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REELI TAMME

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Paper II Tamme R, Jürimäe J, Mäestu E, Remmel L, Purge P, Mengel E, Tillmann V. Physical activity in puberty is associated with total body and femoral neck bone mineral characteristics in males at 18 years of age. *Medicina (Kaunas)* 2019; 55, 203.

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In all papers, Reeli Tamme was involved in the design of the study, assessment of patients, data collection, data analysis, and writing the manuscripts.

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ABBREVIATIONS

BA	bone area
BMAD	bone mineral apparent density
BMC	bone mineral content
BMD	bone mineral density
BMI	body mass index
CV	coefficient of variation
DXA	dual energy x-ray absorptiometry
ER	estrogen receptor
FN	femoral neck
GH	growth hormone
HOMA-IR	homeostasis model assessment-insulin resistance
IGF-1	insulin-like growth factor 1
LAR	leptin to adiponectin ratio
LS	lumbar spine
MVPA	moderate-to-vigorous physical activity
PBM	peak bone mass
PA	physical activity
QCT	quantitative computed tomography
SD	standard deviation
SDS	standard deviation score
TB	total body
TB LH	total body less head
WHO	World Health Organization

1. INTRODUCTION

Osteoporosis is a major public health issue (NIH, 2000). Peak bone mass (PBM) obtained during growth and maturation is an important determinant of skeletal health in adult years (Baxter-Jones *et al*, 2011) and the strongest predictor of later life osteoporosis risk (Ferrari *et al*, 2012). Bone is a dynamic tissue that undergo dynamic changes throughout life and approximately 95% of adult bone mass is accumulated during childhood and adolescence, whereas substantial amount of bone mineral content (BMC) is laid down over the adolescent growth period (Baxter-Jones *et al*, 2011). Factors that alter bone mass accumulation growth and maturation may lead to suboptimal bone strength and higher fracture risk in late adulthood and therefore optimizing bone mineral accrual in puberty is very important for prevention of osteporosis in later life (Ferrari *et al*, 2012). Sex steroids together with growth hormone and insulin-like growth factor 1 (IGF-1) axis, 1,25-dihydroxyvitamin D are the most important hormonal and metabolic factors during that time (Ilich *et al*, 1997; Mauras, 1999; Soyka *et al*, 2000). In addition, several adipokines including leptin and adiponectin play an important role in this process (Liu *et al*, 2013).

This dissertation focuses on sex hormones and two adipokines – leptin and adiponectin, and their associations with bone mineral accrual. Testosterone is positively associated with bone mineral accrual in adolescent males (Yilmaz *et al*, 2005; Pomerants *et al*, 2007) and experimental studies have shown that administration of testosterone has positive effect on bone mineral accrual in boys with delayed puberty (Bertelloni *et al*, 1995) as well as in healthy prepubertal boys (Mauras *et al*, 1994). Nevertheless, limited longitudinal data are available in males regarding the associations between testosterone level and bone mineral parameters through and after puberty. Leptin and adiponectin have been suggested to be potentially linking fat and bone, with conflicting relationships between skeletal outcomes and leptin (do Prado *et al*, 2009; Dimitri *et al*, 2010; Vishnevskaya & Solntsava, 2011; Vaitkeviciute *et al*, 2016a) and adiponectin being reported (Huang *et al*, 2004; Misra *et al*, 2007; Gruodytė *et al*, 2010; Rhie *et al*, 2010; Sayers *et al*, 2010; Parm *et al*, 2012; Soininen *et al*, 2018). In addition to leptin and adiponectin separately, the ratio between those two adipokines has been introduced as a marker of dysfunctional adipose tissue (Frühbeck *et al*, 2017) and has proven to be a more accurate marker of obesity-related complications than adiponectin or leptin alone (Inoue *et al*, 2006; Zhuo *et al*, 2009). Further knowledge about associations between leptin to adiponectin ratio (LAR) and bone mineral characteristics in adolescence would be useful to understand the role of adipokines in bone mineral accrual during growth and maturation.

