THE IMPACT OF FOOD AND NUTRIENT INTAKE ON BONE FROM CHILDHOOD TO EARLY ADULTHOOD

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by
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ABSTRACT

Development of peak bone mass during childhood to early adulthood has been considered as a major determinate of risk of fracture and osteoporosis later in life. The purpose of this project was to determine the impact of food and nutrient intake on bone from childhood to early adulthood using mixed longitudinal data from the University of Saskatchewan Pediatric Bone Mineral Accrual Study (PBMAS).

To determine the role of consumption of milk products and vegetable and fruit on the total body-bone mineral content (TB-BMC) accrual in boys and girls from childhood to late adolescence, seven-year longitudinal data were used. Using a multilevel modeling statistical approach containing major biological and environmental factors, vegetables and fruit intake, calcium intake and physical activity were significant independent environmental predictors of TB-BMC in boys.

Change in the pattern of beverage intake of adolescents as a major component of nutrition transition has aroused health concerns such as obesity, tooth decay, and inadequate bone accrual. Beverage consumption and its relationship with calcium intake of grade 9 students from 1991 to 2004 was evaluated. Percent contribution of milk to total beverage intake was significantly decreased in boys and girls. A significant negative association between milk intake and consumption of non-carbonated soft drinks was observed in both genders. In girls only, a significant negative trend in calcium intake was observed over time.

Milk products, specifically fluid milk, were the major source of dietary calcium from childhood to early adulthood in both genders. There was a substitution of fluid milk by cheese, a decrease in vegetable and fruit intake, and the low intake of vitamin D in young adults, specifically females.

The effect of food and nutrient intake, measured at young adult age and previously in peri-adolescence, on bone mass was investigated. In males, the intake of calcium from peri-adolescence to early adulthood was sustained, whereas in females, there was a significant decrease. Height, weight, protein intake, physical activity and gender were the significant predictors of bone measures only in young adults who had consistent calcium intake from peri-adolescence. Collectively, the results of this study present the

bone protective nutrients and food groups from childhood to early adulthood in our cohort. The food choices and dietary habits of the cohort change by age, but not in the favour of bones, with females more at risk. To prevent risk of osteoporosis, there should be promotion of a healthy dietary plan, not a single food group or nutrient, accompanied with an adequate level of physical activity.

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CHAPTER 1

INTRODUCTION

1.1 Rationale

Osteoporosis is a major public health problem in the world. As the average age of the world's population shifts upward, the incidence and prevalence of osteoporosis and its economic burden on society will increase further. Estimates indicate that the number of osteoporotic hip fractures occurring in the world each year will rise from 1.66 million to 6.29 million by the year 2050 (Iqbal 2000). In Canadian women aged 50 years or older, prevalence of osteoporosis is 15.8% (Tenenhouse et al., 2000). The health costs of osteoporotic hip fracture in Canada will rise from $650 million (1996) to $2.4 billion by the year 2041 (Wiktorowicz et al., 2001). This situation could be worse in this province as Saskatchewan has highest senior population compared to other provinces (Saskatchewan Health, 2003), thereby implying an urgent need for preventive strategies.

Development of peak bone mass is thought to be a major determinant of vulnerability to osteoporosis. Approximately 90 to 95 percent of an adult's bone mineral is achieved by the end of adolescence (Teegarden et al., 1995). Since bone loss is a normal consequence of aging, those who acquire a greater bone mass during the first two decades of life should be at reduced risk for related skeletal health problems later in life (Bachrach et al., 1999; Bailey 1997). Several factors are supposed to influence bone mass accumulation: heredity, sex, diet components, endocrine factors, mechanical forces, and exposure to risk factors (Bonjour, Theintz, Law, Slosman, & Rizzoli, 1995). Although genetic factors play an important role, it is also well established that peak bone mass is influenced by multiple lifestyle factors (Rubin et al., 1999). Therefore the most preventive strategies attempt to reduce or reverse bone loss through dietary intakes and exercise. Dietary factors are implicated in modifying bone (Teegarden et al., 1998). Although there are several cross-sectional studies that indicate the effect of dietary

factors to bone health, to date no studies are available that have measured an individual's bone mass, nutritional background and physical activity during growing years into early adulthood. Using longitudinal data from the University of Saskatchewan Pediatric Bone Mineral Accrual Study (PBMAS) provides a unique opportunity to evaluate the role of diet on bone health during the first three decades of life.

There are different approaches to find the effect of nutrition on bone health. Many researchers have studied the effect of specific nutrients on bone. Due to the fact that over 99% of total body calcium is found in teeth and bone (Institute of Medicine 1997), investigators have studied the effect of dietary calcium intake on bone mineral status of children and adolescents (Chan et al., 1992; Gunnes et al., 1995; Kardinaal et al., 1999; Molgaard et al., 2001; Carter et al., 2001; Matkovic et al., 2005). Studies have found a positive association between dietary calcium intake and bone mineral status, which indicates the importance of calcium intake during childhood and adolescence to bone health later in life. However, results from clinical trials do not support the use of calcium supplementation in healthy children (Abrams, 2005; Winzenberg et al. 2006).

There are fewer studies in early adulthood. Welten et al. (1995) evaluated the literature on the relationship between dietary calcium and bone mass in young adults from 1985-1994. Calcium intake was positively associated with bone mass in premenopausal females. This association was fairly consistent across the different study designs. Teegarden et al. (1998), in a cross-sectional study, investigated the relationship between dietary calcium, protein, and phosphorus to bone mineral density (BMD) and bone mineral content (BMC) in 215 young Caucasian women aged 18-31. This study suggests that nutrients other than calcium contribute to bone health. The study by Lin et al. (2003) on 226 women (age 18-32 years) found that dietary calcium intake was not a significant predictor for the attainment of peak bone mass at measured bone sites. Recently Opotowasky and Bilezikian (2003) using data from the third national health and nutrition examination survey (NHANES III) found that higher calcium and milk intake in adolescence associated with greater BMD at hip sites in 20-39 years old and postmenopausal white women.

The effect of physical activity along with calcium intake on bone measures has been examined in various studies. Recker et al. (1992) found that dietary calcium intake

and physical activity both had a positive effect on bone gain in healthy women aged 18-26 years. There was an interactive effect of calcium intake and physical activity on lumbar spine BMD. However the interactive effect of calcium intake and physical activity is still controversial (Institute of Medicine, 1997; Baxter-Jones, Faulkner & Whiting 2004).

These controversies regarding the effect of calcium intake (alone) during young adulthood on bone or interactive effect with other environmental factors may be due to a number of study design problems, such as: low statistical power, errors in self-reporting of nutrient intake and physical activity, and short duration of study in intervention studies. The PBMAS provide longitudinal data from childhood to early adulthood. The usual intake of the same subjects has been assessed over time; therefore this study can help clarify these controversies.

In using a food group approach to investigate the effect of nutrition on bone, most studies have investigated the effect of milk products on bone measures during growth, as milk is the major source of calcium in North Americans' diet (Chan et al., 1994; Cadogan, Eastell, Jones, & Barker, 1997; Merriless et al., 2000; Do XQ et al., 2002; Kalkwarf et al., 2003). Studies have found a positive association between milk product intake and bone mineral measures. Since milk contains other nutrients essential for bone growth, the results of those studies may be due to nutrients in addition to calcium including protein, phosphorus, magnesium, zinc, vitamin D (if fortified), vitamin A and riboflavin (Cadogan et al.,1997). Previous milk intakes from childhood and adolescence had a beneficial effect on bone measures later in life in evaluating literature which used milk history questionnaires to report previous milk intake (Vatanparast & Whiting, 2004). However, a common conclusion in cross-sectional and retrospective studies is that longitudinal studies are necessary to examine whether the predictions based on cross-sectional results can be supported.

Although a great deal of attention has been given to the importance of milk products, calcium and vitamin D intake, much less in known about the effects of other nutrients and food groups on bone. However, the role of dietary vegetables and fruit has been gaining increasing prominence in the literature. It is hypothised that bone mineral acts as a buffer base and that lifetime buffering of the acid load from the ingestion of

ordinary diet leads to gradual and accumulated bone loss. Therefore it might be worthwhile to consider decreasing the rate of bone attrition by the use of a diet favoring "alkaline". Potassium and magnesium, two nutrients that may have such buffering effect, are found in vegetables and fruit (Tucker et al., 1999). In addition, other bone beneficial nutrients in vegetables and fruit such as antioxidants, phytoestrogens and vitamin K might explain bone protective effects of vegetables and fruit found in cross-sectional studies (New, 2003). The PBMAS is the first prospective study that provides data to evaluate the effect of vegetables and fruit on bone mineral status during the first two decades of life.

Over the last few decades, an overall shift in dietary patterns has occurred, known as Nutrition Transition (Popkin 2002). A critical element of this shift is the increase in consumption of sugared fruit drinks and soft drinks particularly during childhood and adolescents (Guthrie & Morton 2000). This change in beverage intakes has caused several health concerns including links between the high intakes of sugar-sweetened beverages and decreased intake of high-fiber foods and increased obesity (World Cancer Research Fund, 1997; James et al., 2004). Also milk has been increasingly substituted with soft drinks resulting in low calcium intake (Cavadini, Siega-Riz & Popkin, 2000; Whiting et al., 2001). However, Park et al. (2002), studying nationally representative sample of children age 1 to 19 years, for three 2-year periods between 1987 and 1998, reported no difference in milk consumption while the intake of carbonated soft drink intake decreased among 1-5 and 15-19 age groups. By comparing PBMAS data with another study conducted in 2004 (the *FUEL: Fluids Used Effectively for Living* intervention) with subjects at the same age and same school (Henry et al., 2005), we determined whether beverage choices have changed by from 1991 to 2004. This might be helpful in clarifying the inconsistent data.

In addition to change in dietary choices over time, food preference might differ by life stage. Studying subjects in PBMAS, Iuliano-Burns et al. (1999) showed that milk products were the major source of dietary calcium for boys and girls aged from 8-19 years, contributing between 57.2% in girls to 63.3% in boys to total dietary calcium. They found that older boys (age 14-19 year) increased dietary calcium intake compared to younger boys, but the opposite occurred with older girls, whose dietary calcium intake

decreased. Thus for this group in particular, calcium intake declined when skeletal needs were greatest. Less is known about the dietary habits and their effect on bone mineral mass of young adults. Whether food sources of calcium change from childhood to early adulthood and how this change affect bone status are other questions are addressed using PBMAS and its follow-up data.

It should be noted that most studies on nutrition and bone have been done in females. In males, only a few studies have been published to draw firm conclusions about the effect of nutrient and food intake on bone. The above mentioned information represents the areas of research that have been addressed in this thesis through the following research objectives.

1.2 Objectives

The objectives and the related hypotheses of this thesis were:

1. To determine the role of consumption of food groups on total body-BMC around the age of PHV.

   Hypothesis: *Appropriate intake of milk products, and vegetables and fruits has beneficial effect on total body-BMC around the age of PHV in boys and girls.*

The two specific objectives for the first objective were:

1.1 To determine the effect of appropriate intake of milk products on total body-BMC around the age of PHV in boys and girls.

1.2 To determine the effect of appropriate intake of vegetables and fruit on total body-BMC around the age of PHV in boys and girls.

Study one investigates the two specific objectives of the first objectives.

2. To determine how changes in dietary intake, from childhood through adolescence to early adulthood affect bone mineral attainment. In addition, to distinguish whether changes in dietary intake are due to the life cycle and/or are due to a changing food environment.

The hypotheses were:

Hypothesis 1: *Food and beverage choices and eating patterns change from childhood to early adulthood.*

To investigate this hypothesis, two specific objectives were set, further, they were divided to two sub objectives as follows:

2.1 To determine if there have been temporal changes in food intake from 1991 to 2004.

2.1.1 To determine changes in beverage intake in adolescents from 1991 to 2004.

2.1.2 To determine changes in calcium intake from 1991 to 2004.

This objective and its sub objectives were examined in **study two**.

2.2 To determine if there have been life cycle changes in diet and food sources of calcium.

2.2.1 To describe young adult subjects' diet and compare to children and adolescents in PBMAS.

2.2.2 To determine changes in food sources of calcium from childhood to early adulthood.

Study three addresses this objective and its sub objectives.

Hypothesis 2: *Previous calcium intake during adolescence affects bone mass at early adulthood.*

This hypothesis was examined through the following specific objective and its two sub objectives.

2.3 To determine how change in calcium intake from the age of PHV to age of peak bone mass influences bone mass when peak bone mass is achieved.

2.3.1 To determine frequency of change in calcium intake at the age of PHV and as young adults.

2.3.2 To evaluate the relationship between changes in calcium intake by age and bone mass in early adulthood.

Study four explores this specific objective and its two sub objectives.

1.3 Summary

The Impact of Food and Nutrient Intake on Bone from Childhood to Early Adulthood was a multidimensional study that examined nutritional intake, physical activity and body measurements in relation to bone mineral in a mixed longitudinal design. In addition, this study evaluated the change over time and life cycle on dietary intake of healthy individuals from childhood to early adulthood. The objectives of research were tested in four studies, each study designed to contribute to the understanding the relationship between food and nutrient intake, physical activity level and bone and body measures in growing individuals.

Chapter 2 presents a critical review of literature explaining the development of the skeleton and biological and environmental factors affecting it. Nutritional factors, as they are of research interest, were discussed in depth.

Chapter 3 presents an overview of the methodology used in this research.

Chapter 4 presents the evaluation of the effect of vegetable and fruit consumption and calcium intake on bone mineral accrual of boys and girls during growth from childhood to adolescence in the University of Saskatchewan Pediatric Bone Mineral Accrual Study.

Chapter 5 presents an examination of trends of beverage intake from 1991-2004 in grade 9 students living in Saskatoon.

Chapter 6 presents the assessment of changes in food sources and intake of calcium from childhood to early adulthood in the University of Saskatchewan Pediatric Bone Mineral Accrual Study.

Chapter 7 presents the examination of the association between protein intake accompanied by calcium consumption and bone mineral mass achievement of young adults in the three year follow-up of the University of Saskatchewan Pediatric Bone Mineral Accrual Study.

Chapter 8 provides an overall discussion of the key findings presented in the research and examines the linkage between the four studies.

1.4 Definition of terms

For the purposes of this research, the following terms and abbreviations were defined with respect to their usage in this thesis

PBMAS The University of Saskatchewan Pediatric Bone Mineral Accrual Study (1991-1997) and its follow-up (2003-2005)

PHV Peak height velocity, maximum velocity of height gain, which is used as a marker of maturity.

BMC Bone mineral content, the amount of bone mineral contained within the skeletal mass, expressed as grams (g).

BMD Bone mineral density, bone mineral content divided by the scanned area, expressed in grams per centimeter squared (g/cm^2).

DXA Dual energy x-ray absorptiometery, method of measuring bone mass and mineral density using low dose radiation.

PBM Peak bone mass, maximal bone mass achieved by the time of skeletal maturity.

PBMCV Peak bone mineral content velocity, maximum velocity of bone mineral content accrual

Childhood The ages between the initiations of PBMAS (age 7 years) up to and including the age of PHV-2 year. This time period captures the regular bone growth pattern in childhood before starting the effect of maturity.

Adolescence From one year before the age of PHV to three years after it. This time period captures peak bone mineral content accrual.

Early adulthood 20-26 years.

CHAPTER 2

LITERATURE REVIEW

2.1 Overview of lifetime bone health

2.1.1 Bone as an organ

Bone is an organ composed of cortical and trabecular bone, cartilage, haemopoetic and connective tissues (Malina & Bouchard, 2003). These tissues enable the skeleton to serve its main functions that include the protection of internal organs, movement of parts of the body, and the provision of a site for hematopoiesis. In addition, the skeleton is of fundamental importance in mineral homeostasis. Bone is the principal reservoir of calcium, phosphorus, sodium, magnesium and carbonate (Institute of Medicine, 1997).

An individual's bone is formed during the prenatal period through two different processes: bone growth between embryonic membrane identified as intra-membranous bone formation, and endochondral process that is bone formation from cartilage (Malian et al., 2003). There are three types of bone cells. An osteoblast, the bone-forming cell, is of mesenchymal origin and its main function is to produce new bone matrix, osteoid, and to mineralize it. When an osteoblast is entrapped in the bone matrix, it becomes an osteocyte (Stein et al., 1996). Bone resorption is done by the bone-resorbing cell, the osteoclast. Osteoclasts originate from the hematopoietic-macrophage lineage (Rubinacci et al., 1998).

The result of bone formation through those processes is two types of bone, cortical and trabecular. In a healthy adult, cortical (also called compact bone) contributes approximately 80% of total bone mass, and is most abundant in the diaphyses of long bones. It has a high mineral content, and its function is principally mechanical (Lee & Nieman,

2003). Spongy or trabecular bone is composed of a network of fine bone plates filled with hemopoietic marrow, fat containing marrow, or blood vessels. Trabecular bone is located in vertebral bodies, flat bones and in the epiphyses of adult long bones. Therefore, while cortical bone contributes mostly in the appendicular bones, trabecular bone is most abundant in the axial bones (Lee & Nieman, 2003).

After the initial bone formation in the embryonic life, which is for growth of bone tissue as well as adaptation to mechanical loads, large changes in the bone morphology may be needed. This can be done by continuous bone resorption and bone formation. If this process occurs at different bone locations and alters the bone morphology, it is defined as bone modeling (Ruimerman, 2005). If it is done at the same bone location it is defined as remodeling. Bone tissue is under constant reconstruction which is necessary for normal skeletal maintenance particularly during adulthood (Rubinacci et al., 1998). In a homeostatic equilibrium, resorption and formation are balanced. Old bone is continuously replaced by new tissue to ensure that the mechanical integrity of the bone is maintained but it causes no global changes in morphology, in this case, it is defined as bone remodeling. Approximately 30% of bone mass is remodeled in a year (Malina & Bouchard, 2003).

2.1.2 Bone and aging

In the growing individual, a positive relationship between bone mass and age reflects rapid bone deposition (Malina & Bouchard, 2003). In young and middle-aged adults, rates of bone deposition and bone resorption are typically in balance. However, during late adulthood, there is a negative association between age and bone mass in such a way that as age increases bone mass decreases, reflecting a more rapid rate of bone resorption compared to bone deposition (Malina & Bouchard, 2003; Dawson-Hughes, 1996). During menopause, women lose approximately 3% of the total body bone mineral mass per year, followed by around 1% per year bone loss after the age 65 in both females and males (Dawson-Hughes, 1996). This condition results in microarchitectural deterioration of bone tissue and consequently loss of bone strength, thus making bone more fragile and easily susceptible to fracture. These characteristics explain osteoporosis (Brown & Josse, 2002).

Nowadays osteoporosis is a major public health problem in the world. In Canadian women over 50 years of age, the prevalence of osteoporosis is 16 % (Tenenhouse et al., 2000). Osteoporotic hip fracture is an important cause of death and disability, particularly in the developed regions of the world. As the average age of the world's population shifts upwards, the number of osteoporotic hip fractures occurring in the world each year are likely rise from 1.7 million to 6.3 million by the year 2050 (Iqbal, 2000). Annual costs of hip fracture in Canada are expected to rise from $650 million (1995- 96) to $2.4 billion by 2041, hence justifying an urgent need for preventive strategies (Wiktorowicz et al., 2001). Since bone loss is a normal consequence of aging, development of bone mass during the first two decades of life is thought to be a major determinant of vulnerability to osteoporotic fractures in such a way that an increase by 10% in bone mass would reduce the fracture risk by 50% (Teegarden et al., 1998; Bonjour, Ammann, Chevalley, Ferrari, & Rizzoli, 2003). This indicates the importance of optimal bone mass achievement during childhood and adolescence to prevent osteoporosis later in life.

2.2 Bone mass development during growth

Kreipe, in 1992, stated that "the prevention of osteoporosis, often deemed a geriatric disorder, may now be considered the legitimate domain of pediatricians." The underlying assumption is that peak bone mass achieved early in life is one of the major determinants of the risk for future osteoporosis (Bonjour et al., 2003). In recent years, the interest in the bone mineral content (BMC) of children, and in factors affect BMC of children, has increased considerably.

2.2.1 Physiology of bone growth

The bones of the skeleton are formed prenatally in cartilage. The cartilage model is subsequently and gradually replaced by bone tissue as each bone grows. This process begins prenatally and continues into the mid-20s for some bone sites (Malina & Bouchard, 2003). A long bone grows in length as a function of activities in the cartilaginous growth plate (epiphyseal disc). The rate of growth in length varies in different bones and with age. Growth in width simply occurs by the deposition of bone on the outer or subperiosteal surface and resorption of bone on the inner or endosteal

surface. Linear growth of the long bones often results on adding bone to the endosteal surface of the metaphysic when it is modeled to diaphysis. As bone grows in width and length, a need exists for constant remodeling to maintain the shape of bone. The remodeling process involves both internal and external area in the metaphysis (Malina & Bouchard, 2003).

2.2.2 Pubertal accrual of bone

Human growth is a continuous process, a gradual sequencing from one stage of physical and mental development to another. This process is highly regulated and depends on environmental conditions as well as genetic endowment. The tremendous growth during the first two decades of life plays a major role in human development and puberty is a landmark of it (Malina & Bouchard, 2003). Individuals with the same chronological age may be at different levels of maturation (Malina & Bouchard, 2003). Therefore, the gain in BMC during adolescence is more a function of maturational stage than chronological age; accordingly, comparison of adolescence bone accrual rates with reference to chronological age is misleading. This indicates the importance of comparisons based on maturational benchmarks (biological age) for determining the true bone mineral accrual rates (Bailey, Martin, McKay, Whiting & Mirwald 2000).

The three most commonly used indicators for biological maturity status are maturity of skeleton, appearance of secondary sex characteristics, and the timing of maximum growth in height during the growth spurt (Malina & Bouchard, 2003). Age at peak height velocity (APHV) is the most universally used indicator of somatic maturity in longitudinal studies during growth (Malina & Bouchard, 2003). Age at PHV is determined using appropriate longitudinal data for the interval from about 9 to 16 years of age. Therefore with only three or four annual observations during adolescence, the age of PHV can not be determined with accuracy. Ages at PHV in samples of European girls and boys are 11.4 - 12.2 y and 13.8 - 14.4 y, respectively. In North America, the age ranges of PHV range from10.8 - 12.0 y in girls and 13.4 - 14.1 y in boys (Malina & Bouchard, 2003). Age at PHV serves as a landmark against which attained sizes and velocities of other body compartments can be expressed. The University of Saskatchewan Pediatric Bone Mineral Accrual Study (PBMAS) is a mixed-longitudinal study conducted between 1991-1997. It has reported the age at PHV of 11.8 y in girls

which has been attained ~ 2 years earlier compared to boys (13.4 y) (Martin et al., 1997; Bailey, Martin, & McKay 1996; Bailey, McKay, Mirwald, Crocker, & Falkner 1999). In PBMAS, age at PHV has been used as the maturity landmark to express bone mass achievement, measured as BMC, from childhood to adolescence.

The skeleton accounts for 98% of height, and BMC is closely correlated with height in children until the occurrence of the adolescent growth spurt. Maturity affects bone size much more than it does BMC (Malina & Bouchard, 2003). Therefore, there is an asynchrony between the gain in height and the expansion of bone mineral mass during pubertal maturation, where a greater than six months lag time exists between the age at PHV and the age of peak bone mineral accrual velocity (Figure 2.1)(Bailey et al., 1996). Since bone size increases before corresponding increase in bone mass, the rapid longitudinal and circumferential bone growth produces a transient decrease in structural bone density (Bonjour et al., 2003). This phenomenon may be responsible for the occurrence of transient bone fragility in some specific bone sites in adolescence (Bonjour et al., 2003). The increase in risk of fracture at about the time of the pubertal growth spurt has been reported in different studies (Bonjour et al., 2003).

2.2.3 Gender difference in skeletal development

Although there is a dramatic increase in bone mass as a result of normal growth and maturation during childhood and adolescence (Teegarden et al., 1995), before puberty no substantial gender difference has been reported in bone mass of the axial or appendicular skeleton (Bonjour et al., 2003). There is no evidence of a gender difference in bone mass at birth. This absence of a sex difference in bone mass is maintained until the onset of pubertal maturation.

During puberty, a gender difference in bone mass becomes expressed. This difference appears to be due principally to a more prolonged bone maturation period in males than in females (Malina & Bouchard, 2003). In adolescent females, the gain in bone mass declines rapidly after menarche, and no considerable gains are observed even two years later in some bone sites (Bonjour et al., 2003). In adolescent males, bone mineral accrual accelerates particularly from 13-17 years. After age 17 years, the gain in bone mineral mass declines; however, it remains significant between 17-20 years in some bone sites (Bonjour et al., 2003). The prolonged bone maturation period in boys

results in increasing bone size and cortical thickness (Bonjour, Ammann, Chevalley, & Rizzoli, 2001; Bonjour, Theintz, Law, Slosman, & Rizzoli, 1994).

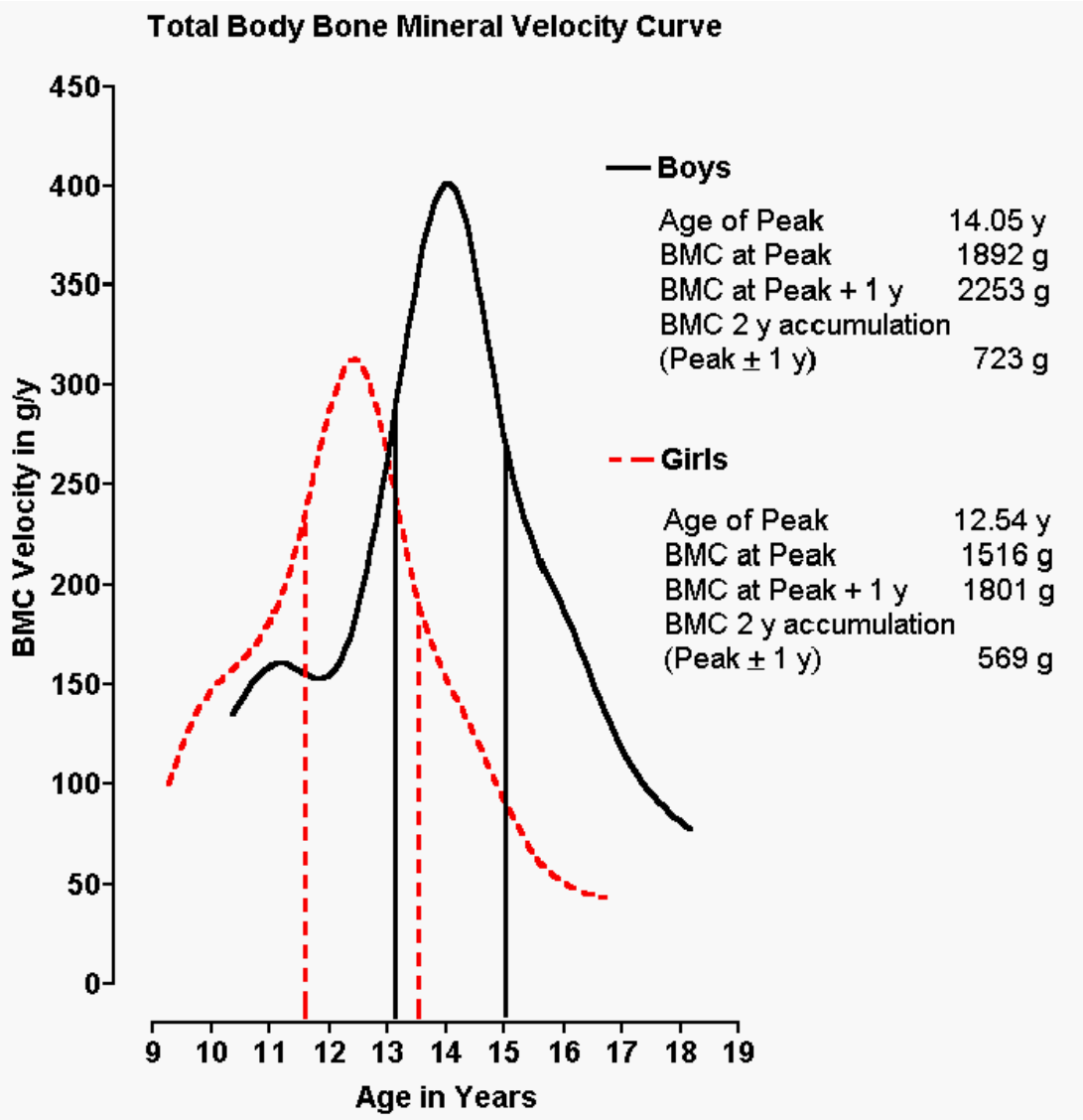

Figure 2.1 Total body bone mineral content velocity during adolescence. Age of peak height velocity (PHV) is 11.8 y in girls and 13.4 y in boys. Peak bone mineral content velocity (PBMCV) is achieved 0.7 year after PHV. Martin et al. (1997).

Baxter-Jones et al. (2003) using longitudinal data from 85 boys and 67 girls aged 8-19 years, observed that the statistical difference in bone mineral accrual due to gender difference was less than the error of the measurement. Using a multilevel modeling approach for growth data analysis, they have shown that the major contributors to the difference in BMC observed between the genders were associated with height and lean mass differences. Since boys, on average, were taller and more muscular than girls, they had greater total body, femoral neck and lumbar spine BMC. They concluded that the gender difference in bone mineral accrual was explained by anthropometric differences. Therefore there was no biologically significant independent difference between boys and girls in BMC of different bone sites. Later, in a cross-sectional study, Arabi et al. (2004) examined 184 boys and 179 girls aged 10-17 y to determine the independent gender-specific contribution of lean mass and fat mass to BMC. They reported that both lean mass and fat mass were consistent predictors of BMC in boys and girls. However, the contribution of lean mass to BMC variance was larger in boys than in girls. These studies suggest that the gender difference in bone mineral accrual is a function of bone and body size.

2.2.4 Time of peak bone mass attainment

Peak bone mass (PBM) has been defined as the maximum strength and density of bone tissue that a person has in his/her life (Heaney, 2000). The age at which PBM is achieved is not certain. Early estimates of attainment of peak total-body BMD and BMC ranged from late adolescence to mid-20s (Teegarden, et al. 1995, Recker, et al. 1992). In a longitudinal study, Theintz et al. (1992) suggested that PBM maybe attained at age 16 y in healthy adolescent females. In contrast, the results of the Fels Longitudinal Study showed that the age at which PBM, measured as BMC and BMD, were achieved ranged between 20 and 25 years (Nguyen et al., 2001). At this age range, the bones of the human skeleton reach 90% to 95% of their peak bone mass. Over the next 10 years, the final 5% to 10 % of bone mineral may be added (Lin et al., 2003).

Different skeletal sites achieve PBM at very different times. For example, the highest level of peak spinal BMD, femoral neck BMD, trochanter BMD, and Ward's triangle BMD was achieved at the age of 23, 18, 14, and 16 years, respectively, in the cross-sectional study by Lin et al. (2003). Females attained their peak earlier than males

(Nguyen et al., 2001). Since puberty occurs approximately two years earlier in girls compared to boys (Malina & Bouchard, 2003), the earlier PBM in girls is more likely related to earlier puberty.

From the information presented, it comes to view that PBM attainment may take place between late in the second decade and early in the third decade of life, depending on the skeletal site. Although most of adults' bone mineral mass is achieved in late adolescence (Bonjour et al., 2003), the whole period from childhood through young adulthood is important for achieving the potential genetically designed bone mass. Therefore, understanding the factors that affect bone mass development during those years is essential in improving bone quality with the potential to prevent osteoporosis later in life.

2.3 Factors affecting bone mass development

Bone mass accumulation is influenced by heredity, sex, diet, hormones, mechanical forces, and exposure to risk factors (Bonjour, Theintz, Law, Slosman, & Rizzoli, 1995; Bonjour et al., 2003). Although genetics is most prominent factor, it is well established that environmental factors such as nutrition and physical activity influence bone mass accrual during adolescence (Heaney et al., 2000; Rubin et al., 1999).

2.3.1 Genetics

There is compelling evidence to suggest that both the development of bone to PBM attainment and subsequent loss depend on genetic, hormonal, environmental and nutritional factors. Studies in twin families have shown that genetic factors play an important role in the regulation of BMD. It has been estimated from twin studies that 50-85% of the variance in BMD is genetically determined (Pocock et al., 1987; Christian et al., 1989; Slemenda et al., 1991). Family- based studies have also found strong heritability estimates for BMD (Gueguen et al.; 1995).

Over last two decades, there have been large number of studies published that reported associations, or lack thereof, between candidate genes and bone markers such as BMD and fracture incidence. These genes encode a wide range of proteins, including receptors for calciotrophic and sex steroid hormones, bone matrix proteins, and

regulators of bone metabolism (Cusack & Cushman, 2003; Ferrari & Rizzoli, 2005). However, heredity and environment are not completely separable. There is increasing evidence that the effects of some of genes on bone health-related variables are modified by certain nutrients and other dietary components (Cusack & Cushman, 2003). Therefore manipulation of an environmental factor (e.g., diet) may influence the expression of genetic influence. Studies investigating the interaction between vitamin D receptor (VDR) genotype, calcium intake and bone integrity were among the first to test gene-nutrient interactions in determining bone health (Ferrari et al., 1995). The area of gene- nutrition interaction in bone health, although a fairly new field, may be able to explain the pathophysiologic basis of the development of osteoporosis.

2.3.2 Physical activity

The concept that physical activity has a positive effect on bone dates back approximately 400 years to the time of Galileo (Murphy & Carroll, 2003). Galileo is known to have discovered the relationship between the mass and form of a bone to the mechanical load applied to it. Bone adapts to the loads applied to it, so that increased mechanical loading leads to an increase in bone density, whereas removal of habitual loads is followed by bone loss. Physical activity intervention studies generally but not uniformly, confirm a training-induced increase in BMD (Heaney et al., 2000). Evidence has been presented that childhood (earlier prepubertal period) may represent a unique opportunity for achieving long-lasting skeletal benefits of physical activity (Wang et al., 2005). A study by Slemenda et al. (1991) was among the first to show that gains in BMD over time were progressively higher with increasing levels of physical activity in a group of children aged 5-14 years. Bailey et al. (1999) using PBMAS longitudinal data observed from 10% to 20% greater 2-year bone mineral accruals across puberty in exercising children than in sedentary controls.

The exact mechanisms by which physical activity increases bone mass are not completely understood. Animal studies suggest that physical activity more likely stimulates osteoblastic activity and thus new bone formation (Malina & Bouchard, 2003; Yeh et al., 1993). Although regular physical activity during childhood and adolescence is associated with increased BMC, this effect is generally specific to the bone sites that mechanical strains occur (Kannus et al., 1996). In addition, weight-bearing physical

activity, compared to non-weight-bearing physical activity, has more effect on bone mineralization in those specific bone sites (Malina & Bouchard, 2003). Nordstrom et al. (1998) evaluated the influence of different types of weight-bearing physical activity on BMD in adolescent boys is three different groups, badminton players, hockey players and controls. They observed that the badminton players had higher BMD at trochanter and distal femur sites compared with hockey players, despite their lower weekly training time. Also, they had higher BMD in most weight-bearing BMD sites. They concluded that weight-bearing physical activity has a great osteogenic potential in adolescent boys.

It is logical to expect that, during the growing years, calcium intake and physical activity are both necessary to optimize bone gain, as the skeleton needs calcium as a major structural component and mechanical loading to stimulate mineral deposition. Calcium intake may have a potentiating effect in allowing physical activity to apply its effect on bone mineral. Studies suggest that calcium intake may influence the impact of physical activity on bone (Iuliano-Burns et al., 2003; Specker & Binkley, 2003). Baxter-Jones et al. (2003) using PBMAS longitudinal data observed that physical activity is quantitatively a more important predicting factor in affecting bone than calcium intake among adolescents; however, no interaction between calcium intake and physical activity was found. In a double-blind randomized placebo-controlled trial on 113 healthy premenarcheal girls, Courteix et al. (2005) reported that that calcium supplementation increased the effect of physical exercise on bone mineral acquisition in the period preceding puberty, and that calcium supplementation without physical activity did not improve the BMD acquisition during this period. They concluded that the physical activity that stimulates bone accretion needs a high calcium intake to be completely effective.

Although most of the adult bone mass has been acquired by the end of the second decade, the bony consolidation occurring in young adults in the third decade may be also be augmented by greater habitual physical activity (Recker et al., 1992; Baxter-Jones et al., 2003). The issue of interactive effect of physical activity and calcium intake has been under investigation in a relatively few studies, and whether calcium and physical activity interact synergistically is an important unanswered questions in the area of lifestyle-related bone health and needs more research (Murphy & Carroll, 2003).

2.3.3 Nutritional aspects of bone growth

Nutrition is an important modifiable factor in the development and maintenance of bone mass. Calcium is the main mineral comprised in bone mineral content. Phosphorus is important both for cellular activities and for mineral deposition (Heaney et al., 2000). Furthermore, the skeleton serves as a very large nutrient reserve for calcium and phosphorus (Tucker, 2003; Institute of Medicine, 1997). Protein is another major component of bone. Other dietary components such as magnesium, zinc, copper, iron, fluoride, and vitamins D, A, C, and K are required for normal bone metabolism (Tucker, 2003).

2.3.3.1 Calcium

Calcium is the most common mineral in the human body. Approximately 80-90% of bone mineral content is comprised of calcium and phosphorus in the form of hydroxyapatite [Ca_{10} $(PO_4)_6(OH)_2$] (Institute of Medicine, 1997). The high velocity of bone mineral accumulation during puberty requires a greater intake of calcium compared to childhood and young adulthood (Institute of Medicine, 1997). The increase in the plasma level of the active form of vitamin D, 1,25-dihydroxy D_3 (1,25DHD) and the stimulation of the renal tubular reabsorption of inorganic phosphate are two adaptive mechanisms to cope with increased bone mineral demand of calcium during pubertal growth spurt (Bonjour et al., 2003). Insulin-like growth factor-1 (IGF-I) presumably is the main hormone responsible this (Bonjour et al., 2003). The plasma level of IGF-I rises during pubertal maturation and reaches to its peak at mid puberty. IGF-I stimulates kidney to produce 1,25DHD and maximize the tubular reabsorption of inorganic phosphate (Caverzasio et al., 1990; Bonjour, 2003). Consequently an elevated plasma level of 1,25DHD intensifies the capacity of intestinal epithelium to absorb calcium and inorganic phosphate (Bonjour et al., 2003). It has been shown in girls that during puberty, calcium absorption and bone calcium deposition rate increase, resulting in more calcium absorption and less overall calcium excretion than adults with the same calcium intake (Abrams & Stuff, 1994; Abrams et al., 2000; Weaver et al., 1995).

