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Institute of Sport Sciences and Physiotherapy, Faculty of Medicine, University of Tartu, Estonia.

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ABBREVIATIONS

APHV	age at peak height velocity
BMC	bone mineral content
BMD	bone mineral density
BMI	body mass index
CTX	C-terminal telopeptide of type I collagen
DXA	dual-energy X-ray absorptiometry
FM	fat mass
FN	femoral neck
LBM	lean body mass
LPA	light physical activity
LS	lumbar spine
MPA	moderate physical activity
MVPA	moderate-to-vigorous physical activity
OC	osteocalcin
PA	physical activity
SD	standard deviation
SED	sedentary time
TPA	total physical activity
VPA	vigorous physical activity
WB	whole body

LIST OF ORIGINAL PUBLICATIONS

I Vaitkevičiūtė D, Lätt E, Mäestu J, Jürimäe T, Saar M, Purge P, Maasalu K, Jürimäe J. Physical activity and bone mineral accrual in boys with different body composition parameters during puberty: a longitudinal study. *PLoS One* 2014; 9(10): e107759.

II Vaitkevičiūtė D, Lätt E, Mäestu J, Jürimäe T, Saar M, Purge P, Maasalu K, Jürimäe J. Adipocytokines and bone metabolism markers in relation to bone mineral values in early pubertal boys with different physical activity. *Journal of Pediatric Endocrinology and Metabolism* 2016; 29(6):723–729.

III Vaitkevičiūtė D, Lätt E, Mäestu J, Jürimäe T, Saar M, Purge P, Maasalu K, Jürimäe J. Longitudinal associations between bone and adipose tissue biochemical markers with bone mineralization in boys during puberty. *BMC Pediatrics* 2016; 16: 102.

In all papers, Donvina Vaitkevičiūtė had primary responsibility for preliminary and final data analyses, and writing the manuscripts.

1. INTRODUCTION

Puberty, or sexual development, is a time of dramatic changes in body size, shape, body composition, including alterations in the relative proportions of muscle, fat, and bone, and result in typical female-male differences (Rogol et al. 2000). The timing of the onset of puberty is determined by a child's skeletal age (biological age) of approximately 11 years in girls and 13 years in boys, however the timing and tempo of puberty can vary widely, even among healthy children (Rogol et al. 2000; Tanner et al. 1975).

Puberty is a critical period of bone mineral content (BMC) accrual that may have long-term consequences for osteoporosis in adulthood (Lappe et al. 2015). It is known that half of peak bone mass is accumulated during pubertal years and estimated peak bone mineral accretion occurs at the age of 14.1 years in boys, 6 months later than peak height velocity (Bailey et al. 1999; Iuliano et al. 2001). During this sensitive period of rapid bone turnover, it is very important to measure bone biochemical markers of formation and resorption to monitor bone-remodeling process (Eeapen et al. 2008). Up to date, there is a disagreement regarding osteocalcin (OC; biochemical marker of bone formation) and C-terminal telopeptide of type I collagen (CTX; biochemical marker of bone resorption) relationships with bone mineral accrual during puberty (Alghadir et al. 2015; Chahla et al. 2015; van Coeverden et al. 2002; Gracia-Marco et al. 2011; Mora et al. 1999; Silva et al. 2011; Yilmaz et al. 2005).

Body mass should also be taken into account when investigating bone mineral accretion. It is known that both, fat mass (FM) and lean body mass (LBM) have an effect on bone mineral parameters (Gracia-Marco et al. 2012; Luo et al. 2006). Lean body mass, through muscle contractions, stimulates bone mechanically and shows strong relationships with different bone mineral parameters (Roblin 2009). However, there are more uncertainties about the effect of FM on bone mineral parameters. Obesity has emerged as a serious problem among children and adolescents in the past years (Ogden et al. 2014). Studies report that obese boys have increased (Leonard et al. 2004; Vandewalle et al. 2013), decreased (Dimitri et al. 2010; Rocher et al. 2008) or similar (Fintini et al. 2011; Hasanoglu et al. 2000) bone mineral density (BMD) compared to normal weight boys. A study of Dimitri et al. (2012) indicated that body fat induced alterations in hormonal factors and cytokines may play an important role in disturbing bone mineral accrual, thus increasing risk for bone fractures during rapid skeletal growth in pubertal period. Moreover, underweight boys have been characterized by lower BMD compared to their normal weight peers and are also at an increased risk of fractures (Galusca et al. 2008). Adipose tissue releases hormones such as leptin and adiponectin that have effects on bone metabolism (Jürimäe et al. 2011). Studies that have investigated relationships between leptin and bone mineralization in adolescents are rather conflicting, reporting no, positive or negalive relationships (Garnett et al. 2004; Jürimäe et al. 2005; do Prado et al. 2009; Roemmich et al. 2003; Tubic et al.

2011; Vyshnevskaya & Solntsava 2011). Adiponectin is less investigated compared to leptin, regarding to the relationships with bone mineralization in adolescents, moreover studies report contradictory results (van Coeverden et al. 2002; Huang et al. 2004; Rhie et al. 2010; Sayers et al. 2011; Tubic et al. 2011; Vyshnevskaya & Solntsava 2011).

Physical activity (PA) is a very important environmental factor that can be controlled and is crucial for healthy bone development and maximizing bone mineral accrual during puberty (Holroyd et al. 2012; Lappe et al. 2015). Weight-bearing activities such as running, stair climbing, jumping rope, racket sports and/or different ball games, give the necessary amount of mechanical loading and enhance BMD in children and adolescents (French et al. 2000; Hind & Burrows 2007). Unfortunately, puberty has been reported as a period where PA sharply decreases (Carroquinio-Salto 2009; Ortega et al. 2013). In addition, World Health Organization (WHO 2015) reported that globally 81% of adolescents aged 11–17 years are not sufficiently physically active. WHO recommendations are 60 min/day of moderate-to-vigorous PA (MVPA) for children and adolescents aged 5–17 years; however, less than one third of this age group population is able to meet the recommended PA guidelines (Gordon et al. 2004; Nader et al. 2008).

Today there is a global problem of adolescents' inactivity and obesity, which could result in osteoporosis during the later years of life. A strong focus should be put into investigation of bone mineral accrual during puberty and to understand all the effects of environmental factors that influences it. To our best knowledge, there are no longitudinal studies about the influence of body composition, bone and adipose tissue biochemical markers and objectively measured physical activity on bone mineral accrual in pubertal boys. Accordingly, the main goal of this dissertation was to evaluate possible associations between body composition, physical activity and bone and adipose tissue biochemical markers with bone mineral development in pubertal boys with different body mass and physical activity values.

2. REVIEW OF THE LITERATURE

2.1. Bone development during puberty

Although bone might seem as a static tissue, it is actually metabolically active and dynamic tissue, with continues remodeling occurring throughout life. Puberty with rapid growth and maturation is a key period for bone mineralization with half of peak bone mass accumulated during pubertal years. It is estimated that peak bone mineral accretion occurs at the age of 12.5 in girls and 14.1 in boys corresponding to pubertal stages 3–5 (Bailey et al. 1999; Bonjour et al. 1991). About 39% of total body bone mineral is acquired during the 4 years surrounding the peak in bone mineral accretion (Baxter-Jones et al. 2011). Furthermore, during pubertal growth bone metabolism reflects both modeling and remodeling of the current bone tissue (Slemenda et al. 1997). Skeleton grows in size and density during the first two decades of life, with half of peak bone mass accumulated during pubertal years (Bachrach 2001). Peak bone mass (maximum strength and density) occurs from early to late twenties for both males and females, with females reaching their peak bone mass significantly earlier than males (Lu et al. 2016). Skeleton growth is not a steady process; bone mineral accrual takes place at different rates at different skeletal sites (Tanner 1976). There is a greater growth in the limbs before the puberty, while after the puberty there is greater growth of the spine and overall growth of skeleton slows down at the end of puberty (Bradney et al. 2000; Zemel 2012).

There are a variety of factors that influence bone accumulation; it is estimated that about 80% of bone development is dependent on endogenic factors: genes, ethnicity, gender and hormone status, while 20% is dependent on exogenic factors: proper nutrition with optimal calcium and vitamin D intake and regular PA with weight bearing exercises (Davies et al. 2005; Rizolli et al. 2010; Weeks & Beck 2010). Environmental factors can be controlled and they are crucial for healthy bone development, especially during puberty (Holroyd et al. 2012, Vicente-Rodríguez et al. 2008).

Gender differences in relation to bone mineralization are not evident before puberty; the length and width of bone increase progressively, while major differences in bone accumulation due to the different effects of sex steroids in males and females appear at the end of puberty (Gilsanz et al. 1997). Estrogen level has stronger contribution to bone mineral acquisition in boys and girls, compared to testosterone level; moreover, the achievement of peak bone mineral accrual was sustained by estrogen only in boys (Yilmaz et al. 2005). Few studies have reported higher BMD values in boys compared to girls (Ausili et al. 2012; Baxter-Jones et al. 2003; Lee et al. 2007). However, Baxter-Jones et al. (2003) concluded that sex differences in BMD values are mostly explained by differences in anthropometric values.

Sexual maturation has also emerged a better predictor of bone mineralization in adolescents compared to chronological age (Yilmaz et al. 2005). Bone mineralization showed high correlations with pubertal stages during pubertal

growth and maturation (Dib et al. 2005). It has been reported that bone mass increases in line with the pubertal development and boys have higher BMD values at each pubertal stage compared to girls (Arabi et al. 2004). Previously, it has been suggested that pubertal maturity predicts variance in the parameters of bone mass in adolescent girls, while PA and muscle power exert most influence on the bones of adolescent boys (Weeks & Beck 2010).

In conclusion, puberty with rapid growth and maturation is a key period for bone mineralization with half of bone mineral accumulated during pubertal years. There are many endogenic and exogenic factors that contribute to bone mineralization during this sensitive period of growth, however, many factors still remain unknown. Accordingly, more longitudinal studies are necessary to investigate the role of environmental factors on bone mineralization in boys during puberty.

2.2. Body composition and bone mineral parameters in boys during puberty

Body composition changes rapidly during puberty and describes the amount of FM, LBM and also BMC in the human body (Wheeler 1991). Human skeleton is composed of two types of bones: cortical bones, which constitute 80 % of total skeleton (long bones, such as femur, tibia and outer surfaces of the flat bones) and trabecular bones (at the end of long bones and in the inner parts of flat bones, for example lumbar spine) (Dempster 2006). Trabecular bones are more metabolically active compared to cortical bones (Bareither et al. 2008); accordingly, they have different sensitivity to mechanical stress (Bakker et al. 2003).

