Bone health and mineral metabolism in 14- to 18-year-old adolescents with usual low intake of milk products: implications of micronutrient intakes and response to a motivational interviewing dietary intervention trial

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To my son, Bassel,

And my family,

The source of my strength

ABSTRACT

Background: Maximizing peak bone mass during growth is an important strategy to prevent osteoporosis. To date, only 5 trials assessed bone health during adolescence; none were Canadian or included males. This dissertation aimed to: (1) assess the nutritional adequacy of bone-relevant nutrients in adolescents with low habitual intakes of MILK and to evaluate the relationship between intakes and bone density and serum biomarkers; (2) test the effect of increased MILK intake on changes in bone density in adolescents with usual MILK intake of < 2 servings per day; and (3) determine whether pentadecanoic acid (C15:0) and heptadecanoic acid (C17:0) can serve as biomarkers of compliance of milk intake.

Design: Data are from the 1-year end-point from the Family Milk Product 2-Year dose-response study, a 2-year randomized controlled trial that took place in Montreal, QC. Eligibility included healthy adolescents 14-18.9 y who consumed < 2 servings of MILK/d, with a healthy body mass index (BMI), and no vitamin D deficiency or anemia. Adolescents (n=94) were randomized to 3 groups: control, Improved (IInt: consumed 3 MILK servings/d); or Recommended (RInt: consumed ≥ 4 servings/d). Visits occurred every 6 months (mo). Motivational interviewing technique was used to improve MILK intake and to optimize adherence. Bone outcomes were assessed by dual-energy X-ray absorptiometry and by peripheral quantitative computed tomography. Dietary intake was assessed by 24 h food recalls and a validated semi-quantitative, food-frequency questionnaire (FFQ). Physical activity (PA) was assessed using the Youth Physical Activity Questionnaire. C15:0 and C17:0 were measured in erythrocytes and plasma to assess compliance to the MILK intervention at 6 and 12 mo.

Results: (Objective 1) Eighty-one adolescents (55 females and 26 males, 16.5 ± 1.6 y) were included. None of the participants met the Estimated Average Requirements (EAR) for potassium

or vitamin D. In males, less than 30% met the EAR for calcium and 23% for magnesium. In females, 15% met the EAR for calcium and magnesium and 40% for phosphorus. FFQ data revealed significant positive associations between fluid milk and WB BMDZ; total MILK with trabecular density; fluid milk with tibia cortical thickness; and fluid milk and yogurt with bone turnover markers, after adjustment for covariates.

(Objective 2) Ninety-four teens 14-18 y were recruited with a BMI z-score of 0.3 ± 0.9. At baseline, there were no differences in MILK intake among study groups, stratified by sex. At 12 mo, WB BMC and BMDZ and TH BMD and BMDZ significantly increased in the female RInt group ($p<0.03$) and greater percentage increases in WB BMC and WB and TH BMD were observed compared to control and IInt groups ($p<0.05$). Intervention effects on DXA outcomes were not significant in males. Furthermore, females in RInt and control groups increased radial trabecular area and cortical density at 66% radius and 38% tibia ($p<0.02$); males in the RInt group significantly increased tibial cortical density at 38% ($p=0.02$). However, intervention effects on pQCT outcomes, bone biomarkers and body composition were not significant in either sex.

(Objective 3) At 12 mo, erythrocyte C15:0 increased in RInt group (+0.37 µg/ml, $p=0.01$). MILK intake improved significantly in both intervention groups from baseline to 12 mo (IInt: 1.65±1.53 vs 3.0±1.60 servings/d; RInt: 1.3±1.0 vs 3.7±1.0 servings/d, respectively). Further, MILK intake positively correlated with erythrocyte C15:0 and C17:0 at 12 mo.

Conclusion: Increasing MILK intake may improve the adequacy of certain nutrients and thus bone health and metabolism in post-pubertal adolescents, specifically females with low habitual calcium intake. Further, erythrocyte C15:0 can detect short and longer-term MILK intakes during adolescence.

RESUMÉ

Contexte: Maximiser la masse osseuse pendant la croissance est une stratégie primaire pour prévenir l'ostéoporose. À date, seuls 5 études ont évalué la santé osseuse pendant l'adolescence; aucun n'est Canadien ou n'incluait de garçon. La thèse visait à: (1) évaluer l'adéquation des apports en nutriments essentiels chez les adolescents ayant un faible apport habituel de LAIT et d'évaluer la relation entre les apports, la densité osseuse et les biomarqueurs sériques; (2) tester l'effet de l'augmentation de l'apport du LAIT sur les modifications de la densité chez les adolescents consommant habituellement < 2 portions de LAIT/j, et (3) déterminer si le C15: 0 et le C17: 0 peuvent servir de biomarqueurs de la consommation de LAIT.

Méthodologie : Les données proviennent du point final d'un an de l'étude Family Milk Product 2-Year dose response study; un essai contrôlé randomisé qui a eu lieu à Montréal, QC. L'admissibilité comprenait des adolescents en bonne santé âgés de 14 à 18,9 ans et ayant consommé moins de 2 portions de LAIT/j, avec un indice de masse corporelle sain, et sans déficit en vitamine D ni anémie. Les adolescents (n=94) ont été randomisés en 3 groupes: contrôle, Amélioré (IInt: consomme 3 portions de LAIT), ou Recommandé (RInt: ≥4 portions de LAIT). Les visites ont eu lieu à tous les 6 mois. La technique d'entrevue motivationnelle a été utilisée pour améliorer l'apport du LAIT et optimiser l'observance. La densité osseuse a été évalués par l'absorptiométrie à rayons X en double énergie et par tomographie informatisée périphérique quantitative. L'apport alimentaire a été évalué au moyen de rappels d'aliments de 24 h et d'un questionnaire semi-quantitatif validé. C15:0 et C17:0 ont été mesurés dans les érythrocytes et le plasma pour évaluer la conformité à l'intervention à 6 et 12 mois.

Résultats: (*1er objectif*) Quatre-vingt-un adolescents (55 femmes et 26 hommes, 16,5 ± 1,6 ans) ont été inclus. Aucun des participants ne répondait pas aux besoins moyens estimés (BME) pour

potassium ou vitamine D. Moins de 30% des hommes répondaient aux BME de calcium et 23% de magnésium. Chez les femmes, 15% avaient des apports en calcium et en magnésium et 40% en phosphore respectaient l'apport recommandé. Des associations significatives ont été observés entre le LAIT et WB BMDZ; le LAIT et la densité trabéculaire; le lait et l'épaisseur corticale; et le lait et yogurt avec les marqueurs de remodelage osseux, après ajuster pour les covariables.

(*2eme objectif*) À 12 mois, WB BMC et BMDZ ainsi que TH BMD et BMDZ ont augmenté au fil d'étude chez les femmes RInt et des augmentations en pourcentage ont été observées par rapport aux contrôle et IInt ($p < 0,05$). Les résultats du DXA n'ont pas changé chez les males. Les femmes RInt et contrôle augmentaient la surface trabéculaire radiale et la densité corticale à 66% du rayon et à 38% du tibia ($p < 0,02$); Les hommes du groupe RInt ont significativement augmenté la densité corticale tibiale à 38% ($p = 0,02$). Cependant, les effets de l'intervention sur les résultats du pQCT, les biomarqueurs osseux et la composition corporelle n'étaient significatifs ni chez les hommes ni chez les femmes.

(*3eme objectif*) À 12 mois, la concentration de C15:0 dans les érythrocytes augmentait dans le groupe RInt (+0,37 µg/ ml, $p = 0,01$). De base à 12 mois, l'apport du LAIT s'est significativement amélioré dans les groupes d'intervention (IInt: 1,65 ± 1,53 au début vs 3,0 ± 1,60 portions/j à 12 mois; RInt: 1,3 ± 1,0 au départ vs 3,7 ± 1,0 portions/j au 12 mois). En outre, la consommation de LAIT a été positivement corrélée positivement avec les érythrocytes C15:0 et C17:0 à 12 mois.

Conclusion : L'augmentation de l'apport de LAIT peut améliorer l'adéquation de certains nutriments et, partant, la santé et le métabolisme osseuse chez les adolescents, en particulier les femmes consommant peu de calcium. De plus, les érythrocytes en C15: 0 peuvent détecter les prises de lait à court et à long terme pendant l'adolescence.

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Preface and Advancement of Scholarly Knowledge

This dissertation is based on the Family Milk Product 2-Year (FAMILY) dose-response study that recruited healthy male and female youth from greater Montreal, Quebec, Canada in 2014-2018. The study aimed at examining the changes among healthy adolescents with habitually low intakes of milk and alternatives (MILK, < 2 servings/d) following the first year of intervention with MILK on various outcomes: (1) bone health; (2) calcium homeostasis; and (3) milk fat biomarkers. The global objective of this research was to generate high-level evidence that increasing MILK intake will enhance bone health in youth. It is important to note that the term MILK in this thesis solely refers to cow's milk products (produced exclusively from healthy cows and are colostrum-free), including fluid milk (plain or flavored), non-processed cheese, yogurt and recipes prepared with milk products.

The study's originality and contribution to the scientific literature are discussed in detail in the thesis conclusions. The FAMILY Study was comprehensive: the work presented in this dissertation include novel biochemical assessments to test compliance to the intervention; assessment of bone sites not currently reported in the literature on adolescents; the use of motivational interviewing techniques to increase MILK intake and improve adherence rather than the provision of products. The findings of this thesis support results from previous trials and are novel by inclusion of young men.

Research articles to be submitted to peer-reviewed journals

- **Slim M,** Vanstone CA, Agellon S, Morin SN, Rahme E, Weiler, HA. Bone-relevant micronutrients and biomarkers in adolescents with habitually low daily intake of milk and alternatives (Chapter 3)
- **Slim M,** Vanstone CA, Morin SN, Rahme E, Weiler, HA. Effects of milk and alternatives on bone properties in 14- to 18-year-old youth: Results from a 1 year randomized controlled trial (Chapter 4)
- **Slim M,** Ha C, Vanstone CA, Agellon S, Morin SN, Rahme E, Weiler, HA. Evaluation of Plasma and Erythrocyte Fatty Acids C15:0 and C17:0 as Biomarkers of Dairy Fat Consumption in Adolescents (Chapter 5)

Abstracts at professional meetings

- **Slim M,** Vanstone CA, Morin SN, Rahme E, Weiler, HA. Forearm bone density is not related to lean body mass in postmenarcheal girls who habitually consume less than 2 servings of dairy/d. Preliminary results of the FAMILY study. American Society of Bone Mineral Research, Seattle, Washington, 2015
- **Slim M,** Vanstone CA, Morin SN, Rahme E, Weiler, HA. Milk and alternatives intervention and bone mineral acquisition in 14 to 18 y postmenarcheal girls: Preliminary results at 12 mo from a 2-y randomized controlled trial. American Society of Bone Mineral Research, Georgia, Atlanta, 2016
- **Slim M,** Vanstone CA, Morin SN, Rahme E, Weiler, HA. Motivational interviewing techniques improve milk and alternatives intake in healthy Canadian youth: Preliminary results at 6 months from a 2-year randomized controlled trial. International Behavioural Trials Network, Montreal, Quebec, 2016

- **Slim M,** Vanstone CA, Morin SN, Rahme E, Weiler, HA. Milk and alternatives intervention improves body composition in 14 to 18 y Canadian adolescents: Preliminary results at 6 months from a 2-year randomized controlled trial. Canadian Nutrition Society, Gatineau, 2016
- **Slim M,** Vanstone CA, Morin SN, Rahme E, Weiler, HA. Motivational interviewing techniques improve milk and alternatives intake in healthy Canadian youth: Preliminary results at 6 months from a 2-year randomized controlled trial. Canadian Nutrition Society, Gatineau, Quebec, 2016
- **Slim M,** Vanstone CA, Morin SN, Rahme E, Weiler, HA. The association among vitamin D status, bone geometry and muscle structure in 14 to 18 y female adolescents with usual intake of < 2 servings of milk and alternatives per day. Experimental Biology Chicago, Illinois, 2017, Finalist in the American Society of Nutrition Emerging Leader Poster Competition.
- **Slim M,** Vanstone CA, Morin SN, Rahme E, Weiler, HA. Milk and alternatives intervention and bone and muscle mass accretion in 14 to 18 y postmenarcheal girls: Preliminary results at 12 mo from a 2-y randomized controlled trial. International Workshop on Musculoskeletal and Neuronal Interactions, Montreal, Quebec, 2017
- **Slim M,** Ha C, Vanstone CA, Agellon S, Morin SA, Rahme E, Weiler, HA. Erythrocyte heptadecanoic fatty acid is positively associated with whole body bone mineral density in healthy female adolescents. International Society for the Study of Fatty Acids and Lipids, Las Vegas, Nevada, 2018 (Winner of New Investigator's Award).
- **Slim M,** Ha C, Vanstone CA, Morin SN, Rahme E, Weiler, HA. Milk and alternatives intervention improves total hip and whole body bone mineral accretion in 14- to 18-year

postmenarcheal females: Results at 12 months from a 2-year randomized controlled trial. American Society of Bone Mineral Research, Montreal, Quebec, 2018

Oral conference presentations

- **Slim M,** Vanstone CA, Morin SN, Rahme E, Weiler, HA. Milk and alternatives intervention and bone and muscle mass accretion in 14 to 18 y postmenarcheal girls: Preliminary results at 12 months from a 2-year randomized controlled trial. International Workshop on Musculoskeletal and Neuronal Interactions, Montreal, Quebec, 2017

Contribution of Authors

Dr. Hope Weiler is the primary investigator of the FAMILY study and conceptualized it in partnership with Dr. Suzanne Morin, Dr. Elham Rahme and Dr. Simon Bacon. The candidate, May Slim, under the guidance of Dr. Hope Weiler, created the intervention platform. The candidate created the study forms under the guidance of Mrs. Catherine Vanstone; Dr. Weiler approved them for use. The candidate, Dr. Weiler and Mrs. Vanstone presented the study protocol to various school boards in Montreal, Quebec, to seek ethics approval for recruitment in public schools. Mrs. Vanstone and Dr. Weiler maintained ethics approval for use of the study data.

This study was funded by the Dairy Research Cluster Initiative, (Dairy Farmers of Canada, Agriculture and Agri-Food Canada, and the Canadian Dairy Commission), Canada Research Chairs Program and the Canadian Foundation for Innovation.

Author study involvement for Manuscripts 1, 2 and 3:

- The candidate, M Slim, actively participated in participant recruitment. The candidate conducted study visits, performed anthropometric assessments, was trained on dual-energy x-ray absorptiometry and peripheral quantitative computed tomography and blood sample procurement. She conducted baseline motivational interviewing and teachings to participants and conducted all FAMILY study interventions. Additionally, the candidate led in the data entry and coordinated data auditing. Finally, the candidate conducted all of the gas chromatography assessments to assess fatty acid composition, assisted by Mrs. Ha and under the supervision of Mrs. Sherry Agellon.
- Mrs. Vanstone actively participated in recruitment, collected the blood, performed bone density assessments and anthropometry.

- Dr. Morin oversaw laboratory results sent to the Montreal Children's Hospital (Montreal, Quebec).
- Dr. Rahme helped oversee statistical analysis.
- Dr. Weiler aided the candidate with the intervention preparation and assisted in overseeing the measurements and interventions. Dr. H Weiler approved all study forms and analyses.

Manuscript 1: The candidate was the primary author, wrote the first draft of the manuscript, conducted all statistical analyses and edited all subsequent drafts. Mrs. Vanstone, Dr. Morin and Dr. Rahme aided with revisions to the manuscript. Dr. Weiler provided guidance on the analytical approach of the manuscript and aided with data interpretation and statistical analysis.

Manuscript 2: The candidate was the primary author, wrote the first draft of the manuscript, conducted all statistical analyses and edited all subsequent drafts. Mrs. Vanstone, Dr. Morin and Dr. Rahme aided with revisions to the manuscript. Dr. Weiler made contributions to the manuscript, including comments regarding statistical analyses.

Manuscript 3: The candidate was the primary author, wrote the first draft of the manuscript, conducted all statistical analyses and edited all subsequent drafts. Mrs. Vanstone, Dr. Morin and Dr. Rahme provided comments on the draft. Dr. Weiler provided comments concerning statistical analyses and interpretation in addition to approving the final draft.

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List of Abbreviations

µg	Microgram
µSv	MicroSievert
$1,25(OH)_2D$	1,25-dihydroxyvitamin D
25(OH)D	25-hydroxyvitamin D
aBMD	Areal bone mineral density
ANOVA	Analysis of variance
AR	Attrition rate
BAP	Bone specific alkaline phosphatase
BMIZ	Body mass index z-score
BIA	Bioelectrical impedance analysis
BMC	Bone mineral content
BMD	Bone mineral density
BMI	Body mass index
C15:0	Pentadecanoic acid
C17:0	Heptadecanoic acid
CaMOS	Canadian Multicenter Osteoporosis Study
CCHS	Canadian Health Measures Survey
CSA	Cross-sectional area
CT	Computed tomography
CONSORT	Consolidated Standards of Reporting Trials
CTX	C-telopeptide of type 1 collagen

CV	Coefficient of variation
d	Day
DXA	Dual-energy x-ray absorptiometry
EAR	Estimated Average Requirements
FFM	Fat-free mass
FFQ	Food Frequency Questionnaire
FM	Fat mass
FMI	Fat mass index
FU	Follow-up
g	Gram (s)
h	Hour (s)
HTZ	Height-for-age z-score
IInt	Improved intervention
IU	International units
ITA	Individual typological angle
j	Jour
kcal	Kilocalorie
kg	Kilograms
L	Liter (s)
LLS	Lateral lumbar spine
LLS vertebrae 3	LLS3
LM	Lean mass

LMI	Lean mass index
LS	Lumbar spine (1-4)
MF	Milk fat
mg	Milligram
MILK	Milk and alternatives
min	Minute (s)
MInt	Motivational interviewing
ml	Milliliter (s)
mo	Month (s)
NAM	National Academy of Medicine
NHANES	National Health and Nutrition Examination Survey
NIST	National Institute of Standards and Technology
OC	Osteocalcin
PA	Physical activity
PAQ	Physical Activity Questionnaire
PBM	Peak bone mass
POMS	Profile of mood state
POMS-A	Profile of mood state for adolescents
pQCT	Peripheral quantitative computed tomography
PTH	Parathyroid hormone
RCT	Randomized controlled trial
RDA	Recommended dietary allowance

RInt	Recommended intervention
RMP	Reference measurement procedures
SSB	Sugar-sweetened beverages
SD	Standard deviation
TBFM	Total body fat mass
vBMD	Volumetric bone mineral density
WTZ	Weight-for-age z-score
WB	Whole body
WC	Waist circumference
WCZ	Waist circumference z-score
WHO	World Health Organization
wk	Week (s)
y	Year (s)

CHAPTER 1

Background and Thesis Rationale

1.1 Background

Osteoporosis is major global health concern affecting more than 200 million people worldwide [1]. In Canada, osteoporosis touches over 1.4 million individuals 40 years (y) of age and older and costs the Canadian health care system over $4.6 billion in 2010-2011, an increase of 83 % from the 2008 estimate [2]. In addition, there is a substantial loss in quality of life and increased morbidity and mortality risk associated with osteoporotic fractures [2]. As the population's age and size continue to rise, the prevalence of osteoporosis is expected to triple by 2050, suggestive of an urgent need for preventative measures. More specifically, hip fractures are estimated to increase from 1.66 million to 6.26 million worldwide [3]. In Quebec, 12,706 men and women are hospitalized annually for osteoporotic fractures [2].

Osteoporosis is a life course disease developing insidiously over many years before overt symptoms arise. Currently, there is a universal consensus that this disease begins during childhood and adolescence secondary to insufficient development of peak bone mass (PBM) and that maximizing PBM during growth is an important strategy to reduce the risk of osteoporosis and fragility fractures [4].

Bone mass accumulates rapidly through childhood and adolescence until it reaches its maximum strength and density, referred to as PBM. The final stages of skeletal maturity reside in late adolescence or early adulthood. About 95% of adult whole body (WB) bone mass is attained by the end of this life stage period [4], of which 33 to 46% is acquired over the 5 y surrounding peak height velocity (PHV) [5]. A longitudinal study in Canada revealed that PBM of the lumbar spine (LS) is reached between 33-40 y in females, but earlier between 16-19 y for the total hip (TH) and femoral neck (FN). Likewise, in males, PBM is achieved at 19-33 y for LS and TH but at 17-19 y for FN [6]. It can, thus, be concluded that the age of PBM attainment varies with skeletal

site and sex. Given that PBM of the FN is achieved at an earlier age in both males and females compared to other bone sites [6], interventions to improve bone density might be late for FN but possible for LS in both males and females and for TH, particularly in males.

The variability in bone mineral density (BMD) at the time of PBM is similar with aging, supporting the importance of optimal PBM to BMD later in life [7]. For example, findings from a cross-sectional study showed that the range of BMD was no wider in women aged 70 to 90 y than in women 20 to 30 y at both LS and FN [8], the two skeletal sites most prone to osteoporotic fracture. These findings were supported by a 22-y longitudinal study showing that, on average, the annual rate of bone loss was relatively constant within women 20 to 94 y of age and that individual baseline BMD was highly correlated with BMD at 22 y [7]. Moreover, in a mathematical model to assess the influences of PBM on the development of osteoporosis, it was estimated that a 10% increase in PBM would delay the onset of osteoporosis by 13 y [9] and reduce hip fracture risk by 50% in postmenopausal women [10]. On the other hand, a 10% increase in the age at menopause would only delay the onset of osteoporosis by 2 y [9]. This pattern of BMD tracking strengthens the postulation that the amount of bone mass acquired at the end of the growth period appears to be more important than bone loss occurring later in life, hence protecting against subsequent fractures [11], and that PBM could be the single most important factor for the prevention of osteoporosis later in life [9]. Thus, understanding the factors that influence skeletal development in this period is important to further develop and reinforce public health strategies for the optimization of PBM.

Overall, 60% to 80% of the variability in PBM is explained by genetics and the remaining is controlled by environmental factors [7]. Earlier family studies found a greater correlation in LS BMD of monozygotic twin pairs compared to dizygotic twins (r=0.92 vs 0.36, respectively) [12]

and a lower radial and tibial trabecular BMD in prepubertal daughters whose mothers had a history of fragility fractures [13]. Moreover, genome-wide association studies have identified about 71 loci associated with adult BMD and osteoporotic fractures [7]. Environmental factors such as diet and physical activity (PA) are the primary modifiable factors associated with bone health [4, 7]. Nutrients involved in bone mineral accretion include calcium, vitamin D and protein, although other nutrients such as potassium, zinc and vitamins A and K also have important physiological functions. Among these nutrients, calcium is the most dominant mineral in bone, with 99% of total body calcium residing in the skeleton. Calcium requirements vary throughout life with greater needs during the growing years as a consequence of the rapid skeletal development and thus adequate calcium intake is critical to reach optimum PBM and to protect against osteoporosis in adulthood [14]. Vitamin D is another important nutrient for optimal bone mineral accretion given its suppressive effects on parathyroid hormone (PTH) and its role in stimulating active intestinal calcium absorption, especially at low calcium intakes [15]. The National Academy of Medicine (NAM) dietary recommendations for calcium were set at 1300 mg/d during adolescence to ensure that individuals are able to maximize peak adult bone mass [16]. Data from a calcium balance study revealed the accretion rate of calcium was 234.7 mg/d in females and 296.3 mg/d in males during adolescence [17]. A recent review [4] of the benefit of calcium on bone throughout the lifespan showed that most of the dietary calcium intervention studies in children and adolescents had a positive impact on bone mineral accretion. The effect varied between 1 and 6% per y depending on the skeletal sites examined, pubertal maturation, and baseline calcium intake. The benefits of calcium supplementation in bone were more apparent in those with low baseline calcium intake and some of these benefits were lost once calcium supplementation was discontinued, suggesting a need for adequate calcium intake throughout life. In late adolescence,

longitudinal bone growth ceases, but consolidation of mineral continues [16]. Data regarding the impact of calcium status on bone mineral accretion in late adolescence are scarce. One randomized trial of 1000 mg of calcium/d among post-pubertal adolescent girls (16-18 y), with initial calcium intake of 600 mg/d, showed greater increases in WB and LS BMD [18]. This study suggests an extended window of opportunity for bone growth during late adolescence and emphasizes the necessity for calcium RCTs during this age period.

MILK products have unique mineral profiles that support bone growth. In addition to being high in calcium, MILK comprise one of the most bioavailable sources of dietary calcium [19] because it contains no phytic acid or oxalates which can bind calcium and prevent its absorption [20]. Further, it contains nutrients that enhance calcium absorption such as vitamin D [21], lactose, casein phosphopeptides [22] and calcium-binding proteins which enables the diffusion of calcium ions into the intestinal cell [23]. MILK is considered a key source of vitamin D in countries as Canada where vitamin D fortification of milk is mandatory [18]. In addition, phosphorus, magnesium, zinc, and potassium are present in MILK and interact synergistically to promote bone health and growth [21]. The Canadian Community Health Survey (CCHS) 2004 [24] showed that MILK made a significant contribution to the population's nutrient intakes, including nearly 50% of the vitamin D, 37% of the calcium and about 10–25% of the potassium, protein, vitamin A, phosphorus, and zinc intakes. In fact, MILK comprises the key food source of several nutrients of public health concern (calcium, vitamin D and potassium) for Canadian adolescents [25].

There is a substantial evidence-base that adequate MILK intake optimizes bone mineral acquisition which, in turn, may reduce subsequent fracture risk [26]. This seems to be even more impactful during adolescence when bone mineral accretion is at its peak [19]. A 2016 systematic review on lifestyle factors that influence maximal PBM (from childhood through young to late

adolescence) concluded that there is good evidence regarding the role of MILK consumption in promoting the development of PBM. A meta-analysis among children and adolescents using BMC as the outcome of interest and including interventions with calcium (n=19) and milk products (n=2, females only) concluded that increased intake of calcium supplements or milk products, with and without vitamin D, significantly increased WB and LS BMC only in participants with low calcium intakes (450–746 mg/day) at baseline [26]. The results of a cross-sectional study in adolescent and young adult females (12-22 y of age) examining the effect of MILK intake on bone health found that females who consumed less than 55 ml of milk/d had an 8% lower BMC and a 7% lower BMD as well as higher PTH concentrations compared to girls who consumed over 260 ml/d [27]. Low milk intake in children and adolescents was associated with smaller body height, a 3% reduction in BMC and BMD as well as with a 2.7-fold increase in fracture risk in adults [28]. Retrospective studies in American postmenopausal women showed that the mean hip BMC and BMD of women who consumed < 1 serving of milk/wk were 3% lower than those of women who consumed > 1 serving of milk/d during adolescence [29].

Despite recognized health benefits of MILK, the majority of Canadians do not meet the recommendations for this food group. Thus, in addition to compromising the status of micronutrients, including calcium, vitamin D, phosphorus, magnesium, zinc, potassium, vitamin B_{12}, and riboflavin, children and adolescents are less likely to achieve their optimal PBM. By 10 to 16 y, 61% of males and 83% of females do not meet the recommended minimum of three daily servings of MILK [30]. To date, only 12 RCTs have investigated the effect of MILK intake on bone BMD, none of which is recent or Canadian. Of the 12 RCTs, only 5 examined the benefits of increased MILK intake on bone mineral acquisition among adolescents, of which only one trial included post-pubertal adolescents. It is worthy to mention that results of these RCTs were

inconsistent and were conducted in females only. The above-mentioned knowledge gaps form the basis of the research encompassed in this thesis.

Further, one MILK RCT showed that increasing calcium intake by consuming cheese (1000 mg calcium daily) had beneficial effects on accrual of cortical bone mineral mass in healthy 10 to 12-y-old girls with low dietary calcium intakes at inclusion [22]. To the author's knowledge, no other MILK RCT has reported bone geometry outcomes (e.g. trabecular and cortical density, total cross-sectional area, cortical area, and cortical thickness) in adolescents. Additional studies are only in older adults. A cross-sectional study in 564 women with a mean age of 84.7 y found that higher intake of MILK (2.8 ± 0.6 servings/d) was associated with greater appendicular bone mass (5.9%, p=0.005) at 15% tibia and total volumetric BMD (6.2%, p=0.0013) [31]. Another recent study in 70-y-old men and women showed that MILK intake was associated with larger trabecular (2.296 (95% CI, 0.552–4.039) mm^2, per dL/d increase, p = 0.01) and cortical cross-sectional areas in the tibia (1.757 (95% CI, 0.683–2.830 mm^2, p=0.001) [32]. This dissertation will examine the effect of MILK intervention on bone geometry in late adolescents and young adults, an outcome that is scarcely included in MILK interventions aimed at this population.

Some of the mechanisms through which MILK may enhance BMD include suppression of PTH, improved vitamin D status based on 25-hydroxyvitamin D (25(OH)D)) and consequently reduced bone resorption and enhanced bone formation [33]. For example, in healthy young women (23 ± 3 y) performing standard resistance exercise over 12 weeks [34], consuming skim milk compared to an isocaloric carbohydrate beverage after exercise improved 25(OH)D, reduced PTH and increased osteocalcin (a marker of osteoblast activity); whereas C-telopeptide (CTX; a marker of osteoclast activity or collagen breakdown) decreased in both groups over time. Similarly, consuming skim milk powder led to suppression of PTH and bone resorption as indicated by

reduced CTX, but also reduced bone formation markers including osteocalcin and propeptide of Type 1 collagen (P1NP) [35]. Such a comprehensive assessment of calcium homeostasis and bone metabolism will be explored in this dissertation.

MILK naturally contain about 400 different fatty acids [36]. Of these fatty acids, pentadecanoic acid (C15:0) and heptadecanoic acid (C17:0) are of great relevance for their ability to objectively assess nutritional intake. Because these fatty acids contain an odd number of carbon atoms, they cannot be synthesized endogenously in humans; therefore, they can serve as reliable and valid biomarkers of MILK fat intake [37]. There is a growing body of evidence that these 2 fatty acids can be considered as good biomarkers of MILK fat intake. More specifically, C15:0 and, to a lesser extent, C17:0 positively correlated with MILK intake in adipose tissue [38-41], erythrocyte lipids [42], and serum/plasma lipids [43, 44]. A recent cross-over RCT has shown a significant increase in plasma-circulating C15:0 and C17:0 following a 4-week intervention with three servings of milk products ranging from 1% to 34% fat content [45]. To the best of the author's knowledge, evidence in healthy adolescents is limited to a study showing that the serum concentration of C15:0 was negatively correlated with total cholesterol concentration in healthy adolescents [46]. This dissertation will explore whether these saturated fatty acids of exogenous origin, predominantly from dairy foods, can serve as biomarkers for short-and long-term intake of MILK fat.

There is evidence that increasing MILK intake by providing milk products throughout the intervention period of the study can enhance bone health in adolescents [18, 22, 47]. It is also evident that high MILK intakes may not be sustained by adolescents [18], indicating that provision of milk products did not positively influence MILK intake over the long term and that self-selection of diet high in MILK intake is hard to achieve. This suggest that professional,

environmental and social support must be considered in new RCT to enable achievement of success. In order to optimize adherence to allocated intakes of MILK, motivational interviewing (MInt) techniques will be incorporated into the counselling framework of this RCT. From a professional counselling perspective, MInt approach can be useful for strengthening self-intrinsic motivation as well as stimulating and sustaining behavior change [48]. This technique has been successfully employed to change a number of health behaviors such as diet and exercise behavior [49] as well as optimized adherence to obesity intervention in adolescents 11 to 18 y [50]. It is anticipated that this approach will be highly applicable to Canadian youth and health care professionals than just provision of MILK. Indeed, personalized treatment that meets the individual's cultural needs and desires revealed more sustained results compared to standard counselling techniques [51]. Further details of this technique follow in the subsequent chapters.

1.2 Thesis Rationale

About 95% of adult PBM is attained by late adolescence of which 33 to 46% is acquired over the 5-y surrounding peak height velocity. MILK consumption has long been widely recognized as an important strategy for optimizing PBM. Therefore, it is assumed that MILK intake, especially during development of PBM, reduces risk for osteoporosis. To date, only 12 RCTs have investigated the effect of MILK intake on bone parameters. None of these studies is recent or Canadian or has investigated the effects of different MILK servings in relation to changes in bone outcomes. Therefore, a study that examines the effects of increasing MILK intake is warranted and would generate high-level evidence that dietary intervention with MILK will enhance bone mineral acquisition in youth.

1.3 Study Objectives and Hypotheses

This study aimed to (1) explore the micronutrient status of bone-relevant nutrients in young males and females with usual low MILK intake; (2) examine the effects of a 12-mo MILK intervention on bone health; and (3) to determine whether C15;0 and C17:0 can serve as biomarkers for short-and longer-term intake of milk fat. Specific objectives and hypotheses for each of these aims are described as follows:

Aim 1: Micronutrient adequacy, bone biomarkers and milk products*: (*Manuscript 1)

To assess the nutritional adequacy of bone-relevant nutrients and to evaluate the relationship to bone density, bone geometry and serum bone biomarkers in adolescents with usual intake of < 2 MILK servings per day.

Hypothesis: Adolescents who consume low usual MILK intake will have inadequate intakes of bone-relevant micronutrients; MILK intake will associate positively with serum biomarkers of bone formation and resorption and BMD.

Aim 2: Milk products' effect on adolescents' bone health*: (*Manuscript 2)

To test the early effect of improving intakes of MILK using motivational interviewing techniques on LS bone properties as measured by DXA. Secondary objectives of the study were to look at changes in DXA-measured outcomes at WB, TH, FN and distal radius. Finally, this study investigated changes in radial and tibial bone geometry using pQCT, bone biomarkers and body composition following 12-mo of the intervention with MILK.

Hypothesis: By the 12-mo time-point of the randomized trial, participants randomized to consume ≥ 4 servings of MILK will have higher LS BMD compared to the control group, whereas those increasing to reach an average of 3 servings (i.e. improved) will be intermediate; volumetric BMD and the cross-sectional area of

the non-dominant radius and tibia will be greater in the those increasing intakes to reach a minimum of 4 daily servings.

Aim 3: MILK fat biomarkers and bone health in youth*: (*Manuscript 3)

To determine whether a relationship exists between milk fat intake and plasma and erythrocyte concentrations of C15:0 and C17:0 and whether these fatty acids can serve as biomarkers for short-and long-term intake of milk fat following a 12-mo trial.

Hypothesis: Participants randomized to increase MILK intake will have significantly higher concentrations of these saturated fatty acids, particularly C15:0, compared to those randomized to the control group. A positive relationship exists between MILK fat and plasma and erythrocyte C15:0 and C17:0.

CHAPTER 2

Literature Review

2.1 Osteoporosis in Canada

Osteoporosis is a disease characterized by decreased bone mass and deteriorated bone geometry, associated with increased fragility and fracture risk [52]. Data from the 2009 Canadian Community Health Survey (CCHS) estimated that approximately 1.4 million Canadians 45 year of age and older are living with osteoporosis, with women being 4 times more likely to be affected than men [53]. The burden of this disease has risen in the last 10 y costing the Canadian health care system over $4.6 billion in the fiscal year 2010-2011, an increase of 83 % from the 2008 estimate [2]. Osteoporosis has therefore become a public health concern in this country.

2.2 Overview of Bone Physiology and Metabolism

Bone tissue consists of a mineralized inorganic (65% of bone weight) and a non-mineralized organic matrix (25%); the remainder is composed of three principal cells (discussed below in detail). The inorganic matrix provides bone with stiffness. The organic matrix, consisting mainly of collagen fibers, confers elasticity. Bone serves as a structural support, protects vital organs and reservoirs essential minerals, especially calcium and phosphorous. It also facilitates locomotion, plays a role in hematopoiesis and acts as an endocrine tissue [54].

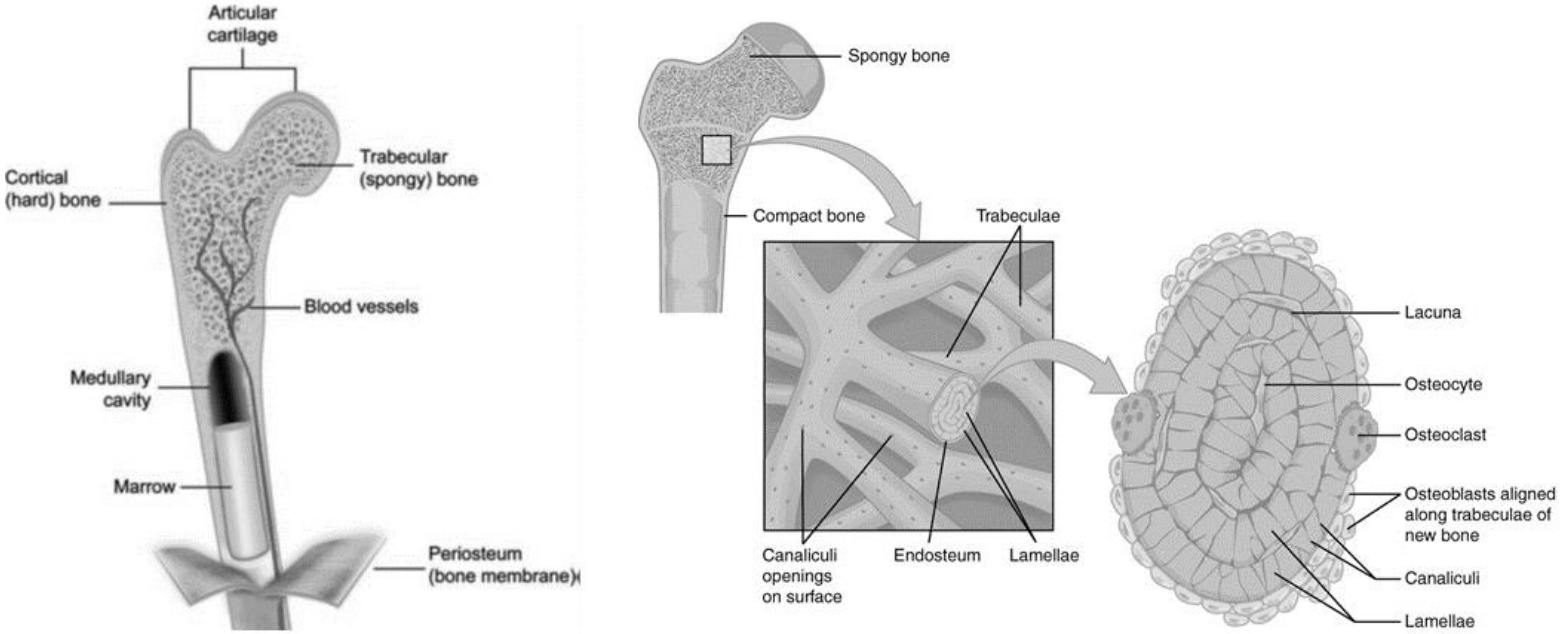
Morphologically, the skeleton consists of two types of bone tissue. Cortical bone contributes approximately 80% of total bone mass and is most abundant in the diaphysis of long bones. Trabecular bone comprises 20% of the skeleton and is primarily found in the metaphysis of long bones, vertebral bodies and flat bones (**Figure 2.1**). Although the composition of the bone matrix is the same in cortical and trabecular bone, it differs in structure and function. The main structural unit of cortical bone is the osteon which consists of longitudinal concentric lamellae of bone matrix. In contrast, trabecular bone is made of interlaced honeycomb-like flat pieces, called trabeculae surrounded by bone marrow (**Figure 2.1**) [54, 55]. Furthermore, 80% to 90% of cortical

bone volume is calcified compared to only 15% to 25% of trabecular bone volume [54]. The remainder of the trabecular bone volume (75% to 85%) is occupied by bone marrow, fat, blood vessels, and connective tissue. These structural differences between the two types of bone are linked to their primary functions: cortical bone has mainly a mechanical and protective function whereas the trabecular bone mainly has a mechanical and metabolic function. Cortical bone is surrounded by the periosteum, a region that plays an active role in bone growth and repair. The endosteum, located on the inner surface of cortical bone, is a thin vascular membrane containing osteogenic cells necessary for bone repair and remodeling [54].

