is voluntary. If you decide to participate in the study, you may withdraw your consent and stop participating at any time without penalty or loss of benefits to which you are otherwise entitled.

WHO WILL SEE THE INFORMATION THAT I GIVE?
The results of the study will be published BUT information will be combined with that of other people taking part in the study and you will NOT be identified by name in any written or oral communication. The only exceptions to this are if we are asked to share the research files for audit purposes with the CSU Institutional Review Board ethics committee, if necessary. In addition, for funded studies, the CSU financial management team may also request an audit of research expenditures. For financial audits, only the fact that you participated would be shared, not any research data.

We will use your original study number to record your DEXA and blood results. You will be given an envelope to fill out your name and address so that we can send your DEXA report to you. We will ask you to access your medical record from your pregnancy to determine the amount of weight gained during your pregnancy and bring that information with you to the laboratory.

WHAT HAPPENS IF I AM INJURED BECAUSE OF THE RESEARCH? The Colorado Governmental Immunity Act determines and may limit Colorado State University's legal responsibility if an injury happens because of this study. Claims against the University must be filed within 180 days of the injury.

WHAT IF I HAVE QUESTIONS?
Before you decide to accept this invitation to take part in the study, please ask any questions that might come to mind. Later, if you have questions about the study, you can contact the investigator, Dr. Mary Harris at 970-491-7462.If you have any questions about your rights as a volunteer in this research, contact the CSU IRB at: RICRO_IRB@mail.colostate.edu; 970-491-1553. We will give you a copy of this consent form to take with you.

Your signature acknowledges that you have read the information stated and willingly sign this consent form. Your signature also acknowledges that you have received, on the date signed, a copy of this document containing 3 pages.

_____
Participant name (printed)

_____ _____
Participant Signature Date

_____ _____
Witness to signature (project staff) Date

APPENDIX C

CONSENT FORM FOR NEW ENROLLEES

Consent to Serve as a Subject in Human Research

Title of Project: Omega Smart Baby Project: Follow Up Study of Moms Treated with DHA During Pregnancy

Principal Investigators: Mary Harris, PhD, RD
Mary.Harris@ColoState.EDU
(970) 491-7462

Co-Investigators: Christopher Bell, PhD
Kimberly Cox-York, PhD

WHO IS DOING THE STUDY? Colorado State University is conducting this research. The study is being funded by the USDA Agricultural Experiment Station.

WHAT IS THE PURPOSE OF THIS STUDY?
The primary purpose of the study is to see if omega-3 DHA status has an effect on body composition and bone density. There is a growing body of research that shows that DHA may be involved in burning body fat and also in promoting bone density. Since body fat increases and bone density decreases during pregnancy, this study will compare the effect of omega-3 DHA in women who have recently been pregnant to those who have not had a recent pregnancy.

WHY AM I BEING ASKED TO PARTICIPATE IN THIS RESEARCH? You are being asked to participate in this follow-up study because you have had children but have not been pregnant for at least three years. Your data will be used to compare to women who were part of our recent Omega Smart Baby Project received supplements of the essential nutrient (an omega-3 fatty acid called DHA, or docosahexaenoic acid) during pregnancy and for the first 3 months of breastfeeding.

WHAT WILL I BE ASKED TO DO? If you agree to be in the study, you will come into the Human Performance Laboratory at Colorado State University to have a DEXA bone density and body composition test. For the DEXA scan all you will need to do is to lay flat on a table fully clothed in comfortable clothing for about 20 minutes while the scanner moves over your body. At that same time, we will ask you to provide a small blood sample (10 ml or about 2 teaspoons) for analysis of your DHA stores and blood markers of inflammation and body fat metabolism and to fill out a one-page food intake and supplement form. We will measure and record your height and body weight.

What is a DEXA?DEXA is an FDA approved device for the measurement of bone density. Dual X-ray absorptiometry (DEXA) is the preferred technique for measuring bone density. The DEXA scanner produces 2 X-ray beams, each with different energy levels. One beam is high energy while the other is low energy. The amount of X-rays that pass through the bone is measured for each beam. This will vary depending on the thickness of the bone. The amount of x-ray exposure is very low. Dose from the DEXA measurement is well below natural background radiation levels. For example, total average x-ray exposure with the DEXA is around 0.2 microSv (units used to describe radiation) compared to dental bitewing xray 60 microSv and 5000 times lower than the recommended daily limit of 1000 micro Sv/day.

WHERE WILL THE STUDY TAKE PLACE?All testing will take place in the Human Performance Laboratory in Moby Gym at Colorado State University.