Among the other modifiable factors that affect bone mineral accrual, weight-bearing physical activity (PA) is one of the most important (Vicente-Rodríguez, 2006). Physical activity has positive influence on bone mineral accrual through impact loading and through strains exerted on bone by muscle contractions,

moreover, PA has indirect effect on bone by increasing muscle mass and hence the tensions generated on bone (Vicente-Rodríguez, 2006). Positive associations between PA and bone mineral characteristics have been found in numerous cross-sectional studies (Tobias *et al*, 2007; Kriemler *et al*, 2008; Ivuškāns *et al*, 2014; Marin-Puyalto *et al*, 2019). In line with the findings from cross-sectional studies, longitudinal studies have confirmed the positive association between PA and bone mineral characteristics (Bailey *et al*, 1999; Vicente-Rodríguez *et al*, 2004; Heidemann *et al*, 2013; Vaitkeviciute *et al*, 2014; Ivuškāns *et al*, 2015; Marin-Puyalto *et al*, 2018). However, majority of the previous studies are either based on subjective PA data or carried out in youger children, therefore further evidence is needed to understand the relationships between PA and bone mineral accrual in puberty and post-puberty.

In 2009, a cohort of healthy boys was recruited at the Institute of Sport Sciences and Physiotherapy at the University of Tartu and followed annually through puberty. As part of this dissertation we have now studied those subjects at the age of 18 years with the main goal to investigate the associations between sex hormones, adipokines and physical activity in puberty and subsequent bone mineral characteristics in those boys.

2. REVIEW OF THE LITERATURE

2.1 Peak bone mass development

Peak bone mass is the amount of bone gained by the time a stable skeletal state has been attained during young adulthood. The concept of PBM more broadly captures peak bone strength, which is characterized by mass, density, micro-architecture, microrepair mechanisms, and the geometric properties that provide structural strength (Weaver *et al*, 2016). The PBM achieved in youth is a major determinant of the future risk of fractures in the elderly (Gilsanz *et al*, 2011) and the strongest predictor of later life osteoporosis risk (Ferrari *et al*, 2012). Bone remodelling simulations have shown that a 10% increase in peak bone mineral density (BMD) is predicted to delay the development of osteoporosis by 13 years (Hernandez *et al*, 2003).

Nonmodifiable factors influencing PBM accrual include gender, ethnicity, heredity and maturation with the hormonal status related to it. Heredity exerts a strong influence on PBM (Szadek & Scharer, 2014), with an estimated 60–80 % of the variability in bone mass and osteoporosis risk being explained by heritable factors (Weaver *et al*, 2016). Inheritance is polygenic and genomic loci have been identified that predict skeletal health in adults (Nina & Gordon, 2012). Primary modifiable factors affecting PBM are diet and PA, although other lifestyle and environmental factors may also be at play (Weaver *et al*, 2016).

The bone first plateaus in area, then roughly 1 to 2 years later, plateaus in mineralization and the age at which PBM is achieved varies by sex and skeletal site (Berger *et al*, 2010; Baxter-Jones *et al*, 2011). Furthermore, estimates of the timing of PBM depend on the specific parameters of bone under consideration (Weaver *et al*, 2016). Trabecular bone and cortical bone undergo different age-related changes in bone mineral accrual and dimensions, for example at the age of attaining PBM, there still may be formation of PBM in one site, a short period of relative equilibrium between formation and resorption in another site, whereas bone loss may have started in another site (Matkovic *et al*, 1994; Szulc *et al*, 2000). In contrast, bones of the skull increase their mass throughout life (Matkovic *et al*, 1994). Certain bones, for example the femur and vertebral bodies, continue to increase in diameter as well (Katzman *et al*, 1991; Matkovic *et al*, 1994). Usually such expansion results in a decrease in true structural density (mass per unit structural volume), despite which there may be no loss, or even an actual increase in mass; however, this periosteal expansion usually results in an increase in stiffness and often in load bearing capacity of the bone (Heaney *et al*, 2000).

A longitudinal study by Baxter-Jones *et al* (2011) has estimated that the plateau in total body (TB) PBM was likely reached by 7 years after peak height velocity in both males and females, which in terms of chronological age, equated to about 18.8 and 20.5 years of age in females and males, respectively. Regarding the differences between skeletal sites, PBM in femoral neck (FN)

was achieved substantially earlier (3 years after peak height velocity) than PBM in the other sites of the skeleton (Baxter-Jones *et al*, 2011). Another longitudinal study presented similar results that hip PBM occured at an earlier age than lumbar spine (LS) PBM (Berger *et al*, 2010). However, gender differences were evident with men achieving LS PBM earlier than women, whereas PBM in hip was achieved earlier in women (Berger *et al*, 2010).