The effect of body mass on bone mineral variables is contributed by both FM (Gracia-Marco et al. 2012) and LBM (Luo et al. 2006) values. It is known that LBM, through muscle contractions, stimulates bone mechanically and presents strong correlation with different BMC and BMD values (Riggs et al. 2002; Robling 2009). Few longitudinal studies have indicated that LBM has a major positive effect on bone health parameters in boys (Gracia-Marco et al. 2012; Pietrobelli et al. 2002). Mora et al. (1994) suggested that weight-bearing and mechanical stresses are determinants of cortical bone in healthy children, while trabecular bones are influenced by hormonal factors. In contrast, Cheng et al. (1999) and Alwis et al. (2008) reported that due to higher metabolic activity, trabecular bones are more responsive to PA intervention in pre-pubertal and pubertal children. Moreover, the contribution of LBM to BMC variance has been found to be larger in 10-to-17-year-old (pubertal stages 1–5) boys than in same age girls (Arabi et al. 2004). Ausili et al. (2012) reported that male sex and LBM seem to represent independent predictors of bone mineral accrual in the cortical bone, while female sex and pubertal maturation are independent predictors of bone mineral accrual in the trabecular bone. Moreover, it was found that proximal femur (cortical bone) of pre-pubertal and early pubertal boys were

more sensitive to mechanical loading than in girls (Kriemler et al. 2008). Such results could be explained by different sensitivity of cortical and trabecular bones on mechanical stress (Bakker et al. 2003) and different sensitivity of bone to physical loading, irrespective of muscle mass in pre-pubertal and pubertal children (Kriemler et al. 2008).

Obesity has emerged as a serious problem among children and adolescents in the past years (Ogden et al. 2014). There is a controversy in the literature as to whether obese boys have higher or lower bone mass compared with normal weight boys. Studies are rather contradictory indicating that obese boys have increased (Leonard et al. 2004; Vandewalle et al. 2013), decreased (Dimitri et al. 2010; Rocher et al. 2008) or similar (Fintini et al. 2011; Hasanoglu et al. 2000) BMD values compared to normal weight peers. It can be argued that the skeleton of obese children has more density, because it has to adapt to high body mass. However, Rocher et al. (2008) found that bone mass to total body mass ratio was significantly lower in pre-pubertal obese children, compared to normal weight children, suggesting that the skeleton is not sufficiently resistant to support the higher body mass of obese children. Moreover, it has been found that overweight adolescents have greater BMC due to higher LBM, but not FM or PA level (Gracia-Marco et al. 2012). Although, a study with 4-to-20-year-old participants showed that overweight or obese children have higher BMC values, they are prone to risk of fractures because visceral FM is inversely associated with BMC (Leonard et al. 2004). Fat induced alterations in hormonal factors and different cytokines may play an important role in disturbing bone mineral accrual, thus increasing risk for bone fractures during rapid skeletal growth in pubertal period (Dimitri et al. 2012). It is needed to understand completely the role of FM on bone mineral accrual during growth and maturation to prevent fracture risks in children and adolescents and to optimize peak BMC to prevent osteoporosis in later years.

Underweight boys have been characterized by lower BMD compared to their normal weight peers and are at an increased risk of fractures (Galusca et al. 2008). Usually, studies have examined differences in BMD between underweight boys attributed to diseases such as anorexia nervosa and their normal weight peers (Castro et al. 2002; Misra et al. 2008). However, to our best knowledge, there are no longitudinal studies performed that have investigated bone mineral accrual during puberty in healthy underweight boys.

In conclusion, both FM and LBM contribute to the effect of body mass on bone development in pubertal boys. It appears that LBM, through muscle contractions, stimulates mechanically bone health and presents strong correlation with BMC and BMD values during puberty. Although overweight or obese children have higher BMC values, they are prone to risk of fractures during rapid skeletal growth in pubertal period.

2.3. Physical activity and bone mineral parameters in boys during puberty

Physical activity (PA) is one of the most important external factors that contribute to bone health throughout life (Fuchs et al. 2001; Jaffre et al. 2001; McKay et al. 2000; Weeks et al. 2008). Therefore, PA together with adequate diet is considered to have a greater osteogenic effect than calcium or protein intake in healthy children (Iuliano-Burns et al. 2005). Moreover, a recent study reported that the beneficial effect of PA on bone, especially high impact PA, applies to the average child and those genetically predisposed to lower adult BMD (Mitchell et al. 2016).

It is known that PA, through muscle contractions, provides bones with mechanical loading and thus stimulates skeletal adaptations (Riggs et al. 2002; Robling 2009). For skeletal adaptations, mechanical load must be of sufficient magnitude, be imposed at significant rates, and be dynamic in application (Robling 2009). It is generally agreed that weight-bearing activities, such as running, stair climbing, jumping rope, racket sports and/or different ball games give the necessary amount of mechanical loading and enhance BMD in children and adolescents (French et al. 2000; Hind & Burrows 2007). Moreover, MacKelvie et al. (2002) described a possible existence of critical period for bone response to weight-bearing activities and reported the existence of peri-pubertal years as a "window of opportunity" for maximal skeletal adaptations in response to PA that can be sustained into later years. During this window of puberty, up to 30 % of total body adult bone mass is gained (Bailey et al. 2000). Therefore, PA has been recommended as a possible preventive strategy against fragility fractures in older age (Karlsson et al. 2007).

To date, most of the studies that report about PA levels or PA relationships to bone mineral accrual in children and adolescents are based on information obtained through questionnaires (Carroquino-Salto 2009; Jackowski et al. 2014; Weeks & Beck 2010). Recent study showed that people tend to report through International Physical Activity Questionnaire less sedentary time and more vigorous PA compared with the accelerometer (Dyrstad et al. 2014). It is very important to objectively measure PA in children and adolescents to avoid imprecise results and understand their actual PA level (Adamo et al. 2009; Janz et al. 2006).

Physical inactivity of children and adolescents has also become a major health problem. The study of Boreham et al. (2001) reported that due to sedentary activities (television, internet, computer games) and increased motorized transport children today expend 600 kcal per day less compared to children 50 years ago. World Health Organization and The U.S. Department of Health and Human Services have released the PA guidelines for children and adolescents. According to guidelines for children and adolescents aged 5–17 years it is recommended to do at least 60 minutes of MVPA daily and at least 3 times per week include activities that strengthen muscle and bone. Unfortunately, less than one third of this age group population is able to meet the recommended PA

guidelines (Gordon et al. 2004; Nader et al. 2008). Moreover, it has been reported that globally 81% of adolescents aged 11–17 years were insufficiently active in 2010 (WHO 2015). Children have become less active with puberty, as study of Carroquinio-Salto (2009) has shown. The levels of daily MVPA declined with age: about 53% of boys and 44% of girls reported of being physically active at the recommended level when they were 11 years old but their PA level decreased to 49.8% and 36.8%, respectively, in 13-year-olds and to 43% and 28.3%, respectively, in 15-year-olds (WHO 2009). Decreased MVPA and increased sedentary time (SED) among pubertal boys (Ortega et al. 2013) is concerning knowing that SED was found to have negative influence on whole body bone mass in growing adolescents (Gracia-Marco et al. 2012). For example, it was reported that adolescents who watched television more than 3 hours/day had an increased risk of low whole body (WB) BMC (Vicente-Rodríguez et al. 2009) or the use of internet for non-study (in boys) and the time spent studying (in girls) are negatively associated with WB and femoral neck (FN) BMC, respectively (Gracia-Marco et al. 2012).

There are cross-sectional (Gracia-Marco et al. 2011; Sayers et al. 2011; Tobias et al. 2007) and longitudinal (Heidemann et al. 2013) data that have demonstrated positive associations between PA and BMD during growth and maturation in adolescents. A recent 7-years long study with school children reported that moderately intense exercise intervention enhanced gains in spine bone mass in girls and knee muscle strength in both genders (Fritz et al. 2016). Another prospective study provided evidence that active male and female adolescents showed 9–17% greater bone mineral accrual in comparison with less active adolescents (Bailey et al. 1999). Moreover, PA has been described as a positive factor for bone mineral accrual also in pre-pubertal boys (Fuchs et al. 2001; McKay et al. 2000). Study of Duckham et al. (2016) showed that higher levels of habitual PA-fitness (measured by pedometer for 7 days) were associated with gains in tibial cortical bone mass in pre-pubertal children, particularly in boys. Also a study with 9.7-to-13.9-year-old children and adolescents showed that increased time in moderate to high-level physical activity as opposed to sedentary and low-level physical activity positively affect BMC, BMD and bone area (Heidemann et al. 2013). In contrast, a study with 15.5-year-old adolescents found that only vigorous PA (VPA) is associated with increased BMC, whereas light to moderate PA had no detectable associations (Sayers et al. 2011). Moreover, Gracia-Marco et al. (2011) reported that 19 min/day of VPA increases FN_BMC and 28–32 min/day of VPA increases FN_BMD. Although high impact exercises promote bone strength, it needs to be aware that too intense training before and after puberty may negatively affect bone development (Klentrou 2016). Physical activity is also known to influence bone turnover values in adolescents (Christo et al. 2008; Eliakim et al. 1997) and it should be considered when analysing the effect of different metabolic markers on bone mineral accrual.

In conclusion, PA, especially weight-bearing exercises, during growth and maturation plays a major role in contribution to bone health. Most of the studies

that investigated the relationships of PA with bone mineral parameters are cross-sectional or without objectively measured PA. Accordingly, there is a strong need for longitudinal studies that investigate the precise amount and intensity of PA that has valuable effect on bone mineral accrual during puberty in boys. In comparison, there are many studies that are carried out on girls or elite sporting populations, while normally active adolescent boys have not been widely studied.

2.4. Bone and adipose tissue biochemical markers in relation to bone mineral parameters in boys during puberty

Bone mineral density itself may not be a sensitive measurement of bone acquisition because of rapid bone turnover and continuous change in bone geometry during childhood and adolescence (Prentice et al. 2006). To monitor bone remodeling processes it is needed to measure bone formation and resorption biochemical markers, which could be used in diagnosis, prognosis and management of bone diseases (Eapen et al. 2008). Bone turnover markers reflect both modeling and remodeling of the current bone tissue during pubertal growth and maturation (Slemenda et al. 1997). However, bone metabolism markers are not site-specific and reflect the turnover of bone tissue of the whole skeleton (Eliakim et al. 1997).

One of the most reliable bone formation markers is OC, which increases during growth spurt and provides data about bone metabolism (Sen et al. 2000). Among bone resorption makers, CTX is a degradation product of type 1 collagen and is recommended to use as a reference standard marker for bone resorption (Vasikaran et al. 2011). There are uncertainties in the findings regarding OC and CTX associations with bone mineral accrual during pubertal growth and maturation (Alghadir et al. 2015; Chahla et al. 2015; van Coeverden et al. 2002; Gracia-Marco et al. 2011; Mora et al. 1999; Silva et al. 2011; Yilmaz et al. 2005). Previous studies have reported negative associations between OC (Mora et al. 1999; Silva et al. 2011), CTX (Silva et al. 2012) and BMC (Mora et al. 1999) or BMD (Silva et al. 2012) in male adolescents. Two studies have found no associations (Gracia-Marco et al. 2011; Yilmaz et al. 2005) between OC (Gracia-Marco et al. 2011; Yilmaz et al. 2005), CTX (Gracia-Marco et al. 2011) and BMD, while one semi-longitudinal study of van Coeverden et al. (2002) found positive correlations between OC and BMC values in 11–13.8 years old boys. However, longitudinal studies regarding bone formation and resorption markers take time effect also into account, which is important during sensitive periods of growth and maturation, especially during puberty. Previous studies have also used quite wide range of participants' age (from 7 to 18 years old) (van Coeverden et al. 2002; Gracia-Marco et al. 2011; Mora et al. 1999; Silva et al. 2011; Yilmaz et al. 2005) and used correlation

coefficient as a statistical method to make conclusions (van Coeverden et al. 2002; Mora et al. 1999; Silva et al. 2011; Yilmaz et al. 2005). Furthermore, there is a lack of studies regarding the influence of PA on the association between bone turnover and BMD values in children during growth and maturation. One cross-sectional study with 8–17 years old children and adolescents reported that higher PA was associated with higher OC level (Chahla et al. 2015). In addition, a study with young adult males (25–30 years old) showed that physically active subjects had significantly higher serum OC level compared with physically non-active males (Alghadir et al. 2015). However, these studies are cross-sectional and long-term conclusions cannot be made. To our best knowledge, there are no longitudinal studies conducted that have reported the associations or effect of PA for CTX in healthy boys during puberty.