2.3.3.1.1 Calcium requirement and bone mass development

The Panel for the Dietary Reference Intakes (DRI) for calcium could not establish an Estimated Average Requirement (EAR) for calcium due to lack of precise data (Institute of Medicine, 1997). The EAR is defined as a data-based, statistically relevant estimate of the average daily nutrient intake level that will meet the requirement of half of the healthy individuals in a given life-stage and gender group (Institute of Medicine, 1997). Currently, only Adequate Intakes (AI) of 800 mg/d calcium for children aged 4-8 y, 1300 mg/d calcium for adolescent boys and girls aged 9-18 y and 1000 mg/d calcium for young adult males and females aged 19-30 y have been set (Institute of Medicine 1997). An AI is derived through experimental or observational data that show a mean intake of nutrient in groups of healthy people. The AI does not bear a consistent relationship to the EAR since it is set without being able to estimate the requirement (Institute of Medicine, 1997). Therefore the ability of nutritionists to provide dietary advice and assess nutrient intake using an AI is limited.

Evidence from balance studies, calcium accretion studies and BMC measurements were used by the Panel for setting the DRI for calcium for children from age 4 through age 8 years. Most balance studies had been conducted prior to 1960. In the meta–analyses of balance studies of young children, a calcium retention of 174 mg/day was obtained which is approximately 21 percent of daily calcium intake (Matkovic & Heaney, 1992). Thus, for the children aged 4 -8 years, the AI for calcium of 800 mg/day has been set (Institute of Medicine, 1997). There were no balance studies available for boys; therefore, the data for girls has been applied to boys (Institute of Medicine, 1997).

In setting an AI for calcium for adolescent boys and girls, the DRI panel for calcium looked at three lines of evidence: a factorial approach, calcium retention using a nonlinear regression model, and results of clinical trials (Institute of Medicine, 1997). The factorial calculation of calcium requirements for adolescents was for the 2 years of maximal peak BMC accrual. In this approach, the estimates of calcium retained in bone together with estimates of calcium losses through skin, urine, and feces was used. Calcium retention values of 212 mg for girls and 282 mg for boys were derived from a cross-sectional analysis of BMAS data within two years of peak BMC accrual (Martin et al., 1997).

Using longitudinal rather than cross-sectional data, Whiting et al. (2004) provided a different picture of BMC accrual. They estimated that during the two years of peak bone accretion calcium requirements increase to 1500 mg for girls and 1700 mg for boys, assuming all other estimates of losses and absorption efficiency remain constant. This demonstrates that the need for calcium is greater during this two-year window of bone accrual than previously estimated. A proper estimate of calcium requirements during the years of greatest need for an adolescent can be obtained by applying two-year peak retention values for calcium. However, it overestimates requirement throughout the whole adolescent period of 9 to 18 y. When the authors applied the average accumulation of calcium during the whole span of the DRI age range for adolescents (ages 9 through 18 y), the factorial calculation resulted in a calcium requirement estimate of ~1000 mg and 1200 mg per day for girls and boys, respectively. In their calculations, they assumed that vitamin D levels are adequate and the absorption efficiencies are equivalent in boys and girls. These data can be proposed for use in setting recommended dietary allowance (RDA) for calcium.

In early adulthood, the longitudinal growth of bones has ceased, but consolidation of bone mass continues (Institute of Medicine, 1997). It indicates a positive calcium balance of the 19- to 30-year age group. The peak bone mass has been suggested to be achieved in these ages. At the time of setting AI for this age group, there was no data on whole body bone mineral accretion. Therefore, the relationship between calcium intake and retention for this age group was computed from a compilation of balance studies between 1922 and 1992. The daily calcium retention of 10 mg/d for females and 50 mg/d for males was determined (Institute of Medicine, 1997). In this age group, since there were no reported randomized clinical trials, a lack of data on whole body bone mineral accretion and uncertainties in reported values of calcium loss, estimates of average calcium requirements were disturbed. Most balance data were in females, therefore estimate of calcium intake from the calcium retention analysis in women was adopted for both genders (Institute of Medicine, 1997). Overall, it was judged to be appropriate to set the AI of 1000 mg/d for both men and women ages 19 to 30 years (Institute of Medicine, 1997).

Calcium is an essential threshold nutrient. The threshold behaviour of calcium means that an intake of calcium at a level below threshold, skeletal accumulation is a function of intake; above the threshold, skeletal accrual is steady, irrespective of further increases in intake (Matkovic & Heaney, 1992). Therefore, calcium retention in bony tissue is not optimal at the intakes below the threshold level. The threshold behaviour of calcium was first reported in animal studies (Forbes et al., 1979). A meta-analysis of balance studies during various stages of human growth and development was conducted by Matkovic and Heaney (1992) to investigate whether that threshold behaviour of calcium occur in human. The threshold behaviour that they found was confirmed later by Jackman et al. (1997). Matkovic and Heaney reported the threshold of 1390 mg/d of calcium for children aged 2- to 8-year, 1480 mg/d of calcium for adolescents aged 9- to 17-year and 957 mg/d calcium for young adults aged 18- to 30-year. For adolescents aged 9 to 17 years, Jackman et al. reported a threshold between 1300-1600 mg/d of calcium. Given this threshold behaviour, an individual's requirement of calcium for skeletal health is the intake at the threshold that is the lowest intake at which calcium retention is maximal.

From the information in this section, it appears that because of the lack of sufficient evidence, an AI was set instead of an EAR. However, it is notable that in setting AIs, most evidence was in females, and male values were extrapolated from female ones. The lack of sufficient evidence based on BMC measurements particularly in males is more pronounced in children and young adults. Additionally, the calcium intake threshold values reported by Matkovic and Heaney suggest higher calcium requirements for children (4-8 y-old) and adolescents (9-17 y-old) than AI values. A probable gender difference in calcium requirements, more specifically during the time of peak BMC velocity, may be an important factor in setting AI values. More research is needed to overcome these uncertainties.

2.3.3.1.2 Dietary sources of calcium

Natural calcium-rich foods, calcium fortified foods, or calcium supplements are dietary sources of calcium. Natural calcium-rich foods are ideal sources of calcium, since they provide a variety of nutrients which are necessary for bone health (Dawson-Hughes 2003; Institute of Medicine, 1997). Dairy products provide the most readily

available sources of dietary calcium, primarily with milk, yogurt, and cheese. Most American adolescents obtain their dietary calcium primarily from milk products (Institute of Medicine 1997; Subar et al., 1998). In a sample of Canadian adolescents studied in Saskatoon, milk products were the major source of dietary calcium (61 %), followed by grain products (9 %), vegetables and fruit (7 %), meat and alternatives (2 %) and other foods (21 %) (Iuliano-Burns et al., 1999) which are close to data from the United States (Institute of Medicine, 1997).

Calcium-fortified food products as another dietary source of calcium may include waffles, orange and other juices, bread, granola and breakfast bars, and cereals. Calcium fortification of foods, such as cereal, fruit juice, bread products, and snack bars, can vary greatly among brands. Some fortified fruit juices contain as much calcium as milk, and other fortified products can have wide-ranging calcium contents (Badenhop-Stevens & Matkovic, 2004). Calcium- fortified foods could be beneficial for individuals who are not willing or cannot consume milk products as the main source of calcium.

A variety of compounds including calcium acetate, carbonate, citrate, citrate malate, lactate lactogluconate, and tricalcium phosphate are available in the market place (Dawson-Hughes, 2003). In healthy individuals, calcium carbonate is the most widely used supplement with adequate absorbability, when consumed with meals (Dawson-Hughes, 2003). Calcium supplements provide additional alkali salts (New, 2003) and since the maintenance of acid–base balance is crucial to preserving bone health, calcium supplements may be important for also providing additional alkali salts (New, 2003; New & Millward, 2003).

2.3.3.1.2.1 Calcium intake in children and adolescents

The intake of calcium during childhood and adolescence varies in different regions of the world. While children in North European countries have calcium intake of 1200 mg/d (Vandenbergh et al., 1995), a calcium intakes as low as 500 mg/d has been reported in Chinese children and adolescents over the last decade (Lee et al., 1993; Lee et al., 2003; Zhu et al., 2004). The data from national surveys in United State indicate that the mean intake of calcium among children and adolescents does not attain the recommended calcium intake for either females or males (Newmark et al., 2004). In the 1994-1996 Continuing Survey of Food Intakes by Individuals (CSFII), children age 5

and younger had an average calcium intake of 809 mg/day. Males age 6 to 11 y and 12 to 19 y had a mean calcium intake of 970 and 1145 mg/day, respectively. Females age 6 to 11 y and 12 to 19 y had a mean calcium intake of 857 and 771 mg/day, respectively (U.S. Department of Agriculture [USDA], 1997). In Canada, Shatenstein et al. (1996) reported a calcium intake less than recommended level in children and adolescent boys and girls except for boys aged 16-18 y with a calcium intake of 1396 mg/d.

In addition to regional variation in calcium intake, a gender difference in calcium intake has been reported in various studies with more calcium intake in boys than in girls (Vandenbergh et al. 1995, Roma-Giannikou et al., 1997; Iuliano-Burns et al, 1999; Rajeshwari et al., 2004; Salamoun et al., 2005). Boys need greater energy intakes as a function of their body size (Malina & Bouchard, 2003); as a result; their overall food intake is more than girls. Hence, the difference in calcium intake between boys and girls may be explained by the greater volume of foods consumed by boys.

The intake of calcium tends to be increased with increasing age in boys. In contrast, studies reported that calcium intake deceases with increasing age in girls (Shatenstein & Ghadirian , 1996; Iuliano-Burns et al, 1999; Rajeshwari et al., 2004; Ruiz et al., 1995; Albertson, Tobelmann, & Marquart. 1997). Food records of 226 boys and girls aged 8-19 y over 6 years of PMBAS study were analyzed by Iuliano-Burns et al. (1999) to determine the intake of calcium and its food sources. Older girls (grade 9-12) consumed significantly less calcium than the younger ones (951 mg/d vs 1021 mg/d). In contrast, older boys had greater dietary calcium intake compared to younger boys (1386 mg/d vs 1179 mg/d). Data from the presented study and other investigations reveal that at the time of growth spurt when the peak BMC velocity takes place and skeletal need for calcium retention increases, girls may not meet the recommendation of calcium intake. Consequently, it may put them at more risk of osteoporosis and its complications later in life.

The trend in calcium intake has been investigated by following the consumption of milk as the main surrogate of calcium intake. In the 1989-1991 (CSFII), milk was the primary source of dietary calcium in individuals aged 2 to 18 years. Milk also provided more than half (>50%) the total amount of calcium in children's intakes in ages 2 to 11 years. This percentage of calcium intake from milk dropped to 46.1% in males and

43.4% in females in the 12- to 18-year age group (Subar et al., 1998). Nielsen and Popkin (2004) analyzing nationwide nutrition surveys in the USA reported a decline from 13.3% of total energy from milk in 1997 to 8.3% in 2001 among children aged 2-18. As it has been discussed later, the alarming downward trend in milk consumption, especially in female adolescents, has been accompanied with the excess intake of other beverages mainly soft drinks.

2.3.3.1.2.2 Calcium supplementation trials

During the last two decades, the facts that calcium is the most abundant mineral in skeleton and that the development of bone mass during childhood through young adulthood is a major determinant of bone health later in life, have encouraged investigators to examine the effect of calcium supplementation on bone mass development. Most studies have been done in girls (Table 2.1); however, calcium supplementation in boys has been investigated in several studies (Table 2.2). In addition, some trials covered both genders without separating the results (Table 2.3). Calcium supplementation trials can be categorized based on the age at entry: pre-pubertal, peri-adolescence, late adolescence and early adulthood.

2.3.3.1.2.3 Calcium supplementation trials in pre-pubertal children

Several randomized, double-blind, controlled calcium supplementation trials in pre-pubertal children have been conducted (Tables 2.1- 2.3). The first trial was the one conducted by Johnston et al. (1992). In a co-twin approach, they found a greater increase in bone mass measured in different sites (Table 2.3) of calcium-supplemented boys and girls. No separate analysis was provided for boys and girls in their study. Lee et al. (1994) then showed that a calcium treatment group had significantly greater gains in distal one-third radial BMC (16.5% vs 14.0%) compared to control subjects. Their subjects had very low habitual calcium intakes and the study group received only 300 mg/d calcium. The positive effect of calcium supplementation on bone mass measures has been reported by other researchers who studied pre-pubertal boys and girls together without separate analysis (Lee et al., 1995; Dibba et al., 2000).

The most persuasive evidence to bear that calcium supplementation increases bone size and bone mass is based on a randomized, double-blind, placebo-controlled

trial in pre-pubertal girls conducted by Bonjour et al. (1997). They reported greater gains in BMD with 850 mg/d of calcium supplementation through calcium-enriched foods at the radius (diaphyses and metaphyses), femoral diaphyses and trochanter. No effect of supplementation was observed for lumbar spine and femoral neck BMD. The main gain from all sites was only significant for participants with calcium intake below the median (880 mg/d). In the girls with low calcium intakes, the greater gains observed for lumbar spine height, and femoral shaft bone area, width, BMC and BMD were evident one year after the cessation of supplementation. Subjects in supplemented group received calcium-enriched foods such as cakes, fruit juice, chocolate bars, and yogurt, instead of a calcium supplement. This study suggests that supplementation with the calcium-enriched foods can significantly increase bone mass accrual in prepubertal girls, with a preferential effect in the appendicular skeleton, and greater benefit at lower spontaneous calcium intake.

The follow up study (Bonjour et al., 2001), analyzing data from 116 of the original 144 girls, reported that the differences in bone mass and bone size acknowledged at the completion of supplementation period persisted and were greater 3.5 years after finishing the study. A very recent published study followed the original subjects after 7.5 years examining 125 of the original 144 girls with the mean age of 16.4 y (Chevalley et al., 2005). They found a greater mean areal BMD gain at six sites (radius metaphysis, radius diaphysis, femoral neck, trochanter, femoral diaphysis, and L2-L4) in the calcium supplemented group than in the placebo group (27 vs. 21 mg/cm^2). Menarcheal age was younger in the calcium supplemented group than in the placebo group. They reported a negative relationship between menarcheal age and calcium intake as well as gain in areal BMD. The positive effect of calcium supplementation on the mean areal BMD gain from baseline remained significantly greater in girls below the median of menarcheal age (13.0 y). The authors suggest an interactive effect of calcium intake and early menarche on bone mineral mass

Table 2.1 Comparison of calcium supplementation trials in female children, adolescents and young adults[1]

Variable / Study	Pre-pubertal			Early adolescence				Late adolescence			Early adulthood
	A	**B**	**C**	**D**		**E**	**F**	**G**	**H**	**I**	**J**
Year of publication	1994	1997	2005	2005		1993	2004	1997	2003	2003	2004
Number of girls	75	144	102	354		94	224	84	100	144	23
Age at entry (year)	**7.2**	**7.9**	**10.3**	**10.9**		**11.9**	**12**	**14**	**14**	**17.3**	**23.7**
Mean Ca intake of un-supplemented controls (mg/day)	278	879	718	830		935	1045	692	578	938	1294
Mean Ca intake with supplements (mg/day)	580	1723	1631	1589		1370	1717	1600	1110	1639	2000
Supplement duration (months)	18	12	24	48	84§	18	12	18	12	15.5	12/18
% Increase in BMD											
Midshaft radius	-	+1.6	-	-	-	-	-	-	-	-	-
Distal radius (DR)	+3.05	+2.4	-	*	NS	-	-	-	-	-	-
Lumbar spine (LP)	-	NS	NS	-	-	+2.9	-	+1.5	+0.66	-	NS
Femoral neck (FN)	-	NS	NS	-	-	-	-	+2.1	NS	-	NS
Trochanter (T)	-	+1.9	NS	-	-	-	-	-	-	-	NS
Femoral diaphysis	-	*	-	-	-	-	-	-	-	-	*
Total body	-	-	-	*	NS	+1.3	+0.57	-	+0.73	-	-
% increase in total body BMC	-	-	+3.69	-	-	-	NS	-	NS	-	-
% increase in Size Adjusted (SA) total body BMC	-	-	-	-	-	-	-	-	-	+0.8	-
SA BMC (DR, LS, FN, T)	-	-	-	-	-	-	-	-	-	*	-

Foot notes for Table 2.1

[1] studies were classified in age groups according to age of subjects at study entry

DR: Distal radius. LS: Lumbar Spine. FN: Femoral Neck. T: Trochanter

*: Significant increase in supplement group compared to the placebo group, but percent change not given. NS: not significant.

§ : Significant at metacarpals & at the forearm of taller girls only.

Studies

A: Lee el al., **B**: Bonjour et al., **C**: Cameron et al., **D**: Matkovic et al., **E**: Lloyd et al., **F**: Molgaard et al., **G**: Nowson et al., **H**: Rozen et al., **I**: Stear et al., **J**: Winter-Stone & Snow

Table 2.2 Comparison of calcium supplementation trials in male children and late-adolescents grouped according to age at study entry

Variable / Study	Pre-pubertal		Late Adolescence
	Lee el al.	Chevalley et al.	Prentice et al.
Year of publication	1994	2005	2005
Number of girls	82	235	143
Age at entry (year)	**7.2**	**7.4**	**16.8**
Mean Ca intake of un-supplemented controls (mg/day)	275	747	1199
Mean Ca intake with supplements (mg/day)	580	1600	1858
Supplement duration (months)	18	12	13
% Increase in BMD			
Midshaft radius	-	NS	-
Distal Radius	+3.2	NS	-
Lumbar spine	-	NS	NS
Femoral neck	-	NS	-
Trochanter	-	NS	-
Total body	-	-	+0.81
Femoral Diaphysis		+17.2	
% increase in total body BMC	-	-	+1.27

NS: Not significant

Table 2.3 Comparison of calcium supplementation trials in pre-pubertal children, where girls and boys results were not analyzed separately

Variable / Study	Lee et al.	Johnston et al.	Dibba et al.
Year of publication	1995	1992	2000
Number of girls	84	88	160
Age at entry (year)	7	9.5	10.1
Mean Ca intake of un-supplemented controls (mg/day)	567	874	338
Mean Ca intake with supplements (mg/day)	800	1612	1056
Supplement duration (months)	18	36	12
% Increase in BMD			
Midshaft radius	-	+5.1	+4.5
Distal radius	-	+3.8	+7.0
Lumbar spine	NS	+2.8	-
Femoral neck	NS	+1.2	-
Trochanter	-	+3.6	-
Total body	-	-	-
% increase in total body BMC	*	-	-

*: Significant BMC in distal radius and lumbar spine

NS: Not significant

development in such a way that the intake of calcium intake during pre-puberty may influence the timing of menarche, and consequently might influence long-term bone mass gain. The interpretation of this study is complex. The authors suggest that the intervention may have altered the trajectory of growth producing the observed persistent effects. It might be possible, however, that the dropouts produced an imbalance in the

level of maturity in the two groups in such a way that by chance alone, those receiving the supplementation were more maturationally advanced.

A co-twin approach allows for greater control of genetic and other endogenous factors, when determining the effect of calcium supplementation on bone mineral mass accrual during pre-puberty. Cameron et al. (2004) examined whether calcium supplementation improved bone accrual in pre-menarcheal females. They recruited 51 pairs of pre-menarcheal female twins aged 10.3 y in their randomized, placebo-controlled trial. One twin of each pair received an average of 914 mg/d calcium as calcium carbonate supplement for a period of 12 months. Bone measurements were included the total body BMC and areal BMD at lumbar spine, trochanter, femoral neck. No within-pair differences in height, weight, calcium intake or areal BMD at different bone sites were observed at baseline. After 6 months, calcium supplementation was associated with increased the total body BMC and areal BMD at all measured sites. Areal BMD at femoral neck was the only insignificant bone measure after 12 months. The BMD at trochanter and total body BMC were the significant bone measures after 18 month; and finally after 24 months, only total body BMC was significantly greater in the calcium supplemented group.

One of the questions rising from the Cameron et al. (2004) study is why the increase in areal BMD in measured bone sites became insignificant over the intervention period. All individuals were pre-menarcheal at baseline; however, over the intervention time, some participants experienced menarche. Due to the rapid growth during puberty, body mass and skeletal mass increase sharply (Malina & Bouchard, 2003). The bone mineral mass of subjects increased over time indicating more demand for calcium relative to the skeletal mass compared to the pre-pubertal stage; however, the amount of calcium intake remained constant during the intervention time. The significant higher total body BMC (3.7 %) in calcium supplemented twins indicates the importance of nutrition as a determinant of bone mass development.

As it was shown in the above studies, pre-puberty might be an opportune time for detecting the benefits of calcium in girls but less is known about boys. The study by Lee et al. (1994), conducted in both pre-pubertal boys and girls found the percent gain in boys and girls was similar (16.65 % vs 16.35 respectively). Whether calcium

supplementation increases bone mass gain in pre-pubertal boys was recently addressed by Chevalley et al. (2005). They conducted a 12-month double-blind, placebo-controlled trial with 1-y follow up in 235 healthy prepubertal boys mean age 7.4 y. Boys in the study group received an additional 850 mg/d calcium in foods. Areal BMD was measured at radius, hip, femoral diaphysis, and L2-L4 vertebrae. They reported a greater gain in areal BMD at the femoral diaphysis and at the mean of the five appendicular skeletal sites, but not at the lumbar spine in the calcium supplemented group. The authors suggest a site specific effect of calcium supplementation in a way that only appendicular bones can get benefit from it. Additionally, the gain in areal BMD at the femoral diaphysis suggests more effect in weight- bearing bone sites. The calcium effect on the femoral diaphysis and the mean of the five appendicular skeletal sites was maintained after one year follow up.

From the studies reviewed, it appears that calcium supplementation in pre-pubertal boys and girls has positive effects on bone mass development, particularly when the baseline calcium intake is low. Follow up studies indicated that there was maintenance of calcium supplementation effects even 7.5 years after intervention. None of the studies reported gain in axial bones, suggesting a site specific manner in the effect of calcium supplementation on bone. Without considering the study on Chinese children (Lee et al. 1994), the baseline calcium intake in all studies was close to AI value of 800 mg/d calcium for children aged 4-8y (Institute of Medicine 1997). The positive effect of calcium intake on bone mass development was observed with the overall calcium intake of >1600 mg/d (~1000 mg/d from supplement) (Tables 2.1 and 2.2) which is over the calcium-threshold value of 1390 mg/d for children aged 2-8 y suggested by Matkovic & Heaney (1992). These lines of evidence suggest the current AI value for children aged 4-8 y needs to be revisited.

2.3.3.1.2.4 Calcium supplementation trials during early adolescence

The pubertal growth spurt is the time for rapid growth in all tissues in the body including skeleton. Therefore calcium supplementation during adolescence has gained the most interest for trials (Table 2.1). It is notable that there is no published study in adolescent boys. In a randomized, double-blind, placebo-controlled trial, Lloyd et al. (1993) evaluated the effect of 18 months of calcium supplementation (500 mg/d

calcium as calcium citrate malate) on bone density among ninety-four girls with a mean age of 11.9 years at study entry. The baseline calcium intake was 960 mg/d for the entire study group. The supplemented group, who received an additional 354 mg/d of calcium, had a greater increases of lumbar spine BMC and BMD, and total body BMD compared to placebo group. They observed an increase of 24 g of bone gain per year among the supplemented group.

The amount of calcium needed to optimize bone mineralization in adolescent girls is not certain. Molgaard et al. (2004) examined how habitual calcium intake influences the effect of calcium supplementation on bone mineral accrual in 224 girls aged 12-14y for 12 months. They divided subjects into a subgroup with habitual calcium intake of 1000-1307 mg/d, and another subgroup who habitually consumed <713 mg/d calcium. The girls from each subgroup were randomly assigned to receive either 500 mg Ca/d or placebo. Calcium supplementation in both groups had a significant effect on total body BMD (0.8%; $P = 0.049$). The authors suggested that habitual calcium intake did not influence the effect of calcium supplementation on bone accretion. It is notable that in the low habitual calcium group, the difference in bone measures between calcium supplemented subjects and placebo subjects was greater than the similarly compared differences in the group with the habitual calcium intake of ≥ 1000 mg/d. The result of this study disagrees with Bonjour et al. (1997) who reported that calcium supplementation had a greater effect on bone measures in pre-pubertal girls with calcium intakes below the median of 880 mg/d. The low number of subjects (≤30 in each group) may have lessened the statistical power in Molgaard et al.'s study. The authors argue that perhaps the mean intake of < 713 mg/d calcium in the low habitual calcium intake group was not low enough to detect the effect of extra calcium intake through supplementation, although it is lower than the median calcium intake of 880 mg/d in Bonjour et al.'s study.

Whether long-term calcium supplementation, started from the onset of puberty, influences bone accretion in young adults has been investigated by Matkovic et al. (2005). They conducted a four-year randomized clinical trial among 354 girls aged 10.9 y. They initially reported that calcium supplementation (670 mg/d) above a habitual dietary calcium intake of 830 mg/d (thus increasing total calcium intake up to 1500

mg/d) positively influenced bone mass acquisition throughout the bone-modeling phase of the pubertal growth spurt. This effect diminished during the skeletal consolidation of late adolescence. The study was extended for three more years, and the positive effect of calcium supplementation was evident at all skeletal regions of interest; however, the only differences that remained significant were those at the metacarpals and at the proximal radius among the subgroup with high calcium intake and in tall persons.

The uniqueness of Matkovic et al.'s study was its long period of supplementation which started at the beginning of adolescence growth spurt and continued to early adulthood. A catch up phenomenon may explain why the positive effect of calcium supplementation diminished as the participants entered to their early adulthood. This phenomenon in bone mass attainment proposes the continuation of a reversible mineral shortage that is acquired during the pubertal growth spurt. As age increases the demand for calcium decreases (AI of 1000 mg/d in young adults vs. 1300 mg/d in adolescents). Additionally the threshold level for calcium balance decreases from 1480 to 975 mg/d (Matkovic & Heaney, 1992). Since the habitual calcium intake of subjects in early adulthood was close to their requirements, it may have allowed bone mass to catch up during the consolidation phase.

The studies presented indicate that calcium supplementation during adolescence beneficially affects bone mass development in girls. However, follow up studies are needed to examine whether the effect remain after discontinuing the supplementation. There are no data in boys to study a possible gender difference in the effect of calcium supplementation on bone mineral mass during early adolescence. Whether there is a bone-site selectivity in calcium supplementation, cannot be judged with the current data (Table 2.2). The mean calcium intake of subject at entry was below the recommendation in this age group; the AI for adolescent boys and girls aged 9 - 18 y is 1300 mg/d (Institute of Medicine, 1997). When the calcium intake was close to the requirement (for which the AI may be a surrogate), such as in the Molgaard et al. study, only a modest effect was observed in the measured bone sites. Therefore, as it was reported in prepubertal children, calcium supplementation may have greater effect when the habitual calcium intake is low.

2.3.3.1.2.5 Calcium supplementation trials in late adolescence

After the pubertal growth spurt, increased secretion of sex steroid hormones results in epiphyseal plate unionization and consequently bone modeling slows down while bone consolidation is continued (Malina & Bouchard, 2003). Therefore the requirement of calcium may be lower during late adolescence compared to early adolescence. In a twin approach, Nowson et al. (1997) evaluated the effect of calcium supplementation on BMD in 42 female pairs with a mean age of 14 y for 18 months. One twin in each pair was randomly assigned to receive 1000 mg/d calcium. There was a 1.62 % increase in BMD at the lumbar spine in those on calcium after 18 months. The significant within-pair differences of 1.53% at the lumbar spine BMD and 1.27% at the hip BMD disappeared after the first six months. The authors concluded that the greatest effect of calcium supplementation was observed at first 6 months; thereafter, the difference was maintained, but there was no accelerated increase in BMD. The wide age range of subjects (10-17 y) in this study may have affected the results, since they had 11 pre-pubertal, and 31 post-menarcheal pairs. The authors found no significant effect of calcium on BMD in pre-menarcheal pairs. However, a significant effect was observed on the 31post-menarcheal girls. One might conclude that post-menarcheal period is the more optimal time for calcium intervention. However, by subdividing the pairs, there were low numbers of subjects thus lowering the statistical power. A reduction in the number of twins completing the study (28 pairs) and the decline in compliance over time might be other reasons why the greatest effect was in the first six months.

Previous data suggest when the baseline calcium intake is low, calcium supplementation may present a measurable effect on bone mass acquisition. The effect of calcium supplementation on bone mass accretion in post-menarcheal adolescent girls with low calcium intakes was assessed by Rozen at al. (2003). One hundred girls aged 14 y with habitual calcium intakes < 600 mg/d completed a 12-month protocol in their double-blind, placebo-controlled study. The treatment group received a daily supplement containing 1000 mg elemental calcium. They observed that the calcium-supplemented group had greater increase in total-body BMD and lumbar spine BMD but not BMC than did the control group. This effect appeared selectively more beneficial for girls who

were 2 y post-menarcheal. In addition, calcium supplementation lowered the serum level of bone resorption biomarkers.

The study by Rozen et al. (2003) is the first trial in adolescent post-menarcheal girls with habitually low calcium intake which shows positive effect. The percentage gain in BMD at the measured bone sites was less pronounced compared to studies in pre-pubertal and pubertal girls (Table 2.1) which might be explained by slowing bone modeling after menarche (Malina & Bouchard, 2003). They found that calcium supplementation had an equal effect on bone gain in girls with >2 years after menarche compared to girls with <2 y after menarche, although, it was expected to observe less bone gain in older girls. The authors confirmed their findings with bone turnover biomarkers. In their follow up study 3.5 y after the end of calcium supplementation (Dodiuk-Gad et al. 2005), the calcium-supplemented group (mean habitual calcium intake of 620 mg/d) tended to have a greater accretion of only total-body BMD (3.8% vs 3.1%; p=0.04) than did the control group (mean habitual calcium intake of 712 mg/d). Since total body BMD represents primarily cortical bone, the effect of calcium supplementation may remains only in cortical bones.

Only one study has been reported in late adolescent boys (Prentice et al., 2005). They conducted a randomized, placebo-controlled trial among 143 boys aged 16-18 y; subjects received 1000 mg/d calcium or placebo for 13 months. The authors observed that calcium carbonate supplementation of adolescent boys increased skeletal growth, resulting in greater stature and bone mineral acquisition measured as BMC at different bone sites. Whether this effect will be maintained into adulthood must be determined by follow-up studies.

The study by Prentice et al. is the first trial that reports the positive effect of calcium supplementation on statural growth in post-pubertal boys. It was unexpected for Prentice et al. (2005) to observe the effect of calcium supplementation on statural growth. As the skeleton accounts for 98% of height and PHV occurs before age 14.4 y in European and North American boys (Malian et al., 2003) and as there is approximately a six months lag between age at PHV and peak BMC velocity (Bailey et al., 1999); then, if there is an association between calcium supplementation and bone size or height, it is more likely to observe it around the age of PHV rather than >2 years after it. An

example for this is the positive effect of calcium supplementation on height of pre-pubertal girls found by Bonjour et al. (1997). Thus the results of Prentice et al. require verification.

The authors (Prentice et al., 2005) in another trial in girls at the same age (16-18 y), found no effect of calcium supplementation on statural growth (Stear et al. 2003). Therefore, Prentice et al. propose a gender difference in the effect of calcium supplementation on bone mass development in comparison to their girls study. The studies in younger subjects reported no gender difference in calcium supplementation, since boys and girls were analyzed together (Johnston et al., 1992; Dibba et al., 2000; Lee et al., 1994; Lee et al., 1995). It is notable that those studies had insufficient sample size to test the gender difference or the subjects' growth affected by the limitation in other nutrients (Dibba et al., 2000).

From the studies reviewed in this section (Tables 2.1, 2.2), it appears that the baseline calcium intake in studies among girls was low, while boys had considerably higher habitual calcium intake. The intervention time was short (> 15.5 months) and only one follow up study has been done (Dodiuk-Gad et al. 2005). The study by Nowson et al. (1997) covered the whole range of pre- peri- and post pubertal years. However, the most recent studies (published during last 2 years) selectively studied only post-pubertal boys and girls in this age group.

2.3.3.1.2.6 Calcium supplementation trials in early adulthood

It has been suggested that skeletal consolidation continues at a lower rate in early adulthood; as a result, peak bone mass is achieved during the third decade of life (Teegarden, et al. 1995; Recker, et al. 1992). However, there is only one published calcium supplementation trial conducted in young adult women age 23.7 y (Winters-Stone& Snow 2004). They found that calcium supplementation with 1000 mg/d calcium as calcium carbonate in athlete subjects with habitually high calcium intake (1006 mg/d) prevented bone loss (~ 2% of BMD) only at the femoral mid-shaft compared to the placebo group. The low number of subjects lowered the statistical power. Additionally, a high habitual calcium intake in subjects, particularly a calcium intake of 1294 mg/d in placebo group, may explain why they observed only a modest effect. The authors found

that calcium supplementation could prevent cortical bone loss in young adult female runners; however, the limitations of the study may compromise their finding.

At a glance, a huge body of research has been done on the effect of calcium intake on bone mass development at different stages of maturation. The major effect of growth spurt on the overall skeletal development justifies the need to consider the maturation stage as the main reference when the participants are chosen for trials. Additionally, the maturational age might be different in the individuals with the same chronological age (Malina & Bouchard, 2003). Overall, studies suggest a positive effect of calcium supplementation on bone mass development from childhood to early adulthood, particularly when the habitual calcium intake is low. A recent comprehensive review on calcium supplementation trials among children suggests that the small effect of calcium supplementation on bone mineral mass in some bone sites do not support the use of calcium supplementation in healthy children (Winzenberg et al., 2006). Differences in genetic regulation of calcium utilization has been offered as an explanation for the findings of calcium supplementation trials among children and adolescence (Abrams, 2005). More research is required to provide evidence where the gaps exist. The optimal amount of supplemented calcium is not certain. Additionally, as suggested in some studies, whether bone selectivity, and gender difference exist in the effect of calcium supplementation on bone mass development from childhood to early adulthood, are the areas for more research (Table 2.4).

Table 2.4 Gaps in the effect of calcium supplementation on bone mass development

	Pre-adolescence	Early-adolescence	Late-adolescence	Early-adulthood
Bone selectivity*	+	-	?	?
Gender difference	-	?	+	?

+: Evidence show the effect, -: Evidence show no effect,?: Not known * More effect on appendicular bones than in axial bones

2.3.3.2 Vitamin D

The active form of vitamin D [1, 25-dihydroxy vitamin D (1,25DHD)], is the metabolite of vitamin D which functions like a steroid hormone (Gropper et al., 2005). The only function of vitamin D was initially believed to be calcium homeostasis, and its target tissues were apparently limited to intestine, bone and kidney (Holick, 2004). However, the presence of specific cell membrane vitamin D receptors (VDR) in many other tissues suggests that vitamin D acts in a wide variety tissues such as cardiac, muscle, pancreas, brain, skin, haematopoietic and immune system tissues (Gropper et al., 2005). The effects of vitamin D in different tissues are cell differentiation, proliferation, and growth. These effects are conducted through genomic pathways and are known as non-calciotropic functions (Gropper et al., 2005). It is believed that the non-calciotropic functions of vitamin D are important in overall health and prevention of a variety of chronic diseases such as type I and type II diabetes, rheumatoid arthritis and multiple sclerosis (Holick, 2004).

The major calciotropic function of vitamin D is providing calcium for bone growth and homeostasis by increasing intestinal calcium absorption (Combs, 2000). During the critical time of peak bone mineral accrual, when the calcium requirement is high, vitamin D status as an adaptive mechanism influences bone mineral accrual (Bonjour et al., 2003). A recent 1-year randomized control trial among Finnish adolescent girls with adequate calcium intake found that bone mineral augmentation in the femur was 14.3% and 17.2% higher in the groups receiving 5 and 10 microgram of vitamin D, respectively, compared with the placebo group (Viljakainen et al. 2006-1). The major source of vitamin D in the world is exposure of skin to sunlight (Combs, 2000). However, cultural, environmental, and physiological factors can impair sun-light induced production of vitamin D; hence, it is important to consider dietary intake of vitamin D (Calvo, Whiting & Barton , 2004).

Although North Americans may have a higher vitamin D intake from both food and supplements compared to people living in other regions of the world, a seasonal insufficiency of vitamin D, during winter, has been reported in adults, adolescents and children (Weiler et al., 2005, Calvo et al., 2004; Calvo & Whiting, 2003; Vieth et al., 2001; Looker et al., 2002; Rucker et al., 2002; Gordon et al., 2004; Nesby-O'Dell et al,

2002; Jones et al. 2004). There are no nationally representative vitamin D intakes data for Canada; however, studies indicate there is the same seasonal variation in serum 25-hydroxy vitamin D (25OHD) concentrations as in the US (Calvo & Whiting, 2003). Considering the higher latitude and a longer winter in Canada than in the US, the vitamin D insufficiency during winter might be in greater extent in Canada than in the US. A recent cross-sectional study among Finnish early and mid-puberty girls reported a seasonal variation of serum 25OHD, serum intact parathyroid hormone, bone turnover and bone mineral density in early and mid-puberty girls (Viljakainen et al. 2006-2). Serum 25OHD is the principal circulating vitamin D metabolite, and it is the biomarker that should be assessed when determining an individual's vitamin D status (Smith & Groff, 2005). Both ingested and endogenously produced vitamin D are converted to 25OHD, therefore serum levels of this metabolite accurately reflect both excess and deficiency states (Reid, 2003).

Current vitamin D fortification practices may not be sufficient to prevent poor vitamin D status during winter (Calvo et al., 2004). However, dietary vitamin D intake per kg body weight was the most important predictor of 25OHD concentration at the end of winter in Edmonton, when adolescents and children were at risk of vitamin D insufficiency (Roth et al., 2004).

2.3.3.2.1 Recommended intake of vitamin D during growth

The current dietary recommendations for vitamin D intakes (200 IU/d for age group 0-50 y) were set in 1997 by the Institute of Medicine. There was insufficient evidence for the DRI panel to set EARs for vitamin D; therefore, AIs were set (Institute of Medicine, 1997). EARs are frequently determined with the factorial approach, which adjusts nutrient demands for accretion and obligatory losses through absorption, or as the intake required for maximal retention. None of these approaches is appropriate for a nutrient such as vitamin D. It is difficult to determine the inputs of vitamin D from sunlight and food. Furthermore, vitamin D is metabolized like a hormone (Smith & Groff, 2005) and the quantitative relationship of vitamin D input to health is uncertain particularly before 1997. In absence of the ability to use common approaches, nutrient requirements can be determined as intakes observed among healthy populations or as those that maximize a functional indicator (Weaver & Fleet, 2004). An AI for vitamin D

was set on the basis of intakes necessary to achieve normal ranges of 25OHD concentrations and it was assumed that there was no cutaneous synthesis of vitamin D through sun exposure (Institute of Medicine, 1997).

Since 1997, much has been learned regarding the calciotropic and non-calciotropic functions, and the metabolism of vitamin D. Furthermore, it is well known that without assurance of sun exposure, requirement and recommendation for vitamin D would be higher than that currently in place (Whiting & Calvo, 2005; Weaver & Fleet, 2004). In addition, high prevalence of vitamin D insufficiency reported in recent studies, are reasons to suggest that the AI values of vitamin D set by Institute of Medicine at 1997 should be revisited (Holick, 2006; Whiting & Calvo, 2006; Calvo & Whiting, 2006).

