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Project Title: Breastfeeding and Bone Mineral Density in Adults Ages 30-46 in The Manitoba Personalized Lifestyle Research (TMPLR) Study

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Summary (250 words max single spaced):

Osteoporosis is a major risk factor for debilitating fractures in geriatric populations. It is increasingly recognized that osteoporosis originates in early life, long before symptoms manifest in late adulthood. Breastfeeding is proposed to "program" skeletal development, however, there is a lack of consensus on the effects of breastfeeding on bone mineral density (BMD), and few studies have investigated this association in adulthood. To address this knowledge gap, we used data from 408 participants ages 30-46 years in the cross-sectional TMPLR study. Feeding in infancy was self-reported (any breastfeeding, breastfeeding duration and any formula feeding) and validated against maternal report for a subset of participants. Dual-energy X-ray Absorptiometry (DXA) was used to measure BMD at the whole body, femoral neck, non-dominant forearm, and lumbar spine (L1-L4 vertebrae). Independent of current age, sex, height, weight, alcohol consumption, physical activity, current and early life socioeconomic status, being breastfed was associated with significantly higher whole body BMD (adjusted beta estimate +22.7 mg/cm^2, 95% CI [0.3, 45.1], equivalent to +1.9%, p=0.046). Similar trends were observed at the femoral neck, forearm and lumbar spine, but these associations were not significant in adjusted models. Unexpectedly, these associations tended to be stronger among those who were partially breastfed (breast milk and formula) than those who were fully breastfed (breast milk only). There was no apparent dose-response relationship according to the duration of breastfeeding. In summary, this study suggests that breastfeeding may contribute to a small but potentially clinically relevant increase in whole body BMD in mid-adulthood.

Student Signature 

Primary Supervisor Signature 

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Introduction

Consequences of Poor Bone Health

A common manifestation of poor bone health is osteoporosis, which is characterized by low bone mass and microarchitectural deterioration[1]. The clinical importance of osteoporosis is the increased risk of fractures which relates to higher rates of morbidity and mortality[2]. The Canadian prevalence of osteoporosis in women over the age of 50 is estimated at 21.3% and 5.5% in men[3]. This translates to a substantial economic burden of $2.3 billion (2008), accounting for 1.3% of the Canadian healthcare budget[4]. Bone mineral density (BMD) measured by central dual-energy x-ray absorptiometry (DXA) is used to diagnose osteoporosis, and on its own is a well-established predictor of future fracture risk[2,5]. Every standard deviation decrease in BMD is associated with a 1.5 to 2.5-fold increase risk of fracture[2].

Bone Development

It is increasingly recognized that osteoporosis and subsequent fractures observed in geriatric populations can have their origins in childhood and adolescence when the body deposits and builds most of its bone mass[6]. Over 90 percent of peak bone mass is acquired by 20 years of age, and the remainder is achieved before the age of 30[7]. Peak bone mass is achieved when bones have reached their maximal density and strength. After this point, bone density is maintained and subsequently declines[8]. The World Health Organization (WHO) reference standard for osteoporosis is a femoral neck T-score of -2.5 or less[5]. One strategy to prevent against low BMD in later life is to optimize peak bone mass accrual. The importance of peak bone mass is indicated by studies that have shown that BMD tracks throughout life, such that those on the high end of the spectrum in childhood remain so in adolescence through to the age of 70[9–11]. Furthermore, it is estimated that even a 2-3% increase in peak bone mass could reduce later fracture risk by 10-20%[12]. Thus, optimizing the attainment of peak bone mass in early life is advantageous and may be protective against the development of osteoporosis and fractures[6].

Breastfeeding and Bone Mineral Density (BMD)

The first year of life may be especially important because exposures during this period of time may “program” bone cells and affect the trajectory of skeletal growth[9,13]. Breastfeeding is an early-life exposure that could influence bone development. Amongst other well-known benefits, breastfeeding is protective against morbidity and mortality from gastrointestinal tract infections in infancy, enhances neurodevelopment in early life, and lowers the risk of obesity and type-2 diabetes in later life[14–16]. Breastfeeding is proposed to have a beneficial effect on BMD through the increased bioavailability of its nutritive contents, the presence of growth factors and hormones, and through its potential epigenetic effects[17–20]. However, there is no consensus on the effects of breastfeeding on BMD at different ages[21]. A recent systematic review of 11 studies found that in children, three studies did not find an association, two studies found a positive association between breastfeeding and BMD, and one study found an inverse relationship[21]. In adolescents, two studies found a positive association between breastfeeding duration and BMD and one reported an absence of any relationship[21]. Only one study in adults was identified, where Pirila et al. found an inverse relationship between breastfeeding duration and BMD in men but not women at age 32 years[22]. Since this review, one other study by Muniz et al. has investigated the association between breastfeeding and the BMD of adults in the third decade of life, finding no association in men or women at age 30[23]. These two adult studies differed methodologically in several ways. Pirila et al. studied 158 individuals and measured BMD at the lumbar spine, femoral neck and whole body and investigated breastfeeding duration categorized into three groups, while Muniz et al. studied two much larger birth cohorts (n=3226

in the 1982 cohort, n=1109 in the 1993 cohort) and investigated breastfeeding as a dichotomous variable (yes/no), as well as breastfeeding duration and breastfeeding exclusivity[22,23]. However, Muniz et al. only investigated effects at the whole body[23]. To our knowledge, no study has examined the association of breastfeeding and BMD beyond 30 years of age.

To address the above knowledge gaps and limitations of previous studies, we explored the long-term relationship between breastfeeding and BMD in adults ages 30-46 (n=410), allowing us to capture maximal peak bone mass. Breastfeeding was assessed as a dichotomous variable (ever/never), breastfeeding duration and breastfeeding exclusivity. In addition, we examined BMD reported as g/cm^2 at the whole body, which is a robust measure of skeletal development, and the femoral neck, forearm and lumbar spine, which are common sites of osteoporotic fractures[24].

Hypothesis

We hypothesized that being breastfed would be associated with higher BMD in adulthood, and that a dose-response relationship exists where breastfeeding duration and exclusivity are positively correlated with BMD.