Adipose tissue is not just a fat depot, it is also known to be an active contributor to whole body homeostasis (Chen et al. 2015). Accordingly, adipose tissue releases hormones such as leptin and adiponectin that have effects on bone metabolism, also adipose tissue itself influences bone metabolism through mechanical loading (Jürimäe et al. 2011; Misra & Klibanski 2013).

Higher concentration of serum leptin is related to obesity in humans (Considine et al. 1996). Moreover, studies have demonstrated that leptin may play a role in bone formation by repressing the development of osteoclasts (Holloway et al. 2002), and accordingly mediate associations between bone and adipose tissue (Jürimäe et al. 2005; Tubic et al. 2011; Vyshnevskaya & Solntsava 2011). While Roemmich et al. (2003) indicated no independent role of leptin on total or regional BMD and BMC in male and female children with different pubertal stages, Vyshnevskaya & Solntsava (2011) reported positive correlations between serum leptin and BMD in obese pre-pubertal, early pubertal, and late pubertal boys and girls. However, these correlations were more expressed in girls. Garnett et al. (2004) also found positive associations between leptin and regional BMD and BMC in adolescent boys. In contrast, do Prado et al. (2009) found inverse associations between leptin and BMD in 13–18 years old boys. However, these studies are cross-sectional and carried out with relatively small sample size (Roemmich et al. 2003) or with pre-pubertal children (Garnett et al. 2004). Accordingly, future studies with longitudinal design are needed to understand the role of FM on bone mineral accrual during rapid growth and maturation.

Adiponectin is related to glucose regulation and has an inverse correlation with obesity in humans (Oh et al. 2007). Recently, it has been reported that adiponectin has no correlations with BMD and BMC in early pubertal girls (Võsoberg et al. 2016) and adults (Jürimäe & Jürimäe 2007; Oh et al. 2005). In contrast, studies with adults have found that adiponectin is negatively associated with BMD (Jürimäe et al. 2005; Peng et al. 2008). Only few studies are carried out in adolescents. Rhie et al. (2010) and Huang et al. (2004) found no associations between adiponectin and bone mineral parameters in female adolescent (Huang et al. 2004) or pre-pubertal girls (Rhie et al. 2010). However, there are studies that report negative (Tubic et al. 2011; Vyshnevskaya &

Solntsava 2011) or positive (van Coeverden et al. 2002) associations between adiponectin and bone mineral parameters in children and adolescents. Only one longitudinal study of Sayers et al. (2011) showed an inverse association between adiponectin and bone mineral parameters in 9.9-to-15.5-year-old girls and boys. However, associations between adiponectin and bone mineral variables are less studied in boys, especially during puberty. Furthermore, the literature is also controversial about PA effect on adipocytokines, reporting no longitudinal associations with adiponectin and leptin in 5 to 8 years old children (Heidemann et al. 2013), or negative associations with leptin in 8 to 18 years old girls but not in boys (Bailey et al. 1999).

In conclusion, adipose and bone tissue biochemical markers and their relationships with bone mineral parameters are understudied in boys during growth and maturation. The used sample age differences and different methods in other studies do not allow making conclusions. Moreover, most of the studies are cross-sectional and there is a strong lack of longitudinal analysis of adipose and bone tissue biochemical markers associations with bone mineral parameters in boys during puberty.

3. AIM AND PURPOSES OF THE STUDY

The general aim of this dissertation was to evaluate possible associations between body composition, physical activity and bone and adipose tissue biochemical markers with bone mineral development in pubertal boys with different body mass and physical activity values.

The specific purposes of this study were to:

1) investigate longitudinal effect of physical activity on bone mineral parameters in boys with different body mass status during puberty (Study I);
2) examine the relationships of bone and adipose tissue biochemical markers with bone mineral parameters in early pubertal boys with different physical activity level (Study II);
3) investigate longitudinal effect of bone and adipose tissue biochemical markers and physical activity level on bone mineral parameters in boys during puberty (Study III).

4. METHODS

4.1. Participants and experimental design

In total, 206 boys aged 12–14 years old from different schools in Tartu (Estonia) and its surrounding areas took part in this longitudinal study. The boys were followed for two years and 3 measurement sessions were performed: at baseline, after 12 and after 24 months (Table 1; Figure 1).

In the first 24-month longitudinal study (Study I), the subjects (n = 206) were divided into underweight (BMI < 15.35 kg/m^2; n = 27), normal weight (BMI ≥ 15.35 – 21.22 kg/m^2; n = 133), overweight (BMI ≥ 21.22 – 26.02 kg/m^2; n = 22) and obese (BMI > 26.02 kg/m^2; n = 24) groups at the baseline. The cut-off points for BMI were set according to Cole et al. (2007).

In the cross-sectional study (Study II), the number of participants was cut to 86 due to lacking data on blood biochemical analysis. The subjects were divided into physically active (MVPA ≥ 60 min/day; n = 46) and physically non-active (MVPA < 60 min/day; n = 40) groups (Gracia-Marco et al. 2011). The cut-off points for being active and non-active were set according to Physical Activity Guidelines for children and adolescents (DHHS).

In the second 24-month longitudinal study (Study III), the number of participants was 96. Boys were put into one group and followed for changes during 24-month period. Number of participants increased in Study III, compared to cross-sectional Study II due to the longitudinal study design, which allows us to include participants who randomly missed some of the measurements.

All participants were free from present or past diseases known to affect skeletal metabolism and none of the boys were receiving medications known to affect bone. Each boy and their parents (or legal guardian) received information about the study and the procedures. The written signed informed consent was obtained from parents, while children gave the verbal assent. All procedures were reviewed and approved by the Medical Ethics Committee of the University of Tartu, Tartu, Estonia.

Table 1. Age and anthoropometric characteristics of participants in Studies I–III.

STUDY I (Longitudinal design)				
	Underweight (n = 27)	*Normal weight* (n = 133)	*Overweight* (n = 22)	*Obese* (n = 24)
Age (years)	11.7 ± 0.47	12.1 ± 0.69	11.9 ± 0.74	12.1 ± 0.95
Tanner stage 1/2/3/4/5	0/19/8/0/0	5/34/75/19/0	1/12/8/1/0	1/7/12/4/0
Height (cm)	152.0 ± 6.86	153.9 ± 8.62	154.6 ± 7.06	160.8 ± 7.46
Body mass (kg)	35.2 ± 3.98	43.6 ± 6.74	56.9 ± 5.43	75.3 ± 11.02
BMI (kg/m^2)	15.1 ± 0.59	18.3 ± 1.44	23.7 ± 1.06	28.9 ± 2.23
STUDY II (Cross-sectional design)				
	Active boys (n = 46)	*Non-active boys* (n = 40)		
Age (years)	11.8 ± 0.6	11.8 ± 0.7		
Tanner stage 1/2/3/4/5	0/15/31/0/0	0/22/18/0/0		
Height (cm)	152.6 ± 5.9	153.5 ± 7.7		
Body mass (kg)	44.9 ± 10.9	51.9 ± 20.5		
BMI (kg/m^2)	19.1 ± 4.1	21.5 ± 6.4		
STUDY III (Longitudinal design)				
	Total group (n = 96)			
Age (years)	11.9 ± 0.6			
Tanner stage 1/2/3/4/5	0/40/48/8/0			
Height (cm)	153.8 ± 7.4			
Body mass (kg)	49.3 ± 16.0			
BMI (kg/m^2)	20.5 ± 5.2			

BMI – body mass index.

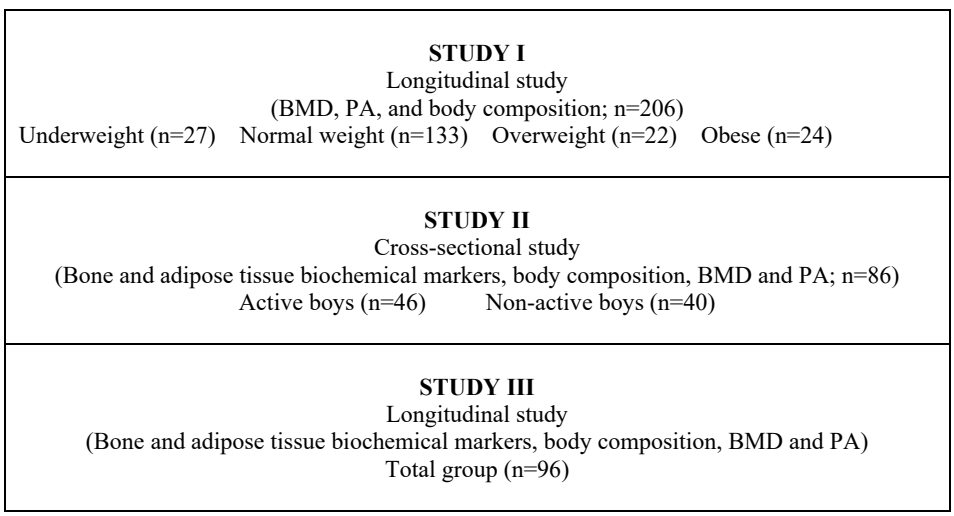

Figure 1. Tracking the study population. BMD – bone mineral density; PA – physical activity.

4.2. Anthropometry and sexual maturation

Body height (cm) was measured with a Martin metal anthropometer to the nearest 0.1 cm according to the standard technique. Body mass (kg) was measured using a medical electronic scale (A&D Instruments, Abingdon, UK) and recorded with 0.05 kg precision with the subject wearing light clothes. Body mass index (BMI; kg/m²) was calculated as body mass in kilograms divided by height in square meters. Pubertal development was assessed by self-reported questionnaire of pubertal stages according to Tanner (1962). Each boy was given line drawings, pictures and descriptions representing genitalia and pubic hair development stages. The subject had to choose the one that most closely matched his own development. In the case of discrepancies between the two variables, greater emphasis for the determination of the pubertal stage was placed on the degree of genitalia development (Duke et al. 1980). The pubertal stage assessment according to the Tanner method, which uses self-assessment of genitalia and pubic hair stage, has been previously validated (Ivuskans et al. 2013). In addition, age at peak height velocity (APHV) was assessed using gender specific anthropometric equations according to Mirwald et al. (2002). Biological age was calculated as the years from APHV (Baxter-Jones et al. 2008).

4.3. Body composition and bone mineral parameters

Whole-body (WB), lumbar spine (LS) and femoral neck (FN) bone mineral density (BMD; g/cm²) and bone mineral content (BMC; g), and body composition (FM and LBM) were measured using dual-energy X-ray absorptiometry (DXA; DPX-IQ densitometer, Lunar Corporation, Madison, USA) equipped with proprietary software. Boys were scanned in a supine position wearing light clothing. The medium scan mode and the standard subject positioning was used for total body measurements, which were analyzed using the extended analysis option. The precision of measurement expressed as coefficient of variation (CV) was less than 2% for all bone mineral and body composition measurements.