2.3.3.2.2 Dietary Sources of vitamin D

There are few natural food sources of vitamin D: primarily fish and fish liver oils (Combs, 2000). Since those foods are not commonly consumed, in North America most of the intake of vitamin D from food comes from fortified food items (Institute of Medicine, 1997). In Canada, the foods that require fortification with vitamin D are milk (100 IU per 250 mL) and margarine (53 IU/10 g); currently, no other fortification of foods with vitamin D is permitted (Calvo et al., 2004).

Although vitamin D is one of the important contributory factors on bone mineral accrual during adolescents' growth spurt, it is difficult to determine vitamin D input from both sources, diet and sun exposure or differentiate them (Weaver & Fleet, 2004). Therefore, in studies investigating the effect of nutrients and dietary factors on bone markers such as PBMAS, it is difficult to include the vitamin D in their analysis.

2.3.3.3 Other nutritional factors

2.3.3.3.1 Protein

The relationship effect of protein intake on bone mass has been investigated in various experimental and clinical studies (Rizzoli et al., 2003). Bone development during growth can be impaired severely by inadequate supply of energy and protein.

Isolated protein deficiency induced in experimental animals leads to reduced bone mass and strength without histomorphologic evidence of osteomalacia (Bourrin, Ammann, Bonjour, & Rizzoli, 2000). Thus an inadequate protein supply appears to play an important role in the pathogenesis of the delayed skeletal growth and reduced bone mass that is observed in under-nourished children (Bonjour et al., 2003).

The detrimental effect of low protein intake on skeletal integrity might be explained by a lowered production of IGF-I. Dietary protein can influence the production of IGF-I (Rizzoli et al., 2003). The plasma level of IGF-I is related to the growth rate during puberty. The variation in the production of IGF-I could explain the changes in bone and calcium metabolism during growth, since it is responsible for the two adoptive mechanisms including inorganic phosphate transport and the production of 1,25DHD in kidneys (Bonjour et al., 2003). Furthermore IGF-I stimulates proliferation and differentiation of chondrocytes in the epiphyseal plate, therefore it is essential factor for bone longitudinal growth (Bonjour et al., 2003). Therefore during the adolescence growth spurt, a relative deficiency in IGF-I may result in reduced bone longitudinal and cross-sectional development.

The relationship between protein intake (within normal range) and bone growth markers in 198 healthy adolescents (98 females and 100 males), aged 9-19 y has been investigated by Theintz et al. (1992). They observed a positive relationship between protein intake and BMC as well as BMD in lumbar and femoral bone sites. This association was particularly dominant in pre-pubertal subjects. Low protein intake in pre-pubertal subjects was associated with a reduced gain in areal BMD and BMC in femoral and lumbar bone sits. This study suggests that high protein intake during prepubertal period could be beneficial for bone mass accrual, although no causal relationship can be derived.

Although calcium requirements are the highest during adolescents' growth spurt (Matkovic et al., 2005), the pre-pubertal period reported to be the optimal time for calcium supplementation to obtain maximum advantages on bone mass accrual (Bonjour et al., 1997). The question arises whether or not an interaction between protein intake and calcium intake exists in their effect on bone mass accrual in pre-pubertal children. Chevalley et al. (2005) reported that in healthy pre-pubertal boys, the response to

calcium supplementation can be modulated by habitual protein intake. The possibility of whether or not the individual's calcium requirement for optimal bone mineral accrual and PBM attainment is less at high protein intake needs further investigation.

In addition to studies in prepubertal and adolescent boys and girls, numerous studies in pre- or post menopausal women and elder men reported the positive relationship between dietary protein intake and bone mass measured in different bone sites (Chiu et al., 1997; Teegarden et al., 1998; Kerstetter et al., 2000). The Framingham Longitudinal study has demonstrated that the rate of bone mineral loss in spinal and femoral bone sites was inversely correlated to dietary protein intake during four years of study in a sample of 615 men and women aged 75 years and older (Hannan et al. 2000). The associations between protein intake and change in BMD in 342 healthy men and women (aged ≥65 y) were examined by Dawson-Hughes and Harris (2002) in a 3-year, randomized, placebo-controlled trial. They found that spontaneous higher protein intake was associated with an increase in BMD in elderly subjects supplemented with calcium citrate malate and vitamin D (Dawson-Hughes & Harris, 2002). This study suggests that the favorable effect of increasing the protein intake on BMD in elderly people requires an adequate supply of both calcium and vitamin D.

It is notable that animal protein as well as purified plant protein, in excess, increases urinary calcium excretion (Massey & Whiting, 2003). There is no evidence to support that vegetable based proteins are healthier for the skeleton than animal proteins (Kerstetter et al., 2003). The effect of excess dietary protein on increasing urinary calcium and adversely affecting bone metabolism may be minimized by other nutrients such as calcium, phosphorus and potassium found in protein sources (meat, milk, mixed dishes) (Massey & Whiting, 2003; Tucker, 2003). Furthermore, the majority of the rise in urinary calcium in response to an increase in dietary protein is related to increased intestinal calcium absorption, as reported recently by Kerstetter et al. (2005).

Overall, low protein intake is associated with less bone formation compared to bone resorption (Rizzoli et al., 2003). Therefore sufficient dietary protein is necessary for bone homeostasis during growth as well as in old age (Massey & Whiting, 2003).

2.3.3.3.2 Potassium

The effect of dietary potassium on bone health is through two synergistic mechanisms, potassium-induced sodium and chloride excretion, and the concurrent diminution in net acid excretion (Institute of Medicine, 2004). Potassium prevents sodium-mediated urinary calcium excretion (Institute of Medicine, 2004, New, 2003). The protective effect of potassium on calcium economy was one criterion used to set the AI for potassium of 4700 mg/d (Institute of Medicine, 2004) for males and females aged ≥ 14 year. Most of studies investigating the relationship between dietary potassium and bone health have been done in adults and peri-menopausal women (New, 2003). The differences in bone metabolism between children and adults (i.e., bone remodeling compared to modeling) may explain why the protective effects of potassium through the mentioned mechanisms are more pronounced in adults. One of the few studies available in children is the one conducted by Jones et al. (2001). They examined the associations between urinary potassium, urinary sodium, usual dietary intake and BMD in 330 boys and girls aged 8 y. In their cross-sectional study, they found a significant relationship between urinary potassium and BMD at all bone sites under investigation, although it decreased after adjusting for lean mass.

It seems that the dietary source of potassium is an important factor in presenting its protective effect. Various population-based studies reported that increased potassium intake through vegetables and fruit is associated with increased BMD (Institute of Medicine, 2004; New, 2003). There is no evidence on beneficial effect of potassium bicarbonate supplementation on bone mass measurements; however, it reduces bone resorption biomarkers and increases bone formation biomarkers in postmenopausal women (Sebastian et al., 1994). A recent study conducted by Rafferty et al. (2005) determined the effects of potassium on the calcium metabolism among 1991 mid-life women. They observed no appreciable net influence of potassium on calcium metabolism. The food sources of potassium in their study protocol were more milk products and meat than vegetables and fruit; hence, they suggested that the food source of potassium may play role in its effect. The effect of vegetables and fruit as a source of potassium on bone development during growth is discussed in Section 2.3.3.4.2.

2.3.3.3.3 Magnesium

Magnesium is another nutrient in addition to potassium that may protect bone contributing to an alkaline environment (Tucker, 2003). Total body magnesium increases from 1 g at birth to 23-27 g in adults. Most of the gain occurs during adolescence, and magnesium output is less than its intake during this time (Heaney et al., 2000). The skeleton contains approximately 60% of the total body magnesium. It has been suggested that magnesium improves bone quality by affecting hydroxyapatite formation (Sojka & Weaver, 1995; Dimai et al. 1998). Magnesium concentration in bone is slightly higher in adolescents than very old people indicating no substantial change by age (Heaney et al., 2000). Periosteal and endosteal surfaces of bone have higher magnesium concentrations equally in both genders (Heaney et al., 2000).

Few data are available regarding the interaction of calcium and magnesium in healthy children. Andon, Ilich, Tzagournis, & Matkovic (1996) conducted a randomized double-blind study to determine the effect of calcium intake on magnesium balance in 26 adolescent girls (mean age 11.3 y) during a 14-d period. A controlled basal diet containing 667 mg calcium and 176 mg magnesium were consumed by subjects. Subjects were randomly assigned to consume 1000 mg/d calcium or placebo. Magnesium intake did not differ between the low-calcium and high-calcium groups. Accordingly, magnesium balance was averaged 21 mg /d for the whole study group. They found that magnesium balance was significantly correlated with magnesium intake and magnesium absorption. They suggested that high-calcium diet in adolescent females does not alter magnesium balance. However, adequate magnesium has been suggested to be important for normal calcium metabolism (Tong & Rude, 2003).

A diet providing enough nutrients to support life likely contains enough magnesium. The richest sources of magnesium are plant products such as whole grains, vegetables and beans (Institute of Medicine, 1997). The beneficial effect of vegetables and fruit on bone health, discussed later, may be partially explained by the presence of magnesium. Potential mechanisms of magnesium deficiency- induced bone loss are decreased bone formation, increased bone resorption and abnormal bone mineralization (Tong & Rude, 2003). A study conducted by Dimai et al. (1998) to examine the effects of daily oral magnesium supplementation on bone turnover in 12 young (27-36 year old) healthy men. They found reductions in serum levels of parathyroid hormone as a bone

turnover biomarker caused by magnesium; however, conflicting results exist regarding relationship between magnesium status and bone turnover biomarkers (Tong & Rude, 2003).

2.3.3.3.4 Other Nutrients

In addition to the nutrients discussed above, other nutrients are required for the cellular processes of bone tissue deposition, maintenance and repair. Vitamin B_{12} (cobalamin) is another nutrient beneficial for bone and it is a required cofactor in metabolic reactions associated with methionine synthesis and it is necessary for synthesis of DNA (Institute of Medicine, 1998). Vitamin B_{12} stimulates osteoblast activity and bone formation (Kim et al., 1996). Vitamin B_{12} treatment of patients with pernicious anemia increases markers of bone formation and improvement in bone mineral density (BMD) (Carmel et al., 1988; Melton & Kochman, 1994). Most studies on the relationship between vitamin B_{12} and bone health indicators have been done on elderly individuals and reported a positive relationship between low vitamin B_{12} status and low BMD and BMC (Tucker et al., 2005; Dhonukshe-Rutten et al., 2004; Stone et al. 2004).

Vitamin K has a role in bone biology as there are vitamin K-dependent proteins in bone including osteocalcin, matrix Gla protein and protein S (Brown & Josse, 2002; Tucker, 2003; Bugel, 2003). Most of the supporting evidence on the bone beneficial effect of vitamin K is based on reported associations between dietary vitamin K and bone measurements among postmenopausal women with osteoporosis (Tucker, 2003). Vitamin C with its effect on collagen formation, and zinc are other nutrients beneficial to bone (Heaney et al., 2000). Adolescents with poor eating behaviour are susceptible for zinc deficiency which may affect their overall growth and potentially their bone mass through IGF-I (Heaney et al., 2000).

Iron is another nutrient that may play a role in bone formation, since it has a critical role in the collagen synthesis which is essential for bone matrix formation (Walsh et al., 2003). There is no report on iron deficiency-induced bone loss. In contrast, iron overload may be detrimental to bone (Walsh et al., 2003). Patients who are suffering from severe iron overload such as hemochromatosis have abnormal bone histology (Walsh et al., 2003). Calcium intake may interfere with the iron utilization,

since it has been reported that iron absorption was reduced by calcium-containing meals and return to normal after 2-4 hours (Gleerup et al., 1993). However, more recent studies suggest that iron indicators remain unaffected with long term high-calcium diets. Ilich-Ernst et al. (1998) conducted a randomized controlled trial among adolescent girls aged 8-13 years to determine the effects of growth, menstrual status, and calcium supplementation on iron status. They found that although growth spurt and menstrual status had adverse effects on iron stores in adolescent girls with low iron intakes (<9 mg/d), long-term supplementation with calcium (total intake of approximately 1500 mg/d for 4 y) did not affect iron status.

In addition to above mentioned vitamins and minerals, animal studies suggest that long chain poly unsaturated fatty acids influence bone mass in various animal models (Watkins et al., 2000; Judex et al., 2000). Their effect on bone might be explained through prostaglandins as mediators of bone cell function (Watkins et al., 2001). Low concentration of prostaglandin E_2 stimulates the bone formation, whereas high concentration inhibits bone formation (Raisz & Fall, 1990). There is no human study to investigate the effect of dietary precursors of prostaglandin on bone mass. A recent study on piglets fed with arachidonic acid and docosahexaenoic acid reported that while low dose of these essential fatty acids increased bone mineral mass, no effect was observed with high dose (Mollard et al. 2005).

Natural health products (NHP) are naturally occurring substances that are consumed for the purpose of diagnosing, treating or preventing illness or maintaining or promoting health (Health Canada, 2005). Dietary NHP supplements based on plant extracts often contain phytoestrogens, compounds that are structurally or functionally related to ovarian or placental estrogens and their active metabolites; these are commonly consumed to promote health or prevent diseases such as osteoporosis. The beneficial effect of phytoestrogens on bone in postmenopausal women might be explained by their hormone replacement effect (New, 2003; Brown & Josse, 2002). The three major groups of phytoestrogens are: isoflavones (in soybeans and other legumes), lignans (in flax seed, fruits and vegetables) and coumestans (in bean sprouts and fodder crops) (Brown & Josse, 2002). Studies show that populations with high phytoestrogens intake such as Asians in Asia have lower rates of hip fracture than North Americans

(Brown & Josse, 2002). Soybean isoflavones may reduce bone resorption in postmenopausal women, although long-term studies are lacking (New 2003; Brown & Josse, 2002). Ipriflavone (a synthetic phytoestrogen) is considered a second-line preventive therapy in postmenopausal women; however, it is not recommended for treatment of postmenopausal women with osteoporosis, nor for use in men or premenopausal women (Brown & Josse, 2002).

Flavonoids are micronutrients, characterized by antioxidant activities, widely present in vegetables and fruit. Wattel et al. (2004) investigated the effects of quercetin, one of the most commonly occurring flavonoids, on osteoclast differentiation. They demonstrated that quercetin exert a potent inhibitory effect on in vitro bone resorption by its suppressive effect on osteoclastic differentiation. Green and black teas contain polyphenol flavonoids (also called tannins) and phytoestrogens as well as fluoride, all of which have probable beneficial effects on bone health (Chen et al., 2003). Epidemiologic studies suggest a positive relationship between tea drinking and BMD in postmenopausal women (Wu et al., 2002) as well as the decreased risk of hip fracture in men and women (Kanis et al., 1999). The positive effect of tea on overall health and bone mass is likely though its polyphenols; however, fluoride in tea may help to maintain BMD (McKay & Blumberg, 2002). There is no study available in children, adolescents and young adults to investigate the effect of phytoestrogens and flavonoids on bone mass development. It appears from the literature reviewed that the effect of vegetables and fruit (discussed in section 2.3.3.4.2.) on bone health may be partially explained by their NHP compounds, although this is an area requiring more research.

In summary, various minerals, vitamins and trace elements and NHPs have been suggested to have a beneficial effect on bone mass development and maintenance. Although some of nutrients such as calcium and vitamin D are substantial for bone health during life span, the evidence for bone protective effect of others such as trace elements during childhood and adolescence are not sufficient. Perhaps difficulty in assessing those nutrients in diet is complicating the situation. Following section describes food patterns as another approach to investigate how nutrition affects bone health.

2.3.3.4 Food groups

Nutrients occur together in foods, and their intake can be detected by assessing dietary patterns and measuring food group intake. Some researchers have assessed the effect of food group intake on bone health. Most research on the effect of food patterns on bone health has been done on milk products, and vegetables and fruit as the major sources of bone protective nutrients.

2.3.3.4.1 Milk products

As milk is the major source of calcium in North American diets, there are several studies of the effect of milk products (as a food group) on bone health during growth (Table 2.5) (Chan et al., 1994; Cadogan et al., 1997; Merriless et al. 2000; Du et al., 2004; Kalkwarf et al., 2003). These studies have found a positive association between milk product intake and BMC. Since milk contains other nutrients essential for bone growth, the results of those studies may be due to nutrients in addition to calcium. Higher milk product intake results in higher intake of protein, calcium, phosphorus, magnesium, zinc, and many vitamins such as vitamin D (if fortified), vitamin A and riboflavin (Cadogan et al.1997).

Although most of the attention has been given to the importance of milk intake during adolescence, in recent years, the pre-pubertal milk consumption has gained the interest of investigators. The effect of pre-pubertal milk consumption on bone mass development has been studied in a 2-year milk intervention study among 757 Chinese girls aged 10 years (Du et al. 2004). They found that a greater increase in change in total body-BMC and total body–BMD was achieved when milk fortified with cholecalciferol compared to the milk fortified with additional calcium.

Another study was conducted by Gibbons et al. (2004) in pre-pubertal children (aged 8-10 y) living in New Zealand. They evaluated the effect of calcium enriched diary drink on bone mass indicators during 18 months of intervention. Subjects in both groups had high habitual dietary calcium intakes (the baseline dietary calcium intake of 934 mg/d and 985 mg/d in the treatment and control groups respectively). No significant

differences in BMC and BMD at the measured bone sites were observed between the intervention and control groups. This might be explained by the threshold behaviour of calcium. Additionally, it might add to the findings in calcium supplementation trials (Section 2.3.3.1.4) that when the habitual intake is low, calcium supplementation through diary products, like elemental calcium, has its maximum effect on bone. Although more studies are needed, these studies suggest that during pre-pubertal period, additional calcium to habitual appropriate milk product intake not necessarily affect bone mass development, rather milk fortification with vitamin D helps to optimize calcium retention in skeleton.

Milk consumption during adolescence has beneficial effect on BMC and BMD at different bone sites, as reported in various studies (Chan et al., 1995; Cadogan et al., 1997; Merrilees et al., 2000; Volek et al., 2003). The effect of calcium supplementation via dairy products on the bone measurements of 48 white girls with the mean age of 11 year were examined in a randomized control trial with 12-month follow-up by Chan et al. (1995). They reported higher intakes of calcium, phosphate, vitamin D, and protein in the dairy group than control subjects. The dairy group had significantly greater increases in BMD at the lumbar spine, and in total body BMC than control subjects. In further analyses, they observed that dietary calcium, phosphate, vitamin D, and protein intakes were positively associated with the lumbar spine bone density and total body BMD. Since milk products contain those nutrients, the authors suggest that by increasing milk products intake, in addition to calcium, one might achieve the benefits from other bone-beneficial nutrients.

Cadogan et al. (1997) investigated the effect of milk supplementation on total body- BMC & -BMD in 82 girls aged 12 year. They reported that an additional 300 mL milk intake in intervention group compared to the intake of 150 mL in control subjects, increased total body -BMC and -BMD in a greater extent. The only study available in adolescent boys is the one conducted by Volek et al. (2003). They examined the effects of increasing milk intake on total body –BMC and -BMD in physically active boys aged 13-17 y. They assigned subjects into two groups with milk intake or non-calcium fortified juice. The authors reported a greater increase in BMD from milk intake compared to juice intake. The demands for calcium and other bone beneficial nutrients

in milk during the time of maximum BMC velocity explain the positive effect in the reviewed studies.

Whether milk intake through the lifespan affects bone health later in life, is an important question addressed in some studies during the last two decades (Sandler et al. 1985; Murphy et al., 1994; Teegarden et al., 1999; Kalkwarf et al., 2003; Opotowsky & Bilezikian, 2003). A critical review on the studies investigated the effect of early milk intake on bone health later in life suggest that in white individuals, milk intake during childhood and adolescence had beneficial effect on bone later in life (Vatanparast & Whiting, 2004). Previous milk intakes from early childhood to 12 y of age and during

Table 2.5 Comparison of milk trials in childhood and adolescents

First Author	Pub. y	Subject (n)	Sex	Age Category	Age (y)	Bon sites	Milk effect	Major findings
Gibbons	2004	150	M/F	Pre-adolescence	8-10	BMC & BMD at TB , LS, H	No	High calcium- dairy drink supplementation in prepubertal boys & girls with high habitual diary intake had no additional effect on bone mass
Du	2004	757	F		10	BMC & BMD at TB	Yes	↑ in milk intake in prepubertal girls who have low intake of calcium & vitamin D, improve their bone mass
Chan	1995	48	F		11	BMC & BMD at R, FN, TB, LS	Yes	Milk intake at or above the recommendation affect bone mineralization
Cadogan	1997	82	F	Adolescence	12	BMC & BMD at TB	Yes	Milk intake supplemented group had ↑ TB-BMD & BMC
Merrilees	2000	91	F		15-18	BMD at LS, T, FN BMC at T, LS	Yes	Milk products increase BMD & BMC at all measured sites with lesser extent at LS-BMC
Volek	2003	28	M		13-17	BMC & BMD at TB	Yes	Milk intake in physically active boys related to ↑ BMD

All studies used dual-energy X- ray absorptiometry (DXA), BMD: Bone Mineral Density, BMC: Bone Mineral Content, F: Female, M: Male, TB: Total Body, LS: Lumbar Spinal, FN: Femoral Neck, R: Radius, T: Trochanter, H: Hip

adolescence (13-19 y) were determined in a cross-sectional analysis in 224 young women (18-31 y) by Teegarden et al. (1999).The milk history questionnaire was used as the source of retrospective dietary intake information; further description of this method is given in Section 2.4.3. Childhood and adolescent milk intakes were positively correlated (r=0.66), and they were correlated with current calcium intakes as well. Current calcium intakes did not independently influence total body-BMD, total body-BMC, radius (R) BMD, and RBMC. It was only correlated with spinal (S)-BMC. Also they found a positive correlation between adolescent milk intake and total body-BMD, total body-BMC after controlling for age, weight and height. The results of this study suggested, for the first time, that adolescent milk intake was a significant predictor of BMD and BMC in young adult white women. In addition, milk intake at a younger age may contribute to similar habits of milk intake later in life suggesting early establishment of healthy eating behaviour. In summary, intake of milk products not only improve bone mineral accrual during the critical years of growth spurt, but also affect bone mass later in life.

However, Opotowsky and Bilezikian (2003) were first investigators who determined milk intake early in life affected BMD differently in white and black women. The subjects were 1675 non-Hispanic white and 735 non-Hispanic black postmenopausal women; and 908 non-Hispanic white and 998 non-Hispanic black women at age range of 20-39 year. BMD data at four hip sites and total hip BMD were analyzed in this study. In postmenopausal women of both races, greater milk intake in childhood and adolescence was associated with high current calcium intake, which is consistent with the results obtained by Teegarden et al. (1999). Black women, however, had lower current calcium intakes overall. Teenage milk intake had significant impact on BMD of white but not black postmenopausal women. The effect of childhood milk intake on bone was less than that teenage milk intake, but again, only for white women. While the results of this study are consistent with previous studies that showed that early milk intake promotes lifelong bone health among white women, more importantly, it indicates a racial difference in the effect of early milk intake on peak and postmenopausal BMD. More research is needed to determine whether this is true for men, as well as in other groups such as Hispanic-Americans.

2.3.3.4.2 Vegetables and fruit

Although a great deal of attention has been given to the importance of calcium and vitamin D intake, much less is known about the effects of other nutrients on bone. However the role of dietary vegetables and fruits has been gaining increasing prominence in the literature. It is hypothized that bone mineral acts as a buffer base and that lifetime buffering of the acid load from the ingestion of ordinary diet leads to gradual and accumulated bone loss (New et al., 2000). Therefore it might be worthwhile to consider decreasing the rate of bone attrition by the use of a diet favoring "alkaline ash". Potassium and magnesium, two nutrients that may have such buffering effect, are found in fruits and vegetables. Hence diets high in fruit and vegetables produce more alkaline urine by contributing a variety of compounds that accept hydrogen ions during their metabolism (Tucker et al., 1999).

Another component found in vegetables is vitamin K which is an essential co-factor for osteoblastic activity (Feskanich et al., 1999). Natural antioxidants and phytoestrogen compounds in some vegetables may also have bone-protective effects (Wangen et al., 2000). There are reports on compounds with an inhibitory effect on bone resorption found in some vegetables such as onion (Muhlbauer et al., 2002).

Tucker et al. (2002), studying subjects from Framingham Osteoporosis Study (FOS), found a protective effect of high fruit and vegetables intake on bone health in men but not in women. They used retrospective data from FOS and analyzed diet history of 907 subjects by grouping in dietary categories. Men who consumed a diet high in fruit, vegetables, and cereal had significantly higher bone density. In anther study, Tucker et al. (1999) investigated association between dietary components contributing to an alkaline environment (dietary potassium, magnesium, and fruits and vegetables) and bone mineral density in elderly subjects. They found greater potassium and magnesium intake were significantly associated with greater BMD for both men and women and greater fruit and vegetable intake was associated with less decline in BMC at one hip site in men.

The association between current and past dietary intake and BMD was investigated in 994 healthy premenopausal women aged 45-49 years by New et al. (1997). They found that high current intakes of the nutrients potassium, magnesium,

vitamin C, fiber, and zinc were associated with a higher bone mass and that a high past consumption of fruit had positive effect on adult bone mass. In a cross-sectional study of 62 healthy women aged 45-55 year, they found a positive link between fruit and vegetable consumption and bone health (New et al., 2000).

A few studies have been investigated the effect of dietary vegetables and fruit intake on bone mass development during childhood and adolescence (Table 2.6). Duff & Whiting (1998) reported that in prepubertal children consumption of potassium citrate providing 32 mmol K (1.2 mmol K/kg body wt), did not change urinary calcium excretion, although net acid excretion decreased. Jones et al. (2001) observed that urinary potassium which represents potassium intake was associated with BMD in different bone sites independent of lean body mass in well-nourished, calcium-replete boys and girls aged 8 year. They suggest vegetables and fruit intake by providing potassium are important for bone health in children. Tylavsky et al. (2004) studying 56 girls aged 8-13 year, found a positive relationship of fruit and vegetable consumption to bone area and BMD. Their analysis indicated that the relationship between fruit and vegetable intake and total body- BMD in these girls remained significant after adjustment for age, body mass index (BMI) and physical activity. Recently McGartland et al. (2004) examined whether usual intake of fruit and vegetable influenced BMD in boys and girls aged 12 and 15. They found a significantly higher heel BMD only in 12 year old girls who consumed high amounts of fruit.

While more studies are needed, these preliminary data suggest that in girls, there is a positive link between fruit and vegetable consumption and bone health as has been shown in adults.

2.3.3.5 Soft drinks

There has been an overall shift in dietary patterns over the last few decades (Popkin, 2002). A critical element of this shift is the increase in consumption of sugared fruit drinks and soft drinks particularly in children and adolescents (Guthrie & Morton, 2000). This change in beverage intakes has caused several health concerns. There are links between the high intakes of sugar-sweetened beverages and decreased intake of high-fiber foods (World Cancer Research Fund, 1997), increased energy intake

Table 2.6 Comparison of potassium and vegetables and fruit intake studies in boys and girls during childhood and adolescence

First Author	Pub. y	Subject (n)	Sex	Age (y)	Outcome measurement	Major findings
Duff	1998	14	F	6.7-10	Urinary calcium excretion	Potassium citrate was not hypocalciuric in children
Jones	2001	330	M/F	8	BMD at FN, TB, LS	Positive association between urinary potassium & both dietary intake & BMD at all sites
Tylavsky	2004	56	F	8-13	BMC & BMD at TB, wrist	Beneficial effects of high fruit & vegetable intakes on bone size of the radius & whole body
McGartland	2004	1345	M/F	12, 15	BMD, forearm, heel	Positive association between high fruit consumption & heel BMD in 12-y-old girls

All studies measured bone useing dual X ray absorptiometry (DXA), BMD: Bone Mineral Density, BMC: Bone Mineral Content, F: Female. M: Male. TB: Total Body. LS: Lumbar Spinal. FN: Femoral Neck.

(Ebbeling et al., 2004) and obesity (James et al., 2004). Also milk has been increasingly substituted with soft drinks resulting in low calcium intake (Cavadini, Siega-Riz & Popkin, 2000; Whiting et al., 2001). Those changes in dietary patterns, known as the Nutrition Transition are an emerging health problem in the world (Popkin, 2002).

As soft drinks become a favorite beverage choice for children, inadequate bone mineral accrual due to decreasing milk consumption might be an important health concern (Whiting et al., 2001). Milk is the principal source of calcium in the typical North American diet and our previous work has shown fluid milk to provide 43.9% of calcium for adolescents (Iuliano-Burns et al., 1999). Cavadini et al. (2000) used Nationwide Food Consumption Surveys (NFCS) and CSFII data to examine adolescents' food consumption trends in US from 1965 to 1996. They found that soft drink intake among adolescents greatly increased and the decreased milk consumption had been replaced by soft drinks and non-citrus juices and drinks. It has been reported that between 1977-1979 and 1994, daily soft drink consumption increased by 65% in adolescent girls and by 74% in adolescent boys while daily milk intake decreased from 72% to 57% in adolescent girls in US (Harnack et al., 1999).

However, Park et al. (2002) studying nationally representative sample of children, age 1 to 19 years, for three 2-year periods between 1987 and 1998, reported no difference in milk consumption over the decade. The intake of carbonated soft drinks remind similar at all age groups except for 1-5 and 15-19 age groups, in which there was a significant decrease in intake of carbonated soft drinks. There was no link between consumption of confectionary purchased from vending machines and poor dietary habits in 12-15 years old children in UK (New & Livingstone, 2003).

Information from most surveys, particularly nationally representative studies, indicates a shift in beverage choices in population from milk intake to soft drinks during growth years. In addition to the negative impact of this shift on other health conditions, bone mass development during growth and its maintenance can be affected adversely by this shift.

2.3.3.6 A dietary plan, the DASH diet

From the information presented, it becomes apparent that a variety of nutrients affect bone health. Hence promoting intake of single nutrients is not the best approach to

improve skeletal health as nutrients occur together in foods. A selection of food and drink regularly consumed mainly for nourishment compromise the diet. A typical Western diet contains inadequate amounts of calcium, vegetables and fruits, and large amounts of salt (NaCl) which harm overall health including bone (Institute of Medicine, 2004).

The DASH (Dietary Approaches to Stop Hypertension) dietary plan, which emphasizes intake of vegetables, fruits and low fat diary products, and avoids consumption of processed foods (Table 2.7)**,** fulfills the need for optimal intake of many bone beneficial nutrients (Institute of Medicine, 2004). The DASH diet provides the amount of calcium needed up to 50 year (Table 2.7), necessitating a calcium supplement to meet the higher needs of those over 50 year. It provides the optimal amount of alkaline potassium equivalents through consumption of fruits and vegetables; these foods also supply magnesium, vitamin C, polyphenols and other antioxidants, all of which play a role in bone metabolism (Institute of Medicine, 2004). Moreover, the DASH diet provides a reasonable level of sodium intake without having to endure unpalatable foods (Table 2.7). An additional study to a randomized feeding trial of the effect DASH diet on blood pressure among middle aged males and females (n=186), Line et al. (2003) reported that the DASH diet significantly reduced bone turnover. Hence, although the DASH diet is designated to control hypertension, it has complementary, beneficial effects on bone health (Doyle & Cashman, 2004). Whether DASH diet has beneficial effect on bone mineral mass measurements in children and adolescent during bone modeling has not been studied yet.

2.4 Dietary assessment methodology

Nutrition assessment is an evaluation of the nutritional status of individuals or populations through measurements of food and nutrient intake, and evaluation of nutrition-related health indicators (Lee & Nieman, 2003). Measurement of nutrient intake or food patterns is the most widely used indirect indicator of nutritional status. There are different techniques measuring diet including food record, 24-hour recall, food frequency questionnaire, diet history, duplicate food collection, food account, food balance sheet and observation (Lee & Nieman, 2003). Approaches to measuring diet

Table 2.7 Daily calcium, sodium, potassium and protein intake from DASH Diet

Food groups (Examples of 1 serving)	DASH diet (minimum servings/day)	Approximate calcium intake (not fortified) (mg)	Approximate sodium intake (mg)	Approximate potassium intake (mg)	Approximate protein intake (g)
Milk Products • Milk, 1% (250 mL- 1 cup) • Cheese (50 g)	2	575	720	520	17
Grain Products • Bread (1 slice) • Cereal (30 g- 1 cup) • Rice (1 cup)	7	160	950	560	21
Vegetable Group (Raw leafy vegetable) • Lettuce (1 cup) • Spinach (1 cup)	4	200	100	970	6
Fruit Group • Banana (medium size) • Orange (medium size) • Orange juice (1/2 cup)	4	95	10	1610	4
Meat • Lean meat (80 g) • Fish (80 g) • Poultry (80 g)	2 or less	50	135	550	19
Alternatives • Egg (1) • Cooked dry beans (125 g) • Tofu (100 g) • Peanut butter (2 tbsp-30 mL)	0.6 (4 servings /week from nuts, seeds, and dry beans)	30	35	180	8
Total	-	1110	1950*	4390	75

(Institute of Medicine, 2004) was used to calculate the values.* Consuming unsalted products reduces sodium intake further.

depend on research design consideration, characteristics of the study participants and available resources (Lee & Nieman, 2003).

2.4.1 Food Frequency Questionnaire (FFQ)

The food frequency questionnaire (FFQ) assesses food intake by determining how frequently a person consumes a limited number of foods that are major sources of nutrients or of a particular dietary component in question. A list of approximately 150 or fewer individual foods or food groups is provided to the respondents to indicate how many times respondents consume the foods. FFQ is the most common method used in epidemiologic studies (Lee & Nieman, 2003). There are three different FFQs including simple or nonquantitative, semiquantitative and quantitative. The simple FFQ records how many times a year, month, week, or day a person eats a food item. The semiquantitative FFQ uses a medium portion size when asking the frequency of consumption. Besides frequency of intake, the quantitative FFQ asks the respondent to describe the size of his or her usual serving as small, medium, or large relative to a standard serving (Lee & Nieman, 2003). The more common questionnaires used in nutritional epidemiology research are Willet Questionnaire, Block Questionnaire and Diet History Questionnaire. The Willet semiquantitative, self- administered and machine-readable questionnaire with 126 food items was used in the Framingham Osteoporosis Study (Tucker et al., 2002).

The FFQ is an inexpensive method for studies with large sample size as it saves considerable time and money. It is representative of usual intake and estimates mean intake of energy and some nutrients for groups. It is useful for ranking persons as having a low, average, or high consumption of energy and certain nutrients (Lee & Nieman, 2003). Although this method has been used in many nutritional epidemiologic studies, it has some disadvantages. The FFQ in some studies has overestimated energy intake and vegetables and fruit intake (Tucker et al., 1999). Subjects are more likely to remember the individual foods than multiple foods grouped in single list. When identical FFQs were repeated within a year, the within subject correlations of measurement error were as high as 53% for nitrogen intake (McKeown et al., 2001).

When the intake of a particular nutrient and its food sources are of interest, a specific FFQ can be used. To assess the intake and food sources of calcium, a FFQ with 36 most common food sources of calcium was used in PBMAS study (Appendix B).

2.4.2 24-hour recall

In this dietary assessment method, an interviewer asks the respondent to recall in detail all the food and drink consumed during previous 24-hours. The information is recorded for further coding and analysis. The 24-hour recall (Appendix A) is the most common method used in surveys (Lee and Nieman, 2003). It is less expensive than food records and requires less than 20 minutes to be administered. Twenty-four-hour recalls can provide detailed information on types of food consumed and can be used to estimate nutrient intake of groups. However, there are disadvantages using this method. Respondents may alter the information because of poor memory. They may underreport consumption of foods perceived unhealthful and overreport name-brand foods and foods considered healthful.

Cross-sectional studies in children and adolescents suggest that underreporting increases with age (Livingstone et al., 1992). Obese children appear to underreport more than do nonobese persons (Bandini et al. 2003). Carter et al. (2001) using PBMAS data reported underreporting of energy intake in adolescent girls in a way that as age increased the rate of underreporting increased. Bandini et al. (2003) conducted a longitudinal study on 26 healthy girls, aged 8–12 year, to determine whether the accuracy of reported energy intake decreases from middle childhood to adolescence. Energy intake and energy expenditure data of girls measured longitudinally at ages 10, 12, and 15 years. They found that errors in reported energy intake increased with age for 90% of the girls studied. These authors concluded that the use of self-reported energy intake data in adolescent girls will result in substantial underestimation of energy intake.

The main limitation of this method is that dietary intake data from only one day, even if with accuracy, can not represent an individual's usual intake because of day-to-day variation in diet (Institute of Medicine, 2003; Lee & Neiman, 2003). As the number of days of data collection increases, it gives a more precise estimation of usual intake. However, the necessary number of days depends on different factors such as sample

size, inter-subject and intra-subject variability, the nutrients of interest and where estimates of usual intake are for individuals or groups (Institute of Medicine, 2003).

2.4.2.1 Adjustment procedure (obtaining usual intake from the actual intake data)

The dietary assessment methods: serial 24-hour recalls and food records, similarly measure the actual intake in a limited period of time which may not represent the usual intake. If dietary intake data do not represent the usual intake, serious mistakes can occur in the assessment of nutrient inadequacy or excess (Institute of Medicine, 2003; Lee & Neiman, 2003). Since assessing diet for many days, to obtain the usual intake, is not economical in most research designs, statistical methods have been developed to estimate the distribution of usual intake in a group by adjusting observed intake data from 24-hour recalls and food records as long as more than one day dietary intake data are available. However, it is important to note that these methods do not estimate the usual intake for a particular individual in the group. Two of those commonly used adjustment methods are the National Research Council (NRC) method and the Iowa State University (ISU) method. Both procedures are conceptually the same; however, the statistical procedures in ISU method is suitable for large sample sizes and in NRC is appropriate for small sample size (Institute of Medicine, 2003).

The NRC method works by partitioning the observed variance into its between- and within-subject components, and then shifting each point in the observed distribution closer to the mean by a function of the ratio of the square roots of the between-subject variance ($V_{between}$) and observed variance ($V_{observed}$) (Institute of Medicine, 2003). In this way, the method attempts to eliminate the effect of within-subject variation on the observed distribution. The four steps of NRC method are as follows:

Step 1: Examining normality of distribution and transforming data if it is necessary.

Step 2: Estimating the within- and between- subject variance.

Step 3: Adjusting individual subjects' mean intakes to estimate the distribution of usual intakes

Step 4: If the original data have been transformed, the adjusted intake data should be transformed back to the original units.

Appendix F illustrates the flowchart and the SAS macro adopted for analyzing data from PBMAS study.