Methods

Study Design and Population

This study used data from The Manitoba Personalized Lifestyle Research study (TMPLR; www.tmplr.ca), a cross-sectional, observational study of 800 Manitobans ages 30-46 years old (born between 1970-1988). Participants were recruited between 2016-2018 to investigate how lifestyle factors (ie. nutrition, sleep, physical activity) as well as genetics and the gut microbiome influence health and chronic disease[25]. A portion of this study addresses the influence of early life factors[25]. Participants completed extensive questionnaires about early and current lifestyle factors and underwent clinical assessment with anthropometric measurements and dual-energy x-ray absorptiometry (DXA) scans. Mothers of participants were also recruited to complete a questionnaire on key pregnancy, birth and postpartum events relating to the participant enrolled in the study. Data from 519 participants were available at the time of this analysis. Of these, 109 participants were missing data on breastfeeding (see below) and were excluded, leaving 410 participants for analysis.

Exposure: Infant Feeding

Participants responded to several questions about feeding in infancy, adapted from the US Nurses' Health Study[26]. Relevant questions included, "Were you breastfed as an infant?", "Were you fed commercial infant formula as an infant (e.g. Similac, Enfamil, SMA, etc.)?", and "If breastfed, do you know to what age you were breastfed?". Participants had the option of responding with "yes", "no", or "don't know" for the former questions and to categories with a range of "less than one week" to "one year or more", "not applicable", or "don't know" to the latter question. Participants who responded, "don't know", "not applicable", or did not respond were excluded from corresponding analyses. Variables used in the analyses included breastfeeding as a dichotomous variable (ever/never), breastfeeding duration categorized into five groups: '<3 months', '3-6 months', '6-9 months', '9-12 months' and '>12 months', and breastfeeding exclusivity classified as 'no breastfeeding' (formula without breastfeeding), 'partial breastfeeding' (breastfeeding and formula), or 'full breastfeeding' (breastfeeding without formula). Other food, water and liquid intake during infancy was not considered.

Participants excluded from this study included 59 participants that "don't know" if they were breastfed as an infant and 50 participants that did not respond. In the analysis of breastfeeding duration, participants that responded with "don't know", "not applicable" or did not respond were excluded (n=237). Analysis on breastfeeding exclusivity excluded participants that could not be classified, including participants that responded "no" to both formula feeding and breastfeeding (n=15), "don't know" to one or both questions (n=194), or were missing responses (n=51).

For a subset of 73 participants where maternal questionnaire data were available, self-reported infant feeding exposures were validated against the participant's mother's response. Mothers responded to corresponding questions about breastfeeding ("Did you ever breastfeed you child?"), breastfeeding duration ("For how many months did you breastfeed?"), and formula feeding ("Did you feed your child a commercial infant formula on a daily basis?"), with regards to the participant in the study. To compare agreement rates for breastfeeding and formula feeding, total congruency was assessed over total number of responses. Agreement for breastfeeding duration was assessed by assigning the median value to self-reported breastfeeding duration categories and this was correlated with the maternal response as a continuous variable using Spearman correlation.

Outcome: Bone Mineral Density g/cm^2 (BMD)

BMD was measured using DXA (GE Lunar Prodigy Advance, GE Healthcare) by a certified Bone Densitometry Technologist. Scans were taken of the whole body, femoral neck, non-dominant forearm and anterior-posterior lumbar spine (L1-L4) using the International Society for Clinical Densitometry (ISCD) official positions for adults and reported as absolute values for analysis (g/cm^2)[5]. Lumbar spine BMD was derived from an average BMD of the L1-L4 vertebrae; participants missing information from at least one vertebra were excluded (n=3). T-scores were generated by comparison of the whole body BMD, femoral neck BMD, total radius BMD, and L1-L4 vertebrae BMD to the USA (Lunar) (ages 20-40) reference populations of the total body (v113), femur (v113), forearm (v113), and AP spine (v113) respectively by the enCORE™ Software Platform (GE Lunar Prodigy Advance, GE Healthcare). Lumbar spine T-score was derived from an average T-score of the L1-L4 vertebrae. T-scores were categorized as "normal" (T-score greater than or equal to -1.0), "low" (T-score less than -1.0 but greater than -2.5), and "osteoporotic" (T-score less than or equal to -2.5) according to World Health Organization definitions[27]. Z-scores were not available at the time of this analysis. Daily quality assurance scans were performed on the DXA scanner using a tub phantom spine in water (LNR6847, GE Healthcare) and a quality assurance block (LNR6847, GE Healthcare).

Covariates

After investigating many covariates identified from the literature and based on biological plausibility, the following were included in our analyses: current sex, age, height, weight, alcohol consumption, level of physical activity, socioeconomic status (household income) and early life socioeconomic status (father's level of education)[17,22,23,28–31]. Other covariates considered that did not result in a change in beta estimate of >10% in our study include ethnicity, smoking status, second-hand smoking in childhood and adulthood, home ownership, education level, personal income, gestational age at birth, method of birth, and comparative financial situation in childhood.

Sex was categorized as a dichotomous variable: "male" or "female", and age was evaluated as a continuous variable. Alcohol consumption was categorized into three categories by pattern of consumption over the past 12 months: "often" (more than one drink a week), "sometimes" (less than one drink a week to more than one drink a month), and "rarely" (less

than one drink a month to none). Level of physical activity was categorized by the highest level of physical activity in the past seven days: “strenuous activity”, “moderate activity”, “light activity”, or none. Household income was used as a proxy for current socioeconomic status and was defined as the total income received by all household members from all sources (before taxes and deductions) in the past 12 months. Household income was categorized as: “<$50,000”, “$50,000-$100,000”, “$100,000-$150,000”, or “more than $150,000”. Father’s level of education was used as a proxy for early life socioeconomic status and was characterized by the level of education achieved by the participant’s father, categorized into two groups: <4 or ≥4 years of post-secondary education. All of these factors were self-reported by participants through TMPLR questionnaires. Height (cm) and weight (kg) were measured using a stadiometer and an electronic scale respectively, by TMPLR staff, and evaluated as continuous variables.