Bone is further composed of three cell types accounting for up to 10 % of total mass (**Figure 2.1**). The osteoblast arises from mesenchymal origin in the bone marrow [56]. Its main function is to produce a non-mineralized bone matrix, called osteoid, that eventually surrounds the cells. Osteoblasts later promote the deposition of calcium salts, converting osteoid to mature bone. Once entrapped in the bone matrix, an osteoblast becomes an osteocyte. During bone formation, 5% to 20% of the osteoblasts become osteocytes [55]. In adults, osteocytes are the most abundant cells accounting for > 90 % of bone matrix cells. Their unique location in the bone matrix allows it to sense local changes in mechanical signals (e.g. stress) and hormonal fluctuations and consequently to transduce messages to cells on the bone surface through gap junctions (mainly connexin 43), influencing bone modeling or remodeling (discussed in greater detail later) [57]. The osteoclast originates from the monocyte-macrophage lineage [55, 56]. The interaction and cross-talk between these various cells influence the rate of bone turnover and the remodeling process. A number of transcription factors, hormones and other metabolites regulate the action of these cells and are discussed in detail later.

Figure 2.1 Bone structure

*Authored by: OpenStax College. Provided by: Open Stax College. Located at: https://cnx.org/contents/FPtK1zmh@9.1:kwbeYj9S@5/Bone-Structure Project: Anatomy & Physiology. License: CC BY: Attribution. License Terms: Download for free at http://cnx.org/contents/14fb4ad7-39a1-4eee-ab6e-3ef2482e3e22@9.1.



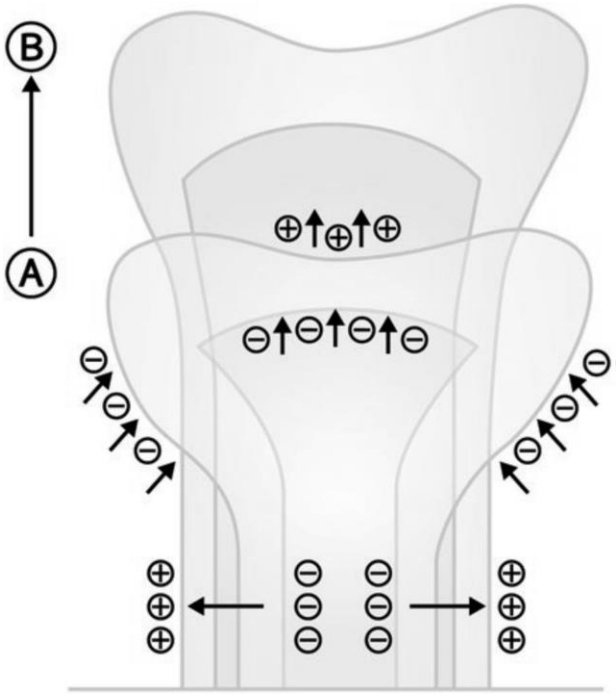
2.2.1 Skeletal Growth and Bone Modeling

Bone modeling is a process during which bone is formed by osteoblasts without prior resorption (formation modeling) or resorbed by osteoclasts on an existing bone surface (resorption modeling). Modeling exists during growth and before peak bone mass (PBM) achievement. Its primary role is to increase bone mass and size and to alter bone shape and position to optimally adapt to mechanical loading [55]. It occurs on the subperiosteal, endocortical, and trabecular bone surfaces, and thus is essential for longitudinal and radial growth and cortical bone drifts [58].

Skeletal growth begins during prenatal life and continues into late adolescence through two different processes, intramembranous and endochondral ossification [58]. During intramembranous ossification, embryonic skeleton develops from mesenchymal cells that differentiate directly into osteoblasts, which eventually form intramembranous bone. The transcription factor Runt-related transcription factor 2 (RUNX2) plays a crucial role in directing mesenchymal stem cells to the osteoblast lineage [55, 58]. During endochondral ossification, mesenchymal cells condensate into chondroblasts by the transcription factor sex-determining region Y-Sox 9 (Sox9). The chondroblasts produce a hyaline cartilage model that serves as a template to eventually be replaced by new bone [50]. This process initiates at the circumference of the diaphysis of long bones (primary ossification center). Two secondary ossification centers eventually form at both ends of the long bone. The primary and secondary ossification centers are separated by the epiphyseal plate which is responsible for longitudinal bone growth. Bone continues to grow in length through endochondral ossification until the cartilage template is fully ossified **(Figure 2.2)** [55, 56, 58]. The ossification of the cartilage plate signals the closure of the growth plate and thus, height growth cessation. This closure is facilitated mainly by estrogen in females and testosterone in males.

Figure 2.2 Skeletal Growth: Example of bone modeling of long bone

*With Dr. Gasser's permission to reprint (see Appendix 1).



2.2.2 Bone Remodeling

Bone remodeling is a process during which osteoblast and osteoclast activity occur sequentially in a coupled manner at the same location [55] **(Table 2.1**). Remodeling occurs at anatomically distinct sites called basic multicellular units (BMUs) consisting of bone-resorbing osteoclasts, bone-forming osteoblasts and mechano-sensing osteocytes [58]. Remodeling persists throughout life and predominates by early adulthood. After the attainment of PBM, bone remodeling is balanced (i.e. the amount of tissue resorbed is equal to the amount formed at each site), and bone mass remains stable until age-related bone loss starts. Studies have shown that the stable period is shorter for the hip compared to other bone sites, where it begins to decrease shortly after PBM is reached [6]. Bone loss is a normal process of aging caused by increased resorptive activity and reduced bone formation. For example, bone loss in postmenopausal women is caused by an imbalance between osteoblast and osteoclast activity resulting from estrogen deficiency [57]. The latter leads to enhanced formation of osteoclasts, which causes increased bone turnover and progressive loss of trabecular bone. Such a negative balance at each BMU causes reduced bone strength and can lead to osteoporosis. The spatial organization of BMU differ in cortical bone compared to trabecular bone. Trabecular BMU conversely are more metabolically active than the cortical units due to the larger bone surface area they occupy [59], resulting in a more accelerated trabecular bone loss relative to cortical bone [60].

Remodeling can occur at the periosteal, endocortical, intracortical or trabecular bone surfaces, and thus is central to bone maturation, maintenance of the mechanical skeletal integrity and mineral homeostasis [58]. The remodeling cycle begins with recruitment of osteoclasts for the degradation of old mineralized bone and ends with osteoblast-mediated bone formation, with bone formation lasting approximately three times as long as bone resorption [55]. This balance between

resorption and formation ensures skeletal integrity while allowing up to 10 % of the adult skeleton to be replaced each year [61]. In growing children and adolescents, bone turnover can be 2 to 10 times greater than in adults because it reflects three physiological processes: bone modeling, remodeling and growth [58]. In normal bone, the whole process of the remodeling cycle in cortical bone is shorter than in trabecular bone occurring over 120–200 d in cortical and trabecular bone, respectively [62]. The changes in bone turnover are reflected by bone markers produced during the remodeling cycle (further details in section 2.2.3).

Table 2.1 Characteristics of Modeling and Remodeling

	Modeling	Remodeling
Goal	Shape bone, increase bone mass	Renew bone
Cells	Osteoclasts or osteoblasts and precursors	Osteoclasts, osteoblasts, and precursors
Bone envelope	Periosteal, endocortical, trabecular	Periosteal, intracortical endocortical, trabecular
Mechanism	Activation-formation or activation-resorption	Activation-resorption-formation
Timing	Primarily childhood but continues throughout life	Throughout life
Net effect on bone mass	Increase	Maintain or slight decrease

2.2.3 Markers of Bone Metabolism

While DXA remains the gold technique for bone mineral mass and density measurements in children and adolescents, the use of bone biomarkers has attracted much attention in the clinical assessment in the past decade. Direct serum measures of bone biomarkers offer sensitive and accurate methods for assessing skeletal metabolism. Moreover, bone biomarkers, as well as other assessments of bone health (e.g., vitamin D metabolites and PTH), have been used to effectively monitor the response and adherence to intervention [63] and have allowed further understanding of the pathogenesis and the clinical management of bone disease [64].

Bone biomarkers are proteins secreted into blood circulation from the modeling and remodeling processes that can be classified as markers of bone formation or markers of bone resorption [52]. Formation markers are mainly derived from osteoblast activity during the formation of bone matrix whereas bone resorption markers reflect the activity of osteoclasts and are products of bone type 1 collagen degradation [52]. Bone markers are commonly elevated during adolescence due to the high skeletal growth velocity and rapid bone modeling [65-67], and its expression is sex-specific starting at puberty [67-70]. During growth and development, biomarkers of bone metabolism illustrate the cumulative effects of bone formation, longitudinal growth, growth in bone size, as well as bone remodeling [66]. After this period, the epiphyseal plate closes and consequently bone modeling slows down while bone consolidation is continued, resulting in a reduced expression of bone biomarkers thereafter. It is important to note that bone biomarkers decrease more slowly and later in males compared to females as the growth spurt occurs later in males [66]. Previous studies demonstrated higher concentrations of circulating bone biomarkers in early- and mid-puberty compared to advanced puberty [68, 69]. In a cross-sectional study among 101 adolescent females between 10 and 20 y, higher concentrations of bone

remodeling variables (bone alkaline phosphatase, BAP; osteocalcin and C-telopeptide; CTX) were observed between 10 and 12 y of age in females [69] and 13 and 15 y of age in males [68], with those between 16 and 18 y having the lowest concentrations in both sexes. A previous study in peri-pubertal girls found that bone formation and resorption markers can predict changes in BMD at LS but not at FN, possibly due to higher biological activity of trabecular bone compared that of cortical bone [70]. Authors of this study concluded that bone biomarkers can be a useful tool for monitoring bone health in maturing children at least in the LS region [70]. Therefore, the relationship between bone biomarkers and bone outcomes depends on the skeletal site measured [64].

To date, different bone biomarkers are used in research to evaluate bone remodeling processes. To the best of the author's knowledge, evidence on the changes in bone markers during adolescence is limited to few MILK trials [71-73]. Also, no trial assessed the impact of increasing MILK on bone biomarkers during post adolescence. The following provides a simplified and brief overview of the bone biomarkers described in this dissertation:

1. Markers of Bone Formation

- *Bone alkaline phosphatase (BAP)*

Bone alkaline phosphatase is a bone-specific glycoprotein found on the osteoblast cell membrane and is involved in the regulation of bone mineralization [52]. BAP can be measured in serum, has a half-life of 1 to 2 days and is not affected by circadian variation [65]. Further, serum BAP is stable when stored frozen at -80 ºC and is not affected by repeated freeze-thaw cycles [65]. During the growing period, serum BAP concentrations increase until mid-puberty whereby females peak before males and decreases in late puberty, reflective of a positive correlation with growth velocity [65, 69]. Reference values for serum BAP are available for

preterm infants [74], healthy Caucasian children, adolescents and young adults aged 0.1 mo to 21 y [75]. In a study examining the effects of 18-mo milk intervention in healthy girls (n=82; mean age 12.2 y), BAP and osteocalcin concentrations peaked approximately 20 mo before menarche; no changes in BAP or osteocalcin concentrations were noted between the milk and the control groups despite significant gains in BMD. These results corroborate the findings from a study by Chan et al. [47]. To the best of this author's knowledge, those have been the only published MILK trials assessing BAP in peri-pubertal adolescents.

- *Osteocalcin*

Osteocalcin is a non-collagenous bone matrix protein and is a sensitive and specific marker of bone formation [52, 76]. Serum osteocalcin concentration is elevated in response to high bone modeling (e.g. rapid skeletal growth) [65]. Unlike BAP, osteocalcin is an unstable molecule, sensitive to freeze-thaw cycles and has a large diurnal variation, peaking in the early morning [65, 66]. Further, it has a short half-life (~5 min) and may decrease up to 70% if left at room temperature for 6 to 24 h [77]. Therefore, careful handling of samples is required: samples should be collected on ice, separated within the hour of collection and frozen immediately at -80 °C. To quantify osteocalcin, an enzyme-linked immunosorbent assay is the most commonly used method of assessment [65]. Circulating osteocalcin has been widely used in clinical investigations as a marker of bone formation being a late marker of osteoblastic activity [52]. Reference values for osteocalcin have been established for healthy Caucasian children aged 0-18 y, peaking between 13.1 and 14 y in both sexes [78]. In a recent systematic review to assess the quality, methodologies, and findings of MILK intervention trials on body size, no body composition, and bone properties in children and adolescents, only one milk trial showed that serum osteocalcin changed following a 18-mo intervention in healthy children [79].

2. Marker of Bone Resorption

- *C-terminal telopeptide of type I collagen*

C-terminal telopeptide of type I collagen is a bone resorption marker specific to bone and dentin. It is released into blood circulation during collagen degradation by osteoclasts [52]. CTX can be measured in urine and in serum by immunoassay; however, serum is the most commonly measured sample type. It is stable even after exposure to numerous freeze-thaws. CTX should be measured in a fasting state because food intake substantially decreases its concentration [52] and its concentration peaks in the morning then nadir concentrations occur by mid-day [80]. As of 2010, the International Osteoporosis Foundation recommends serum CTX as a bone resorption marker for use in observational and intervention studies [81]. Rauchenzauner et al. [82] provided reference curves for serum CTX in healthy children aged 2 mo to 18 y, whereby serum CTX concentrations reflect growth patterns, peaking first in the first 2 y of life then typically a second peak occurs during the pubertal growth spurt (11-15 y). To the author's knowledge, no trial has assessed to date the effect of MILK supplementation on serum CTX concentrations in adolescents.

3. Other Measured Markers Important to Bone Health

- *Vitamin D*

Vitamin D is important for skeletal growth and development in children and adolescents [83]. Vitamin D can be synthesized endogenously through exposure of skin to sunlight, which means that its cutaneous synthesis is limited to April through October for those residing at latitudes ≥40°N [84]. Briefly, solar ultraviolet beta radiation (UV-B) triggers vitamin D synthesis in the skin by converting 7-dehydrocholesterol to cholecalciferol (vitamin D_3), then vitamin D enters circulation bound to vitamin D binding protein (VDBP). Vitamin D_3 is modified by 2 hydroxylation steps to the active form. The first is in the liver, whereby it is converted to 25-

hydroxyvitamin D (25[OH]D). The second is in the kidneys, where 25(OH)D is converted to 1,25-dihydroxyvitamin D ($1,25[OH]_2D$) [85]. Clinically, vitamin D status is best measured by quantifying serum 25(OH)D concentration which reflects both total exogenous intake and endogenous synthesis. Different measurement techniques exist such as immunoassays or chromatographic techniques. Only small amounts of vitamin D are obtained exogenously from diet (e.g., oily fish, eggs and meat, and fortified milk and milk products) [85].

There appears to be a discrepancy between vitamin D requirements among various public health agencies and professional societies. For instance, the Canadian Paediatric Society recommends that children 4-18 y of age consume 400-800 international units (IU/d) [86] whereas the NAM advises an Estimated Average Requirement (EAR) of 400 IU/d, a Recommended Dietary Allowance (RDA) of 600 IU/D and a tolerable upper intake level of 3000 IU/d . The NAM reference ranges were set to meet the needs of the general healthy population in the absence of UV-B exposure. In practical terms, the aim behind those recommendations is to provide enough vitamin D that can facilitate calcium absorption from the dietary sources and minimize PTH synthesis without causing any adverse health effects [87]. Vitamin D deficiency, characterized by a low plasma 25(OH)D concentration, is associated with decreased calcium absorption from the intestine and a tendency towards hypocalcemia.

2.3 Peak Bone Mass

From birth to late adolescence, skeletal mass accumulates steadily from approximately 70-95 g at birth to 2400-3300 g in young adult women and men, respectively [88]. The rate of skeletal growth is not uniform and is in parallel with height velocity [8]. Differences in the timing of skeletal mass accumulation are sex- and site-dependent. PBM is defined as the maximum amount of bone and strength acquired when accrual ceases or plateaus before bone loss occurs. Moreover,

accumulation of skeletal mass during the growing period varies depending on the skeletal segment considered. For example, before puberty much of the skeletal gain is of the appendicular skeleton while axial growth predominates after puberty [22]. Quantitative computed tomography (CT) showed that PBM follows different patterns in cortical and trabecular bone compartments. Trabecular losses in the peripheral skeleton occurs among young adults in their early 20s, but cortical losses occur later, well after the age of 40 y [8]. The role of cortical and trabecular bone in optimizing PBM has not been well established in either childhood or adulthood [4].

Although the timing of PBM attainment is still controversial, PBM is generally believed to be reached in the late teenage years or young adult years [5, 28, 89], with ~ 50% of the skeletal mineral content acquired during the peripubertal growth period [5]. Earlier cross-sectional studies concluded that PBM at most of the skeletal regions is attained by late adolescence or by the average age of 18 y [90]; however, other cross-sectional studies revealed that bone mass peaks in the thirties or even in the forties depending on the site of bone measurement [91]. In contrast, data from the Fels Longitudinal Study of 186 healthy Caucasians 6 to 36 y of age showed that in females, PBM occurs at 20 y whereas at 25 y in males in total body, arms, pelvis and spine regions. In a more recent Canadian longitudinal study [6], data revealed that in females, PBM of the lumbar spine (LS) is reached between ages 33 and 40 y, but earlier between ages 16 and 19 y for the total hip (TH) and femoral neck (FN); similarly in males PBM is achieved at ages 19 to 33 y for LS and TH, but at 17-19 y for FN [6]. Most of the skeletal mass (90-95%) is attained by the age of 16 y [6] or 18 y [90] in both women and men, particularly in regions of the LS and FN. Data from an early longitudinal study in young adult women (mean age 21.4 ± 1.7 y) noted substantial gains in bone mass and density in the third decade of life (e.g., 6.8% increase in LS areal BMD (aBMD) and 1.2% increase in WB aBMD) [92]. Overall, although the age at which PBM is reached is still

debatable, it is undeniable that the greatest gains in bone mass occur during the peri-pubertal growth period and is bone site-, bone compartment- and sex-specific.PBM is achieved through bone growth, modeling and remodeling processes. Several interrelated factors affect maximal bone mass accretion. These include unmodifiable factors such as genetics, ethnicity, hormones and sex and modifiable factors pertaining to PA and nutrition [8]. Therefore, understanding the factors that affect bone mass development during the growing years is essential in improving bone health.

2.3.1 Genetics

Overall, 60% to 80% of the variation in PBM is explained by genetics [8]. Genome-wide association studies have identified 71 loci associated with adult BMD and osteoporotic fractures [8]. However, it is important to keep in mind that genetic and environmental factors are not completely independent. The genetic factors, *per se*, affects the efficiency of absorption and utilization of bone-building nutrients [93]. Moreover, there is increasing evidence that some nutrients might interact with genetic polymorphisms to regulate BMD [7, 8]. All this to say that manipulation of an environmental factor such as diet can modulate genetic phenotype.

2.3.2 Endocrine Hormones

Several endocrine hormones modulate skeletal growth depending on the stage of puberty. During puberty, skeletal growth is driven by growth hormone (GH), which is stimulated by the increased release of sex hormones as testosterone and estrogen [7]. GH, directly or via its stimulation of insulin-like growth factor I (IGF-1) secretion, induces osteoblastogenesis, and osteolclastogenesis, thereby enhancing bone modeling [7]. IGF-I, in turn, induces chondrocyte proliferation and endochondral ossification, thereby promoting linear bone growth [94]. Moreover, IGF-I indirectly favors bone mineralization by stimulating intestinal calcium absorption, increasing renal phosphate excretion and $1,25(OH)_2D$ production [94]. In a trial in short non-GH-

deficient children, GH supplementation resulted in dose-dependent increments in IGF-1, leading to an increase in adult height [95]. During adolescence, sex steroids (estrogens and androgens) play a major role in bone development and the attainment of PBM [7], as well in the sexual differences in bone growth and development [96]. Estrogen is responsible for the normal closure of the epiphyseal plate in both sexes, whereas testosterone has profound effects on longitudinal bone growth. Any deficiency in aromatase, an enzyme responsible for the conversion of testosterone to estrogen, results in tall stature in men [7, 96]. A negative relation was observed between the timing of puberty and peak BMD in male and female children, most likely due to a low presence of sex steroids during growth. Moreover, administration of testosterone before closure of the growth plate resulted in greater bone calcium accretion in prepubertal boys [96]. Sex steroids play an influential role not only on BMD, but also on bone quality. In a study among pubertal girls, it was shown that estrogen, but not testosterone, positively associated with tibial cortical thickness and total volumetric BMD (vBMD). This was explained by the role of estrogen in suppressing bone turnover at the endocortical surface during the early pubertal period [97]. Other studies showed that both estrogen and testosterone influence tibial cross-sectional area [97].

2.3.3 Sex

Between the age of 8 and 18 y, a BMC increase of 146 g per y was recorded at multiple skeletal sites, equivalent to 58 g/y (or 160 mg/d) of total body calcium reaching approximately 900 g by late adolescence or early adulthood [90]. Despite this dramatic increase in bone mass during the growing life stage, no significant sex difference has been observed [98]. Sexual variability in bone mass only emerges during puberty, mainly attributed to the delayed puberty onset and the prolonged pubertal period in males which results in bones with larger size and thicker cortex [91, 98]. Cortical thickness in males increases by increased bone deposition at the periosteal surface

whereas in females more bone is deposited at the endosteal inner surface. During puberty, aBMD increases significantly, mainly explained by increases in bone size given the two-dimensional nature of aBMD. On the other hand, vBMD remains constant in males and females until late puberty and then increases in both sexes to a similar degree, attributed mainly to an increase in trabecular thickness which continues for a while after cessation of growth of the vertebral bodies [91, 98, 99]. In different terms, a longer period of bone maturation results in longer and wider rather than denser bone. In a study among 82 twins of opposite sex aged between 18 and 80 y, Nagathan et al. found significant sex differences in BMC and aBMD of LS, FN and distal radius, however only small differences in vBMD occured between the sexes [100]. Another study among healthy individuals aged 5 to 35 y showed lower cortical BMC and periosteal circumference in the 38 % site of the tibia for females compared with males, however, cortical vBMD was greater and endosteal circumferencewas lower [4]. About 33% to 46% of the adult BMC is accrued in the circumpubertal period (-2 to +2 y from PHV which is usually achieved ~ 2 y earlier in females compared to males (11.8 y vs. 13.4 y respectively) [89]. It is also important to note that a 6- to 12-mo lag period exists between PHV and peak mineralization, resulting in comparatively a low aBMD and increased incidence of fractures given the desynchronization between the bone mineral accrual and the increase in bone size.

2.3.4 Physical Activity

The effect of PA on bone health dates to the 17th century when Galileo first proposed a relationship between bone size and the mechanical loads, also called strains, applied to it. There is an extensive body of knowledge that PA increases bone mineral accretion and/or improves bone strength during growth [8]. In a longitudinal study of pre-pubertal children, Baxter et al. found that high impact activities such as jumping resulted in 3.6% and 1.4% gain in hip BMC 7 mo and 8 y

respectively compared to controls who were exposed to stretching activities [101]. Similarly, 9 mo of school-based exercise resulted in higher WB (6.2%), FN (8.1%) and TH (7.7%) BMC compared with their non-exercising counterparts. Three years after the trial completion, the benefits persisted, with a sustained 7–8% gains in BMC of the FN and TH [102].

Not all types of physical activity benefit bone equally. The frequency, intensity, magnitude and duration of the load are all factors that influence optimal bone adaptations [8]. For example, high impact activities such as jumping appear to hold more osteogenic properties than lower-impact activities such as walking [103]. Robinson et al. showed that LS and FN BMD were significantly greater in gymnasts compared to distance runners as estimated by dual-energy x-ray absorptiometry (DXA). Furthermore, PA-induced skeletal benefits are greater and more consistent before and at puberty than in adulthood [5, 103]. A study of male tennis athletes 10-19 y of age revealed a 17% greater BMC in the playing arm than in the nonplaying arm of the prepubertal players, coupled with a 12–21% greater cortical area in the loaded arm and attributed that to increased periosteal deposition and less endosteal expansion. Peri-pubertal athletes demonstrated a 27% greater BMC and 20 to 33% thicker cortex, whereas post-pubertal players had 18% greater BMC and 18 to 22.5% thicker cortical are in the playing arm than in the nonplaying arm [104].

The exact mechanisms by which PA increases bone mass are not completely understood. Animal studies suggest that the PA strains are sensed by osteocytes which respond by stimulating the Wnt-β-catenin canonical signaling pathway on the osteoblast activity, and thus bone formation. PA also stimulates the synthesis of GH and IGF-1, which exert anabolic effects on muscle and bone tissue [102]. A recent study found that PA increased the circulating level of PTH which, in turn, downregulated the expression of sclerostin in osteocytes and upregulated fibroblast growth

factor-23 (FGF-23). Altogether these findings support the anabolic role of Wnt-β-catenin canonical signaling in bone adaptation response to PA [102].

An examination of the literature points out a potentiating interaction between PA and dietary calcium on bone mass, particularly during the growing period [105] which is rational as PA and dietary calcium are both necessary to optimize bone gain; the skeleton needs calcium as a major structural component and mechanical loading as a stimulus for mineral deposition. In a meta-analysis of PA trials in adults (mean age range of 30-65 y), a minimum of 1 g of daily calcium intake was needed for LS BMD to respond optimally to PA, with no difference among exercise groups at the lower range of calcium intake (< l000 mg) [106]. Participants performed wide ranges of exercises including resistance training and low- to high-impact exercises. Similarly, a meta-analysis in children and adolescents, concluded that weight-bearing exercise, coupled with a high calcium intake (1300 mg/d), improves BMC (effect size, 0.17; 95% CI, 0.02–0.32) [107]. The 2016 National Osteoporosis Foundation position statement reinforced that the two lifestyle factors-calcium intake and PA– demonstrated consistently strong evidence (Grade A), with both positively associated with improved bone mass and BMD [4].

2.3.5 Nutrition

Nutrition plays a critical role in the growth, maintenance and repair of the skeleton. Nutrients involved in bone mineral deposition and accrual include, but not limited to, calcium, magnesium, phosphorus, potassium and vitamins D and K, in addition to macronutrients, mainly protein and fat [4]. Although the impact of calcium in promoting bone health has been commonly researched, Canadians, particularly adolescents, consume it in quantities below age-specific recommendations [87]. Therefore, more attention will be devoted to this nutrient in this review.

2.3.5.1 Protein and Bone

Dietary protein plays a key role in the development and the maintenance of bone health and thereby serves to protect against osteoporosis. About 30% of bone weight and 50% of its volume is comprised of protein [108]. Several studies support a positive association between protein intake and bone health over the life span [109, 110]. These studies found that individuals with the highest BMD are those who reported the highest protein intake [111]. One mechanism by which protein can promote bone health is explained by its role in increasing circulating levels of IGF-1, a key mediator of linear bone growth [76]. Protein intake also stimulates acid release in the stomach, and consequently, enhances calcium absorption [87]. Recently, an animal study demonstrated that a low protein intake (< 10% of energy intake) suppressed bone mineral acquisition and bone strength in growing male rats (5 weeks of age) [112]. On the other hand, it was reported that protein can be harmful to bone health depending on the amount of protein in the diet, the amount of calcium in the diet and the protein source [108]. However, the potential detrimental effect of protein on urinary calcium excretion is a concern only when the diet is very high in protein intake (i.e. > 2.0 g/kg body weight/d) coupled with low calcium intake (< 600 mg/d) [108]. Furthermore, many protein-rich foods such as meat and MILK are also high in phosphorus and potassium, both of which decrease urinary calcium losses [108]. Overall, it is generally agreed that diets containing moderate protein (i.e. 1.0 to 1.5 g/kg body weight/day) promote normal calcium metabolism and do not adversely alter bone metabolism [108]. National data showed that more than 95% of all adolescents consumed protein within the recommended ranges [113], which may protect them from calciuria. It is noteworthy that increased urinary excretion of calcium does not necessarily lead to negative calcium balance or reduced bone mass [111].

2.3.5.2 Phosphorus and Bone

Phosphorus is an essential component of the skeleton. Approximately 85% of total body phosphorus is present in bone. Phosphorus is, therefore, necessary for maintaining skeletal integrity [93, 114]. Low intakes of phosphorus result in rickets and stunted growth in children [74]. However, most people do not have difficulty meeting intake requirements because phosphorus is present in a large array of foods (e.g. meat, poultry, fish, eggs, dairy products, nuts, legumes, cereals, grains, cola beverages …etc.) and is readily absorbed (65 to 90 % in children and 55-70% in adults) in the small intestine [74]. In contrast, excessive intake of phosphorus is problematic for bone and mineral metabolism [114]. In a recent review involving both animal and human studies, it was concluded that negative effects of high dietary phosphorus on bone health have been observed, mediated through elevated PTH and increased osteopontin, a bone resorptive protein [114]. Data from the NHANES survey 2003-2006 showed that MILK products accounting for 29% of the total phosphorus intake in children and adolescents 3-17 y [115].

2.3.5.3 Magnesium and Bone

Magnesium is another mineral that constitutes a part of the bone hydroxyapatite crystal [93], of which one third is in cortical bone [116]. About 60% of the total body magnesium is stored in bone [116]. At term birth, the human body contains 1 g of total body magnesium that reaches up to 23-27 g by adulthood, with most of the magnesium gained during adolescence [93]. Magnesium plays a key role in bone and calcium metabolism, through calcium-sensing receptors which require magnesium for its action. The mechanisms explaining the effects of magnesium deficiency on bone density in humans include: (1) alteration of hydroxyapatite crystals and subsequent bone stiffness; (2) reduction of PTH concentrations, along with a decrease in the serum concentration of $1,25(OH)_2D$; (3) and enhancement of pro-inflammatory cytokine secretion which

stimulates bone remodeling and osteopenia [116]. In spite of its beneficial role, hypermagnesemia can be harmful for bone. Indeed, it can inhibit the formation of hydroxyapatite crystals by competing with calcium ions [116]. Thus, because calcium and magnesium are both essential for maintaining bone integrity, adequate intakes at appropriate ratios of these minerals must be met. Despite the wide distribution of magnesium among plant and animal foods, data from CCHS 2004 found that 41.5% male adolescents and 66.3% female adolescents had magnesium intakes below the EAR for their age [113]. MILK alone contribute about 20% of dietary magnesium among adolescents [93]. In a modeling study to assess the impact of meeting daily recommended amounts of MILK on population-based nutrient intakes, it was shown that MILK intake increased magnesium consumption by 8–13 % in those 9 y of age and older [117]. In line with these findings, an earlier study using data from the U.S. National Health and Nutrition Examination Survey (NHANES, 1999-2004) showed that children and adolescent 9-18 y of age would need to consume 3 MILK servings/d in order to meet 100% of EAR for magnesium [118].

2.3.5.4 Vitamin D and Bone

Vitamin D is either consumed in the diet through vitamin D-enriched food or synthesized in the skin from 7-dehydrocholesterol by UV-B exposure (**Figure 2.3**) [87]. Calcitriol, the active form of vitamin D, is involved in calcium homeostasis and is essential for normal bone mineralization. In addition, calcitriol can directly affect bone health through vitamin D receptors found in osteoblasts and osteoclasts, by stimulating matrix formation and bone maturation and enhancing osteoclastic activity. Together with PTH and PTH receptor as well as serum ionized calcium and the calcium-sensing receptor, calcitriol tightly regulates calcium metabolism via three main mechanisms: intestinal absorption, renal reabsorption, and bone resorption. As the major stimulus of active calcium absorption, it induces calcium influx via the apical channel known as

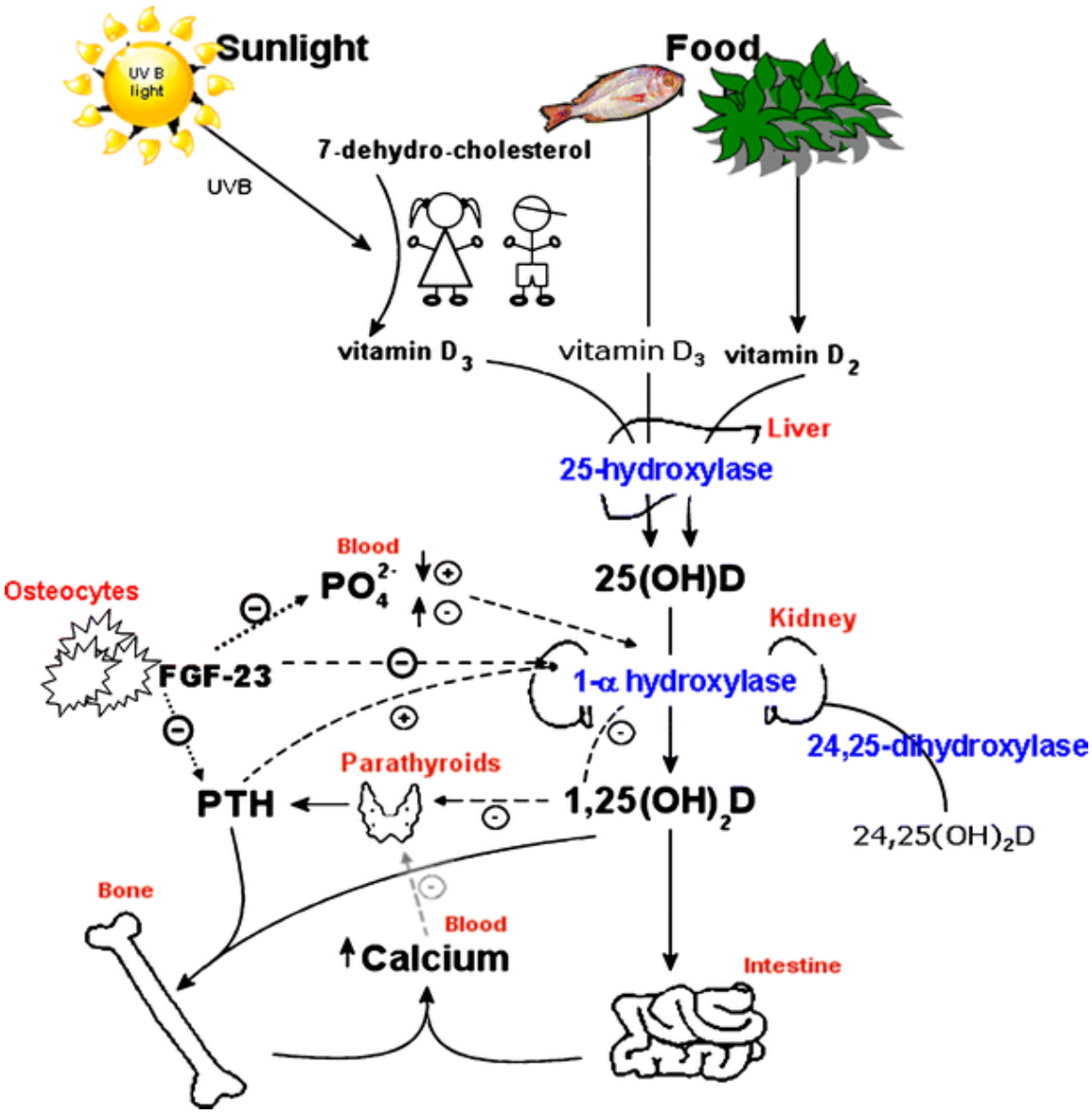
transient receptor potential vanilloid type 6. This latter then induces the expression of calbindin-D9k which, in turn, facilitates calcium translocation through the enterocyte and extrude it from the enterocyte against electrochemical gradient to avoid cell toxicity [15, 119].