2.4.3 Milk history questionnaire

Over the past 20 years, researchers have studied the effect of milk intake through the lifespan on BMD later in life. A unique tool to assess milk intake in childhood, adolescence, early adulthood and middle age period in those studies is the retrospective milk history questionnaire in which the frequency of milk intake is categorized in six descending categories from more than once a day to never (Appendix C). The first study conducted using the milk history questionnaire was that of Sandler et al. in 1985. This tool has been used in national surveys such as the Third National Health and Nutrition Examination Survey (NHANES III) and the Ongoing NHANES (Kalkwarf et al., 2003; Vatanparast & Whiting, 2004). While the milk history questionnaire has proven to be a valuable tool in demonstrating the role of early diet on BMD of adulthood, it does have limitations. One cannot assign intakes of milk greater than 2 glasses per day. The size of the glass is assumed to be a standard serving and similar from person to person (Vatanparast & Whiting, 2004).

Despite these shortcomings, the milk history questionnaire is a useful, rapid, retrospective assessment tool. It continues to be incorporated into the Ongoing NHANES. Over time, however, other calcium sources such as calcium-fortified fruit juices will displace milk as important sources of dietary calcium in different age and gender groups.

2.5 Bone mineral mass measurements

The increase in bone strength throughout growth and development is the result of changes in bone geometry and bone mineral mass (Rauch & Schoenau, 2001). There is no precise measure of overall bone strength. The measurement of bone mineral mass is quantified as BMC or BMD. Bone mineral content is the amount of mineral in gram at a particular skeletal site such as the femoral neck, lumbar spine, or total body (Institute of Medicine, 1997). Areal BMD (g/cm^2) can be calculated by dividing BMC (g) by bone area (cm^2) (Institute of Medicine, 1997). Volumetric BMD is a three-dimensional

measurement of (g/cm^3) that includes bone volume and thickness (Maricic & Chen, 2000).

2.5.1 Bone mineral density measurement methods

Measuring BMD is the most commonly used approach in studies investigating the effect of environmental factors on bone. Five main approaches are used to quantify bone mineral density including single-photon absorptiometry, dual-photon absorptiometry, dual-energy X-ray absorptiometry, quantitative computed tomography (CT) and ultrasound (Lee & Nieman, 2003). Other methods are under-development or not easily available including neutron activation analysis, Compton scattering, ultrasonic transmission velocity and magnetic resonance (Lee & Nieman, 2003).

2.5.2 Dual-Energy X-Ray absorptiometry

Dual-energy X-ray absorptiometry (DXA) is the most popular method of bone measurement in research as well as clinics. The fundamental basis of DXA is passing two dimensional X-rays through the bone and assessing the X-rays absorbed by calcium atoms in the bony tissue (Lee & Nieman, 2003). DXA bone densitometry records the areal BMD (g/cm^2) which is basically BMC divided by the area of the scanned region (Institute of Medicine, 1997). Several advantages of DXA compared to other methods have made it the method of choice in bone studies. DXA has low radiation dose and it is easy to use. The lowest precision error, compared to other methods, allows the detection of changes in BMC over short periods of time and makes DXA the most sensitive method for detecting changes in BMD in the axial skeleton (Lee & Nieman, 2003).

Standard DXA software provides measurement of bone area (BA, cm^2), BMC (g) and areal BMD (g/cm^2). Each bone outcome has advantages and disadvantages depending on its use in children and adolescents. During growth, BMC is affected by both bone mineralization and bone size. However the use of BMC as an outcome measure is often the primary choice in longitudinal or serial studies among children, (Nelson & Koo, 1999). In studies investigating the effect of environmental factors on BMC (as the outcome variable), including BA, weight, and height as individual covariates in the statistical models has been recommended (Fulkerson et al., 2004). In this way, bone size-related artifacts can be avoided.

Since DXA measures bone in only two dimensions, areal BMD does not represent a true volumetric bone density (Fulkerson et al., 2004). The three-dimensional volumetric bone density accounts for approximately 60% to 80% of the variation in bone strength (Maricic & Chen, 2000; Cummings et al., 2003; Lee and Nieman, 2003). Recent studies have questioned the accuracy of DXA measurements (Wren et al., 2005a) DXA areal BMD measurements overestimate volumetric BMD in tall children and those with large bone size, and they underestimate volumetric BMD in short children or those with small bones (Fulkerson et al., 2004). There is evidence that areal BMD maybe useful as lower areal BMD is related to increased odds of wrist and forearm fractures among children (Goulding et al., 1998; Goulding et al., 2001). However, a recent study comparing the DXA BMC and BMD data with the results of QCT which provides volumetric measurement of bone, reported that DXA BMC was a more accurate and reliable measure than DXA BMD in 60 girls (6-17 y) (Wren et al., 2005b).Since BMD appears to be more reproducible than BMC, some longitudinal studies use it as outcome measure (Prentice et al., 1994).

Bone mass measured as areal BMD is only one surrogate marker of bone strength. Recently the focus of many bone researchers is shifting away from bone mineral mass measured as BMD or BMC to bone geometry or bone strength (Heaney, 2003). Recent studies suggest that bone mass should be related to the height to provide a better measure of bone dimensions and strength (Leonard et al. 2004; Schonau, 2004). Furthermore, recent literature suggests peak bone strength might by more relevant parameter for bone health than peak bone mass (Heaney, 2003; Schonau, 2004). Moreover, as the relationship between bone densitometric data and fracture risk has not been confirmed, other clinical findings such as fragility fracture should be considered in evaluating bone status of children (Heaney, 2006).

Collectively, it seems that a combination of clinical findings, bone formation and resorption biomarkers, and bone mineral measurements provides an optimal evaluation of bone status.

2.6 Statistical methods to analyze the effect of biological and environmental factors on bone

In studies of the effect of environmental factors on body development during growth such as PBMAS study, there is need to high quality data as well as appropriate analytic methods. For many years investigators has been using growth data; however, the major complexity is that they has not used a uniform method for data analysis and presentation.

Growth modeling or growth curve fitting is adjusting a mathematical model to a set of growth data (Baxter-Jones & Mirwald, 2004). There is a huge body of literature on growth models. The advantages of those models in describing the pattern of human growth depends on the quality of data, type of growth variables included in the model, age range and frequency of measurements and the ability of the model. In studying effects of environmental factors such as nutrition and physical activity on growth, it is important to consider each individual's own growth characteristic. In biostatistics language, it means that each subject's growth curve has its own intercept and slope. The advantage of this approach is capturing the wide variation observed among each subject's growth parameters at any age, and at the rate of change of these parameters from one age to the next (Baxter-Jones et al., 2004).

Regression equations are the mathematical methods which have been used to explain relationship between an outcome variable and an independent measure of growth traditionally. Analysis of covariance (ANCOVA) is usually used for inter-group comparisons. ANCOVA cannot indicate which within individual growth characteristic(s) explains the between individual differences (Norman & Streiner, 2000). Repeated measures analysis of variance (ANOVA), available in SPSS or SAS statistical softwares, is another method to analyze growth data. There are two major disadvantages of this method. Firstly, time dependent independent variables such as height cannot be fitted. Secondly, for using repeated measures ANOVA data have to be balanced (Norman & Streiner, 2000). Therefore this method cannot handle missing data which is common in longitudinal studies.

The analysis of data with a hierarchical or clustered structure has been described in the literature as hierarchical modeling, random coefficient modeling, latent curve

modeling, growth curve modeling or multilevel modeling. Multilevel modeling is an extension of ordinary multiple regression, where the data have a hierarchical or clustered structure. In this technique, time dependent covariates can be fitted and missing data does not constitute a problem as long as it is missing at random (Goldstein 1995). Each subject has his/her own growth trajectory, with intercepts and slope coefficients varying between subjects (Figure 2.2: A & B). Also it is possible to identify independent between subject effects while simultaneously controlling the effects of growth and maturation within each subject (Baxter-Jones et al. 2004). The modeling procedure for analyzing PBMAS data has been described in Appendix E.

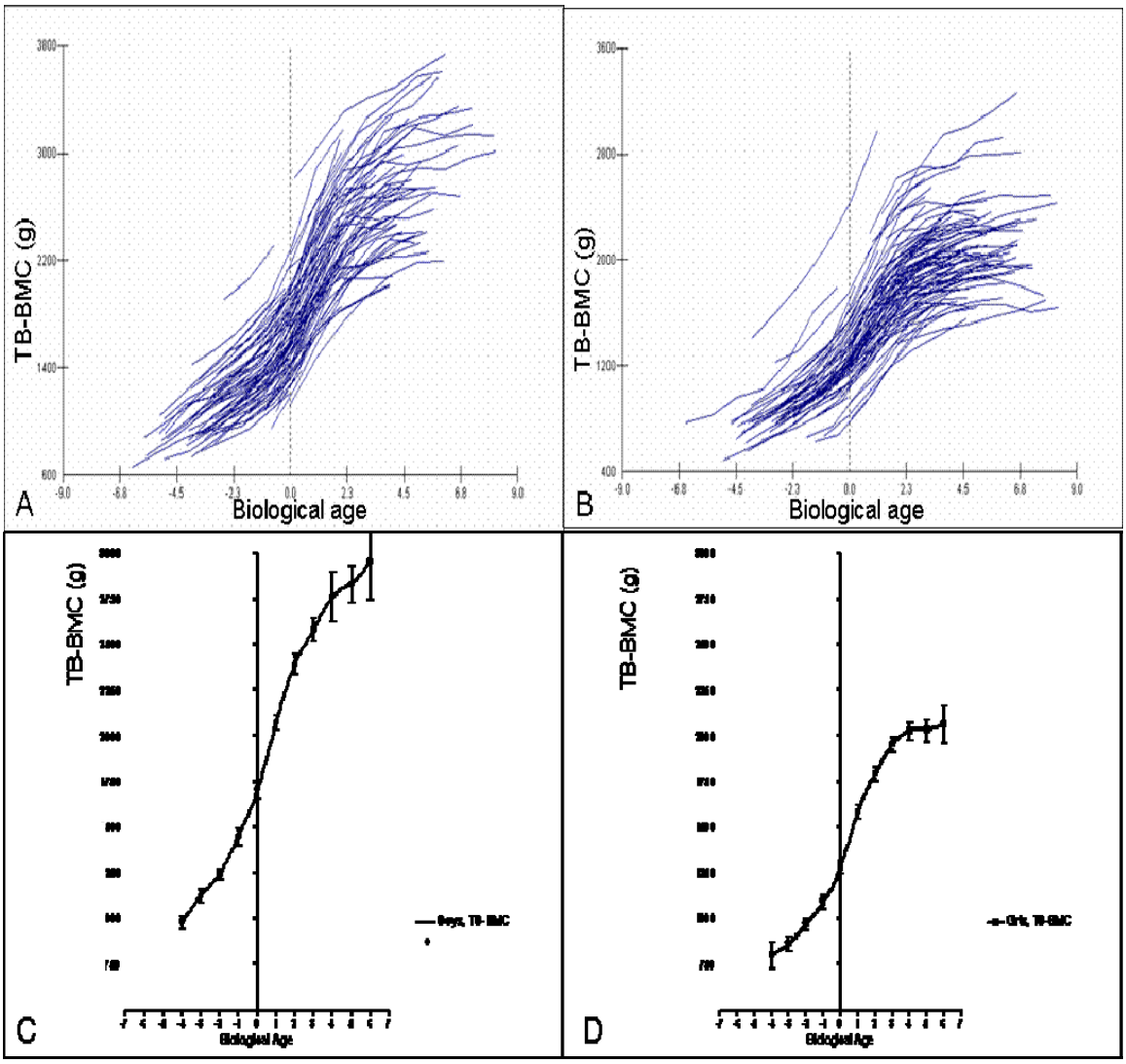

Figure 2.2 A & B: Total body- bone mineral content (TB-BMC) plotted (MLwiN) against biological age in boys (n=85) (A) and girls (n=67) (B).Each line represents an individual's repeated growth trajectory (Vatanparast, unpublished). C & D: TB-BMC distance curves (cubic spline procedure- Graph Pad Prism version 4, mean ± SEM) by biological age in boys (n= 85)-(C) and girls (n=67) (D).Values on y-axis represent mean TB-BMC in gram (Vatanparast et al. 2005).

CHAPTER 3

OVERVIEW OF METHODOLOGY

3.1 Diet and bone status from childhood to early adulthood

The purpose of this research was to examine the effect of diet, as food group and as nutrient intake, on bone mineral status during growth, from childhood to early adulthood. Research was conducted in four studies to address the objectives. While studies 1 and 4 evaluated the effect of different aspects of diet on bone, studies 2 and 3 examined how changes in food environment and life cycle affect the food choices which are important in development and stabilization of bone. These studies were conducted using data from subjects enrolled in the University of Saskatchewan Pediatric Bone Mineral Accrual Study (original and follow up studies).

3.2 Design

The University of Saskatchewan Pediatric Bone Mineral Accrual Study (PBMAS) is a prospective mixed –longitudinal study in which subjects were annually scanned for 7 consecutive years (1991-1997), with a small sample (n = 13) scanned in 1998 to complete the dataset. The age range at study entry (1991) was 8 to14 years and at the conclusion (1998) 14 to 21 years. From 2003 to 2005, the investigators conducted a 3-year follow-up on PBMAS subjects. The age range of the cohort in 2003 was 17 to 27 years. At the completion of the follow up study, in 2005, subjects in PBMAS ranged in age from 19 to 28 years. Combining the original data with the follow up data resulted in an age span of 8-28 years (Table 3.1). According to the study protocol began in the

Table 3.1 Age and gender distribution of PBMAS subjects in original cohort and its follow up (bolded)

Age	year (*Seq*)	1991 (2)	1992 (4)	1993 (6)	1994 (8)	1995 (10)	1996 (12)	1997 (14)	1998 (16)	**2003 (24)**	**2004 (26)**	**2005 (28)**	Total M(F)	Total
8		3(7)	5(11)	(2)									8(20)	28
9		10(15)	3(9)	5(10)	(2)								18(36)	54
10		19(16)	10(18)	3(10)	5(10)	(2)							37(56)	93
11		17(12)	18(15)	12(18)	3(9)	5(10)	(2)						55(56)	111
12		21(18)	17(12)	16(15)	12(14)	3910)	5(10)	(2)					74(81)	155
13		17(21)	21(16)	17(11)	14(16)	11(14)	3(10)	5(7)					88(95)	183
14		17(16)	15(20)	20(15)	14(11)	14(16)	10(12)	3(9)	2(4)				95(103)	198
15		3(8)	17(15)	14(20)	14(15)	12(11)	10(16)	8(8)	1(3)				79(96)	175
16			3(7)	17(14)	13(12)	13(14)	11(7)	8(11)	1				66(65)	131
17				3(7)	13(11)	13(12)	12(12)	9(6)	2	**(2)**			52(50)	102
18					3(6)	13(8)	12(11)	8(9)		**3(3)**	**(2)**		39(39)	78
19						3(6)	8(6)	7(8)		**4(13)**	**3(2)**	**(2)**	25(37)	62
20							4(3)	5(3)		**6(13)**	**2(12)**	**5(4)**	22(35)	57
21								2(2)		**6(9)**	**5(7)**	**2(9)**	15(27)	42
22										**12(9)**	**6(11)**	**6(10)**	24(30)	54
23										**8(10)**	**10(7)**	**7(9)**	25(26)	51
24										**14(13)**	**9(13)**	**10(7)**	33(33)	66
25										**8(6)**	**11(13)**	**8(14)**	27(33)	60
26										**3(5)**	**9(6)**	**10(10)**	22(21)	43
27										**1**	**4(4)**	**9(5)**	14(9)	23
28												**3(3)**	3(3)	6
Total		107(113)	109(123)	107(122)	91(106)	87(103)	74(90)	55(65)	6(7)	**65(83)**	**63(77)**	**60 (73)**	821(951)	1772
Total		220	232	229	197	190	164	120	13	**148**	**136**	**133**	1772	1772

original study, data collection included anthropometric and physical activity data every six months, dietary data two to four times per year in different seasons and annual DXA scans. In the follow-up study, annual measurements of anthropometric, physical activity, dietary assessment and annual DXA scan were also conducted.

3.3 Recruitment

3.3.1 Original study

In the original PBMAS subjects, 113 boys and 115 girls were recruited as a population –based sample of children in Saskatoon at 1991 (Bailey 2000). Almost all subjects were Caucasian, living in a middle class area of Saskatoon. During the second (1992) and third (1993) years of study, new subjects were added to increase the cell size to 251 subjects. By 1994, 197 individuals have been repeatedly measured on more than one occasion. In 1998, 13 individuals were bone scanned to complete the data set of individuals who needed 1 more measurement to identify their peak velocities (Table 3.1). Eligible children had no history of chronic disease or chronic medication use, and had no medical conditions, allergies or medication known to influence bone metabolism or calcium balance.

3.3.2 Follow-up study

In 2001, the parents of the 197 individuals who were measured on more than one occasion and who had not withdrawn from the study, were contacted and asked to provide contact details for their children. The subjects were then contacted and invited to participate in the PBMAS study for 3 years (2003 to 2005). Of the 197 contacted, 173 subjects were willing to participate, and 161 subjects were measured on at least one occasion in the follow up study (Table 3.1). Eighty percent of subjects participated on all three bone and body measurements. Inclusion criteria to the follow-up study were having at least two measurements in childhood at the original study.

3.4 Maturational assessment

The mean age of biological maturity for these subjects was defined by the age of peak height velocity (PHV). Age of peak height velocity is the most commonly used indicator of somatic maturity in longitudinal studies of adolescence. It is the age at maximum growth in stature during the adolescent growth spurt. Fitting growth curves either graphically or mathematically to individual growth record of stature derives age at PHV (Malina & Bouchard, 2003). By using the age of PHV as a reference, which is determined as a zero point, the ages were valued before PHV by minus numbers and after PHV by positive numbers. This is the same procedure that other investigators have used in the PBMAS study (Bailey et al., 1996; Bailey et al., 1999). Using this method, total body BMC distance and velocity curves were fitted at different levels of food groups and nutrient intake by biological ages in males and in females. Further, in the studies described in this thesis, age of PHV has been used as a landmark of maturity. A greater than six months lag time exists between the age at PHV and the age of peak bone mineral content velocity (PBMCV) (Bailey et al., 1996). During the two years around PBMCV, 26% of adults' calcium is thought to be laid down in skeleton (Bailey et al., 2000). Due to the importance of PHV in bone mass achievement, in **Study Four,** bone and body measures, dietary intake and physical activity at PHV was compared with data when peak bone was achieved.

3.5 Bone measurements

Bone measurements were obtained by annual DXA (Hologic QDR 2000; Hologic, Waltham, MA) scans by one of two experienced operators across seven years of the original study. The same technician conducted DXA scans during three years of follow-up. DXA provides an accurate and precise quantification of bone mass with low radiation exposure. Array mode was used for bone mineral acquisition and enhanced global software version 7.10 was used for analysis. To minimize operator-related variability, the same person analyzed all total body scans using software version 5.67 A. Short-term precision in vivo for total body (TB) BMC, expressed as coefficient of variation (%), was 0.60 %. A Victoreen ion chamber survey meter (model 450p;

Victoreen, Inc., Cleveland, OH, U.S.A.) measured entrance radiation dose. When this surface dose was corrected for body attenuation, subject age, and type and volume of tissue being irradiated, the effective dose equivalent was less than 1 mrem. The TBBMC is thought to be more accurate than other anatomical regions in estimating general bone mass and has been a good correlation with total body calcium content (Institute of Medicine, 1997). Change in BMC is a useful indicator for calcium retention in children while BMD misses change in skeletal size. However in adults with generally stable skeletal size, both BMC and BMD are useful outcome measures (Institute of Medicine, 1997). Since the change in bone mass from childhood to early adulthood was studied, BMC measured in young adults was considered as an indicator for calcium retention. However, total body (TB) BMD, bone area and the net gain of TBBMC from peri-adolescence to early adulthood were other bone indicators that were entered to statistical models to investigate the effect of possible factors on bone of young adults.

3.6 Anthropometrics

Standing height was measured using a Holtain stadiometer. A subject was required to stand barefoot, with hands by his or her side and heels touching the wall. The head was tilted forward so the eyes were in line with ears. Subjects were required to breath in, and the measurement taken. At least two measurements were taken, and the average recorded. Body mass of the subject wearing only light clothing (shorts and T-shirts with shoes and jewelry removed) was measured using a SECA electronic scale. Height and weight were recorded to the nearest 0.1 centimeter and 0.1 kilogram, respectively.

3.7 Dietary intake

The main tool for dietary assessment was 24 h recall (Appendix A) for both the original and follow-up studies, however, there were some differences in procedures. In original study and its follow-up, the milk history questionnaire was used as the source of retrospective dietary intake information. This is a unique tool to assess milk intake in childhood, adolescence, early adulthood and middle age period (Vatanparast & Whiting 2004). The frequency of milk intake is categorized in six descending categories from

more than once a day to never (Appendix B). Further description of this method is given in Section 2.4.3. of the literature review. Data from the milk history questionnaire were compared to data from 24-h recalls. A specific food frequency questionnaire was used to assess food sources of calcium in PBMAS follow-up (Appendix C).

3.7.1 Original study

Intake was assessed via serial 24-hour recalls conducted both at the participation schools and in the study room at the Royal University Hospital at the time of the bone scans. All days of the week, except Friday and Saturday were included. Food intake from the 24-hour recalls was analyzed using the nutritional assessment software package (NUTS Nutritional Assessment System, version 3.7 Quilchena Consulting Ltd., Victoria, BC), which used the 1988 Canadian Nutrient File information. This software categorized every food according to servings from the 1982 Canada's Food Guide, which, with the exception of names and recommended servings of the food groups and graphic to illustrate the Guide, was similar to the current version of the Food Guide (Iuliano-Burns et al., 1999). For foods categorized as "other foods", two separate groups were used: fats and oils, and sweets and desserts. Nutrient supplement use was included in nutrient intake data when supplement use was considered consistent, that is at least two-thirds of the time (Iuliano-Burns et al., 1999). The same individual coded, checked all the forms and analyzed dietary intake data according to procedures described elsewhere (Whiting et al., 1995). To obtain usual intake of subjects, intake of food and nutrients from serial 24 hour recalls were averaged for each year of study.

3.7.2 Follow-up study

Nutritional quantity and quality were measured via repeated 24-h recalls from year 2003. Intakes from at least three recalls were averaged to determine usual intake in PBMAS young adults. Two of 24-h recalls were obtained at the time of bone scans and the third one was a recall by a phone interview by a dietitian or trained senior nutrition student. Food intakes of PBMAS subjects were analyzed using Food Processor (version 8.0, ESHA Research Inc., Salem OR) that contained foods from the Canadian Nutrient File (1997).

The following procedures were done to obtain the usual intake for each subject.

1: Entered data to Food Processor software to obtain food list and nutrient values; supplement intakes were included.

2: Comparison of food lists to the 24-h recall by Dr. Whiting; any discrepancies were noted and corrected.

3: Exported data from Food Processor to EXCEL to obtain spreadsheets for the nutrients of interest.

4: Missing values for nutrients were found using US Department of Agriculture (USDA) food composition web site or other available valid sources.

For each subject, the estimated resting energy expenditure (REE) based on WHO equations (Formulas 1, 2) was calculated to define the limits for acceptable total energy intake (Lee & Nieman 2003).

Females 18- 30 years old (14.7 * Weight) + 496 ±121 (SD) *Formula 1*

Males 18- 30 years old (15.3 * Weight) + 679 ±151 (SD) *Formula 2*

Recalls with total energy intake below the standard deviation of calculated REE were eliminated and if the total number of recalls were not enough, an arrangement was made to obtain another 24 h recall by telephone. Three valid 24-h recalls were needed to obtain the usual intake of the subjects, however in 21 cases there were only two valid recalls. The software "Food Processor" classifies the food items based on the US food pyramid. Therefore, to obtain food group data, we had to use data for the US Food Pyramid, which is slightly different from the food group classification in Canada's Food Guide to Healthy Eating (Health Canada, 2005; USDA, 2005). The details of their difference were described in Section 2.3.3.4 of the literature review. In food group analyses, the Canadian food items were converted to the closest equivalent US food item to obtain food group data based on Food Pyramid.

A specific FFQ was used to assess calcium and vitamin D intake from different food sources. The two FFQs for each subject were obtained during the first and second bone scan. A milk history questionnaire was used only when subjects were measured in 2003.

3.8 Physical activity

General levels of physical activity of subjects in original PBMAS study were assessed using the Physical Activity Questionnaire for Older Children (PAQ-C) and Physical Activity Questionnaire for Adolescents (PAQ-A). Subjects rated their physical activity level during their spare time in the previous seven days, resulting in a rating from one to five. Higher scores suggest higher levels of physical activity. The PAQ-C was modified for high school students by omitting one item regarding activity at recess. Using the average of 2 or 3 scores collected over a year shows an acceptable measurement properties and sensitivity to determine activity differences between boys and girls and differences across seasons (Crocker et al., 1997; Kowalski et al., 1997). Overall, this tool is a cost efficient method for assessing general levels of children's physical activity during the school year (Crocker et al., 1997). In follow-up study, the Physical Activity Questionnaire for Adults (PAQ-AD) was used to rate the subjects' physical activity level during their spare time in the previous seven days. The PAQ-AD is an adult version of the PAQ-C, so that the same scoring system as the PAQ-C is used in PAQ-AD. Significant correlations were reported between data from self-report PAC-AD and direct measurements of physical activity in validity studies (Copeland et al., 2005).

3.9 Ethical approval

Ethical approval for the original PBMAS study was obtained from the University of Saskatchewan and Royal University Hospital Advisory Committee on Ethics in Human Experimentation in 1991. Ethical re-approval for the follow-up study was received in 2001 to continue the PBMAS cohort and resume testing (Appendix D).

3.10 Statistical analysis

Different statistical approaches were used to address the objectives of the study as they are presented in Studies One through Four. In all studies, for descriptive data, values are reported as mean ± standard deviation (SD). In all analyses, alpha was set to a value of 0.05. Following is the overview of statistical approaches. The details of statistical approaches have been described in respective chapter for each study.

Study One was designed to address the first objective, to determine the role of consumption of food groups on TBBMC around the age of PHV. For longitudinal analysis of the effect of food groups and calcium intake on TBBMC, hierarchical linear models were constructed using a multilevel (random effects) modeling approach. In brief, models were built in a stepwise procedure with predictor variables added one at a time. Log likelihood ratio statistics were used to determine if the new model was a significant improvement over the previous model. Since multi-level modeling is a complex method for analyzing longitudinal data, in addition to Chapter 4, a detailed explanation on this method has been provided in Appendix E.

The specific objective 2.1 was to determine if there have been temporal changes in food intake from 1991 to 2004; this was addressed in **Study Two**. Beverage and calcium intakes of grade 9 students were obtained at three time points, 1993, 1997 and 2003. Data from PBMAS subjects and the *FUEL: Fluids Used Effectively for Living* study (Henry et al., 2005) were used for this analysis. To examine any possible trend in beverage intake over time, one way ANOVA method or its non-parametric equivalent, the Kruskal- Wallis test, was used. Pearson and Spearman correlation methods were used to investigate association between beverages and calcium intakes of parametric and nonparametric data (respectively). Chapter 5 contains a detailed explanation.

To determine if there have been life cycle changes in diet and food sources of calcium in PBMAS subjects (specific objective 2.2) was investigated in **Study Three** (Chapter 6). Food sources of calcium in peri- and late adolescent subjects were already determined (Iuliano-Burns et al. 1999). Unpaired student's t-test was used to determine gender difference in calcium and vitamin D intakes of young adults. One-way ANOVA and Bonferroni pair wise comparison were used to investigate differences in food sources of calcium between the three age groups, peri- and late adolescent and young adults.

Study Four explored how changes in calcium intake from the age of PHV to age of peak bone mass influenced bone mass (specific objective 2.3). A two-sided paired Student's t-test was used to compare the variables of interest in two time points, peri-adolescence and young adulthood. Pearson's correlation was used to examine the relationship between variables of interest. A multiple regression model and stepwise

procedure was used to investigate the effect of calcium intake on bone measures in presence of other potential factors.

All computations were conducted using Microsoft EXCEL (2000), Statistical Package for the Social Sciences (SPSS) version 13. The software SAS V8 for Windows was used for statistical adjustment of nutrient intake from two 24 h recalls to obtain the usual nutrient intake of PBMAS subjects (Appendix F). To plot the distance curves of TBBMC, nutrients and food groups intake, Microsoft Graph Pad PRISM (version 4.0) was used. The software MlwiN version 1.0 (Multilevel Models Project, Institute of Education, University of London, London, UK) was used for statistical model used in Study One.

Table 3.2 outlines the four research studies of this thesis, and the dataset used to perform analyses.

Table 3.2 Summary of the research studies: The impact of food and nutrient intake on bone from childhood to early adulthood

Study	Project Title	Data Used
One	The effect of vegetables & fruit, and milk product intake on total body bone mineral content around the age of peak height velocity (PHV)	The original University of Saskatchewan Pediatric Bone Mineral Accrual Study (PBMAS) (1991-1997)
Two	The patterns of beverage and calcium intake from 1991-2004 in grade 9 students living in Saskatoon	The original PBMAS study (1991-1997) and the *FUEL: Fluids Used Effectively for Living* study
Three	Food sources of calcium from childhood to early adulthood	The original PBMAS study (1991-1997) and its Follow-up
Four	The effect of diet around the age of PHV on bone mineral status of young adults	The original PBMAS study (1991-1997) and its Follow-up

CHAPTER 4[1]

THE POSITIVE EFFECT OF VEGETABLE AND FRUIT CONSUMPTION AND CALCIUM INTAKE ON BONE MINERAL ACCRUAL OF BOYS DURING GROWTH FROM CHILDHOOD TO ADOLESCENCE IN THE UNIVERSITY OF SASKATCHEWAN PEDIATRIC BONE MINERAL ACCRUAL STUDY.

4.1 Introduction

Nutrition is an important modifiable factor in the development and maintenance of bone mass. Approximately 80-90% of bone mineral content is comprised of calcium and phosphorus (Institute of Medicine, 1997); protein is another major component of bone. Other dietary components such as magnesium, zinc, copper, iron, fluoride, and vitamins D, A, C, and K are required for normal bone metabolism (Ilich & Kerstetter, 2000). These nutrients occur together in foods, and their intake can be detected by assessing dietary patterns and measuring food group intake. Our group previously showed in a non-nationally representative study that during adolescence that milk products were the major source of dietary calcium (61 %), followed by grain products (9 %), vegetables and fruit (7 %), meat and alternatives (2 %) and other foods (21 %) (Iuliano-Burns et al., 1999). Food groups that contribute to calcium intake may also contribute other important bone forming nutrients; conversely, food groups other than those contributing calcium may be beneficial to bone growth for other reasons.

Although most studies have focused on the effect of calcium and/or milk product intake on bone accrual (Chan, 1992; Gunnes & Lehmann, 1995; Kardinaal et al., 1999; Molgaard et al., 2001; Carter et al., 2001; Chan et al.,1995; Cadogan et al., 1997;

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Merriless et al., 2000; Du, 2002), the role of dietary vegetables and fruit is emerging in the literature. Jones et al. (2001) first reported cross-sectional data that showed a positive link between fruit and vegetable consumption and Bone Mineral Density (BMD) in 10-y girls. Tylavsky et al. (2004) studying 56 girls aged 8-13 y, found a positive relationship of fruit and vegetable consumption to bone area and BMD. Their analysis indicated that the relationship between fruit and vegetable intake and total body BMD in these girls remained significant after adjustment for age, body mass index (BMI) and physical activity. Recently McGartland et al. (2004) examined whether usual intake of fruit and vegetable influenced BMD in boys and girls aged 12 and 15 years. They found a significantly higher heel BMD only in 12 years old girls who consumed high amounts of fruit. While more studies are needed, these preliminary data suggest that in girls, there is a positive link between fruit and vegetable consumption and bone health as shown in adults (New et al., 2000; Macdonald et al., 2004; New, 2004; Tucker et al., 2002; Tucker et al., 1999). There is no reported association in boys.

During childhood and adolescence there is a dramatic increase in bone mass, measured as either Bone Mineral Content (BMC) or BMD, which results from normal growth and biological maturation (Whiting et al., 2001). Change in BMC is a useful indicator of calcium retention in children. Since areal BMD can misrepresent bone mass changes in the growing skeleton, BMC is considered to be a better indicator of bone accrual than BMD in growing children (Institute of Medicine, 1997). Children of the same chronological age can differ by several years in their biological maturity (Malina & Bouchard, 2003); therefore, assessment of biological maturity is critical in controlling for the effects of maturation on outcome variables in studies during adolescents. The age of attainment of peak height velocity (PHV), a measure of somatic maturity, is the most commonly used indicator of biological maturity in longitudinal studies (Bailey, 1997). Hence subjects in the present study were aligned on a biological maturity age range (years from age at PHV). The purpose of this study was to determine the role of consumption of food groups, specifically milk products and of vegetables and fruit, on total body-BMC accrual in boys and girls from childhood to late adolescence.

4.2 Methods

4.2.1 Study participants and design

Subjects were participants in the University of Saskatchewan Pediatric Bone Mineral Accrual Study (PBMAS). PBMAS used a mixed-longitudinal study design incorporating 8 age cohorts (age 8 to 15 years at study entry). The initial phase of the study ran from 1991-1997, during this time span the clustering of the age cohorts remained the same, as they overlapped a developmental age range of 8 to 21 years of age was assessed. Over 251 subjects enrolled in the study. They were selected as a population–based sample of children in Saskatoon (Bailey, 1997). The majority of them were Caucasian, attending to two public schools in a middle socio-economic class area of the city. Eligible children had no history of chronic disease or chronic medication use, and had no medical conditions, allergies or medication known to influence bone metabolism or calcium balance. Subjects and their parents provided written consent for their children to participate in the study. Ethical approval was obtained from the University of Saskatchewan and Royal University Hospital Advisory Committee on Ethics in Human Experimentation.

According to the study protocol, data collection included anthropometric and physical activity data every six months, dietary data two to four times per year in different seasons and annual DXA scans. In the present analysis, seven-year longitudinal data were used from 85 boys and 67 girls. The sample represents an age range of 8 to 20 years.

4.2.2 Dietary analysis

Intake was assessed via serial 24-hour recalls conducted both at the participation schools and in the hospital at the time of the bone scans. All days of the week, except Friday and Saturday were included. Food intake from the 24-hour recalls was analyzed using a nutritional assessment software package (NUTS Nutritional Assessment System, version 3.7 Quilchena Consulting Ltd., Victoria, BC), which used the 1988 Canadian Nutrient File information. This software categorized every food according to servings from the 1982 Canada's Food Guide, which, with the exception of names and recommended servings of the food groups and graphic to illustrate the Guide, was

similar to the current version of the Guide (Health Canada, 2003). For foods categorized as “other foods”, two separate groups were used: fats and oils, and sweets and desserts. Nutrient supplement use was included in nutrient intake data when supplement use was considered consistent (Iuliano-Burns et al., 1999). The same individual coded, checked all the forms and analyzed dietary intake data according to procedures described elsewhere (Whiting et al., 1995). To obtain usual intake of subjects, intake of food and nutrients from serial 24 hour recalls were averaged for each year of study.

4.2.3 Bone measurements

Bone measurements were obtained by annual DXA (Hologic QDR 2000; Hologic, Waltham, MA) scans of the whole body, posterior -anterior lumbar spine and proximal femur by one of two experienced operators across 7 years. Array mode was used for bone mineral acquisition and enhanced global software version 7.10 was used for analysis. To minimize operator-related variability, the same person analyzed all total body scans using software version 5.67 A. Short-term precision in vivo for total body BMC, expressed, as coefficient of variation (%), was 0.60. A Victoreen ion chamber survey meter (model 450p; Victoreen, Inc., Cleveland, OH, U.S.A.) measured entrance radiation dose. When this surface dose was corrected for body attenuation, subject age, and type and volume of tissue being irradiated, the effective dose equivalent was less than 1 mrem. Total body-bone mineral content (TB-BMC) data were used in this study (Whiting et al., 2001).

4.2.4 Anthropometric, physical activity and maturity assessments

Height and weight were measured every six months by trained study personnel using the same scale and stadiometer. Subjects wore t-shirts and shorts during measurement, with shoes and jewelry removed. Height and weight were measured twice and recorded to the nearest 0.1 centimeter and 0.1 kilogram respectively.

General levels of physical activity of subjects were assessed using the Physical Activity Questionnaire for Older Children (PAQ-C). Subjects rated their physical activity level during their spare time in the previous seven days, resulting in a rating from one to five. Higher scores suggest higher levels of physical activity. The PAQ-C was modified for high school students by omitting one item regarding activity at recess.

Details regarding our derivation of PHV have been described elsewhere (Baxter-Jones et al., 2003; Bailey et al., 1999). In brief, a cubic spline procedure fitted a curve to each individual's velocity data (cm/year) and the age at the peak value was extrapolated from the curve. Biological maturity was calculated as measurement age minus age at PHV, with biological maturity at age of PHV equal to 0. Biological maturity age prior to age of PHV is measured in negative years and after PHV in positive years. Individuals were clustered into yearly biological maturity age categories. Using this method we fitted total body BMC distance curves at different food group intakes by biological maturity age in boys and in girls**.**

4.2.5 Statistical analysis

Values are reported as mean ± standard deviation (SD). Data analyses were conducted using Microsoft EXCEL (2000) and Statistical Package for the Social Sciences (SPSS, version 11.5). Microsoft Graph Pad PRISM (version 4.0) was used to produce the distance curves for TB-BMC, nutrient and food group intake in boys and girls by chronological and biological maturity age. As biological maturity age controls the effect of maturation, the chronological age curves were not analyzed further. A two-sided unpaired Student's t-test was used for cross-sectional analysis. Alpha was set to a value of 0.05.

For longitudinal analysis, hierarchical linear models were constructed using a multilevel (random effects) modeling approach (MlwiN version 1.0, Multilevel Models Project, Institute of Education, University of London, London, UK). Multilevel modeling is an extension of ordinary multiple regression, where the data have a hierarchical or clustered structure. The modeling procedure has been described in detail elsewhere (Baxter-Jones & Mirwald, 2004). In brief, models were built in a stepwise procedure with predictor variables added one at a time. Variables were considered significant if the estimated mean coefficient (β) was greater than twice the standard error of the estimate (SEE). Loglikelihood ratio statistics were used to determine if the new model was a significant improvement over the previous model (Baxter-Jones & Mirwald, 2004). The following additive polynomial models to describe the developmental changes in accrual of bone mineral content accrual were adopted.

$$y_{ij} = \alpha_j + \beta_j x_{ij1} + \beta x_{ij2} + \ldots + \beta x_{ijp} + \varepsilon_{ij} \quad \text{[Eq.1]}$$

where y is *TBBMC* at assessment occasion i in the j-th individual, α_j is the constant for the j-th individual, $\beta_j x_{ij1}$ is the slope of *TBBMC* with biological maturity age for the j-th individual, and x_2 to x_p are the coefficients of various explanatory variables at assessment occasion i in the j-th individual (see Table 4.3); ε_{ij} is the level-1 residual (within individual variance) for the i-th assessment of *TBBMC* in the j-th individual. The details of statistical models has been described in Appendix E.