Statistical Analysis

Analysis was done using R Studio (Version 1.1.453). Descriptive statistics were used to describe the distribution of demographic characteristics. Continuous variables were checked for normality by visualizing the data plotted as a histogram. Height, weight, whole body BMD, femoral neck BMD, forearm BMD and lumbar spine BMD were all normally distributed. The mean and standard deviation were reported for normally distributed continuous variables. Continuous variables with a skewed distribution including age and body mass index (BMI) were reported using the median and interquartile range (IQR). Categorical variables were reported as number and percentages (excluding missing responses).

Univariate analyses were used to assess associations between relevant covariates and the main exposure and outcome variables. Chi-square test was used to test categorical variables for significant differences in proportions and Fisher’s exact test was used when there was at least one cell frequency less than 5. T-test was used to compare the means between two groups for normally distributed continuous variables, and Wilcoxon test was used to compare the medians between two groups for non-normally distributed continuous variables. Analysis of variation (ANOVA) was used to compare the means between more than two groups.

Multivariable linear regression was used to investigate associations between breastfeeding and BMD in crude (unadjusted), basic (adjusted for current age and sex), and adjusted (further adjusted for important covariates) models. Covariates were identified by their association with the exposure and outcome variable based on previous studies or based on univariate analyses (described above)[17,22,23,28–31]. Covariates were considered important if their inclusion resulted in a change in beta estimate of >10% in multivariable analyses associating breastfeeding with BMD. We also considered the overall model fit using the adjusted r^2. Sex differences were considered by testing an interaction term between sex and breastfeeding; this was not significant (p=0.98), therefore we did not stratify by sex for subsequent analyses.

Results

Demographic Characteristics

Table 1 describes our study population. Most participants were white (82.5%) and females were overrepresented (62.5%). The median age was 38 and ranged from 30 to 46 years old. Participants tended to consume alcohol sometimes (43.5%) or often (31.5%) and about half engaged in strenuous physical activity (51.2%). Few participants reported a household income less than $50,000 (8.8%). Most participant’s fathers had less than four years of post-secondary

education (61.8%). The median BMI of participants was 25.7 m/kg^2 (IQR 23.3, 29.9), over half were overweight (BMI 25-30 m/kg^2) (31.9%) or obese (BMI >30 m/kg^2) (24.8%).

Compared to the 410 participants included in the study, those excluded due to missing breastfeeding data (n=109) were not significantly different according to these characteristics (results not shown).

Breastfeeding

Participants excluded from subsequent analyses included 59 participants that "don't know" if they were breastfed as an infant and 50 participants that did not respond (Table 2). Of participants that reported that they were breastfed as an infant (285 of 410), 111 participants could not confirm their duration of breastfeeding and 25 participants could not be classified by exclusivity of breastfeeding.

The majority of participants included in the study reported that they were breastfed as an infant (69.5%) (Table 2). Of these, over half (n=173) reported on their duration of breastfeeding, with similar proportions reporting "<3 months" (20.8%), "3-6 months" (16.2%), "6-9 months" (22.5%), "9-12 months" (23.1%), and ">12 months" (17.3%) (Table 2). Breastfeeding exclusivity was inferred in participants that had complete data for breastfeeding and formula feeding (n=259). Of these participants, 37.8% were classified as "no breastfeeding", 29.0% were classified as "partial breastfeeding", and 33.2% were classified as "full breastfeeding" (Table 2).

In a subset of participants where maternal responses were available (n=73), there was a high agreement rate between self-reported and maternal-reported responses (88% agreement for breastfeeding and 84% for formula feeding). Self-reported and maternal reported breastfeeding duration were also highly correlated (Spearman r=0.74).

Compared to the never breastfed group, the ever breastfed group had a lower proportion of females (56.2% vs. 76.8%), were younger (median age 36 years old (IQR 33, 41) vs. 40 years old (IQR 37, 44)) and taller (172.3$\pm$9.5 cm vs. 167.4$\pm$8.6 cm), and experienced higher early life socioeconomic status (45.0% vs 23.0% had fathers with $\geq$4 years of post-secondary education) (Table 1). The association with age is reflective of secular trends in breastfeeding where the percentage of mothers who initiated breastfeeding increased dramatically from 25% in the mid-1960s to 75% in the early 1990s[32]. Current weight, household income, alcohol consumption, and level of physical activity were not associated with breastfeeding status (Table 1).

Bone Mineral Density (BMD)

The mean BMD were: whole body 1.25$\pm$0.13 g/cm^2, femoral neck 1.03$\pm$0.13 g/cm^2, forearm 0.70$\pm$0.09 g/cm^2, and lumbar spine 1.22$\pm$0.14 g/cm^2. T-scores at the femoral neck, forearm and lumbar spine followed standard normal distribution as expected with a majority of participants having a "normal" T-score (86.5%, 84.0% and 85.0%, respectively), a few participants had "low" T-scores (13.2%, 15.5% and 13.0%, respectively), and "osteoporotic" T-scores were rare with <2% of participants falling within this range. T-scores at the whole body did not follow standard normal distribution and had a disproportionately high distribution of "normal" T-score (98.3%) and a disproportionately low distribution of "low" T-score (1.7%), with no participants falling within the "osteoporotic" T-score range.

Whole body BMD was significantly associated with sex, where males had expectedly higher BMD than females (1.33$\pm$0.12 g/cm^2 vs. 1.21$\pm$0.11 g/cm^2, p<0.001) (Table 1). Whole body BMD was also significantly associated with frequency of alcohol consumption (p=0.001), level of physical activity (p=0.03) and household income (p=0.001) and was positively correlated

with height (Pearson r=0.50, p<0.001) and weight (Pearson r=0.62, p<0.001) (Table 1). Whole body BMD was not significantly associated with age or father's level of education (Table 1). Some of these associations differed at other body sites (not shown); for example, while height and weight were positively correlated with BMD at all sites, age was negatively correlated with BMD at the femoral neck only, and sex differences were observed at the femoral neck and forearm, but not at the lumbar spine.