HOW LONG WILL THE STUDY LAST? The study will take about 30 minutes of your time.

WHAT ARE THE POSSIBLE RISKS AND DISCOMFORTS? It is not possible to identify all potential risks in a research procedure, but the researchers have taken reasonable precautions to minimize any known and potential, but unknown, risks**.** There are no anticipated physical risks for participating in the DEXA test other than a very small amount of radiation (equal to about 15 minutes outside on a sunny day). This is a commonly used clinical test to measure bone density and risk for osteoporosis in women. The risks of blood drawing are: a possible hematoma (bruise) at the site where blood was drawn and the remote risk of infection. The results of the study will be published BUT information will be combined with that of other people taking part in the study and you will NOT be identified by name in any written or oral communication.There are no other known or anticipated risks associated with the study other than those addressed above.

WHAT ARE THE BENEFITS OF PARTICIPATION IN THIS STUDY? You will receive the results of your free DEXA scan (both your body fat composition and your bone density compared to expected values for your age). It is anticipated that this study will contribute understanding of the role of omega-3 DHA status in regulating body weight and bone density.

WILL I RECEIVE ANY COMPENSATION FOR TAKING PART IN THIS STUDY? You will not be compensated other than to receive the results of your DEXA.

WHAT WILL IT COST ME TO PARTICIPATE? There will be no costs to you. You will receive a prepaid permit for parking on campus.

DO I HAVE TO TAKE PART IN THE STUDY?/ CAN MY TAKING PART IN THE STUDY END EARLY? Your participation in this research is voluntary. If you decide to participate in the study, you may withdraw your consent and stop participating at any time without penalty or loss of benefits to which you are otherwise entitled.

WHO WILL SEE THE INFORMATION THAT I GIVE?
The results of the study will be published but the information will be combined with that of other people taking part in the study and you will NOT be identified by name in any written or oral communication. The only exceptions to this are if we are asked to share the research files for audit purposes with the CSU Institutional Review Board ethics committee, if necessary. In addition, for funded studies, the CSU financial management team may also request an audit of research expenditures. For financial audits, only the fact that you participated would be shared, not any research data.

We will use a coded study number to record your DEXA and blood results. You will be given an envelope to fill out your name and address so that we can send your DEXA report to you.

WHAT HAPPENS IF I AM INJURED BECAUSE OF THE RESEARCH? The Colorado Governmental Immunity Act determines and may limit Colorado State University's legal responsibility if an injury happens because of this study. Claims against the University must be filed within 180 days of the injury.

WHAT IF I HAVE QUESTIONS?
Before you decide whether to accept this invitation to take part in the study, please ask any questions that might come to mind. Later, if you have questions about the study, you can contact the investigator, Dr. Mary Harris at 970-491-7462.If you have any questions about your rights as a volunteer in this research, contact the CSU IRB at: RICRO_IRB@mail.colostate.edu; 970-491-1553. We will give you a copy of this consent form to take with you.

Your signature acknowledges that you have read the information stated and willingly sign this consent form. Your signature also acknowledges that you have received, on the date signed, a copy of this document containing 3 pages.

_____
Participant name (printed)

_____ _____
Participant Signature Date

_____ _____
Witness to signature (project staff) Date

APPENDIX D

FOLLOW-UP DEMOGRAPHIC DATA COLLECTION FORM

Omega Smart Baby: Follow-Up Study DATA Collection Form

Subject Code Number: Date:

Date of Birth:
Participation in Any Other Study? Yes No
Height (inches):
Weight (pounds):
Months since birth of last child:

For Omega Smart Baby Participants Only
Pregnancy Weight Gain (pounds):
Months of Breastfeeding:
Months since cessation of breastfeeding:

<u>Checklist</u>
___ Dexa completed
___ Blood Drawn
___ Food Frequency questionnaire completed and verified

APPENDIX E

BONE DENSITY STUDY QUESTIONNAIRE

Bone Density Study Questionnaire

Date of Birth_____ Study Number_____

Are you pregnant?	Yes	No
Are you breastfeeding?	Yes	No
Have you been diagnosed with:		
Rheumatoid arthritis?	Yes	No
Celiac Disease?	Yes	No
Hyperparathyroidism?	Yes	No
Osteopenia?	Yes	No
Osteoporosis?	Yes	No
Diabetes (type 1)	Yes	No
Asthma	Yes	No
Multiple Sclerosis or Lupus	Yes	No
Liver Disease	Yes	No
Kidney Disease	Yes	No

Please list all medications and supplements (vitamins, minerals, herbals) which you are currently taking:

APPENDIX F

FOOD FREQUENCY QUESTIONNAIRE (FFQ)

Food Frequency Questionnaire **Study Number _____**

Please provide information regarding the consumption of the following foods over the past 12 months.