4.3 Results

Table 4.1 shows age, height, weight and BMI at the age of PHV (biological maturity = Zero). Chronological age corresponding to age of PHV was 11.8 years for girls and 13.5 years for boys, not different to what we have reported for smaller samples of this cohort (26). Girls' heights, weights and BMI at age of PHV were less than those for boys ($p < 0.05$). Food group intakes are given in Table 4.2.The majority of subjects had an appropriate intake of milk products; however, girls consumed less milk product than boys ($p < 0.05$). Gender difference could be seen in meat and alternatives intakes as well (Table 4.2). In boys, the mean intakes of all food groups at overall age span were significantly more than in girls ($p<0.001$) (Table 4.2). The majority of boys and girls consumed vegetables and fruit less than the recommended amounts. In contrast, more than 40% of them consumed high amount (> 3 servings/day) of sweets and desserts.

Figure 4.1 illustrates the TB-BMC and calcium intake distance curves of boys and girls aligned by biological age. As biological age increased, TB-BMC increased in boys and girls. The higher magnitude of this increase in boys was related to their greater body size. Before the age of PHV, the intake of calcium was not different in males and females. After the age of PHV, there was a dramatic increase in calcium intake in males, while for girls, the intake of calcium dipped. At age of PHV, boys had significantly higher intake of calcium than girls ($P< 0.05$) (Table 4.2).

Table 4.3 show the results of the additive polynomial model described previously (Eq. 1). Two models were fitted, one for (a) boys and one for girls (b). By making our time variable (biological maturity) random at level-2, the variance of TB-BMC accrual over increasing biological maturity was estimated in two parts. Level-1 variance was the variance associated with an individual's regression line of TB-BMC development on

biological maturity. The second part of the variance (level-2) was variance representing the deviation of each individual's line from the average line for the whole group. The fixed effects in both models indicated that TB-BMC increased with increasing biological maturity (114.8 ± 10.7 g per year and 80.4 ± 7.1 per year for boys and girls, respectively). The power functions for biological maturity were included to shape the curves, essentially forcing a sigmoidal shape to a linear model. In boys a 1 cm increase in stature predicted 22.6 g of TBMC, whereas as 1 cm increase in stature predicted 18.9 g TBBMC in girls. For body mass the coefficients were 4.3 and 7.3 for boys and girls, respectively. Once the confounding effects of growth and biological maturity were controlled, the effects of physical activity, calcium intake and vegetable and fruit intake were shown to be gender specific. In girls physical activity, calcium intake and vegetable and fruit intake were not significant independent predictors of TBBMC ($p>0.05$). However in boys a score of 1 on the physical activity scale (1 low, 5 high) predicted 22.2 g TBBMC. For every 1 mg of calcium consumed 0.017g of TBBMC was accrued and for every serving of vegetable and fruit intake 5.4g of TBBMC were accrued.

The coefficient can also be used to predict percentage of TBBMC accrued at any particular time point. For example, in boys at peak height velocity (biological maturity = zero) height accounted for 91.6 % of the TB-BMC prediction, body mass accounted for 5.7 % of predicted TB-BMC. Physical activity, calcium intake and vegetable and fruit intake accounted for 1.7%, 0.5% and 0.5% of the predicted TB-BMC, respectively, for boys. While in girls, percent contribution of covariates were 89.3 %, 9.8%, 0.8%, 0.09% and 0.009% for height, body mass, physical activity level, calcium intake and vegetable and fruit intake, respectively (Table 4.4). Energy intake and other food groups including grain products, meat and alternatives, fat and oils, and sweets and desserts were not significant predictors of TB-BMC in males or females and thus were not included in the final models.

4.4 Discussion

The unique aspect of the design of this study is the alignment of subjects along an axis related to age of attainment of PHV. Age at PHV is a very useful biological maturity indicator, and size and velocities of tissues such as bone attained during

Table 4.1 Characteristics of subjects at the age of peak height velocity

	Boys (n = 85) Mean (SD)	Girls (n = 67) Mean (SD)
Age of PHV, y	13.5 (1.0)*	11.8 (0.9)*
Height, cm	164.1 (6.9)*	153.3 (7.7)*
Weight, kg	52.31 (8.68)*	43.27 (9.72)*
BMI, kg/m^2	19.4 (2.6)*	18.3 (3.0)*

PHV = peak height velocity, BMI= Body Mass Index, *= $p < 0.05$ (two-sided unpaired Student's t-test)

maturity can be expressed against this landmark (Malina & Bouchard, 2003). As we have previously described (Baxter-Jones & Mirwald, 2003) the magnitude of TB-BMC accrual in females was less than in males (Figure 1), and this gender difference in accrual can be explained by anthropometric differences (Baxter-Jones & Mirwald, 2003). Height and weight of this cohort were within normal reference standard ranges (between 50th and 90th percentile) for all chronological ages in both genders (Mundt et al. 2006). Males were taller and heavier than females across biological maturity ages.

Although our subjects, particularly males, had a relatively better food group intake compared to other published studies (Table 4.5), they did not all meet the recommendations (Munoz et al., 1997; Brady et al. 2000; Starkey et al., 2001). The percentage of subjects meeting the guidelines for each of the food groups ranged from about 18% in meat and alternatives (females) to about 90% for milk products (males) in our cohort. There was a marked gender difference in food choices and the high intake by boys increased from childhood to adolescence in almost all instances.

Despite milk product being the major source of calcium for these subjects (Iuliano-Burns et al. 1999), our current results do not show a specific effect of milk product intake compared to that for calcium intake, on TB-BMC accrual. Most subjects in our cohort met the recommendation for milk products. As we were able to show that calcium intake was a significant predictor of TB-BMC in boys (Baxter-Jones et al.,

Table 4.2 Mean intake of nutrient and food groups at the age of peak height velocity (PHV) and overall in boys and girls

	Canadian Recommendations‡	Boys [Mean (SD)] (n=85)		Girls [Mean (SD)] (n=67)	
		Age of PHV (13.4 y)	Overall age span (age 8-20)	Age of PHV (11.8 y)	Overall age span (age 8-20)
Milk Products (servings/d)	2-4	3.1 (1.8)*	3.3 (1.9) §	2.6 (1.1)*	2.3 (1.3) §
Grain Products (servings/d)	5-12	5.9 (2.2)	6.7 (3.2) §	5.5 (1.6)	5.3 (1.9) §
Meat & Alternatives (servings/d)	2-3	2.3 (1.0)*	2.6 (1.6) §	1.7 (0.7)*	1.6 (0.8) §
Fat & Oils (servings/d)	N/A	5.2 (3.7)	5.8 (5.3) §	4.2 (2.9)	4.5 (3.3) §
Vegetables & Fruit (servings/d)	5-10	4.1 (2.7)	4.5 (3.3) §	3.8 (2.0)	3.8 (2.4) §
Sweet & Desserts (servings/d)	N/A	3.3 (2.1)	3.7 (2.9) §	3.1 (1.6)	2.8 (1.9) §
Calcium (mg/day)	1300	1199 (569)*	1292 (634) §	1030 (360)*	958 (423) §

* = $P < 0.05$, mean intake of food groups and calcium of boys (n=85) compared to girls' (n=67) at age of PHV,

§ = $p < 0.001$, mean intake of food groups and calcium of boys compared to girls' at overall age span (two-sided unpaired Student's t-test),‡ = The intake of our subjects was compared with the lower value in the range

Table 4.3 Multilevel regression analysis of total body bone mineral content (TB-BMC) aligned on biological maturity, adjusted for height, body mass, physical activity, calcium intake and vegetable & fruit intake)

Fixed effect	Estimates ± SEE			
	(a)Boys(n=85)		(b)Girls(n=67)	
Constant	-2306.6 ± 233.4*		-1898.4±184.1*	
Biological maturity	114.8±10.7*		80.4±7.1*	
Biological maturity2	12.8±0.8*		10.2±0.9*	
Biological maturity3	-2.3±0.2*		-2.0±0.1*	
Height	22.6±1.5*		18.9±1.3*	
Body mass	4.3±0.8*		7.3±0.6*	
Physical activity**	22.2±9.0*		NS	
Calcium intake**	0.017±0.008*		NS	
Vegetable & fruit intake**	5.4±1.3*		NS	
Random effects	Level 1(within individuals)			
Constant	4330.8±316.8*		2204.7±156.5*	
	Level 2 (between individuals)			
	Constant	Biological maturity	Constant	Biological maturity
Constant	36851.0±5175.5*	4186.3±806.1*	20204.9±2798.0*	1650.1±112.6*
Biological maturity	4186.3±806.1*	1026.6±188.3*	1650.1±112.6*	669.2±112.6*

Fixed effects values are Estimated Mean Coefficients ± SEE (Standard Error Estimate) (TB-BMC, g). Random effects values Estimated Mean Variance ± SEE (TBBMC, g). Biological maturity – years from age at PHV (years), height (cm); body mass (kg); physical activity score (1 low, 5 high); calcium intake (mg/day), vegetable & fruit intake (servings/day). * = $p < 0.05$ if Estimate > 2x SEE; Not Significant (NS). ** = Significant predictors only in boys. (Vatanparast et al. 2005).

Table 4.4 Percent contribution of each variable (Table 4.3) on the prediction of total body bone mineral content (TB-BMC) at the age of biological maturity [peak height velocity (PHV) =0] in boys (n=85) and girls (n=67).

	Boys (n=85)	Girls (n=67)
	%	%
Height	91.6	89.3
Body weight	5.7	9.8
Physical Activity	1.7	0.8
Calcium Intake	0.5	0.091
Vegetable & Fruit Intake	0.5	0.009

2003), it suggests that calcium intake from other sources was important. Our derivation of number of servings of milk products was not as precise as finding mg of calcium, which may have reduced our power to detect a significant effect of milk products compared to calcium.

Vegetable and fruit intake had a significant independent effect on TB-BMC development in boys. For subjects of the same biological maturity age, height, body mass, physical activity and calcium intake, TB-BMC would be 48.6 g higher in the subject with 10 servings/day intake of vegetable and fruit compared to 1 serving /day of intake. Bone mineral acts as a buffering base, and a lifetime of buffering the acid load from the ingestion of "western-type" diets is believed to lead to bone loss (Tucker et al., 2002). Fruit and vegetables provide organic salts of potassium and magnesium which have such a buffering effect. Another component found in vegetables is vitamin K which is an essential co-factor for osteoblastic activity (Feskanich et al., 1999). Lower BMD and higher hip fracture have been reported in patients with low vitamin K levels (Feskanich et al., 1999). Natural antioxidants and phytoestrogen compounds in some vegetables may also have bone-protective effects (Wangen et al., 2000). There are reports on compounds with an inhibitory effect on bone resorption found in some vegetables such as onion (Muhlbauer et al., 2002).

Table 4.5 Percentage of subjects who meet dietary food group recommendation

Food Groups	Grains	Vegetables	Fruit	Milk Product	Meat & Alternatives
Recommendation[1] (serving / day)	6-11	3-5	2-4	2-3	2-3
Munoz et al. 1997					
Male 6-11 y	36.2	35.4	27.8	40.6	23.4 [2]
Male 12-19 y	42.5	50.3	17.1	48.9	44.9 [2]
Female 6-11 y	35.1	34.3	34.1	31.5	25.8 [2]
Female 12-19 y	20.6	46.1	19.1	21.7	31.8 [2]
Brady et al. 2000					
Male 7-14 y	49	30	6	11	30
Female 7-14 y	43	13	3	8	22
Recommendation (serving/ day) [3]	5-12	5-10 Vegetables & fruit		2-4	2-3
Starkey et al. 2001					
Male 13-17 y	71.5	42.9		77.4	61.9
Female 13-17 y	55.9	56		53.8	55.5
Present Study					
Male, 8-20 y	82.7	30.1		87.8	74.5
Female, 8-20 y	52.9	17.7		62.6	18.4

[1] US Food Guide Pyramid

[2] The percent of subjects who met between 5-7 ounces

[3] Canada's food guide to healthy eating. Food groups are similar to US food pyramid except vegetables and fruit are added.

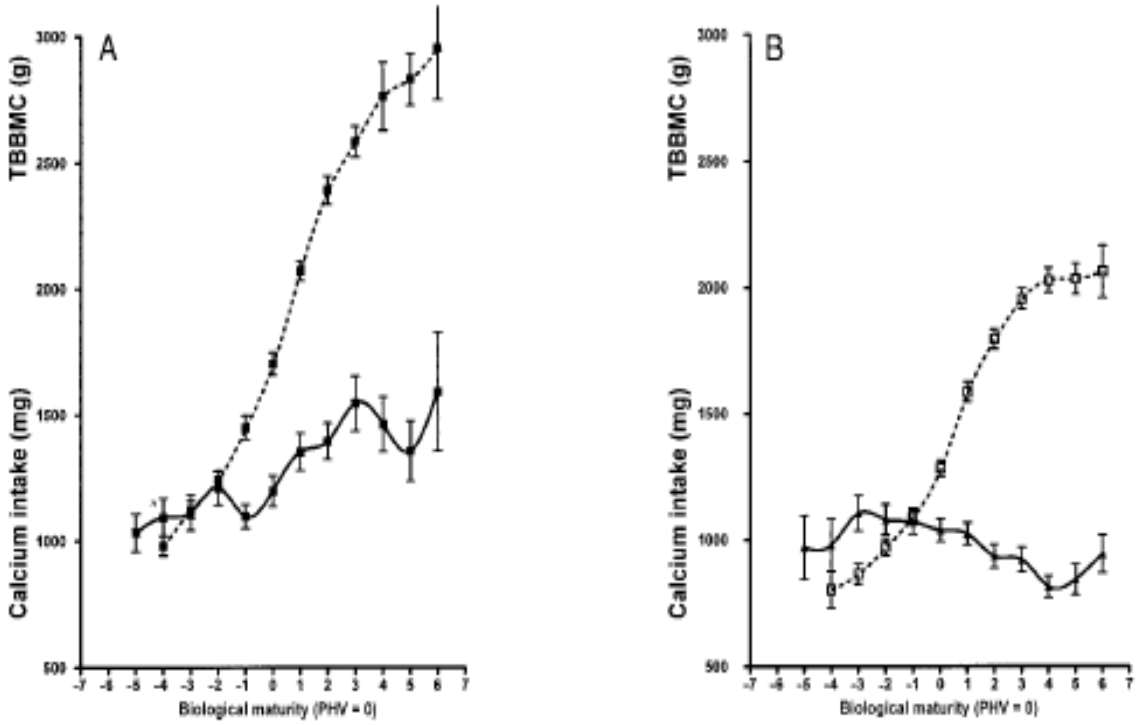

Figure 4.1 Total body bone mineral content (TB-BMC) and calcium (Ca) intake distance curves (cubic spline procedure, mean ± SEM) by biological maturity [age of peak height velocity (PHV) as a reference] in boys (n=85) and girls (n=67). Values on y-axis represent both mean calcium intake in milligram (mg) and amount of mean TB-BMC in gram (g). Solid lines = Calcium intake, Dashed lines = TB-BMC

Most research on the effect of fruit and vegetables on bone health has been conducted in peri-menopausal women (New et al., 2000; Macdonald et al., 2004; New, 2004; Tucker et al., 2002; Tucker et al., 1999; Feskanich et al., 1999; Wangen et al., 2000; Muhlbauer et al., 2002). Three cross-sectional studies in children and adolescents found a positive relationship between vegetable and /or fruit intake and bone mass markers in girls (Jones et al., 2001; Tylavsky et al., 2004; McGartland et al., 2004). We demonstrated vegetable and fruit, as a significant predictor of TB- BMC in boys aged 8-20. Overall these studies support the concept of lifetime beneficial effect of vegetables and fruit intake on bone health.

Studies show a positive relationship between moderate physical activity and BMC accrual (Scerpella et al. 2003; Sundberg et al., 2001; Sundberg et al., 2002) including results from our previous analysis (Bailey et al., 1999). In our final model physical activity was a significant predictor of TB-BMC in the presence of calcium and vegetable and fruit intakes. However there was no interaction between those factors. The concept of a threshold intake of calcium during childhood and/or adolescence that optimizes the effect of physical activity on adult bone status has been supported by several studies (VandenBerg et al., 1995; Tylavsky et al., 1992; Wosje et al., 2000).

We could not demonstrate the effect of physical activity, calcium and vegetable and fruit intake on TB-BMC in girls. We have previously shown, using data from the first year of our study, that underreporting was greater in girls than in boys and that older girls underreported more than younger girls did (Carter at al. 2001). This pattern of underreporting can be seen in calcium intake plotted in Figure 1. Our data are in agreement with energy intake and energy expenditure data of girls measured longitudinally at ages 10, 12, and 15 years by Bandini et al. (2003) who found that as the age of girls increased, they tended to report energy intake less accurately. These authors concluded that the use of self-reported energy intake data in adolescent girls will result in substantial underestimation of energy intake. In our cohort, the calcium intake distance curve in girls (Figure 1) was identical of their energy intake distance curve.

There are limitations in our study. Our subjects were not drawn from nationally representative sample. Dietary intakes were self reported, which can lead to underestimation due to underreporting. Physical activity was also self-reported, and was

determined as frequency of various activities. The longitudinal nature of the study and the determination of biological maturity age, however, are unique features of the study. Use of multi-level modeling method specifically developed for growth data analysis permitted determination of factors affecting TB-BMC during biological maturation.

Major biological, psychological, social, and cognitive changes occur during adolescence. These changes can affect the nutritional needs of teenagers (Heaney et al., 2000). Although biological factors play the most important role in bone mineral accrual during those critical years, nutrition and physical activity are important factors to achieve genetically designed potential peak bone mass. The beneficial effect of vegetable and fruit intake on bone mass measure as TB-BMC in boys aged 8-20 years, adds to literature indicating this effect occurs in girls. Likely all adolescents would benefit the effect of appropriate intake of this food group on their bone health beside its other health benefits.

4.5 Summary

Nutrition is an important modifiable factor in the development of bone mass during adolescence. Recent studies of children and adolescents have examined the effects of foods such as milk products and fruit and vegetables on bone growth, however, few have studied both boys and girls. The purpose of the study was to determine the role of consumption of milk products and vegetable and fruit on the total body-bone mineral content (TB-BMC) accrual in boys and girls from childhood to late adolescence. Seven-year longitudinal data were used from 85 boys and 67 girls (8-20 y). Biological maturity was defined by the years from age at peak height velocity (PHV). Dietary intake was assessed via serial 24-hour recalls. Anthropometrics and physical activity were assessed every six months. TB-BMC assessed using DXA in the fall of each year, was the indicator of bone mass. Most boys (87.8 %) met Canadian recommendations for milk product intake. Few subjects (< 30 %) consumed vegetables and fruit in recommended amounts. Using a multilevel modeling statistical approach containing major biological and environmental factors, we found that vegetable and fruit intake, calcium intake and physical activity were significant independent environmental predictors of TB-BMC in boys but not girls. In addition to dietary calcium, increased vegetable and fruit intake has a beneficial effect on TB-BMC in boys aged 8-20 y. Underreporting of dietary intake by girls in our study may explain why this effect was not apparent in girls.

CHAPTER 5[2]

A NEGATIVE TREND IN CALCIUM INTAKE WITHIN 15 YEARS (1991-2004) WAS ACCOMPANIED BY A SHIFT FROM MILK TO NON-CARBONATED SOFT DRINKS IN GRADE 9 FEMALE STUDENTS LIVING IN CANADA

5.1 Introduction

Over the past few decades, an increase in the consumption of sugared fruit drinks and soft drinks has been documented, particularly in children and adolescents (Popkin & Nielsen, 2003; Rampersaud et al., 2003; French et al., 2003; Bowman, 2002). Adolescents have a higher nutrient requirements compared to other age groups because of their rapid growth and development; further, they may develop unhealthy eating habits that lead to inadequate nutrient intake (Spear, 2002). This situation places adolescents in a nutritionally vulnerable position, particularly for obesity, tooth decay, and inadequate bone accrual (Bray, Nielsen, & Popkin, 2004).

There are direct links between the high intakes of sugar-sweetened beverages and decreased intake of high-fiber foods, increased energy intake and obesity (St-Onge et al., 2003; Gillis et al,. 2002; Mrdjenovic & Levitsky, 2003). Although soft drink intake may not be the primary cause of obesity, it has been identified as one of contributory factors in school children (Berkey, Rockett, Field, Gillman & Colditz, 2004; Ludwig et al., 2001; Weiss et al., 2004). A prospective study among 11-12 years old children showed that the risk of becoming obese was raised 1.6 times for each additional can or glass of soft drink consumed everyday (Weiss et al., 2004). This is a concern because obesity in adolescence is a risk for early establishment of chronic diseases such as coronary heart diseases, hypertension and type 2 diabetes (Gidding et al., 2004). It is difficult to directly

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attribute the rise in soft drink intake to increasing obesity as total energy intake reported by adolescents decreased from 1965 to 1996 (Cavadini et al., 2000). However, soft drink consumption by participants aged 2 years to 18 years increased from less than 3 % (52 kcal) of total energy in 1977/78 and to more than 5 % (105 kcal) in 1994/96 (Nielsen & Popkin, 2004).

Another health concern associated with high soft drink consumption is inadequate bone mineral accrual due to decreasing milk consumption as soft drinks become a favorite choice for children (Whiting et al., 2001). Milk is the principal source of calcium in the typical North American diet and our previous work has shown fluid milk to provide 44 % of calcium for adolescents (Iuliano-Burns et al., 1999). Using nationwide food consumption trends in the United States from 1965 to 1996, Cavadini et al.(2000) found that soft drink intake among adolescents greatly increased with a corresponding decline in milk consumption. Replacing milk with soft drinks occurs from the third grade (Bowman, 2002; Lytle et al., 2000; Forshee & Storey, 2003). It has been reported that between 1977-1979 and 1994, daily soft drink consumption increased by 65% in adolescent girls and by 74% in adolescent boys while daily milk intake decreased from 72% to 57% in adolescent girls in US (French et al., 2003; Cavadini et al., 2000). Several studies have shown that the result of diminished milk consumption and consequently low calcium intake of adolescents is less accrual of bone mass at a critical time in life (Whiting et al., 2001; McGartland et al., 2003).

Since there were no national data available for determining the trend of beverage intake in Canadian adolescents, the objective of this study was to examine the trend in contribution of beverages to total caloric beverage intake and its relationship with calcium intake of grade 9 students, based on gender, at three time points from 1991 to 2004. Adolescence is the time of maximum bone mineral accrual velocity (Bailey et al., 2000). Therefore, understanding the pattern of trend in beverage consumption and its effect on calcium intake during these critical years will assist nutrition policy makers in designing appropriate interventions to establish healthy dietary behaviors. We hypothesized that there would be a similar relationship as reported by US studies; that is, that milk (and therefore calcium intake) would decline as a result of shift from milk to soft drinks.

5.2 Methods and Materials

5.2.1 Participants

Data from the University of Saskatchewan Pediatric Bone Mineral Accrual Study (PBMAS) and the University of Saskatchewan *Fluids Used Effectively for Living Study* (FUEL) study were used for this analysis. PBMAS is a mixed-longitudinal study that ran from 1991-1997. The original subjects of PBMAS, over 251 boys and girls, were selected as a population–based sample of children attending to two schools in Saskatoon. Eligible children had no history of chronic disease or chronic medication use, and had no medical conditions known to influence bone metabolism or calcium balance. Subjects and their parents provided written consent to participate in the study. Details about the PBMAS study have been described elsewhere (Bailey, 2000). Dietary intake data were collected two to four times per year in different seasons during the years of PBMAS study. Ethical approval was obtained from the University of Saskatchewan and Royal University Hospital Advisory Committee on Ethics in Human Experimentation. For this new analysis, calcium and beverage intake data were used from 98 subjects (45 boys and 53 girls) who were grade 9 students during 1991-93, and 62 subjects (31 boys and 31 girls) who were grade 9 students between 1995-97. The age range of these grade 9 students was 14-16 years.

FUEL is a nutrition education program designed to promote healthy beverage consumption among high school students. This program started in fall 2003 at the same high schools as PBMAS. Subjects were 58 grade 9 students (40 boys and 18 girls aged 14-16 years) who voluntarily enrolled in the study. School divisions and principals of the selected schools gave permission to conduct the study in their schools. Written consent was obtained from students, parents and guardians of eligible participants. Ethical approval was obtained from the University of Saskatchewan Committee in Behavioral Science Research. Dietary intake was assessed using serial 24-hour recalls and a specific beverage frequency questionnaire. Data from the first two 24-hour recalls (taken before and one week after starting the program) were used in this study. The majority of subjects in PBMAS and FUEL studies were Caucasian. The schools are located in a middle class area of the city.

5.2.2 Dietary assessment

In both studies, intake was assessed via serial 24-hour recalls (2-4 per subject). The nutritional assessment software package for PBMAS (NUTS Nutritional Assessment System, version 3.7 Quilchena Consulting Ltd., Victoria, BC, 1988) and FUEL (Food Processor version 8.0, ESHA Research Inc., Salem OR, 2003) both contained foods from the Canadian Nutrient File which included information on food items having fortification levels unique to Canada..

Beverage intake was determined directly from recalls by classifying intake in three main categories of beverages called "caloric beverages": fluid milk used as beverage (including hot chocolate and milk shakes), fruit juice (at least 50 % real fruit juice) and soft drinks (any sugary beverage). Soft drinks were subdivided into carbonated (CSD) and non-carbonated (NCSD) soft drinks. The uses of noncaloric beverages such as diet drinks, and tea or coffee without sugar, as well as water, were not included in this analysis, the former being negligible and the latter not measured in PBMAS.

5.2.3 Statistical analysis

The mean and standard deviation (SD) of intakes, and the percent contribution of each beverage category (fluid milk, fruit juice and soft drinks) to total caloric beverage intake, were calculated for each of the three time points for boys and girls separately, to examine any possible trend in beverage intake over time. One way ANOVA method or its non-parametric equivalent the Kruskal- Wallis test was used to assess the significance of differences over time. Pearson and Spearman correlation methods were used to investigate association between beverages and calcium intakes of parametric and nonparametric data (respectively). Alpha was set to a value of 0.05. Data analyses were conducted using Microsoft EXCEL (2000) and Statistical Package for the Social Sciences (SPSS) version 11.5.

5.3 Results

Figures 1and 2 illustrate the percent contribution of each category of caloric beverages (fluid milk, fruit juice and soft drinks) to total caloric beverage intake at three time points in boys and girls. Percent contribution of milk to total caloric beverage

intake was significantly decreased in boys and girls from 1991 to 2004. However, the mean intake of milk significantly decreased over time only in girls. Percent contribution of fruit juice has significantly increased in boys and girls from 1991 to 2004. Calcium-fortified fruit juices entered the Canadian market in the late 1990s. These were not available for the PBMAS subjects. In FUEL study (2003-04), approximately 50% of boys and girls reported intake of calcium-enriched fruit juices at least some of the time.

There was a significant negative association between milk intake and consumption of non-carbonated soft drinks in boys and girls [$r = -0.197$ ($p < 0.035$) and $r=-0.322$ ($p=0.001$) respectively] but not for carbonated soft drinks or total soft drinks. Mean intake of all caloric beverages in boys was significantly more than in girls except for NCSD. Both genders had similar mean intakes of NCSD. Percent contribution of soft drinks to total caloric beverage intakes increased overtime in girls, but not in boys (Figures 1 and 2). In year 2003-04, soft drinks contributed to approximately 50% of overall caloric beverage intakes in girls, while 34% of total caloric beverage intakes were from soft drinks in boy.

In girls, there was a significant negative trend in total daily calcium intake from 1991 to 2004. Results from post hoc analysis indicated that the main difference was between the second (1995-97) and third (2003-04) time points. Mean intake of calcium decreased from 997 mg/d in 1995-7 to 772 mg/d in 2003-04. No significant trend was observed in boys. There was an overall positive correlation between calcium intake and fluid milk consumption ($r = 0.685$ and $r = 0.697$, $p < 0.01$) in girls and boys respectively.

5.4 Discussion

Milk, soft drinks, and fruit juice are consumed in high amounts by adolescents (Rampersaud et al., 2003; Lytle et al. 2000) yet these beverages differ in nutrient composition. Milk contributes energy, protein, fat, calcium, vitamin C, vitamin A, riboflavin , and vitamin D (if fortified), while fruit juice contributes energy, calcium (if

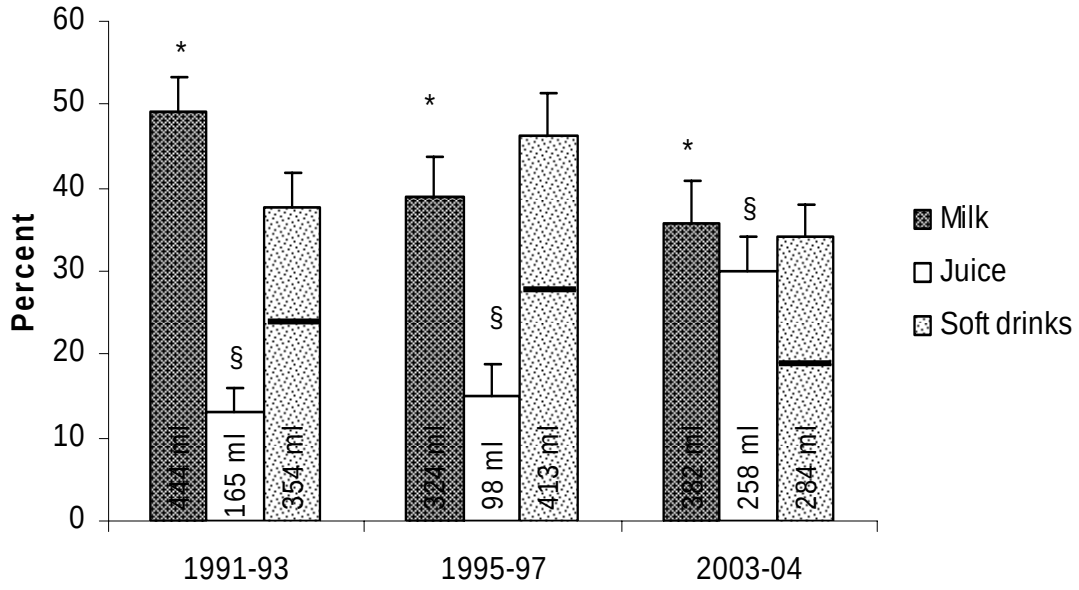

Figure 5.1 Percent contribution of milk, juice and soft drinks to total caloric beverage intakes over time in boys. Mean intakes of beverages in mL are provided inside columns. In soft drinks bar, percent contributions of non-carbonated soft drinks and of carbonated soft drinks are presented above and below the dividing line, respectively. The symbols * and § represent significant trend over time for milk and fruit juice intakes, respectively ($p < 0.05$, Kruskal- Wallis test).

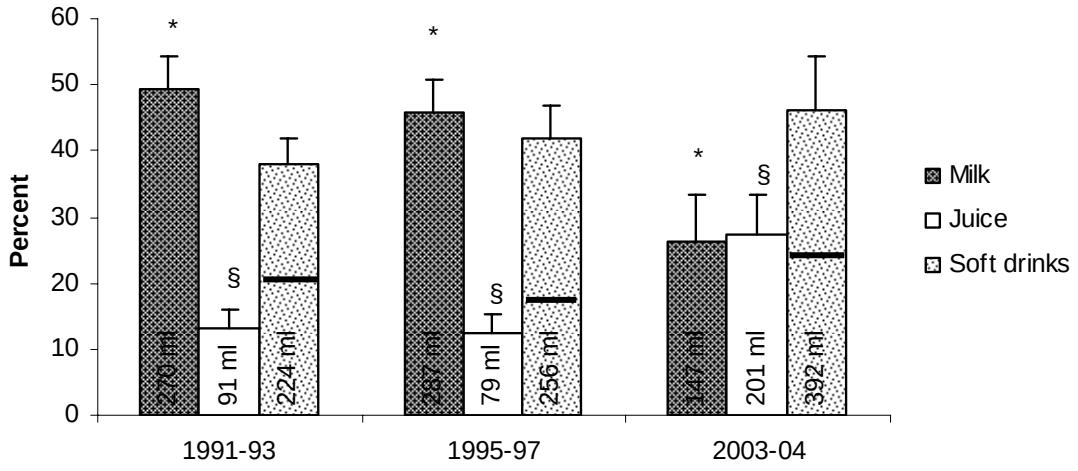

Figure 5.2 Percent contribution of milk, juice and soft drinks to total caloric beverage intakes over time in girls. Mean intakes of beverages in mL are provided inside columns. In soft drinks bar, percent contributions of non-carbonated soft drinks and of carbonated soft drinks are presented above and below the dividing line, respectively. The symbols * and § represent significant trend over time for milk and fruit juice intakes respectively ($p < 0.05$, Kruskal- Wallis test).

fortified), vitamin C, and vitamin A, and soft drinks contribute only energy (unless fortified) (Phillips et al., 2004). We evaluated both mean intake of beverages and the percent contribution of each beverage to total caloric beverage intake. Our results that the percent contribution of milk to total caloric beverage intake significantly decreased in both genders since 1991, is in agreement with other studies showing a decline in milk intake in the last 40 years (Popkin & Nielsen, 2003; Rampersaud et al., 2003; French et al., 2003; Bowman, 2002; Cavadini et al., 2000). These studies reported that decrease in milk intake was accompanied by the decrease of calcium intake (Popkin & Nielsen, 2003; Cavadini et al., 2000). In our study we found a significant decrease in calcium intake over time in girls.

Percent contribution of soft drinks to total caloric beverage intake increased over time in girls, but not in boys. In 2003-04, soft drinks contributed approximately 50% of total caloric beverage intake in girls. However, no significant trend over time was observed in non-carbonated or carbonated soft drink consumption in girls and boys in our study. Three major studies using nationwide data in the United States were consistent in showing that for the past 27 years, soft drink intake has increased (Popkin & Nielsen, 2003; French et al., 2003; Cavadini et al., 2000). In those data, boys and girls aged 14-17 years were the greatest soft drink consumers in the population of 6-17 year olds (French et al., 2003; Heller et al., 2001). One study (not a nationally representative one) observed a decline in soft drink consumption from 1988 to 1998 (Park et al., 2002). That cohort as well as our cohort, had smaller sample sizes which explain why neither observed a trend in total soft drink intake.

The negative trend over time in calcium intake found in girls indicates that they are more at risk of substituting milk with soft drinks than boys. Adequate calcium intake is important for bone mineral accrual which reaches its peak velocity during adolescence (Martin et al. 1997). In two studies of the effect of soft drinks on bone accrual of adolescents, girls were shown to have less bone accrual when intakes of soft drinks were high; boys were spared an adverse effect on bone (Whiting et al., 2001; McGartland et al., 2003). As females lose bone mineral mass due to menopause and aging more than males later in life, it is important to achieve maximal bone accrual and attainment of peak bone mass during adolescence (Whiting et al., 2004).

It has been observed that raw fruit consumption has been substituted by fruit juice, making juice one of the main choices of beverages in adolescents (Cavadini et al., 2000; Lytle et al., 2000). Although there was a trend in fruit juice consumption in our study, no inverse relationship was observed between milk intake and fruit juice consumption. We have reported in other study that juice beverage consumption had no apparent negative or positive effect on bone accrual (Whiting et al., 2001). Since fruit juice has become a preferred beverage for adolescents, calcium-fortified fruit juice might be considered as an important vehicle for dietary calcium. However, there was little use of this beverage by our subjects.

Although homes are still the largest source of soft drink access, studies have shown that soft drink access from vending machines and school cafeterias has increased (French et al., 2003). Soft drink companies have focused on schools since their potential customers spend most of their active time at school (Nestle, 2002). They reached almost every middle and senior high school through vending machines in the United States in 2003 (Molnar, 2004). Health promotion strategies have targeted carbonated soft drinks because of evidence on relationship between CSD and health conditions including obesity, and bone and dental health. Recently in Canada, it has been decided not to sell CSDs in elementary and middle schools beginning 2005 (Leith, 2004). However, what is not appreciated is that high intakes of NCSD can raise the same health issues as CSDs. Furthermore, a shift from milk intake to NCSDs observed in this study shows the importance of controlling high intake of this beverage, particularly in girls. Including beverages such as chocolate milk in vending machines may increase the intake of milk, since flavored-milk is more favorable than white milk for children and adolescents (Johnson et al., 2002).

Our study had some limitations. Two different nutrient composition software programs were used as the studies were done at different times. The potential for seasonal variations in intake were addressed in PBMAS study but not the FUEL study. The average of intake from two to four 24-hour recalls was assumed to represent usual intakes of subjects.

In conclusion, healthy behaviors, including consuming nutritious beverages, are important for adolescents to improve their school performance, growth, developmental

tasks and future health (He et al., 2004). Furthermore, eating behaviors and dietary patterns established during childhood and adolescence may be maintained through adulthood (Lytle, 2002). Hence, adolescents need the skills to make healthy choices. School is the best place to reach most adolescents for health intervention programs (Hoelscher et al. 2002; Pate & Sirard, 2004; Story et al., 2002). The nature of foods available at home and schools have been identified as significant influences on eating habits in children and youth (Gillman et al., 2000; Fisher et al., 2001; Fried & Nestle, 2002). Parents and schools can provide this opportunity for adolescents to practice healthy eating behaviors.

5.5 Summary

Over the past 25 years, adolescents' intake of soft drinks doubled, arousing health concerns such as obesity, tooth decay, and inadequate bone accrual (Popkin & Nielsen, 2003). In the absence of national data for determining a trend of beverage intake in Canadian adolescents, we examined beverage consumption and its relationship with calcium intake of grade 9 students living in Saskatoon, at three time points from 1991 to 2004. Intake data from two studies at the University of Saskatchewan were used. Subjects were 98 (45 M, 53 F), 62 (31 M, 31 F), and 58 (40 M, 18 F) grade 9 students during 1991-93, 1995-97 and 2003-04, respectively; the age range spanned 14-16 years. Dietary intakes were assessed via serial 24-hour recalls, during which the name and quantity of each food and beverage consumed in last 24 hours were reported. One way ANOVA or its non-parametric equivalent was used to assess differences over time. Beverages were classified into 3 groups: milk, juice and soft drinks (excluding noncaloric beverages). Percent contribution of milk to total beverage intake was significantly decreased in boys and girls from 1991 to 2004. In girls only, a significant negative trend in calcium intake was observed over time. Percent contribution of fruit juice significantly increased in boys and girls over time. There was a significant negative association between milk intake and consumption of non-carbonated soft drinks (NCSD) in both genders ($p < 0.001$). Milk was replaced by NCSD more than by carbonated beverages, and girls were more at risk of this shift than boys.