Breastfeeding and BMD

In univariate analyses, participants who were ever breastfed had significantly higher BMD than those who were never breastfed, at the following skeletal sites: whole body (1.27±0.13 g/cm^2 vs. 1.22±0.12 g/cm^2, p<0.001), femoral neck (1.05±0.13 g/cm^2 vs. 1.00±0.13 g/cm^2, p=0.001), and forearm (0.71±0.09 g/cm^2 vs. 0.68±0.09, p=0.002) (Figure 1A). Breastfeeding was not associated with BMD at the lumbar spine (1.23±0.14 g/cm^2 vs. 1.21±0.14 g/cm^2, p=0.07). We did not observe a clear dose-response relationship according to breastfeeding duration (Figure 1B) or exclusivity (Figure 1C), although we had lower power for these analyses due to higher proportions of missing data. Partial breastfeeding was associated with significantly higher BMD than no breastfeeding, but there was no further increase with full breastfeeding (Figure 1C). For example, whole body BMD was 1.22±0.13 g/cm^2, 1.27±0.15 g/cm^2 and 1.24±0.12 g/cm^2 among non-breastfed, partially breastfed and fully breastfed participants, respectively.

Three linear regression models were used to control for potential confounders and to further investigate the association between breastfeeding and BMD: (1) crude model, without adjusting for any covariates; (2) basic model, adjusted for age and sex; (3) adjusted model, adjusted for current age, sex, height (cm), weight (kg), alcohol consumption, level of physical activity, socioeconomic status (household income), and early life socioeconomic status (father's level of education).

The association between any breastfeeding and higher whole body BMD was attenuated, but remained significant in adjusted models (crude β = +49.5 mg/cm^2, 95% CI [22.0, 76.1], p<0.001; basic β = +33.1 mg/cm^2, 95% CI [7.8, 58.4], p=0.01; adjusted β = +22.7 mg/cm^2, 95% CI [0.3, 45.1], p=0.046) (Figure 2; Table 3). Similar trends were observed at other skeletal sites, but these associations were not significant in adjusted models. For example, at the femoral neck, independent of age and sex, ever breastfeeding was significantly associated with higher BMD (β basic = +35.0 mg/cm^2, 95% CI [6.3, 63.7], p=0.02). However, this association was attenuated and became non-significant in the adjusted model (adjusted β = +19.9 mg/cm^2, 95% CI [-9.2, 49.1], p=0.18). Breastfeeding was not significantly associated with lumbar spine BMD in the crude or adjusted analyses.

There was no apparent dose-response relationship according to the duration of breastfeeding and BMD in adjusted models among participants that reported this information (n=173).

Unexpectedly but consistent with univariate analyses, the positive trend observed between breastfeeding and BMD tended to be stronger among those who were partially breastfed than those who were fully breastfed (Table 3). At the whole body, partial breastfeeding tended to be associated with higher BMD than no breastfeeding (adjusted β = +25.3 mg/cm^2, 95% CI [-7.6, 58.1], p=0.13), while the effect of full breastfeeding was much smaller (adjusted β = +3.7 mg/cm^2, 95% CI [-27.6, 35.0], p=0.81). Similar trends were observed at the femoral neck, forearm and lumbar spine.

Discussion

Our main finding is that being breastfed was associated with significantly higher whole body BMD in adults ages 30-46 years old (adjusted β = +22.7 mg/cm^2, 95% CI [0.3, 45.1], p=0.046), independent of current age, sex, height, weight, alcohol consumption, level of physical activity, as well as current and early life socioeconomic status. Similar trends were observed at the femoral neck, forearm and lumbar spine, but these associations were not significant in adjusted models. Unexpectedly, these associations tended to be stronger among those who were partially breastfed (breast milk and formula) than those who were fully breastfed (breast milk only). There was no apparent dose-response relationship according to the duration of breastfeeding, although we had limited power to examine these potential "dose effects".

Despite the relatively small estimated effect of ever breastfeeding on whole body BMD (equivalent to +1.9%), the association we have observed could be clinically significant. This effect is similar in magnitude to the well known effect of calcium intake in older adults, which increases BMD by 0.6-1.8%[33]. Moreover, it is estimated that even a 2-3% increase in peak bone mass could reduce later fracture risk by 10-20%[12].

Breastfeeding in Infancy (never vs. ever)

Our results expand upon previous studies that have presented conflicting findings on the effects of breastfeeding on BMD at different ages. Consistent with our findings, the Generation R study of children at the age of 6 years old (n=4919; Netherlands; born 2001-2005) also reported a positive association between breastfeeding and whole body BMD, where never breastfeeding was associated with lower BMD (adjusted β = -4.62 mg/cm^2, 95% CI [-9.28, -0.97], p<0.05). Similarly, in adolescents (n=415; Tasmania; born 1988-1989), Jones et al. observed that breastfeeding was associated with a 2-3% increase in BMD at the spine, hip and whole body and a one third reduction in fracture risk[29]. The effect size that we observe of ever breastfeeding on whole body BMD in adults ages 30-46 years old (adjusted β = +22.7 mg/cm^2, 95% CI [0.3, 45.1], p=0.046), is similar to the effect size observed by Muniz et al. in young men ages 18 years old (n=1109; Brazil; born 1993) (adjusted β = +0.026 g/cm^2, 95% CI [0.001, 0.050])[23]. However, the same authors did not observe an association in women at 18 years old (n=1681) nor in men or women at 30 years old (n=3226; Brazil; born 1982)[23]. These differences between studies might be related to differences in study period and/or location. Breastmilk composition varies between different parts of the world especially in concentrations of certain proteins, minerals and vitamins which are essential nutrients for bone mineralization[21,34,35]. In addition, the composition of infant formula (i.e. the diet of non-breastfed infants) differs geographically according to country-specific regulations, and has changed markedly over time[36].

Comparing different skeletal sites, the magnitude of effect for breastfeeding was greatest at the whole body and femoral neck and smaller at the forearm and lumbar spine. Consistent across all skeletal sites, breastfeeding tended to be positively associated with BMD, although this association was only significant at the whole body. This finding suggests that like genetics, environmental factors may have site specific effects[24].