4.4. Physical activity

A uniaxial accelerometer GT1M (ActiGraph, Pensacola, USA) was used to assess physical activity. GT1M accelerometer is a small (3.8 x 3.7 x 1.8 cm) and lightweight (27 g) device that detects vertical accelerations ranging in magnitude from 0.05 to 2.00 G with a frequency response of 0.25 – 2.50 Hz. The ActiGraph accelerometer has been previously validated in laboratory and free-living conditions in children and adolescents (Freedson et al. 2005). All participants wore the accelerometer on the right hip attached by an elastic belt and adjustable buckle for 7 consecutive days. Boys were instructed to remove the

devices during showering, bathing, swimming and during sleep period. The used interval of time (epoch) was set at 15 s. Data were uploaded to a computer after the measurements and were analyzed later. At least two workdays and one weekend day of recording with minimum 8 hours/day was set as an inclusion criterion and all sequences of 10 min or more of consecutive epoch with 0 counts were removed from the analyzes (Ekelund et al. 2007). Total daily physical activity (TPA; counts/min) was calculated as the total number of counts divided by total daily registered time. The following PA thresholds were used: sedentary time (SED; < 100 counts/min), light intensity PA (LPA; 100 – 1999 counts/min), moderate intensity PA (MPA; 2000–4000 counts/min) and vigorous intensity PA (VPA; > 4000 counts/min) (Ekelund et al. 2007; van Coeverden et al. 2002). The time spent in moderate and vigorous intensity PA (MVPA; ≥ 2000 counts/min) was calculated as the sum of MPA and VPA.

4.5. Blood analysis

A 10 mL blood sample was obtained from an antecubital vein with the participant sitting in the upright position between 07.30 and – 08.30 A.M. after an overnight fast. The blood serum was separated and then frozen at –80 ^{0}C for further analysis. Leptin was determined by radioimmunoassay (RIA) (Mediagnost, Reutlingen, Germany). This assay has intra- and interassay CVs less than 5%, and the least detection limit was 0.01 ng/ml. Adiponectin was also determined with a commercially available RIA kit (cat. no. HADP-61HK; Linco Research, St. Charles, MO, USA). The intra- and interassay CVs were less than 7%, and the least detection limit was 1 µg/ml. Testosterone, osteocalcin (OC) and C-terminal telopeptide of type I collagen (CTX) were analysed using Immulite 2000 (DPC, Los Angeles, USA). The intra- and interassay CVs for OC and CTX were less than 7%.

4.6. Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics for Macintosh, version 21.0 (IBM Inc., Armonk, NY, USA) and SAS 9.2 (SAS Institute, Inc. Cary, NC, USA). Descriptive statistics were performed for all variables and were presented as means and standard deviations (± SD). Shapiro-Wilks test and q-q plots controlled normality of parameters.

Analysis of variance (ANOVA) with Tukey *post hoc* tests were used to determine:

a) differences between underweight, normal weight, overweight and obese groups at baseline for anthropometric, body composition, maturity, bone mineral and physical activity parameters (Study I);

b) differences between physically active and non-active groups for maturity, body composition, bone mineral, PA and adipose and bone tissue biochemical parameters (Study II).

Analysis of variance for repeated measures was used to determine differences between three measurement sessions (baseline, after 12 months and after 24 months) (Study III).

Partial correlation analysis was performed to assess the relationships of BMD parameters with bone and adipose tissue biochemical markers after controlling for age, pubertal stage and BMI (Study II).

Stepwise multiple regression analyses were used to identify the effects of OC, CTX, leptin and adiponectin (together with chronological age, pubertal stage, BMI and TPA) on WB, LS and FN_BMD and BMC (Study II).

For longitudinal analyses, multilevel fixed effects regression models were constructed using PROC MIXED method (SAS version 9.2) (Studies II-III). Multilevel modeling allowed us to include participants who randomly missed some of the measurements. The coefficients of fixed variables were used to predict WB_BMC/BMD, FN_BMD and LS_BMD.

A P-value less than 0.05 was considered significant for all analyses.

5. RESULTS

5.1. Physical activity and bone mineral density in pubertal boys with different body mass values (Study I)

5.1.1. Mean characteristics of the subjects according to body mass values at the first measurement occasion

Anthropometric, maturational, BMD and PA characteristics of the subjects are presented in Table 2. No difference was found in chronological age among all four groups with different body mass parameters. However, biological age in underweight boys was significantly lower compared to other subgroups ($P<0.05$). Underweight boys were significantly shorter compared to obese boys. All groups differed significantly from each other by body mass, FM and LBM ($P<0.05$). In addition, WB_BMD was significantly different in all four groups. LS_BMD was significantly lower in underweight and normal weight boys when compared to obese boys ($P<0.05$), while FN_BMD was significantly lower in underweight boys compared to normal weight and obese boys. Even though no differences were found in SED time among all groups of boys, underweight and normal weight boys showed higher MVPA and VPA levels compared to obese boys ($P<0.05$) (Table 2).

Table 2. Mean (± SD) characteristics of the subjects at baseline measurement.

	Underweight (n = 27)	*Normal weight* (n = 133)	*Overweight* (n = 22)	*Obese* (n = 24)
Age (years)	11.7 ± 0.47	12.1 ± 0.69	11.9 ± 0.74	12.1 ± 0.95
Biological age	-2.03 ± 0.48^{bcd}	-1.50 ± 0.73^{ad}	-1.39 ± 0.54^{ad}	-0.66 ± 0.78^{abc}
Tanner stage I/II/III/IV/V	0/19/8/0/0	5/34/75/19/0	1/12/8/1/0	1/7/12/4/0
Height (cm)	152.0 ± 6.86	153.9 ± 8.62	154.6 ± 7.06	160.8 ± 7.46^{abc}
Body mass (kg)	35.2 ± 3.98^{bcd}	43.6 ± 6.74^{acd}	56.9 ± 5.43^{abd}	75.3 ± 11.02^{abc}
BMI (kg/m^2)	15.1 ± 0.59^{bcd}	18.3 ± 1.44^{acd}	23.7 ± 1.06^{abd}	28.9 ± 2.23^{abc}
FM (kg)	4.4 ± 1.17^{bcd}	7.9 ± 2.78^{acd}	18.6 ± 4.28^{abd}	30.9 ± 6.54^{abc}
LBM (kg)	28.7 ± 3.52^{bcd}	33.3 ± 5.97^{ad}	34.5 ± 5.08^{ad}	40.8 ± 6.36^{abc}
LBM/height (kg/cm)	0.18 ± 0.143^{bcd}	0.21 ± 0.212^{acd}	0.23 ± 0.271^{abd}	0.25 ± 0.289^{abc}
WB_BMD (g/cm^2)	0.932 ± 0.053^{bcd}	0.979 ± 0.057^{ad}	1.001 ± 0.047^{ad}	1.056 ± 0.067^{abc}
LS_BMD (g/cm^2)	0.796 ± 0.088	0.829 ± 0.098	0.849 ± 0.094	0.898 ± 0.084^{ab}
FN_BMD (g/cm^2)	0.852 ± 0.077^{bd}	0.906 ± 0.099	0.913 ± 0.092	0.938 ± 0.088
SED (min/day)	416.0 ± 54.2	410.5 ± 65.5	406.1 ± 72.9	411.7 ± 62.9
MVPA (min/day)	64.1 ± 34.8	60.6 ± 25.8	50.6 ± 15.2	40.9 ± 25.8^{ab}
VPA (min/day)	15.3 ± 16.1	12.8 ± 11.7	7.2 ± 4.6	5.1 ± 7.7^{ab}

[a] Significantly different from underweight boys; [b] significantly different from normal weight boys;
[c] significantly different from overweight boys; [d] significantly different from obese boys ($P<0.05$); Biological age is years from age at peak height velocity; BMI, body mass index;

FM – fat mass; LBM – lean body mass; LBM/height – lean body mass and height ratio; WB_BMD, whole body bone mineral density; FN_BMD, femoral neck bone mineral density; LS_BMD, lumbar spine bone mineral density; SED, sedentary time; MVPA, moderate and vigorous physical activity; VPA – vigorous physical activity.

5.1.2. Longitudinal effect of body composition and physical activity on bone mineral parameters

Subjects varied significantly at each measurement occasion in their level of WB_BMD (P<0.001), FN_BMD (P<0.001) and LS_BMD (P<0.001) (Tables 3 and 4). Multilevel models indicated that biological age, height and LBM had significant effect for explanation of WB_BMD, FN_BMD and LS_BMD in pubertal boys with different body composition values. However, the effect of LBM on WB_BMD was not seen, but very close of being significant in the model if testing for the effect of SED time and VPA (P=0.050; Table 5). SED time and MVPA (P=0.002 and P=0.006, respectively; Table 3) and SED time and VPA (P=0.001; P<0.001, respectively; Table 4) had a significant effect only in the explanation of FN_BMD. However, time (testing at baseline, after 12 months and after 24 months) had no effect on bone mineral parameters.

The group effect was significant only for WB_BMD (estimates = 0.02520; P<0.001). Because four different BMI groups form group effect, we had to run further mixed procedure (Table 5) to find which groups vary differently from each other. The further mixed procedure indicated that there was no significant difference for longitudinal model effect on WB_BMD between normal weight and overweight groups (P=0.084), but the rest grouping had significant differences for longitudinal model effect on WB_BMD.

The significant variance of biological age suggests that with the increasing biological age, boys varied by the changes in WB_BMD, FN_BMD and LS_BMD. The tendency of variance could be seen in Figure 2, which presents changes of WB_BMD during puberty, where 0 is APHV. We used the third year data to present this variance of changes and it indicated that all obese boys reached their APHV of being approximately 14 years old (variation range of biological age was 0 - 3 APHV for obese boys). While other groups had greater range of variation, their biological age varied from –2 to 3 APHV.

SED time increased for all body composition groups during the 24-month study period. In addition, a significant decrease was seen in light PA at all measurement points. No change was seen in MPA, except for underweight boys where MPA had decreased at 24-month follow-up compared to baseline. Vigorous PA increased significantly for all weight groups (except for underweight boys) at 24 months follow-up compared to baseline.

Table 3. Multilevel regression model for WB_BMD, FN_BMD and LS_BMD controlling for biological age, height, lean body mass and testing for SED and MVPA.