CHAPTER 6

CHANGES IN FOOD SOURCES AND INTAKE OF CALCIUM FROM CHILDHOOD TO EARLY ADULTHOOD IN THE UNIVERSITY OF SASKATCHEWAN PEDIATRIC BONE MINERAL ACCRUAL STUDY

6.1 Introduction

Osteoporosis is a major public health problem in the world because of the aging population, particularly in the developed regions of the world (Iqbal, 2000). In Canadian women over 50 y of age, prevalence of osteoporosis is 16 % (Tenenhouse et al., 2000). Development of bone mass during the first two decades of life is thought to be a major determinant of vulnerability to osteoporotic fractures in such a way that an increase by 10% in bone mass would reduce the fracture risk by 50% (Teegarden et al. 1998; Bounjor et al 2003). The high velocity of bone mineral accumulation during puberty requires a greater intake of calcium (Institute of Medicine, 1997). Calcium absorption and bone calcium deposition rate increase during puberty, resulting in more calcium absorption and less overall calcium excretion than adults with the same calcium intake (Abrams & Stuff, 1994; Abrams et al., 2000; Weaver et al., 1995). In early adulthood, the longitudinal growth of bones is ceased, but consolidation of bone mass continues (Institute of Medicine, 1997). These indicate a positive calcium balance from childhood to early adulthood, the time that peak bone mass is achieved.

The intake of calcium during childhood and adolescence varies in different regions of the world. While children in North European countries have calcium intake of 1200 mg/d (Vandenbergh et al., 1995), a calcium intakes as low as 500 mg/d has been reported in Chinese children and adolescents over the last decade (Lee et al., 1993; Lee et al., 2003; Zhu et al., 2004). The data from national surveys in the United States

indicate that the mean intake of calcium among children and adolescents does not achieve the recommended values for either females or males (Newmark et al., 2004). In 1994-1996 Continuing Survey of Food Intakes by Individuals (CSFII), children age 5 and younger had an average calcium intake of 809 mg/day. Males age 6 to 11 and 12 to 19 had a mean calcium intake of 970 and 1145 mg/day, respectively. Females age 6 to 11 and 12 to 19 had a mean calcium intake of 857 and 771 mg/day, respectively (U.S. Department of Agriculture [USDA], 1997). In Canada, Shatenstein et al. (1996) reported a calcium intake less than recommended level in children and adolescent boys and girls except for boys aged 16-18 y with a calcium intake of 1396 mg/d. Analyzing data from the University of Saskatchewan Pediatric Bone Mineral Accrual Study (PBMAS), we reported that the calcium intake of our cohort was reasonably higher compared to other studies during peri-adolescence (Vatanparast et al., 2005).

In addition to regional variations in calcium intake, a gender difference in calcium intake has been reported in various studies with more calcium intake in boys than in girls (Vandenbergh et al. 1995, Roma-Giannikou et al., 1997; Iuliano-Burns et al, 1999; Rajeshwari et al., 2004; Salamoun et al., 2005). Boys need greater energy intakes as a function of their body size (Malina & Bouchard, 2003); as a result; their overall food intake is more than girls. Hence, the difference in calcium intake between boys and girls may be explained by the greater volume of foods consumed by boys.

The intake of calcium tends to rise with increasing age in boys. In contrast, studies reported that calcium intake deceases by increasing age in girls. (Shatenstein & Ghadirian , 1996; Iuliano-Burns et al, 1999; Rajeshwari et al., 2004; Ruiz et al., 1995; Albertson et al., 1997). Food records of 226 boys and girls aged 8-19 y over 6 years of the PMBAS study were analyzed by Iuliano-Burns et al. (1999) to determine the intake of calcium and its food sources. Older girls (grade 9-12) consumed significantly less calcium than the younger ones (951 mg/d vs 1021 mg/d). In contrast, older boys had greater dietary calcium intake compared to younger boys (1386 mg/d vs 1179 mg /d). These data reveal that at the time of growth spurt when the peak BMC velocity takes place and skeletal need for calcium retention increases, girls may not meet the recommendation of calcium intake.

The trend in calcium intake has been investigated by following the consumption of milk as the main surrogate of calcium intake. In the 1989-1991 Continuing Survey of Food Intakes (CSFII), milk was the primary source of dietary calcium in individuals aged 2 to 18 years. Milk provided more than half (>50%) the total amount of calcium in children's intakes in ages 2 to 11 years. This percentage of calcium intake from milk dropped to 46 % in males and 43 % in females in the 12- to 18-year age group (Subar et al., 1998). Nielsen and Popkin (2004), analyzing US nationwide nutrition surveys, reported a decline from 13 % of total energy from milk in 1997 to 8 % in 2001 among children aged 2-18. As discussed later, the alarming downward trend in milk consumption, especially in female adolescents, has been accompanied by an excess intake of other beverages mainly soft drinks. Comparing grade 9 students at three time points within last 15 years, 1991, 1997 and 2004, we found a negative trend in calcium intake of female subjects accompanied with milk substitution by non-carbonated soft drinks (Vatanparast et al., 2006).

Milk products have been the main sources of dietary calcium in North Americans' diet (Institute of Medicine, 1997; Subar et al., 1998). In PBMAS cohort, we found that milk products were the major source of dietary calcium (61 %), followed by grain products (9 %), vegetables and fruit (7 %), meat and alternatives (2 %) and other foods (21 %) which were close to data from the United States (Iuliano-Burns et al. 1999; Institute of Medicine 1997). In our peri-adolescent cohort, the mean intake of milk products was 3.1 servings/day and 2.6 servings/day in males and females respectively (Vatanparast et al. 2005). In the Bogalusa Heart Study examining dietary intake of 246 at age 10 and young adulthood, although milk consumption decreased as the subjects entered to young adulthood (Demory-Luice et al. 2004), no significant difference was reported in their calcium intake (Rajeshwari et al. 2004).

Increased bone mineral demand of calcium during pubertal growth spurt can be facilitated by the increase in plasma level of the active form of vitamin D, 1,25-dihydroxy vitamin D_3 (1,25DHD) (Bonjour et al., 2003). Insulin-like growth factor-I (IGF-I) stimulates kidney to produce 1,25DHD (Caverzasio et al., 1990; Bonjour, 2003). Consequently an elevated plasma level of 1,25DHD intensifies the capacity of intestinal epithelium to absorb calcium and inorganic phosphate (Bonjour et al., 2003). Although

the major source of vitamin D in the world is exposure of skin to sunlight (Combs, 2000), cultural, environmental, and physiological factors can impair sun-light induced production of vitamin D. Hence, it is important to consider dietary intake of vitamin D (Calvo et al., 2004). In North America, most of the intake of vitamin D from food comes from fortified food items (Institute of Medicine, 1997). In Canada, the foods that require fortification with vitamin D are milk (100 IU per 250 mL) and margarine (53 IU/10 g) (Calvo et al., 2004). The difficulty of differentiating vitamin D input, from diet and/or sun exposure, has limited the ability of investigators to include vitamin D intake in their analysis (Weaver & Fleet, 2004). Despite the relatively higher intake of vitamin D in North Americans compared to people of other regions, a seasonal insufficiency of vitamin D, during winter, has been reported in adults, adolescents and children (Weiler et al., 2005; Calvo et al., 2004; Calvo & Whiting, 2003; Vieth et al., 2001; Looker et al., 2002; Rucker et al., 2002; Gordon et al., 2004; Nesby-O'Dell et al, 2002; Jones et al. 2004). No nationally representative data exist on vitamin D intakes of Canadians, however, studies indicate the same seasonal variation as the US in serum 25-hydroxy vitamin D (25OHD) concentrations (Calvo & Whiting, 2003). Furthermore, a higher latitude, longer winter and less available vitamin D-fortified foods in Canada justifies assuming a greater extent of vitamin D insufficiency during winter in Canada than in the United States (Calvo & Whiting, 2003). Dietary vitamin D intake per kg body weight was the most important predictor of 25OHD concentration at the end of winter in Edmonton, when adolescents and children were at risk of vitamin D insufficiency (Roth et al., 2004).

Behavioral and physiological changes from childhood to early adulthood may affect food choices and dietary patterns of young adults. However, the majority of data regarding dietary patterns are from cross-sectional samples of specific age groups such as data from NHANES and CFSII. Further, less is known about the dietary intakes of young adults. Therefore, the objective of this study was to determine changes in intake and food sources of calcium of PBMAS males and females from childhood through adolescence to early adulthood. In addition, the gender difference in food sources of vitamin D in PBMAS young adults was assessed.

6.2 Materials and methods

6.2.1 Study participants and design

For this study, data has been obtained from the PBMAS. PBMAS is a prospective mixed –longitudinal study in which subjects were annually scanned for 7 consecutive years (1991-1997), with a small sample (n = 13) scanned in 1998 to complete the dataset. The age range at study entry (1991) was 8 to15 years and at the conclusion (1998), 14 to 21 years. Over 251 subjects enrolled in the original study. The PBMAS subjects were contacted and invited to participate in 3-year follow- up study for 3 years (2003 to 2005). Of the 197, 173 subjects have been contacted, 154 have agreed to participate in the new study. The age range of the cohort in 2003 was 17 to 27 years. At the completion of the follow up study, in 2005, subjects in PBMAS ranged in age from 19 to 28 years. Combining the original data with the follow up data resulted in an age span of 8-28 years. Subjects were categorized into three age groups, peri-adolescents 8-14 y (130 boys, 126 girls), late adolescents 15-19 y (79 boys and 96 girls) and young adults 19-27 y (67 males and 87 females).

The majority of subjects were Caucasian, attending to two public schools in a middle socio-economic class area of the city during their childhood and adolescence. No data is available on socio-economic status of the subjects during their early adulthood. Eligible subjects had no history of chronic disease or chronic medication use, and had no medical conditions, allergies or medication known to influence bone metabolism or calcium balance. Subjects and their parents (during their childhood) provided written consent to participate in the study. Ethical approval was obtained from the University of Saskatchewan and Royal University Hospital Advisory Committee on Ethics in Human Experimentation.

6.2.2 Dietary analysis

In peri-adolescence and late adolescence groups, the intake was assessed via serial 24-hour recalls conducted both at the participation schools and in the hospital at the time of the bone scans. Food intake from the 24-hour recalls was analyzed using a nutritional assessment software package (Nutritional Assessment System, NUTS, version 3.7 Quilchena Consulting Ltd., Victoria, BC), which used the 1988 Canadian

Nutrient File information. This software categorized every food according to servings from the 1982 Canada's Food Guide, which, with the exception of names and recommended servings of the food groups and graphic to illustrate the Guide, was similar to the current version of the Guide (Iuliano-Burns et al, 1999; Health Canada, 2006). Nutrient supplement use was included in nutrient intake data when supplement use was considered consistent (Iuliano-Burns et al, 1999). The same individual coded, checked all the forms and analyzed dietary intake data according to procedures described elsewhere (Iuliano-Burns et al, 1999; Whiting et al. 1995). To obtain usual intake of subjects, intake of food and nutrients from serial 24 hour recalls were averaged for each year of study (Iuliano-Burns et al. 1999)

In young adults, a specific food frequency questionnaire (FFQ) designed to assess calcium and vitamin D intakes from 36 food items which are the main sources of calcium and vitamin D in Canadian diet (Shrestha et al., 1998). Data from two FFQs, obtained during 2002-03, entered to a specific EXCEL spreadsheet designed to compute the calcium and vitamin D values. The average of calcium and vitamin D intakes data from two FFQs was used for further analysis. In addition, serial 24h recalls were used to assess young adult's overall dietary intake. The total energy intake values were obtained averaging data from three 24-h recalls in year 2002-03. Further, the mean calcium intake values from FFQs were validated using statistically adjusted calcium intake values from adjusted intake values that were computed from two 24-h recalls (Appendix F). Food items categorized according to the Food Guide Canada for Healthy Eating to 5 groups as follows: milk products, grain products, meat and alternatives, vegetables and fruit, and others. For foods categorized as "other foods", three separate subgroups were defined: combined dishes, fats and oils, and sweets and desserts.

6.2.3 Statistical analysis

Food sources of calcium in peri- and late adolescent subjects have already been determined ((Iuliano-Burns et al. 1999). Values are reported as mean ± standard deviation (SD). Statistical analysis performed by Microsoft EXCEL (2000) and Statistical Package for the Social Sciences (SPSS, version 13).Unpaired student's t-test was used to determine gender difference in calcium and vitamin D intakes of young adults. One-way ANOVA and Boneferroni pairwise comparison were used to investigate

differences in food sources of calcium between the three age groups. Hypothesis testing was based on the Type-I error rate of 5% (alpha=0.05).

6.3 Results

6.3.1 Calcium intake by young adults

The mean calcium intake of peri- and late adolescent males were 1178 mg/d and 1386 mg/d respectively. In females, calcium intake decreased from 1042 mg/d in peri-adolescence to 951 mg/d in late adolescence. The mean calcium intake of young adult males was 1140 mg/d which is above the Adequate Intake (AI) value of 1000 mg/d for age range 19-30 y. However, 46% of them did not meet the AI for calcium. In young adult females, the mean calcium intake (824 mg/d) was below the AI and 79% did not meet the AI for calcium. However, expressing total dietary calcium intake relative to 1000 kcal energy intake, no significant gender difference in calcium intake was observed (432 mg/ 1000 kcal and 447 mg/1000 kcal for males and females, respectively). There was a positive relationship between calcium intake and energy intake in young adult males ($r=0.39$, $p=0.002$), but not in females. Young adult males and females had the lowest nutrient density for calcium in comparison to the peri- and late adolescent males (578 mg/1000 kcal and 551 mg/1000 kcal) and females (531mg/1000 kcal and 575 mg/1000 kcal), respectively.

6.3.2 Food sources of calcium from childhood to early adulthood

Table 6.1 presents the percent contribution of food groups to total dietary calcium intake from food sources. Milk products, specifically milk, were the major source of dietary calcium from childhood to early adulthood in both genders. In all age groups, milk products contributed to more than 800 mg/d and approximately 600 mg/d dietary calcium in male and females respectively (Figure 6.1). In males, although milk products contributed to more than 60% of calcium intake in all age groups, contribution of fluid milk to total dietary calcium intake significantly decreased as age increased ($p<0.05$). In stead, the contribution of cheese significantly increased from 10% of total dietary calcium in peri-adolescent males to 22% in young adults ($p<0.05$). The similar pattern of significant increase can be seen in the intake of yogurt ($p<0.05$), where its

contribution to calcium intake increased 10 times in young adults compared to late adolescents (Table 6.1). In females, the contribution of fluid milk to calcium intake in late adolescent group was significantly less than in peri-adolescents ($p<0.05$), while no significant decline was observed in young adult females. There was a significant rise in cheese and yogurt intake in young adult females, while the intake of other milk products decreased significantly ($p<0.05$). When the mean intakes of calcium from various milk products were compared (Figure 6.2), a significant decline in fluid milk consumption ($p<0.05$) was accompanied with an increase in yogurt intake in both genders in young adult males and females ($p<0.05$).

After milk products, combined dishes were the second major source of dietary calcium which their contribution to dietary calcium increased by age. However, it was only significant in young adult females ($p<0.05$) comparing to the contribution of combined dishes in peri-adolescent females (Table 6.1). Vegetables and fruit, grain products, and meat and alternatives were the other food sources of calcium respectively (Table 6.1). While the intake of calcium from vegetables in young adult males and females was the lowest among the three age groups ($p<0.05$), the contribution of fruit to total dietary calcium intake was the highest in young adult males ($p<0.05$), but not females (Table 6.1). In young adult males and females, grain products contributed to the lowest amount of total dietary calcium intake among the three age groups ($p<0.05$). The decline in calcium intake from meat and alternatives (meat and eggs) was only significant in young adult females ($p<0.05$).

6.3.3 Vitamin D intakes by young adults

The mean intakes of vitamin D for young adult males and females (330 IU/d and 213 IU/d respectively) were above the AI value of 200 IU/d. No vitamin D intake from supplements was reported in males (Table 6.2). When dietary vitamin D intake was expressed relative to energy intake, young adult males demonstrated a nonsignificantly higher nutrient density for vitamin D (123 mg/1000 kcal) than females (109 mg/ 1000 kcal). Milk products, mainly milk, were the main source of dietary vitamin D in young adult males and females (Table 6.2, Figure 6.3). Fish and combined dishes were the two other major sources of dietary vitamin D respectively. When percent contribution of different food items to vitamin D intake were compared, fluid milk, fish and combined

dishes were the first three major contributors to dietary vitamin D intake (83% in males and 82% in females) (Figure 6.3). There was no gender difference in vitamin D intake from dietary sources except for fish, and fat and oils. Males had significantly greater vitamin D intake from fish ($p<0.05$), while female had more of it from fat and oils compared to males ($p<0.05$) (Figure 6.3).

6.4 Discussion

Whether the intakes of calcium and its food sources are changing by age from childhood to early adulthood and its relationship with bone health has been of interest of researchers for many years (Vatanparast & Whiting, 2004). Due to the difficulties in conducting longitudinal studies, most studies have assessed the intake of milk as the main source of dietary calcium retrospectively (Vatanparast & Whiting, 2004). In our male cohort, the significant decrease in milk consumption was observed from late adolescence to early adulthood accompanied with a probably adequate calcium intake from childhood to early adulthood. In contrast, the significant lower intake of milk in females was presented at late adolescence compared to peri-adolescence resulting in low dietary calcium intake for a longer period of time during the critical time of peak bone mass achievement. These changes in milk intake were accompanied with significantly higher intake of calcium from cheese in both genders of our young adult cohort compared to peri- and post adolescence suggesting milk substitution by cheese. Although cheese is another valuable food source for calcium (Appendix G), the high concentration of sodium may interfere with the calcium status (Institute of Medicine, 1997).

The only regional comparable data to our study was data from the Saskatchewan Nutrition Survey (1993-1994). The mean intakes of calcium in young adults (18-34 y) were 822 mg/d and 1251 mg/d (females and males respectively) which were similar to our data (Stephen & Reeder, 2001). Percent contribution of milk products to calcium intake in our cohort was remarkably similar to the Saskatchewan Nutrition Survey data (Table 6.3). The main difference was in the contribution of yogurt to intake of calcium.

Table 6.1 Percent Contribution of food groups (SD) to total dietary calcium intake by gender and age group

	Male			Female		
	Group 1 (n= 130)	Group 2 (n=79)	Group 3 (n= 67)	Group 1 (n=126)	Group 2 (n=96)	Group 3 (n=87)
Milk Products	63.3	62.3	67.7	62.1	57.2	68.6
-Fluid milk	49.6(26)	48.2(27)	38.8(20)[a]	48.4(24)	39.5(29)[c]	41.0(20)
-Cheese	10.0(17)	11.7(18)	21.8(14)[a,b]	9.5(16)	14.6(21.2)	17.9(12)[d]
-Yogurt	0.9(5)	0.4(3)	4.0(5)[a,b]	0.9(6)	0.7(8)	7.7(8)[d,e]
-Other	2.8(8)	2(7)	3.1(6)	3.3(9)	2.4(7)	1.8(4)[d]
Vegetables & Fruit	6.5	6	7.5	6.7	7.6	5.2
-Fruit	2.9(5)	2.2(4)	6.2(12)[a,b]	3.1(5)	2.8(4)	3.4(3)
-Vegetables	3.6(5)	3.8(6)	1.3(1)[a,b]	3.6(6)	4.8(7)	1.7(1)[d,e]
Meat & Alternatives	2.5	3.0	2.4	2.0	2.1	1.8
-Meat/Eggs	2.1(4)	2.5(4)	1.3(2)	1.7(3)	1.9(3)	0.8(1)[d,e]
-Fish	0.4(3)	0.5(4)	0.6(1)	0.3(2)	0.2(1)	0.4(1)
Grain Products	9.0(9)	8.6(9)	4.6(6)[a,b]	8.9(9)	8.5(9)	3.9(3)[d,e]
Other Foods (Combined dishes)	11.7(18)	12.0(18)	17.7(11)	12.7(19)	14.7(21)	20.5(14)[d]

Footnote for Table 6.1

Group 1: Peri-adolescents (8-14 y). Group 2: Late adolescents (15-19 y). Group 3: Young adults (19-27 y). Results of multiple comparisons between groups are presented as a: between groups 1&3 in males, b: between groups 2&3 in males, c: between groups 1&2 in females, d: between groups 1&3 in females, e: between groups 2&3 in female ($P < 0.05$ Bonferroni comparison test).

Table 6.2 Contribution of food groups and supplement to intake of vitamin D [IU/day (SD)] by gender

	Male (n= 67)	Female (n= 87)
Milk Products	**175(138)[a]**	**130(90)**
-Fluid milk	165(137)[a]	121(89)
-Soy milk	0(2)	1(9)
-Cheese	8(6)	6(6)
-Other	2(3)	2(9)
Vegetables	1(1)	0
Meat & Alternatives	**115(192)[c]**	**32(46)**
-Meat/Eggs	11(19)[b]	5(5)
-Fish	104(184)[c]	27(45)
Grain Products	**4(3)**	**3(2)**
Other Foods	**35(33)**	**32(18)**
-Fat & Oils	9(14)	9(7)
-Combined dishes	26(22)	23(17)
-Supplement	0	15(94)
Food & Supplement	**330(281)[c]**	**213(134)**

a, b, & c represent significant differences (males versus females) as a = $p < 0.05$, b = $p < 0.01$, c = $p < 0.001$. One microgram vitamin D is equal to 40 International Units.

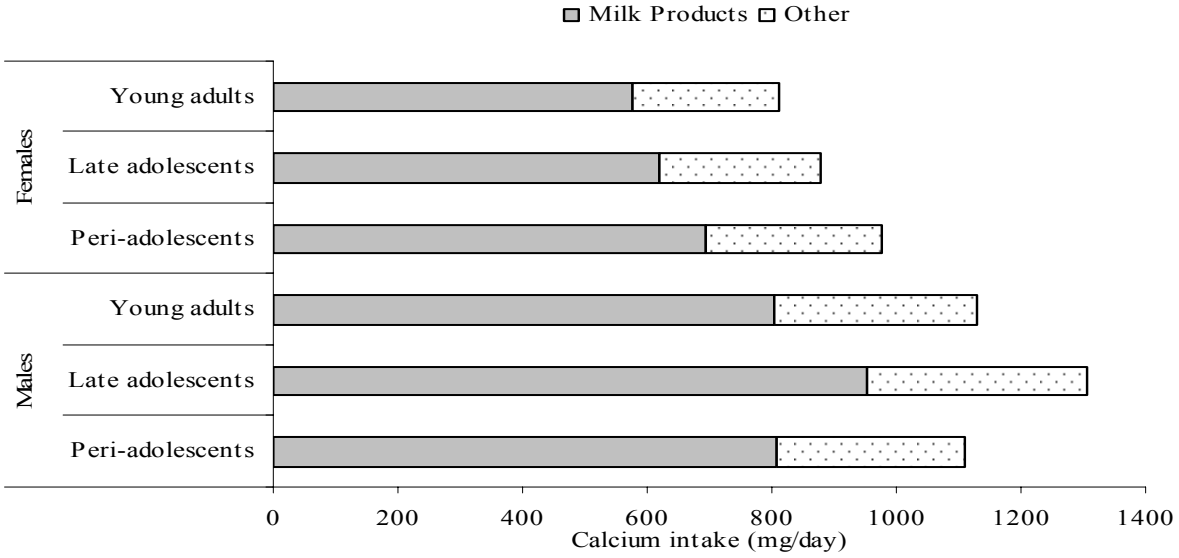

Figure 6.1 Changes in contribution of food groups in total calcium intake by gender from peri-adolescence to young adulthood
Peri-adolescents (8-14 y). Late adolescents (15-19 y). Young adults (19-27 y).

Subjects in our cohort had more intake of calcium from yogurt and less intake calcium intake from vegetables in compare to individuals in Saskatchewan Nutrition Survey. The similarities in calcium intake and food sources of calcium indicate that during last 10 years, from 1994 to 2004, there was not considerable change in dietary intake of young adults with regard to food sources of calcium. This suggests that change in food environment during the last decade have not affected dietary habits of young adults in relation with calcium intake. Hence, decrease in dietary calcium intake from childhood to early adulthood in our cohort could have been a function of age rather than time.

Low intake of milk products is suggested to be the primary reason for low calcium intake during adolescence (Brady et al., 2000). Comparing beverage intake of PBMAS grade 9 students in 1991 through 1997 with other grade 9 students in 2004, we found

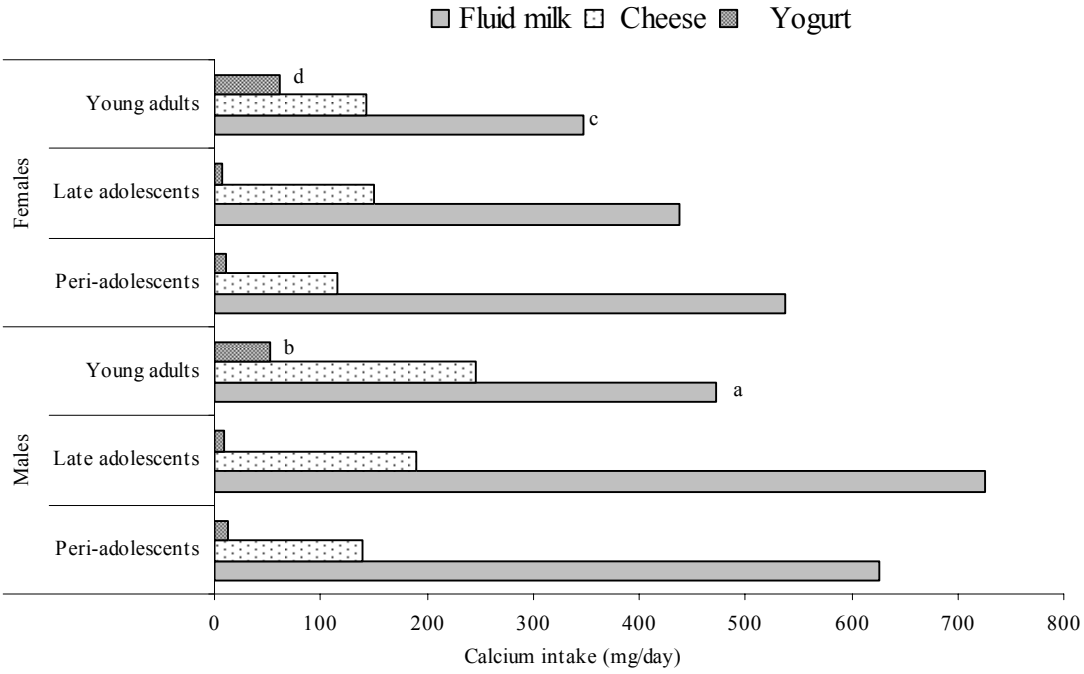

Figure 6.2 Changes in contribution of fluid milk, cheese, and yogurt in calcium intake from milk products from peri-adolescence to young adulthood in males and females

Peri-adolescents (8-14 y), late adolescents (15-19 y) and young adults (19-27 y). Significant differences between groups are presented as **a**: between late adolescent and young adult males, **b**: between peri-adolescents and young adults; late adolescents and young adults in males, **c**: between groups peri-adolescents and young adults in females, **d**: between groups peri-adolescents and young adults; late adolescents and young adults in females ($P < 0.05$ Bonferroni comparison test).

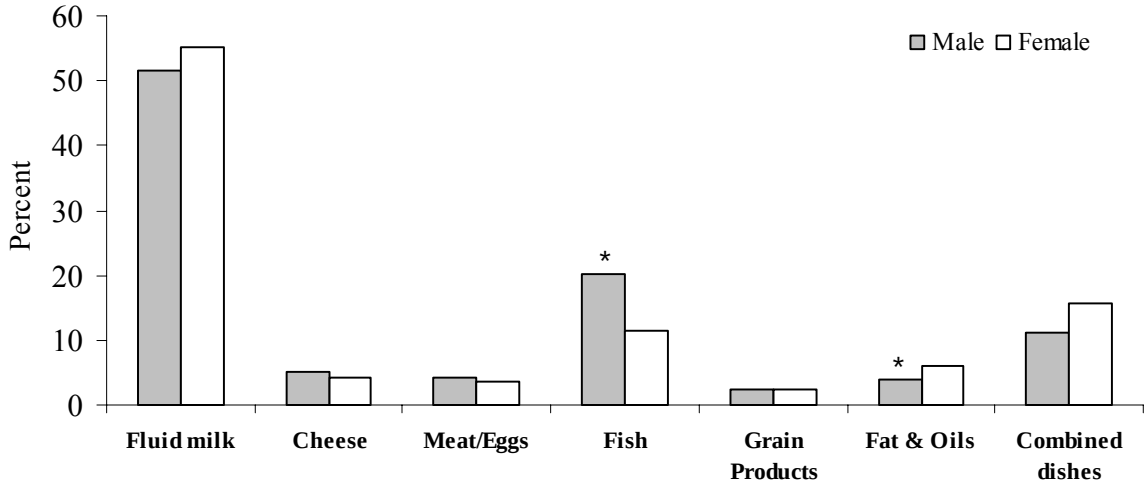

Figure 6.3 Percent contribution of foods to dietary vitamin D intake of young adults. * P < 0.05 (males vs females)

milk substitution by non-carbonated soft drinks accompanied with a negative trend in calcium intake from 1991 to 2004 in females (Vatanparast et al., 2006). These data suggest the established dietary habits during early adulthood, therefore, change in food choices and dietary habits may occur earlier, during adolescence. Total calcium intake of 237 young adult subjects (ages 19-28 years) at Bogalusa Heart Study was not significantly different with their calcium intake at age 10 y (Rajeshwari et al. 2004). In young adult cohort of Bogalusa Heart Study, although the main source of dietary calcium in was milk, the intake of fluid milk was close to one half of milk intake in our cohort (Table 6.3). Moreover, grain products and meat/eggs contributed to calcium intake more than four times and 10 times of the same foods in our cohort respectively. This indicates a major difference in dietary pattern of our subjects compared to those from Bogalusa Heart Study. It is notable that dietary intake in Bogalusa Heart Study was assessed through a single 24-h recall (Rajeshwari et al. 2004) which is not a representative of usual intake (Lee & Nieman, 2003).

More than half of female and two fifth of male young adults did not meet the AI value for vitamin D of 200 IU. The mean daily intake of vitamin D in PBMAS cohort from age 8 y to 15 y was 225 IU and 269 IU in females and males respectively (Whiting

et al. 1995). No statistical significant difference was observed in vitamin D intake from childhood to early adulthood in PBMAS subjects. During the last decade, other health benefits of vitamin D have been reported regarding prevention and control of chronic diseases (Holick, 2005), as well as high prevalence of vitamin D insufficiency (Holick, 2006). Moreover, researchers suggest that the AI values set by Institute of Medicine at 1997 are needed to be revised considering the findings of recent studies (Calvo et al., 2004; Calvo & Whiting, 2005; Whiting & Calvo, 2006; Calvo & Whiting, 2006). Therefore, there is likely a high number of our subjects, especially females, who did not have appropriate intake of vitamin D. Less is known about vitamin D intake and its dietary sources in young adults. The mean daily intake of vitamin D intake in young adults of Bogalusa Heart Study (n=504, ages 19-28 years) was 248 IU and 204 IU in males and females respectively (Zive et al. 1996). These values are slightly less than vitamin D intake of our young adult cohort (Table 6.2). Fluid milk contributed to 23% of dietary vitamin D in Bogalusa Heart Study compared to more than 50% in our cohort. Similar to food sources of calcium, grain products and meat/eggs were major contributors of dietary vitamin D after fluid milk in Bogalusa Heart Study (Figure 6.4).

A limitation of this study was the reliance on mean and standard deviations of calcium and vitamin D values in three age groups during childhood to early adulthood instead of following the intake values of each individual from childhood to early adulthood. However, it may not have affected the results as there was no new entry in our young adult cohort in this mixed longitudinal data set. The other issue is comparing data obtained from the FFQ in young adults to those from serial 24-h recalls in peri-adolescents and late adolescents. Although calcium intake values of young adults were available through serial 24-h recalls, food sources data were more easily obtained through the FFQ. Hence, because of internal consistency in method, we used all data from FFQ for young adults; we were confident this was similar to recall data as we had compared calcium intake data from FFQs to adjusted 24-h recall intakes and found no significant difference.

At the time of completing this study there was no study to investigate dietary calcium intake and its food sources from peri-adolescence to early adulthood in a prospective design in Canadians. In addition, no study reported vitamin D intake form

childhood to early adulthood and food sources of vitamin D in young adults. The mixed longitudinal design of study provided the opportunity to investigate changes by age in intake of these two important nutrients. However, it is notable that these data may not be generalizable.

This study shows that although fluid milk was the major source of dietary calcium, the contribution of milk to total dietary calcium deceased from peri-adolescence to early adulthood accompanied with increased cheese intake. In females, the early start of low milk intake in late adolescence resulted in significant decrease in dietary calcium intake during the critical years of bone mineral consolidation. In addition to this, lower than current recommendation of vitamin D intake in more than half of females may put them in a vulnerable situation regarding their peak bone mass achievement and bone health later in life. Shift from fluid milk to cheese as another dairy product may negatively affects the calcium status, because of high sodium concentration, in females with low total dietary calcium intake. In addition, substitution of milk as a beverage with non-carbonated soft drinks (Vatanparast et al., 2006) may worsen the situation. Hence, the food choices and dietary habits of young adult females have changed in a way that their food choices may not afford their needs for calcium and vitamin D. These findings argue the importance of developing appropriate nutrition policies promoting healthy eating behavior in adolescents and young adults.

Table 6.3 Contribution (%) of food groups to dietary calcium in PBMAS young adults and data from other studies

	SK Nutrition Survey[1] 1993-94 (n=549)	PBMAS Study[2] 2003-04 (n=154)	Bogalusa Study[3] 1988-91(n=504)
Age range (y)	18-34	19-27	19-28
Milk Products	63.1	68.2	-
-Fluid milk	37.8	39.6	20.5
-Cheese	19.8	19.6	14.2
-Yogurt	1.1	6.1	-
-Other	2.2	3.0	-
Grain Products	10.5	4.2	17.6
Vegetables & Fruit	8.7	6.2	10.1
- Fruit	3.7	4.6	2.2
- Vegetables	5.0	1.6	7.9
Meat & Alternatives	5.7	2.1	-
-Meat/Eggs	3.3	1.0	10.51
-Fish	1.0	0.5	3.14
-Beans, Tofu	1.4	0.6	-
Other foods	12.0	19.3	24.0

[1] Saskatchewan Nutrition Survey (2001). [2] The University of Saskatchewan Pediatric Bone Mineral Accrual Study. [3] Zive et al. 1996

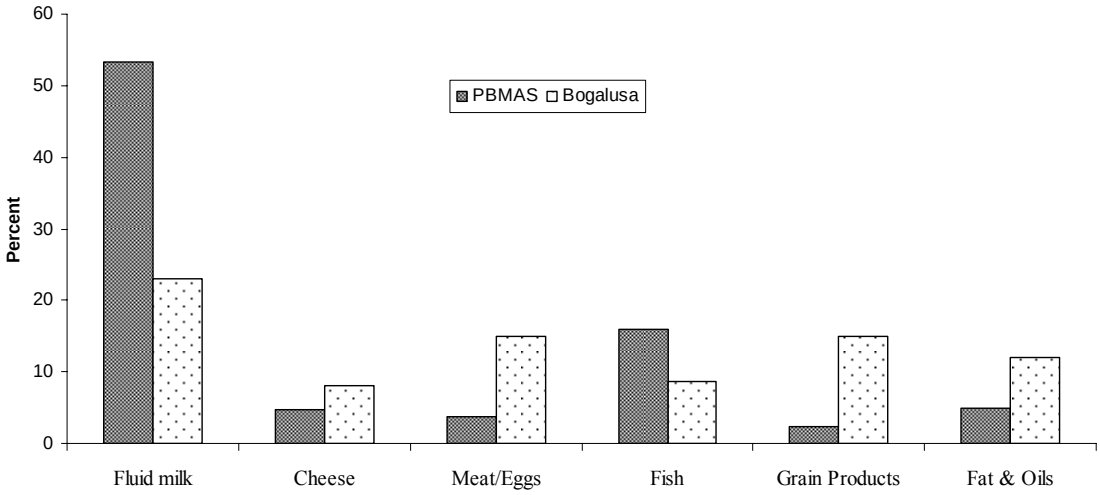

Figure 6.4 Percent contribution of food groups in dietary vitamin D intake of young adults in PBMAS and Bogalusa Heart Study (data obtained from Zive et al. 1996)

6.5 Summary

Changes in intake and food sources of calcium of males and females from childhood through adolescence to early adulthood were examined using seven-year longitudinal (1991-97) and three-year follow up data (2003-05) from the University of Saskatchewan Pediatric Bone Mineral Accrual Study (PBMAS). Subjects' dietary intakes were compared in three age groups: peri-adolescents, 8-14y (130 M, 126 F); late adolescents, 15-19y (79 M, 96 F); and young adults 19-27y (67 M, 87 F). Dietary intake from serial 24-hour recalls and a food frequency questionnaire were used in PBMAS (ages 8-19 y) and follow up study (ages 19-27y), respectively. One-way ANOVA was used to investigate differences in food sources of calcium among age groups. Mean calcium intake of young adults was 1140 mg/d and 824 mg/d in males and females respectively, with 46% of males and 79% of females not meeting the AI (1000 mg). Milk products, specifically fluid milk, were the major source of dietary calcium from childhood to early adulthood in both genders. In males, the contribution of fluid milk to total dietary calcium intake significantly decreased as age increased ($p<0.05$). In females, the contribution of fluid milk to calcium intake in late adolescent group was significantly less than in peri-adolescents ($p<0.05$), while no significant decline was observed in young adults. The contribution of cheese to calcium intake increased in both genders ($p<0.05$). In young adults, combined dishes, vegetables and fruit, grain products, and meat and alternatives were the other food sources of calcium. The intake of calcium from vegetables in young adult males and females was the lowest among the three age groups ($p<0.05$). In young adults, 51% of females and 38% of males did not meet the AI value (5 µg) for dietary vitamin D; milk was the main food source of dietary vitamin D. There were significant changes in dietary behavior of subjects from childhood to adulthood, especially in females. The substitution of fluid milk by cheese, the decrease in vegetable and fruit intake, and the low intake of vitamin D may put young adult females more at risk of osteoporosis later in life.

CHAPTER 7

THE EFFECT OF PROTEIN ON BONE MINERAL MASS OF YOUNG ADULTS, IN THEE-YEAR FOLLOW-UP OF THE UNIVERSITY OF SASKATCHEWAN PEDIATRIC BONE MINERAL ACCRUAL STUDY

7.1 Introduction

Bone mass accumulation is influenced by heredity, sex, hormones, diet and mechanical forces (Bonjour et al.,1995; Bonjour et al., 2003). Although genetics is the most prominent factor, it is well established that environmental factors such as nutrition and physical activity influence bone mass development and maintenance (Heaney et al., 2000; Rubin et al., 1999). Calcium is the main mineral comprising bone, and the skeleton serves as a large nutrient reserve for calcium and phosphorus (Tucker, 2003; Institute of Medicine, 1997). Other dietary components such as protein, essential fatty acids and most micronutrients are directly or indirectly associated with normal bone metabolism (Tucker, 2003).