1) Salmon/Trout 
___ times per day, week or month
(circle one)

2) White tuna 
(also called albacore tuna)
___ times per day, week or month
(circle one)

3) Light tuna 

___ times per day, week or month
(circle one)

4) Sardines/ 
Herring/Anchovies
___ times per day, week or month
(circle one)

5) Pork/Beef/Lamb 
___ times per day, week or month
(circle one)

6) Chicken/Turkey 
___ times per day, week or month
(circle one)

7) Eggs 
___ times per day, week or month
(circle one)

8) Goldcircle or Store 
brand Omega-3 eggs
___ times per day, week or month
(circle one)

9) Milk 

With DHA? Yes No
___ times per day, week or month
(circle one)

10) Cheese 
___ times per day, week or month
(circle one)

11) Cod liver oil 
or other fish oils
___ times per day, week or month
(circle one)

12) DHA 
(docosahexanoic acid) or Omega 3
fatty acid or fish oil supplement
Brand _____
Amount of DHA/EPA_____
___ times per day, week or month
(circle one)

Thank you for your time and assistance with this study. Your input will help other women in the future.

~ From The Omega-3 Smart Baby Project Follow-Up Team!

APPENDIX G

PHYSICAL ACTIVITY QUESTIONNAIRE (PAQ)

ID Number | | | | | | | | |

PHYSICAL ACTIVITY QUESTIONNAIRE

This questionnaire is designed to find out about your physical activity in your everyday life.

Please try to answer every question, except when there is a specific request to skip a section.

Your answers will be treated as strictly confidential and will be used only for medical research

CAMB/PA/4/1201

Page 2

THE QUESTIONNAIRE IS DIVIDED INTO 3 SECTIONS

- **Section A** asks about your physical activity patterns in and around the house.
- **Section B** is about travel to work and your activity at work. It may be skipped by people who have not worked at any stage during the last 12 months.
- **Section C** asks about recreations that you may have engaged in during the last 12 months.

What is your date of birth? day / month / year

What is today's date? day / month / year

Your sex *(Please tick (✓) appropriate box)*? Male ☐ Female ☐

Section A HOME ACTIVITIES

GETTING UP AND GOING TO BED

*Please put a time in **each** box*

	Average over the past year	
	At what time do you normally get up?	At what time do you normally go to bed?
On a weekday		
On a weekend day		

GETTING ABOUT — Apart from going to work

Which form of transport do you use **most often** apart from your journey to and from work?

*Please tick (✓) one box **ONLY** per line*

Distance of journeys	Usual mode of transport			
	Car	Walk	Public transport	Cycle
less than one mile				
1–5 mile(s)				
More than 5 miles				

TV OR VIDEO VIEWING

*Please put a tick (✓) on **every** line*

Hours of TV or Video watched per day	Average over the last 12 months					
	None	less than 1 hour a day	1 to 2 hours a day	2 to 3 hours a day	3 to 4 hours a day	More than 4 hours a day
On a weekday before 6 pm						
On a weekday after 6 pm						
On a weekend day before 6 pm						
On a weekend day after 6 pm						

STAIR CLIMBING AT HOME

*Please put a tick (✓) on **every** line*

Number of times you climbed up a flight of stairs (approx 10 steps) each day at home	Average over the last 12 months					
	None	1 to 5 times a day	6 to 10 times a day	11 to 15 times a day	16 to 20 times a day	More than 20 times a day
On a weekday						
On a weekend day						

ACTIVITIES IN AND AROUND THE HOME

*Please put a tick (✓) on **every** line*

Approximate number of hours each week	Average over the last 12 months						
	None	Less than 1 hour a week	1 to 3 hours a week	3 to 6 hours a week	6 to 10 hours a week	10 to 15 hours a week	More than 15 hours a week
Preparing food, cooking and washing up							
Shopping for food and groceries							
Shopping and browsing in shops for other items (e.g. clothes,toys)							
Cleaning the house							
Doing the laundry and ironing							
Caring for pre-school children or babies at home (not as paid employment)							
Caring for handicapped, elderly or disabled people at home (not as paid employment)							

Section B ACTIVITY AT WORK

Please answer this section **only** if you have been in paid employment at any time during the last 12 months or you have done regular, organised voluntary work.

If not please go to page 9

TYPES OF WORK DURING THE LAST TWELVE MONTHS

- We would like to know what full or part-time jobs you have done in the last 12 months.
- You may have held a single job or have held two jobs at once.
- If you have changed jobs with the same employer, you should enter it as a change of job **only** if it entailed a substantial change in physical effort.