Breastfeeding Duration

We hypothesized that breastfeeding duration would be positively correlated with BMD but we did not find a dose-response relationship according to breastfeeding duration. Some previous studies have found a positive correlation between the duration of breastfeeding and BMD, some have reported an inverse relationship and others did not find an asoociation[22,30,31]. A Copenhagen cohort study (n=109) reported a positive correlation between the duration of exclusive breastfeeding and lumbar spine BMD at 17 years old[30]. In contrast, Pirila et al. found

an inverse relationship between breastfeeding duration and whole body Z-score, where breastfeeding for shorter (≤3 months) was associated with +4.7% higher BMD at the age of 32 compared to breastfeeding for longer (≥ 7 months)[22]. Meanwhile, a large prospective cohort based in the Netherlands (n=4919) did not find a significant association between the duration of breastfeeding and BMD at 6 years old, but did report that ever breastfeeding was associated with higher BMD[31]. This is consistent with our findings that any breastfeeding was associated with higher whole body BMD but we did not detect an association with breastfeeding duration. It is possible that we are underpowered to detect an association between breastfeeding duration and BMD because fewer participants (61% of participants that were breastfed) reported their duration of exposure (n=173). In addition, a limitation of our cross-sectional study is the difficulty in attaining accurate information through recall about details on breastfeeding duration. Our findings suggest that a critical early period of (potentially brief) exposure rather than a sustained duration of exposure is more important for the programming of skeletal development.

Breastfeeding Exclusivity

We found that partial breastfeeding, although not significant, tended to be associated with higher BMD than no breastfeeding, which further supports our hypothesis of the beneficial effects of any exposure to breastfeeding. Interestingly and contrary to our hypothesis, the effect of full breastfeeding, although still positive, was weaker than partial breastfeeding.

This finding suggests that the combined effect of formula feeding with exposure to breastfeeding somehow maximizes the accrual of BMD. A possible explanation for this is the differential composition and nutrient bioavailability in breast milk compared to formula milk[37–39]. Previous studies have found that calcium absorption from breast milk was higher than formula (76% vs. 47%) but formula fed babies had higher total protein intake[38,39]. Thus, in partially fed babies, the intake of protein and calcium, which are essential nutrients for bone development, may be optimized allowing for the interaction of these nutrients to provide greater strength and density to bone. Despite this finding, it is important to note that the World Health Organization (WHO) recommends exclusive breastfeeding for 6 months as the gold standard to meet the demands of infant growth[40]. Exclusive breastfeeding for 6 months has additional short and long-term benefits to infants and their mothers that should be taken into consideration beyond impact on BMD[15,40,41]. Further research is needed to clarify the relationship between breastfeeding and formula feeding on BMD, specifically the duration of exclusive breastfeeding, timing when formula feeding was initiated, frequency of formula use, and the proportion of formula milk to breast milk intake. Unfortunately, these details are not available in our study.

Potential Mechanisms of Action

There are several possible mechanisms for the association that we and others have observed between breastfeeding and BMD. Genetic factors are estimated to account for 72-92% of BMD[8]; however, lifestyle factors, including childhood nutrition and exercise play an important role in achieving maximum bone mass accrual and reaching full genetic potential[42]. Breastfeeding is a major postnatal exposure that is proposed to affect the trajectory of skeletal development through the “programming” of bone cells[9,21]. Breast milk is hypothesized to have a positive effect on this trajectory resulting in greater bone mass in later life[43]. It is proposed that the higher proportions of fatty acids esterified in the sn-2 position in breastmilk compared to the sn-1 and sn-3 positions in formula explain the higher bioavailability of calcium from breastmilk[44]. Additionally, it is proposed that the bioactive non-nutrient factors, such as growth factors and hormones may program bone cells to develop greater bone mass later in life[18]. One such hormone is leptin, which is a metabolism-regulating hormone found in breastmilk that has also been shown to have a direct effects on bone[19]. Leptin has been found to increase proliferation

of isolated fetal rat osteoblasts and plasma leptin levels have also been positively associated with BMD[45,46]. Alternatively, breastfeeding has been suggested to influence the expression of genes in the short and long-term through epigenetic modifications[20]. Previous studies have found that breastfeeding is associated with promoter methylation of *LEP* and *CDKN2A* genes in humans and *Npy* and *Slc2a4* genes in rats which sets precedence on the epigenetic effects of breastfeeding[47]. Breastfeeding might act in a similar way with genes that influence bone development to establish changes in skeletal growth from infancy. In summary, breastfeeding is proposed to positively influence bone development through the increased bioavailability of its nutritive contents compared to infant formula, the presence of growth factors and hormones, and through its potential epigenetic effects.

Strength and Limitations

A key strength of our study is the focus on adults ranging from ages 30-46 years old, which allowed us to study the long-term association between breastfeeding and BMD in a population expected to have reached peak bone mass, but prior to the onset of osteoporosis. In addition, we assessed BMD at multiple skeletal sites, which is advantageous because there is considerable heterogeneity in bone mineral accrual at different skeletal sites that can be differentially affected by genetic and environmental factors over the lifespan[24,47,48].

The use of self-reported infant feeding data is the main limitation of our study. We were, however, able to verify participant responses against responses from their mothers in a subset of the sample (n=73) and found a very high agreement rate (88% for breastfeeding and 84% for formula feeding). Other studies investigating the validity of self-reported breastfeeding history in women 27-44 years old also found a high agreement rate between participants and their mothers[49]. Not surprisingly, self-reported responses to breastfeeding (yes/no) and formula feeding (yes/no) were more accurate and more likely to be recalled than the specific duration of breastfeeding, although we still observed a strong correlation for this measure (Spearman r=0.74), which is consistent with findings from previous studies[49]. While we were able to investigate breastfeeding exclusivity classified by formula feeding and breastfeeding, we were unable to evaluate the association between “truly exclusive” breastfeeding on BMD, because of a lack of information on the intake of food, water and other liquids in infancy. Furthermore, we were limited by our current sample size to investigate the dose effect of breastfeeding on BMD according to breastfeeding duration.

Another limitation is that participants for TMPLR study were recruited in a non-random, voluntary manner, which can introduce selection bias. Comparing our study population with demographic data from the general Manitoba population, our sample was comparable to the general Manitoba population on ethnicity (82.5% white), and BMI (31.9% overweight, 24.8% obese)[50,51]. On the other hand, females (62.5% vs. 50.0%) and the highest income category (27.8% vs. 9.1%) were overrepresented in our study, while the lowest income category was underrepresented (8.8% vs 27.3%)[50]. These differences might affect the external generalizability of findings from this study. It is also important to acknowledge that changes in formula composition and trends in breastfeeding over the last 30-46 years might also compromise the generalizability of these findings to modern times.