Variables	WB_BMD		FN_BMD		LS_BMD	
Fixed effect	Estimates ± SE	P value	Estimates ± SE	P value	Estimates ± SE	P value
Intercept	2.3717 ± 0.2727	<0.001	1.8821 ± 0.5465	<0.001	2.9526 ± 0.4983	<0.001
Time	0.002705 ± 0.0036	0.459	-0.00057 ± 0.0066	0.931	0.000131 ± 0.0064	0.983
Biological age (years)	-0.01940 ± 0.0034	<0.001	-0.01412 ± 0.0068	0.042	-0.02969 ± 0.0063	<0.001
Biological age^2 (years^2)	0.000065 ± 0.00001	<0.001	0.000048 ± 0.00002	0.032	0.000099 ± 0.00002	<0.001
Height (cm)	0.02135 ± 0.0057	<0.001	0.02826 ± 0.0108	0.010	0.03115 ± 0.01032	0.003
Lean body mass (kg)	0.001090 ± 0.0005	0.049	0.002926 ± 0.0010	0.006	0.004438 ± 0.0010	<0.001
SED (min/day)	-0.00001 ± 0.00001	0.528	-0.00010 ± 0.00003	0.002	-0.00001 ± 0.00003	0.871
MVPA (min\day)	0.000049 ± 0.00006	0.421	0.000331 ± 0.0001	0.006	0.000015 ± 0.0001	0.892
Group	0.02520 ± 0.0043	<0.001	0.007300 ± 0.0073	0.321	0.001637 ± 0.0071	0.818

SE – standard error. Biological age – years from age at peak height velocity (APHV). WB – whole body; FN – femoral neck; LS – lumbar spine; BMD – bone mineral density; SED – sedentary time. MVPA – moderate to vigorous physical activity. Group effect is four different groups according to baseline BMI.

Table 4. Multilevel regression model for WB_BMD, FN_BMD and LS_BMD values when controlling for biological age, height, lean mass and testing for SED and VPA.

Variables	WB_BMD		FN_BMD		LS_BMD	
Fixed effect	Estimates ± SE	P value	Estimates ± SE	P value	Estimates ± SE	P value
Intercept	2.3514 ± 0.2715	<0.001	1.8034 ± 0.5430	<0.001	2.9229 ± 0.4971	<0.001
Time	0.002310 ± 0.0036	0.528	-0.00285 ± 0.0066	0.666	-0.00036 ± 0.0064	0.956
Biological age (years)	-0.01912 ± 0.0034	<0.001	-0.01289 ± 0.0068	0.061	-0.02939 ± 0.0063	<0.001
Biological age^2 (years^2)	0.000064 ± 0.00001	<0.001	0.000043 ± 0.00002	0.049	0.000098 ± 0.00002	<0.001
Height (cm)	0.02115 ± 0.0057	<0.001	0.02823 ± 0.0107	0.009	0.03055 ± 0.01029	0.003
Lean body mass (kg)	0.001078 ± 0.0005	0.050	0.002920 ± 0.0010	0.005	0.004354 ± 0.0010	<0.001
SED (min/day)	-0.00001 ± 0.00002	0.519	-0.00010 ± 0.00003	<0.001	-0.00001 ± 0.00003	0.936
VPA (min\day)	0.000214 ± 0.00013	0.108	0.001033 ± 0.00025	<0.001	0.000265 ± 0.0002	0.284
Group	0.02574 ± 0.0043	<0.001	0.009473 ± 0.0073	0.196	0.002946 ± 0.0071	0.679

SE – standard error. Biological age – years from age at peak height velocity (APHV). WB – whole body; FN – femoral neck; LS – lumbar spine; BMD – bone mineral density; SED – sedentary time. VPA – vigorous physical activity. Group effect is four different groups according to baseline BMI.

Table 5. Differences between groups on the effect of model to WB_BMD.

Effect	Group	Group	Estimates ± SE	P value
Group	Underweight	Normal weight	-0.03831 ± 0.01187	<0.001
Group	Underweight	Overweight	-0.06080 ± 0.01615	<0.001
Group	Underweight	Obese	-0.1056 ± 0.01577	<0.001
Group	Normal weight	Overweight	-0.02250 ± 0.01294	0.084
Group	Normal weight	Obese	-0.06726 ± 0.01247	<0.001
Group	Overweight	Obese	-0.04476 ± 0.01660	0.008

WB_BMD – whole body bone mineral density.

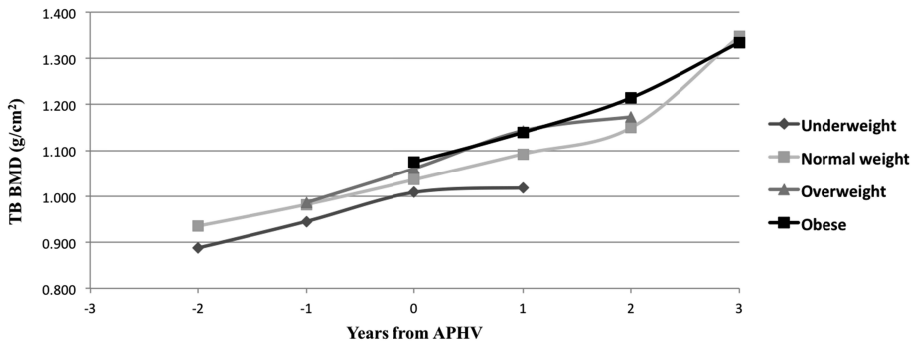

Figure 2. Whole/total body (TB_BMD**)** changes during pubertal growth spurt. Comparing four different groups – underweight, normal weight, overweight and obese pubertal boys. APHV – age at peak height velocity.

5.2. Bone and adipose tissue biochemical markers and physical activity in relation to bone mineralization (Studies II and III)

5.2.1. Body composition, bone mineral parameters, physical activity and blood biochemical characteristics in physically active and non-active boys (Study II)

There were no differences in chronological age and height between physically active and non-active groups that were categorized according to MVPA (Table 6). Non-active subjects had significantly higher ($P<0.05$) body mass, BMI, and FM. No differences were found in LBM between two groups. Estimated WB and LS bone mineral parameters (BMC, BMD) were not different ($P>0.05$) between active and non-active subjects. However, active subjects had higher FN_BMD ($P<0.05$). Active subjects had lower leptin levels ($P<0.05$), while testosterone, adiponectin and bone metabolism markers (OC and CTX) did not differ ($P>0.05$) between the groups. Active group accumulated 554.6 counts/ min and were 82.5 min/day in MVPA compared to non-active group 340.2 counts/min and 45.2 min/day, respectively. VPA was significantly higher and SED significantly lower in active group of boys ($P<0.05$).

5.2.2. Relationships of body composition and bone and adipose tissue biochemical markers with bone mineral parameters in physically active and non-active boys (Study II)

OC and CTX were correlated with WB_BMC and LS_BMC in active pubertal boys ($P<0.05$). In contrast, there were no correlations of bone mineral values

with OC and CTX while controlled for age, pubertal stage and BMI in non-active group. From two body composition parameters (FM and LBM) only LBM showed significant association with most of bone mineral parameters in both groups of pubertal boys (Table 7).

Stepwise linear regression models revealed that OC contributed to the models only in physically active group and explained 6.6 % of the variance (R^2 x 100) in WB_BMC [F(5,44)=10.847; P<0.001], and 9.7 % of the variance in LS_BMC [F(5,44)=4.158; P=0.004] (Table 8).

Table 6. Mean (± SD) body composition, bone mineral parameters, physical activity and blood biochemical characteristics of the subjects.

Variable	*Active boys* (n = 46)	*Non-active boys* (n = 40)
Age (years)	11.8 ± 0.6	11.8 ± 0.7
Tanner stage 1/2/3/4/5	0/15/31/0/0	0/22/18/0/0
Height (cm)	152.6 ± 5.9	153.5 ± 7.7
Body mass (kg)	44.9 ± 10.9	51.9 ± 20.5*
BMI (kg/m^2)	19.1 ± 4.1	21.5 ± 6.4*
Fat mass (kg)	10.3 ± 7.7	16.3 ± 13.7*
Lean body mass (kg)	32.1 ± 4.5	32.8 ± 6.9
WB_BMC (g)	1650.6 ± 288.6	1696.8 ± 386.5
WB_BMD (g/cm^2)	0.973 ± 0.056	0.979 ± 0.075
LS_BMC (g)	26.3 ± 5.2	25.5 ± 6.1
LS _BMD (g/cm^2)	0.819 ± 0.088	0.821 ± 0.094
FN_BMC (g)	4.1 ± 0.5	3.9 ± 0.2
FN _BMD (g/cm^2)	0.906 ± 0.080	0.868 ± 0.086*
Testosterone (nmol/l)	3.5 ± 5.3	2.8 ± 4.2
Leptin (ng/ml)	4.9 ± 6.1	10.5 ± 12.9*
Adiponectin (μg/ml)	9.4 ± 4.4	8.6 ± 5.2
Osteocalcin (ng/ml)	106.8 ± 37.3	107.5 ± 34.2
CTX (ng/ml)	1.6 ± 0.3	1.6 ± 0.4
TPA (counts/min)	554.6 ± 106.0	340.2 ± 65.0*
MVPA (min/day)	82.5 ± 18.6	45.2 ± 10.2*
VPA (min/day)	23.6 ± 12.6	9.2 ± 4.8*
SED (min/day)	504.6 ± 62.6	575.0 ± 69.4*

BMI – body mass index; WB – whole body; LS – lumbar spine; FN – femoral neck; BMD – bone mineral density; BMC – bone mineral content; CTX – C-terminal telopeptide of type I collagen; TPA – total daily physical activity; MVPA – moderate-to-vigorous physical activity; VPA – vigorous physical activity; SED – sedentary time.
* Significantly different from active boys, (P<0.05).

Table 7. Partial correlation coefficients between measured bone mineral parameters and osteocalcin (OC), C-terminal telopeptide of type I collagen (CTX), leptin, adiponectin, FM and LBM in active and non-active pubertal boys. Controlling for age, pubertal stage, BMI and TPA.

Variable	WB_BMD (g/cm^2)		WB_BMC (g)		LS_BMD (g/cm^2)		LS_BMC (g)		FN_BMD (g/cm^2)		FN_BMC (g)	
	Active	*Non-active*	*Active*	*Non-active*	*Active*	*Non-active*	*Active*	*Non-active*	*Active*	*Non-active*	*Active*	*Non-active*
Osteocalcin (ng/ml)	0.200	0.092	0.369*	0.203	0.221	0.133	0.359*	0.065	-0.120	0.248	0.177	0.134
CTX (ng/ml)	0.285	-0.055	0.365*	0.126	0.248	0.045	0.325*	0.160	0.012	0.178	0.119	0.132
Leptin (ng/ml)	-0.002	0.001	0.157	-0.249	0.097	-0.085	0.154	-0.277	-0.126	-0.145	0.127	-0.242
Adiponectin (µg/ml)	0.211	0.182	0.164	-0.094	0.011	0.054	-0.026	-0.096	0.018	-0.003	0.125	-0.071
FM (kg)	-0.081	-0.014	0.042	-0.179	-0.058	-0.154	0.009	-0.199	0.166	-0.165	0.139	-0.210
LBM (kg)	0.475*	0.364*	0.760*	0.732*	0.490*	0.308	0.704*	0.670*	0.099	0.318	0.500*	0.595*

BMI – body mass index; WB – whole body; LS – lumbar spine; FN – femoral neck; BMD – bone mineral density; BMC – bone mineral content, CTX – C-terminal telopeptide of type I collagen; FM – fat mass; LBM – lean body mass, TPA – total physical activity.

* Statistically significant correlation: $P<0.05$.

Table 8. Stepwise multiple regression models for bone mineral density (BMD) and bone mineral content (BMC) of whole body (WB), lumbar spine (LS) and femoral neck (FN) in pubertal boys.