The high rate of bone mineral accrual during the period of growth and development requires a greater intake of calcium [120, 121]. To cope with the increased calcium needs, calcitriol stimulates active intestinal absorption of calcium by inducing the synthesis of a calcium-binding protein in the proximal small intestine [87, 122]. In general, the amount of calcium absorbed through active transport is inversely proportional to calcium intakes and the fraction of calcium absorbed rises adaptively as intake drops [15, 93]. This adaptive mechanism occurs within 1 to 2 wk subsequent to a decline in serum calcium concentration and a rise in serum PTH and calcitriol concentrations [87]. A review suggests that calcium absorption is increased to 30–40% of intake with adequate vitamin D status (i.e. 25(OH)D concentration of $\geq$ 50 nmol/L as per NAM) compared with a 10–15% absorption without adequate vitamin D [85]. The importance of vitamin D supplementation on bone mineral accrual has been recognized in children and adolescents [4]. In a trial of 179 females between 10 and 17 y of age, weekly doses of 14,000 IU compared to 1400 IU vitamin D_3 over 1 y resulted in greater total hip BMC and bone area, as well as trochanter BMC in premenarcheal, but not postmenarcheal females [123]. Moreover, 1-y supplementation with either 200 or 400 IU vitamin D_3 among Finnish females (mean age, 11.4 ± 0.4 y) resulted in greater increases in LS and femur BMC after adjusting for bone area, weight gain, and changes in maturation compared to a placebo group [4]. Due to the important role that vitamin D plays in bone growth and maintenance, consuming adequate vitamin D is essential [4]. This is of particular importance if living in countries at $\geq 40^{\circ}$ latitude, such as Canada, where cutaneous vitamin D cannot be synthesized year-round, and thus must be obtained from dietary or supplemental sources

[84]. In Canada, around 75% to 93% of adolescents (14 to 18 y of age) fail to achieve the EAR for vitamin D, coupled with up to 70% calcium intake inadequacy, thereby adversely affecting bone mineral accretion [113]. Despite the 2010 revisions for vitamin D intake, recent data from the Canadian Health Measure Survey (CHMS) found that serum 25(OH)D status continues to decline among Canadian children and adolescents 6-18 y (+ 2.2% deficiency from CHMS Cycle 1 to Cycle 3) [124]. It was estimated that consuming milk more than once a day had an average increase of 12 nmol/L, compared with those doing so less than once a day. To alleviate the concerns and consequences of vitamin D inadequacy, Health Canada has recently proposed increasing the mandatory vitamin D fortification of fluid milks from 1 µg/ /100 ml (actual fortification policy) to 2 µg/100 mL [125].

Figure 2.3 Overview of vitamin D synthesis and metabolism

by courtesy of Dr. Shroff to reprint (see appendix 2)



2.3.5.5 Calcium and Bone

Calcium is the fifth most abundant mineral in the human body [15]. Over 99% of total body calcium is in the skeleton primarily in the form of hydroxyapatite, providing rigidity and structural integrity of the skeleton [15]. Calcium in the skeleton acts as a reservoir that is tapped repeatedly through bone remodeling to maintain stable extracellular calcium concentrations, thereby masking calcium deficiency until skeletal fragility or fractures occur [87]. The remaining calcium (< 1%) is located in body fluids and soft tissues either bound to protein (~40% of the calcium in the fluids), or complexed with other anions (~10%) or as a free ionized cation (~50%) [119]. Although quantitatively small, this pool of ionized calcium plays an essential role in many health conditions as cardiovascular diseases, blood pressure, weight management, skeletal and smooth muscle contraction as well as nerve and neuromuscular transmission [87]. Calcium is only available through dietary sources which makes it an essential nutrient [15]. Blood calcium concentration is tightly maintained with a normal total calcium of 2.2-2.6 mmol/L and an ionized calcium of 1.1-1.35 mmol/L [15].

A transient drop in ionized calcium concentration is normalized by serum proteins, mainly albumin, that dislodges calcium from its calcium binding sites thereby acting as a calcium buffer [126]. On the other hand, long-term regulation of ionized calcium concentration is achieved through an intricate hormonal interplay between PTH, $1,25(OH)_2D$ and FGF-23 with specific target tissues (kidney, bone and intestine) [15]. A fall in ionized calcium concentration is immediately sensed by calcium-sensing receptor located on the surface of parathyroid cells which responds by transducing an intracellular signal that triggers the synthesis and secretion of PTH from the chief cells of the parathyroid gland [15]. PTH acts to normalize calcium concentration through three different biological mechanisms: (a) stimulation of osteoclastic bone resorption and

release of calcium and phosphate from bone, (b) stimulation of calcium reabsorption and inhibition of phosphate reabsorption from the renal tubules, and (c) stimulation of renal production of $1,25(OH)_2D$, which increases intestinal absorption of calcium and phosphate. More specifically, PTH binds to its receptors on osteoblasts upregulating the expression of receptor activator of nuclear factor kappa-B ligand (RANKL) and inhibiting the secretion of osteoprotegerin (OPG). Low OPG and increased RANKL act synergistically to promote osteoclastogenesis and hence the release of calcium and phosphorus from the bone matrix into circulation [127]. PTH also stimulates calcium reabsorption in the distal tubule and collecting duct and decreases the reabsorption of phosphate in the proximal tubule. Further, PTH increases the expression of 1α-hydroxylase in proximal renal tubules which converts 25(OH)D to its active metabolite, $1,25(OH)_2D$ [15, 128]. Once transported into the enterocyte, $1,25(OH)_2D$ then binds to its vitamin D receptor, enhancing active calcium and phosphate uptake [15]. Similar to PTH, $1,25(OH)_2D$ acts directly on the skeleton, specifically on osteoblasts, modulating calcium resorption by increasing RANKL and decreasing OPG expression [127]. Calcium-sensing receptors found on the basolateral membrane of the thick ascending limb of the loop of Henle also senses the change in ionized calcium ions and inhibits calcium reabsorption independent of PTH and $1,25(OH)_2D$ [127].

FGF23 is a newer concept in calcium homeostasis [129]. It is secreted by osteocytes and signals through an FGF receptor/Klotho co-receptor complex in tissues, including proximal renal tubules in the kidney and chief cells in the parathyroid gland. It contributes to calcium homeostasis by regulating phosphate homeostasis and vitamin D metabolism. FGF-23 production is increased in the presence of high phosphate concentrations inhibiting renal phosphate reabsorption. Excess FGF-23 also decreases $1,25(OH)_2D$ concentrations via downregulation of renal 1-α hydroxylase activity and thereby reduce both calcium and phosphorus absorption [129].

2.3.5.5.1 Paracellular and transcellular calcium absorption

Calcium is absorbed in the intestine by two independent pathways, active transcellular (i.e., the ion moves through the cell) and passive paracellular (i.e. the ion is transported between two adjacent epithelial cells in the lumen) pathways [127, 130]. It is the amount of calcium ingested that determines the transport route. When calcium intake is low, the active transcellular route is activated permitting the uptake of calcium ions into the enterocytes against an electrochemical gradient. In contrast, passive diffusion dominates at high calcium intakes and occurs down an electrochemical gradient i.e. it requires a high luminal calcium concentration to occur [131]. Paracellular absorption occurs passively throughout the small intestine, predominantly in the jejunum and ileum [122]. It is facilitated by tight junctions named claudins and situated between adjacent epithelial cells, thereby allowing Ca^{2+} flux between intestinal cells. Although still debatable, recent murine studies show consistent evidence that calcitriol, traditionally known as the main stimulator of active calcium transport, can increase paracellular calcium flux through upregulation of claudins expression [131].

Conversely, transcellular absorption is prevalent in the proximal small intestine (i.e. duodenum and jejunum) where the VDR is expressed in the highest concentration and is under nutritional and physiological regulation. Habitual low calcium intake is a major stimulus of this pathway resulting in an increased efficiency of intestinal calcium absorption, a process mediated by increased renal production of $1,25(OH)_2D$ [20, 122, 128]. Another determining factor is the physiological state (e.g. growth period). Transcellular active transport requires 3 sequential steps: entry, intracellular diffusion and extrusion into the blood circulation [20, 122]. The entry of luminal calcium across the brush border into the enterocyte is thought to be mediated by a calcium channel or transporter known as TRPV6 [122]. The intracellular diffusion of calcium ions through

the cytosol is strongly mediated by a calcium-binding protein named calbindin D_{9K} whose biosynthesis *per se* is controlled or dependent on $1,25(OH)_2D$ [15]. Calbindin D_{9K} transports calcium ions from the apical membrane to the basolateral edge of duodenal cells [132]. The translocation step of calcium transport with the aid of vitamin D-dependent calbindin D_{9K} is believed to be the rate-limiting step in a sense that calcium ions entering the vitamin D-depleted enterocytes were shown to stay localized at the brush border incapable of crossing the cytosol [122]. The active extrusion of calcium from enterocytes into the lamina propria and later into the blood circulation takes place against an electrochemical gradient and is mainly controlled by calcium-ATPase located in the basolateral membrane of the intestinal cell [122]. During adolescence, calcium needs peak and are higher as compared to childhood and young adulthood [18]. During this period of rapid growth, a greater proportion of dietary calcium is absorbed, associated with reduced urinary excretion to conserve the amount available for bone mineralization [122].

2.3.5.5.2 Calcium requirement in adolescents

Calcium is required for normal skeletal growth and maturation [5, 17, 133]. During childhood and adolescence growth i.e. up to the age of 20 to 30 y, the rate of bone formation exceeds that of resorption, resulting in the rapid accretion of bone mass. Following this period, bone density remains relatively stable with small variations until the age of 50 y. With aging there is a bone-remodeling imbalance, with the rate of resorption exceeding that of formation, and bone loss begins to occur. [120]. Calcium is a threshold nutrient, meaning that PBM can be attained only with an intake of calcium at or above this threshold where calcium retention is maximal [93]. A metabolic balance study reported that the calcium threshold for children aged 2- to 8-y is 1390 mg/d, 1480 mg/d for adolescents aged 9- to 17-y and 957 mg/d for young adults 18- to 30-y [134].

Based on this information it is clear that dietary calcium requirements peak (1300 mg/d) in the pre and post-pubertal period subsequent to the rapid longitudinal growth and periosteal expansion with significant reductions thereafter as the bone modeling process slows. [122]. The opportunity to support PBM during this stage could be compromised if dietary intakes of calcium are below these thresholds [93]. Moreover, the University of Saskatchewan's Paediatric Bone Mineral Accrual Study, a 6-y longitudinal study of 228 Canadian males and females between the age of 9-18 y, found a peak calcium accretion of 296.3 mg/d in males at age 14.0 y and 234.7 mg/d in females at age 12 y [17].

In response to growing public concern and uncertainty, the NAM set a committee to assess the current data on health outcomes associated with calcium and vitamin D and update the existing Dietary Reference Intake values (DRIs) [87]. The committee in charge evaluated more than 1000 studies of skeletal and non-skeletal nature and found that available scientific evidence from skeletal studies was consistent with a causality relationship between calcium and vitamin D and skeletal health and provided a strong basis to establish an EAR covering requirements of 50% of the healthy population at a given life stage and a RDA covering requirements of ≥ 97.5% of the population for all age groups except infants less than 12 mo. The RDA for calcium was set at 700 mg/d calcium for children aged 1-3 y, 1000 mg/d for those 4-8 y, 1300 mg/d calcium for adolescent males and females aged 9-18 y and 1000 mg/d calcium for adult males and females aged 19-50 y. The tolerable upper intake level was set at 2500 mg/d for people between 1 and 50 y of age except for the age group of 14-18 y where a tolerable upper intake level of 3000 mg/d calcium was set. The new recommendations for children and adolescents were based on evidence from calcium balance studies using bone health as an indicator.

2.3.5.5.3 Current calcium intake

The most recent data on calcium intakes in the Canadian population is available from the 2004 CCHS. To date, the 2015 CCHS nutrition data is not available to the public. A significant proportion of Canadian adolescents do not attain their need of calcium intake. Data from CCHS 2004 reported that more than one-third of adolescent males and as much as 70% of females aged 14- to 18-y do not meet the EAR of calcium [30]. The prevalence of inadequacy was more problematic in females than in males. The mean calcium intake (from food sources only) in Canada was 1287 ± 28 mg/d in 14- to 18-y males versus 913 ± 20 mg/d in age-matched females [87]. The sex difference in calcium intake becomes more pertinent across puberty [135] and is attributed to several factors: (1) males generally consume more food than females to accommodate the rapid growth and puberty-related physical changes [135]; (2) females tend to skip meals, especially breakfast, more than males which typically contains calcium-containing foods (e.g. cereal with milk or cheese on a toast) [92]; and (3) they follow energy-restricted diets because they fear gaining weight, resulting in inadequate calcium intake; and (4) tend to consume less MILK as they grow because they perceive MILK as a fattening food [136]. A study by Larson et al. associated the decline of dietary calcium intake in females (mean age 15.9 y) with increased hours of watching television [137]. Moreover, data from CCHS 2004 showed that consumption of beverages in Canadian children and adolescents is in favor of sugar-sweetened beverages (SSB), contributing negatively to calcium intake likely because of milk displacement [138]. In a 10-y longitudinal study (1987-1997), annual changes in consumption of 6 types of beverages were studied in 2379 females (age 9-10 y at study entry) from childhood to late adolescence. A substantial change in beverage intakes was noted with a decrease in milk intake by > 25 % along the study course. Results of this study also revealed ethnic differences in beverage intake with lower milk intake in

Black compared to White females. On the other hand, consumption of sodas tripled during this 10-y study among all participants. The increase in soda intake coupled with reduced milk consumption was associated with a considerably decreased intake of calcium and an increase in BMI [139]. In line with these findings, a study among Canadian youth (ages 13-18 y, n=10188, 2009-2010) in 3 geographically distant regions of Canada found that more than 80% of youth consumed at least 1 SSB in the previous day, with 44% consuming 3 or more SSBs. Males and those who were less physically active consumed significantly more SSBs [140]. This decline in calcium intake, particularly among adolescent females, could be highly problematic because the skeletal needs are greatest during adolescence, thus putting them at increased risk of meeting their calcium needs and at subsequent risk of fracture and osteoporosis later in life.

2.3.5.5.4 Calcium sources

Total calcium intake comes either from food sources (natural or fortified) or from supplements [87]. Milk products such as milk, cheese and yogurt are the primary and the most bioavailable sources of dietary calcium in the Canadian diet [141]. There is about 300 mg of calcium in a 250-ml serving of fluid milk; 360 mg in 50 g of cheese; and 250 mg in a 175-ml serving of yogurt [87]. Calcium is also derived from other dietary sources, such as fish (e.g., sardines or canned salmon with bones), legumes (e.g., lentils or beans), certain green vegetables (e.g., spinach, broccoli) and grains (e.g., whole wheat bread or cereal) [87]. In a Canadian study among 8 to 19 y male and female participants, MILK contributed 57-63% of total calcium intake, followed by grain products (9%), vegetable and fruits (7%) and meat and substitutes (2%) [142].

Calcium-fortified foods constitute another dietary source of calcium such as orange juice, plant-based milk beverages and some ready-to-eat cereals and breads [87]. Another contributor to calcium intake in the Canadian population includes various dietary calcium supplements. There

are two main types in the Canadian market, calcium citrate and calcium carbonate. Calcium carbonate is more commonly available and contains more calcium than calcium citrate (40% versus 21% respectively). Moreover, it is more readily bioavailable when consumed with food whereas the citrate form is absorbed equally with or without food. Recent data from CCHS 2015 showed that about a third of Canadian adolescents consume nutritional supplements, with the lowest rates reported in Quebec, at 22.1% for children and teenagers aged 9 to 18 y [143].

The bioavailability of calcium and thus its effect on bone health depends not only on the amount of calcium ingested, but also on the food source [144]. Calcium bioavailability is subject to the components of a given food which interfere with the enterocytes' ability to absorb and/or excrete calcium[87]. For example, phytates in spinach and oxalates in whole-grain products, beans, seeds and nuts, bind to calcium forming complexes too large to be taken by enterocytes, thus inhibiting its absorption [87]. Furthermore, phytic acid can alter the pH of the intestinal lumen reducing the binding affinity of calcium to its carrier protein and, hence affecting the transport of calcium through the mucosal cell membrane [145]. This is why these foods are considered to be poor sources of dietary calcium despite their high calcium content [87]. The absorption of calcium from milk products is about 30% to 35% depending on age, with higher absorption observed during the period of growth spurt. This is attributed to elements found in milk that enhance calcium absorption as lactose, lactulose, and casein phosphopeptides formed during digestion of caseins from milk [122, 144]. It is thought that these elements chelate dietary calcium, thus preventing its precipitation and subsequently increasing its bioavailability [87]. This explains the high bioavailability of calcium from milk products.

2.4 Milk and Alternatives Intake and Bone Health

Much of the early research on bone health focused narrowly on the role of single nutrients, particularly calcium and vitamin D [146]. However, humans consume foods rather than single nutrients [147]. Studies that focus on intake of foods rather than single nutrients are more helpful in revising dietary guidelines and exploring the relationship between dietary patterns and disease risk [146]. MILK have been long identified as nutrient-dense foods that support bone growth and development especially during the period of childhood and adolescence [148]. The nutritional composition of MILK, the recommendations, current intake as well as the effect of MILK intervention on bone mineral accrual at various skeletal sites in youth will be discussed in the following sections.

2.4.1 Nutritional Composition of MILK

MILK provide energy, protein, fat and a unique combination of essential micronutrients such as calcium, phosphorus, magnesium and potassium that are considered as bone building nutrients and, when consumed together, may have synergistic effects on bone. MILK are significant contributors to multiple nutrient intake recommendations in the North American diet [117, 118]. In the US diet, MILK contributes only 13% of the total energy, yet it provides nearly 60 % of the vitamin D, 53% of the calcium and about 15–30 % of potassium, protein, vitamin A, vitamin B_{12}, phosphorus, zinc and riboflavin intakes for children and adolescents 3-17 y [115]. Similar data from Canada are not currently available [87]. A diet modeling study using data from the National Health and Nutrition Examination Survey (NHANES 2007-2010) concluded that increasing MILK intake to age-recommended amounts would result in a significant improvement in the population's adequacy for certain vitamins and minerals that currently fall below EAR, particularly calcium, vitamin D and magnesium [117].

Energy and carbohydrates

The energy content of milk products varies depending on fat content. For example, whole milk (3.25% milk fat) provides 160 kcal per 250 ml; reduced fat (2% milk fat) milk provides 130 kcal per 250 ml; low fat (1% milk fat) milk provides 100 kcal per 250 ml; and skim milk provides 80 kcal per 250 ml. Lactose is the main carbohydrate found in milk, contributing about 30% of the total energy content. The energy content of yogurt varies depending on the nutritional composition of the milk from which the yogurt is derived and whether it is plain or flavored. For example, 1 serving of fat-free plain yogurt (175 g or ¾ cup) provides 98 kcal compared to 150 kcal in fat-free fruit yogurt; and 1 serving of reduced fat (2% milk fat) plain yogurt provides 110 kcal compared to 175 kcal in 2% fat yogurt with fruit. Similarly, the variability in the energy composition of the different types of cheese depends on the type of fat content of the milk used (double cream, cream, full fat, three quarters fat, half fat, quarter fat) such as 1 serving (1 ½ oz or 50 g) provide 36 kcal (fat free cottage cheese) to 200 kcal (regular fat cheddar cheese) [149].

Protein

MILK constitutes an excellent source of high-quality protein, contributing about 3.2% of its weight and 21% of its energy content [150]. MILK proteins exist in two major forms, soluble whey and insoluble casein proteins [150]. Of the total milk proteins, about 20% is whey and 80% is casein. Whey protein is rich in branched chain amino acids, especially leucine, that play an important role in muscle anabolism [151]. Supplementation with either a 45-g whey protein or isocaloric maltodextrin for 18 mo significantly increased truncal lean mass, CTX and IGF-1 without affecting renal function in healthy older adults [152]. Another trial among healthy young women (21.3 ± 1.2 y) showed that supplementation with 40 mg/d of milk basic protein for 6 mo improved LS BMD by 1.57 %. Moreover, urinary cross-linked N-telopeptides of type-I collagen

were significantly decreased and serum osteocalcin was significantly increased [153]. The functional fraction of whey consists predominantly of alpha-lactalbumin and beta-lactoglobulin, in addition to albumin, immunoglobulins, lactoferrin and transferrin. Each has its unique role in human metabolism (e.g. retinol carrier, antioxidant and anticancer effects, iron absorption, immunity enhancer …etc) [150]. Casein is a calcium and phosphorus carrier that forms a coagulum thereby enhancing calcium and phosphorus digestibility in the stomach. The four major components of milk caseins are alpha-, beta-, gamma- and kappa-casein [150]. Overall, milk protein is considered of a high quality because of its content of essential amino acids that humans cannot synthesize in proportions that meet needs.

Fat

MILK fat is an important source of energy, essential fatty acids and fat-soluble vitamins, contributing about 48% of the energy of whole milk [151]. Fat is present in MILK as an oil-in-water emulsion of microscopic globules. MILK fat consists mainly of triacylglycerol (about 98%), while the remaining includes diacylglycerol, phospholipid, cholesterol and free fatty acids. The milk fatty acids are derived from two sources, either from the feed or from the microbial activity in the rumen of the cow [36]. Milk products are complex foods that naturally contain more than 400 different fatty acids, ranging in length from 4 to 26 carbons [151]. MILK fat contains certain fatty acids with an odd number of carbons, such as pentadecanoic acid (C15:0) and heptadecanoic acid (C17:0). These two fatty acids are synthesized through microbial fermentation in the ruminant gut [36]. The mean concentrations of C15: 0 and C17: 0 in milk are ~1.2 and 0.54% of total fatty acids, respectively [154]. Due to the odd carbon length of these fatty acids, it cannot be synthesized endogenously in humans, and thus may serve as reliable and valid biomarkers of MILK fat intake [37, 42, 43].

Around 62% of total milk fat is comprised of saturated fatty acids, 30% is monounsaturated fatty acid, primarily oleic acid (18:1), 4% is polyunsaturated fatty acids, mainly linoleic acid (18:2) and alpha-linolenic acid (18:3). Approximately 2.7% of the fatty acids in milk are trans fatty acids with one or more trans-double bonds, with vaccenic acid being the main one [151]. MILK fat is also a dietary source of conjugated linoleic acid (0.34%–1.37%), which has been the focus of several research studies, including bone research, in the recent years [155]. MILK fat also contains fat-soluble vitamins, A, D, E and K.

2.4.2 MILK Recommendation and Actual Intake in Youth

MILK is implicated as part of a nutrient-rich and balanced diet and have been related to several health benefits including bone growth and development [115]. Canada's Food Guide [156] previously linked MILK intake to improved bone health in children and adolescents and offers age-specific recommendations on the number of daily servings that are necessary to allow Canadians to meet their nutritional need [87] for the achievement of optimal PBM. Recommendations in Canada are 2 servings per day for children 4-8 y and 3 to 4 servings per day for children and adolescents 9 to 18 y of age **(Table 2.2)** [156]. In addition to being a major source of many nutrients, such as calcium, vitamin D, protein and phosphorus, which play a key role in the formation and maintenance of optimal bone health, other mechanisms by which increased MILK intakes enhance BMD, and thus PBM, exist. Combined ingestion of calcium and vitamin D, such as fluid milk and other vitamin D-fortified milk products, reduces excessive bone remodeling or turnover by reducing PTH secretion, enhances vitamin D status based on serum 25(OH)D and consequently reduces bone resorption and enhances bone formation [33]. MILK protein increases calcium absorption and stimulates production of IGF-I, a bone anabolic agent [152]. For example, in healthy young women (23 ± 3 y) performing standard resistance exercise

over 12 weeks consuming vitamin D-fortified skim milk compared to a maltodextrose isocaloric beverage pre- and post-exercise improved 25(OH)D, reduced PTH and increased osteocalcin whereas CTX decreased in both groups over time [34]. Similarly, supplementation with 500 ml semi-skimmed milk over 6 weeks lead to suppression of PTH and bone resorption as indicated by CTX, but also reduced bone formation markers including osteocalcin an P1NP in postmenopausal women [35]. In that study, osteocalcin was highly significant compared to P1NP. In contrast, in a 16-week trial where milk prevented loss of BMD at the total hip in postmenopausal women, PTH and a bone resorption marker (urinary hydroxyproline) were not different compared to placebo [157].

MILK intake by Canadian children and adolescents has waned in recent decades, with a substantial proportion of adolescents failing to meet age-recommended intakes. Recent data from the Canadian Dairy Information Centre (CDIC 2018) reported that daily consumption per capita of total fluid milk is 182 ml, total cheese is 38 g, and yogurt is 28 ml. This represents, on average, 1.65 servings of MILK/d. Several observational and longitudinal studies observed a decrease in MILK consumption with age, particularly fluid milk [115]. In a study among 1521 adolescent males and females (mean age 15.9 y at baseline) describing longitudinal changes in intakes of milk products as they transition to young adulthood, MILK intake decreased on average by 0.5 serving in both sexes. This decline in MILK intake was associated with females who spent more time watching television and with males who perceived being lactose intolerant [158].

Table 2.2 Canada's Food Guide serving sizes of milk and milk products (weight or volume) [159]

Food Item	Serving Equivalent
Milk, fluid, skim, 1%, 2% or 3.25% milk fat	250 ml, 1 cup
Milk, fluid, chocolate	250 ml, 1 cup
Milk, fluid, evaporated, canned	125 ml, ½ cup- undiluted
Milk, fluid, goat, enriched	250 ml, 1 cup
Milk, fluid, lactose reduced	250 ml, 1 cup
Milk, powdered	25 g, 75 ml, ⅓ cup
Buttermilk	250 ml, 1 cup
Cheese, block (i.e., cheddar, Mozzarella, Swiss, feta)	50 g, 1 ½ oz
Cheese, cottage or quark	250 ml, 1 cup
Cheese, goat	50 g, 1 ½ oz
Kefir	175 g, 175 ml, ¾ cup
Paneer	50 g, 1 ½ oz
Pudding/custard (made with milk)	125 ml, ½ cup
Yogurt (plain and flavored), any milk fat	175 g, 175 ml, ¾ cup
Yogurt drinks, any milk fat	200 ml
Fortified soy beverage*	250 ml, 1 cup

*recommended when not able to consume cow's milk

2.5 Assessing Bone Densitometry and Bone Metabolism

Assessing BMD in children and adolescents serves as a baseline for monitoring BMD changes over time and allows tracking changes in response to intervention [160, 161]. Numerous techniques to assess bone density in pediatrics have been developed, all presenting with strengths and limitations. A brief review of those described in this dissertation are summarized below:

2.5.1 Dual-Energy X-ray Absorptiometry

DXA was introduced in 1987 and has become the "gold standard" densitometric technique for assessing bone health in pediatrics because of it is speed, precision, ease of use, low cost and low doses of radiation [162]. DXA provides a 2-dimensional analysis of BMD; i.e. it uses 2 different X-ray beams, one with high energy and one with low energy, enabling the separation of the mineralized and soft tissue components of the sites scanned. The lower energy beam (70 peak kilovoltage) is attenuated by soft tissue while the higher energy beam (140 peak kilovolts) by bone; what is not attenuated is then detected by the instrument and areal BMD (aBMD) is calculated using an algorithm [163]. This method is considered safe as the effective radiation dose from a DXA examination is very low, ranging from 1 to 10 microSieverts (µSv). For example, in a 15-y-old child, a WB fan-beam DXA scan deliver an effective radiation dose of about 4.2 µSv, much less than the average natural background radiation exposure (~2,400 µSv/y or 6.6 µSv/d) [164]. The procedure is painless and relatively noninvasive.

DXA estimates bone mass, defined as bone mineral content and expressed in grams (g), as well as aBMD, expressed in g/cm^2 [162]. These measurements are used to assess fracture risk or monitor response to treatment/intervention. In children and adolescents, the interpretation of the DXA results are made in comparison to z-score generated from healthy youth with similar age, sex and ethnicity using a reference database from NHANES; 1999–2004 [161, 162]. The use of

DXA in pediatrics is supported by the International Society for Clinical Densitometry (ISCD 2013) which has defined a BMC or aBMD z-score < –2.0 as "low for age and sex" [8]. Limitations of the DXA technique include the provision of two-dimensional projection images. This means that it doesn't take into consideration the depth or the volume of the bone, hence a smaller bone can be falsely interpreted as having a low BMD. This is particularly a problem in growing children and adolescents because their bone is continually changing in size and in shape and thus could affect the analysis of longitudinal changes in aBMD [6]. In addition to being size-dependent, DXA is also affected by changes in body composition in longitudinal studies. DXA corrects for soft tissue around the bone by assuming a homogenous distribution; however, the change in the soft tissue of growing children will cause some inaccuracies in measurement. Further, when comparing repeated DXA scans, great care should be taken to assure validity of positioning to enable comparison. For example, the interval growth changes and the increases in bone size in growing children and adolescents are 2 aspects that should be considered when interpreting DXA results in pediatrics [161]. An interval of 6 to 12 mo is recommended to allow for the detection of meaningful changes in children and adolescents [162]. Also, measurement errors can occur because of the inter-individual heterogeneity in soft tissue composition. Positioning and region of interest selection also have to be consistent and uniform and no movement artifacts has to be present [160]. Moreover, DXA provides limited measures of bone geometry.

2.5.2 Peripheral Quantitative Computed Tomography

Peripheral quantitative computed tomography (pQCT) is another imaging technique based on x-ray absorption. It provides a three-dimensional image enabling investigation of the geometrical properties and composition of the bone, typically the distal forearm (radius) and the distal tibia [165-167]. pQCT estimates a true volumetric BMD (vBMD, expressed in g/cm^3), which

is independent of bone size. Unlike DXA, it can measure cortical and trabecular bone compartments separately and provide information about the bone geometrical properties and strength, making it ideal for evaluation of bone in growing children and adolescents [167]. Standard outcome measurements include cortical and trabecular vBMD and area whereas derived measures include cortical thickness and CSA of muscle and fat. Also, other strength indices can be extracted such as muscle density. Therefore, pQCT has the potential to evaluate the functional 'muscle-bone unit', defined as BMC/muscle CSA ratio. Such functional approach allows for assessing whether bone strength is normally adapted to muscle force, and if muscle force is adequate for body size [168]. Trabecular bone is best measured at a metaphyseal site and cortical bone at a diaphyseal site of the radius or the tibia. A recent review found that trabecular bone outcomes was obtained through a scan at 4% of the bone length in the distal region of long bones whereas information cortical bone parameters were obtained through a scan at 15-65% of the bone length in the distal direction of the proximal end of the bone [166]. Similar to DXA, the radiation exposure from pQCT is relatively low depending on the model and device (i.e., the Stratec 2000 XCT (<1.0 μSv) or the Xtreme CT (3.0 μSv)) [164]. Limitations of pQCT are that it is expensive, not easily accessible, used mainly for research purposes, requires a trained technician to operate, easily affected by motion artifacts, requires proper positioning and most importantly, the normative data created so far are not representative to all age groups and/or ethnicities, making it difficult to generalize the results [166].

2.6 Milk and Milk Products and Bone Health

During adolescence, approximately 15-25% of adult height [121] and 33 to 46 % of total adult bone mineral mass is accumulated over the 2 y surrounding peak height velocity depending on the skeletal site [5]. Nutrition, particularly calcium, during this period is a key environmental factor to establish optimum PBM and thus to protect against later bone loss [28] because calcium needs are at its highest (~ doubling) during adolescence [14]. Most calcium intervention studies in prepubertal children [169-173], early adolescents [134, 174, 175] and late adolescents [176-179] revealed positive relation with bone mineral accretion. The effect varied between 1 and 6%/y depending on the skeletal sites measured, pubertal maturation, and the initial calcium intake. In a meta-analysis of 21 randomized controlled trials (RCTs) of 3,821 participants aged 4-17 y, the authors concluded that increased intake of dietary calcium or milk products, with and without vitamin D, significantly increased LS BMC in participants with low baseline calcium intakes [26]. Data regarding the impact of calcium status on bone mineral accretion in late adolescence are scarce. Compared to adolescent girls (16-18 y) with initial calcium intake of 600 mg/d, those that were supplemented with 1000 mg of calcium had greater WB BMD and LS BMD but not BMC, selectively in girls who were 2 y postmenarcheal, providing an extended window of opportunity for bone acquisition.

As reviewed previously, the NAM recommends 1300 mg of calcium daily for males and females ages 9 to 18 y in order to meet their increased needs during this period of growth [16]. Despite these recommendations, the CCHS 2004 identifies calcium, phosphorus, magnesium, vitamin D and other as shortfall nutrients [113]. Because milk and milk products are rich in these nutrients and others, particularly calcium, vitamin D and protein, regular consumption of milk and milk products is recommended to maintain normal skeletal growth and development [19, 21, 148],

especially during late adolescence and young adulthood, the last window of opportunity. Moreover, the 2016 position statement reported by the National Osteoporosis Foundation graded the evidence for nutrients and food that can maximize bone accrual. The evidence was strongest for calcium and more mixed for milk products. However, evidence was based on 16 RCTs for calcium but only on 3 RCTs for MILK intake published since 2000 [4]. However, these 3 MILK RCTs were not generalizable because they included only white females, except for one study [180] conducted in Asian females. These findings implicate the necessity for much more research on MILK intake and bone health, to ascertain its beneficial effect on BMD during the growing phase.

2.6.1 Evidence from Human Intervention Trials

Data from RCTs provide a higher level of evidence evaluating relations between a dietary change and health outcomes [181]. In preparing this thesis, only 12 RCTs addressed the relationship between milk products consumption and BMD, the clinically accepted indicator of bone strength [182, 183].This estimate only included healthy participants and did not include studies of milk supplements or powders fortified with high amounts of calcium and vitamin D or whey-based interventions which are not as relevant to the food supply or milk industry in Canada. Of the 12 RCTs, only 5 examined the benefits of increased MILK intake on bone density outcomes among adolescents [18, 22, 47, 184, 185]. Three trials were conducted in children 10 y and younger [180, 186, 187] and the remaining included adult females 24 to 70 y of age [105, 157, 188-191]. Of the 5 RCT, 4 showed positive relation between MILK intake and BMD [18, 22, 47, 184] and one resulted in statistically nonsignificant changes. **Table 2.3** includes a more detailed review of these trials.

A relevant study by Cadogan et al. evaluated the efficacy of MILK intervention on bone mineral acquisition [184]. This trial examined the effect of milk supplementation in healthy

adolescent females (n=82; mean age 12.2 y) over a period of 18 mo. Participants were randomized using randomised permuted blocks stratified by pubertal stage, into a milk group and a control group. The milk group consumed an additional 568 ml of whole or reduced fat milk according to one's preference, which was delivered to all participants' houses every morning whereas those in the control group were asked to continue with their habitual diet (containing, on average, 150 ml of daily milk intake). WB BMC and BMD were assessed at baseline and every 6 mo. Compliance to milk intake was assessed by 7-day weighed food records completed at intervals of 3 mo throughout the study. At the end of the trial, the milk supplementation group had greater percentage increases in WB BMC (27% v 24.1%, p = 0.009) and BMD (9.6% v 8.5%, p = 0.017) compared to control [184]. Other bone measures included bone turnover markers (osteocalcin, BAP, deoxypyridinoline, N-telopeptides of type-I collagen) and hormones important to skeletal growth (PTH, estradiol, IGF-1). From baseline to 18 mo, bone turnover was not affected by milk supplementation, whereas serum concentrations of IGF-I increased in the milk group compared with the control group (35% v 25%, p= 0.02). Height, weight and body composition did not significantly differ between the intervention and the control groups. In addition, the milk group had higher intakes of protein, calcium, phosphorus, magnesium, zinc, and a range of other micronutrients. This study suggests that a modest increase in milk consumption augments bone mineral acquisition in adolescent girls. The small difference in bone mass observed in this trial (1-3% per y), if maintained, could have a substantial impact on future incidence of fractures.

A similar trial that tested the effects of MILK intervention on bone outcomes in children and adolescents resulted in similar findings. In this 12-mo trial, Chan et al. [47] randomly assigned 48 female adolescents (9-13 y of age) to either a control or an intervention group. The control group continued to consume their usual diet whereas the intervention group was supplemented

weekly with MILK products, including milk, cheese, and yogurt, to at least 1200 mg calcium daily. BMC and BMD of the WB, LS, FN and DR were assessed. Bone biomarkers were also evaluated, including serum 25(OH)D and BAP. By the end of the trial, the intervention group had higher intakes of calcium, phosphate, vitamin D and protein than the control group. The intervention group had significantly greater gains in BMD at the LS (22.8 ± 6.9% vs 12.9 ± 8.3%) and the WB (14.2 ± 7.0% vs 7.6 ± 6.0%) compared to the control. There were no differences in serum biochemical values between groups. Increased MILK intake did not increase total energy intake as well as total or saturated fat intake.

In contrast, another study by Matkovic et al. [185] to assess the impact of MILK intervention on bone health among pubertal females, resulted in inconsistent findings. Matkovic et al. randomized 31 females (14 y) into 2 intervention groups allocated to receive either 900 ml milk a day or 1000 mg calcium carbonate (4 pills of 250 mg calcium) and a control group. BMD of the distal forearm and the LS were assessed. No significant changes in any of the measured bone outcomes were noted after 2 y of supplementation. However, the author concluded that the study was not statistically powered to detect differences between study the groups. Moreover, neither vitamin D nor protein intakes were controlled or accounted for in the analyses [185].

A 2-y trial in adolescent females (n=195; age 10-12 y with usual calcium intake > 900 mg/d) tested the supplementation effects of either 1000 mg calcium carbonate or 1000 mg calcium carbonate plus 200 IU vitamin D_3 or 1000 mg calcium from cheese or placebo on BMC and aBMD of the TH, LS and WB and volumetric BMD and cortical thickness of the radius and tibia at 6-mo interval [22]. Tablets and cheese were provided. Tablets were consumed twice a day whereas cheese was advised to be consumed throughout the day. Leptin, serum 25(OH)D, intact PTH concentrations were measured by enzyme immunoassay in addition to 24 h urine samples to assess

calcium excretion. Compliance was assessed every 3 mo over the phone from 2 sources: a special diary was provided to document intakes of the study calcium tablets, vitamin D drops, and cheese products and by counting the returned tablets. After 24 mo, no overall significant differences were found in iPTH or 25(OH)D between the groups. Higher WB BMD and cortical thickness of the tibia were noticed in the intervention group compared to control when comliance was > 50, indicative of the importance of adequate MILK intake during the consolidation process.