Skeleton grows in size and density during the first two decades of life, the greatest accretion of bone occurs in puberty and the accrual of bone mass in puberty is a major determinant of PBM (Saggese *et al*, 2002). Therefore, abnormal pubertal development may result in decreased bone mineralization – delayed puberty is a cause for reduced PBM in male (Finkelstein *et al*, 1992; Yap *et al*, 2004) and in female (Chevalley *et al*, 2008).

2.2 Bone development during maturation

Bone is a dynamic tissue that is constantly adapting its structure (Katsimbri, 2017). Bone modelling, the formation and shaping of bone, is the formation of bone at one site and removal of bone at another site within the same bone, resulting in bone mass increase and bone shape modification (Szadek & Scharer, 2014). Bone remodelling, the replacement or renewal of old bone at the same site, is the process to repair microdamages, renew the skeleton and thereby maintain the strength of a bone (Andersen *et al*, 2013). In remodelling resorption and formation processes are tightly coupled, and imbalance of those processes leads to bone mineral diseases such as osteoporosis when there is excess bone loss and osteopetrosis when there is excess bone formation (Andersen *et al*, 2013; Katsimbri, 2017). During normal bone modelling, bone resorption by osteoclasts and formation by osteoblasts are not tightly coupled (Katsimbri, 2017). In childhood, both bone modelling and bone remodelling occur (Katsimbri, 2017), however, from infancy through late adolescence the activity of bone formation predominates, resulting in steady accumulation of bone mass (Saggese *et al*, 2002). During the pubertal growth spurt modelling is responsible for most of the bone formation and resorption; thereafter, remodelling prevails and modelling activity gradually decreases as longitudinal growth ceases (Mora *et al*, 1999). In adults bone modelling is less frequent than bone remodelling (Katsimbri, 2017).

Bone mineral accrual takes place at different rates at different skeletal sites (Tanner *et al*, 1976). Different temporal sequence of growth in axial and appendicular skeleton is seen both in males and females, with appendicular growth being more rapid before puberty, wherease during puberty, axial growth accelerates compared to appendicular growth (Bass *et al*, 1999; Bradney *et al*, 2000). Bone mineral accrual continues even after linear growth has ceased, as up to approximately 10% of maximal observed TB BMC is accrued in late adolescence (McCormack *et al*, 2017). However, pubertal period is characterized by high fracture rate, which may result from the inability of the

mineralizaton process to keep pace with the growth in length of the long bones due to the high magnitude of the sex steroid-driven growth spurt (Saggese *et al*, 2002).

Aproximately 95 % of adult bone mass is accumulated during childhood and adolescence and maximizing bone mineral mass gain during this period of growth and maturation is essential to develop and maintain a healthy skeleton (Baxter-Jones *et al*, 2011). Puberty plays a fundamental role in the accumulation of bone mass, as substantial amount of BMC is laid down over the adolescent growth period – depending on the skeletal site, 33% to 46% of the adult BMC is accrued over the entire 5 years of adolescent growth surrounding peak height velocity (Baxter-Jones *et al*, 2011). Peak bone mineral accrual occurs at 14 years for boys and 12.5 years for girls, that is approximately half year after the age of peak height velocity (Bailey *et al*, 2000). However, most gains in bone mass during childhood and puberty are attributed to bone expansion rather than to an increase in bone mineral per unit volume (Katzman *et al*, 1991).

Bone mass values at sexual maturity are predicted by values in early puberty (Loro *et al*, 2000). However, independent of bone mass at early puberty, earlier beginning of puberty is Associated with higher PBM at skeletal maturity, while variations in pubertal length do not significantly influence bone accretion as both slow and fast sexually maturing male and female teenagers achieve similar PBM (Gilsanz *et al*, 2011).

Skeletal gender differences are not evident before puberty, but with the progression of puberty gender differences regulated mainly by sex steroids are established (Vanderschueren *et al*, 2014). Superior bone size with both a larger diameter and greater cortical thickness in the long bones in boys compared to girls during maturity has been documented (Gabel *et al*, 2017a), but it has been suggested that gender differences in BMD values are in general explained by anthropometric differences (Baxter-Jones *et al*, 2003).