Finally, although we controlled for a number of important confounders, we cannot rule out the potential of residual confounding as an explanation for the associations that we observed. For example, we have not accounted for the timing of introduction of solid foods, types of solid food introduced and vitamin D supplementation, which are early nutritional factors that can affect bone development[29,52]. Current dietary intake and weight-bearing exercise were also not considered[42]. As more data from TMPLR study becomes available from the mother’s

questionnaire and the diet history questionnaires, we will be able to reconsider the effects of these covariates on the association between breastfeeding and BMD.

Future Directions

Due to conflicting evidence of breastfeeding on BMD and the lack of studies investigating this effect in mid-adulthood, more research is needed to clarify this association. Specifically in TMPLR study, breastfeeding can be studied in relation to BMD Z-scores as an additional measure of bone health, and the one-third radius site rather than total forearm can be used for greater clinical significance as suggested by the ISCD[5]. Generally, since randomized controlled trials are unethical in breastfeeding, prospective cohorts with measures of infant feeding collected in infancy will offer the best evidence, although long-term prospective studies are challenging and expensive. To better evaluate the dose-effect of breastfeeding on BMD, breastfeeding duration should ideally be collected as a continuous variable; the duration of exclusive breastfeeding, duration of any breastfeeding as well as timing, amount and frequency of introduction of other liquids and solids should also be collected[53]. Beyond BMD, other measures of bone strength and bone geometry can be incorporated to further assess the clinical importance of breastfeeding on overall bone health. Findings from epidemiological studies such as this one can help to inform areas of research focused on understanding the mechanism of breastfeeding on BMD. To this end, biological samples of breastmilk can be used in *in vitro* and *in vivo* studies to further study the effects of breastfeeding on bone development.

Conclusion

In summary, this study provides new evidence that being breastfed is associated with significantly higher whole body BMD in mid-adulthood, independent of age, sex, and key measures of lifestyle and socioeconomic status. Similar trends were observed at the femoral neck, forearm and lumbar spine, but these associations were not significant in adjusted models. Unexpectedly, these associations tended to be stronger among those who were partially breastfed (breast milk and formula) than those who were fully breastfed (breast milk only), and there was no apparent dose-response relationship according to the duration of breastfeeding. More research is needed to confirm this association, determine the relative importance of breastfeeding duration and exclusivity, and characterize the underlying biological mechanisms. Our results suggest that breastfeeding may contribute to a small but potentially clinically relevant increase in whole body BMD in mid-adulthood. These findings point to the importance of investigating and promoting breastfeeding, and possibly other early life factors, to optimize bone mass accrual as a strategy to protect against osteoporosis and fractures.

Table 1. Demographic characteristics and univariate associations with breastfeeding in infancy and bone mineral density (BMD) in adulthood

Variables	Included	Breastfed as an Infant			Whole Body BMD (g/cm²)	
	n (%)	Ever	Never			
	Median (IQR)	n (%)	n (%)	p-value[2]	Mean (SD)	p-value[3]
	Mean (SD)	Mean (SD)	Mean (SD)		Pearson r	
Total	410	285	125		408	
Sex				<0.001*		<0.001*
Female	255 (62.5)	159 (56.2)	96 (76.8)		1.21 (0.11)	
Male	153 (37.5)	124 (43.8)	29 (23.2)		1.33 (0.12)	
Missing	2	2	0		-	
Age	38 (34, 42)	36 (33, 41)	40 (37, 44)	<0.001* (†)		0.62
30-35	147 (36.2)	124 (44.1)	23 (18.4)		1.26 (0.13)	
36-40	123 (30.3)	83 (29.5)	40 (32.0)		1.25 (0.12)	
41-46	136 (33.5)	74 (26.3)	62 (49.6)		1.25 (0.14)	
Missing	4	4	0		-	
Alcohol Consumption				0.31		0.001*
Rarely	100 (25.0)	66 (23.7)	34 (27.9)		1.23 (0.11)	
Sometimes	174 (43.5)	118 (42.4)	56 (45.9)		1.25 (0.12)	
Often	126 (31.5)	94 (33.8)	32 (26.2)		1.29 (0.14)	
Missing	10	7	3		-	
Level of Physical Activity				0.10		0.03*
None	40 (9.8)	24 (8.5)	16 (12.9)		1.25 (0.11)	
Light activity	28 (6.9)	22 (7.7)	6 (4.8)		1.21 (0.11)	
Moderate activity	131 (32.1)	84 (29.6)	47 (37.9)		1.24 (0.13)	
Strenuous activity	209 (51.2)	154 (54.2)	55 (44.4)		1.27 (0.13)	
Missing	2	1	1		-	
Household Income				0.34		0.001*
<$50,000	35 (8.8)	29 (10.4)	6 (5.0)		1.20 (0.11)	
$50,000-$100,000	116 (29.1)	81 (29.0)	35 (29.2)		1.23 (0.12)	
$100,000-$150,000	137 (34.3)	95 (34.1)	42 (35.0)		1.26 (0.13)	
More than $150,000	111 (27.8)	74 (26.5)	37 (30.8)		1.28 (0.13)	
Missing	11	6	5		-	
Father's Level of Education				<0.001*		0.67
< 4 years of post-secondary	247 (61.8)	153 (55.0)	94 (77.0)		1.26 (0.13)	
>4 years of post-secondary	153 (38.3)	125 (45.0)	28 (23.0)		1.25 (0.13)	
Missing	10	7	3		-	-
Height (cm)	170.8 (9.5)	172.3 (9.5)	167.4 (8.6)	<0.001*	0.50	<0.001*
Missing	2	1	1		-	
Weight (kg)	78.7 (16.8)	79.7 (16.3)	76.5 (17.5)	0.08	0.62	<0.001*
Missing	2	1	1		-	

[2]Differences in subject characteristics between ever and never 'Breastfed as an Infant' groups were evaluated using t-test for normally distributed continuous variables, Wilcoxon test for continuous variables that were non-normally distributed (†), and $\chi 2$ test for categorical variables.