Although the effects of MILK intake during the pre-pubertal and pubertal phase gained most of the research interest, one trial investigated the effects of post-pubertal MILK consumption on BMD. A MILK trial among 91 females 15 to 18 y of age with a mean baseline dietary calcium intake below 800 mg/d tested the effects of supplementation with milk products (e.g. plain milk, flavored milk, cheese, yogurt or dessert prepared with milk products) to at least 1000 mg calcium a day on changes in bone outcomes assessed by DXA [18]. All the participants were examined at the beginning of the study then every 6 mo for the first two y and then followed up 1 y after the cessation of the study. Milk products were delivered home biweekly and all participants were required to fill in a compliance questionnaire at 6, 12, 18 and 24 mo to assess compliance. BMC and BMD of WB, LS and TH were assessed by DXA. Lipid profiles, hydroxyproline excretion and urinary calcium and sodium excretion measurements were performed as well at each visit. At the end of the supplementation period, there was a significant increase in trochanter (4.6 %), LS (1.5 %) and FN (4.8%) BMD ($p < 0.05$) in the intervention group. Dietary calcium, posphate and protein intake were higher in the intervention group as well ($p<0.001$). Further, the increase in MILK intake achieved did not adversely affect body weight, fat and lean mass, blood lipid profiles or bone biomarkers at the end of the trial. However, 12 mo after the supplementation finished

participants returned to their baseline diet, indicating that provision of MILK products did not foster a sustained behavior change or self-selection of a MILK-rich diet.

Recently, a systematic review among healthy children and adolescents (9-18 y) participating in MILK intervention trials up to December 2016 (including the 5 RCTs discussed above) was conducted to examine the outcomes and the methodologic quality of MILK intake intervention studies in children and adolescents. Overall the findings suggested significant positive effects of MILK consumption on bone properties [79], with an average 8% increase in BMD after 16 mo of supplementation. They attributed the positive effects either to: (1) significant increases in dietary calcium intake or (2) enhanced absorption of calcium from MILK due to the presence of lactose, casein phosphopeptides, or vitamin D or (3) synergistic effect of calcium, vitamin D and protein on bone mineralization and collagen formation. Moreover, on the basis of the Jadad scale, Kouvelioti rated the methodologic quality of the MILK RCTs as good despite certain design and methodological limitations in some studies. (Further details about the RCTs methodological quality are depicted in **Table 2.4**). Overall, there is little evidence regarding the effects of MILK intake on bone mineralization among adolescents generally and late adolescents particularly. Results of MILK trials completed today are inconsistent, and most importantly, do not use treatment regimens that are advisable or sustainable for adolescents.

Table 2.3 Summary of MILK intervention trials on BMD in adolescents

Author (y) Country	Length; Age (y); Sex	Groups: dose	n; (AR%)	Scan Method	End of Trial Results
Matkovic (1990) [185] USA	2-y 14 y F	Int 1: 3x 300 ml/d milk Int 2: 4x 250 mg/d Ca Control: placebo	31 (0%)	SPA and DPA	At 2-y F/U: nonsignificant increase in BMD at LS or FA in the Int groups, possibly due to type II error
Chan (1995) [47] USA	1-y 9-13 y F	Int: 1200 mg Ca/d from milk products Control: usual diet	48 (0%)	SPA for FA and DXA for WB, FN, LS	At 1-y F/U: Int had greater gains in WB and LS BMD ($p<0.001$), not FA and FN
Cadogan (1997) [184] UK	18-mo 11-13 y F	Int: 568 ml milk Control: usual diet	74 (3%)	DXA	At 18-mo F/U: Int had greater WB BMD % compared to control (Δ%BMD: 9.6 vs 8.5, $p=0.017$)
Merrilees (2000) [18] New Zealand	2-y + 1-y F/U 15-16 y F	Int: 1000 mg Ca/d from milk products Control: usual diet	105; (14%)	DXA	At 2-y F/U: Int had greater gains in LS and FN BMD ($p<0.05$)
Cheng (2005) [22] Finland	2-y 10-12y F	Int 1: 1000 mg Ca from cheese Int 2: 1000 mg Ca Int 3: 1000 mg Ca + 200 IU vitamin D Control: placebo	195; (26%)	DXA for WB, FN and LS and pQCT for cortical thickness	At 2-y F/U: Int 3 had higher WB BMD ($p=0.04$) but not FN and LS compared to control Int 3 had higher tibial cortical thickness ($p=0.01$) compared to other groups

Abbreviations: AR: attrition rate; BMD: bone mineral density; Ca: calcium; Control: control; d: day; Δ: change; DPA: dual photon absorptiometry: DXA: dual-energy x-ray absorptiometry; F: female; F/U: follow-up; FA: forearm; FN: femoral neck; Int: Intervention; IU; international unit; LS: lumbar spine L1-4; mo: month; n: sample size; pQCT: peripheral quantitative computed tomography; SPA: single photon absorptiometry; y: year.

Table 2.4 Methodologic characteristics of MILK intervention trials on BMD in adolescents

Author (y); Country	Sample Size Estimation; (power)	Adjusting for Confounding	Diet Assessment Method	Limitations	Recommendations
Matkovic (1990) [185] USA	Yes (80%)	No	3-d food records (5 times)	Not stated	Not stated
Chan (1995) [47] USA	No	No	3-d food records (4 times) and FFQ	Not stated	Not stated
Cadogan (1997) [184] UK	No	Yes (pubertal stage)	7-d weighed food records (2 times) and 4-d food records (5 times)	Not stated	Not stated
Merrilees (2000) [18] New Zealand	No	No	3-d food records, calcium FFQ	Not stated	Not stated
Cheng (2005) [22] Finland	Yes (90%)	Yes (pubertal stage)	3-d food record	Not stated	Future studies in calcium deficient participants

Abbreviations: FFQ: food frequency questionnaire

2.6.2 Changing Behavior Towards Higher MILK Intakes

If Canadians are to realize the benefits of increasing MILK intake, behavior change will be necessary. There is some evidence that increasing milk and/or milk products is a proven approach to achieving calcium intake with benefits to bone health in postmenopausal women [188]. Similarly, 2 RCTs of MILK intake in young women encouraged participants to meet target intakes for calcium by providing milk and/or milk products; preferences were established, and product provided every 1 or 2 weeks [18, 47]. In line with these studies, a study among premenopausal women showed that a 2-y educational intervention (either a leaflet information or osteoporosis prevention and self-management course) increased total hip BMD over a 2-y period [192]. These RCTs and others that demonstrated failure to self-select a high MILK diet when provision of milk products stopped after the trial [18]suggest that professional, environmental and social support must be considered in new RCT to enable sustained behavior change associated with long-term bone health benefits.

From a professional counselling perspective, the motivational interviewing (MInt) technique has recently emerged as a useful model for stimulating and sustaining behavior change. This technique is a person-centered communication approach for strengthening self-intrinsic motivation and commitment to change [48]. It has been successfully employed to change a number of health behaviors (e.g. diet and exercise, smoking, drinking issues and medication adherence) [193]. Berg-Smith et al. showed that adolescents (13-17 y) with hyperlipidemia were able to significantly reduce their fat intake, and hence cholesterol following 2 face-to-face MInt sessions over 3-mo period [194]. In another recent trial among male and female adolescents (11-18 y) with 6-mo obesity intervention (a combination of dietary intervention and physical activity), MInt, resulted in better adherence to the obesity intervention compared to controls (received obesity

intervention only) [50]. In this thesis, MInt technique will be incorporated to improve MILK intake and to optimize adherence. MInt is anticipated to be highly applicable to Canadian adolescents and health care professionals (and possibly guide new public health messages i.e. the use of techniques that empower patients and strengthen their motivation commitment to change) rather than just provision of MILK products. Such counseling technique used during the intervention is important, as it supports changes in lifestyle behaviors that can be sustained even after the completion of the study.

2.6.3 Assessing Compliance to MILK Interventions

Different methods were used in the previously mentioned studies to assess compliance to the MILK interventions. This included either 3- to 4-day food diaries [184, 185], a special compliance questionnaire [18], a combination of 3-day food diaries and food frequency questionnaire [47], and a combination of 1-day diary and counting the returned supplementation tablets [22]. Such traditional methods are susceptible to forgetfulness, bias and inaccuracies in reporting actual intake [44, 195]. Moreover, MILK fat is not only found in whole foods as milk, cheeses and yogurts but it is also sourced from recipes prepared with milk products. This makes it more challenging to accurately estimate the intake of MILK fat from all food sources [196]. Therefore, epidemiological studies may benefit from the assessment of biochemical markers that can objectively provide a more complex measurement of MILK fat intake [5-11] and monitor intervention compliance [197].

Milk and milk products are naturally rich in fatty acids that have been used as biomarkers of MILK fat intake in several observational studies [37, 38, 40, 42, 44]. These biomarkers are objective and are free of measurement bias possibly associated with dietary recall error or analysis [156], especially when assessing dietary intake in teens [157], as adolescents have difficulty

estimating portion sizes, often eats out and constantly form new dietary patterns [198]. Plasma [199], erythrocyte [42, 200] and serum saturated fatty acids [201] are considered valid biomarkers for assessing MILK intake, specifically C15:0 and, to a lesser extent, C17:0 [37]. For instance, Wolk et al showed that C15:0 reflected MILK fat consumption in both men and women with correlations ranging from 0.74 to 0.45 for adipose tissue and 0.5 to 0.45 in serum or plasma lipids. Levels of C17:0 were also associated with total MILK fat intake, but to a lesser extent with milk products intake [38]. In a recent Canadian cross-over trial, 124 healthy adults consumed 3 servings/d of commercial milk products ranging from 1% to 34% fat content or energy-equivalent control products for 4 weeks each, separated by a 4-week washout period. Plasma levels of C15:0 and C17:0 were higher (0.26 vs 0.22% and 0.42 vs 0.39% of the total identified fatty acids, respectively) after the MILK phase than after the control phase (p=0·0001), indicating that C15:0 and C17:0 may have potential role as short-term biomarkers of MILK intake [45]. So far, dietary biomarkers of MILK fat intake have been investigated in adult men and women. However, to the best of author's knowledge, evidence in healthy adolescents is limited to a study showing that the serum level of C15:0 was negatively correlated with total cholesterol level in this population [46].

2.7 Summary

In this review, many factors have been shown to affect the development of PBM, making this a complex topic. It has been revealed that the variation in BMD at the time of PBM is similar to that seen with aging, suggesting that efforts to enhance BMD early in life may also benefit individuals later in life. Indeed, it has been estimated that 5 to 10% higher BMD early could translate to a 20% reduction in osteoporotic fracture and 50% reduction in hip fracture after the age of 40-50 y.

Among the determinants of PBM, dietary factors, particularly milk and milk products, represent an important and growing area of research for bone health. The existing evidence for the effects of MILK consumption on bone mineralization specifically in adolescents is limited to 5 trials which were conducted between 1990 and 2005 and are methodologically and statistically disparate. None of those existing trials were recent or Canadian or included males. Further, none tested the effects of different servings of milk products (i.e. as a food group) in relation to changes in BMD, necessitating a high-level evidence to support nutrition guidelines.

Though BMD is a useful assessment and DXA is the gold standard, this technique measures aBMD and does not take into consideration bone size and, thus, does not give information about the geometrical structure of the bone and partially explains bone strength and fracture resistance. This is particularly an issue in growing skeletons given the potential bone alterations in geometry in the pediatric population. This explains ongoing discussions regarding the appropriateness of the use of projectional techniques such as DXA in children and growing young adults. This is where quantitative imaging such as pQCT has the advantage in pediatric research; bone measurement is independent of bone size. pQCT provides information beyond aBMD, including measures of vBMD, bone geometry (area, cortical thickness) and bone strength; it improves fracture risk

prediction and define the skeletal response to therapy [166]. It is also increasingly used to study the interaction between muscle and bone systems. Recent cross-sectional work in elderly suggested a positive association between MILK intake and appendicular bone mineralization and muscle mass. To date, only one intervention with cheese among adolescents included pQCT imaging technique in their analyses. It is not known if increasing MILK to meet nutritional guidelines will favorably affect skeletal geometry in adolescents.

Moreover, current interventions typically assess compliance using standard dietary recall measures and have not considered using objective measures to assess milk fat concentrations. Finally, the use of the motivational interviewing technique, instead of providing milk products, to improve MILK intake is unique to this trial. Such counseling technique incorporated during the intervention is important as it supports changes in lifestyle behaviors that can be sustained even after the completion of the study.

Several gaps have been identified in the literature and are important to consider for PBM optimization during adolescence: well-designed trials are needed to generate high-level evidence that dietary intervention with milk and milk products will enhance PBM in young men and women.

Bridge Statement 1

The literature review of this dissertation has outlined the different factors influencing bone mineral gains and peak bone mass acquisition, focusing particularly on the relevance of MILK products in optimizing bone health in adolescents. Today, there is a substantial evidence that adequate MILK intake during the growing period optimizes bone mineral acquisition which, in turn, may reduce subsequent fracture risk later in life. MILK products have a unique profile of bone-relevant nutrients that are under consumed (e.g. bioavailable protein, calcium, vitamin D and phosphorus). For decades, food guide recommendations in North America have included at least 3 servings per day of MILK for adolescence. However, milk products are underconsumed by teenagers in both Canada (Garriguet 2008) and the U.S. [117]. A diet modeling study using data from the National Health and Nutrition Examination Survey (NHANES 2007-2010) has shown that increasing MILK intake to age-recommended amounts would reduce the prevalence of "shortfall nutrients", particularly calcium, vitamin D and magnesium [117]. Moreover, recent data from the NHANES 2011-2014 in children and adolescents 2-18 y demonstrated that MILK products, particularly milk remain the main source of calcium, vitamin D, and potassium for this age group. Similar data in Canada is lacking, particularly for adolescents with low MILK intake.

The following chapter explores the nutritional adequacy of bone-relevant nutrients and the relationship to bone density, bone geometry and serum biomarkers in adolescents with usual intake of < 2 MILK servings/d.

CHAPTER 3

Manuscript 1: Micronutrients adequacy, bone biomarkers and milk products

Bone-relevant micronutrients and biomarkers in adolescents with habitually low daily intake of milk and alternatives

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3.1 ABSTRACT

Objective: To assess the nutritional adequacy of bone-relevant nutrients and to evaluate the relationship among usual nutrient intake and bone mineral density (BMD), bone geometry and serum bone biomarkers in adolescents with low usual intake of milk and milk products (MILK), < 2 servings per day (d).

Methods: Healthy adolescents 14-18 y of age were studied using adjusted nutrient intakes from 24 h food recalls to evaluate nutrient adequacy. Usual dietary intake was assessed using a semi-quantitative food frequency questionnaire (FFQ). Bone biomarkers were also measured. Dual-energy X-ray absorptiometry and peripheral quantitative computed tomography were used to assess BMD and geometry.

Results: MILK intake was 1.6 (0.9, 2.3) servings/d among the 81 participants (16.5 ± 1.6 y). Based on adjusted 24 h intakes, none met the Estimated Average Requirements (EAR) for dietary potassium or vitamin D. For both calcium and magnesium, < 30% of males and < 15% of females met the EAR. Phosphorus intake was adequate in males, however 40% of females met the EAR. Males had higher bone biomarker concentrations yet lower BMD at the lumbar spine, 33% radius and whole body compared to females. FFQ data showed positive correlations among tertiles of MILK and bone biomarkers and 4% radial and tibial trabecular density; tertiles of fluid milk and tibial cortical thickness and whole body BMD z-score, after adjusting for covariates.

Conclusion: Consuming nutrient-rich food such as MILK may improve the adequacy of certain nutrients and thereby exert a positive impact on bone health and metabolism in post-pubertal adolescents.

3.2 INTRODUCTION

Adolescence is a critical period for the achievement of peak bone mass (PBM) [7]. This growth period requires an adequate supply of several nutrients to support skeletal growth and mineralization. The 2004 Canadian Community Health Survey (CCHS) showed that a high proportion of adolescents do not meet the EAR of several bone-relevant nutrients such as vitamin D, calcium and vitamin A [113]. Milk and milk products (MILK) constitute an important source of these nutrients [202]. For decades, food guide recommendations in North America have included at least 3 servings per day of MILK for adolescence [203, 204] . However, milk products are underconsumed by teenagers in both Canada [30] and the U.S. [117].

The positive effect of MILK consumption on bone mineral accretion during growth has been -reported in a recent systematic review of several clinical trials in children and adolescents (Kouvelioti et al. 2017). Furthermore, increasing MILK consumption to recommended targets resulted in improved intake of several shortfall nutrients in adolescents (Campmans-Kuijpers et al. 2016). Several trials in children and adolescents have shown that MILK intake may exert a regulatory role on different bone formation (osteocalcin, and bone-specific alkaline phosphatase; BAP) and resorption (C-telopeptide of Type 1 Collagen; CTX) biomarkers (Josse et al. 2010). In a randomized trial among 8-year-old boys, the intake of a milk-based drink (540 ml/d), containing concentrated whey and casein, significantly increased serum osteocalcin after 7 days (Mark et al. 2010). Similarly, the consumption of vitamin D-fortified milk (500 ml twice a day) by healthy young women over 12 weeks reduced serum concentrations of parathyroid hormone (PTH) and CTX and increased serum concentration of osteocalcin (Josse et al. 2010), suggestive of possible bone-enhancing effects.

The aim of the present study was to assess the nutritional adequacy of bone-relevant nutrients and to evaluate the relationship among usual nutrient intake and bone density, bone geometry and serum bone biomarkers in adolescents with usual low MILK intake.

3.3 METHODS

Participants

Baseline data were obtained from a randomized, parallel-arm intervention trial (NCT02236871) that investigated the impact of MILK intake on bone health in adolescent males and females from Montreal, Canada. Healthy adolescents 14-18 y (n=94) with usual low intake were recruited. Of the 94 recruited adolescents, 81 participants provided complete dietary intake data used in the present analysis.

Anthropometry

Weight was recorded to the nearest 0.1 kg using a balance-beam scale (Detecto, Webb City, MO, USA). Height was measured to 0.1 cm using a stadiometer (Seca model 226; new manufacturer Ayrton 226 Hite-Rite Precision Mechanical Stadiometer); body mass index (BMI; kg/m2), BMI for-age z-scores (BAZ) and height-for-age z- scores (HAZ) were computed using the WHO Anthro-Plus Software. Waist circumference (WC) was measured to 0.1 cm according to Health Canada guidelines and WC z-scores (WCZ) were calculated using the LMS method based on National Health and Nutrition Examination Survey (NHANES) III reference curves [205]. Physical activity (PA) was captured using the Youth Physical Activity Questionnaire reflecting activities performed the week prior to the study visit [206]. Skin type was assessed using a spectrophotometer (CM-700d/600d; Konica Minolta) and calculation of individual typological angle (ITA) of the inner upper arm as a proxy for constitutive skin pigmentation and potential to endogenously synthesize vitamin D [207]. ITA was calculated using the L*a*b* colorimetric system of the Commission Internationale de I'Eclairage and classified into skin types based on the Fitzpatrick scale [207].

Dietary Intake Assessments

Dietary intakes were collected using a 24 h recall for the day prior to study visit. Nutrient composition was determined using Nutritionist Pro™ (Axxya Systems LLC, Stafford, US) with the Canadian Nutrient File version 2010b. MILK intake (servings/d) was the total of milk, yogurt, cheese as well as recipes with milk products and was calculated according to Canada's Food Guide (e.g. 1 serving is equivalent to 250 ml of milk, 50 grams of cheese and 175 grams of yogurt). A second 24 h recall was repeated one month later in a subsample of 26 participants to estimate the within-individual variance of nutrients [208]. The variance estimates from this subsample were estimated using the National Cancer Institute method and were applied to adjust the 1-day intake data for the 81 participants. The EAR cut-point method was used to estimate the proportion of the group with inadequate intakes of nutrients. Usual dietary intake over the past year was captured using a modified semi-quantitative validated food frequency questionnaire (FFQ) [209]. This FFQ has been validated in English and in French speaking adults [155], with nutrient output derived using the Canadian Nutrient File.

Bone Assessment

Whole body (WB), lumbar spine 1-4 (LS), one-third distal radius, femoral neck (FN) and total hip (TH) bone mineral content (BMC, g) and areal BMD (g/cm^2) were determined using dual-energy x-ray absorptiometry (DXA, Hologic 4500A, APEX software, v13.3:3, MA). BMD z-scores (BMDZ) were obtained using the NHANES reference data for white children and adolescents (Gordon et al. 2008). Quality control was performed using the manufacturer's proprietary LS bone phantom with a coefficient of variability (CV) of 0.5% for BMC and 0.3% for BMD.

Bone geometry was examined using pQCT scans (XCT-2000; Stratec, Pforzheim, Germany) of the non-dominant tibia (4%, 38% and 66% sites) and radius (4% and 66%) providing trabecular density and CSA (mm^2), volumetric BMD (vBMD, mg/cm^3), cortical thickness (mm), muscle density (mg/mm^3) and muscle area (mm^2). The reference point was the most proximal line of the growth plate if open and through the middle of the ulnar border of the articular cartilage if closed (Neu et al. 2001). Scan slice thickness was 2.0 mm, voxel size 0.2 mm, and speed set at 10 mm/sec. Scans were analyzed using contour mode 2 (45%) and peel mode 1 at the 4% site and with separation mode 1 and a threshold of 710 mg/cm^3 at the 66% site. Segmentation of muscle from radial and trabecular bone was performed using a density threshold of 280 mg/cm^3 with contour mode 1 and peel mode 2.

Biochemistry

Fasted blood samples were collected between 0700 h and 1030 h to control for diurnal variation. Ionized calcium was measured immediately in a blood gas tube (ABL80 FLEX, Radiometer Medical A/S, Denmark) with a CV of ≤ 5%. Manual enzyme-linked immunosorbent assays were used to measure plasma CTX (CrossLaps ELISA, Intermedico, Canada, nmol/L), which had a CV of < 9.8 %. Serum total 25(OH)D (nmol/l), osteocalcin (ng/ml), BAP (µg/l), intact 1-84 PTH (pg/ml) was measured using automated chemiluminescent immunoassays (Liaison, Diasorin, Ontario, Canada) with a CV of < 7.0%. Other quality control measures for 25(OH)D included participating in the Vitamin D External Quality Assurance Scheme; and using the National Institute of Standards and Technology (NIST) guidelines for standardization of serum 25(OH)D concentrations in study samples [210, 211]. NIST control samples had an in inter-assay CV of 3.4 % and accuracy of 104.2 %.

Statistical Analysis

Data were analyzed using SAS 9.4 (SAS Institute Inc, Cary, NC, USA). Participants were stratified into 4 groups based on sex and age (14 to 16.9 y males, 17 to 18.9 y males, 14 to 16.9 y females and 17 to 18.9 y females) to control for physical maturity. All data were tested for normality by using the Kolmogorov-Smirnov and for homogeneity of variances by using Bartlett test. Significance was set at < 0.05. A mixed-model ANOVA was used to compare groups for continuous data with Bonferroni post hoc testing and Chi-square test for differences in proportions. MILK intake was estimated by FFQ to explore longer term usual intakes and was classified into tertiles. Multivariate linear regression models with a stepwise forward selection were constructed to assess the association between tertiles of MILK intake and bone health parameters adjusting for age, sex, height, weight, skin tone, energy intake and PA. Season was also explored as a covariate since 46 participants were recruited between April 1 and October 31 i.e. during the cutaneous vitamin D synthesizing period. A cross-classification analysis was used to determine agreement between the FFQ and 24 h recall dietary methods. Misclassification was defined as the percentage of participants classified in the lowest tertile in the FFQ and the highest tertile in the 24 h recall and vice versa.

3.4 RESULTS

Baseline Characteristics

A total of 81 healthy adolescents (55 females and 26 males) participated in this study. The average age of the study population was 16.5 ± 1.6 y **(Table 3.1).** The average HAZ was not significantly different among males, however older females were shorter for their age compared to younger females. BMI and WC z-scores did not significantly differ between sexes or with age. Male groups were not different in terms of their physical activity, whereas older females were less active compared to the younger ones **(Table 3.1).** Overall, ethnic distribution was 75% Caucasian, 19% Asian, 5% African or Black and 1% Hispanic. Socioeconomic analysis showed that 43% of the study families had an annual household income > $75,000 Canadian dollars, with 37 % of unknown income.

Nutrient Intakes Compared to Dietary Reference Intake Values

Usual Intakes Based on FFQ data

FFQ data showed an overall median MILK intake of 1.4 (IQR: 0.8, 2.1) servings/d. Total MILK intake of males, 1.9 (IQR: 1.2, 2.8) serving/d, was substantially higher than that of females, 1.2 (IQR: 0.5, 1.9) servings/d. Males drank significantly more fluid milk (1.0 serving/d) compared to females (0.4 serving/d) (Figure 1). All the participants reported milk-based alternatives. Cheese and yogurt intakes were similar for both sexes. In comparing MILK intake for age and sex, younger males consumed the most MILK. Younger males consumed more fluid milk than older males; however, cheese and yogurt intake did not differ by age. Females did not differ by age in their MILK intake.

Intakes of Nutrients

Based on the adjusted 24 h recall data, none of the females met the EAR for vitamin D or adequate intake for potassium; approximately 15% met the EAR for calcium and magnesium intakes; and approximately 40% met the EAR for phosphorus intakes. The pattern did not differ by age. In males, none met the EAR for vitamin D or adequate intake for potassium intakes; Approximately 30% met the EAR for calcium and magnesium and 65% for phosphorus, with adequacy being lower in the older age group (**Table 3.2**).

Cross-classification assessment showed that 90% of participants were in the same or adjacent MILK tertile. Further, the percentages of participants cross-classified in the same or adjacent tertile was 84% for calcium, 94% for vitamin D, 91% for potassium, 100% for phosphorus and 85% for magnesium.

Percentage Contributions of MILK to the Intake of Bone-relevant Nutrients

Usual intake of MILK, based on FFQ, makes a substantial contribution to the daily intake of several bone-building nutrients, particularly nutrients of concern such as calcium, vitamin D, potassium, phosphorus and magnesium (**Figure 3.2**). In males, MILK contributed to more than 60% of vitamin D intake, approximately 50% of calcium intake and one third of daily riboflavin, phosphorus and vitamin B_{12} intakes. MILK consumption contributed less to potassium, magnesium, vitamin B_6, folate, niacin and iron intakes. In females, MILK contributed to more than 40% for calcium and vitamin D and accounted less, between 1.5% and 30%, for the intake of phosphorus, riboflavin, vitamin B_{12} and other nutrients **(Figure 3.2).**

Biochemical Assessments

No significant difference in serum concentrations of PTH, ionized calcium or 25(OH)D was observed among groups by sex or age **(Table 3.3)**. Seasonal variations were, generally,

reflected in vitamin D status, with lower 25(OH)D concentrations in winter and higher concentrations in summer (data not shown). Serum BAP, osteocalcin and plasma CTX concentrations were higher in the younger male group. In females, bone biomarkers were not significantly different by age. Overall, males had greater concentrations of bone biomarkers than females ($p<0.001$). Fluid milk tertiles were positively associated with BAP ($\beta=0.2$, adjusted R-sq, 0.64, $p=0.01$) and osteocalcin ($\beta=0.1$, adjusted R-sq, 0.71, $p=0.03$). Yogurt tertiles associated negatively with CTX ($\beta=-0.1$, adjusted R-sq, 0.63, $p=0.04$) only after adjustment for covariates.

DXA assessments

There was no significant pattern or heterogeneity by age for BMC or BMD at any of the skeletal sites studied. Compared to males, females had greater LS and TH BMC as well as greater BMD at the LS and FN **(Table 3.3)**. With regard to z-scores, mean values of LS BMD were lower across age groups in males but not in females. Females also had higher BMD z-scores than males at all skeletal sites measured **(Table 3.3)**. No associations were found between total MILK tertiles and bone outcomes, both in unadjusted and adjusted multiple regression models. After adjustment for age, weight, height, sex, energy intake, physical activity and skin color, a positive association was found between tertiles of fluid milk and WB BMDZ **(Table 3.4)** such that an increase of one milk serving was associated with a change of 0.33 in WB BMDZ ($p=0.03$). In both crude and adjusted models, no association was found between yogurt tertiles and BMC, BMD or BMDZ at any of the bone sites tested. Cheese tertiles negatively associated with distal radius and WB BMD, however this relationship was lost after adjusting for potential covariates in the final model.

pQCT Assessments

There were no significant differences in any of the radial bone parameters across age, but females had greater radial cortical density and smaller muscle area compared to males **(Table 3.5)**.

With regard to tibia bone properties, cortical density at 38% and 66% was higher in older than younger males. The remaining bone outcomes did not vary across age. According to sex, mean values of 4% radial trabecular area as well as tibial cortical density at 38% and 66% sites were higher in females than in males; however, males had larger muscle CSA than females at both the radius and tibia **(Table 3.5)**.

The associations between tertiles of MILK intake and pQCT bone parameters are presented in **Table 3.4**. In the unadjusted model, no positive associations were observed between tertiles of MILK and any of the radial or tibial bone or muscle variables except at the 4% radial trabecular density. Further, negative associations were found between MILK intake tertiles and cortical densities at 38% and 66% bone sites that were lost after adjusting for covariates (age, weight, height, sex, energy intake, physical activity and skin color). In the fully adjusted model, tertiles of MILK consumed showed a positive association with 4% radial and tibial trabecular density. Tertiles of fluid milk were a significant positive correlate of tibia cortical thickness after adjustment for confounder variables **(Table 3.4).**

With regards to the relationship among MILK products and pQCT outcomes, no association was observed between tertiles of cheese or yogurt and any measure in the radius and tibia as assessed by pQCT **(Table 3.4).**

3.5 DISCUSSION

The present study demonstrated a higher prevalence of nutrient inadequacy of bone-relevant nutrients than national estimates among 14-18-year-old adolescents with low usual MILK consumption and provided a valuable insight into the important contribution of MILK to shortfall bone-relevant micronutrients and its relationship with bone biomarkers in youth. Consistent with our hypothesis, all adolescents failed to meet the recommendations for vitamin D and potassium, more than 70% did not meet the recommendations for calcium and magnesium and more than one-third reported inadequate intake of phosphorus. Nutrient inadequacy was, overall, more pronounced in females than males. These findings exceed national estimates of the prevalence of nutrient inadequacy [113] which is likely due to the exclusive inclusion of adolescents with low MILK consumption. In line with this, the observation that females had greater nutrient inadequacy compared to males may be explained, at least in part, by their lower consumption of total MILK (1.2 vs 1.9 servings/d).

A remarkable observation from this analysis is the important contribution of total MILK intake to shortfall micronutrients (> 40% of daily calcium, ~ 60% of vitamin D and > 30% of riboflavin, vitamin B_{12} and phosphorus intake). So, by choosing nutrient rich foods such as MILK and others containing calcium and vitamin D, adolescents might be able to meet the EAR for calcium and vitamin D and to reduce the prevalence of inadequate intake of potassium, magnesium and phosphorus. In a modeling study, increasing MILK consumption to age-sex recommended amounts significantly improved the adequacy of several under-consumed vitamins and minerals in 2 to 19 y participants [117]. Educational strategies to meet the dietary recommendations, within the context of food and lifestyle preferences, could be useful in the achievement of skeletal health.

Total MILK and fluid milk intake tertiles were positively associated with BAP and fluid

milk intake tertiles were positively associated with osteocalcin. In agreement with the present findings, a cross-sectional study among healthy prepubertal males (8.1±0.1 y) showed a tendency toward a positive association with BAP (p=0.07) (Budek et al. 2007). Another interesting finding in this study is the inverse association between yogurt tertiles and CTX. CTX is a bone resorption marker released during degradation of type I collagen, reflective of osteoclastic activity (Kuo and Chen 2017). No significant associations between MILK intake and 25(OH)D, PTH or ionized calcium were observed, which is most likely attributed to recruiting participants without vitamin D deficiency. Together, this suggests that MILK in general supports bone modeling and thus might have protective effects on peak bone mass and bone health in this age group. Such a finding warrants further investigation.

No significant heterogeneity by age was observed for areal bone measures at any of the skeletal sites except for LS BMDZ which was higher in the younger males than their older counterparts (Table 3). This might be explained by the higher intake of total MILK observed in the younger male cohort [2.7 (1.6, 2.9) vs 1.1 (0. 9, 1.8)]. Between sexes, WB, LS and distal radius BMDZ were higher in females compared with males. These findings corroborate the results of a previous longitudinal study among adolescents (Bachrach et al. 1999). Puberty occurs 1-2 years earlier in females compared to males (Yilmaz et al. 2005), suggesting that gains in BMD for WB and LS might have levelled off in both female groups and in older male group. Indeed, younger males in this study were still growing, and some had not reached their final height, which is directly related to skeletal growth.

The present study explored MILK intake with bone and muscle geometry. Overall, tertiles of MILK revealed positive associations with trabecular and cortical thickness, implying that MILK has implications to bone geometry independent of muscle CSA or density that showed no

significant relationship with MILK tertiles. Tertiles of fluid milk were also associated with a higher WB BMDZ. Although extensive research on the effects of MILK consumption on bone health has been conducted, the mechanism or nutrient linking the two is not clear. Besides its calcium and vitamin D content, MILK intake can positively affect bone health through different mechanisms involving intakes of bone-beneficial nutrients such as protein. Milk whey proteins, particularly leucine, increase secretion of insulin-like growth factor-1 which stimulates the activity of the osteoblasts and increases calcium and phosphorus absorption in the intestine and increases the renal reabsorption of phosphorus. MILK whey proteins are rich in branched-chain amino acids which exert anabolic effects on muscle tissue [102]. Such advantages might help explain the benefits of MILK intake on musculoskeletal health in this age group.

This study is limited by its cross-sectional design, the use of self-reported dietary intake including the FFQ which may overestimate intakes, and a white majority of participants. However, the strengths include the adjustment of within-individual variances and the estimation of usual intakes, a critical step in the assessment of nutrient adequacy. Another strength was the use of a validated FFQ to complement actual dietary intake collected with 24 h recalls and the use of cross-classification analysis to determine the extent of agreement between these two dietary assessment methods. Further, this study provided a unique occasion exploring the relationship between MILK intake within three assessment methods of bone health- biomarkers, DXA and pQCT outcomes.

In conclusion, MILK products are an important source of a range of shortfall micronutrients that are implicated in bone health. Low consumption of MILK contributes to inadequate intake for some nutrients critical for bone health, particularly among female adolescents. Therefore, nutritional education and intervention programs appear to be needed to help adolescents meet the DRI values for many bone relevant micronutrients.

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Table 3.1 Characteristics of participants, stratified by age and sex

	Males			Females		
	14-16.9 y (n=17)	**17-18.9 y (n=9)**	**All (n=26)**	**14-16.9 y (n=27)**	**17-18.9 y (n=28)**	**All (n=55)**
Age (y)	14.7±0.9[a]	17.8±0.4[b]	15.8±1.7[1]	15.5±0.8[a]	18.0±0.5[b]	16.8±1.5[2]
Height (cm)	164.7±11.7[a]	178.0±7.7[b]	169.3±12.2[1]	166.1±6.4	161.5±6.6	163.7±6.9[2]
Height z-score	-0.3±1.2	0.3±1.0	-0.1±1.1	0.6±1.0[a]	-0.2±1.0[b]	0.2±1.1
Weight (kg)	51.6±9.1[a]	72.6±10.8[b]	58.9±14.0	59.4±9.9	58.9±10.9	59.1±10.3
BMI (kg/m^2)	18.9±1.7[a]	22.9±2.3[b]	20.3±2.7	21.5±3.1	22.5±3.3	22.0±3.2
BMI z-score	-0.4±0.8	0.4±0.7	-0.1±0.8	0.2±0.9	0.3±0.9	0.2±0.9
WC (cm)	70.4±5.5[a]	79.9±9.0[b]	73.7±8.2	73.8±8.1	74.1±7.3	73.9±7.6
WC z-score	0.2±0.01	0.2±0.01	0.2±0.01[1]	0.2±0.01	0.2±0.03	0.2±0.02[2]
PAQ (min/wk)	627±342	426±414	558±373[1]	477±290[a]	264±105[b]	369±240[2]
Ethnicity, White (%)[*]	76	89	81	89[a]	57[b]	73
Family income (%)						
< 75000[ǂ]	12	22	15	22	21	23
≥ 75000	59	0	39	63	29	44
Not disclosed	29	78	46	15	50	33

Data are expressed as mean ± SD or counts and percentages.
Differences among means for age and sex groups were tested using mixed model ANOVA with lower case letters ([a,b]) indicating differences among age groups within sex, and numbers ([1,2]) indicating differences among sexes, $p<0.05$).
Differences among proportions were tested using Chi-square tests.
[*] Non-white includes Asian (n=15), African or black (n=4), Hispanic (n=1).
[ǂ] Annual household income

Table 3.2 Adjusted nutrient intakes and percentage of participants meeting estimated average requirements (EAR), stratified by age and sex

Intake/d[1]	Males				Females			
	EAR	14-16.9 y (n=17)	17-18.9 y (n=9)	All (n=26)	EAR	14-16.9 y (n=27)	17-18.9 y (n=28)	All (n=55)
Calcium (mg)	**1100**	957.2±226.7 30%	843.4±241.6 22%	917.7±233.7 27%	**1100**	854.3±189.9 15%	812.0±201.0 14%	833.1±194.8 15%
Vitamin D (IU)	**400**	165.1±52.2 0%	136.0±51.6 0%	155.0±52.9 0%	**400**	131.0±65.1 0%	102.9±50.4 0%	116.9±59.4 0%
Potassium[2] (mg)	**4700**	2208.4±248.1 0%	2032.2±266.2 0%	2147.4±263.4 0%	**4700**	2120.5±285.5 0%	2072.5±297.8 0%	2096.5±290.0 0%
Magnesium (mg)	**340**	281.7±103.6 23%	221.1±50.3 0%	260.8±92.4 15%	**300**	225.8±55.5 15%	257.7±110.4 18%	241.7±88.1 16%
Phosphorus (mg)	**1055**	1120.3±151.0 70%	1054.3±206.5 55%	1097.5±171.1 65%	**1055**	994.0±156.9 45%	1012.1±193.9 36%	1003.1±174.9 40%
Zinc (mg)	**8.5**	10.2±2.9 65%	11.7±7.9 78%	10.7±5.2 69%	**7.3**	8.4±2.7 67%	9.2±4.6 64%	8.8±3.8 65%
Iron (mg)	**7.7**	14.7±3.5 100%	13.7±3.7 100%	14.4±3.6 100%	**7.9**	12.7±2.9 100%	13.2±3.2 100%	12.9±3.0 100%
Niacin (mg)	**12**	20.7±4.9 100%	19.8±6.3 100%	20.4±5.3 100%	**11**	16.8±3.0 100%	17.4±4.6 100%	17.1±3.8 100%
Thiamin (mg)	**1.0**	1.8±0.6 100%	1.6±0.5 100%	1.8±0.6 100%	**0.9**	1.5±0.5 96%	1.5±0.4 93%	1.5±0.5 95%
Riboflavin (mg)	**1.1**	1.6±0.2 100%	1.5±0.4 100%	1.6±0.3 100%	**0.9**	1.5±0.3 100%	1.4±0.2 100%	1.4±0.2 100%
Vitamin B_6 (mg)	**1.1**	1.7±0.4 100%	1.8±0.8 100%	1.7±0.6 100%	**1.0**	1.6±0.5 93%	1.6±0.8 85%	1.6±0.6 89%

Data is presented as mean ± SD followed by % meeting EAR or AI.