Although protein is a major component of bone matrix, debate exists on the effect of dietary protein on bone mass. Some studies suggest a detrimental effect of high protein intake on bone (Anderson et al., 1995; Sellmeyer et al., 2001; Sebastian et al., 1994; Frassetto et al., 2000) or no association (Henderson et al., 1995; New et al., 1997; Nieves et al., 1995; Wang 1997; Ballard et al., 2005a). However, other studies have found beneficial effects of increasing protein intake on bone measures, in different stages of life (Calvo et al., 1998; Chiu et al., 1997; Cooper et al., 1996; Kerstetter et al., 2000; Lau et al., 1998; Hannan et al., 2000; Dawson-Hughes et al., 2002; Dawson-Hughes et al., 2004; Alexy et al., 2005; Devine et al., 2005; Theintz et al., 1992;

Chevalley et al., 2005; Michaelsson et al., 1995; Teegarden et al., 1998). An inadequate protein supply appears to play an important role in the pathogenesis of the delayed skeletal growth and reduced bone mass that is observed in under-nourished children (Rizzoli et al., 2003; Bonjour et al., 2003). Further, increasing protein intake among those who have inadequate dietary protein reduces the risk of hip fracture in men and women (Brown & Josse, 2002).

Among the studies investigating a relationship between protein intake and bone parameters, most have been conducted in pre- or post menopausal women and older men (Calvo et al., 1998; Chiu et al., 1997; Cooper et al., 1996; Kerstetter et al., 2000; Lau et al., 1998; Hannan et al., 2000; Dawson-Hughes et al., 2002; Devine et al. 2005). Only a few studies are available in children and adolescents (Alexy et al., 2005; Theintz et al., 1992; Chevalley et al., 2005). Theintz et al. (1992) observed a positive relationship between protein intake and BMC and BMD in lumbar and femoral bone sites in healthy adolescents aged 9-19 y. Chevalley et al. (2005) reported that in healthy pre-pubertal boys, the positive response to calcium supplementation can be influenced by habitual protein intake. A recent study found that dietary protein in children and adolescents aged 6-18 y, has a beneficial effect on diaphyseal bone strength during growth (Alexy et al., 2005); these authors caution, however, that the anabolic effect of dietary protein only occurs with adequate intake of alkali equivalents, such as potassium and magnesium found in fruits and vegetables.

Although studies show additional dietary protein improves bone formation and homeostasis during growth and in old age (Rizzoli et al., 2003; Massey & Whiting, 2003), less is known about the effect of protein on bone parameters in young adults in whom peak bone mass been attained. Calcium, protein, phosphorus, and the calcium-protein or calcium-phosphorus ratios together had significant effects on the spine and total body (TB) BMD and TBBMC in females aged 18-31 y measured cross-sectionally (Teegarden et al., 1998). In a longitudinal prospective study among young adult females, Recker et al. (1992) reported that the rate in bone gain in spine BMD had a positive correlation with calcium-protein ratio. Recently, Ballard et al. (2005a) reported no effect of protein supplementation on areal and volumetric BMD during a 6- month exercise program in females aged 18-25 y, however, protein increased bone formation

biomarkers (Ballard et al. 2005a; Ballard et al. 2005b). Further, longitudinal studies are needed to evaluate the effect of dietary protein on bone parameters in healthy young adults.

In a young adult cohort that was the follow up to the University of Saskatchewan Pediatric Bone Mineral Accrual Study (PBMAS), measures of diet, physical activity and body and bone parameters have been recorded from childhood. This allows for evaluation of the cumulative exposure to foods and nutrients of interest (Willet, 2002). Accordingly, the primary purpose of this study was to examine the association of selected dietary factors, specifically protein, calcium and fruit and vegetable on bone mineral mass parameters of young adult males and females who have reached peak bone mass. Further, as calcium intake is known to modulate the protein response (Massey & Whiting, 2003), subgroups of the cohort based on calcium intake, were analyzed separately.

7.2 Subjects and Methods

7.2.1 Study participants and design

Subjects were participants in PBMAS and its three-year follow-up. PBMAS used a mixed-longitudinal study design incorporating eight age cohorts (age 8 to 15 years at study entry). The initial phase of the study ran from 1991-1997, and during this time span the clustering of the age cohorts remained the same; due to overlapping of age groups, a developmental age range of 8 to 21 years of age was assessed. Over 251 subjects enrolled in the original study. The majority of subjects were Caucasian, selected as a population–based sample of children in Saskatoon (Bailey et al., 1997). From 2003 to 2005, a follow up study was conducted on the original PBMAS subjects who were then 17-28 years of age. Subjects provided written consent to participate in the original and follow up study. Ethical approval was obtained from the University of Saskatchewan and Royal University Hospital Advisory Committee on Ethics in Human Experimentation for the original study and its follow-up.

In the follow-up study protocol, data collection included anthropometric and physical activity data every year, several 24-hour dietary assessments, and annual DXA scans.

7.2.2 Dietary analysis

Details of dietary assessment in original PBMAS study have been described elsewhere (Whiting et al., 1995; Vatanparast et al., 2005). In the follow-up study, dietary intake was assessed via three to five 24-hour recalls (in-person or by telephone) over the three year period. Dietary data were analyzed using Food Processor (version 8.0 and its revisions, ESHA Research Inc., Salem OR) that contained foods from the 2000 Canadian Nutrient File. Recalls were considered valid when energy intake was more than the basal metabolic rate and less than 4000 kcal/day. Intakes from at least three valid recalls were averaged to determine usual intake of subjects. Nutrient data includes supplement use. Food sources of calcium were determined using a food frequency questionnaire containing 37 common food sources of calcium (Shrestha et al., 1998).

7.2.3 Bone measurements

Bone measurements were obtained by annual DXA (Hologic QDR 2000; Hologic, Waltham, MA) scans of the whole body, posterior -anterior lumbar spine and proximal femur in the original and follow up studies. Array mode was used for bone mineral acquisition and enhanced global software (version 7.10) was used for analysis. To minimize operator-related variability, the same person analyzed all total body scans using software version 5.67 A. Short-term precision in vivo for total body BMC, expressed, as coefficient of variation (%), was 0.60 (Baxter-Jones et al., 2003).
The same procedures were used in the follow up as in the original study (Baxter-Jones et al., 2005). For each subject TBBMC and TBBMD data during the three year follow-up were averaged to represent bone measures of young adults. The averaged TBBMC values of young adults were used in this study.

7.2.4 Anthropometric, physical activity and maturity assessments

Height and weight were measured every six months in the original PBMAS study (Baxter-Jones et al., 2003). The age at PHV was considered as a biological maturity age, and details regarding our derivation of PHV have been described elsewhere (Vatanparast et al., 2005). In the follow-up study, height and weight were measured every year by trained study personnel using the same scale and stadiometer. Subjects wore t-shirts and shorts during measurement, with shoes and jewelry removed. Height and weight were

measured twice and recorded to the nearest 0.1 centimeter and 0.1 kilogram, respectively. The averages of three measurements of height and weight during early adulthood were used in this study.

A measure of physical activity of subjects in the original PBMAS study was assessed using the Physical Activity Questionnaire for Older Children (PAQ-C) and PAQ for Adolescents (Baxter-Jones et al., 2003). In the follow-up study, subjects used an adult version called PAQ-AD to rate their physical activity level during their spare time and due to strenuous occupational activity during the previous seven days, resulting in a rating from one to five, where a higher score reflected a higher level of physical activity (Baxter-Jones et al., 2005). The averaged physical activity data during the three years of follow-up study was defined as the physical activity pattern of our young adult cohort.

Two time points were of interest, that around the age of PHV and that in early adulthood which was close to time of peak bone mass achievement. To characterize dietary and physical activity patterns of peri-adolescent subjects, the dietary and physical activity data from four years surrounding age of PHV were averaged. To calculate net gain in TBBMC, height and weight from age of PHV to early adulthood, bone and body measures at age of PHV (-0.5 to +0.5) were subtracted from their equivalents at early adulthood. The closest bone and body measures to the age of PHV were used in subjects with no data within one year of age of PHV. The sample comprised 133 subjects (59 males and 74 females).

7.2.5 Statistical analysis

Values are reported as mean ± standard deviation (SD). Data analyses were conducted using Microsoft EXCEL (2000) and Statistical Package for the Social Sciences (SPSS, version 13). A two-sided paired Student's t-test was used to compare the variables of interest in the two time points, peri-adolescence and young adulthood. Comparison of bone measures between oral contraceptive users and non users was conducted using independent two-sided Student's t-test. Further, the differences of bone measures, food and nutrient intakes and physical activity patterns between those with a consistent low calcium intake at two time points (age of PHV and young adulthood) and all other subjects were investigated independent two-sided Student's t-test . Pearson's

correlation was used to examine relationships between variables of interest. Alpha was set to a value of 0.05.

Repeated measures ANOVA was used to investigated the effect of selected factors on bone measures at two time points, age of PHV and early adulthood (within subject variables) considering other covariates. Only height, weight and sex were significant covariates in relation to TBBMC ($p<0.05$). In this method, factors had to be categorical variables to enter into the model, which limited the ability to include continuous nutrient intake measures as factors (Munro & Batten, 1993).

A multiple regression model (Formula 1) was used to investigate the effect of intake of nutrients of interest, calcium and protein on bone mass measures in the presence of other potential factors:

$$y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \ldots + \beta_i X_i + e_i \qquad \textit{Formula 1}$$

where y is TBBMC or TBBMD at early adulthood, or TBBMC net gain from peri-adolescence to early adulthood, β_0 is the coefficient for the intercept, β_1 is the coefficient for the variable X_1 and e_i is the residual. Forward stepwise procedures were used in adding the potential covariates into the model including sex, current height and weight, physical activity level, calcium intake, protein intake, vegetables and fruit intake, vitamin D and potassium; and peri-adolescent intakes of vegetables and fruit, calcium and protein, and physical activity. When TBBMC net gain was considered as the outcome variable, current height and weight were replaced by their respective net gain from age at PHV to early adulthood.

Using PBMAS longitudinal data, Whiting et al. (2004) estimated calcium requirements of ~1000 mg and 1200 mg/d for girls and boys during the whole age range of adolescence (9-18 years). In addition, the AI value of calcium for young adult males and females is 1000 mg/d. Therefore, the calcium intake of 1000 mg/d might be an appropriate cutoff value. Hence, subjects were classified into two groups: those with adequate calcium intake (≥ 1000 mg/d) and individuals with low calcium intake (<1000 mg/d). Table 7.1 represents the distribution of PBMAS subjects according to their calcium intake in peri-adolescence and early adulthood. The intake of calcium was defined "consistent" when it was in the same category at peri-adolescence as at early adulthood (group A) (Table 7.1). Group A consists of subjects who had consistent

calcium intake either low (LL) or adequate (AA) at both time points. Group B includes subjects with inconsistent calcium intake at both time points, labeled LA and AL (Table 7.1). In the next step, subgroup analyses, first Group A vs. Group B, then within group A, LL vs. AA, were conducted to investigate any possible differences in the relationship between dietary factors in those groups and bone measures, considering other possible covariates. Finally, differences in predictors of TBBMC net gain were determined in females alone as there was an appropriate number of female subjects who had low (LL) vs. adequate (AA, AL, LA) calcium intake (AA, AL, LA) (Table 7.1). In all analyses, the significance level for entry and removal from the model were 0.05 and 0.10, respectively.

Table 7.1 Distribution of PBMAS subjects according to their calcium intake[1] at peri-adolescence and early adulthood

Group	Calcium intake at peri-adolescence and early adulthood	N (%)		TBBMC (g)		Protein intake (g)	
		M	F	M	F	M	F
A	Low-Low (LL)	5 (9.6)	30 (46.1)	3123	2135	83	63
	Adequate-Adequate (AA)	26 (50)	12 (18.5)	2919	2358	137	82
B	Low-Adequate (LA)	14 (26.9)	12 (18.5)	3033	2236	116	77
	Adequate-Low (AL)	7 (13.5)	11 (16.9)	3260	2191	106	66

1. Low represents calcium intake <1000 mg/d, Adequate represents calcium intake≥1000 mg/d

TBBMC=Total body bone mineral content. TBBMC net gain was calculated by subtracting mean TBBMC at PHV from the mean TBBMC in early adulthood.

7.3 Results

Table 7.2 provides descriptive data for all subjects. Males had significantly higher bone and body measures than females ($p<0.05$) and their intakes of protein, calcium, potassium and vitamin D and milk product were higher than females ($p<0.05$). In males, the mean intake of calcium was similar at peri-adolescence and early adulthood, whereas

in females, a significant decrease was observed in mean calcium intake (15%, $p<0.05$). The intake of vegetables and fruit in peri-adolescence was not different in males and females. The level of physical activity declined significantly by 21% ($p<0.001$) from peri-adolescence to early adulthood, and no gender difference was observed in current physical activity level. In both genders, changes in bone and body measures from age of PHV to early adulthood showed significant increases for TBBMC, height and weight ($p<0.05$). The percent increase in TBBMC was 41% for males and 37% for females from peri-adolescence to early adulthood. In young adults, a strong relationship between current calcium and protein intakes ($r= 0.71$ in males and $r= 0.43$ in females, $p<0.001$) was observed, which is reasonable as milk products were the main source of dietary calcium for males and females (68% and 69%, respectively). No statistically significant difference in bone and body measures was observed among oral contraceptive user and non-user females.

In multiple regression analysis, when all subjects were included (Table 7.3), height, weight and sex were significant predictors of all three bone measures (TBBMC, TBBMD and TBBMC net gain). While peri-adolescence physical activity significantly predicted TBBMC and TBBMC net gain, current physical activity was a significant predictor of only TBBMD. Protein intake at early adulthood was a significant predictor of TBBMC net gain.

Fifty-five percent of subjects (53% M, 57% F) had a consistent pattern of calcium intake from peri-adolescence to early adulthood. A consistent pattern of calcium intake represents an established dietary habit regarding food sources of calcium and allows us to evaluate the cumulative effect of calcium intake on bone measures. In analysis among these subjects (Group A), height, weight, and protein intake were significant predictors of TBBMC, while only height and protein intake were significant predictors of TBBMD (Table 7.4). The significant predictors of TBBMC net gain were height and weight net gains, sex, physical activity and protein intake (Table 7.4). When calcium-to-protein ratio was added into the models, no significant association was observed with the bone measures in either all-subject or subgroup analysis. No association between protein intake and bone measures were observed in Group B

Table 7.2 Characteristics and measurements of the participants at early adulthood

	Males (n=59)	Females (n=74)
Age (y)	23.5 (2.2)	22.6 (2.2)
Height (cm)	179.8 (7.4)*	166.6 (6.6)
Weight (kg)	83.4 (13.5)*	70.2 (17.2)
BMI	25.8 (3.9)	25.3 (5.8)
TBBMC (g)	3009 (433)*	2220 (357)
TBBMC (g) at age of PHV	1770 (386)*	1404 (326)
TBBMC net gain (g)	1239 (350)*	816 (261)
TBBMD (g/cm^2)	1.18 (0.09)*	1.04 (0.08)
Physical activity score	2.4 (0.5)	2.3 (0.5)
Calcium intake (mg/d)	1367 (674)*	900 (4.6)
Calcium intake (mg/d) at peri-adolescence	1217 (432)*	1052 (391)
Protein intake (g/d)	119 (53)*	68 (22)
Calcium-protein ratio	11.7 (4.1)	13.4 (5.1)
Vitamin D intake (IU/d)	387 (368)*	206 (192)
Potassium intake (mg)	3298 (1198)*	2234 (713)
Previous vegetable & fruit intake (servings/d) at peri-adolescence	4.0 (1.7)	4.1 (2.1)
Vegetable & fruit intake (servings/d)	5.1(2.7)	3.9 (1.9)
Previous Milk products intake (servings/d) at peri-adolescence	3.1(1.4)	2.6(1.1)
Milk products intake (servings/d)	2.7(1.9)*	2.0(1.3)

* Significant difference between genders by two-tailed independent t-test ($p<0.05$)

TBBMC: Total body bone mineral content. TBBMC net gain: calculated by subtracting mean TBBMC at the age of PHV from the mean TBBMC in early adulthood. TBBMD: Total body bone mineral density. Physical activity score: rating from one to five, where a higher score reflected a higher level of physical activity

Table 7.3 Factors associated with bone mineral measures in regression analysis among all subjects (n=133)

Outcome variable	Constant	Regression coefficient					Total R^2
		Sex	Height	Weight	Protein intake	Physical activity	
TBBMC	-2354± 576	-0.275±0.054	0.362±0.057	0.414±0.045	NS	0.143±0.040*	0.808
Partial R^2/p value		-0.41 p<0.001	0.49 p<0.001	0.63 p<0.001	-	0.25 p<0.001	
TBBMC net gain**	60±99	-0.227±0.046	0.506±0.041	0.345±0.042	0.148±0.046	0.107±0.038*	0.821
Partial R^2/p value		-0.39 p<0.001	0.73 p<0.001	0.58 p<0.001	0.27 p=0.006	0.24 p=0.006	
TBBMD	0.608±171	-0.390±0.19	0.178±0.089	0.261±0.0	NS	0.161±0.97	0.538
Partial R^2/p value		-0.27 p<0.001	0.17 P=0.049	0.22 p<0.001	-	0.16 p=0.01	

TBBMC=Total body bone mineral content, TBBMD= Total body bone mineral density, * the net gain of TBBMC, and the net gains in height and weight from age of peak height velocity to early adulthood entered to the model. Peri-adolescence was considered as two years before the age of peak height velocity to two years after it. Early adulthood considered from age 20-28 y. Variables in the model were sex, current height, weight, physical activity level, protein intake, vitamin D, vegetables and fruit intake, and potassium; and peri-adolescence intakes of vegetables and fruit, and protein, and physical activity. For calcium intake, subjects categorized as those who had ≥ 1000 mg /d or < 1000 mg/d calcium intake from peri-adolescence to early adulthood. NS= Not significant

Table 7.4 Factors associated with bone mineral measures in regression analysis of subjects with consistent calcium intake from peri-adolescence to early adulthood (Group A, n=73)

Outcome variable	Constant	Regression coefficient					Total R^2
		Sex	Height	Weight	Protein intake	Physical activity	
TBBMC	-3359 ± 551	NS	0.532 ± 0.068	0.354 ± 0.067	0.183 ± 0.064	NS	0.793
Partial R^2/p value	-	-	0.69 p<0.001	0.54 p<0.001	0.32 p<0.001	-	
TBBMC gain*	49 ± 96	-0.175 ± 0.063	0.531 ± 0.056	0.320 ± 0.058	0.239 ± 0.068	0.115 ± 0.05	0.847
Partial R^2/p value	-	-0.32 p=0.008	0.76 p<0.001	0.56 p<0.001	0.40 p=0.001	0.26 p=0.034	
TBBMD	0.207 ± 0.164	NS	0.484 ± 0.097	NS	0.306 ± 0.000	NS	0.470
Partial R^2/p value	-	-	0.51 p<0.001	-	0.35 p=0.003	-	

TBBMC=Total body bone mineral content, TBBMD= Total body bone mineral density, * the net gain of TBBMC, and the net gains in height and weight from age of peak height velocity to early adulthood entered to the model. Peri-adolescence was considered as two years before the age of peak height velocity to two years after it. Early adulthood considered from age 20-28 y. Variables in the model were sex, current height, weight, physical activity level, protein intake, vitamin D, vegetables and fruit intake, and potassium; and peri-adolescence intakes of vegetables and fruit, and protein, and physical activity. For calcium intake, subjects categorized as those who had ≥ 1000 mg /d or < 1000 mg/d calcium intake from peri-adolescence to early adulthood. NS= not significant.

Table 7.5 Factors associated with bone mineral measures in regression analysis of females with adequate calcium intake at peri-adolescence and/or early adulthood

Outcome variable	Constant	Regression Coefficient			Total R^2
		Height	Weight	Protein intake	
TBBMC	-2954± 840	0.47± 0.096	0.46± 0.097	0.21± 0.095	0.606
Partial R^2/p value	-	0.61	0.60	0.33	
		p<0.001	p<0.001	p=0.02	
TBBMC net gain*	73± 91	0.72± 0.081	0.26± 0.082	0.17± 0.33	0.781
Partial R^2/p value	-				
		p<0.001	p=0.003	p=0.033	
TBBMD	0.94± 0.04	0.29± 0.19	NS	0.32± 0.32	0.222
Partial R^2/p value	-	0.31	-	0.34	
		p=0.046		p=0.027	

TBBMC=Total body bone mineral content, TBBMD= Total body bone mineral density, * the net gain of TBBMC, and the net gains in height and weight from age of peak height velocity to early adulthood entered to the model. Peri-adolescence was considered as two years before the age of peak height velocity to two years after it. Early adulthood considered from age 20-28 y. Variables in the model were, current height, weight, physical activity level, protein intake, vitamin D, vegetables and fruit intake, and potassium; and peri-adolescence intakes of vegetables and fruit, and protein, and physical activity. For calcium intake, subjects categorized as those who had ≥ 1000 mg /d or < 1000 mg/d calcium intake from peri-adolescence to early adulthood. NS= not significant.

Table 7.6 Summary of the results of regression analysis of factors associated with bone mineral status at young adulthood

	Factors					Outcome
	Ht	Wt	Sex	Protein	Physical Activity	
All Subjects (n=133)	*	*	*	NS	NS	TBBMC
	*	*	*	*	*	TBBMC net gain
	*	*	*	NS	*	TBBMD
Consistent calcium intake (LL, AA) (n=73)	*	*	NS	*	NS	TBBMC
	*	*	*	*	*	TBBMC net gain
	*	NS	NS	*	NS	TBBMD
Females with adequate calcium intake (LA, AL, AA) (n=35)	*	*	-	*	NS	TBBMC
	*	*	-	*	NS	TBBMC net gain
	*	NS	-	*	NS	TBBMD

* Significant ($p<0.05$). NS=not significant. TBBMC= Total body bone mineral content. TBBMD= Total body bone mineral density, * TBBMC net gain= the net gains in TBBMC from age of peak height velocity to early adulthood. L=low (calcium intake < 1000 mg/d), A= adequate (calcium intake ≥ 1000 mg/d). Any combinations of L and/or A represent calcium intake at two time points, peri-adolescence and early adulthood.

comprising all subjects with inconsistent calcium intake between peri-adolescence and early adulthood.

In Group A (those with consistent calcium intake), protein was a significant predictor of TBBMC net gain only in subgroup AA (consistent adequate calcium intake). There was a very low number of male subjects in LL group and low number of female subjects in AA group (Table 7.1). While 30 females had consistent low calcium intake in peri-adolescence and early adulthood, only five males were in that situation. As a more appropriate analysis, females with consistent low calcium intake (LL) were compared to all other female subjects who had adequate calcium intake either or both the peri-adolescence and young adulthood (AA, LA and AA). Current and peri-adolescence mean calcium intake in LL group were 659 mg/d and 766 mg/d respectively. In group with adequate calcium intake (AA, LA and AA) the mean intakes of calcium were 1064 mg/d and 1247 mg/d in early adulthood and peri-adolescence respectively. Milk product and vegetables and fruit consumption at peri- adolescence and early adulthood and current protein intake were significantly lower ($p<0.5$) in LL females compared to all other females; however, there was no significant difference in bone measures between these two groups. In the multiple regression model, height and weight net gains were significant predictors of TBBMC net gain for all females, whereas protein intake was significant predictor of TBBMC,TBBMC net gain and TBBMD only in females who had adequate calcium intake either or both the peri-adolescence and young adulthood (Table 7.5). No association was observed between protein intake and TBBMC net gain among females with consistent low calcium intake in this analysis. Table 7.6 presents a Summary of the results of regression models in three levels, all subjects, subjects with consistent calcium intake and females categorized based on their calcium intake.

7.4 Discussion

In this longitudinal data set, among nutrients and food groups that were hypothetically bone protective, only protein was a significant predictor of net gain in TBBMC from the age of PHV to early adulthood, the most likely time of peak bone mass achievement (Lin et al., 2003). TBBMC provides more accurate measurement of

bone mineral mass than TBBMD during the time of bone mass development (Institute of Medicine, 1997; Wren et al. 2005a). Further, the net gain of TBBMC from age of PHV to early adulthood excludes the amount of bone mineral mass accumulated before the age of PHV, hence provides an appropriate and specific outcome variable in our analysis. Calcium intake seemed to influence the extent of the association of protein intake and bone measures, as this was demonstrated more clearly when subjects were classified according to their calcium intake.

The anabolic effect of protein on bone is due to provision of amino acids for bone matrix formation as well as production of insulin-like growth hormone I (IGF-I) which is responsible for bone mass development (Bonjour et al., 2003). These two mechanisms may explain the positive association between bone measures and protein intake in men and women (Calvo et al., 1998; Chiu et al., 1997; Cooper et al., 1996; Kerstetter et al., 2000; Lau et al., 1998; Hannan et al., 2000; Dawson-Hughes et al., 2002; Dawson-Hughes et al., 2004; Alexy et al., 2005; Devine et al., 2005; Theintz et al., 1992; Chevalley et al., 2005; Michaelsson et al., 1995; Teegarden et al., 1998 ; Ballard et al., 2003 ; Bonjour 2005). Other studies state that a high protein diet induces metabolic acidosis followed by bone mineral dissolution, and urinary calcium excretion (Sebastian et al., 1994; Frassetto et al., 2000; New at al., 2002); however, if protein intake is accompanied by a low rather than high potential renal acid load (PRAL), the effect on bone is anabolic rather than catabolic (Alexy et al., 2005; Chevalley et al., 2005; Massey & Whiting 2003; Sebastian et al., 2001; Sebastian 2005).

Milk products are the main source of dietary calcium, and we previously showed that the intake of milk product in our cohort is higher than in subjects of similar age in other studies in North America (Vatanparast et al., 2005; Iuliano-Burns et al., 1999). Further, milk products were the second main food source of protein after meat and accounted for 15% of protein in the Canadian diet in a study by Johnson-Down et al. (2006). In addition to containing calcium, milk contains potassium and phosphorus levels that results in a nearly neutral PRAL compared to a highly positive (thus acidogenic) value for meat (0.3 mEq vs 26 mEq) (Massey & Whiting 2003). Hence, the alignment of subjects based on consistent daily calcium intake at peri-adolescence and early adulthood mainly from milk products allowed us to investigate

the cumulative effect of calcium intake on bone measures as well as the effect of other possible factors on bone measures when calcium intake is adequate. Furthermore, a consistent pattern of calcium intake at peri-adolescence and early adulthood might represent a reliable and established dietary behavior as in addition to calcium, a significant correlation in intakes of calcium, protein, vegetables and fruit, and milk products between two time points, peri- adolescence and early adulthood was observed. In subgroup analysis, protein intake was the only consistent significant predictor of bone measures among all nutrients of interest after height and weight.

Only 3% of male subjects had lower than age- and weight-matched recommended dietary allowance (RDA) for protein intake (0.8 g/kg) providing a homogenous sample regarding protein intake, while 24% of females did not meet the RDA for protein. Considering that athletes require more protein (Thompson, Manore & Sheeshka, 2005), it might be reasonable to assume that a protein intake of 1 g/kg/day would provide a more appropriate estimation of protein requirement of young adults, considering their physical activity pattern. Using this higher cutoff, 55 % of females did not meet a higher recommendation for protein intake. Hence, the effect of protein was more demonstrable in females. In addition, approximately half of females had consistent low calcium intake at peri-adolescence and/or early adulthood. Therefore, this provided a unique opportunity to examine the effect of protein on bone measure of young adult females in relation to calcium intake. The finding that among subjects with consistent low calcium intake at peri-adolescence and early adulthood, there was no association between protein intake and TBBMC net gain provides support for the hypothesis that only in the situation of adequate calcium intake, protein intake has a positive effect on bone mineral mass (Massey & Whiting 2003; Dawson-Hughes 2002).

Although a high calcium-to- protein ratio has been reported as a significant predictor of bone mass (Teegarden et al., 1998), it did not explain the changes in bone measures of our cohort. From peri-adolescence to early adulthood, the intake of protein significantly increased in both males and females ($p<0.05$), while the intake of calcium decreased in females or was unchanged (Table 7.2) in males providing a descending trend in calcium-to- protein ratio. Therefore calcium-to- protein ratio was not a

significant predictor on bone measures in our cohort. Instead, our data suggest an adequate intake of calcium is more important than a relative intake.

Vegetables and fruit provide organic salts of potassium and magnesium. Higher intakes of this food group produce more net base which is reflected as a negative PRAL (New et al., 2002; New 2003). In addition, vitamin K, an essential co-factor for osteoblastic activity, is found in vegetables (Feskanich et al., 1999). Natural antioxidants and phytoestrogen compounds in many vegetables may also have bone-protective effects (Wangen et al., 2000). We previously demonstrated vegetable and fruit intake as a significant predictor of TBBMC in males aged 8-20 (Vatanparast et al., 2005), however, this effect was not maintained to early adulthood in this analysis.

We previously reported a positive relationship between physical activity and bone mineral mass in males analyzing data from original PBMAS (Baxter-Jones et al., 2003; Vatanparast et al., 2005). Data from all subjects reveal that current physical activity was a significant predictor of TBBMD, whereas physical activity at peri-adolescence was a significant predictor of TBBMC and TBBMC net gain (Table 7.4). This suggests that physical activity during peak bone mineral content velocity (peri-adolescence) is important and its effect can be expressed on adult TBBMC. In subgroup analysis, physical activity was a significant predictor of TBBMC net gain (Table 7.4). In our cohort, data reveal a positive association between physical activity and bone mineral mass from childhood to early adulthood. A recent study examined the effect of protein supplementation and physical activity on bone parameters of 52 young adults aged 18-25 y during a trial of 6 months. Although no effect was found on volumetric bone parameters (Ballard et al. 2005a), protein supplementation during a strength and conditioning program significantly increased IGF-I concentrations (Ballard et al. 2005b). Those results suggest that a six-month period is too short to observe the effect of physical activity and protein intake on bone mass measures, while it can be assessable in bone biomarkers. In our cohort of subjects with habitually adequate intakes of protein, even though physical activity level decreased from peri-adolescence, its effect on TBBMC net gain was maintained to early adulthood.

Studies have shown that contraceptives such as Depo-Provera and ultra-low dose oral contraceptives (20 μg ethinyl estradiol) may interfere with achieving optimal peak

bone mass in young women (Cromer, 2003). Teegarden et al. (2005) reported that adequate dietary calcium intake protects oral contraceptive users from spine and hip bone loss. In our cohort, females who used oral contraceptives had no detectable differences in bone and body measures compared to non-users.

There are limitations in our study. The sample size was small. Dietary intakes were self reported, which can lead to underestimation due to underreporting. We have previously shown, using data from the first year of our study, that underreporting was greater in girls than in boys and that older girls underreported more than younger girls did (Carter et al., 2000). It has been reported that as adolescent females age, they tend to report energy intake less accurately (Bandini et al., 2003), and this phenomenon may partially explain the difference in intakes of calcium and protein among males and females. Physical activity was also self-reported, and was determined as frequency of various activities. One strong aspect of our study is its longitudinal nature. Data from the same subjects were analyzed at two important time points, age of PHV and early adulthood as the time of peak bone mass achievement.

Our data suggest that dietary protein intake should be considered among other bone beneficial nutrients in peri-adolescence and early adulthood, as these are two important time points regarding bone mineral mass development. Peak bone mineral content velocity occurs around the first time point (Martinet al. 1997) and peak bone mass is achieved in early adulthood (Lin et al. 2003). In our cohort of healthy young adults longitudinal data from childhood indicates that their habitual appropriate calcium intake mainly from milk products, along with biological factors height and weight, current protein intake and physical activity during peri-adolescence were significant predictors of TBBMC net gain from age of PHV to young adulthood. Among young adult females who had long term low calcium intake at peri-adolescence and early adulthood, no effect of protein on TBBMC net gain was observed. The results of this study suggest that to benefit the anabolic effect of protein during the critical years of bone mineral mass development, appropriate intake of calcium is necessary. This adds to the existing data presented a positive effect of protein on bone in children, postmenopausal women and elderly people.

7.5 Summary

Debate exists on the effect of protein intake on bone mass measures in different life stages. Although traditionally considered a risk factor for bone, recent studies in children and elderly people report a positive association of dietary protein with bone measures. The purpose of the study was to investigate the influence of protein and calcium intake, current and during peri-adolescence, on total body bone mineral content (TBBMC) and density (TBBMD) in young adults. Longitudinal data from 133 young adults, mean age of 23 y (59 M, 74 F), enrolled in the University of Saskatchewan Pediatric Bone Mineral Accrual Study (BMAS, 1991-1997) and its 3-year follow up study (2002-2004) were used. Dietary intake was assessed via yearly serial 24-hour recalls. Anthropometrics and physical activity were assessed every six months. TBBMC and TBBMD were assessed annually using DXA. Peri-adolescence was defined as 2 years before the age of peak height velocity to 2 years after it. TBBMC net gain from age of peak height velocity (PHV) to early adulthood was defined as difference between current and PHV mean TBBMC. Multiple regression was used to analyze data from all subjects as well as subsets of subjects who had consistent intake of calcium (low vs. high) at peri-adolescence and young adulthood (31 M, 42 F). In both genders, there was a significant increase in TBBMC, from age at PHV to early adulthood (41% and 37% in M and F respectively). In males, the intake of calcium at peri-adolescence and early adulthood was similar, whereas in females, there was a significant decrease (15%, $p<0.05$).No significant association was observed between calcium-to-protein ratio and the bone measures in all subjects and in subgroups analyses. Bone and body measures were not statistically different among oral contraceptive users and non-users. Height, weight, physical activity and gender were the significant predictors of TBBMC, TBBMC net gain and TBBMD among all subjects and subgroups with consistent calcium intake from peri-adolescence ($p< 0.05$). Protein intake was a significant predictor of TBBMC net gain in all subjects. In a subgroup with consistent calcium intake at peri-adolescence and early adulthood, protein intake significantly predicted TBBMC, TBMC net gain and TBBMD ($p< 0.05$). Females with consistent low calcium intake did not benefit from the positive effect of protein on bone mass. In the situation of adequate intake of calcium primarily from dairy products, protein intake has a beneficial effect on bone mass of young adults.

CHAPTER 8

GENERAL DISCUSSION

8.1 Scientific contribution of studies

The designs of four studies (Chapters 4-7) included in this thesis allowed for appropriately addressing the two objectives. This body of research individually and in combination has significantly added to the previous knowledge of the impact of diet on bone health. The individual and overall scientific contributions of this research are discussed below.

Study one (Chapter 4), was designed to examine the hypothesis that milk products and vegetables and fruit intake have beneficial effects on TBBMC around the age of peak PHV. This was the first study that presented vegetables and fruit intake as a predictor of TBBMC in boys aged 8 to 21 years. The longitudinal setting of our data and the specific statistical model designed to analyze data from growing children made our study unique. Most studies on the effect of diet as food groups or nutrients on bone measures had cross-sectional designs and had been conducted in adult females. The results of this study filled an information gap in males and added to the existing data on the positive effect of vegetables and fruit intake in children and adults (Tylavsky et al., 2004; New, 2003). Further, a positive effect of calcium and physical activity on TBBMC in boys found in our study supported data from cross-sectional studies.

The original PBMAS study and its follow-up provided the opportunity to examine the effect of diet, physical activity and body measures on bone mineral mass at two critical time points, adolescence, the time of peak bone mineral content velocity, and young adulthood, when peak bone mass is achieved. Study four presents this part of my research (Chapter 7). No effect of previous and current calcium intake was observed

on bone measures. This can be partially explained by the threshold behavior of calcium. Below the threshold, calcium retention increases by increasing calcium intake. Recent data suggest that calcium intake should be in the range of 1000 mg/d up to age 50 years (NIH News, 2006). To be able to demonstrate the effect of calcium, the calcium intake of subjects should be distributed appropriately between low and adequate intakes. Our cohort was a relatively homogenous sample, as calcium intakes of most subjects was around 1000 mg. However, results of Study four (Chapter 7) is the first to show that protein intake is a predictor of bone mineral measures among young adults. As females with low calcium intake at peri-adolescence and early adulthood did not show the benefit of the positive effect of protein, it is likely the protein effect is seen only with adequate calcium intake. Although protein intake was traditionally believed to cause bone resorption by accelerating calcium excretion, there is emerging evidence that it has a positive impact on bone modeling and maintenance (Bonjour, 2005). When calcium intake is adequate, it can compensate for the probable calcium excretion induced by protein intake as well as providing mineral for the anabolic effect of protein on bone.

While the above studies investigated the effect of nutrition on bone measures, Study two and Study three were designed to evaluate the dietary patterns of PBMAS in relation to time and age. Study two was the first study investigating the trend in beverage consumption in relation to calcium intake in Canadian adolescents. The negative trend in calcium intake found in our study in combination with milk substitution with non-carbonated soft drinks is alarming. Adolescence is the optimal time for peak bone mineral accrual requiring a healthy dietary behavior providing bone beneficial nutrients. Further, females are more at risk of osteoporosis later in life than males. Soft drinks contributed to nearly 50% of total caloric beverage intakes of our adolescent females. Although there are now some restrictions in availability of carbonated soft drinks in schools, non carbonated soft drinks are frequently accessible by children and adolescents and might be mistakenly consumed as fruit juice. The results of this study will be informative for nutrition policy makers to establish appropriate intervention programs for school age students.

Many studies of nutrition and bone are now completed involving children, middle age and elderly people, yet less is known about the dietary intake of young adults with regard to their bones. Study three describes changes in calcium and vitamin D intakes and corresponding food choices of the PBMAS cohort from childhood to early adulthood. Although fluid milk was the major source of dietary calcium from childhood to early adulthood in both genders, there were significant changes in dietary behavior of our cohort, especially in females. The substitution of fluid milk by cheese in both genders is not a change that is in favour of bone. The high quantity of sodium in cheese accelerates calcium excretion and provokes calcium resorption in those with low calcium intake. When data from childhood, adolescence and early adulthood were compared, a significant lower intake of milk occurred in adolescent females and was maintained to early adulthood. The decrease in vegetable and fruit intake is another concern. The low intake of vitamin D in our subjects is in agreement with the recent studies concerning a high rate of vitamin D insufficiency in North America (Calvo & Whiting, 2003). All these factors may put young adult females more at risk of osteoporosis later in life. In the absence of current data at the national level, this study provides evidence for poor dietary behavior of young adults. Nutrition policy makers need to establish appropriate programs to promote healthy eating behavior in this neglected population. The results of the four studies presented in this thesis indicate that the biological factors height and weight are the major determinants of bone mineral mass from childhood to early adulthood. However, calcium intake and consumption of vegetables and fruit from childhood to adolescence and protein intake in young adults had a beneficial effect on bone. Physical activity is another important environmental factor that positively related to bone mineral mass from childhood to early adulthood. However, food choices and dietary behavior have changed in two aspects, over time and by age. The choices of beverages in adolescents 15 years ago were healthier than their peers in the year 2004. In addition, as adolescents aged and entered to early adulthood, the food choices changed, but not in the favour of their bones. The decrease in physical activity by age is another important change observed in our young adult cohort. Further, the positive effects of vegetables and fruit, calcium and physical activity during adolescence were demonstrated only in males and no protein effect was observed in

young adult females with habitual low calcium intake. Collectively, the negative change in dietary patterns was more dominant in females who are more at risk of osteoporosis and its complication later in life than males.