[3]Differences in 'BMD' were evaluated using t-test between two groups and ANOVA between more than two groups.

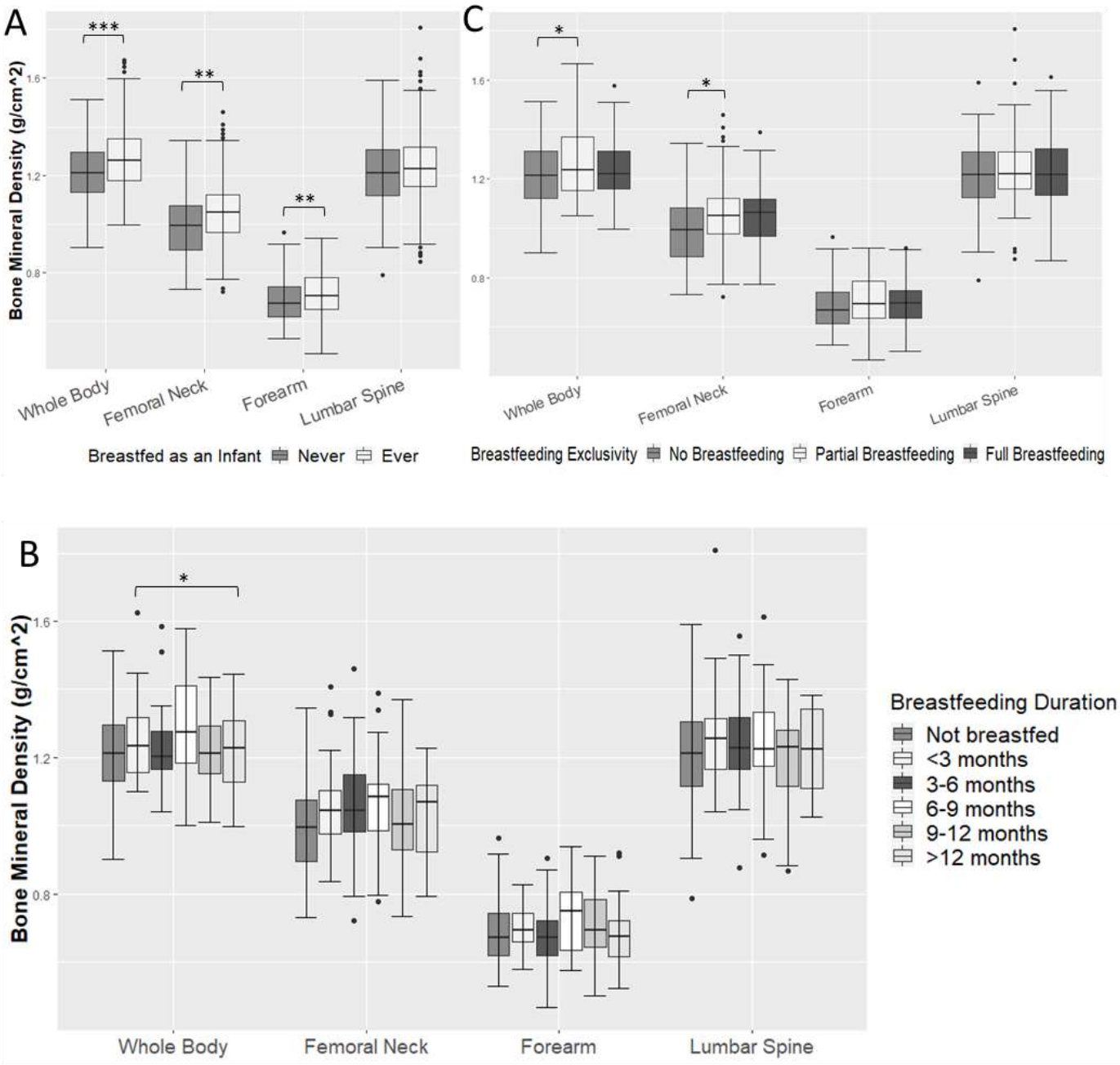

Figure 1. Crude association and distribution of bone mineral density (BMD) in participants that were (A) ever vs. never breastfed in infancy, (B) according to breastfeeding duration, and (C) breastfeeding exclusivity. T-test was used to compare ever vs. never breastfed groups, ANOVA used to compare between 'breastfeeding duration' groups ('Not breastfed' group excluded) and 'breastfeeding exclusivity' groups. Boxes indicate median, upper (Q3) and lower (Q1) quartiles, whiskers indicate range, horizontal lines indicate 1.5 IQR from Q1 and Q3, dots are outliers.
***p-value<0.001, **p-value<0.01, *p-value<0.05.

Table 2. Demographic characteristics: breastfeeding exposure variables

Variables	Exposure n (%)
Breastfed as an Infant	
Never	125 (30.5)
Ever	285 (69.5)
Don't Know (Excluded)	59
Missing (Excluded)	50
Breastfeeding Duration	
<3 months	36 (20.8)
3-6 months	28 (16.2)
6-9 months	39 (22.5)
9-12 months	40 (23.1)
>12 months	30 (17.3)
Don't Know	108
Missing	129
Breastfeeding Exclusivity	
No breastfeeding	98 (37.8)
Partial breastfeeding	75 (29.0)
Full breastfeeding	86 (33.2)
Could not be classified	260