[1] Adjusted nutrient intakes were obtained using two 24 h food recalls using a subgroup of 26 participants.

[2] No EAR available for potassium, these data reflect Adequate Intake values.

Table 3.3 Bone health markers of participants, stratified by age and sex

	Males			Female		
	14-16.9 y (n=17)	**17-18.9 y (n=9)**	**All (n=26)**	**14-16.9 y (n=27)**	**17-18.9 y (n=28)**	**All (n=55)**
Biochemistry						
Serum osteocalcin (ng/ml)	79.6±25.9	56.1±46.4	71.5±35.3[1]	39.5±15.4	25.0±5.9	32.1±13.6[2]
Serum BAP (µg/l)	86.7±43.1[a]	35.0±29.0[b]	68.8±45.7[1]	29.9±19.6	17.0±7.7	23.3±16.0[2]
Plasma CTX (nmol/l)	2.1±0.6[a]	1.1±0.5[b]	1.8±0.7[1]	0.9±0.4	0.6±0.3	0.7±0.4[2]
Serum 25OHD (nmol/l)	65.8±17.9	48.7±16.6	59.9±19.0	66.4±29.6	54.9±26.7	60.5±28.4
Serum PTH (pg/ml)	17.8±5.0	20.9±10.4	18.9±7.2	17.4±6.3	17.9±8.6	17.7±7.5
iCa (mmol/l)	1.27±0.03	1.25±0.04	1.26±0.04	1.27±0.04	1.26±0.03	1.27±0.04
DXA measurements						
WB BMC (g)	2004.54±568.08	2841.16±563.13	2294.14±687.58	2273.32±343.42	2143.87±353.26	2207.42±345.76
BMD (g/cm^2)	1.080±0.149	1.258±0.120	1.141±0.162	1.168±0.102	1.160±0.082	1.163±0.091
BMDZ	0.571±1.226	0.911±1.199	0.688±1.204[1]	1.533±1.134	0.900±1.004	1.211±1.107[2]
LS BMC (g)	48.21±19.03	67.01±16.99	54.72±20.18[1]	60.91±10.89	56.33±10.74	58.58±10.96[2]
BMD (g/cm^2)	0.803±0.192	0.977±0.139	0.863±0.192[1]	1.005±0.120	0.986±0.109	0.995±0.114[2]
BMDZ	-0.418±1.491[a]	-0.489±1.131[b]	-0.442±1.354[1]	0.396±1.054	-0.200±1.063	0.100±1.100[2]
33% radius BMC (g)	1.66±0.39	2.14±0.29	1.83±0.43	1.73±0.39	1.67±0.19	1.69±0.30
BMD (g/cm^2)	0.643±0.107	0.752±0.074	0.681±0.109	0.670±0.05	0.688±0.043	0.680±0.047
BMDZ	-0.147±1.654	-0.133±1.320	-0.142±1.519[1]	0.281±0.967	0.136±0.822	0.206±0.889[2]
FN BMC (g)	4.18±1.12	5.02±0.97	4.47±1.13	4.42±0.76	3.95±0.86	4.19±0.84
BMD (g/cm^2)	0.799±0.160	0.935±0.120	0.847±0.159[1]	0.905±0.135	0.866±0.114	0.886±0.125[2]
BMDZ	-0.700±1.436	-0.050±0.956	-0.492±1.318[1]	0.496±1.176	0.155±1.018	0.326±1.102[2]
TH BMC (g)	31.68±11.10	42.80±8.71	35.53±11.50[1]	31.37±5.11	28.63±5.92	29.98±5.66[2]
BMD (g/cm^2)	0.906±0.195	1.089±0.114	0.969±0.191	0.998±0.144	0.962±0.113	0.980±0.130
BMDZ	-0.476±1.561	0.287±0.980	-0.232±1.427[1]	0.507±1.254	0.163±0.974	0.335±1.126[2]

Data are expressed as mean ± SD and adjusted for height, weight, physical activity, skin color and season (with 25OHD).
Differences among means for age and sex groups were tested using mixed model ANOVA with lower case letters ([a,b]) indicating differences among age groups within sex, and numbers ([1,2]) indicating differences among sexes, $p<0.05$).
Abbreviations: BAP: bone specific alkaline phosphatase; BMC: bone mineral content; BMD: bone mineral density; CTX: C-terminal telopeptide of type 1 collagen FN; Femoral neck; iCa: ionized calcium; LS: Lumbar spine L1-4; PTH: parathyroid hormone; TH: total hip; WB: Whole body; 25OHD: 25-hydroxyvitamin D

Table 3.4 Multiple linear regression models for tertiles of total MILK and fluid milk intake and bone measures, assessed by DXA and pQCT

Dependent Variable	Regression Model[1] (n=81)	Adj R-sq	β	SE	p-value
DXA measurements					
WB BMDZ		0.34			
	Fluid milk tertiles (servings/d)		0.33	0.15	0.029
	Weight (kg)		0.06	0.01	<.0001
	Sex		1.01	0.26	0.0002
	PA (60 min/wk)		0.05	0.02	0.02
	ITA (º)		-0.02	0.01	0.009
pQCT measurements					
		0.20			
4% radius trabecular density (mg/cm^3)	MILK tertiles[2] (servings/d)		14.9	5.1	0.005
	Weight (kg)		1.3	0.4	0.0004
		0.34			
4% tibial trabecular density (mg/cm^3)	MILK tertiles (servings/d)		15.0	5.2	0.006
	Weight (kg)		1.2	0.4	0.001
	Age (y)		8.1	2.9	0.006
	Sex		24.5	8.9	0.008
		0.13			
66% tibia cortical thickness (mm)	Fluid milk tertiles (servings/d)		0.15	0.07	0.03
	Weight (kg)		0.02	0.005	0.001

[1] Multiple linear regression models were used with a stepwise forward selection including the following covariates (age, height, weight, sex, energy, skin color and physical activity).
[2] MILK intake included milk (plain and flavored milk, milk drinks and recipes with milk), cheese and yogurt.
BMDZ; bone mineral density z-score; ITA: individual topological angle; PA; physical activity; WB: Whole body.

Table 3.5 Bone and muscle parameters of the radius and tibia at baseline, assessed by pQCT

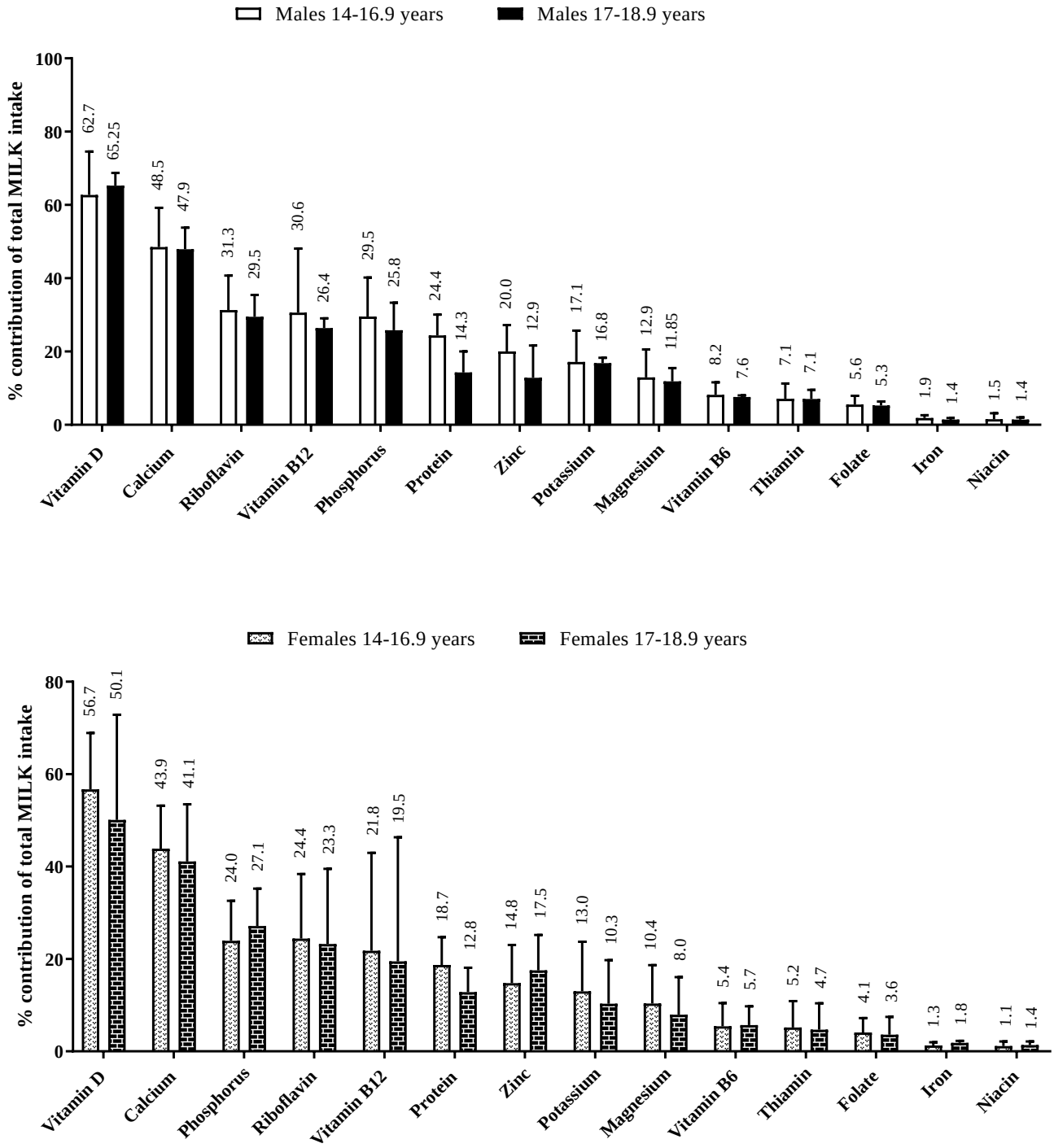
	Males			Females		
	14-16.9 y	**17-18.9 y**	**All**	**14-16.9 y**	**17-18.9 y**	**All**
Radius Geometry						
4% Trabecular density, mg/cm^3	191.6±33.6	227.7±14.9	204.1±33.1	194.5±42.6	190.5±36.8	192.6±39.5
4% Trabecular CSA, mm^2	125.5±32.2	152.5±29.6	134.9±33.4	131.0±17.7	115.3±17.5	123.3±19.1
66% Cortical density, mg/cm^3	1056.3±46.6	1085.2±42.9	1066.8±46.5[1]	1121.0±26.9	1139.9±30.0	1130.6±29.8[2]
66% Cortical CSA, mm^2	73.2±19.9	90.0±11.8	79.3±19.0	74.0±9.6	71.1±10.1	72.5±9.9
66% Cortical thickness, mm	1.5±0.5	1.7±0.5	1.5±0.5	1.4±0.1	1.4±0.2	1.4±0.2
66% Muscle density, mg/cm^3	78.3±1.5	78.8±0.8	78.5±1.3	78.1±1.5	78.3±3.0	78.2±2.3
66% Muscle CSA, mm^2	2871.7±488.3	3766.0±406.1	3196.9±629.7[1]	2362.0±293.5	2205.9±591.7	2282.4±471.8[2]
Tibia Geometry						
4% Trabecular density, mg/cm^3	211.3±46.9	222.6±42.6	220.6±45.3[1]	243.1±35.2	252.2±34.4	248.1±34.7[2]
4% Trabecular CSA, mm^2	413.2±97.4	423.6±57.3	424.9±92.3	408.6±72.3	411.5±55.0	410.2±58.0
38% Cortical density, mg/cm^3	1096.0±32.8[a]	1125.5±43.8[b]	1110.8±40.3[1]	1166.5±22.9[c]	1186.9±20.1[d]	1176.3±23.8[2]
38% Cortical CSA, mm^2	269.8±52.8	301.5±49.2	291.9±61.0[1]	266.7±36.4	264.8±33.5	265.8±34.7[2]
66% Cortical density, mg/cm^3	1043.7±34.4[a]	1074.1±43.9[b]	1059.1±40.2[1]	1115.1±23.8	1132.6±21.9	1124.0±24.3[2]
66% Cortical CSA, mm^2	285.1±59.3	319.9±30.5	307.0±64.5	280.7±38.1	276.6±37.3	278.7±37.4
66% Cortical thickness, mm	3.3±0.5	3.2±0.7	3.4±0.5	3.4±0.5	3.2±0.5	3.3±0.5
66% Muscle density, mg/cm^3	77.0±1.0	75.7±2.9	77.1±1.4	77.4±1.1	76.8±1.3	77.1±1.2
66% Muscle CSA, mm^2	5288.8±1026.0	5692.8±923.4	5791.0±1178.3[1]	5178.8±830.4	5480.2±991.0	5332.3±919.9[2]

Data are expressed as mean ± SD and adjusted for height, weight, physical activity and skin color. Abbreviations: CSA: cross-sectional area
Differences among means for age and sex groups were tested using mixed model ANOVA with lower case letters (^{a,b}) indicating differences among age groups within sex, and numbers (^{1,2}) indicating differences among sexes, $p<0.05$).

Figure 3.1 Usual daily intake of MILK in adolescents 14-18 years of age

Data are median and interquartile range; estimated by food frequency questionnaire.
[a, b] Differences in mean values among groups, based on age and sex, were analyzed using mixed model ANOVA ($p < 0.05$)
Total MILK and fluid milk intake were significantly higher in males than females ($p<0.03$)

Figure 3.2 Percentage contribution of MILK intake to bone-relevant nutrient intakes in adolescents



Data are median and interquartile range; measured by food frequency questionnaire
Dietary folate equivalents.

Supplemental table 3.1 **Adjusted nutrient intakes and percentage of participants with macronutrient intakes within the Acceptable Macronutrient Distribution Ranges, stratified by age and sex**

	Males			Females			
	14-16.9 y (n=17)	**17-18.9 y (n=9)**	**All (n=26)**	**14-16.9 y (n=27)**	**17-18.9 y (n=28)**	**All (n=55)**	**AMDR (% of total energy intake)**
Energy intake (kcal/d)	2080±265	1794±378	1981±331[1]	1714±399	1769±391	1742±392[2]	
Protein intake (g/d)	86.8±10.91 6.7%	81.8±24.9 18.2%	85.1±16.7[1] 17.2%	70.1±13.51 6.4%	75.4±23.5 17.0%	72.8±19.2[2] 16.7%	10-30%
Carbohydrate intake (g/d)	249.6±37.1 48.0%	218.6±37.2 48.7%	238.9±39.4 48.2%	222.1±51.6 51.8%	227.5±43.1 51.4%	225.8±47.2 51.8%	45-65%
Total fat intake (g/d)	78.8±20.5 34.1%	65.7±12.2 32.9%	74.3±18.9[1] 33.7%	63.3±14.7 33.2%	64.8±14.8 33.0%	64.1±14.6[2] 33.1%	25-35%

Data is presented as mean ± SD followed by acceptable macronutrient distribution range (AMDR).
Adjusted 24 h dietary nutrient intakes were obtained using two 24 h food recalls using a subgroup of 26 participants.
[1,2] Differences in mean values among groups, based on sex, were analyzed using mixed model ANOVA ($p < 0.05$)

Bridge Statement 2

Chapter 3 provided new insight into the nutritional adequacy of bone-relevant nutrients and its relationship to bone density and serum biomarkers among adolescents with usual below-recommended MILK consumption. It provided an overall view of the baseline nutrient status of study participants before they started the MILK intervention (intervention will be discussed in detail in the subsequent chapter). A remarkable observation from this analysis was the extent of nutrient inadequacy among this particular group of participants, mainly the female participants and the important contribution of total MILK intake to shortfall bone-relevant micronutrients.

Children and adolescents with habitual low MILK intake are at greater risk of bone fragility and fractures. Therefore, the need to consume adequate MILK intakes at a young age is justified and the effects of MILK intake on bone health has been established by previous studies. However, a high-level evidence is needed to support current guidelines of Canada's Food Guide for the MILK food group (i.e. 3-4 MILK servings/d in adolescents 14-18 y). The following chapter (chapter 4) aims to address these uncertainties by assessing bone density using three different methods, DXA, pQCT and bone health biomarkers to further appreciate the impact of meeting current Canada's Food Guide guidelines on bone health in youth. The primary objective is to assess the impact of increasing MILK intake to age recommendations on bone outcomes in post-pubertal adolescents over 12-mo participation. This following study highlights a research gap in males and add to the existing literature on the positive effect of MILK intake in adolescents. Further, the use of client-centered counseling technique, in this case motivational interviewing techniques, to improve MILK intake and intervention adherence adds to the originality of this study.

CHAPTER 4

Manuscript 2: Milk products' effect on adolescents' bone health

Effects of milk and alternatives on bone properties in 14- to 18-year-old youth: Results at 12 months of a 2-year randomized controlled trial

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ABSTRACT

Background: Adequate nutrition is important for bone health during growth. This study tested whether increasing milk and milk alternatives (MILK) improves bone health in post-pubertal adolescents with usual MILK intake of < 2 servings per day, participating in a randomized trial.

Methods: Participants were stratified by sex and randomized to 1 of 3 groups: Control (receiving no intervention), Improved (IInt: 3 MILK servings /d), or Recommended (RInt: 4 or more MILK servings/d). Baseline and 12-month (mo) measurements included: whole body (WB), lumbar spine, lumbar lateral spine of vertebrae 3, total hip (TH), distal radius bone mineral content (BMC), density (BMD) and BMD z-scores (BMDZ) using dual-energy X-ray absorptiometry (DXA); and bone geometry using peripheral quantitative computed tomography (pQCT). Biomarkers of bone metabolism were also assessed.

Results: Ninety-four adolescents 14-18 y were recruited with a body mass index z-score of 0.3 ± 0.9. At baseline, MILK and calcium intake were not different among study groups. Seventy-six adolescents (80.9%) completed the 12 mo-visit. Compliance to intervention was 70% to 75%. Compared to baseline, females in RInt group had significantly higher calcium and vitamin D intakes ($p< 0.001$). Further, WB BMC and BMDZ and TH BMD and BMDZ significantly increased over time ($p<0.03$) in RInt group and greater percentage increases in WB BMC and WB and TH BMD were observed compared to control and IInt groups ($p<0.05$). None of the DXA measurements differed among male groups at 12 mo. Females in RInt and control groups increased 4% radial trabecular area and cortical density at 66% radius and 38% tibia ($p<0.02$); all male groups significantly increased 66% tibial bone area ($p=0.009$). Intervention effects on pQCT outcomes were not significant in either sex. Bone biomarkers and body composition did not change by time or among groups.

Conclusion: Increasing MILK intake to meet age recommendation favors bone mineral acquisition during late adolescence in females, particularly when calcium intake is low.

4.1 INTRODUCTION

Osteoporosis is a major public health issue, affecting 1.4 million individuals 45 y of age or older in Canada and incurring over $ 4.6 billion in yearly healthcare costs [2]. Maximizing peak bone mass (PBM) during skeletal growth is widely recognized as a primary prevention strategy for osteoporosis and related fratures. Almost 95% of adult bone mass is achieved by late adolescence of which 33 to 46% is acquired over a 5-y period of peak skeletal growth [4]. This developmental period is also characterized by changes in bone geometry (size and shape) and composition (trabecular and cortical), thereby influencing bone strength. Moreover, skeletal mineral accumulation and structural changes during growth varies depending on the bone compartment and skeletal site examined [6].

There is a growing evidence that adequate MILK intake is important in optimizing bone mineral acquisition [79] as it contains significant amounts of calcium, protein, and other bioactive nutrients beneficial for bone health [212]. MILK consumption by Canadian children and adolescents has waned in recent decades. Recent data from the Canadian Dairy Information Centre [213] reported that daily MILK consumption per capita is, on average, 1.65 servings/d. Consequently, adolescents may be consuming insufficient calcium to meet the demands of rapid skeletal growth. Studies tesing the effect of MILK intervention on bone outcomes showed a favorable impact on bone acquisition not only during adolescence [47, 184], but also into the third decade of life [185], reflecting the final stages of growth and consolidation of bone.

Most studies investigating the effect of MILK intake on bone health have used DXA to assess skeletal changes. Recent research shows that MILK intake affects bone geometry and is compartment-specific i.e. affects cortical and trabecular bone differently [22, 31, 32]. pQCT estimates volumetric peripheral BMD and enables the distinction between cortical and trabecular

bone. However, few studies investigating the effects of MILK intake have used pQCT for the assessment of bone health [22, 31, 32]. A recent study found that higher intake of MILK is associated with higher trabecular volumetric BMD (vBMD) of the vertebral bodies in men [214]. In addition to changes in bone properties, bone biomarkers may also be altered during growth. Bone-specific alkaline phosphatase (BAP) increases until mid-puberty and decreases in late puberty. Post-pubertal adolescents tend to have higher concentrations of BAP, osteocalcin and C-terminal telopeptide of type 1 collagen (CTX) than adults, but lower than pubertal children [66, 215].

The primary objective of this study was to test the early effect of improving intakes of MILK using motivational interviewing techniques on LS bone properties as measured by DXA. Secondary objectives of the study were to look at changes in DXA-measured outcomes at WB, TH, FN and distal radius. Finally, this study investigated changes in radial and tibial bone geometry using pQCT, bone biomarkers and body composition following 12-mo of the intervention with MILK.

4.2 METHODS

Study design

This manuscript reports data at 12 mo from a 2-y randomized controlled trial (ClinicalTrials.gov; Trial Registry No. NCT02236871) including healthy adolescents from the Greater Montreal, Canada. The trial was constructed in accordance with CONSORT (Consolidated Standards of Reporting Trials) guidelines for non-pharmacological studies [216], with baseline assessments starting in August 2014 until December 2016.

Adolescents were randomized (allocation ratio 1:1:1) to one of three groups: a control group (control, received no intervention), Improved intervention (IInt; consumed 3 MILK servings/d) or Recommended intervention (RInt: consumed 4 or more MILK servings/d) by a computer-generated list that was stratified by sex and age (14-16.9 y and 17 to 18.9 y) to help control for differences between sexes and maturity. Randomization was done by the registered dietitian who conducted the interventions. Participants were informed of their group at the end of their baseline visit. All other research staff were blinded. At each visit, the dietitian applied the motivational interviewing technique (MInt) as a counseling technique to improve MILK intake and to optimize adherence as modeled by Miller [217]. MInt is an individualized, supportive counseling style where the counselor helps people achieve their goals by enhancing intrinsic motivation for positive change [19]. MInt has been successfully employed to change a number of health behaviors such as weight loss, dietary modification, exercise, and smoking cessation [49, 218]. Assessments occurred at the Mary Emily Clinical Nutrition Research Unit of McGill University (Sainte-Anne-de-Bellevue, QC, Canada) at baseline and every 6 mo for 2 y. At each time-point, anthropometric measures, fasting venous blood samples, sun exposure, physical activity and dietary intake data were collected. Bone outcome measures were obtained at baseline,

12 mo and 24 mo. At baseline, sociodemographic information was also obtained, and all participants received a basic teaching of Canada's Food Guide, specifically the milk and alternatives group [159]. Canada's Food Guide recommends this age-specific population to consume three to four MILK servings/day [e.g., one serving= one cup (250 ml) fluid milk; 50 g (1.5 oz.) cheese; and ¾ cup (175 g) yogurt].

Subjects

Participants were recruited through public and private high schools and colleges, clinics, postal mailings, local advertisements (newspapers, magazines), radio commercials, word of mouth, health and sports organizations as well as social media (Facebook). The participants were healthy with a normal body mass index (BMI) (± 1SD of 50% percentile WHO curves; which aligns with adult targets of 18.5 to 24.9 kg/m^2) [219], 14-18 y of age who consumed < 2 servings of MILK per day. Teenagers with MILK allergy or intolerance, vitamin D deficiency (< 30 nmol/L), anemia, unwilling to stop any nutritional supplements (i.e. calcium and vitamin D) 2 weeks prior to and during the study, or with a preexisting medical condition affecting bone mass were excluded (e.g. asthma, history of fractures). Participants were screened for eligibility by a nurse during the initial telephone call. To determine eligibility, participants or their parents were about the average daily consumption of milk and milk product (including fluid milk, cheese and yogurt). Participant and/or parents provided written consent forms at the start of baseline visit.

Assessments

Anthropometry

Body weight was measured using a standard balance-beam scale (Detecto, Webb, USA) in the fasted state. Height (ht) was measured to the nearest 0.1 cm using a wall-mounted stadiometer (Seca 216, Seca Medical Scales and Measuring Systems, Hamburg, Germany) and used to

calculate height velocity (height at 12 mo minus height at baseline divided by age at 12 mo minus age at baseline). BMI (kg/m^2) was then calculated and z-scores for weight (WTZ), height (HTZ) and BMI (BMIZ) were computed using the WHO AnthroPlus Software version 3.2.2 [219]. Waist circumference (WC) was also measured using a non-stretchable measuring tape (to the nearest 0.1cm) as per Health Canada's guidelines and WC z-scores (WCZ) were calculated using the LMS method based on NHANES III reference curves [205]. All anthropometric measurements were performed by a research-trained nurse and registered dietitian.

Blood sampling and bone biomarkers

Fasting venous blood samples were obtained between 7 and 11 am by a registered nurse, following a 12-hour fast. Immediately thereafter, about 0.1 ml of whole blood was transferred into a capillary tube to measure ionized calcium using a blood gas analyzer (ABL80 FLEX; Radiometer Medical A/S). Plasma samples were immediately centrifuged (1500 g, 15 min at 4ºC) for measurements of CTX, while serum samples were centrifuged 30 min after collection (4000 g, 15 min at 4 ºC) to measure 25-hydroxyvitamin D (25(OH)D), PTH, osteocalcin and BAP. All samples were stored at -80 ºC until biochemical analyses.

Manual enzyme-linked immunosorbent assays were used to measure CTX (SA, Intermedico, Ontario, Canada, measured in nmol/L), resulting in coefficients of variation (CV) of < 9.8 % intra-assay and 9.75 % inter-assay CV. The accuracy using the mid-range of manufacturer specifications was between 90-105%. Serum total 25(OH)D (nmol/l), BAP (µg/l), intact 1-84 PTH (pg/ml), and osteocalcin (ng/ml) were quantified using automated chemiluminescent immunoassays (Liaison, Diasorin, Ontario, Canada). The CV of internal standards for 25(OH)D were 4.8 % (39.6 ± 1.9) for low controls and 3.8% (123.9 ± 4.7) for high controls. Serum BAP, PTH, and osteocalcin had the following CV and accuracies: BAP (intra-assay CV < 2.7%, inter-

assay CV < 4.6 %, accuracy: 90-98%), PTH (intra-assay CV <3.6%, inter-assay CV 4.4%, accuracy 102–104%), and osteocalcin (intra-assay CV <4.4%, inter-assay CV 7.1%, accuracy 96-101%). Other quality control measures included participating in the Vitamin D External Quality Assurance Scheme; and using the National Institute of Standards and Technology (NIST) guidelines. Control samples from the NIST resulted in inter-assay CV of 3.4 % and accuracy of 104.2 %.

Bone assessments

DXA is the preferred method for assessing bone mineral content (BMC) and areal bone mineral density (aBMD) in the pediatric population [220]. In accordance with the International Society for Clinical Densitometry [221] and common sites of fracture in adolescents [222], BMC and aBMD for WB, LS, FN, TH, distal one-third radius were measured at baseline and 12 mo using DXA [Hologic Discovery A fan beam with APEX software (version 13.3:3), Hologic Inc, Bedford, MA, USA]. DXA scans were also performed at lateral lumbar spine of vertebrae 3 (LLS3) because this region reflects trabecular bone of the vertebral bodies [223]. All scans were completed with the participants wearing light clothing and no jewelry or metal. Longitudinal stability of DXA was monitored using a LS phantom no. 14,774; CVs were 0.5% for BMC and 0.3% for BMD. Global standard deviations for the low-air and high-air measures of radiographic uniformity were always <2.0.

In order to assess skeletal geometry and muscle variables, pQCT (XCT-2000; Stratec, Pforzheim, Germany) scans of the nondominant radius and tibia were performed by the same operator at baseline and 12 mo. pQCT enables separate measurement of trabecular and cortical bone compartments which may allow earlier detection of changes in bone mass in response to intervention [166]. To date, there are no standard pQCT methods for pediatric assessments [25];

thus, our methods were based on the protocol used by the Canadian Multicenter Osteoporosis Study (CaMOS) [224]. A scout view was carried out before the CT scan to position the scanner at the reference line of the limb being measured. The reference line was placed at the most proximal line of the growth plate if the latter was still open and through the middle of the ulnar border of the articular cartilage if closed. For the radius, forearm length was measured from the olecranon process to the tip of the ulnar styloid process. Scans were obtained at the 4% and the 66% radius site (defined as percentage of the radial length from the distal to the proximal end). For the tibia, the length of the tibia was measured between the inferior border of the medial malleolus and the medial tibial plateau. Scans at 4%, 38% and 66% of the tibial length proximally form the distal tibial end were obtained. A single tomographic slice of 2.0 mm thickness was taken at a voxel size of 0.2 mm with a scan speed of 10 mm/s for both the radius and the tibia. The scans were analyzed using contour mode 2 (45%) and peel mode 1 to assess total and trabecular bone parameters at the 4% site. At the 66% site, cortical bone is detected with separation mode 1 and a threshold of 710 mg/cm^3. Radial and trabecular cross-sectional area (CSA) was determined after detecting the outer bone contour at a threshold of 280 mg/cm^3.

Diet assessment and compliance

Dietary intake of adolescents was assessed by a registered dietitian using single-day 24 h food recalls which were completed at each visit at baseline, 6, 12, 18 and 24 mo. These recalls reflected dietary intake consumed during the day prior the visit. Two additional 24 h recalls were conducted over the phone at 1 and 3 mo for educational purposes and to help achieve the goals of the group targets for MILK intakes. Food items were entered and analyzed in the Nutritionist Pro software (Axxya Systems, Woodinville, WA, USA) using the Canadian Nutrient File 2010b. An average of the four days from food recalls conducted at 1, 3, 6 and 12 mo was used to estimate the

dietary intakes of the adolescents over the course of the study. Specifically, mean values for macro- and micronutrients of interest (i.e. energy, protein, calcium and vitamin D).

Although 4 d of 24-h intake assessments may be sufficient to measure macronutrient intakes, to represent usual intakes of certain micronutrients, a longer-term assessment provides better estimates for adolescents aged 16 y of age and older [225, 226]. Thus, to complement actual dietary intake collected with 24-h recall, usual dietary intake over the past y was captured using a validated semiquantitative, food-frequency questionnaire (FFQ) [209] at baseline and 12 mo. Participants and/or parents were provided with a pre-stamped addressed envelope at the end of the visit and were asked to mail back completed FFQs to the study center. The FFQ collects information on the intake of 131 food items with nutrient output derived using the Canadian Nutrient File. It has been validated for English and French speaking adults [155]. Estimates for MILK intake (servings/d) for both the FFQ and food recall were calculated according to Canada's Food Guide [227] (e.g., 1 serving is equivalent to 250 ml of milk, 50 grams or 1½ ounces of cheese, 175 grams of yogurt) and the mean values of these four time points were also computed. The average of four 24 h dietary recalls was used to assess compliance of participants to the MILK intervention at 6- and 12-mo of the study. Participants were classified as always compliant if the mean MILK intake met the allocated MILK servings, exceeding compliance if the mean MILK intake exceeded the allocated servings or non-compliant within their trial group when their MILK intake fell below the allocated servings.

MInt and profile of mood state

At each visit, MInt counseling techniques were employed among participants in the intervention groups to assess their motivation/readiness to change and to help increase their intrinsic motivation to adhere to allocated MILK servings. There is accumulating evidence that

behavior change is influenced by mood [228]. To explore that, the profile of mood state for adolescents (POMS-A) questionnaire was obtained from participants at baseline and yearly. POMS-A is a 24-item questionnaire that assesses six mood subscales (anger, confusion, depression, fatigue, tension and vigor). Participants were asked to rate `How are you feeling right now' in terms of the 24 mood adjectives such as `sleepy' and `panicky'. Responses were provided on a scale from 0 ('not at all') to 4 ('extremely').

Demographic characteristics, physical activity, skin pigmentation, and UV-B exposure

At baseline, self-reported sociodemographic questionnaires were obtained from the parent/participants. This self-reported questionnaire provided information about parental occupations, cultural and ethnic origins, highest education completed and gross income. At each visit, physical activity was captured using the Youth Physical Activity Questionnaire (YPAQ) which reflects activities performed the week prior to the study visit [206]. Data regarding sun exposure 30 d prior to the visit, frequency of sunscreen use, and hours spent in direct sunlight per day based on the Canadian Health Measures Survey was obtained [229]. To compliment the Canadian Health Measures Survey, skin type was identified using a spectrophotometer (CM-700d/600d; Konica Minolta) that measured pigmentation at the inner upper arm, forehead, mid-forearm, and lower leg. Changes in pigmentation over time allows for an estimation of exposure to ultraviolet B radiation. The individual typological angle (ITA) was then calculated by using the L*a*b* colorimetric system of the Commission Internationale de I'Eclairage and then classified into one of 6 skin types according to the Fitzpatrick scale [207].

Ethics

Ethics approval was obtained from McGill University Faculty of Medicine Institutional Review Board, Lester B. Pearson School Board and the English Montreal School Board.

Sample size calculation

Initially a sample size of 270 adolescents was calculated using the longitudinal data from the CaMOS study of youth. The CaMOS study started at 16 y of age and demonstrated that LS in particular had increments in BMD into the 4th decade (i.e. > 30 y) [6]. This sample size estimate provided 80% power at a 5% significance level (two-sided) to detect a mean change of 5% in LS 1-4 BMD over 2 y (or 2.5%/y). However, data on WB BMD was not available from that study and the study began at 16 y of age whereas the CFG dietary recommendations for teens begin at 14 y of age, consistent with our entrance criteria (14.0 to 18.9 y) [6]. After the pilot phase of the trial, data analysis suggested that in males the increments in LS BMD were much greater (13%/y) than 2.5%/y originally used in the calculations. As a result, the sample size was re-estimated using preliminary data from the trial. Accretion rate for WB BMC, instead of LS BMD, was used to re-calculate the sample size estimates. Based on a difference of 199.5 g/y for reaching 4 servings/d of MILK compared to 51.2 g/y for the control with SD of 132.4 g/y, power of 80% and alpha of 0.05, a sample of 13 per sex per group would be required i.e. 78 in total. With a 20% drop out rate, the overall sample size target would thus be 94.

Statistical Analysis

Data analyses were conducted using SAS (version 9.3, SAS Institute, Cary, NC, USA). Differences at baseline among groups for continuous variables (body composition, anthropometry, bone biomarkers and bone outcomes) were assessed using mixed model ANOVA. Fisher's exact tests were used for differences in proportions (ethnicity, income, education). Due to sex-related differences in growth velocity, separate analyses were performed for males and females. Data were tested for normality by using the Kolmogorov-Smirnov test and homogeneity of variance using the Bartlett test. Non-normal data were log-transformed where applicable [e.g. 25(OH)D]. A

mixed-model ANOVA was used to determine if there were significant effects of group, time or group-by-time interactions for bone outcomes, accounting for fixed effects (group and age) and random effects (e.g. demographic characteristics, body composition), with Tukey *post hoc* testing where necessary. Dietary data were also analyzed by mixed model ANOVA for mean dietary intakes from 1-, 3-, 6- and 12-mo food recalls and compared among groups for differences for total energy, macronutrient, dietary calcium, and vitamin D intakes as well as food group servings. Participants who withdrew or didn't comply with the allocated servings were transferred to intent-to-treat analysis, which included all randomized study participants in the groups to which they were randomized, independently of receiving the allocated treatment or having dropped out of the study. Spearman's partial correlation coefficients of mood subscales with MILK intake at 6 mo were generated to determine the degree of association between mood at the start of the study and adherence to MILK intervention. All analyses were performed as intent-to-treat and presented as mean ± SD, unless otherwise stated. A p-value of < 0.05 was considered significant.

4.3 RESULTS

Demographic characteristics

Ninety-four adolescents (30 males and 64 females with a mean age of 16.6 ± 1.5 y) completed the baseline visit **(Figure 4.1)**. No differences were observed in baseline characteristics **(Table 4.1)** among allocation groups with exception of age at menarche. Participants were primarily white (75%). No significant differences were seen among all groups in weekly hours engaged in physical activity at baseline (419.8±296.4 min/wk) nor at 12 mo (544.7±414.6 min/wk) **(Table 4.1)**. At baseline, z-scores of height and BMI were not significantly different among groups. No differences in WB FM and LM were observed among groups at baseline (**Table 4.1**) or over time (data not shown). Neither male groups nor female groups were different in terms of their WCZ. Height velocity and HTZ change did not differ within groups of the same sex. Seventy-six (80.9%) adolescents completed the 12 mo follow-up visit. The main reasons for not completing the study (n=18) were lack of time or interest (n=11), perceived lactose intolerance (n=2) and loss to follow up for unknown reasons (n=5); these participants did not differ in terms of anthropometry or socioeconomic status compared to those who completed the study at 12 mo.