Active boys (n = 46)				
	β	SE	P	R^2
WB_BMD	-	-	-	-
WB_BMC	F= (5,44) = 10.847		<0.001	
Osteocalcin	340.7	137.4	0.018	0.066
LS_BMD	-	-	-	-
LS_BMC	F= (5,44) = 4.158		0.004	
Osteocalcin	7.5	3.1	0.021	0.097
LS_BMAD	-	-	-	-
FN_BMD	-	-	-	-
FN_BMC	-	-	-	-

Chronological age, Tanner stage, body mass index (BMI) and total daily physical activity (TPA) were included as covariates using enter method. Osteocalcin (OC), C-terminal telopeptide of type I collagen (CTX), leptin and adiponectin were entered into a model as independent variables using stepwise method.

5.2.3. Longitudinal changes in body composition, bone mineral parameters, physical activity and bone and adipose tissue biochemical markers in boys during puberty (Study III)

Mean pubertal stage, height, body mass, BMI, FM, LBM and all bone mineral parameters increased significantly during every measurement session (Table 9). Adiponectin concentration increased significantly after 12 months of the study, however, it decreased significantly after 24 months showing the peak concentration during the second measurement session. OC and CTX also increased significantly over the first 12 months of the study and remained significantly higher after 24 months compared with baseline level. MVPA decreased over the 24-month study period with significant decrement between baseline and after 24 months measurements. SED increased during the study period with significant increment between baseline and after 24 months measurements.

Table 9. Mean (± SD) characteristics of the subjects at every measurement occasion.

Variable	Baseline (n = 96)	After 12 months (n = 96)	After 24 months (n = 96)
Age (years)	11.9 ± 0.6	12.9 ± 0.6*	13.9 ± 0.6*♯
Tanner stage 1/2/3/4/5	0/40/48/8/0	0/17/47/22/10	0/3/24/42/25
Height (cm)	153.8 ± 7.4	161.5 ± 8.4*	168.3 ± 8.3*♯
Body mass (kg)	49.3 ± 16.0	55.9 ± 17.7*	62.1 ± 18.8*♯
BMI (kg/m^2)	20.5 ± 5.2	21.1 ± 5.3*	21.7 ± 5.3*♯
Fat mass (kg)	13.4 ± 10.5	14.7 ± 11.2*	15.1 ± 11.7*
Lean body mass (kg)	33.2 ± 6.5	38.5 ± 8.3*	43.7 ± 9.4*♯
WB_BMC (g)	1721.1 ± 384.5	1986.7 ± 474.4*	2265.9 ± 533.9*♯
WB_BMD (g/cm^2)	0.983 ± 0.069	1.018 ± 0.081*	1.060 ± 0.098*♯
FN _BMD (g/cm^2)	0.895 ± 0.086	0.940 ± 0.103*	0.985 ± 0.120*♯
LS _BMD (g/cm^2)	0.831 ± 0.097	0.890 ± 0.121*	0.966 ± 0.147*♯
Leptin (ng/ml)	7.1 ± 8.3	6.3 ± 7.2	5.4 ± 7.3
Adiponectin (μg/ml)	8.9 ± 4.7	10.5 ± 4.7*	8.3 ± 4.4 ♯
Osteocalcin (ng/ml)	110.4 ± 37.6	136.4 ± 48.0*	132.1 ± 44.6*
CTX (ng/ml)	1.6 ± 0.4	1.9 ± 0.5*	1.9 ± 0.5*
MVPA (min/day)	64.5 ± 23.5	60.4 ± 25.8	56.5 ± 26.1*
SED (min/day)	537.2 ± 72.7	556.4 ± 78.8	574.0 ± 93.2*

BMI – body mass index; WB – whole body; LS – lumbar spine; FN – femoral neck; BMD – bone mineral density; BMC – bone mineral content; CTX – C-terminal telopeptide of type I collagen; MVPA – moderate-to-vigorous physical activity; SED – sedentary time;
* Significant changes from baseline, (P<0.05);
♯ Significant changes from 12 months, (P<0.05).

5.2.4. Longitudinal effect of bone and adipose tissue biochemical markers on bone mineral parameters in boys during puberty (Study III)

Subjects varied significantly at each measurement occasion in their level of WB_BMC, WB_BMD and FN_BMD (Table 10). OC had the significant inverse effect for explanation WB_BMD (P<0.001) and LS_BMD (P=0.021) changes in pubertal boys. However, the effect of CTX was inversely significant only for FN_BMD change (P=0.011). Leptin had a significant negative effect in the explanation of WB_BMD/BMC (P=0.001), FN_BMD (P=0.002) and LS_BMD (P=0.001) changes, while the effect of adiponectin was not significant in the models. Results from Table 11 indicated that MVPA had a negative effect in the explanation for leptin (P=0.030) changes. No effect of MVPA to OC, CTX or adiponectin changes were found. Additionally, it was found that SED had no longitudinal effect to OC, CTX, leptin and adiponectin in boys during puberty (Table 11).

Table 10. Multilevel regression models for variables that contribute to the change in WB_BMC, WB_BMD, FN_BMD and LS_BMD.

Variables	WB_BMC		WB_BMD		FN_BMD		LS_BMD	
Fixed effect	Estimates ± SE	P value	Estimates ± SE	P value	Estimates ± SE	P value	Estimates ± SE	P value
Intercept	-1669.01 ± 140.83	**<0.001**	0.4690 ± 0.0267	**<0.001**	0.3354 ± 0.0508	**<0.001**	0.0754 ± 0.058	0.198
Osteocalcin	-0.6044 ± 0.3273	0.069	-0.0003 ± 0.0001	**<0.001**	0.00004 ± 0.0001	0.743	-0.0003 ± 0.0001	**0.021**
CTX	7.6320 ± 32.3889	0.814	0.0014 ± 0.0063	0.827	-0.0307 ± 0.0118	**0.011**	-0.0155 ± 0.0125	0.219
Leptin	-9.9625 ± 2.5970	**0.001**	-0.0019 ± 0.0005	**0.001**	-0.0031 ± 0.0010	**0.002**	-0.0037 ± 0.0010	**0.001**
Adiponectin	2.7653 ± 1.9866	0.168	0.0006 ± 0.0004	0.138	0.0010 ± 0.0007	0.173	0.0004 ± 0.0008	0.587

SE is standard error. BMI - body mass index; WB – whole body; LS – lumbar spine; FN – femoral neck; BMD – bone mineral density; BMC – bone mineral content; CTX - C-terminal telopeptide of type I collagen; MVPA – moderate-to-vigorous physical activity; Model was controlled by age, BMI and Tanner stage.

Table 11. Multilevel regression models for variables that contribute to the change in osteocalcin, CTX, leptin and adiponectin.

Variables	Osteocalcin		CTX		Leptin		Adiponectin	
Fixed effect	Estimates ± SE	P value	Estimates ± SE	P value	Estimates ± SE	P value	Estimates ± SE	P value
Intercept	6.713 ± 39.312	0.865	0.413 ± 0.391	0.294	1.304 ± 3.500	0.710	20.724 ± 3.725	**<0.001**
MVPA	-0.0368 ± 0.1095	0.738	-0.0012 ± 0.0011	0.304	-0.0219 ± 0.099	**0.030**	-0.01942 ± 0.0114	0.093
Intercept	-16.886 ± 38.968	0.658	0.1384 ± 0.377	0.715	-3.0598 ± 3.522	0.387	16.109 ± 3.600	**<0.001**
SED	0.0611 ± 0.0324	0.063	0.0004 ± 0.0003	0.189	0.0051 ± 0.0029	0.079	0.0066 ± 0.0033	0.052

SE – standard error; BMI – body mass index; CTX – C-terminal telopeptide of type I collagen; MVPA – moderate-to-vigorous physical activity; SED – sedentary time; Model was controlled by age, BMI and Tanner stage.

6. DISCUSSION

6.1. Longitudinal effect of physical activity on bone mineral accrual in boys with different body mass parameters during puberty

The findings of multilevel regression models for longitudinal data indicated that MVPA, VPA and SED time had an effect on FN_BMD over the 24-month period. However, no effect in the current study was found on WB_BMD or LS_BMD in boys during the puberty. Our findings confirm the results from the cross-sectional study of Kriemler et al. (2008), who also found that VPA had significant effect on FN_BMD in boys during pubertal growth spurt. Different authors have argued that FN is the skeletal site that is most responsive to PA, as loading forces have the most direct impact (Baptista et al. 2012; Ivuskans et al. 2015). Our study results also indicated a decrease in overall PA in boys at the beginning of puberty and during further maturation. Such decrease in habitual PA and increase in SED time with age is well known (Knuth & Hallal 2009). Differently from our results, Sherar et al. (2007) found the faster decrease in VPA compared to MVPA in 8-to-13-year-old children. However, in contrast, we found the increase of VPA between the time points in every BMI group. This might be due to the increase of participation in sports clubs or at higher exercise intensities during the physical education lessons.

In the current study, underweight boys had significantly lower absolute LBM values compared with normal weight, overweight and obese boys. LBM/height ratio showed that their low LBM is a result of true body composition difference rather than their shorter stature (Table 2). The results of our longitudinal study confirm the findings that LBM had a major positive effect on bone mass parameters in boys as previously reported (Gracia-Marco et al. 2012; Pietrobelli et al. 2002). We indeed found the positive effect of LBM on FN_BMD and LS_BMD parameters in pubertal boys with different body mass status. However, for underweight boys, the effect of LBM was not seen if controlling for the effects of SED time and VPA (Table 4). This might be explained by an additional loading of the LBM, as it has been found in cross-sectional design that for overweight boys even moderate PA may be significantly related to bone mineral parameters (MacKelvie et al. 2004).

In the current study to assess PA levels we used accelerometry, which could measure the amount, but not the type of PA. Although the association of activity duration has found to be greater compared to the frequency of the activity (Farr et al. 2011), there is also indication that the high strain eliciting or weight-bearing PA has a greater effect on bone mineral parameters, compared to the amount of PA (Vicente-Rodríguez 2006). At baseline only underweight and normal weight boys fulfilled the recommendation of Physical Activity Guidelines for children and adolescents to spend at least 60 min in MVPA per day (Table 2) (WHO recommendation). Longitudinal multilevel model indicated

that a boy with 60 min of MVPA had 0.00993 g/cm^2 higher FN_BMD compared to a boy of the same biological age, height and LBM, but with 30 min of MVPA a day (0.00331 x 30; Table 3). In comparison, after 24 months we had 35.4% of boys with ≤ 30 min/day and only 5.4% of boys with ≥ 60 min/day of MVPA in average. In contrast, Gracia-Marco et al. (2011) reported that more than 78 min/day of MVPA (>3 METs) or 32 min/day of VPA (>6 METs) was associated with increased FN_BMD in adolescents, however, the study of Gracia-Marco et al. (2011) used epoch of 15 sec in accelerometry, while it was 60 sec in the current study, which might estimate differently, for example, the amount of vigorous PA. Our longitudinal analysis indicated that a boy with ≥ 32 min/day of VPA had 0.02273 g/cm^2 higher FN_BMD compared to a boy of the same biological age, height and LBM, but with less than 10 min VPA a day (0.001033x32)-(0.001033x10) (Table 4). Furthermore, our data confirm the findings of Cardareiro et al. (2012) who found that 10 min/day of VPA would be expected to result in a ≈ 1% higher FN_BMD in boys. Results from our study show ≈ 1.2% higher FN_BMD in boys with every 10 min/day of VPA and ≈ 3.7% higher FN_BMD in boys with 32 min/day of VPA.