EXAMPLE

Someone who worked full-time for 6 months, then retired, rested for 3 months and then started a voluntary job for 6 hours a week, would complete the questions as follows.

	Job 1	Job 2
Name of occupation	nurse	shop work
How many hours **per week** did you usually work?	38	6
For how many months in the last 12 months did you do this work?	6	3

EXAMPLE

ACTIVITY LEVELS AT YOUR WORK

Now we would like you to take the total number of hours you worked per week in each job and divide them up according to your activity level.

Please complete EACH line

	Job 1			Job 2		
	No	Yes	Hours per week	No	Yes	Hours per week
Sitting — light work e.g. desk work, or driving a car or truck		✓	6	✓		
Sitting — moderate work e.g. working heavy levers or riding a mower or forklift truck	✓				✓	2
Standing — light work e.g. lab technician work or working at a shop counter		✓	30		✓	4
Standing — light/moderate work e.g. light welding or stocking shelves		✓	2	✓		

EXAMPLE

The number of hours in each activity should add up to the number of hours that you worked in each job e.g. 6+30+2=38 (nurse)

What jobs have you held in the last 12 months, and how many months in the year did you do them?

Please complete EACH line

	Job 1	Job 2
Name of occupation		
How many hours **per week** did you usually work?		
For how many months in the last 12 months did you do this work?		

ACTIVITY LEVELS AT YOUR WORK

Now we would like you to take the total number of hours you worked per week in each job and divide them up according to your activity level.

Please complete EACH line

	Job 1			Job 2		
	No	Yes	Hours per week	No	Yes	Hours per week
Sitting — light work e.g. desk work, or driving a car or truck						
Sitting — moderate work e.g. working heavy levers or riding a mower or forklift truck						
Standing — light work e.g. lab technician work or working at a shop counter						
Standing — light/moderate work e.g. light welding or stocking shelves						
Standing — moderate work e.g. fast rate assembly line work or lifting up to 50 lbs every 5 minutes for a few seconds at a time						
Standing — moderate/heavy work e.g. masonry/painting or lifting more than 50 lbs every 5 minutes for a few seconds at a time						
Walking at work — carrying nothing heavier than a briefcase e.g. moving about a shop						
Walking — carrying something heavy						
Moving, pushing heavy objects objects weighing over 75lbs						

STAIR OR STEP CLIMBING AT WORK

Please put a tick (✓) on EACH line where appropriate

Number of times you climbed up a flight of stairs (10 steps) at work	AVERAGE OVER THE LAST 12 MONTHS					
	None	1 to 5 times a day	6 to 10 times a day	11 to 15 times a day	16 to 20 times a day	More than 20 times a day
Job 1						
Job 2						

Please put a tick (✓) on EACH line where appropriate

Number of times you climbed up a ladder at work	AVERAGE OVER THE LAST 12 MONTHS					
	None	1 to 5 times a day	6 to 10 times a day	11 to 15 times a day	16 to 20 times a day	More than 20 times a day
Job 1						
Job 2						

KNEELING AND SQUATTING AT WORK IN JOB 1

In an average working day in Job 1 did you

kneel for more than one hour in total? No ☐ Yes ☐ Don't know ☐

squat for more than one hour in total? No ☐ Yes ☐ Don't know ☐

get up from kneeling or squatting more than 30 times? No ☐ Yes ☐ Don't know ☐

KNEELING AND SQUATTING AT WORK IN JOB 2

In an average working day in Job 2 did you

kneel for more than one hour in total? No ☐ Yes ☐ Don't know ☐

squat for more than one hour in total? No ☐ Yes ☐ Don't know ☐

get up from kneeling or squatting more than 30 times? No ☐ Yes ☐ Don't know ☐

TRAVEL TO AND FROM WORK

JOB 1

Please complete EVERY line

Roughly how many miles was it from home to Job 1?	
How many times a week did you travel from home to Job 1?	

Please tick (✓) one box ONLY per line

How did you normally travel to Job 1?	Always	Usually	Occasionally	Never or rarely
By car				
By works or public transport				
By bicycle				
Walking				

JOB 2 *(if appropriate)*

Please complete EVERY line

Roughly how many miles was it from home to Job 2?	
How many times a week did you travel from home to Job 2?	

Please tick (✓) one box ONLY per line

How did you normally travel to Job 2?	Always	Usually	Occasionally	Never or rarely
By car				
By works or public transport				
By bicycle				
Walking				

Section C RECREATION

The following questions ask about how you spent your leisure time.