Dietary characteristics

There were no differences at baseline among intervention groups for dietary intakes of nutrients (i.e. energy, protein, calcium and vitamin D) and MILK servings based on 24 h recall and 12-mo FFQ data from the year prior to joining the study **(Table 4.2).** At 12 mo, dietary data derived from four 24 h recalls (1, 3, 6 and 12 mo) showed that intervention groups increased MILK intake and consequently calcium, but not total energy intake in both males and females **(Table 4.2)**. Only female intervention groups reported increased protein intake compared to control **(**91.4±17.5 vs 67.5±12.3 g/d, p=0.018). In contrast, FFQ data after 12-mo showed that only female

intervention groups increased MILK intake significantly, with calcium and vitamin D intake being greater in the female RInt group compared to control **(Table 4.2)**. Energy intake did not differ by groups or by time.

Estimates of compliance for MILK intake at 6 mo in the intervention groups were distributed as follows: in males, 25% were not compliant, 46% always complied and 29% exceeded allocated MILK intake, with no differences among groups (p=0.42). In females, 32% were non-compliant, 42% always complied and 26% exceeded the MILK target, with no differences among groups (p=0.41). At 12 mo, 15% of males did not comply, 58% did and 27% exceeded target MILK intakes. Similarly, in females, 28 % were not compliant, 54% always did and 18% reported exceeded MILK intakes. Correlation between mood state at baseline and MILK intake at 6 mo revealed a significant association between "vigor" and MILK intake in females RInt group (r=0.47, p=0.04).

Biochemical assessments

Mean concentrations of 25(OH)D, total osteocalcin, BAP and CTX were not different among groups at baseline. Further, baseline PTH concentration and ionized calcium did not differ among allocated groups. At 6 and 12 mo, 25(OH)D, osteocalcin, BAP, CTX and PTH did not vary among groups of the same sex or compared to baseline (**Supplemental Table 4.1**). Average ionized calcium was within normal limits (1.15–1.38 mmol/L) and did not vary by time or among sex-stratified intervention groups. No overall significant differences (group x time) were found for urinary calcium (mg/dl): creatinine (mg/dl) ratio among intervention groups for both sexes.

Bone assessments

At baseline, male groups did not differ for any DXA-measured bone outcome except for FN BMDZ where values in RInt were greater compared to IInt (p=0.005). From baseline to 12 mo,

bone area in males only varied by time for LS area in RInt group (baseline: 63.29±11.23 vs 12-mo: 67.32±10.22 cm^2, p=0.003). In males, BMC of WB, LS, 33% radius improved significantly (p<0.02) over the study period among all 3 groups. Mean values for BMD at all skeletal sites and WB BMD z-score were higher at 12 mo compared to baseline values. However, no significant intervention effect for any of the bone outcomes were observed among the male intervention groups at 12 mo (**Table 4.3**).

In females, there were no differences in any of the bone area measures among the intervention groups. LS 1-4 BMC, BMD and BMDZ did not vary by time and were lower in IInt compared to RInt at both baseline and 12 mo. In contrast, WB BMC and BMD were greater at 12 mo compared to baseline in RInt group and the mean values for WB BMC and BMDZ were greater in RInt compared to control and IInt at 12 mo (p<0.01) **(Table 4.4)**. Furthermore, females in the RInt group had greater WB BMC accretion rate and percentage increases of WB BMC and BMD over 12-mo compared to control and IInt groups (p<0.05) **(Table 4.5).** Likewise, compared to baseline, 12-mo values of TH BMD and BMDZ in RInt significantly increased by time (p<0.03) **(Table 4.4)** and greater percentage increases in TH BMD were observed compared to the control group (p=0.03) **(Table 4.5)**. No significant interactions of group by time were found in any of the bone measures for LLS, FN and 33% radius.

With regard to bone geometry, females in RInt and control groups increased radial trabecular area and cortical density at 66% radius and 38% tibia (p<0.02); males in the RInt group significantly increased tibial cortical density at 38% (p=0.02). However, the effect of MILK intervention on bone geometry was not significant for any of the study groups (**Supplemental Table 4.2**).

4.4 DISCUSSION

The present study investigated multiple bone health outcomes using DXA, pQCT and bone biomarkers over a 12-mo MILK-based intervention in healthy 14 to 18-y-old adolescents with usual MILK consumption below age recommended levels. In this study, the beneficial effect of increasing MILK intake was noticeable at the WB and TH in females who were allocated to consume 4 or more MILK servings/d compared to those in the control group. However, the predicted increase in LS BMD was not observed after the 12-mo MILK intervention period. Such finding does not corroborate the results of previous work among females of comparable age [18]. The main difference from the present report is the shorter duration of follow-up (2 y vs 1 y). Since the rate of accrual of BMD decelerates with age, it is highly probable that longer periods of follow-up are desirable to reveal the benefit of MILK intake on changes in BMD. It is also possible that the effect of MILK intervention is bone site-specific, acting faster on certain sites than other. An area of research that requires further investigation.

Overall, compliance among study participants ranged between 70 and 75%. The use of MInt techniques to improve MILK intake resulted in significant increases in MILK intake among intervention groups. This finding corroborates results of a dietary and exercise intervention among adolescents 11-18 y where MInt resulted in lower attrition and better adherence to obesity treatment over a 6-mo period [50]. In the present study, mood also seemed to play a role in adherence to intervention, and that the ability to increase MILK intake was explained by feelings of vigor. Morgan et al. found that the elite athletes who scored higher for vigor prior to a competition were the ones who performed better during the competition [230].

With regard to males, although improved from baseline, none of the bone outcomes differed among intervention groups by the end of the 12-mo study period. Several reasons may

explain this. One main reason is that the effect of MILK intervention in males may have been masked by growth patterns over 12 mo, as reflected by height velocity of 3.6 ± 3.5 cm/y vs 0.4 ± 0.7 cm/y in females. Second, males had high baseline calcium intake (average intake of 1052± 404 mg/d, n=26), mainly attributed to increased overall food intake, which may have limited the effect of MILK intervention on bone accrual. In a recent systematic review on predictors of PBM in youth [4], the MILK intervention trials that had the greatest benefit on WB bone mineral acquisition was in those with a baseline calcium intake of 455 mg/d [180].

In the present study, pQCT together with DXA was used to evaluate the effect of increased MILK intake on bone and muscle geometry. As a three-dimensional technique [166], pQCT enables in-depth examination of bone properties and bone-muscle interactions. To the best of the authors' knowledge, this study is the first to investigate the effects of MILK intake on bone geometry and muscle properties in late adolescence. However, no differences among groups were detected following the 12-mo of study. The null findings might have resulted from the measurement error that is associated with the inherent difficulty of repeated measures in growing adolescents or that any differences among groups observed in the DXA-based assessments were size-dependent and not ascribed to true changes in density. Another explanation, especially in the male group, could be the small sample size. Only 26 male participants completed the 12-mo visit which represented 72% of the estimated male sample size originally set for this study (n=36). Thus, it is largely possible that such a sample size was inadequate in terms of statistical power for detecting BMD differences among male groups over 12 mo, with a high likelihood of type II error.

Regarding the effect of the MILK intervention on bone biomarkers, no significant differences among study groups were observed in both bone formation and resorption biomarkers by 12 mo among all study groups. These results agree with comparable trials [18, 47, 180, 184].

Previous studies demonstrated higher concentrations of bone biomarkers in early puberty compared to advanced puberty [68, 69], with peak concentrations observed between 10 and 12 y of age in females [69] and 13 and 15 y of age in males [68], and those between 16 and 18 y having the lowest concentrations in both sexes. A recent systematic review that looked at the effects of MILK consumption on bone properties proposed evaluating bone biomarker changes over periods less than 6 mo due to their sensitivity to a variety of factors as puberty, growth and others [79]. Previous intervention trials with antiresorptive agents showed that bone biomarkers changes occur as early as two weeks and reach a plateau within 3 to 6 mo [79]. This indicates that markers of bone turnover are likely to change over short time period, i.e. hours or days, versus months or a y and may explain why no differences were observed at the intervals tested in the present study.

In this trial, the intervention did not influence any measures of body composition and did not lead to a difference in weight gain despite the freedom to consume milk products of any fat content. Throughout the intervention period, dietary calcium and vitamin D intake were significantly greater in participants randomized to the RInt intervention group compared to control, whereas total energy intake did not vary. These results indicate that participants in the intervention group integrated MILK intake into their diet rather than adding it. This is interesting as sometimes milk products are avoided, particularly in female adolescents, for fear of gaining weight [136]. More interestingly, females in the RInt group were able to increase their calcium intake to meet the dietary recommendation allowance for their age over the 12-mo period. Specifically, percentage of females meeting calcium RDA in RInt group increased from 7% at baseline to 30% at 12 mo compared to 7% to 8% in control group, p=0.0009). These results are promising; adolescents were able to meet their age recommendations for MILK and calcium intakes, all while maintaining their mean energy intakes and a healthy body composition.

The present study is novel by its inclusion of young males and the use of the motivational interviewing technique in lieu of provision of milk products. Additional strengths of the study are embodied in the inclusion of measures of bone quality, assessed by pQCT, in addition to DXA bone measures at multiple skeletal sites (WB, LS1-4, LLS3, TH, FN and 33% distal radius). As with all studies, this study also presents with limitations. This study had a lengthy recruitment period (2 y) and the majority of the participants were white. Study visits, particularly at baseline, were quite lengthy due to the multiple bone scans and questionnaires collected which might have been a reason behind the high attrition rates (19%). Dietary intakes are self-reported which can lead to under- or over-reporting, particularly those done over the phone and at screening. Another limitation is the use of single-day 24 h recalls to assess MILK intake which may fall on an atypical day and thus is subject to under- or over-reporting error. Additionally, due to school schedules, most of the visits occurred on a weekend day and thus the majority of the 24 h recalls were recounts of Fridays and Saturdays, which are days that may have atypical dietary intakes.
Furthermore, the inability to blind the MILK intervention was another potential limitation.

In summary, the consumption of 4 or more MILK servings/d improved bone accrual or density at the WB and TH and increased calcium and vitamin D intakes, particularly in females. The present study provides high-level scientific evidence in support of the beneficial effects of consuming 4 or more MILK servings/d on bone mineralization during the consolidation process. It would be of interest to pursue multiple follow-ups after trial completion to determine whether the positive MILK effects are sustained or improved and whether self-selection of a high MILK diet is achievable. Future longer-term trials with larger sample sizes are necessary, particularly in male adolescents.

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Table 4.1 Participant baseline characteristics according to randomization

Variable	Males			Females		
	Control (n=9)	**IInt (n=10)**	**RInt (n=11)**	**Control (n=20)**	**IInt (n=22)**	**RInt (n=22)**
Age (years)	15.8 ± 1.8	16.1± 1.7	16.0± 1.7	16.8± 1.4	16.9±1.5	16.9 ± 1.4
Ethnicity, White (%, n)[1]	67% (6)	80% (8)	91% (10)	70% (14)	68% (15)	68% (15)
Education, college or more						
Mother (%, n)	78% (7)	40% (4)	73% (8)	70% (14)	59% (13)	82% (18)
Not disclosed (%, n)	11% (1)	40% (4)	19% (2)	25% (5)	18% (4)	9% (2)
Family Income,						
>$75,000/y (%, n)	33% (3)	40% (4)	36% (4)	40% (8)	41% (9)	32% (7)
Not disclosed (%,n)	44% (4)	60% (6)	45% (5)	35% (7)	45% (10)	41% (9)
Age at menarche (y)	N/A	N/A	N/A	11.6±1.2[a]	12.5±1.6[ab]	12.8±1.4[b]
Weight (kg)	59.6±11.0	55.8±8.3	63.1±18.6	59.1±10.0	60.4±11.9	60.6±11.0
Height (cm)	168.8±8.0	170.6±11.4	170.4±14.9	162.5±7.2	163.4±6.1	165.9±7.2
Height z-score	-0.14±0.8	0.10±1.1	-0.02±1.3	-0.02± 1.1	0.1±1.0	0.5±1.1
BMI (kg/m^2)	20.8±2.5	19.1±1.2	21.2±3.5	22.3±3.2	22.6±4.2	22.0±3.2
BMIZ	0.1±0.6	-0.6±0.5	0.1± 1.0	0.4±0.9	0.4±1.0	0.2±0.9
WC (cm)	74.3±7.2	72.4±3.8	75.3± 11.6	74.7±8.7	75.6±9.3	73.9±7.2
WCZ	0.2±0.01	0.2±0.00	0.2± 0.02	0.2±0.03	0.2±0.02	0.2±0.02
Whole body fat mass (kg)[2]	10.4±2.7	9.1±12.85	9.8±5.3	41.8±3.1	17.4±8.0	16.0±4.9
Whole body lean mass (kg)[2]	46.8±8.7	45.0±8.4	50.8±14.1	16.8±7.9	40.8±5.8	42.1±6.3
Physical activity (min/week)	573.0±438.1	572.5 ± 417.1	446.4± 270.5	353.8±209.1	349.6±244.8	404.7±275.5

Data are Unadjusted mean ± SD; Abbreviations: IInt: Improved intervention group; RInt; Recommended intervention group
BMI: Body mass index (kg/m^2); BMIZ: Body mass index z-score; WCZ: waist circumference z-score
p-values were derived by testing for differences among groups with the use of a mixed-model ANOVA or Chi-square test
[1] Non-white includes Asian (n = 20), African or black (n = 5), Hispanic (n=1); [2] Assessed by dual-energy x-ray absorptiometry.
[a, b] Different superscript letters denote significant differences among groups at each time point

Table 4.2 Intakes of energy, protein, calcium and vitamin D across the 12-mo study

	Males			Females		
Variable	**Control**	**IInt**	**RInt**	**Control**	**IInt**	**RInt**
24 h dietary recall						
Energy, kcal/d						
Baseline	2218±685	2318±389	2476±839	1671±632	2036±842	1853±652
6 mo	2249±742	3504±1591	2560±761	1618±567	2357±562	2073±555
12 mo	2530±1059	3090±1032	2808±1031	1808±415	1803±567	2315±663
Average over 12 mo*	2103±297	2507±568	2538±654	1604±272	2133±472	2057±356
Protein, g/d						
Baseline	77.6±18.7	102.7±20.8	110.5±39.5	61.4±24.5	82.4±50.4	74.2±28.3
6 mo	106.6±33.5	145.6±36.3	129.6±47.6	60.7±22.3	92.7±24.5	95.6±24.3
12 mo	107.0±60.8	128.4±41.3	133.4±52.7	81.8±26.4	77.8±33.5	103.8±30.1
Average over 12 mo	94.4±13.9[a]	111.4±11.1[b]	121.9±34.4[b]	67.5±12.3[a]	90.9±23.6[ab]	91.4±17.5[b]
Calcium, mg/d						
Baseline	979.4±416.1	1128.1±624.9	958.5±584.8	705.3±445.4	884.4±532.1	760.8±433.1
6 mo	561.6±224.1[a]	1967.3±787.4[b]	1719.8±787.4[b]	877.0±389.0[ab]	1317.8±294.6[ab]	1363.2±443.7[b]
12 mo	1151.4±526.9[a]	1719.4±685.7[b]	1790.5±563.0[b]	944.4±406.5[ab]	1067.1±483.5[ab]	1458.3±317.6[b]
Average over 12 mo	876.6±110.8[a]	1501.3±323.7[b]	1412±310[b]	813.6±196.5[a]	1178.4±232.9[b]	1261.9±253.1[b]
Vitamin D, IU/d						
Baseline	220.7±151.0	191.1±128.5	126.3±93.0	92.1±92.6	104.2±112.4	131.5±119.4
6 mo	118.7±102.7	373.9±239.4	332.2±129.3	184.6±130.0	276.9±129.1	284.9±120.4
12 mo	305.2±261.2	358.8±200.3	307.6±105.9	224.0±138.8	211.1±149.8	293.7±127.7
Average over 12 mo	196.3±63.6	324.8±89.4	286.1±138.0	161.0±73.7	228.8±83.0	251.0±69.0
MILK, servings/d						
Baseline	1.9±1.6	2.3±1.7	1.5±1.3	1.2±0.9	1.3±1.3	1.2±0.9
6 mo	0.8±1.1[a]	4.5±1.7[b]	3.9±1.5[b]	1.2±1.0[a]	2.9±1.5[ab]	3.6±1.4[b]
12 mo	2.0±1.1	3.4±1.3	3.8±0.7	1.7±1.3[a]	2.7±1.7[ab]	3.7±1.1[b]
Average over 12 mo	1.6±0.5[a]	3.9±0.9[b]	3.5±0.7[b]	1.8±0.8[a]	3.1±0.9[b]	3.6±0.9[b]

Table 4.2 Continued

Food Frequency Questionnaire						
Energy, kcal/d						
Baseline	1947(1675,2307)	1805(1522, 2063)	2009(1484, 2414)	1815(1458,2122)	1466(1129,1796)	1488(1109,1964)
12 mo	2047(1897,2979)	2129(1722, 2231)	1897(1486, 2658)	1544(1385,2054)	1728(1355,2015)	2025(1437,2217)
Protein, g/d						
Baseline	82.3(75.5,87.4)	72.3(62.0, 81.3)	97.8(60.2, 126.1)	81.1(55.8,87.3)	61.9(45.9,75.3)	54.3(45.5,86.1)
12 mo	94.2(89.8,97.6)	92.4(69.6, 105.3)	95.4(53.1, 114.6)	67.9(59.4,82.3)	78.1(70.7,91.7)	88.8(70.3,110.2)
Calcium, mg/d						
Baseline	813.6(749.6,1083.8)	1093.3(824.0, 1223.5)	1147.1(756.6, 1531.6)	775.8(646.0,925.8)	641.3(483.4,798.6)	689.5(493.3,1126.0)
12 mo	1042.2(890.2,1264.8)	1248.1(1071.8,1448.5)	1179.7(917.0,1786.1)	909.7(622.4,1137.1)[a]	1062.7(953.5,1304.8)[ab]	1311.6(936.1,1665.3)[b]
Vitamin D, IU/d						
Baseline	220.0(171.9,297.8)	221.2(182.3,249.2)	190.6(120.1,357.8)	145.9(101.6,237.0)	127.1(106.7,196.6)	180.0(114.4,267.3)
12 mo	267.8(215.9,319.5)	317.2(251.8,353.7)	309.9(271.4,385.4)	179.2(131.3,243.9)[a]	321.9(302.3,369.1)[ab]	373.9(275.9,461.2)[b]
MILK, servings/d						
Baseline	1.8 (1.6, 2.7)	2.3(1.6, 2.9)	1.2 (1.0, 3.8)	1.1(0.6,1.8)	1.0(0.4,1.4)	1.5(0.6,2.0)
12 mo	1.7 (1.3, 2.4)	3.3(2.7, 3.5)	2.8 (2.3, 3.6)	1.5(1.1,2.1)[a]	2.7(1.4,3.2)[b]	3.9(2.8,4.4)[b]

Data are mean±SD or median and interquartile; Abbreviations: MILK: milk and alternatives intake estimated as per Canada's Food Guide;

* derived from the average of 4 one-day food recall at 1,3,6 and 12 mo.

[a, b] Different superscript letters denote significant differences among groups at each time point

Table 4.3 Characteristics of male participants at baseline and 12-mo assessed by DXA

	Control		Improved		Recommended		p-value		
	Baseline	12-mo	Baseline	12-mo	Baseline	12-mo	Group	Time	Group *time
Whole Body									
BMC (g)	2203.85±501.68[a]	2456.25±448.77[b]	2170.36±550.00[a]	2423.53±572.68[b]	2547.03±824.64[a]	2750.43±854.83[b]	0.26	<.0001	0.67
BMD (g/cm^2)	1.117±0.117[a]	1.183±0.109[b]	1.105±0.113[a]	1.157±0.128[b]	1.209±0.197[a]	1.261±0.219 [b]	0.18	0.01	0.19
BMDZ	0.466±1.176[a]	0.689±0.995[b]	0.220±0.805[a]	0.337±0.913[b]	1.209±1.368[a]	1.400±1.585[b]	0.13	0.005	0.43
LLS									
BMC (g)	1.58±0.37	1.53±0.47	1.74±0.77	1.71±0.82	1.98±0.49	1.84±0.56	0.52	0.42	0.89
BMD (g/cm^2)	0.713±0.097	0.770±0.089	0.657±0.168	0.692±0.212	0.821±0.104	0.843±0.125	0.10	0.08	0.94
Lumbar spine									
BMC (g)	51.49±14.29[a]	59.19±12.39[b]	51.74±17.8[a]	57.63±19.55[ab]	61.84±23.40[a]	67.32±10.22[b]	0.32	0.0003	0.09
BMD (g/cm^2)	0.843±0.140[a]	0.928±0.113[b]	0.817±0.166[a]	0.858±0.181[ab]	0.945±0.216[a]	0.994±0.222[b]	0.20	0.002	0.07
BMDZ	-0.533±1.474	-0.344±1.266	-1.070±1.016	-1.212±1.191	0.145±1.276	0.133±1.330	0.09	0.12	0.34
33% distal radius									
BMC (g)	1.77±0.42[a]	1.95±0.37[b]	1.81±0.36[a]	1.98±0.36[ab]	2.02±0.45[a]	2.16±0.42[b]	0.13	0.0001	0.58
BMD (g/cm^2)	0.679±0.117[a]	0.720±0.105[b]	0.671±0.079[a]	0.697±0.008[b]	0.717±0.118[a]	0.750±0.123[b]	0.48	<.0001	0.59
BMDZ	-0.144±1.629	-0.044±1.628	-0.500±0.819	-0.625±0.889	0.300±1.803	0.389±1.683	0.33	0.67	0.66
Total Hip									
BMC (g)	33.25±6.74	38.22±6.36	33.38±10.18	38.96±9.95	41.23±13.83	45.79±14.36	0.09	0.16	0.34
BMD (g/cm^2)	0.967±0.133[a]	1.034±0.133[b]	0.902±0.164[a]	0.992±0.167[ab]	1.060±0.227[a]	1.115±0.235[c]	0.11	0.001	0.61
BMDZ	-0.089±1.155	0.156±1.056	-1.044±1.143	-0.443±1.314	0.545±1.513	0.622±1.624	0.08	0.26	0.53
Femoral Neck									
BMC (g)	4.29±0.70	4.50±0.82	4.20±0.89	4.61±0.93	5.04±1.38	5.39±1.43	0.08	0.30	0.32
BMD (g/cm^2)	0.833±0.100[a]	0.890±0.115[b]	0.780±0.125[a]	0.845±0.136[ab]	0.933±0.192[a]	0.978±0.202[c]	0.06	0.007	0.96
BMDZ	-0.444±0.991[ab]	-0.200±0.960[ab]	-1.322±0882[a]	-0.929±1.110[a]	0.209±1.451[b]	0.355±1.561[b]	0.02	0.43	0.83

Values are unadjusted means ± SDs. Abbreviations: BMC, bone mineral content; BMD, bone mineral density; BMDZ; BMD z-score; DXA, dual-energy X-ray absorptiometry; LLS: Lateral lumbar spine of vertebrae 3

[#]p-values are shown for differences between baseline and 12 mo, differences among groups, and differences among groups by time in each variable.

A mixed-model ANOVA was used and adjusted for age strata, skin color, height velocity, weight and length of study.

[a, b, c] Different superscript letters denote significant differences among groups at each time point.

Table 4.4 Characteristics of female participants at baseline and 12-mo assessed by DXA

	Control		Improved		Recommended		p-value		
	Baseline	12-mo	Baseline	12-mo	Baseline	12-mo	Group	Time	Group *time
Whole Body									
BMC (g)	2185.78±330.34[a]	2327.57±269.08[cd]	2133.81±281.11[a]	2186.70±337.44[ac]	2367.5±384.95[b]	2509.24±333.67[d]	0.004	<.0001	0.03
BMD (g/cm^2)	1.155±0.096[ab]	1.193±0.094[ab]	1.129±0.078[a]	1.136±0.086[a]	1.205±0.101[b]	1.242±0.090[c]	0.002	0.005	0.09
BMDZ	1.1±1.0[ab]	1.4±1.2[ab]	0.8±1.0[a]	0.7±1.1[a]	1.7±1.1[b]	2.0±1.0[c]	0.001	0.50	0.03
LLS									
BMC (g)	1.76±0.49	1.65±0.52	1.71±0.57	1.81±0.55	1.97±0.56	2.01±0.65	0.07	0.33	0.25
BMD (g/cm^2)	0.735±0.088[a]	0.751±0.103[a]	0.750±0.186[a]	0.735±0.148[a]	0.840±0.114[b]	0.877±0.136[b]	0.007	0.41	0.74
Lumbar spine									
BMC (g)	57.75±9.16[ab]	61.54±8.93[ab]	54.27±8.67[a]	55.50±10.56[a]	63.29±12.74[b]	65.76±11.93[ab]	0.003	0.43	0.38
BMD (g/cm^2)	0.987±0.010[ab]	1.021±0.084[ab]	0.945±0.100[a]	0.937±0.113[a]	1.045±0.123[b]	1.075±0.111[b]	0.001	0.19	0.60
BMDZ	0.0±1.0[ab]	0.2±0.8[ab]	-0.4±1.0[a]	-0.6±1.1[a]	0.6±1.1[b]	0.7±1.0[b]	0.001	0.20	0.67
33% distal radius									
BMC (g)	1.68±0.44	1.70±0.21	1.68±0.16	1.69±0.18	1.73±0.24	1.82±0.22	0.38	0.85	0.54
BMD (g/cm^2)	0.671±0.053[a]	0.683±0.056	0.678±0.039[a]	0.678±0.041	0.690±0.053[a]	0.703±0.051	0.35	0.03	0.99
BMDZ	0.0±1.0	0.1±1.1	0.2±0.8	-0.0±0.9	0.4±0.9	0.5±0.9	0.33	0.13	0.96
Total Hip									
BMC (g)	29.01±5.72	30.98±5.08	29.3±5.21	30.29±4.89	31.68±6.08	33.33±5.26	0.06	0.09	0.403
BMD (g/cm^2)	0.967±0.125[ab]	1.002±0.102[ab]	0.950±0.128[a]	0.951±0.137[a]	1.021±0.137[b]	1.062±0.131[c]	0.05	0.01	0.01
BMDZ	0.2±1.1[ab]	0.5±0.9[c]	0.1±1.1[a]	0.1±1.2[ac]	0.7±1.2[b]	1.0±0.1[d]	0.059	0.02	0.03
Femoral Neck									
BMC (g)	4.09±0.83	4.36±0.62	4.00±0.70	4.07±0.73	4.45±0.98	4.60±0.70	0.06	0.37	0.95
BMD (g/cm^2)	0.862±0.124	0.897±0.103	0.866±0.126	0.865±0.139	0.924±0.131	0.951±0.111	0.12	0.45	0.68
BMDZ	0.1±1.1	0.4±0.9[12]	0.2±1.1	0.1±1.2	0.7±1.1	0.9±1.0	0.11	0.47	0.66

Values are unadjusted means ± SDs. Abbreviations: BMC, bone mineral content; BMD, bone mineral density; BMDZ; BMD z-score; DXA, dual-energy X-ray absorptiometry; LLS: Lateral lumbar spine of vertebrae 3

[#]p-values are shown for differences between baseline and 12 mo, differences among groups, and differences among groups by time in each variable.

A mixed-model ANOVA was used and adjusted for age strata, skin color, height velocity, weight and length of study.

[a, b, c] Different superscript letters denote significant differences among groups at each time point.

Table 4.5 Accretion rate and percentage change of bone variables in male and female adolescents at 12-mo assessed by DXA

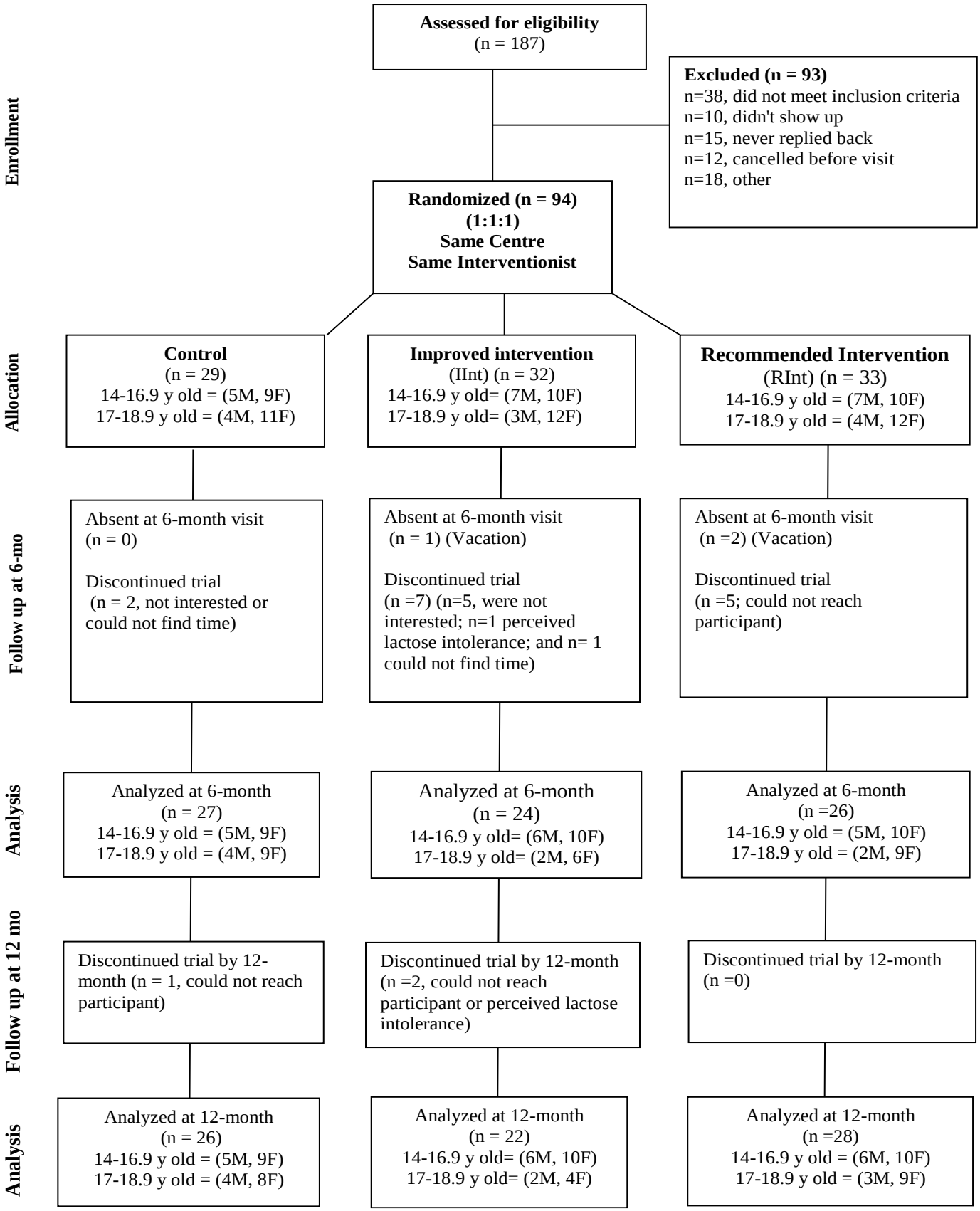
Variable	Males				Females			
	Control	Improved	Recommended	p (group)	Control	Improved	Recommended	p (group)
WB BMC, g/y	252.39±144.65	198.30±128.01	210.31±109.37	0.31	58.19±83.06[a]	71.22±107.42[a]	125.89±120.30[b]	0.02
LS BMC, g/y	7.69±4.06	4.62±3.18	6.09±4.13	0.12	1.71±2.6	1.50±3.28	1.53±2.17	0.26
LLS BMC, g/y	-0.034±0.47	-0.03±0.03	-0.02±0.41	1.00	-0.16±0.41	-0.07±0.72	0.08±0.52	0.25
33% distal radius BMC, g/y	0.19±0.10	0.16±0.11	0.19±0.13	0.69	-0.04±0.41	0.03±0.05	0.06±0.07	0.57
TH BMC, g/y	4.97±3.26	4.14±2.12	4.93±2.31	0.63	0.69±1.52	1.08±2.47	1.40±1.59	0.23
FN BMC, g/y	0.20±0.37	0.30±0.26	0.38±0.29	0.51	0.05±0.23	0.11±0.30	0.07±0.3	0.70
WB								
BMC	12.80±9.51	9.90±7.10	10.76±8.39	0.28	2.68±4.08[a]	3.41±5.33[a]	5.78±5.54[b]	0.02
BMD	6.047±3.623	3.367±3.857	4.883±2.663	0.16	1.618±3.216[a]	1.547±3.132[a]	3.128±3.766[b]	0.02
LS								
BMC	17.05±10.94	10.47±8.09	13.60±12.22	0.12	2.73±4.01	3.11±7.39	2.61±3.46	0.21
BMD	11.00±7.95	4.951±7.240	6.822±6.310	0.11	1.370±2.987	1.132±3.127	1.348±2.798	0.47
LLS								
BMC	-0.74±28.08	0.15±20.27	1.09±19.94	0.99	-6.369±22.094	3.16±46.30	5.55±31.34	0.36
BMD	9.026±12.341	7.519±7.034	6.893±15.457	0.88	2.292±13.121	-3.944±32.902	5.978±16.053	0.26
33% distal radius								
BMC	12.07±8.24	9.27±6.56	11.22±10.72	0.57	0.84±13.40	1.95±3.29	3.92±4.49	0.63
BMD	6.649±5.107	4.933±4.285	6.707±4.488	0.64	1.291±2.814	2.014±3.409	1.323±2.960	0.85
TH								
BMC	16.46±13.16	14.10±9.68	15.48±12.41	0.52	2.48±5.07	4.80±10.04	4.58±5.07	0.36
BMD	7.241±5.619	7.990±5.530	7.211±5.007	0.92	0.683±2.073[a]	2.866±5.249[ab]	3.135±3.307[b]	0.04
FN								
BMC	4.60±8.32	7.39±6.84	9.19±7.81	0.45	1.50±6.53	3.65±7.80	1.62±3.88	0.86
BMD	6.576±5.723	14.100±9.682	5.929±5.052	0.92	0.676±2.489	2.478±5.391	4.585±5.072	0.46

Values are unadjusted means ± SDs. Abbreviations: BMC, bone mineral content; BMD, bone mineral density; BMDZ; BMD z-score; DXA, dual-energy X-ray absorptiometry; LLS: Lateral lumbar spine of vertebrae 3

[#]p-values are shown for differences between baseline and 12 mo, differences among groups, and differences among groups by time in each variable.
A mixed-model ANOVA was used and adjusted for age strata, skin color, height velocity, weight and length of study.

[a, b, c] Different superscript letters denote significant differences among groups at each time point

Figure 4.1 CONSORT diagram of the FAMILY Study



Supplemental Table 4.1 Bone health markers of participants after 12 mo of study, stratified by sex and group

	Males				Females			
	Control	**IInt**	**RInt**	**p-value***	**Control**	**IInt**	**RInt**	**p-value**
Serum OC (ng/ml)	70.0±38.0	74.8±33.0	70.3±39.5	0.30	27.7±7.2	29.6±7.5	30.6±8.8	0.12
Serum BAP (μg/l)	61.8±50.7	72.6±53.2	57.8±57.6	0.30	14.5±5.7	18.2± 10.6	20.0±11.0	0.65
Plasma CTX (nmol/l)	1.5±0.5	1.6±0.6	1.6±0.8	0.46	0.6±0.2	0.8±0.3	0.8±0.4	0.88
Serum 25OHD (nmol/l)	55.0±28.4	57.1±21.0	58.2±18.4	0.44	58.1±24.2	69.1±22.6	57.9±30.5	0.46
Serum PTH (pg/ml)	23.1±16.4	16.8±3.2	18.8±7.9	0.33	22.1±9.7	16.3±6.3	18.4±5.0	0.25

Data presented as mean±SD; A mixed-model ANOVA was used and adjusted for height velocity, weight, skin color and length of study.
p-value denotes significant differences among groups at 12 mo, $p<0.05$
Abbreviations: BAP: bone specific alkaline phosphatase; CTX: C-terminal telopeptide of type 1 collagen PTH: parathyroid hormone; 25OHD: 25-hydroxyvitamin D

Supplemental Table 4.2 Bone and muscle variables of the radius and tibia after 12 mo of study assessed by pQCT

	Males				Females			
	Control	IInt	RInt	p-value*	Control	IInt	RInt	p-value
Radius Geometry								
4% Trabecular density, mg/cm^3	221.6±19.7	188.5±46.9	206.1±40.8	0.30	195.4±30.8	184.2±33.9	194.3±46.4	0.06
4% Trabecular CSA, mm^2	134.3±23.9	155.9±15.2	138.0±38.4	0.46	120.7±14.5	130.2±16.6	128.0±13.9	0.84
66% Cortical density, mg/cm^3	1078.3±61.9	1075.0±22.4	1097.5±43.6	0.44	1142.3±24.3	1144.6±25.7	1139.9±24.9	0.60
66% Cortical CSA, mm^2	85.1±16.4	85.2±15.0	91.6±18.5	0.77	70.4±6.7	70.7±7.7	89.4±38.8	0.06
66% Cortical Thickness, mm	1.5±0.2	1.5±0.2	1.5±0.3	0.34	1.4±0.1	1.4±0.1	1.8±1.1	0.15
66% Muscle Density, mg/cm^3	78.4±1.1	78.3±1.2	78.9±1.6	0.85	77.4±1.8	78.7±1.7	77.7±1.1	0.29
66% Muscle CSA, mm^2	3645.3±578.6	3213.1±722.8	3712.8±928.3	0.39	2442.5±365.2	2496.5±323.1	2548.0±421.0	0.38
Tibia Geometry								
4% Trabecular density, mg/cm^3	214.5±26.6	222.6±42.6	240.8±61.1	0.47	254.2±31.1	233.8±39.1	255.6±37.0	0.84
4% Trabecular CSA, mm^2	432.7±60.7	423.6±57.3	466.3±100.5	0.11	428.1±72.3	404.1±50.8	415.6±52.7	0.52
38% Cortical density, mg/cm^3	1123.3±37.3	1125.5±43.8	1135.3±41.5	0.94	1190.6±24.1	1179.9±19.6	1181.0±18.4	0.05
38% Cortical CSA, mm^2	301.3±36.2[1]	301.5±49.2[1]	333.8±80.5[1]	0.94	273.3±34.0	268.1±37.3	280.5±32.5	0.31
66% Cortical density, mg/cm^3	1073.1±37.3	1074.1±43.9	1076.8±46.5	0.19	1133.5±25.1	1122.2±16.9	1122.9±16.8	0.49
66% Cortical CSA, mm^2	371.9±161.8	319.9±30.5	343.0±80.3	0.37	283.7±36.8	266.1±38.7	293.3±32.4	0.15
66% Cortical Thickness, mm	3.5±0.9	3.2±0.7	3.4±0.6	0.99	3.2±0.5	3.3±0.6	3.3±0.5	0.97
66% Muscle Density, mg/cm^3	77.0±2.4	75.7±2.9	77.5±1.7	0.59	76.4±1.6	76.7±1.0	76.7±1.4	0.29
66% Muscle CSA, mm^2	6226.9±1024.0	5692.8±923.4	6239.7±1089.0	0.24	5249.9±598.7	5087.3±622.7	5717.0±1017.2	0.62

Data presented as mean±SD; A mixed-model ANOVA was used and adjusted for height velocity, weight, skin color and length of study. Abbreviations: CSA; cross sectional area

*p-value denotes significant differences among groups at 12 mo, $p<0.05$.