Sedentary behavior is becoming more important and interesting topic to investigate in relation to bone mineralization, as it could result a higher bone resorption leading to reduced BMC (Tremblay et al. 2008). Results from our study showed significant increase in SED time over the 24-month period in all four groups of the boys. Pate et al. (2008) reported that subject has to stay in low energy expenditure zone (<1.5 MET) for at least several hours to call it sedentary behavior. The last year of the present study boys accumulated more than 9 hours of SED time (studying, watching television or surfing the internet). The study of Vicente-Rodriguez et al. (2009) indicated that watching television for 3 or more hours a day increases the risk for low BMC in male adolescents, however it is important to emphasize that this association is mediated by participation in PA and authors suggest that negative consequences of sedentary behavior on adolescent bone health could be counteracted by sport participation. Similarly, Gracia-Marco et al. (2012) found that the use of the internet for non-study purposes was negatively associated with WB_BMC and FN_BMC in adolescent boys even after controlling for LBM and MVPA, also it was reported that total SED time was negatively associated with WB_BMC in boys, but after controlling for LBM the association disappeared. Our longitudinal models showed that SED time has a significant effect only on FN_BMD (Tables 3 and 4) and supports the main findings of the Gracia-Marco et al. (2012) study where they found negative association between total SED time and FN_BMC after controlling for LBM and MVPA. Our previous study regarding one year observation period indicated that SED time has an impact on bone mineral parameters, however using a longitudinal model the effect was stronger, which further indicates the need for longer observation periods in order to study the effect of sedentary time to bone mineral parameters (Ivuskans et al. 2015). Current and previously reported studies (Gracia-Marco et al. 2012; Ivuskans et al. 2015; Vicente-Rodriguez et al. 2009) provide important evidence of negative

effect of SED time on bone mineralization in adolescents. However, it is needed to carry out more longitudinal studies' to investigate the interaction between SED time and bone mass parameters.

Our presented models indicated that the group effect is not significantly important for FN_BMD and LS_BMD in boys, and that does not show differences between the groups when looking for effect of MVPA, VPA and SED time on FN_BMD or LS_BMD (Tables 3 and 4). This indicates that being underweight, normal weight, overweight or obese boy at baseline (12 years old in our case) has no effect on future (2 years of follow up) FN_BMD or LS_BMD. However, there was a significant group effect of SED time, MVPA and VPA on WB_BMD ($P<0.001$; Tables 3 and 4). Further mixed procedure showed that being an underweight boy at baseline is related to higher chance to have lower WB_BMD in the future (2 years at follow up) development compared to normal weight, overweight and obese boys (Table 3). We have recently found that the cut-offs for PA, taking into account its effect on fitness and fatness parameters should be at least 59 min MVPA including 14 min VPA (Lätt et al. 2015). As the underweight boys fulfilled the criteria for healthy PA (64.1 ± 34.8 min/day, that even included 15.3 ± 16.1 min/day for VPA), it can be considered that the PA guidelines for healthy bone development may not be sufficient for underweights. This result is very relevant, taking into account that FN is very important skeletal site for its clinical relevance to osteoporosis. Unfortunately, due to too small sample size in underweight, overweight and obese groups we could not run multilevel model for sufficient power for each group separately to check the effects of MVPA, VPA and SED time on WB_BMD. However, there was a tendency that MVPA and VPA had a significant effect on WB BMD in overweight boys (data not shown). In underweight and normal weight boys, only LBM had a strong positive effect on WB_BMD and in obese boys nor LBM, MVPA or SED had an effect on WB_BMD (data not shown).

Our research has some limitations. Our major limitation is a relatively small number of subjects in underweight, overweight and obese groups. Because of that we could not run group-based longitudinal multilevel models for sufficient power. Further, the use of 1-min epoch in accelerometry might have some effect on short bouts of VPA and therefore, might probably underestimate the amount of VPA (Welk 2002). However, the strength of the current study is the relatively long investigation period that covers two years during the puberty ending with PHV with objective measures of PA and BMD.

In conclusion, LBM had a positive significant effect on WB_BMD, FN_BMD and LS_BMD in boys during pubertal growth spurt. MVPA, VPA and SED time had significant effect only on FN_BMD. Being an underweight boy at the baseline indicated greater chance to have lower WB_BMD in the future (2 years at follow up) development, compared to normal weight (estimates=−0.038), overweight (estimates=−0.061) and obese boys (estimates= −0.106).

6.2. Bone and adipose tissue biochemical markers in relation to bone mineral parameters in early pubertal boys with different physical activity

In the present study, possible differences in the associations of bone and adipose tissue biochemical markers with bone mineral parameters were studied in early pubertal boys with different PA level. The main finding was that OC and CTX were significantly associated with bone mineral values in physically active boys, while these associations were not found in non-active boys. Physical activity has been described as a positive factor for bone mineral accrual in pre-pubertal (Fusch et al. 2001; McKay et al. 2000), pubertal (McKay et al. 2000; Tobias et al. 2007) and adolescent (Gracia-Marco et al. 2011; Sayers et al. 2011; Weeks et al. 2008) boys that was also the case in our study, where significantly higher FN_BMD was seen in active boys compared with non-active boys. Leptin and FM, but not LBM were significantly lower in active boys, while there was no relation in adipocytokines and bone mineral values. These results rather demonstrate that it is PA that triggers the main effect on bone mineralization during puberty, however the cross-sectional design limits the conclusions.

It appears that early pubertal boys who are regularly active accumulating at least 60 min of MVPA daily present significantly higher VPA and FN_BMD and significantly lower SED time, FM and leptin values in comparison with non-active boys (Table 6). It was reported by Gracia-Marco et al. (2011) that 19 min/day of VPA increases FN_BMC and 28–32 min/day of VPA increases FN_BMD; accordingly active boys in our study accumulated 23.6 min/day of VPA.

Our study reports a positive association of OC and CTX with WB and LS_BMC in active 11–13-year-old early pubertal boys confirming the results of van Coeverden et al. (2002) who also found positive correlation between OC and BMC (LS, FN and WB) values in 11–14 years old boys. According to these results, it could be argued that bone turnover markers are more related to trabecular bone. In contrast, Mora et al. (1999) found negative association between OC and cortical BMD in 7–18 years old boys and girls. Moreover, Silva et al. (2011) reported negative correlation of serum OC and CTX with BMD (different evaluated locations) in a heterogeneous group of 10–18 years old boys. However, results from non-active group showed no relationship between OC and CTX with bone mineral parameters in early pubertal boys (Table 7) indicating the importance of PA. In addition, FN_BMD, which is the skeletal site that is most affected by mechanical loading during PA, was significantly higher in active early pubertal boys compared with non-active boys. Accordingly, it could be argued that PA is an important cofactor mediating the effect of circulating OC and CTX concentrations on bone mineral parameters. This could also be the explanation why regression analysis indicated osteocalcin as bone formation marker to be most significant in active boys during early puberty (Table 8).

Previous cross-sectional study of Gracia-Marco et al. (2011) reported that OC and CTX concentrations have no association with bone mineral parameters in adolescent boys. Although PA was entered into the model as independent variable (Gracia-Marco et al. 2011), there were still some differences from our study that could have impact for the different results. Sample size consisted of only 25 boys whose age varied largely from 12.5 to 17.5 years. In contrast, our data is from a relatively narrow age range and maturity group of early pubertal boys (pubertal stages 2 and 3). It has also been reported that OC and CTX levels were lower in older and more sexually matured groups, compared to younger groups of children (van Coeverden et al. 2002) and that peak concentrations of OC and CTX appear at pubertal stage 3 (Yilmaz et al. 2005) or 4 (van Coeverden et al. 2002; Gracia-Marco et al. 2010) in boys. This could explain OC contribution to WB_BMC, and LS_BMC in active group, where more boys were in pubertal stage 3 (31 vs. 18), compared to non-active group. To our best knowledge, this is the first study that indicates the importance of PA and pubertal stage in contribution of bone metabolism markers (OC and CTX) to bone mineral parameters in early maturing boys. Our study indicated that the relationship between bone turnover markers and bone mineral parameters were not uniform in boys with different PA level during early puberty (pubertal stages 2 and 3). Puberty demands to conduct specific studies, with relatively low variation in age and maturity, and analysis procedures. To date, there is still a lack of studies regarding the influence of specific bone turnover markers on bone mineral acquisition during puberty. Especially, longitudinal studies are required to clarify the role of bone turnover markers and PA on bone mineral acquisition in boys entering puberty.

It is well known that in addition to mechanical loading that is caused by PA on bone development, body mass plays a major role in changes in BMD (Fuchs et al. 2001). Therefore, we included BMI into the statistical analyses. Adipose tissue produces different adipocytokines such as leptin and adiponectin, which participate in energy homeostasis (Jürimäe et al. 2011, 2015) and may contribute to the development of bone mineral parameters in boys during growth and maturation (Luo et al. 2006; Sayers et al. 2010; Vyshnevskaya & Solntsava 2011). Our study demonstrated no associations between leptin and adiponectin with bone mineral parameters in pubertal boys with different PA levels, supporting the results of Huang et al. (2004) and Rhie et al. (2010). However, there are studies that report negative (Sayers et al. 2010; Tubic et al. 2011; Vyshnevskaya & Solntsava 2011) or positive (Misra et al. 2007) associations between adiponectin and bone mineral parameters in children (Sayers et al. 2010; Tubic et al. 2011) and adolescents (Misra et al. 2007). Accordingly, although our results demonstrated no associations between adipocytokines and bone mineral development in early pubertal boys with different PA level, further longitudinal studies are needed to clarify the possible role of leptin and adiponectin on bone mineral acquisition in active boys entering into puberty as the current PA might not be very reliable regarding historic PA. This issue is

important to consider, since puberty has been reported as a period where PA sharply decreases (Ortega et al. 2013).

Our study has some limitations. A major limitation is a relatively small number of subjects in active and non-active groups. However, the number of subjects was similar to previous studies in children (Jürimäe et al. 2015; Rhie et al. 2010; Vyshnevskaya & Solntsava 2011). Further limitation is a cross-sectional study design, which cannot provide the definite information about cause and effect relationships. However, the strengths of the current study are measured biochemical markers of bone formation (OC), resorption (CTX) and adipocytokines (leptin, adiponectin). The use of accelerometer (with 15s epoch) to monitor objective PA level and the use of DXA to measure body composition add more strength to the present study.

In conclusion, PA has the important role in mediating the relationship between bone tissue biochemical markers, especially OC, and bone mineral parameters.

6.3. Longitudinal effect of bone and adipose tissue biochemical markers on bone mineralization in boys during puberty

The important finding of this study was that bone formation marker OC had an inverse longitudinal effect on changes in WB_BMD/BMC ($P<0.001$) and LS_BMD ($P=0.021$). Moreover, we found that bone resorption marker CTX had an inverse longitudinal effect on changes in FN_BMD ($P=0.011$). To our best knowledge, there are no longitudinal studies regarding the associations between bone metabolism markers and bone mineral accrual in boys during puberty.