Although the focus of this thesis was on the impact of diet on bone, it can be seen that the changes in dietary pattern over time and by age have raised other health concerns such as risk of cardiovascular diseases, cancer and diabetes. These indicate the need for appropriate strategies to promote healthy nutrition and lifestyle. Although a nutrient approach in research provides valuable information, promoting the intake of a single nutrient or a group of nutrients would not be a good approach, as nutrients occur together in foods. Pharmaceutical companies are those that benefit most from a single-nutrient promotion. Food is the main source of nutrients and a selection of different foods appears in diet. The best approach is promoting a diet which provides appropriate amount of nutrients. An example of that type of diet is the DASH diet. Although the DASH diet was designed to prevent risk of hypertension, studies show that it has a positive impact on bone (Doyle & Cashman, 2004). Considering the body as a whole, this shows that a DASH diet is beneficial for some compartments, and most likely benefits other compartments of the body as well.

8.2 Limitations

Despite the longitudinal nature of this study that makes PBMAS unique for the field of nutrition and bone, there were some limitations. Our cohort was a small sample of healthy children who were followed to young adulthood. A habitual higher intake of milk products compared to other studies might reflect a learning effect due to repeated bone and body measurements in our cohort for a long period of time. Dietary intake and physical activity data were self-reported, therefore were subject to under- and over reporting. In self-reported dietary assessments, there is a tendency in subjects to underreport low quality foods and overreport nutrient dense foods (Lee & Nieman, 2003). The underreporting of energy intake has already been reported in PBMAS female subjects (Carter et al., 2001). The five years gap in measurements (1999-2003) has limited the ability to develop longitudinal statistical models and determine the peak bone mass in our cohort.

8.3 Future Research

The PBMAS data set has provided the opportunity to improve the knowledge and understanding of relationship between diet, physical active and bone status during critical years of bone modeling. In this study only total body DXA bone scans were used. Probable bone site selectivity and gender differences in the effect of nutrients and food groups on site specific bone measures during bone mass development, as suggested in some studies (Vatanparast & Whiting, 2006) warrant further research using PBMAS data. Further, the peak bone mass of our cohort has not been determined yet. In the absence of subjects with appropriate age range to fill the 5 years gap in measurement, appropriate statistical models can predict the peak bone mass values in our cohort.

The fact that DXA measurements of bone provide information on only bone mineral status not overall bone quality (Heaney, 2003) encourages examining other bone quality indicators in relation to diet and physical activity in the PBMAS cohort. The risk of fracture has been considered an appropriate indicator of bone status (Heaney, 2006). The incidence of fracture in PBMAS subjects can be obtained retrospectively.

Data from the PBMAS cohort were used in a factorial approach to determine the requirements of dietary calcium in adolescence (Institute of Medicine, 1997). Due to the lack of sufficient data, the EAR and RDA values for calcium have not been determined yet. Reanalyzing data from all subjects can provide a better estimation of calcium requirement to set the RDA values for calcium intake during adolescence. In addition, different threshold values have been suggested for calcium for different age groups (Institute of Medicine, 1997; Jackman et al, 1997; Matcovic &Heaney, 1992). These values can be reevaluated using calcium intake and calcium retention values of the PBMAS cohort.

A negative trend in calcium intake observed in adolescent girls accompanied with milk substitution by non-carbonated soft drinks (Chapter 5). This can be compensated by calcium fortified fruit juice. Another component of milk is protein. It has been reported that milk products were the second main food source of protein after meat and accounted for 15% of dietary protein in a sample of Canadian subjects (Johnson-Down et al., 2006). A nearly neutral PRAL of milk compared to a highly positive (thus acidogenic) value for meat as the main source of dietary protein in North

Americans' diet (0.3 mEq vs. 26 mEq) (Massey & Whiting 2003) makes milk a more favourable source of dietary protein with regard to bone health. Hence, whether the substitution of milk by non-carbonated soft drinks affects the protein intake of adolescent females needs further investigation.

Although a milk history questionnaire has been frequently used in various studies and surveys (Vatanparast & Whiting, 2004), whether this tool provides accurate data has not been examined yet. PBMAS data provide the opportunity to evaluate the accuracy of data obtained from milk history questionnaires.

In addition to the above mentioned fields of further research that can be addressed using PBMAS data, there are other general issues regarding the relationship of nutrition and bone. As was presented in Chapter 2 and Chapter 6, recent data on seasonal insufficiency and other health benefits of vitamin D reported in the recent decade have raised a question of inadequacy of current AI values of vitamin D. There are considerable publications suggesting that the AI values of vitamin D are not sufficient to overcome the seasonal insufficiency and need to be increased. Currently there is no study to evaluate the vitamin D status at the national level in the Canadian population. These data are needed to realize the extent of vitamin D insufficiency suggested by recent studies. Further, this will be crucial in setting up the new DRI values for vitamin D and preventing situations such as the calcium craze that happened in the 1990s.

The other issue is a lack of randomized control trials (RCT) examining a diet containing all bone beneficial nutrients. Most RCTs have investigated the effect of calcium on bone measures (Vatanparast &Whiting, 2006). Although there are a few trials that investigated the effect of milk on bone measures (Table 2.5), to date no study has been conducted to evaluate the effect of a diet comprising bone beneficial nutrients. Such a trial can fill the gap between knowledge and practice on the relationship between nutrition and bone, particularly at a community level.

Studies have reported a gender difference in timing of PHV, peak TBBMC velocity and peak bone mass (Chapter 2, Section 2.2). The gender difference in the effect of calcium on bone during growth and development from childhood to early adulthood is not certain (Chapter 2, Section 2.3.3.1). Further, whether bone selectivity in

relation to gender exists in the effect of calcium on bone during the years of bone modeling, considering maturity as a landmark, needs to be determined.

Less is known about the relationship between nutrition and bone in other races as most studies in this regard have been conducted in Caucasians. Consequently, dietary recommendations are based on the findings of those studies. Although Blacks have the most bone mineral mass (Lee & Nieman, 2003), data from NHANES III suggest that the current and past milk intake in Black females are less than Caucasians (Opotowsky & Bilezikian, 2003). Further research needs to be conducted to determine the racial differences in dietary intake in relation to bone. Further, whether a race specific dietary recommendation should take place is an unanswered question.

Finally, it is important to choose an appropriate bone measure as an outcome variable to be able to demonstrate the effect of nutrition and physical activity as the major environmental factors on bone. While bone mineral mass measures such as BMC and BMD represent the amount of mineral accumulated in bone, bone absorption and resorption biomarkers represents recent changes in bone modeling and remodeling processes. Recent literature suggests that none of these measurements alone provide an optimal indicator of bone quality (Chapter 2, Section 2.5). It seems that a combination of bone mineral mass measures and bone biomarkers along with clinical findings such as risk of fracture that might be translated into scores in a well-designed scoring system can provide an appropriate evaluation of bone status. This is another important area of further research that can prevent controversies in literature that used different bone measures.

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Appendix A

24-hour Recall a common dietary assessment tool

PLEASE LIST Every FOOD and DRINK You Ate Yesterday

UNIVERSITY OF SASKATCHEWAN

Name: _____ **Age:** _____ **Date:** _____

Time	Food Items	Type & Preparation	Amount	Brand Name or Where Bought
Morning				
Mid-morning				
Noon Meal				
Midday				
Evening Meal				
Before Bed				
EXAMPLE	***CEREAL***	***CORN FLAKES***	***1 cup***	***Kellogs***

Was this intake usual? Circle one: Yes No (*if No, explain why not* _____)
*Did you take any vitamins/minerals during this time?*Circle one: Yes No (if Yes, list names: _____

Appendix B

Food Frequency Questionnaire to assess food sources of calcium

UNIVERSITY OF SASKATCHEWAN
FOOD FREQUENCY QUESTIONNAIRE

Name _____ Today's Date _____
[Subject code:_____]

Please list nutritional supplements used:

_____
_____
_____
_____
_____
_____

1. **We want to know how often you eat or drink certain foods each week.**

2. Think about a ***typical week***, not what you ate this week which might be different.

3. **Medium** portion sizes are given to help you determine the usual size of the food or drink.

4. If you eat *much less* than the medium portion size described, then give a fraction. For example, a small glass of milk is "2" the medium, so "2" describes your usual intake. If you drink 2 small glasses of milk every day, this is the same as drinking 7 medium drinks each week.
If you eat *much more* than a medium size portion, then indicate this by giving the number of portions your size is equal to. For example, a very large plate of spaghetti would be 2 or 3 medium portions.

5. Fill out the form similar to this **example**:

- If you drink a Large Chocolate milk (500mL carton) three times a week, then choose 6 medium portions per week because it is 2 times the size of the medium portion, and you have it 3 times (i.e., 3 x 2).

Food Type	Medium Size Portion	How Many Times You Have a *Medium* Portion Each Week
1. **MILK** *How much milk do you drink milk each week?*	glass or drink carton	**7** *[this is what you fill in]* *You may write in daily values e.g.,* **1/day** *(please indicate per day@ if you do)*

Food Type	Medium Size Portion	How Many Times You Have a *Medium* Portion Each Week
1. **MILK** *How much milk do you drink milk each week?* (Treat white or chocolate milk the same)	Glass or Drink carton (8 oz or 250 mL)	
1.a **SOY MILK** ____ regular soy milk ____ Ca fortified soy milk (E.g. *So Good* brand)	Glass or Drink carton (8 oz or 250 mL)	
2. **MILK IN Coffee/Tea** *How often do you have milk (or cream) in coffee or tea ?*	milk(or cream) in one cup of coffee or tea	
3. **MILK ON CEREAL** *How often do you eat cereal with milk each week?*	½ cup milk per bowl of cereal	
Milkshake Purchased at _____	10 oz	
Milk- Based DESSERT Ice Cream/ice milk, soy dessert, pudding, custard	½ cup (one scoop or pudding cup)	
Yogurt (also mini-go, Yop etc.)	one container (175 mL)	
4. **CHEESE** cheese single slice (in sandwich or as snack)	single slice	
----->hard cheese (such as cheddar)	piece (1 oz)	
----->soft cheese	Serving Size: _____	
----->cottage cheese	Serving Size: _____	
5. **BREAD** *How often do you eat bread each week? (Remember sandwiches)* Bread, roll, bun	1 slice bread 1 small roll 1/2 bagel	
Food Type	**Medium Size Portion**	**How Many Times You Have a *Medium* Portion Each Week**
6. **BUTTER/MARGARINE** Butter	one pat (1 teaspoon)	
Margarine	one pat (1 teaspoon)	
7. **LUNCH and DINNER ITEMS**	**Medium Size Portion**	**How Many Times You Have a *Medium* Portion Each Week**
____ Tofu	one piece (1 inch cube)	
____ Spaghetti with tomato sauce or noodles and sauce	1 plate (1 cup)	

7. **LUNCH and DINNER ITEMS** (continued)	**Medium Size Portion**	**How Many Times You Have a *Medium* Portion Each Week**
____ Macaroni and cheese	1 plate (1 cup)	
____ Canned Salmon (in sandwich or casserole)	1 serving (1 oz)	
____ Canned sardines	1 serving = 4 small fish	
____ Tuna (in sandwich or casserole)	1 serving (1 oz)	
____ Seafood (shrimp, lobster, salmon steak etc.)	3 oz	
____ Lasagne	1 square	
____ Perogies ____ Do you have sour cream?	Give usual number eaten: Circle: Yes or No?	
____ Tacos, burritos with cheese, beans, lettuce etc.	1 regular	
____ Pizza -->take-out -->frozen mini pizza	one slice one round	
____ Baked beans or other beans or lentils	½ cup	
____ Green salad	1 bowl (1 cup)	
____ Potatoes: mashed with milk	one scoop (½ cup)	
____ Eggs: any type	one whole (with yolk)	
____ Cream soups (made with milk)	one bowl (1 cup)	
____ Orange	one medium	
____ Orange Juice - regular ____ Orange Juice with Calcium	one juicepack (1 cup)	
____ Eggo-type waffle or pancake	one	
____ Homemade or restaurant Pancake, Waffle, French Toast	one 5"across	
____ Broccoli, Spinach, Beet greens or kale	½ cup	
____ Taco chips, Nacho chips	28 g (½ small bag) or small bowl	

Questions or Comments:

Appendix C

Milk intake history questionnaire used in NHANES III for subjects aged 20+ y

N10. REFER TO AGE OF SP. HAND VARD HAQ-6.
READ RESPONSE CATEGORIES TO SP IF NECESSARY.

Now I am going to ask how often you drank milk over your lifetime. Try to remember whether you were a milk drinker or a non-drinker during different times in your life. Then think of certain events that might have occurred during each time period; for example, were you in school, at home with children, on a farm, or in the service.

How often did you drink any type of milk, including milk added to cereal, when you were a _____? Do not count small amounts of milk added to coffee or tea.

Time period (age)	more than once per day	once per day	less than once per day but more than once per week	once per week	less than once per week	never	don't know
a. Child (5-12)	1☐	2☐	3☐	4☐	5☐	0☐	9☐
b. Teenager (13-17)	1☐	2☐	3☐	4☐	5☐	0☐	9☐
c. Young adult (18-35)	1☐	2☐	3☐	4☐	5☐	0☐	9☐
d. Middle –aged adult (36-65)	1☐	2☐	3☐	4☐	5☐	0☐	9☐
e. Older adult (over 65)	1☐	2☐	3☐	4☐	5☐	0☐	9☐

Appendix D

Appendix D1: Original PBMAS study

Appendix D2: Follow-up study

Appendix D1

Ethics Certificate (Original PBMAS study)

UNIVERSITY ADVISORY COMMITTEE ON ETHICS IN HUMAN EXPERIMENTATION

Name and E.C. File #: D.A. Bailey 88-102 April 15, 1991

Your project entitled: A mixed longitudinal study of bone density changes during the adolescent years in boys and girls with reference to physical activity patterns and nutritional factors.

has been approved by the Committee.

1. Therefore you are free to proceed with the project subject to the following conditions:

Letter of consent submitted March 20, 1991 approved.

2. Please submit the revisions requested above to the Director of Research Services, Room 50, Murray Building.

3. Any significant changes of your protocol should be reported to the Director of Research Services for Committee consideration in advance of its implementation.

Sincerely,

ROLAND MUIR
Director of Research Services
University of Saskatchewan

Dr. E.A. McKenna, Chairman
University Advisory Committee on
Ethics in Human Experimentation

Appendix D2

Ethics Certificate (Follow-up study)

University Of Saskatchewan, Biomedical Research Ethics Board (Bio-REB)
Office of Research Services
Room 3403, 110 Gymnasium Place
Box 5000 RPO University
Saskatoon, SK S7N 4J8

RECEIVED
OCT 2 2 2004
University of Saskatchewan
Research Ethics Board (REB)

STATUS REPORT FORM

This form is submitted for the following purpose

1st Annual status report and re-approval request. When was the ethics approval for this study due to expire? Date: 1st Nov 2004

__N/A__ Notice of study closure.

Report Prepared by: Adam Baxter-Jones Date: Oct 19th , 2004

ONGOING REVIEW REQUIREMENTS: This approval is valid for up to one year. The REB will require the submission of an annual status report at least one month prior to the expiration date indicated below. Please note if the Status Report Form is not submitted by the one-year expiry date, the ethics certificate will automatically expire.

1. PRINCIPAL INVESTIGATOR

Dr Adam Baxter-Jones

NOTE: An investigator who does not maintain a physical presence at the trial site in proportion to the inherent level of risk that subjects will be exposed to cannot continue to be identified as the principal investigator. The responsibility must be transferred to a new principal investigator.

2. DEPARTMENT/DIVISION
College of Kinesiology

3. REB FILE #
88-102 (NHRDP#6608-126-OS)

4. STUDY SITE(S) Physical Activity Complex, 87 Campus Drive

5. TITLE OF PROTOCOL AND PROTOCOL # (where applicable) Relationship of growth and lifestyle to peak bone mass

6. SPONSOR (where applicable)
CIHR MOP-57671

7. BRIEF SUMMARY OF PROGRESS OF STUDY (projected completion date for recruitment and data collection, number of subjects admitted to date, target enrollment, anticipated end-date). Are subjects currently receiving study treatment or interventions, or is the study only active for follow-up to endpoints?

Between Oct 2002 and August 2003 we contaced the original 251 subjects recruited into the first phase of the study, which ran from 1991 to 1993. We were unable to trace nine individuals, 72 withdrew from the study. 170 indicated their willingness to continue. Between November 2002 and August 2003, 148 of this 170 were tested at RUH. Four of the 170 were pregnant and were not tested; the remaining 18 were unable to be tested. During the second year of the study an attempt was made to contact the 170 again. 135 participants were tested this time; 8 of whom were not tested the previous year. 33 participants could not be tested due to scheduling conflicts. 3 participants verbally withdrew their participation. We are now entering the third year of the follow-up study and will be contacting the 167 remaining participants to invite them for a further annual assessment.

8. ARE THERE ANY ASPECTS OF THIS STUDY WHICH SHOULD BE BROUGHT TO THE ATTENTION OF THE REB (i.e., any new information or knowledge bearing on the anticipated risks or anticipated benefits, and therefore possibly affecting subjects' ongoing decision to participate in this study. Clinical trialists should reflect upon adverse events associated with their protocol).

No

9. WHAT ARE YOUR CURRENT SAFETY REVIEW PROCEDURES FOR THIS RESEARCH PROJECT (i.e., drug safety monitoring board (DSMB), clinical end-point committee (CEC), hotline, periodic reporting to the ethics board)?

All bone measurements are done in the PAC by qualified technicians from Dept. of Medical Imaging, RUH. A Safety and preventive maintence inspection report of the DXA has just been completed (Oct 8th 2004)

10. PRINCIPAL INVESTIGATOR

A Baxter-Jones
Signature

Oct 14th 2004
Date:

For Administrative Use Only:

Approved On: OCT 2 6 2004

Expiry Date: November 1, 2005

Signature of Chair or designate: Barry D. McLennan

Please note that this form, once signed by the chair or designate, serves as your official re-approval certificate.

02June03

Appendix E

Analyzing PBMAS data using MLM approach[3]

At the study entry participant boys and girls were aged between 8 and 15 (Table 3.1). After 7 years of annual data collection the composition of these clusters remained the same. As there were overlaps in ages between the clusters it is possible to estimate a consecutive 12 years of developmental pattern (8-21) over the shorter period of 7 years (Table 3.1). Subjects required complete measurements of age, height, weight, total body-BMC (TB-BMC), dietary intake and physical activity for more than one measurement sequence. In addition, they were required to have experienced peak height velocity (PHV).

In PBMAS model the outcome or dependent variable is TB-BMC (y). Since there are repeated measurements within individual, therefore the outcome variable (TB-BMC) has two subscripts (y_{ij}). Subscript ij refers to measurement sequence *i* in subject *j*. The objective is estimating the contribution of fixed variables (height, weight, milk product intake, vegetables and fruit intake, calcium intake, and physical activity) and random variable (biological age) to determine the relationship of TB-BMC accrual overtime within and between subjects. Like other human growth longitudinal data, PBMAS data have a hierarchical or clustered structure. It means that units grouped at different levels. The measurement sequences (within subject) are the level 1 unit in a 2-level structure, where the level -2 units are the subjects themselves.

The data structure in multilevel analysis is generally different from statistical packages such as SPSS and SAS. In MLM, each row represents an individual's measurements on a single occasion (sequence). Therefore there is more than one row for each individual. Subject ID and sequence number of each measurement occasion within subject have been defined to structure the data in this particular way. Therefore sequence number is a unique identifier for level-1 units and subject ID is the unique identifier for level-2 units.

---

[3] In this Appendix information from MlwiN software manual and also Baxter-Jones et al. (2004) were used.

TB-BMC is repeatedly measured annually for 7 consecutive years during the study period from 1991-1997 (Bailey 1997). By making the time variable (biological age) random at level-2, the variance of TB-BMC accrual over increasing biological age will be estimated in two parts. Level-1 variance is the variance associated with an individual's regression line of TB-BMC development on biological age. The second part of the variance (level-2) is variance representing the deviation of each individual's line from the average line for the whole group. It means that each subject has the same predictor variable (biological age, height, weight, physical activity, food group intake and calcium intake) and the same outcome variable (TB-BMC), but each individual has different regression coefficients.

The software program MlwiN version 1.0 (Multilevel Models Project, Institute of Education, University of London, London, UK) was used to analyze our data set. The program uses the following equation to estimate both the fixed and random parameters.

$$y_{ij} = \alpha + \beta_j x_{ij1} + \beta x_{j2} + \ldots\ldots\ldots\ldots + \beta x_{ijp} + (u_j + v_j x_{ij} + \varepsilon_{ij})$$

Where y is TB-BMC at measurement sequence *i* in the *j*-th subject, $\beta_j x_{ij1}$ is the slope of TB-BMC with biological age for the j-th individual, and x_2 to x_p are the coefficients of various explanatory variables at measurement sequence *i* in the *j*-th subject . ε_{ij} the level-1 residual (within subject variance) for the *i*-th measurement of TB-BMC in the *j*-th subject. Overall the constants α and β are fixed values, the average intercept and slope, which are estimated from the data. u_j and v_j are both random variables at level-2 and represent the extent to which the *j*-th subject's intercept and slop departs from the average.

There are four phases in human growth: rapid growth in infancy and early childhood, steady but constant growth during middle childhood, rapid growth during the adolescent spurt, and slow and eventual cessation of growth after adolescence (Malina et al. 2003). Therefore the growth pattern of height, weight and most external dimensions of the body is "S "-shaped (sigmoid) (Figure 2.1). Hence, a 'non-linear' function of linear multilevel models is needed when applying them to growth data. The '*S*' shape can be fitted mathematically by adding 2nd and 3rd order polynomials of our time variable 'biological age'.

In multilevel models the goodness of fit is measured by the deviance between two models, analyzed using the likelihood ratio statistic. The difference in likelihood ratio's between the model prior to inclusion of a new variable and after it follows a chi-square distribution with degrees of freedom equal to the number of new variables included. If the difference in likelihood ratio statistics between two models is significant, it indicates that the new variable should be included in the model and the new model is a significant improvement in fit of the data over the previous model.

First a null model was fitted containing only our outcome variable (TB-BMC) and the mean intercept and the likelihood ratio statistic calculated. The null model is used as a baseline to estimate explained versus unexplained variances. Models were built in a stepwise procedure, in this way independent variables are added to the models and are retained if the difference in likelihood ratio statistics between two models is significant. Variables are significant if the estimated mean coefficient (β) is greater than twice the standard error of the estimate (SEE). The intercept in null model represents the average value of TB-BMC (1878.5 g in boys' model and 1632.2g in girls' model) at biological age 0 within our subjects. The log likelihood statistic takes a value of 9596.2 in boys' model and 10089.8 in girls' model.

Table E.1 illustrates the development of the multilevel model of TB-BMC with biological age in boys. Null model was extended by adding an independent factor (biological age). The change in likelihood ratio statistic indicates that this model was a better fit of the data than null model (Table E.1, Model A). The difference in likelihood ratio statistic between those two models with 1 degree of freedom is $p<0.001$.

In next step, biological age was made random at level-2, it allowed individuals to have separate intercepts and slopes. It can be seen that the goodness of fit of this model has significantly improved ($p < 0.05$) from the previous model (Table E.1, Model C). The likelihood ratio statistic drops by 165.4 units. The biological age coefficient (221.8±6.4) indicates that TB-BMC increase by 221.8 g per biological age year in boys. For example if biological age=1 then the model would predict a TB-BMC of 2002.2 g (1780.4 + 1*221.8). When we added other variables one at the time we observed the gradual development of the model. The inclusion of variables in the final model depends on whether they are biologically or statistically significant. The power functions of

biological age (biological age^2 and biological age^3) were significant and were included in the linear model to shape the curves. There was no independent sex difference after controlling for growth and body size confounders. Therefore separate models were conducted for boys and girls.

The coefficients that significantly predicted TB-BMC (Table E.2) in boys and girls, were biological age (-years from age at PHV), height (cm) and weight (kg). Physical activity (score), calcium intake (mg/day), and vegetable and fruit intake (servings/day) were environmental factors that had significant independent effect on TB-BMC only in boys (Table E.2). Any possible interaction terms between environmental variables were examined; none of them were significant predictors of TB-BMC in both genders. Milk product intake (servings/day) and other food groups were not significant predictors of TB-BMC in both boys and girls. Figure E.1 illustrates the final model in MlwiN printout. The regression equation shown in Figure E.1 can be used to predict TBBMC for each male subject at each measurement occasion.

Table E. 1 Development of the multilevel model of Total Body Bone Mineral Content (TB-BMC) with biological age in boys

Variables	Null Model	Model A	Model B	Model C	Model D	Model E	Model F	Model G	Model H	Final Model
	Estimates	Estimates	Estimates	Estimates	Estimates	Estimates	Estimates	Estimates	Estimates	Estimates
Fixed Effects										
Constant	1878.5±53.1	1778.2±27.5	1780.4±30.5	1780.5±30.5	1750.8±27.6	-2510.6±217.0	-2314.2±213.4	-2287.4±235.9	-2268.35±235.9	-2306.6±233.4
Biological age		219.9±3.6	221.8±6.4	221.8±6.4	277.3±5.5	116.6±9.3	106.7±9.3	114.6±10.9	115.7±10.9	114.8±10.7
Biological age^2				-3.8±1.1	5.2±0.8	12.5±0.8	12.1±0.8	13.5±0.8	13.2±0.9	12.8±0.8
Biological age^3					-3.5±0.2	-2.1±0.1	-2.0±0.2	-2.3±0.2	-2.3±0.2	-2.3±0.2
Height (cm)						25.9±1.3	23.4±1.4	22.7±0.5	22.6±1.5	22.6±1.5
Weight (kg)							4.3±0.8	4.6±0.8	4.4±0.8	4.3±0.8
Physical activity (score)								17.9±9.2	17.5±9.1	22.2±9.0
Calcium (mg/day)									0.020±0.008	0.017±0.008
Vegetable & fruit (servings/day)										5.4±1.3
Random Effects	**Level 1 (within individual)**									
Constant	209016.4 ±13063.4	26766.3 ±1672.8	13458.0 ±935.9	13238.3 ±920.9	7458 ±517.8	4691.7 ±325.9	4781.8 ±335.4	4555.4 ±333.0	4480.0 ±327.7	4330.8 ±316.8
	Level 2(between individual)									
	Con / B age	Con / B age	Con / B age	Con / B age	Con / B age	Con / B age	Con / B age	Con / B age	Con / B age	Con / B age
Constant	266924.6 ±41567.4	77021.8 ±11137.6	99085.9 ±14165.1	513.2 ±2002.7	95612.7 ±13681.5	2720.4 ±1998.6	80043 ±11216	4979.3 ±1309	44793 ±6314	4892.9 ±965.9	36517.0 ±5145.0	4133.2 ±805.8	36004.9 ±5078.1	4311.7 ±832.1	36643.6 ±5155.7	4267.2 ±825.5	3651.0 ±5157.5	4186.3 ±809.1
Biological age			513.2 ±2002.7	3068.9 ±555.7	2720.4 ±1998.6	3136.9 ±536.7	4979.3 ±1309	1366.4 ±258.2	4892.9 ±965.9	1276.4 ±221.5	4133.2 ±805.8	1039.1 ±187.9	4311.7 ±832.1	1111.6 ±202	4267.2 ±825.5	1076.4 ±196.9	4186.3 ±809.1	1026.6 ±188.3
2*log likelihood (difference)	9596.2	8400.2	8234.8	8223.8	7873.1	7581.5	7468.9	7060.9	7054.0	7037.4

Footnotes for Table E.1

Fixed effect values are estimated mean coefficients ±SEE(Standard Error Estimate) (TBBMC,g), Random effect values are estimated mean variance ±SEE (TBBMC,g^2), Con= Constant, B age= Biological age (-years from age at PHV), $p < 0.05$ if mean > 2*SEE, $p < 0.05$ for change in log likelihood between models

Start | More | Stop | IGLS | Estimation control..

$$\text{tbbmc}_{ij} \sim \text{N}(XB,\ \Omega)$$

$$\text{tbbmc}_{ij} = \beta_{0ij}\text{cons} + \beta_{1j}\text{bio age}_{ij} + 12.837(0.838)\text{bioage2}_{ij} + -2.268(0.215)\text{bioage3}_{ij} + 22.587(1.466)\text{ht}_{ij} + 4.339(0.827)\text{wt}_{ij} + 22.193(9.041)\text{act.score}_{ij} + 0.017(0.008)\text{CALCIUM.MG}_{ij} + 5.371(1.302)\text{fruit\&veg}_{ij}$$

$$\beta_{0ij} = -2306.636(233.447) + u_{0j} + e_{0ij}$$

$$\beta_{1j} = 114.830(10.736) + u_{1j}$$

$$\begin{bmatrix} u_{0j} \\ u_{1j} \end{bmatrix} \sim \text{N}(0,\ \Omega_u)\ :\ \Omega_u = \begin{bmatrix} 36850.990(5175.497) & \\ 4186.323(809.071) & 1026.585(188.265) \end{bmatrix}$$

$$\begin{bmatrix} e_{0ij} \end{bmatrix} \sim \text{N}(0,\ \Omega_e)\ :\ \Omega_e = \begin{bmatrix} 4330.806(316.792) \end{bmatrix}$$

*-2*loglikelihood(IGLS)* = 7037.362(581 of 621 cases in use)

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Figure E.1 Final Model containing all significant predictors of TB-BMC in boys

Appendix F

The adjustment procedure to obtain usual intake from actual intake data (NRC method)

The details of four steps of NRC method are as follows:

Step 1: Examining normality of distribution and transforming data if it is necessary.

Step 2: Estimating the within- and between- subject variance.

Partitioning the variance of the observed data into the within- and between-subject variance components can be done using the ANOVA procedures. Both the mean square model and the mean square error from the ANOVA table need to be divided by the mean number of days of intake data per subject to obtain $V_{observed}$ and V_{within} (e.g., $V_{observed}$ = mean square model/*n* and V_{within} =mean square of error/*n*. Therefore V between can be obtained from the following formula:

$$V_{between} = (\text{mean square model} - \text{mean square error}) / n \qquad \textbf{[Eq. 1]}$$

Where *n* is the mean number of days of intake data and $V_{between}$ represents the "true" variance of the distribution of usual intakes. Standard deviations are the square root of the variances.

Step 3: Adjusting individual subjects' mean intakes to estimate the distribution of usual intakes

Now, each subject's nutrient intake is adjusted by the following formula:

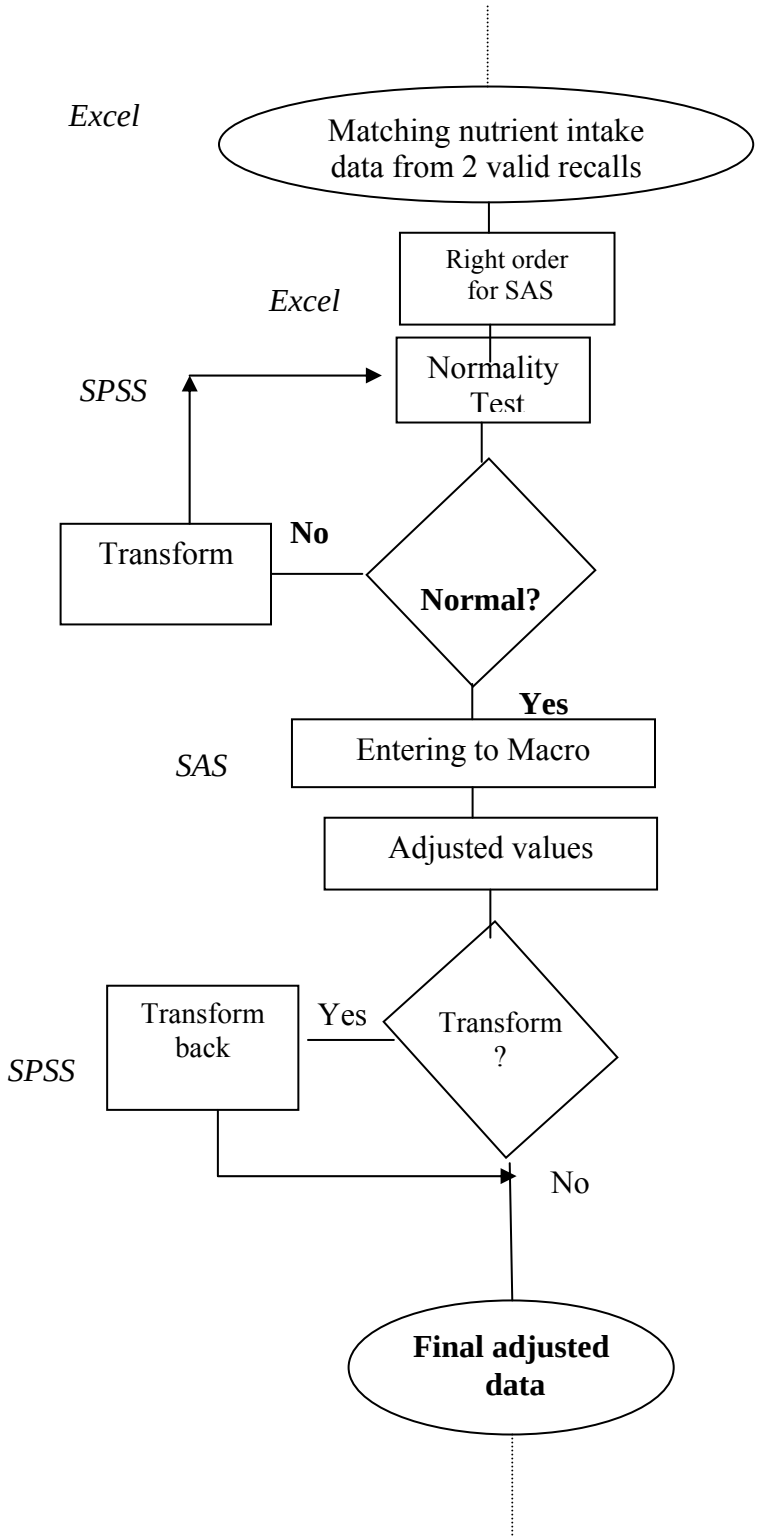
$$\text{Adjusted intake} = [(\text{subject's mean} - \text{group mean}) \times (SD_{between} / SD_{observed})] + \text{group mean} \qquad \textbf{[Eq. 2]}$$

where $SD_{between}$ is the square root of $V_{between}$ and $SD_{observed}$ is the square root of $V_{observed}$. This equation efficiently moves each point in the distribution of observed intakes closer to the group mean, but it does not shift the group mean.

Step 4: If the original data have been transformed, the adjusted intake data should be transformed back to the original units.

Figure F.1 illustrates the flowchart for the steps taken in adjusting the BMAS nutrient data followed by the SAS macro adopted for analyzing calcium intake data from PBMAS study.

Figure F.1 The algorithm for the adjustment procedure to estimate the distribution of usual intake in PBMAS study



The SAS macro to adjust the observed calcium intake data to estimate the distribution of usual intake in PBMAS study

```
set dir.ca;
run;
proc print data=dir.ca;
run;

PROC ANOVA DATA= dir.ca OUTSTAT=ANOVASTAT;
CLASS SUBJID;
MODEL Ca1= SUBJID;

*<< CHANGE VARIABLE NAME TO NUTRIENT OF INTEREST;

DATA PARTIT1;
SET ANOVASTAT;
MS=SS/DF;
MSERROR=MS; MSMODEL=MS;
DFERROR=DF; DFMODEL=DF;
IF _TYPE_='ERROR' THEN MSMODEL=.;
IF _TYPE_='ANOVA' THEN MSERROR=.;
IF _TYPE_='ERROR' THEN DFMODEL=.;
IF _TYPE_='ANOVA' THEN DFERROR=.;
KEEP MSMODEL DFMODEL MSERROR DFERROR;
proc print data=PARTIT1;run;
PROC UNIVARIATE NOPRINT;
VAR MSMODEL DFMODEL MSERROR DFERROR;
OUTPUT OUT=PARTIT2 MEAN=MSMODEL DFMODEL MSERROR DFERROR;

DATA PARTIT3;
SET PARTIT2;
MEANREPL=(DFMODEL+DFERROR+1)/(DFMODEL+1);
ERRORDIF=MSMODEL-MSERROR;
IF ERRORDIF LT 0 THEN ERRORDIF=0;
SDINTRA=MSERROR**0.5;
SDINTER=(ERRORDIF/MEANREPL)**0.5;
SDTOTAL=(SDINTRA**2+(SDINTRA**2/MEANREPL))**0.5;
INDEX=1;
KEEP SDINTER SDTOTAL INDEX;

PROC MEANS NOPRINT DATA=dir.ca;
   VAR Ca1 Ca2; BY SUBJID;
```

```
   OUTPUT OUT=SUBJMEAN MEAN=SMEAN;
DATA SUBJMEAN; SET SUBJMEAN; INDEX=1;

PROC UNIVARIATE NOPRINT; VAR SMEAN;
OUTPUT OUT=MEANS MEAN = GMEAN;
DATA MEANS; SET MEANS; INDEX=1;
DATA ADJUST;
MERGE SUBJMEAN PARTIT3 MEANS;
BY INDEX;
NRCADJ=GMEAN+(SMEAN-GMEAN)*SDINTER/SDTOTAL;
KEEP SUBJID NRCADJ;
RUN;
DATA FINAL; MERGE dir.ca ADJUST; BY SUBJID;
PROC PRINT;
TITLE 'NUTRIENT DATA SHOWING INDIVIDUAL OBS, MEAN, NRC ADJUSTED';
RUN;
```

Appendix G

The amount of calcium/ 100 kcal energy from food sources[1]

Food item	Amount	Energy (kcal)	Calcium (mg)
Milk, Whole	159 ml	100	196
Milk, 2%	194 ml	100	243
Milk, skim	276 ml	100	353
Cheese, hard, goat	22 g	100	198
Cheese, soft, goat	37 g	100	52
Cheese, feta	38 g	100	34
Cheese, Mozzarella	35 g	100	183
Cheese, hard, parmesan	25 g	100	303
Cheese, cottage, creamed	97 g	100	58
Yogurt, low fat 1%, Vanilla	95 g	100	104
Yogurt, low fat, plain	101 g	100	139
Broccoli, raw	232 g	100	111
Orange juice, with calcium	215 ml	100	273

[1] Nutrient values obtained from Food Processor Software (Esha, version 8.4)