Please indicate how often you did each activity on average over the last 12 months.

For activities that are seasonal, e.g. cricket or mowing the lawn, please put the average frequency during the season when you did the activity.

Please indicate the average length of time that you spent doing the activity on each occasion.

EXAMPLE

If you had mowed the lawn every fortnight in the grass cutting season and took 1 hour and 10 minutes on each occasion.

If you went walking for pleasure for 40 minutes once a week.

You would complete the table below as follows:

Please give an answer for the AVERAGE TIME you spent on each activity and the NUMBER OF TIMES you did that activity in the past year.

	Number of times you did the activity in the last 12 months								Average time per episode	
	None	Less than once a month	Once a month	2 to 3 times a month	Once a week	2 to 3 times a week	4 to 5 times a week	Every day	Hours	Mins
Mowing the lawn				✓					1	10
Walking for pleasure					✓					40

EXAMPLE

Now please complete the table on pages 10 and 11

Please give an answer for the NUMBER OF TIMES you did the following activities in the last 12 months and the AVERAGE TIME you spent on each activity.

Please complete EACH line

	Number of times you did the activity in the last 12 months								Average time per episode	
	None	Less than once a month	Once a month	2 to 3 times a month	Once a week	2 to 3 times a week	4 to 5 times a week	6 times a week or more	Hours	Mins
Swimming — competitive										
Swimming — leisurely										
Backpacking or mountain climbing										
Walking for pleasure — you should not include walking as a means of transportation as this was included in Sections A & B										
Racing or rough terrain cycling										
Cycling for pleasure — you should not include cycling as a means of transportation										
Mowing the lawn — during the grass cutting season										
Watering the lawn or garden in the summer										
Digging, shovelling or chopping wood										
Weeding or pruning										
DIY e.g. carpentry, home or car maintenance										
High impact aerobics or step aerobics										
Other types of aerobics										
Exercises with weights										
Conditioning exercises e.g. using an exercise bike or rowing machine										

Please continue on the next page

Please complete EACH line

	Number of times you did the activity in the last 12 months								Average time per episode	
	None	Less than once a month	Once a month	2 to 3 times a month	Once a week	2 to 3 times a week	4 to 5 times a week	6 times a week or more	Hours	Mins
Floor exercises e.g. stretching, bending, keep fit or yoga										
Dancing e.g. ballroom or disco										
Competitive running										
Jogging										
Bowling — indoor, lawn or 10 pin										
Tennis or badminton										
Squash										
Table tennis										
Golf										
Football, rugby or hockey (during the season)										
Cricket (during the season)										
Rowing										
Netball, volleyball or basketball										
Fishing										
Horse-riding										
Snooker, billiards or darts										
Musical instrument playing or singing										
Ice-skating										
Sailing, wind-surfing or boating										
Martial arts, boxing or wrestling										

You have finished the questionnaire — *Thank you*

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APPENDIX H

INFORMATION SHEET FOR INTERPRETATION OF DEXA RESULTS

Interpretation of Your Bone Density Test

Your bone density scores in your hip and spine, which are printed on the DEXA report, are shown as “T-scores” and “Z-scores”.
The T-score represents deviation from the average bone density of healthy adult women.
The Z-score represents deviation from the average bone density compared to women your exact age.
The World Health Organization defines Osteoporosis as a T-score of - 2.5 or more and Osteopenia (low bone density) as a T-score of -1 to -2.5.
Since deviations in T-scores increase your risk of bone fractures, you should consult your family physician if you have negative scores.
Your Z-score is used to classify the type of osteoporosis.
A Z-score of <1.5 is usually indicative of age related bone loss (or primary osteoporosis) seen in older women.
A Z-score of 1.5 and higher indicates secondary osteoporosis, which can be caused by too much or too little parathyroid hormone, rheumatoid arthritis,diabetes, celiac disease or the use of certain medications.

Interpretation of Your Percent Body Fat

Your percent body fat is dependent upon a number of factors including age and fitness level, but there is no one "ideal" number. The American College on Exercise (ACE) has published recommended ranges of body fat for healthy individuals. In women these ranges are:

Athletes	14 -20%
Fitness	21 – 24%
Average	25 – 31%
Above Average	>32%

The ACE ranges do not take age into consideration. For adults, a range of 21 – 33% body fat is generally considered to be "healthy". It is thought that "underfat" may be unhealthy. For a woman between 20 – 40 years of age, less than 21% represents "underfat". You can also use the chart provided as a guide based on age but remember that an active person with higher body fat can be healthier that an inactive lean person.