Few cross-sectional studies have reported positive associations between OC and BMC values (van Coeverden et al. 2002), negative associations between OC and BMD values (Mora et al. 1999; Silva et al. 2011), and no associations between OC (Gracia-Marco et al. 2011; Yilmaz et al. 2005) or CTX (Gracia-Marco et al. 2011) and bone mineral values. There is only one five-year longitudinal study that was conducted with young men (18.9 years old at baseline) (Darelid et al. 2015) and reported that OC predicts increment in BMD and BMC of WB, LS and radius.

According to our longitudinal results, it could be suggested that bone formation marker (OC) and bone resorption marker (CTX) negatively affect bone mineralization in healthy pubertal boys. The different results between our longitudinal study and that of Darelid et al. (2015) could be explained by the different age range of studied males. It has been suggested previously that while bone turnover markers are higher in early puberty, greater bone mineral accrual occurs later puberty when markers are declining (Alghadir et al. 2015; Slemenda et al. 1997). Specifically, it has been reported that lower concentrations of bone formation and resorption markers predict increased BMD values in

children during the development of peak skeletal mass (Slemenda et al. 2007). Cross-sectional study with 12.5–17.5 year-old adolescents supports our findings and reports lower bone formation and resorption markers in older adolescents compared to younger ones (do Prado et al. 2009). Furthermore, decreasing bone turnover in adolescence results in longer mineralization of bone tissue during early adulthood (Alghadir et al. 2015). Finally, it is important to measure more than one bone formation and resorption markers together with densitometric parameters to better detect effects in bone growth (Alghadir et al. 2015). Accordingly, further longitudinal studies during growth and early adulthood are needed to characterize the influence of specific bone turnover markers on bone mineral acquisition.

Multilevel regression indicated that leptin has inverse longitudinal effect in the explanation of increases in WB_BMC/BMD (P=0.001), FN_BMD (P= 0.002) and LS_BMD (P=0.001). In contrast, adiponectin had no longitudinal effect in the explanation of WB_BMC/BMD, FN_BMD and LS_BMD changes in healthy boys during puberty. While the association between leptin and bone mineral values has previously been studied in children (Garnett et al. 2004; Huang et al. 2004; Roemmich et al. 2003; Vyshnevskaya & Solntsava 2011), there is only one longitudinal research about the associations between adiponectin and bone mineralization that reported an inverse association between adiponectin and bone mineral parameters in children (Sayers et al. 2010). However, our findings of associations between leptin and bone mineralization are similar to the results of do Prado et al. (2009) study, where inverse associations between leptin and BMD were found in 13–18 years old boys. Other cross-sectional studies have reported positive (Huang et al. 2004) or no (Roemmich et al. 2003) associations between leptin and WB or regional BMD and BMC in adolescent boys. However, these studies are cross-sectional and carried out with relatively small sample size (Roemmich et al. 2003) or with pre-pubertal children (Huang et al. 2004). Girls are more studied than boys (Rhie et al. 2010; Shimizu et al. 1997), however our study does not confirm the findings of the study with pubertal girls where no relationships between leptin and WB_BMC and WB_BMD were found (Shimizu et al. 1997). Furthermore, according to our results, it can be argued that FM, which is directly correlated with leptin in healthy subjects (Cole et al. 2012), has an inverse longitudinal effect for bone mineralization in boys during puberty. These results are in accordance with a cross-sectional study with 6-year old children, which concluded that FM is negatively associated with volumetric bone density independent of LBM (Viljakainen et al. 2011). However, a cross-sectional study with similar age range subjects (12.5–17.5 years) to our study concluded that FM is positively associated with bone mass independent of LBM, but after controlling for LBM, the associations between FM and bone mass became inverse (Ortega et al. 2013). Also a study with 7–19 years old boys and girls concluded that low or high body FM could influence skeletal development, while normal level of FM is necessary for bone health in growing children (Gracia-Marco et al. 2012). Taken together, our longitudinal study suggests that

leptin but not adiponectin significantly affects changes in bone mineral parameters (BMC and BMD) in boys during puberty.

The role of PA impact on bone mineralization during growth and maturation is well known (Gracia-Marco et al. 2011; Heidemann et al. 2013; Sayers et al. 2011) and the decrease in MVPA and increase in SED during puberty has been reported before (Riddoch et al. 2009). Increased SED among pubertal boys is concerning knowing that SED was found to have negative influence on whole body bone mass in growing adolescents (Jiménez-Pavón et al. 2012). Our longitudinal study also reports the significant decrease in MVPA and significant increase in SED over the 24-month period in boys during puberty.

The effect of PA on bone turnover markers during pubertal maturation is highly understudied (Chahla et al. 2015; Christo et al. 2008; Eliakim et al. 1997). While the association between PA and OC has been studied in young adult males (Alghadir et al. 2015), to our best knowledge, there are no studies that reported the longitudinal associations of the effect of PA on CTX in adolescents. While a cross-sectional study with 8–17-year-old children and adolescents reported that higher PA is associated with higher OC level (Chahla et al. 2015), our longitudinal study did not find the longitudinal effect of MVPA or SED to OC after controlling for age, BMI and pubertal stage. Such disagreement could be a result of different study design (cross-sectional versus longitudinal), applied PA measurement methods (questionnaire versus accelerometer) and sample age, whereas we conducted a longitudinal study with objectively measured PA and our sample age range was very narrow during puberty. In addition, our longitudinal analysis showed that MVPA and SED did not influence changes in CTX in boys during puberty. However, further longitudinal studies with adolescents are necessary to look for a longitudinal effect of habitual or special physical training exercises on changes in bone turnover markers during puberty.

In agreement with results of a longitudinal study with 5-year old boys at baseline (Metcalf et al. 2009), our study found no associations between objectively measured MVPA and adiponectin in boys during puberty. However, we found a significant longitudinal effect of MVPA for leptin ($P=0.030$). Such results were highly expected as it is known that PA level is associated with lower FM in children and adolescents (Riddoch et al. 2009). The influence of PA on leptin level in children and adolescents is controversial. The study of Romon et al. (2004) found no associations between leptin and PA in a group of 8–18 years old males; while in females such associations were found, however, the PA was not measured objectively, authors used questionnaires and sample size was with a wide range in age. Our study supports the results of Jiménez-Pavón et al. (2012), which reported that PA decreases the level of leptin circulation in 12.5–17.5 years old male and females. Accordingly, our longitudinal study reports that MVPA has an effect in the explanation of leptin, suggesting that MVPA, but not SED is an important factor that reduces the excess amount of FM and further reduces the negative influence of FM on bones in healthy pubertal boys.

The major strength of our study is a longitudinal design (24-month observation period with 3 measurement sessions); other strengths of the current study are the measured biochemical markers of bone formation (OC), resorption (CTX) and adipocytokines (leptin, adiponectin). The use of accelerometer with 15s epochs to objectively monitor PA level and the use of DXA to measure body composition add more strength to the present study. However, a relatively small sample size (96 pubertal boys) is a major limitation to our study.

In conclusion, bone formation (OC) and bone resorption (CTX) markers negatively affect bone mineralization in healthy boys during puberty. Leptin, but not adiponectin was inversely associated with BMC and BMD increment. Finally, MVPA negatively influenced leptin level in pubertal boys indicating a strong effect against adolescents' obesity problem.

7. CONCLUSIONS

1) Lean body mass has a positive longitudinal effect on bone mineral values in boys during puberty. Being an early pubertal underweight boy at the baseline indicates greater chance to have lower bone mineral density during future pubertal development compared to normal weight, overweight and obese boys;
2) Physical activity has an important role in mediating the relationship between bone and adipose tissue biochemical markers and bone mineral parameters in early pubertal boys;
3) Bone formation and resorption markers are negatively associated with bone mineralization in boys during puberty. Leptin, but not adiponectin is inversely associated with bone mineralization in boys during puberty.

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SUMMARY IN ESTONIAN

Muutused luutiheduses puberteedieelistel poistel: seosed keha koostise, kehalise aktiivsuse ja luukoe ning rasvkoe biokeemiliste näitajatega

Sissejuhatus

Kehaline inaktiivsus ja rasvumine on kujunenud rahvatervise seisukohalt ülemaailmseks probleemiks, omades sealhulgas mõju ka organismi luukoe arengule. Eriti ulatuslik luukoe juurdekasv toimub organismis puberteediperioodi jooksul, mistõttu võib seda perioodi pidada eriti kriitiliseks hilisema luukoe tervise suhtes. Mitmetes uuringutes on leitud, et luukoe arengut mõjutavad nii keha koostise ja kehalise aktiivsuse, kui ka vere biokeemilised näitajad. Seega on oluline uurida, millised faktorid mõjutavad luukoe juurdekasvu just sellel kriitilisel kasvuperioodil. Meile teadaolevalt puuduvad varasemad uuringud luukoe juurdekasvu määramisel, kus hinnatakse erinevate keha koostise, kehalise aktiivsuse ja luukoe ning rasvkoe vere biokeemiliste markerite pikaajalist mõju puberteedieelistele poiste luukoe arengule.

Uurimustöö eesmärk ja ülesanded

Antud uurimistöö eesmärgiks oli hinnata erinevate keha koostise, kehalise aktiivsuse ning luukoe ja rasvkoe biokeemiliste markerite seoseid luukoe juurdekasvuga erineva keha koostise ja kehalise aktiivsusega puberteedieelistel poistel. Lähtuvalt eesmärgist olid uurimistöö ülesanneteks:

1) uurida kehalise aktiivsuse pikemaajalist mõju luutiheduse suurenemisele puberteediperioodil erineva kehamassiga poistel;
2) uurida luukoe ja rasvkoe biokeemiliste markerite seoseid luutihedusega erineva kehalise aktiivsusega varases puberteedieas poistel;
3) uurida luukoe ja rasvkoe vere biokeemiliste näitajate ning kehalise aktiivsuse pikemaajalist mõju luutiheduse suurenemisele poistel puberteediperioodil.

Uuritavad ja metoodika

Uuringus osales kokku 206 12–14 aastast poissi Tartu linna ning ümbruskonna koolidest. Vaatlusaluseid uuriti kahe aasta jooksul ning mõõtmised toimusid uuringu alguses ning peale 12 ja 24 kuu möödumist. Kõigil uuritavatel määrati keha pikkus ja kehamass ning arvutati kehamassiindeks. Bioloogiline vanus määrati Tanneri metoodikaga. ning luuline vanus käelaba röntgeniga. Keha koostise ja luutiheduse näitajad määrati DXA meetodiga ning kehaline aktiivsus aktselerомeetritega. Samuti määrati veeniverest erinevad luukoe ja rasvkoe biokeemilised näitajad.

Järeldused

1) Keha rasvavaba mass omas pikaajalist positiivset mõju poiste luutiheduse suurenemisele puberteediperioodil. Alakaal puberteediperioodi alguses on tervise riskifaktor madalamale luutihedusele hilisemas puberteedieas võrreldes normaalkaaluliste, ülekaalulisete ja rasvunud poistega;
2) Kehaline aktiivsus mõjutab puberteediealistel poistel rasvkoe ja luukoe vere biokeemiliste markerite seost luutihedusega;
3) Luukoe vere biokeemilised näitajad on poistel puberteediperioodil negatiivselt seotud luutiheduse suurenemisega. Samuti oli leptiini kontsentratsioon veres negatiivselt seotud luutiheduse kasvuga puberteediealistel poistel.

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