Bridge Statement 3

Manuscript 2 described the changes in bone outcomes and biomarkers of bone health in adolescents with usual low MILK intake participating up to the 12-mo point of a 2-y trial. Despite a 70% compliance rate, bone health significantly improved in females while bone biomarkers and body composition were unchanged. MILK intervention over 12-mo improved several bone-relevant nutrients, all without increasing energy intake; the results of this study are reassuring that an extended window of opportunity for bone growth still exists during the late adolescence and early adulthood period.

The literature review of this dissertation discussed studies that included a milk intervention using standard methods of dietary assessment to test compliance (i.e., 24-h recalls, food diaries, food frequency questionnaires); objective methods of assessment would provide a more accurate insight to the changes in dietary practices as they are free of measurement bias possibly associated with dietary recall error or analysis. This is of particular importance especially during adolescence as adolescents have difficulty estimating portion sizes, often eat take-out foods and are constantly forming new dietary patterns.

Biomarkers of MILK fat intake have been investigated in adult men and women. However, to the best of the authors' knowledge, no previous studies have explored these biomarkers in healthy adolescents. This would be highly useful information in view of the low rates of return for food records in many studies. In the present thesis, 24 h recall data were used making it possible to quantitatively assessment the relationship between dietary intakes and biological measurements. The following chapter (chapter 5) explores the role of MILK fat as biomarkers in MILK intervention, in addition to standard methods of dietary assessment.

CHAPTER 5

Manuscript 3: MILK fat biomarkers and bone health in youth

Evaluation of plasma and erythrocyte fatty acids C15:0 and C17:0 as biomarkers of dairy fat consumption in adolescents

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ABSTRACT

Introduction: To investigate the relationship between milk and alternatives (MILK) intake and plasma and erythrocyte pentadecaenoic acid (C15:0) and heptadecaenoic acid (C17:0) in adolescents.

Material and methods: Healthy adolescents were randomized to one of three groups (Group 1: control; Group 2: consume 3 MILK servings/day; and Group 3: consume ≥ 4 MILK servings/d). Plasma and erythrocyte C15:0 and C17:0 were quantified using gas chromatography. Dietary intakes were assessed by 24 h diet recalls over 12-month (mo) period.

Results: No difference was observed in plasma and erythrocyte fatty acids at baseline and 6 mo, however, at 12 mo, erythrocyte C15:0 increased in group 3 (+0.37 μg/ml, $p=0.01$). MILK intake increased in both intervention groups (Group 2: +1.4 servings/d; Group 3: +2.4 servings/d, $p<0.0001$). MILK intake positively correlated with erythrocyte C15:0 and C17:0 at 12 mo.

Conclusion: Erythrocyte fatty acids appear to be associated with short-term and yearly MILK intakes during adolescence.

Keywords: pentadecaenoic and hexadecaenoic fatty acids, milk products, adolescents, compliance

5.1 INTRODUCTION

Participant compliance to an intervention in randomized controlled trials (RCT) significantly affects the interpretation of the trial results [231]. Studies involving dietary interventions traditionally measure compliance using self-reported methods such as food frequency questionnaires, multiple-day food records and 24-hour dietary recalls [199]. Such methods are susceptible to forgetfulness, bias and inaccuracies in reporting actual intake [44, 195]. The accurate assessment of dietary intakes, particularly during adolescence, is a challenge, as adolescents have difficulty estimating portion sizes, often eat take-out foods and are constantly forming new dietary patterns [198]. Therefore, epidemiological studies may benefit from biochemical indicators that can objectively assess compliance to dietary intake [5-11], monitor intervention compliance [197] and investigate diet-health associations [43, 44]. Accordingly, the blood concentrations of an ideal dietary biomarker should accurately reflect compliance to dietary intakes and should not be influenced by endogenous biosynthesis [199, 232].

Health Canada's Surveillance Tool revealed that children and adolescents consume low usual MILK intake [233]. MILK intakes, particularly in adolescents, has been encouraged by most dietary guidelines as part of a healthy diet to optimize nutrition and development [159]. MILK products are complex foods that naturally contain up to 400 different fatty acids, of which the C15:0 and C17:0 fatty acids are of particular interest [36]. Due to the odd number of carbon atoms, these fatty acids cannot be synthesized endogenously in humans and, thus, may serve as reliable and valid biomarkers of MILK fat intake [37]. Furthermore, given the diversity of MILK products consumed with different milk fat proportions, C15:0 and C17:0 concentrations in body tissues are expected to vary considerably according to MILK types consumed. There is a growing body of evidence suggesting that these fatty acids can act as good biomarkers of MILK fat intake, as they

are found to be significantly correlated with MILK consumption in multiple tissues including adipose tissue [38-41, 154], erythrocytes [42], serum [38, 41, 43, 44] and plasma [42, 45]. For instance, Wolk et al showed that C15:0 and C17:0 in adipose tissue were significantly associated with MILK intake in adults with correlations of 0.74 and 0.42 [37, 38]. A recent Canadian cross-over RCT has shown a significant increase in plasma C15:0 and C17:0 (0·26 vs 0·22% and 0·42 vs 0·39% of the total fatty acids) following a 4-week intervention with three MILK servings ranging from 1% to 34% fat content [45]. So far, dietary biomarkers of MILK fat have been investigated in adult men and women. However, no previous studies have assessed whether C15:0 and C17:0 are associated with MILK intake in healthy adolescents.

Nutrient needs are higher during adolescence because of the rapid growth rate and biological and body composition changes. Whether these changes affect the functioning of 15:0 and C17:0 as a marker of MILK fat intake in the diet is still unknown. Moreover, the extent to which dietary biomarkers can be used to detect diet-induced changes in plasma and erythrocyte fatty acids as a function of time is rarely examined. In this study, we examined whether C15:0 and C17:0 fatty acids in plasma (i.e. shorter-term intake) and in erythrocyte lipids (i.e. longer-term intake) can serve as biomarkers for the intake of MILK products in adolescents. We also examined whether their relative concentrations are affected by changes in the amount of MILK fat intake over a period of 12 mo, thereby serving as a measure of adherence to dietary intervention.

5.2 MATERIALS AND METHODS

Study design

This study is a secondary analysis of data and samples obtained from an RCT for evaluating the impact of MILK intake on bone health (www.clinicaltrial.gov as NCT02236871). At baseline, 94 participants were randomized to 1 of 3 groups and stratified based on sex and age (15.0-16.9 y, 17.0-18.9 y). Group 1 received no intervention and maintained their current consumption of < 2 servings of MILK/day, whereas intervention group 2 and 3 were counselled to increase their MILK consumption to 3, and 4 or more servings/d, respectively. There were no restrictions placed on the type of milk products, whether it be skim, 1%, 2% or whole milk, unprocessed cheeses (e.g. regular cheddar, mozzarella of any milk fat percent) and yogurts varying in milk fat content. Study visits took place at 6 and 12 mo in addition to obtaining two 24 h dietary recalls over the telephone at 1 and 3 mo.

Study population

Participants were recruited (August 2014-2016) through public and private high schools (grade 9 through 11) and colleges, postal mailings, local advertisements (newspapers, magazines), radio commercials, word of mouth, health and sports organizations, as well as social media. Eligible participants were healthy 14- to 18-y-old youth with a normal body mass index (BMI) (± 1 SD of 50% percentile WHO curves; which aligns with adult targets of 18.5 to 24.9 kg/m^2) [234] and usual MILK consumption of < 2 servings/d. Exclusion criteria included any pre-existing medical condition affecting bone mass and known allergy or intolerance to milk products.

A written informed consent was obtained from the parents and the participants prior to participation in the study. Ethics approval from the Institutional Review Board of the Faculty of Medicine at McGill University was obtained before the study commenced.

Anthropometric and demographic assessments

Height (ht) was measured to 0.1 cm using a wall-mounted stadiometer (Seca 216, Seca Medical Scales and Measuring Systems, Hamburg, Germany), and body weight was measured using a standard balance-beam scale (Detecto, Webb, USA) in the fasted state. BMI (kg/m^2) was then calculated and z-scores for weight (WAZ), height (HAZ) and BMI (BMIZ) were computed using the WHO AnthroPlus Software version 3.2.2.

At baseline only, the parent/participant completed a general history questionnaire in order to obtain socio-demographic information. This self-reported questionnaire consisted of open-ended questions about parental current occupations and ages when participant was born, in addition to multiple choice questions asking about parents' highest education, ethnic and cultural origins, single or multi-parent home, size of family, and gross family income.

Dietary intake assessments

Dietary intakes were assessed using 24 h recalls at each study visit. At baseline, 6 and 12 mo, dietary recalls were completed on site while at 1 and 3 mo, 24 h dietary intake assessments were completed over the telephone by the dietitian (5 recalls in total). Nutrient intakes were analyzed using Nutritionist Pro™ (Axxya Systems LLC, Stafford, TX, US) and the Canadian Nutrient File version 2010b. MILK intake was expressed as total number of servings per day. MILK intake was estimated from the intake of MILK products (including milk, yogurt, chocolate milk, cheese as well as recipes with milk and cheese) and was calculated according to standardized serving sizes (examples: 1 serving is equivalent to 250 ml of milk, 50 grams or 1½ ounces of cheese, 175 grams of yogurt, etc.). Similarly, meat and alternatives servings were calculated (examples: 1 serving is equivalent to 75 g of meat, fish and poultry, 175 ml of cooked legumes or

tofu, 2 eggs, 30 ml peanut butter, and 60 ml of shelled nuts, etc.) to help evaluate ruminant sources of C15:0 and C17:0

Plasma and erythrocyte fatty acid analysis

At each visit, fasted blood samples were provided by the participants between 7:00 and 11:00 am after an overnight fast of at least 12 h. Heparinized venous blood (4 ml) was centrifuged (1500 x g, 10 min, 4 ºC) and separated into aliquots of plasma, buffy coat, and erythrocytes, which were then stored at – 80 ºC until extraction to minimize oxidation and degradation of the samples. After the removal of the buffy coat layer, the erythrocytes layer was washed twice with isotonic saline. Erythrocytes were then re-suspended with an equal volume of saline, flash frozen in liquid nitrogen and purged with nitrogen gas prior to storage at −80 °C.

Plasma and erythrocyte samples were directly methylated using a 1:10 ratio of acetyl chloride-methanol according to the method published by Bell et al. [200]. With the addition of an internal standard of heneicosanoic acid and a reference standard of methyl nonadecanoic (Nu-Chek Prep, Inc. Elysian, MN, USA) added to the samples at concentrations of 0.5 μg/ml of each standard, it enabled the quantification of the fatty acid peaks. The FAMEs were analyzed by gas-liquid chromatography (Varian Inc, Walnut Creek, CA, USA) equipped with a flame ionization detector. A column of 60 m X 0.25 mm HP-88 0.2 um film thickness (Agilent Technologies, Santa Clara, CA, USA) was used, with hydrogen as carrier gas and nitrogen as make-up gas. The injection port temperature was 270 ºC, the detector was 280 ºC and the temperature program was from 60 -120 °C at 20 °C/min, to 180 °C at 10 °C /min, to 200 °C at 2 °C /min and then to 220 °C at 5 °C /min. After the samples were run through the column, the fatty acid profile and peaks were identified through comparison with the SUPELCO 37 component FAME Mix (Millipore Sigma Canada, Oakville, ON, Canada), and expressed as percentage by weight of total fatty acids and

microgram/ml of erythrocytes. Total fatty acids ranged from C14:0 to C22:6n3. Precision was established through repeated measures of a pooled plasma or pooled erythrocyte sample. These pooled samples were added to each batch of fatty acid methylations. The coefficients of variation (CV) for erythrocytes and plasma C15:0 were 8.5% and 7.4%, and 4% and 9.4% for C17:0.

Statistical analysis

An estimated sample size of 66 participants provided ≥80% power (given $\alpha = 0.05$ and a 2-sided test) to detect differences to detect a correlation as low as 0.25 between MILK intake and plasma and erythrocyte C15:0 and C17:0 [45] and to detect changes in C15:0 and C17:0 concentrations in plasma and erythrocytes within and between populations over a period of 12 mo [42] .

Data was analyzed using SAS software (version 9.4, SAS Institute Inc., USA). For each of the study groups, normality and homogeneity of variances were explored using Kolmogorov Smirnov and Levene's tests respectively, and non-normal variables were log-transformed to meet the assumptions of normality. Mixed-model ANOVA was used to test differences at baseline among groups for anthropometry, MILK intake and plasma and erythrocyte proportions of fatty acids. To test the validity of plasma and erythrocyte C15:0 and C17:0 as MILK fat biomarkers, Spearman's partial rank-correlation coefficients, including the entire study sample, were generated to test the relationships between plasma and erythrocyte C15:0 and C17:0 and MILK intake (expressed as servings/d) at 12 mo, captured either by one 24 h recall (to reflect short-term) or four-averaged 24 h recalls (to reflect longer-term), adjusting for age, sex, BMIZ and other possible sources of C15:0 and C17:0 such as ruminant meat intakes [235]. Mixed model ANOVA for repeated measures was used to analyze changes in plasma and erythrocytes fatty acid composition over time between baseline, 6 and 12 mo with Tukey *post-hoc* tests. Random effects included age,

sex and BMIZ, whereas fixed effects included group and time. Confounders were chosen *a priori* based on their reported relation with blood fatty acid content [236]. An alpha of 0.05 was considered significant.

5.3 RESULTS

Study population

Ninety-four adolescents (31% males) participated in the study with a mean age of 16.6±1.5 y and an average BMI of 21.7±3.4 kg/m2. No significant differences among study groups were observed at baseline for any of the anthropometric or demographic characteristics. Detailed baseline participant characteristics and plasma and erythrocyte concentrations of C15:0 and C17:0 are summarized in **Table 5.1**. Of the 94 participants recruited, 77 returned for the 6 mo and 76 returned for the 12 mo assessments.

Dietary intakes at baseline, 6 and 12 mo

At baseline, the mean 24 h intakes of energy, protein, total fat and saturated fat were not different among groups **(Table 5.2)**. There were also no differences in mean intake of meat and alternatives (servings/d) and ruminant meat (servings/d) among groups according to 24 h recalls(s). At 6 and 12 mo, macronutrient intake was not different among groups except for Group 2 which revealed higher intakes of protein and saturated fat compared to group 1 at 6 mo, mostly because of increased overall energy intake **(Table 5.2)**. Total meat and ruminant meat (i.e. beef, veal, lamb) were not different among groups at any study time point. However, according to the averages derived from the 24 h dietary intake assessments at 1, 3, 6 and/or 12 mo, both intervention groups had higher macronutrient intakes (energy, protein and total fat) compared to group 1; intake of meat and alternatives generally and ruminant meat specifically was maintained over the 12 mo study period **(Table 5.3)**.

MILK intakes and associations with plasma and erythrocytes C15:0 and C17:0

At baseline, no difference in MILK intake was observed among groups **(Table 5.2)**; however, at 6 and 12 mo, both intervention groups consumed significantly higher amounts of MILK than the group 1 according to 24 dietary recall(s) ($p<0.0001$, **Table 5.2**).

Plasma and erythrocyte fatty acids were analyzed at baseline (n=94), 6 mo (n=77) and 12 mo (n=76) serving as an objective measure of compliance to the MILK intervention. Plasma and erythrocyte C15:0 and C17:0 were not different among groups at baseline and 6 mo. At 12 mo, Group 3 showed higher mean values of erythrocyte C15:0 in comparison to group 1 (2.68±0.66 vs 2.43±0.63 µg/ml, $p=0.03$). No change over time was observed in any of the plasma or erythrocyte fatty acids (percentage of total fatty acids) at 6 or 12 mo **(Table 5.4).** For plasma, Spearman correlation coefficients between MILK intake and C15:0 and C17:0 were not significant both in the short or longer-term. Concerning erythrocyte fatty acids, it was observed that long-term MILK intake, derived from the mean of four 24 h dietary recalls, was positively correlated with both C15:0 ($r=0.27$, $p=0.03$) and C17:0 ($r=0.29$, $p=0.02$) at 12 mo. However, short-term MILK intake, captured by one 24 h recall at 12 mo, only related to erythrocyte C15:0 ($r=0.25$, $p<0.03$). Spearman correlation between MILK intake and plasma and erythrocyte fatty acids, expressed as percentage of total fatty acids, were not significant **(Table 5.5).**

5.4 DISCUSSION AND CONCLUSION

The present study assessed the effect of consuming increasing intakes of MILK on plasma and erythrocyte fatty acids. In comparison with group 1, 12-mo intake of 4 or more MILK servings resulted in higher erythrocyte C15:0. This was accompanied by a positive correlation between MILK intake and erythrocyte fatty acids in late adolescents. The approach taken for the fatty acid analyses were in line with recent guideline [236]. To the authors' knowledge, this is the first RCT to assess the association between C15:0 and C17:0 in plasma and erythrocyte and MILK intake and to explore the effects of increasing MILK products intake on C15:0 and C17:0 concentrations in healthy adolescents over a 12-mo period. While previous work showed that erythrocyte fatty acids are good MILK fat biomarkers in adults [42, 237], the present study demonstrated that erythrocytes C15:0 can also serve as a compliance biomarker to increasing MILK servings in adolescents, as shown by changes in erythrocytes C15:0 fatty acids over the study period.

As per the study objective, daily intakes of MILK, derived from the averaged four 24 h recalls, increased over the study and correlated positively with C15:0 and C17:0 within erythrocyte membranes, indicating that these might be considered as valid biological markers of long-term stable intakes of MILK fat. Moreover, erythrocyte C15:0 was positively associated with MILK fat intake estimated by one 24 h recall, suggesting a potential role as a short-term biomarker as well. These findings are consistent with prior studies [15, 16, 27] which observed that erythrocyte C15:0, and to a lesser extent C17:0, reflect habitual MILK fat intake. In contrast, correlation analysis in this study showed no significant association between MILK intake and any of the plasma fatty acids. This is inconsistent with previous studies in adults [7, 16]. Such inconsistency could be attributable to several factors such as the amount of fat per fixed quantity in MILK products (ie, reduced-fat milk versus whole milk). Although the intervention in this study focused on allocating

the number of MILK servings without choosing the percentage of milk fat, over 90% of the participants consumed reduced-fat milk rather than whole milk, and fat-free products were not commonly reported. Further, the MILK foods used to calculate the number of servings in our study was limited to fluid milk, yogurt and regular cheese, whereas Sun et al. [7] and de Otto et al. [16] added contributions from sour cream, sherbet, ice cream and butter. This discrepancy could also be attributed to the reliance on one self-reported 24 h recall in determining the short-term relationship between plasma fatty acids and MILK intake as well as to the increased energy requirements during growth.

Furthermore, according to the secondary objective, a 12-mo intervention with 4 or more servings/d of commonly consumed MILK products resulted in higher erythrocyte concentrations of C15:0, but not C17:0, in comparison with group 1 (< 2 MILK servings/d) and group 2 (3 MILK servings/d) groups. MILK fat is found in several mixed dishes with amounts varying within the same recipe itself depending on the percent of milk fat being used, which reflects the measurement errors of self-reported dietary assessments. MILK-derived fatty acids within erythrocyte membranes are not subject to these measurement errors and thus are believed to be a more accurate means of dietary compliance assessment. A recent study observed a significant increase in plasma C15:0 and C17:0 following a 4-wk intervention with 3 MILK servings [17]. Moreover, a 2017 review concluded that C15:0, and to a lesser extent C17:0, reflect both habitual and changes in MILK intake in various circulating and tissue lipid fractions [15].

According to recent guidelines on the reporting of fatty acids, the preferred manner for presenting fatty acid data is as relative weight % of the total fatty acids rather than absolute concentrations [236]. In the present study, percentages of C15:0 and C17:0 in plasma and erythrocytes neither correlated significantly with MILK intake nor changed over time. These

findings can be attributed to the fact that the use of relative percentage units can mask the changes in the lipid pool size, particularly when dealing with relatively less abundant fatty acid as C15:0 and C17:0 [238].

A strength of the present study is the use of both plasma and erythrocyte fatty acids as objective means to assess compliance to the dietary intervention of increasing the daily MILK servings instead of relying solely on the traditional subjective dietary assessment methods. Human erythrocytes circulate for 100 to 120 days before being recycled and replaced [239], thus erythrocytes markers may be a useful reflection of longer-term dietary intakes when no changes in dietary patterns occurs [42, 240]. Additionally, the values obtained for the fatty acid profile of the erythrocytes and for the correlation analysis are comparable to those obtained from previous studies [42, 237]. Moreover, although the use of these MILK fat biomarkers has previously been explored in adult studies, to our knowledge, this was the first study to report on a MILK intervention trial in adolescents. A limitation to the present study is the use of single-day 24 h recalls to assess MILK intake which may fall on an atypical day and thus is subject to under- or over-reporting error. Additionally, due to school schedules, most of the visits occurred on a weekend day and thus the majority of the 24 h recalls were recounts of Fridays and Saturdays, which are days that may have atypical dietary intakes. In the current trial, only the quantity of MILK servings was fixed and the use of low-fat or fat-free MILK products was allowed, although less commonly used.

In conclusion, erythrocyte fatty acids, particularly C15:0, can serve a useful biomarker of MILK intake in adolescents and can detect short- and longer-term changes in the absolute MILK intake. Future controlled feeding studies are warranted to evaluate these biomarkers and associations with different percentages of dairy fat.

5.5 ACKNOWLEDGEMENTS

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Table 5.1 Participant baseline characteristics stratified by intervention groups

Variable	Group 1 n=29	Group 2 n=32	Group 3 n=33	Total Sample n=94
Age (y)	16.5 ± 1.6	16.6 ± 1.5	16.6 ± 1.5	16.6 ± 1.5
Ethnicity, White (%) *	71.9%	75.8%	69%	72.3%
Male (%, n)	37.5%	33.3%	29.2%	31.9%
Waist circumference (cm)	74.6 ± 8.1	74.6 ± 8.1	74.4 ± 8.7	74.5 ± 8.2
Weight (kg)	59.4 ± 10.0	58.9 ± 11.0	61.5 ± 13.7	59.9 ± 11.7
Height z-score	-0.05 ± 1.0	0.1 ± 1.0	0.3 ± 1.2	0.1 ± 1.1
BMI (kg/m^2)	21.7 ± 3.0	21.5 ± 3.9	21.7 ± 3.3	21.7 ± 3.4
BMIZ	0.3 ± 0.8	0.1 ± 1.0	0.2 ± 1.0	0.2 ± 0.9
Physical activity (min/week)	421.8 ± 308.5	419.3 ± 319.6	418.6 ± 270.3	419.8 ± 296.4
Plasma C15:0				
μg/ml	3.9 ± 1.4	4.5 ± 1.2	4.2 ± 1.6	4.2 ± 1.4
% of total fatty acid	0.20 ± 0.04	0.21 ± 0.04	0.21 ± 0.04	0.21 ± 0.04
Plasma C17:0				
μg/ml	5.1 ± 1.6	5.5 ± 1.2	5.3 ± 1.7	5.3 ± 1.4
% of total fatty acid	0.27 ± 0.04	0.26 ± 0.03	0.27 ± 0.04	0.27 ± 0.04
Erythrocyte C15:0				
μg/ml	2.2 ± 0.5	2.4 ± 0.4	2.4 ± 0.4	2.3 ± 0.4
% of total fatty acid	0.19 ± 0.05	0.20 ± 0.04	0.20 ± 0.04	0.20 ± 0.04

Erythrocyte C17:0				
μg/ml	3.7 ± 0.5	3.8 ± 0.6	3.8 ± 0.6	3.8 ± 0.6
% of total fatty acid	0.32 ± 0.04	0.33 ± 0.04	0.32 ± 0.04	0.32 ± 0.04

Data are mean ± standard deviation (n) unless stated otherwise; Abbreviations: BMI: Body mass index (kg/m^2); BMIZ: Body mass-for age-z-score; C15:0 pentadecanoic acid; C17:0 heptadecanoic acid.

* Non-white includes Asian (n = 20), African or black (n = 5), Hispanic (n = 1).

Table 5.2 Macronutrient intakes and Canada's Food Guide Groups[*] assessed by food recall[#] at baseline, 6- and 12-mo

	Control			Improved			Recommended		
	BL	**6-mo**	**12-mo**	**BL**	**6-mo**	**12-mo**	**BL**	**6-mo**	**12-mo**
n=	*29*	*27*	*26*	*32*	*24*	*22*	*33*	*26*	*28*
Total energy (kcal)	1840±687	1828±686[a]	2058±770	2124±736	2739±1131[b]	2271±978	2081±761	2204±640[ab]	2473±814
Protein (g/d)	66.4±23.8	76.0±34.0[a]	90.5±42.2	88.7±44.0	110.3±38.0[b]	96.2±43.4	86.7±36.4	104.7±34.7[ab]	113.3±40.3
Fat (g/d)	67.4±27.7	65.5±34.1	65.7±26.4	82.1±37.0	107.1±42.3	92.8±47.5	79.3±44.6	82.7±38.1	89.3±32.9
Saturated fat (g/d)	24.8±10.0	23.7±20.5[a]	23.0±11.1	28.4±12.1	34.5±14.5[b]	33.1±16.1	26.4±16.1	34.1±16.7[ab]	34.4±16.2
Total MILK intake (servings/d)	1.4±1.2[a]	1.5±1.1[a]	1.8±1.2[a]	1.6±1.5[a]	3.7±1.4[b]	3.0±1.6[b]	1.3±1.1[a]	3.7±1.4[b]	3.7±1.0[b]
Meat and alternatives (servings/d)	1.5±1.2	2.3±2.1	1.9±1.6	2.3±1.9	2.2±2.4	2.0±1.6	2.3±2.2	2.1±1.9	2.2±2.4
Ruminant meat (servings/d)	0.4±0.9	0.7±1.3	0.8±1.5	0.7±0.8	0.2±0.5	0.8±1.0	0.5±1.2	0.5±0.9	0.4±1.0

Data are mean ± standard deviation. n=94 at baseline, 77 at 6 mo and 76 at 12 mo

[*] Canada's Food Guide groups: servings/day

[#] Dietary data at each time-point was obtained from one single-day 24 h recall reflecting the day prior to study visit

[a, b] Different superscript letters denote significant differences among groups at 6 mo. No difference in dietary intake or food groups was observed at baseline or 12 mo among groups

Table 5.3 Average macronutrient intakes and Canada's Food Guide Groups[*] assessed by food recall[‡] at 6 and 12 mo

	Group 1			Group 2			Group 3		
	BL	**6-mo**	**12-mo**	**BL**	**6-mo**	**12-mo**	**BL**	**6-mo**	**12-mo**
n=	*29*	*27*	*26*	*32*	*24*	*22*	*33*	*26*	*28*
Total energy (kcal)	1840±687	1828±686[a]	2058±770	2124±736	2739±1131[b]	2271±978	2081±761	2204±640[ab]	2473±814
Protein (g/d)	66.4±23.8	76.0±34.0[a]	90.5±42.2	88.7±44.0	110.3±38.0[b]	96.2±43.4	86.7±36.4	104.7±34.7[ab]	113.3±40.3
Fat (g/d)	67.4±27.7	65.5±34.1	65.7±26.4	82.1±37.0	107.1±42.3	92.8±47.5	79.3±44.6	82.7±38.1	89.3±32.9
Saturated fat (g/d)	24.8±10.0	23.7±20.5[a]	23.0±11.1	28.4±12.1	34.5±14.5[b]	33.1±16.1	26.4±16.1	34.1±16.7[ab]	34.4±16.2
Total MILK intake (servings/d[*])	1.4±1.2[a]	1.5±1.1[a]	1.8±1.2[a]	1.6±1.5[a]	3.7±1.4[b]	3.0±1.6[b]	1.3±1.1[a]	3.7±1.4[b]	3.7±1.0[b]
Meat and alternatives (servings/d[**])	1.5±1.2	2.3±2.1	1.9±1.6	2.3±1.9	2.2±2.4	2.0±1.6	2.3±2.2	2.1±1.9	2.2±2.4
Ruminant meat (servings/d)	0.4±0.9	0.7±1.3	0.8±1.5	0.7±0.8	0.2±0.5	0.8±1.0	0.5±1.2	0.5±0.9	0.4±1.0

Data are mean ± standard deviation. n=94 at baseline, 77 at 6 mo and 76 at 12 mo

[*] Example of 1 serving size = 250 ml of milk, 50 grams or 1½ ounces of cheese, 175 grams of yogurt

[**] Example of 1 serving size = 75 g of meat, fish or poultry, 175 ml of cooked legumes or tofu, 2 eggs, 30 ml peanut butter, and 60 ml of shelled nuts

[#] Dietary data at each time-point was obtained from one single-day 24 h recall reflecting the day prior to study visit

[a, b] Different superscript letters denote significant differences among groups at 6 mo. No difference in dietary intake or food groups was observed at baseline or 12 mo among groups

Table 5.4 Plasma and erythrocyte C15:0 and C17:0 at 6 and 12 months, stratified by intervention groups

		Control		Improved		Recommended	
		6-mo	**12-mo**	**6-mo**	**12-mo**	**6-mo**	**12-mo**
	n=	*27*	*26*	*24*	*22*	*26*	*28*
Plasma C15:0							
µg/ml		3.74±0.91	3.75±1.16	3.76±0.89	4.26±1.09	4.13±0.91	4.31±2.24
% of total fatty acids		0.19±0.03	0.20±0.04	0.21±0.04	0.21±0.04	0.22±0.03	0.22±0.06
Plasma C17:0							
µg/ml		4.83±0.99	4.66±1.19	4.52±0.80	5.15±1.32	5.03±1.03	5.25±2.01
% of total fatty acids		0.25±0.02	0.25±0.05	0.25±0.03	0.26±0.04	0.26±0.03	0.27±0.05
Erythrocyte C15:0							
µg/ml		2.17±0.44	2.43±0.63[a]	2.21±0.31	2.56±0.48[ab]	2.31±0.28	2.68±0.66[b]
% of total fatty acids		0.20±0.05	0.22±0.07	0.21±0.05	0.24±0.06	0.22±0.05	0.24±0.07
Erythrocyte C17:0							
µg/ml		3.55±0.48	3.47±0.66	3.61±0.56	3.72±0.48	3.58±0.72	3.62±0.46
% of total fatty acids		0.34±0.05	0.31±0.04	0.33±0.03	0.34±0.03	0.34±0.06	0.32±0.05

Abbreviations: C15:0 pentadecanoic acid; C17:0 heptadecanoic acid; mo: months.
Data are means and adjusted for age, BMI z-score, skin color, sex, and meat and alternatives intake.
[a, b] Groups with different superscripts are significantly different at 12 mo using a mixed model ANOVA ($p < 0.05$).

Table 5.5 Spearman's correlation coefficients between C15:0 and C17:0 in plasma and erythrocyte and MILK intake captured at 12 mo by one (short-term) or averaged four (long-term) 24 h recalls

	Plasma				Erythrocyte			
	Short-term		Long-term		Short-term		Long-term	
	Unadjusted	Adjusted	Unadjusted	Adjusted	Unadjusted	Adjusted	Unadjusted	Adjusted
C15:0								
µg/ml	-0.04	-0.04	-0.04	0.06	0.2	0.28*	0.14	0.27*
% of total fatty acids	0.02	0.00002	0.02	0.09	0.11	0.21	0.04	0.20
C17:0								
µg/ml	-0.1	-0.07	-0.1	0.06	0.12	0.07	0.25*	0.29*
% of total fatty acids	-0.03	0.01	-0.02	0.05	0.006	-0.12	0.09	0.08

n=76 completed the study at 12 mo; Abbreviations: C15:0 pentadecaenoic acid; C17:0 heptadecaenoic acid; mo: months.

[1]Spearman's correlation coefficients were adjusted for age at blood drawing (y), sex, BMI z-score and meat and alternatives intake (servings/d).

ǂ Short-term: reflects dietary intake a day prior visit.

Long-term: reflect mean dietary intake averaged from four 24 h recalls collected at 1,3,6 and 12 mo.

* $p < 0.05$

CHAPTER 6

General Discussion and Conclusions

6.1 General Discussion

The global objective of this dissertation was to: (1) explore the micronutrient status of bone-relevant nutrients in young males and females with usual low milk and milk alternatives (MILK) intake; (2) examine the effects of a 12-mo MILK intervention on bone health; and (3) explore whether plasma and erythrocyte C15:0 and C17:0 fatty acids can serve as biomarkers of compliance. As such, this dissertation comprises 3 original research papers (Chapters 3-5). The study design included in this dissertation allowed for appropriate investigation of the abovementioned objectives. The questionnaires used in this study were designed specifically to capture multiple factors that affect bone health.

Overall, this dissertation sought to respond to a number of research gaps related to the benefits of MILK intake during the skeletal consolidation period and generated high-level evidence that a sustainable dietary intervention with MILK, i.e. motivational interviewing to increase volitional intakes, will enhance bone mineral acquisition in youth. The present chapter will highlight the general thesis findings and discuss additional considerations pertaining to the study findings. This dissertation presents with important public health inferences and is positioned to extend recommendations for future investigation.

Hypothesis 1: ***Micronutrients adequacy, bone biomarkers and milk products***

Adolescents who do not meet the recommendations for MILK intake will have inadequate intakes of bone-relevant micronutrients; MILK intake will associate positively with serum biomarkers of bone formation and resorption and BMD.

Study one (chapter 3) examined the hypothesis that adolescents who do not meet the recommendations for MILK intake will have inadequate intakes of bone-relevant micronutrients and that MILK intake will associate positively with serum biomarkers of bone formation and

resorption, areal bone mineral density (BMD) and bone geometry. This study provided a unique opportunity to explore the MILK intake-bone health relationship with 3 assessment methods of bone health, i.e. bone biomarkers, dual-energy x-ray absorptiometry (DXA) and peripheral quantitative computed tomography (pQCT). In this study, all adolescents failed to meet the Estimated Average Requirements (EAR) for vitamin D and the Adequate Intake of potassium, more than 70% did not meet the recommendations for calcium and magnesium and more than one-third reported inadequate intake of phosphorus. For the majority of nutrients analyzed in this study, nutrient inadequacy was more pronounced in females compared to males. This is mostly attributed to sex differences in dietary patterns; males tend to consume more food than females. These findings are corroborated by a wide body of existing literature [25, 117, 118]. The 2004 Canadian Community Health Survey found that, on average, males 12 to 19 y consume 2,800 kilocalories a day compared to 2000 kilocalories in age-matched females [30]. Generally, body size and growth velocity are the main stimuli for the increased dietary intakes of males compared to females.

In the present study, MILK intake also contributed to more than 40% of daily calcium, almost 60% of vitamin D, more than 25% of riboflavin, vitamin B_{12} and phosphorus intake and up to 15% of potassium and zinc intake. Further, this analysis revealed a positive association between tertiles of MILK products and bone formation and resorption markers, with bone properties of the trabecular and cortical compartments and with BMD z-scores (BMDZ) at the whole body (WB). These positive associations may be attributed to the naturally high content of essential nutrients in MILK that are required for optimizing bones mass in childhood and adolescence and for their maintenance during adulthood with the aim to reduce osteoporosis and bone fractures later in life. This package of key nutrients is difficult to obtain in diets with limited or no milk products. A study conducted on 9- to 18-y old adolescents concluded that it is not possible to achieve

recommended daily calcium intakes with a MILK-free diet [241]. The high calcium, vitamin D and protein intakes achieved via MILK products can affect bone mineralization and collagen formation. It can also decrease circulating parathyroid hormone (PTH) and reduce bone turnover [79]. It is reasonable to believe that more food is consumed during this life phase, contributing to a greater adequacy of these bone-relevant nutrients.

Taken together, this analysis indicates that low consumption of MILK products contributes to inadequate nutrient intakes for several bone-relevant nutrients, particularly for adolescent females, posing an elevated risk of suboptimal bone growth and mineralization. Therefore, recommendations to the public should continue to emphasize increasing intake of nutrient-rich foods such as MILK in order to provide adequate nutrients necessary for skeletal modelling and consolidation. A recent study among Canadian adolescents identified several factors affecting MILK intake during this life stage including personal knowledge about milk products, misconceptions about milk products and their association with adverse health effects, social and physical influence, and others [136]. Addressing these factors should be the potential target for interventions to increase MILK intake.

<u>Hypothesis 2</u>: ***Milk products effect on adolescents' bone health***

By the 12-mo time-point of the randomized trial, participants randomized to consume ≥ 4 servings of MILK (i.e. Canada's Food Guide age-recommended servings) will have higher LS BMD compared to the control group, whereas those increasing to reach an average of 3 servings (i.e. improved, but not yet at recommended servings) will be intermediate; volumetric BMD and the cross-sectional area of the non-dominant radius and tibia will be greater in the those increasing intakes to reach a minimum of 4 daily servings.