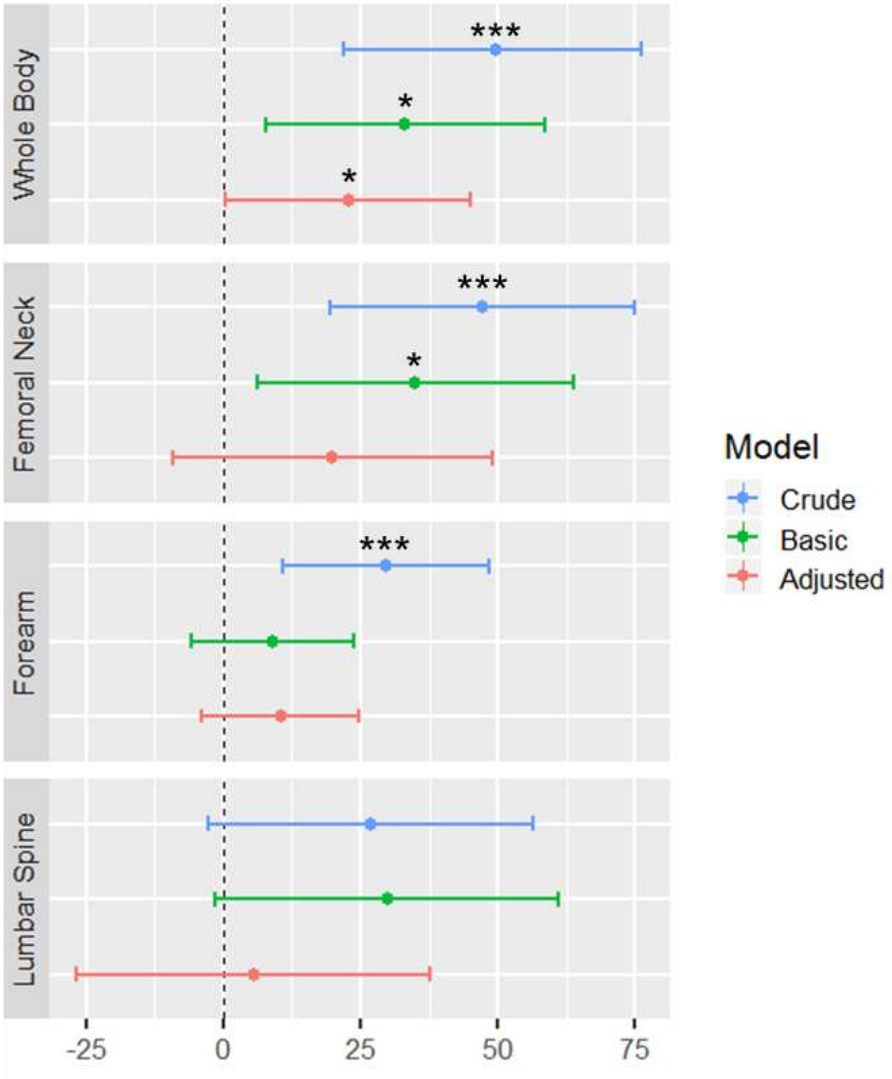

Figure 2. Crude and adjusted association between ever breastfeeding and BMD at skeletal sites. Blue is crude model, no adjustments; green is basic model, adjusted for current age and sex; red is adjusted model, adjusted for current age, sex, height, weight, alcohol consumption, level of physical activity, socioeconomic status (household income), and early life socioeconomic status (father's level of education). Dot indicates β (mg/cm^2), horizontal lines indicate 95% CI, vertical line indicates reference group 'never breastfed'.
***p-value<0.001, **p-value<0.01, *p-value<0.05.

Table 3. Crude and adjusted coefficients of the association between breastfeeding and bone mineral density (BMD)

	Whole Body BMD (mg/cm^2)			Femoral Neck BMD (mg/cm^2)			Forearm BMD (mg/cm^2)			Lumbar Spine BMD (mg/cm^2)		
	n	β (95% CI)	p-value	n	β (95% CI)	p-value	n	β (95% CI)	p-value	n	β (95% CI)	p-value
Breastfed as an Infant												
Never		Ref.			Ref.			Ref.			Ref.	
Ever (Crude)	408	49.5 (22.0, 76.1)	<0.001*	408	47.2 (19.6, 74.8)	<0.001*	406	29.7 (10.9, 48.5)	0.002*	408	26.9 (-2.8, 56.5)	0.08
Ever (Basic Model)[1]	404	33.1 (7.8, 58.4)	0.01*	404	35.0 (6.3, 63.7)	0.02*	402	9.0 (-5.7, 23.7)	0.23	404	29.9 (-1.4, 61.1)	0.06
Ever (Adjusted Model)[2]	375	22.7 (0.3, 45.1)	0.046*	375	19.9 (-9.2, 49.1)	0.18	373	10.5 (-3.9, 24.8)	0.15	375	5.5 (-26.6, 37.6)	0.74
Breastfeeding Duration (Adjusted Model)[2]	158			158			158			158		
<3 months		Ref.			Ref.			Ref.			Ref.	
3-6 months		7.6 (-42.0, 57.1)	0.76		26.6 (-42.8, 95.9)	0.45		12.1 (-20.6, 44.8)	0.46		-3.7 (-75.7, 68.4)	0.92
6-9 months		13.5 (-32.1, 59.0)	0.56		12.3 (-51.5, 76.0)	0.70		9.5 (-20.5, 39.6)	0.53		-9.9 (-76.1, 56.3)	0.77
9-12 months		-36.0 (-83.0, 10.9)	0.13		-32.1 (-97.8, 33.7)	0.34		-0.7 (-31.7, 30.3)	0.97		-37.9 (-106.2, 30.3)	0.27
>12 months		-13.5 (-62.8, 35.9)	0.59		-9.3 (-78.3, 59.7)	0.79		-16.5 (-49.0, 16.0)	0.32		-11.7 (-83.4, 60.0)	0.75
Breastfeeding Exclusivity (Adjusted Model)[2]	234			234			234			234		
No Breastfeeding		Ref.			Ref.			Ref.			Ref.	
Partial Breastfeeding		25.3 (-7.6, 58.1)	0.13		42.8 (-0.9, 86.4)	0.05		20.9 (0.6, 41.3)	0.04*		3.9 (-43.1, 50.8)	0.87
Full Breastfeeding		3.7 (-27.6, 35.0)	0.81		32.7 (-8.9, 74.3)	0.12		5.9 (-13.5, 25.3)	0.55		-16.5 (-61.2, 28.2)	0.47

β, regression coefficient. 95% CI, 95% confidence interval; p-value<0.05 is significant.

[1]Adjusted for current age and sex

[2]Adjusted for current age, sex, height, weight, alcohol consumption, level of physical activity, socioeconomic status (household income); and early life socioeconomic status (father's level of education)

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