While the above study assessed the nutritional adequacy of bone-relevant nutrients and evaluated the relationship to BMD and serum biomarkers of bone formation and resorption in adolescents with a usual intake of < 2 MILK serving per day, the main trial (Chapter 4) was designed to demonstrate in healthy young males and females (14-18 y) with habitually low MILK intake (< 2 servings/d) that increasing MILK to meet current recommendations will improve bone outcomes as assessed by DXA, pQCT and biomarkers of bone metabolism.

In females, there was no evidence for a group-by-time interaction effect for lumbar spine (LS) bone accretion or density. In contrast, WB bone mineral content (BMC) and BMD were greater at 12 mo compared to baseline in all groups. Females who consumed 4 or more MILK servings/d had more rapid gains in WB BMC and BMDZ than the control (< 2 MILK servings/d) and IInt group (3 MILK servings/d) ($p < 0.01$)**.** It was also demonstrated in this trial that females in the RInt group had greater WB BMC accretion rate and percentage increases of WB BMC and BMD over 12-mo compared to control and IInt groups ($p<0.02$) as well as greater percentage increases in TH BMD were observed compared to the control group ($p=0.03$). The RInt intervention group had also significantly higher calcium and protein intake compared to baseline

and to the control group at 12 mo. As for males, none of the bone outcomes differed among the trial groups by 12 mo, although intervention effects on LS BMD tended to be significant over the study period (explained in detail later in this chapter). Further, the intervention had no significant effects on pQCT outcomes, bone biomarkers and body composition.

Among the determinants of peak bone mass (PBM), nutrition, particularly milk products, represents an important and growing area of research for bone health. In a recent review of 12 MILK intervention trials, 5 trials were conducted in adolescents [79]. These were methodologically and statistically disparate and none of these trials was recent or Canadian or included males. Further, none tested the effects of different servings of milk products (i.e. as a food group) in relation to changes in BMD, necessitating a high-level evidence to support current nutrition guidelines. Therefore, the results of this intervention trial filled an information gap in males and added to the existing literature on the positive effect of MILK intake in female adolescents [4, 79, 115].

In this study, no group-by-time interaction effect for LS bone mineral accretion or BMD was observed despite a significant effect at WB and TH in female RInt groups at 12 mo. Indeed, the sample size calculation for this study was set based on a WB BMC accretion rate of 199.5±132.4 g/y for reaching 4 servings per day of MILK compared to 34.1±57.3 g/y for the control. However, the mean change of WB BMC at 12-mo was 125.9±120.3 and 58.2±83.1 in RInt and control respectively. It is possible that the size effect was over estimated based on the small pilot group. In this study, although there was no evidence for a group-by-time interaction effect for LS bone accretion or density in neither males or females, it did significantly result in greater BMD of WB and TH in females in the RInt group. These findings are of great relevance, particularly for TH BMD, given the increased prevalence of hip fractures and the greatest costs to

individuals and health care it incurs [1]. Therefore, increased consumption of MILK intake, particularly for female adolescents, can help optimize WB BMD generally and TH BMD specifically, and hence can delay or prevent osteoporotic fractures later in life. Today, despite the numerous research that has been conducted on milk products and its association with bone health, the results are contradictory. These variations in data could be a result of differences in assessment methods (e.g. DXA vs single-photon absorptiometry), analysis procedure, outcome variables (BMC vs BMD), confounding variables, inaccurate subject reporting **(Tables 2.6.1 and 2.6.2)**, and the type of MILK product consumed, all of which prevents the generalization of the results and the support of public health.

The present study demonstrated that meeting current increasing MILK intake improve bone health in female adolescents, with previously low habitual MILK intake. This finding is consistent with 3 previous meta-analysis of numerous MILK intervention studies where increasing milk products resulted in a statistically and clinically higher gains of WB and LS BMC in participants with baseline calcium intake lower than 750 mg/d [26, 79, 242]. This can be partially explained by the threshold behavior of calcium, i.e. increasing intake of MILK products or dietary calcium intake above requirements is unlikely to add further benefit to BMD in adolescents [4, 93]. It also helps to put the observations in the male participants into better context as their total calcium intake, despite lower milk intakes, were already in accordance with the EAR, and consistent with no differences among groups following the motivational interviewing interventions to enhance MILK intake.

The FAMILY Study focused on examining bone outcomes and included bone biomarkers that reflected both formation by osteoblasts and resorption by osteoclasts. Specifically, this study assessed the bone formation markers bone specific alkaline phosphatase (BAP) and osteocalcin,

and C-terminal telopeptide of type I collagen (CTX), a bone resorption marker, in addition to 25-hydroxyvitamin D (25(OH)D) and PTH. Previous studies suggest that these biomarkers may change in response to MILK intake, however, this study did not see significant differences among groups by 12-mo despite the significant effects of increased MILK consumption on BMC and BMD. Suggested reasons include the duration of the study (12 mo); bone biomarkers are short-term biomarkers that respond to intervention faster (< 6 mo) than BMD or BMC do. Other considerations that may explain the null findings may be related to mode of handling of samples (e.g. the time of sample collection i.e. 8:00 am versus 11:00 am), the rapid rates of growth as well as the number of males already meeting calcium EAR at baseline. Indeed, the time of the day during which the sample is collected affects the variability of the biomarkers concentration due to the circadian rhythm. Previous work has shown that fasting significantly reduces circadian variations. Fasting specimens collected early in the morning are advisable for optimal results [243].

Effects of MILK intake on nutrient intake and body composition

Overall, all participants started the study with a healthy BMI for their age, with no difference between males or females **(Table 5.1)**. WC was also within the normal range according to Health Canada guidelines (< 102 cm for males and < 88 cm for females) **(Table 5.1)**. In this trial, the intervention did not influence any measures of body fat and did not lead to a difference in weight gain despite the freedom to consume milk products of any fat content. These findings are consistent with a recent systematic review of 15 RCT that examined the effects of MILK intake on body size and body composition in children and adolescents [79]. One reason for not showing significant effects on body fat could be that participants replaced other energy-containing beverages with MILK products. In a previous trial among 98 children 8-10 y who regularly consume sugar-sweetened beverages (SSB), participants were assigned to a control (i.e. continue

their usual consumption of SSB) or to an intervention group who drank milk servings/ of milk and no SSBs. After 16 week (wk), replacing habitual consumption of SSBs with milk improved lean body mass in children without affecting percentage body fat [244].

Moreover, increasing MILK intake to 4 or more servings/d significantly increased dietary calcium and protein intake in female participants randomized to the RInt intervention group compared to control, whereas total energy intake did not vary. This insignificant change in total energy intake reflected the integration of MILK products into the diet rather than adding it. This finding contradicted with that from a recent trial among pubertal males and females where intervention with 3 servings of MILK per day resulted in higher intakes of energy after 18 mo [245]. One possible explanation might be due to choosing more MILK intake as fluid milk or rather than as cheese **(Table 5.2)**. Specifically, the majority of the study participants consumed fluid milk with fat content ranging from 0% to 2%. Unique to this study, another reason for MILK integration might be the use of MInt techniques to improve MILK intake rather than provision of MILK products as in the abovementioned trial [245]. MInt has shown promise in changing a number of health behaviors (e.g. weight loss, diet, exercise, diabetes care, IBM management and adherence) [246]. Participants who received MInt counseling (i.e. the intervention groups) learned about the importance of MILK intake during the growing y, the different MILK products and their appropriate portion sizes and the different preference-tailored strategies to increase MILK intake (e.g. fluid milk with oatmeal instead of water, sprinkle their salads with cheese, drink chocolate milk (milk vs milk beverage) after exercise instead of juices…etc), all of which might have helped in the integration of MILK in their daily routine.

In addition, the use of MInt techniques to improve MILK intake resulted in significant increases in MILK intake among participants randomized to intervention groups despite that the

increases, although significant, did not meet the targets of 4 or more servings of MILK/d in the RInt group. Further research should be conducted to expand upon this work simply to provide more evidence on the effectiveness of MI in improving MILK intake. This is a critical gap to fill since the majority of Canadian teens do not get the suggested minimum number of 3 to 4 servings of milk product [156].

In this study, the general message was to bring high-level evidence that increased MILK intake is important for optimal bone mineralization during the consolidation phase. As mentioned above, previous MILK trials in adolescence suggest that milk product intakes mediate changes in bone mineral accretion at different skeletal sites, however, their effects are conflicting. In this study, the female milk intervention group (RInt) resulted in significant increases in BMD at WB and TH at 12-mo, indicative of the importance of adequate MILK intake during the consolidation phase. A discussion as to how milk products may have mediated these changes in bone outcomes is discussed in the subsequent paragraph.

Potential mechanisms

There are several postulated mechanisms for the effect of MILK products on bone health. Besides their richness in calcium, MILK products contain other nutrients that interact synergistically to promote bone health, such as vitamin D, phosphorus and protein. For example, calcium and protein in MILK products play a key role in the formation and mineralization of bone organic matrix through the formation of hydroxyapatite crystals and collagen respectively [147]. MILK products also contain nutrients that enhance intestinal calcium absorption such as vitamin D, lactose and casein phosphopeptides [22].

Figure 6.1 shows the interaction of vitamin D and dietary protein in stimulating intestinal calcium absorption and, thus, bone mineralization. Briefly, whey proteins, besides being a key

component of the bone matrix, stimulate increased secretion of insulin-like growth factor-1 (IGF-1) by osteoblasts, resulting in an increase in osteoblastic cell proliferation and collagen synthesis [21]. Circulating IGF-I also enhances the renal production of $1,25(OH)_2D$. The latter binds to vitamin D receptor in the enterocyte stimulating the transcription of several factors that are implicated in the transport of calcium across either the luminal or basolateral membranes or through the intracellular compartment of the enterocyte. This in turn stimulates the intestinal absorption of both calcium and phosphorus and triggers the tubular reabsorption of phosphorus. Therefore, IGF-I plays a dual role, directly and indirectly, in calcium and phosphorus homeostasis, thereby positively influencing bone mineralization [147]. Furthermore, phosphorus also plays an important role in bone mineralization because they produce fibroblast growth factor 23, a hormonal regulator of renal phosphorus reabsorption and $1,25(OH)_2D$ production [147]. It is remarkable that the beneficial effect of dietary protein on BMC and BMD is more sustained in presence of adequate intakes of both calcium and vitamin D which supports MILK products intake for optimal bone health.

MILK trial effect on bone health in males

Previously in this dissertation, it was mentioned that this study has highlighted a research gap by including male adolescents. By 12 mo, male participants in the intervention groups revealed no significant differences in any of the bone parameters measured. This lack of different is largely explained by the growth rate observed in males over the 12-mo period, as reflected by height velocity over the 12 mo; males 3.6 ± 3.5 cm/y vs females 0.4 ± 0.7 cm/y ($p=0.0004$). Based on World Health Organization (WHO) growth data, younger teens of 14 y would experience greater increments in height than older ones (**Table 6.1**). Further, PBM accretion lags behind growth in bone size by 6 to 12 mo [8], resulting in a transient decrease in bone density. Thus, this rapid height

velocity observed in males versus females of this study would not result in increases in BMD until growth is complete as mineral consolidation follows growth [5]. As height velocity slows with increasing age, the benefits of MILK might become more apparent in males as it was in females. As the trial was designed with a follow-up of 2 y, such differences can be further evaluated.

Longer term evaluation is indeed justified as there were more pronounced increases in LS BMD over time in the RInt male group (4 or more MILK servings) than in the control group (<2 MILK servings/d), and while the differences failed to achieve significance (p=0.07), the results show promise for the longer-term follow-up. These changes in LS BMD coincided with increased gains in LS areas in the RInt group **(Figure 4.3)** which might have resulted in a transient non-significant change in the ratio of BMC to area. Second, the 2-y recruitment phase of this study closed with 30 males of which 26 completed the 12-mo visit; this represented 72% of the estimated male sample size originally set for this study (n=36, 12/group). It is largely possible that such a small sample size was inadequate in terms of statistical power for detecting BMD differences among male groups over 12 mo, with a high likelihood of type II error. This also helps to put the significance of BMD changes in the female participants into better context as the number of females who completed the 12-mo visit (n=50) was larger than what was initially estimated (n=36), therefore the study seemed to be adequately powered for females.

Table 6.1 Height velocity over the 12-mo study period and comparison with WHO growth charts in adolescents 14 to 18 y old [219]

	Males					Females				
Age (y)	WHO 50th percentile (cm)	Height (cm)	Height z-score	WHO Height Increments (cm/y)	Velocity (cm/y)	WHO 50th percentile (cm)	Height (cm)	Height z-score	WHO Height Increments (cm/y)	Velocity (cm/y)
14	163.2	161.6±10.3	-0.5±1.2	7.2		159.8	167.2±7.9	-0.5±1.2	3.4	
15	169.0	175.6±3.6	0.6±0.6	5.8	6.3±3.3	161.7	165.0±4.9	0.6±0.6	1.9	1.1±0.9
16	172.9	181.0±4.2	0.8±0.6	3.9	2.3±2.7	162.5	166.5±6.1	0.8±0.6	0.8	0.6±0.3
17	175.2	176.0±7.2	0.02±0.9	2.3	0.7±0.6	162.9	162.1±6.5	0.02±0.9	0.4	0.2±0.5
18	176.1	178.0±9.2	0.7±0.8	0.9	1.1±1.3	163.1	162.1±7.7	0.70.8	0.2	0.3±0.6

Abbreviation: WHO: World health Organization

Figure 6.1 Interaction of MILK nutrients to improve bone health [147]
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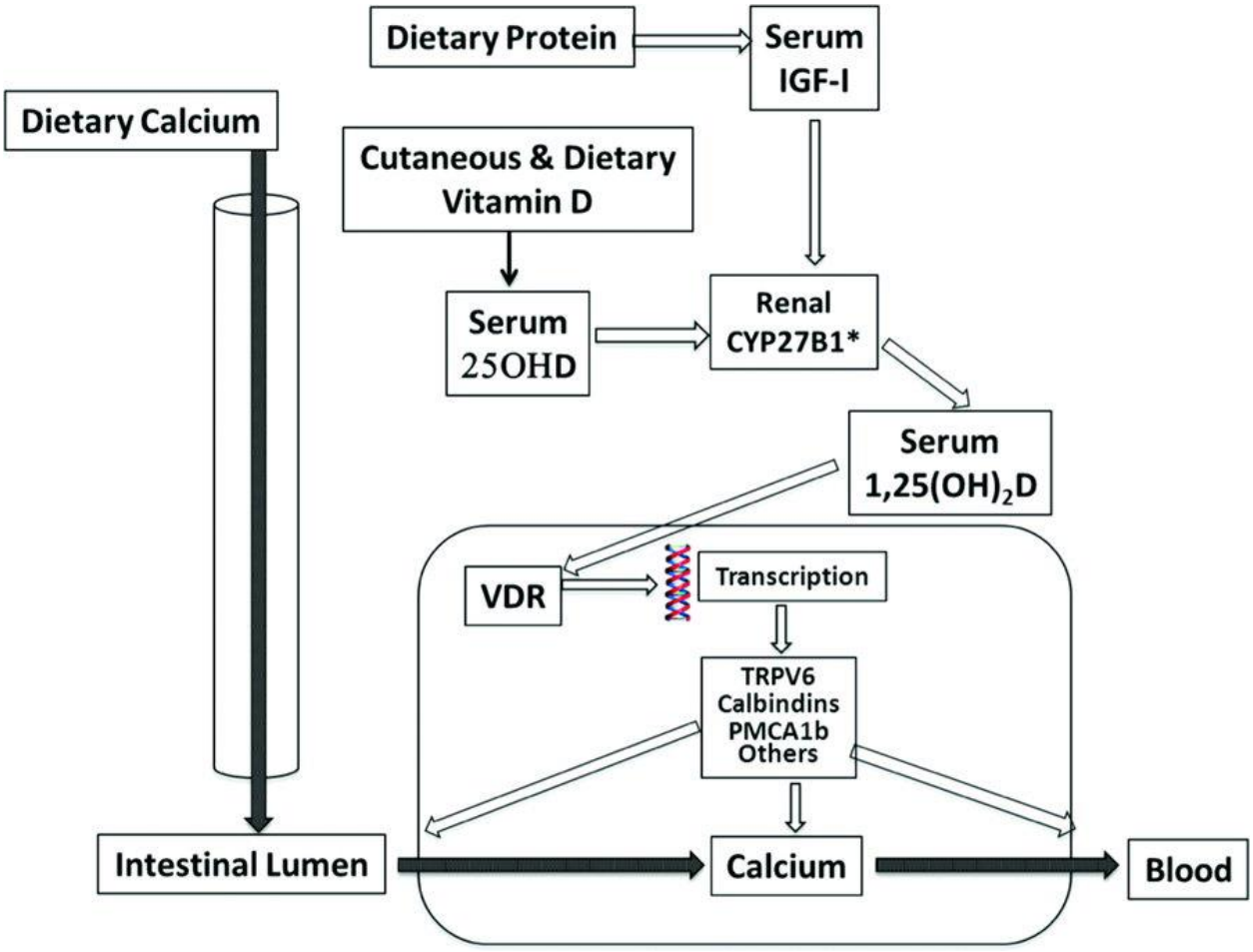

Legend: $1,25(OH)_2D$= 1,25-dihydroxyvitamin D; *VDR*= nuclear vitamin D receptors; CYP27B1= 25 hydroxyvitamin D-1α hydroxylase, TRPV6 = transient receptor potential cation channel, subfamily V, member 6, Calbindins = Ca binding proteins, PMCA1b = plasma membrane Ca ATPase 1b.

<u>Hypothesis 3</u>: MILK fat biomarkers and bone health in youth

Participants randomized to increase MILK will have significantly higher intakes of these saturated fatty acids, particularly C15:0, compared to those randomized to the control group. A positive relationship exists between MILK fat and plasma and erythrocyte C15:0 and C17:0.

In the final manuscript (chapter 5), compliance to dietary intervention of increasing daily MILK servings was assessed using traditional subjective and novel objective methods. Specifically, during each study visit a single-day 24 h dietary recall was collected from participants, in addition to 2 other recalls performed over the phone at 1 and 3 mo. Odd chain fatty acids from erythrocytes and plasma, particularly C15:0 and C17:0, were measured at baseline, 6 and 12 mo to serve as biomarkers of MILK intake and to reflect compliance during the study intervention phase.

By 12 mo, erythrocyte C15:0 increased in RInt group and MILK intake positively correlated with erythrocyte C15:0 and C17:0 at 12 mo. Plasma fatty acids neither correlated with MILK intake nor significantly changed over time. This implied erythrocyte fatty acids can serve as useful biomarker of MILK intake in adolescents and can detect short- and longer-term changes in the absolute MILK intake.

Although the use of these milk biomarkers has previously been explored in adult studies [42, 44, 237] to the author's knowledge, this was the first study to report on a MILK trial in adolescents. This novel approach of assessing compliance is largely beneficial when used in an intervention such as the one presented in the FAMILY Study i.e. intervention with adolescents. Adolescence is a critical period in the life span and implies multiple physiological and psychological changes that increase nutritional needs and affect dietary patterns. At this stage of

life, adolescents are more autonomous in their food choices, often eat take-away foods, spend more time at school/work thus, skipping meals and eating more junk fast food, and are less knowledgeable of portion sizes estimates, all of which questions the reliability of the traditional self-reported dietary assessment methods (e.g. 24 h recalls) [136, 198]. A recent study among adolescents 10-17 y old indicated that between 8 to 32 dietary recalls would be necessary to achieve an acceptable level of ≥80% reliability for total energy, fat, fruit, or vegetable intake in this age group [247]. Despite the open-ended nature of food recalls, they represent high participant burden. Estimation of nutrient or number of food servings, in this case MILK servings, from these recalls may be impeded by the participants' tendency to respond consistent with allocated servings; and adolescents may seek approval and avoid criticism [248]. Currently, research is exploring novel methods of assessing dietary intakes; these are considered at the end of this chapter.

In this study, correlation analysis showed no significant association between MILK intake and any of the plasma fatty acids. This is inconsistent with previous studies in adults [42, 232]. One possible explanation of this inconsistency could be attributed to the nature of the intervention per se. Specifically, the intervention used in this study specified the quantity of MILK servings to be consumed but not the quality i.e. MILK products of any fat content were allowed in this study, all while increasing the servings of MILK/d. Moreover, the MILK foods used to calculate the number of servings in the present study was limited to fluid milk, yogurt and regular cheese, of any fat content, whereas Sun et al. [42] and de Otto et al. [232] added contributions from sour cream, ice cream and butter. Another explanation could be related to the difficulties in accurate assessment of MILK consumption and the reliance on one self-reported 24-h recall. Food items were also not reviewed for specific details (i.e., % milk fat), but were rather categorized by food group. Furthermore, the concentrations of plasma fatty acids are influenced by the type and source

of fatty acids consumed in the diet [249, 250]. Postprandial plasma lipid responses were investigated by comparing plasma phospholipids profiles which revealed that phosphatidylcholine containing C15:0 showed a significantly larger plasma increase after the consumption of MILK-based meal compared to soy oil-based meal in men 40-60 y participating in a cross-over trial [249], implying that different fat meals affect plasma lipid composition differently. It is also plausible that the pattern of change over time would differ between different fatty acids [251]. For example, substituting a high saturated fatty acid diet by a high-n-6 polyunsaturated fat one was reflected in the plasma and erythrocyte fatty acid composition within 1-3 d [251]. A supplementation trial with n-3 polyunsaturated fatty acids showed that eicosapentaenoic acid can induce a faster lipemic response compared to docosahexaenoic acid, with a turnover period of 4 and 19 d for plasma and 6 and 65 d for erythrocytes. In a recent cross over trial among 7 males consume either a saturated fatty acid-rich or n–6 polyunsaturated fatty acid-rich diet for 8 wk, the same pattern of change over time occurred for the C15:0 composition of plasma and erythrocyte lipids. Together, this suggests a complex variability at which dietary fatty acids are incorporated and lost in different tissues and raise a question as to whether other phospholipid classes exist that can be more specific to plasma.

Recent fatty acid reporting standards recommended expressing fatty acids concentrations as percentage of total [236]. This study expressed erythrocyte and circulating fatty acids as a percentage of total and as concentration in solution (µg/ml of erythrocyte or plasma). However, the correlation between MILK intake and these fatty acids as well as the changes over the study period were significant when expressed in terms of concentration in a fluid or tissue (i.e. erythrocyte and plasma) but not as percentage of total fatty acids. These data reveal that the different methods of expressing fatty acids lead to dissimilar results, at least with specific fatty acids that are found in MILK fat in very small amounts (C15:0 at 1.0% and C17:0 at 0.6%) [36,

235]. Another concern was recently raised is related to the fact that these fatty acids are also present in many common food sources at amounts comparable to MILK fat such as beef, veal, lamb, fish and some vegetable oils as canola oil [235]. Therefore, changes in fatty acid concentrations may be mediated by both % milk fat and these food sources. In this study, fatty acids were quantified using gas chromatography, which is considered a valid method of identifying fatty acid peaks, however, identification of C15:0 and C17:0 may be challenging as these fatty acids are found in very small amounts in tissues and, may easily co-elute with other fatty acids in gas chromatography analysis [235]. This study raises important questions about how such reversals in association patterns impact the interpretation of numerous association studies evaluating fatty acids and their relationships with dietary intake. Despite these limitations, findings from this study are relevant to future trials that consider using saturated fatty acids, particularly erythrocyte C15:0, as a MILK fat biomarker. It would be also interesting to look at changes in milk, yogurt and cheese intake distinctly and the degree of correlation with such biomarkers i.e. C15:0 and C17:0 than intake of MILK collectively. Further, since C15:0 fatty acid is found in small amounts in erythrocytes and both reporting methods can be obtained simultaneously from the same laboratory assay, the use of both metrics is warranted to better understand fatty acid-diet relationships.

This study highlights the importance of using additional dietary assessment methods to associate and validate the findings. For example, in addition to C15:0 and C17:0, researchers are now using other metabolomics for assessing MILK intakes such as urinary citrate, hippurate, urea and others [252]. This technique of profiling low-molecular-weight metabolites has been successfully employed as a tool to differentiate different dietary patterns and is considered a potential tool for assessment of MILK intake instead of traditional methods, although further

validation needs to be established [252]. Nonetheless, if feasible, future work may wish to consider using metabolomics in conjunction with other valid and reliable biomarkers of MILK fat intakes.

6.2 Strengths and Limitations

This MILK intervention was the first intervention that targeted young males with the intention to ameliorate their bone density and had added to the existing body of literature in young females. The present study is unique as it employed, for the first time, the motivational interviewing technique as a counselling tool to improve MILK intake and adherence instead of the provision of milk products used in previous comparable trials. The study recognizes that a person is more likely to change a behavior if intrinsically motivated. Another major strength of the study is embodied in its longitudinal nature. Moreover, in addition to the common methods used to assess bone outcomes, i.e. DXA and bone biomarkers, this intervention included a bone assessment method that allows to assess changes in bone geometry i.e. pQCT at radius and tibia.

The assessment tools used in this study were considered valid for anthropometric measures, and biomarkers analysis, DXA, pQCT and physical activity (PA). This study considered the time commitment of participating thus conducted study visits at times that were convenient to participants, including weekends, all while maintaining a professional and clinical setting. This is particularly important when dealing with this age group where social responsibility is low; scan outcomes were always discussed with participants and parents, with an emphasis on developing strong bones. The MInt counselling sessions increased the awareness of the participants of the importance of optimizing bone health early in life, improved their knowledge or MILK portion size estimates; during each study visit, the interventionist insured consistent flow of the session and the topics of discussion, all of which made the experience enjoyable, unique and informative. The assessment of mood state is also another strength of this trial.

As will all studies, this research work also presents with limitations. One of the acknowledged limitations of this study is the lengthy recruitment. The recruitment period for the

FAMILY study was ~ 2 y and involved both active and passive methods of recruitment. More specifically, active methods included visiting >50 medical clinics, including dental and pediatric clinics, presenting and running recruiting booths at public and private high schools, colleges and universities from both French and English school boards and reaching out adolescents at public transport bus stops; passive methods included bilingual newspaper advertisements, mailing bilingual study brochures by Canada Post (n=>20,000), dropping >10,000 study brochures at schools, CLSCs, City Halls, City libraries, Sports complexes, in addition to online advertisements on Facebook (English). Specifically, 55% were recruited from schools (including high schools, colleges, and universities), 21% through word of mouth by enrolled participants, 14% from sports complexes, 7% through magazines, and 3% from Canada Post. These recruitment methods were time-consuming and costly. The recruitment-phase closed with 94 participants; however, only 76 participants completed the 12-mo period i.e. an attrition rate of 19%. Study visits, particularly at baseline, were quite lengthy due to the multiple bone scans and questionnaires collected which might have been a reason behind the high dropout rate. Comparable trials cited in this thesis had attrition rates ranging from 0% to 26%.

Another important aspect to be considered is the study location. The FAMILY study was carried out in Ste-Anne-de-Bellevue, located about 35 minutes away from downtown Montreal and 50-60 minutes away from East Montreal where a large youth population resides. Given the long-term nature of the study, almost 75% of the visits took place during the academic year. To accommodate participants, the visits were offered at convenient times (weekends, early morning).

Dietary intakes were self-reported which might have led to under- or over-estimation of dietary intake, particularly those done over the telephone at 1 and 3 mo. At baseline, some of the participants reported MILK intakes > 2 servings/d (n=12) which were inconsistent with their

reported intake at screening. At screening, participants were asked over the phone about how much milk, cheese and yogurt they usually consume per day. It is highly probable that some of the additional MILK found within mixed dishes was not evident on screening, even with prompting. Also, in many cases, screening for MILK intake of adolescents was reported by the parents who might not know what was eaten outside of home. This could be also affected by the sporadic intakes of MILK-containing foods such pizza, which was, possibly, not recalled at the time of screening. Additional reasons behind such discrepancy might be the different methods used to collect MILK intake at each time point (i.e. screening and baseline) and the different time-frame. At baseline, participants were given a detailed food frequency questionnaire. For each milk product and milk-containing food, a standard portion size was specified, and the participants were asked how often, on average, they consumed of that specified amount in the previous year. Another reason might be the time difference between screening and baseline visit which ranged from days to several weeks. Additionally, over-reporting of MILK intake can be elicited by participation in the intervention itself *per se*, for example, by the desire of participants to appear compliant or by their tendency to please the interventionist [253].

Although the use of pQCT technology allow a more comprehensive assessment of bone geometry and its relationship to muscle mass than DXA does, it presents with important limitations particularly in growing adolescents. As with any medical imaging device, proper positioning is essential to ensure accuracy, reproducibility and scan-rescan repeatability. In order to define the region of interest, pediatric protocols require that a reference line be placed at either the most proximal line of the growth plate if the latter is still open and through the middle of the ulnar border of the articular cartilage if closed [167]. However, the changing appearance of the growth plate in the growing adolescents made it difficult to exactly reposition the reference line during follow up,

which might be, at least partly, the reason for the lack of significant intervention effects on pQCT assessments.

Further, although food preferences, such as specific likes towards MILK, were discussed at baseline and an individualized plan was developed to help improve their MILK intake, up to 25% did not comply with the allocated MILK servings. Despite not achieving the recommended MILK intake, a significant positive trend was clear and meaningful results seen. Moreover, PA measures were self-reported; ideally, objective assessments such as accelerometers, would have been a more reliable assessment method especially that PA is an important modifiable factor to bone mass. Another important aspect to consider is the degree of sensitivity of the questionnaire used in this study to capture subtle differences in PA between MILK intake subgroups to exclude the possibility of confounding, even after adjustment. Although the youth physical activity questionnaire (YPAQ) has been validated in 4 to 17-year-old children and adolescents, the strength and statistical significance of correlation varied by sex and age. Moreover, YPAQ was validated in British adolescents which might engage in different sports compared to North American adolescents. For example, weight training and soccer were not an option in YPAQ while these were very common in the study participants especially the 16 to 18.9 group. Finally, the inability to blind the MILK intervention to the participant was another potential limitation.

6.3 Future Directions

In the present trial, DXA and pQCT were used to assess bone outcomes and are considered valid in pediatric trials [162, 167]. However, the use of these methods presents with challenges in the growing skeleton. DXA-derived BMD is an areal density and thus is affected by bone size changes. Similarly, pQCT repeatability over time is affected by the changing appearance of the growth plate which can make comparison of follow-up and baseline scans challenging to interpret and can increase the likelihood of a type II error. In order to reduce the variability and to detect significant and meaningful BMD changes in this age group, a larger sample size or a longer period of follow are advisable in the future studies. In fact, using the data from this thesis, 44 subjects would be needed in each group to detect the observed WB BMC accretion rate at 12 mo of 125.89 g/y in RInt vs 58.19 g/y in the control group. Another approach might be recruiting participants that either have finished growing at baseline of the study period. Additionally, the use of spinal computed tomography might be useful to reduce the variability as it provides a more refined characterization of bone given its increased sensitivity and accuracy and is independent of bone size, unlike DXA [166].

In the present study, the consumption of 4 or more MILK servings/d had positive effects on BMD, particularly in females. To date, several studies have examined the effect of MILK products combined on bone health. Fermented milk products like yogurt exhibit prebiotic properties that can enhance intestinal calcium absorption, intestinal permeability modulation and increase IGF-I, and, thus, can improve bone metabolism during pubertal growth [254]. Intervention with 125 g of yogurt for 5 times/wk (vs usual diet) over a 9-mo period demonstrated a favorable influence on forearm BMD among 3-5 y Chinese children [255]. Therefore, it would

be of interest for future studies to investigate the effect of increased yogurt intake on skeletal health in post-pubertal adolescents.

Engaging in PA is widely acknowledged to have a beneficial effect on BMD during growth [8]. There is growing evidence that benefits of PA undertaken early in life persist well beyond activity cessation [103]. In the present study, PA reflected the total weekly minutes of moderate to vigorous activities but did not take into account the different types of PA exercised. However, it is important to be cognizant that not all types of PA affect skeletal health equally. For example, high impact activities such as jumping appear to hold more osteogenic properties than lower-impact activities such as walking [256]. Moreover, evidence suggests that PA and calcium intake have a synergistic effect on bone [93]. Therefore, future studies that combine increased MILK intake (specifically 4 or more servings/d) with weight-bearing types of activities would be of interest to evaluate their effects on bone outcomes in post-pubertal adolescents.

MILK consumption declines as children transition into adolescence. Thus, effective interventions are needed to address this inadequacy. Dietary interventions that aim to increase MILK intake in adolescents should consider preferences, lifestyle and current trends. In the present study, the MInt technique has been used for the first time as a tool to improve MILK intake and adherence. Results of this technique showed promise as illustrated by a significant increase in MILK intake over 12 mo. A systematic review among adolescents 12 to 18 y [257] found that targeting adolescents at a group level (such as in school) is a successful strategy for changing dietary behavior. Improving knowledge is another target of intervention. The use of technology, although still in its infancy, seems to be impactful. Examples included web- and internet-based interventions that provide general encouragement and plan for social support [257]. Research also has started to adapt technology to improve dietary compliance such as mobile image-assisted and

web-based food recording [258]. Therefore, these novel mobile methods could be the future of dietary assessment although further research is needed to estimate their degree of error and cost.

6.4 Public Health Implications

The intervention presented in this study was based on Canadian health guidelines developed in 2007. The aim of this research was to provide evidence to enhance Health Canada's statement that "*Having milk ... every day provides the nutrients that you need for healthy bones and optimal health"[156]*. However, Canada's food guide has been recently updated and the MILK food group, as well as the meat and alternatives food group, have been lumped into the protein-rich category instead of their previous standalone food groups. Results from this dissertation supported the importance of MILK intake to improve bone health outcomes in 14 to 18 y old healthy adolescent females, particularly these with habitually low MILK intake. Therefore, nutritional education and intervention programs appear to be needed to help adolescents meet the dietary recommendations for bone-relevant nutrients, within the context of food and lifestyle preferences, and thus improve their skeletal health.

It has been noticed in this research that the current packaging of cheese (21 g) and yogurt (100 g) misleads the consumer into thinking that the packet is equivalent in quantity to one MILK serving compared to 0.4-0.6 servings, respectively. This poses an elevated risk of suboptimal intake of bone-relevant nutrients as protein and calcium. Adequate calcium intake is essential for PBM attainment in adolescents and for the prevention of osteoporosis in later years of life. Further, the present study demonstrated beneficial effects of MILK on bone health only in participants who consumed 4 or more servings/d. Therefore, public health messages should promote the importance of adequate MILK intake contributing to the optimization of calcium intake, and hence a maximal PBM. Taking into account all these considerations, packaging guidelines developed by public authorities as well as national legislation might need to be reconsidered.

6.5 Conclusion

In summary, this dissertation tested the effect of 12-mo MILK intervention in healthy post-pubertal adolescents and young adults on changes in bone health outcomes; explored the micronutrient status of bone-relevant nutrients in young males and females with usual low MILK intake; and the role of MILK fat as objective biomarkers of compliance.

The results presented throughout the dissertation suggested that usual low consumption of MILK products contribute to inadequate nutrient intakes for several bone-relevant nutrients, particularly for adolescent females, posing an elevated risk of suboptimal bone growth and mineralization. Further, female adolescents who were randomized to consume 4 or more MILK servings resulted in favorable changes in bone outcomes particularly at the WB and TH over the 12-mo study period. This dissertation confirmed those with suboptimal baseline nutrient status (as was the case of females in this study), especially in the case of a threshold nutrient such as calcium, are the ones who benefited more from this intervention. In different terms, this dissertation provided evidence that late adolescence is an extended window of opportunity for bone acquisition. It was also identified in this study that erythrocyte fatty acids, particularly C15:0, can serve a useful biomarker of MILK intake in adolescents and can detect short- and longer-term changes in MILK intake. Lastly, individualized nutrition education provided by health professionals was an effective strategy to promote MILK intake generally, and calcium and protein intake particularly, all of which contributes favorably to bone health.

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Appendix

Appendix 1: Permission to use Figure 2.2

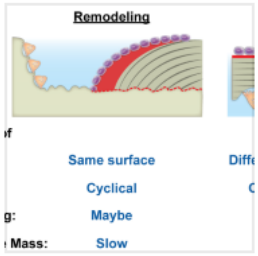
GJ

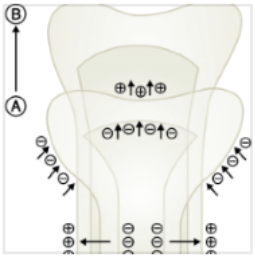
Gasser, Juerg <juerg.gasser@novartis.com>

7/2/2018 7:30 AM

To: May Slim

Save all attachments





Dear May,
Thank you for your e-mail and please accept my sincere apologies for not responding to your request any earlier but I was ooo for the last 10 days.

Attached please find the original files for Figures 2.11 and 2.14 for use in your PhD thesis. I hope that your progressing well with writing up your work (which I remember was a real hassle for me) and wish you all the Best for the viva and future. Please let me know in case you need anything else.

Kind regards
Jürg

Jürg Andreas Gasser, PhD
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Appendix 2: Permission to use Figure 2.3

RS

Fri 6/22/2018 10:25 AM

Rukshana Shroff <Rukshana.Shroff@gosh.nhs.uk>

Re: Permission to use a figure from a publication

To May Slim

Sure you can May!
Good luck with the PhD.

Rukshana

-------- Original Message --------
Subject: Permission to use a figure from a publication
From: May Slim <may.slim@mail.mcgill.ca>
Date: 22 Jun 2018, 14:58
To: Rukshana Shroff <Rukshana.Shroff@gosh.nhs.uk>
Hello Dr. Shroff,

This is May Slim from McGill University, Canada. I am writing to you today to request the permission to use your figure from Pediatric Nephrology publication titled: *The virtues of vitamin D—but how much is too much?* In my PhD thesis. I would appreciate if I can cite Figure 1 (Vitamin D sources and metabolism) and use it in my dissertation.

Thank you
May