There is a close association between bone mass and body mass gain during growth and maturation, and the direct pathway for body mass to influence bone is via mechanical loading and as a component of body mass, fat mass is a major contributor to this relationship (Iwaniec & Turner, 2016). In addition to mechanical loading, the influence of fat mass on bone can be mediated also via hormonal factors linked to adipose tissue (Reid, 2010). Adipose tissue has dual effects on the growing skeleton, specifically adequate adipose tissue is required for normal bone mass development, but excess adipose tissue is harmful (Viljakainen *et al*, 2011). For example, negative association has been found between bone mineral apparent density and fat mass in prepubertal children (Cole *et al*, 2012). Those associations are supported by a study demonstrating that body fat mass has a negative effect on the BMD and BMC in 6-10 year old children (Liang *et al*, 2020). Obesity and prior fracture were associated with decreased bone mass in children (Dimitri *et al*, 2010) wheras no evidence of a deleterious effect of body fat on children's growing bones was found by Streeter *et al* (Streeter *et al*, 2013) and increased bone mineral characteristics have been

found in obese boys (Leonard *et al*, 2004b; Vandewalle *et al*, 2013) and girls (El Hage *et al*, 2010).

2.3 Bone mineral characteristics

The macrostructural charateristics of the skeleton can be evaluated by using dual-energy x-ray absorptiometry (DXA), quantitative computed tomography (QCT) (including peripheral QCT, high-resolution peripheral QCT), quantitative ultrasonography, magnet resonance imaging, or X-ray films (radiogrammetry) (Baroncelli, 2008; Bachrach & Gordon, 2016). DXA is the most commonly used technique for bone mineral status assessment worldwide (Baroncelli 2008), because of its availability, feasibility, low cost, reproducibility, speed and low exposure to ionizing radiation (Crabtree *et al*, 2014).

Dual-energy x-ray absorptiometry measurements generate information about bone mass and bone area (BA) (Wasserman *et al*, 2017). The bone mass is the BMC expressed in grams, and measured calibrating the transmitted intensity of X-rays through the entire structure. BMC depends on bone length, width and density. Bone area is the projected area of the bone tissue that is expressed in cm^2. BMC and BA are used to calculate BMD, a characteristic indicating the amount of mineral in bone projected area, expressed in g/cm^2. The BMD value obtained by DXA is derived from a projected two-dimensional area of a three-dimensional bone structure, and can therefore also be called areal BMD in contrast with the true volumetric BMD (Messina *et al*, 2018). True volumetric bone density is a function of BMC per unit volume of bone and can be assessed only by QCT (Baroncelli *et al*, 1998). Because of its two-dimensional nature, which is unable to account for the depth of the bone, smaller bones appear to have a lower areal BMD than larger bones despite equal volumetric BMD (Wasserman *et al*, 2017). However, BMD is the easiest parameter to quantify bone strenght in clinical practice and it accounts for 60–80% of bone mechanical resistance (Rossini *et al*, 2016).

Bone mineral apparent density (BMAD), a characteristic calculated using DXA scan outcomes, is a considerable alternative to volumetric BMD measured with QCT, a technique known to involve high radiation exposure (Baroncelli *et al*, 1998). BMAD is defined as mineralized tissue mass per total tissue volume, expressed in g/cm^3 (Messina *et al*, 2018), and calculation of BMAD includes transformation of BA measured with DXA to estimate the volume of bone tissue to approximate the effects of bone depth and body size (Crabtree *et al*, 2014).

2.3.1 Assessment of bone mineral density in children

The use of threedimensional densitometry methods (QCT and magnet resonance imaging) in paediatric population in clinical practice is limited in large part by the lack of standardized scanning protocols and paediatric normative data

(Gordon *et al*, 2014). Therefore, DXA remains the preferred method for clinical measurements of bone density in paediatric population because of the availability of large amount of age appropriate reference data from healthy children (Crabtree *et al*, 2014).

The use of volumetric BMD could be accurate to assess BMD in paediatric patients, particularly during pubertal development when rapid growth takes place influencing significantly also the size of bones and thereby the results obtained by areal scans (Messina *et al*, 2018). In terms of, DXA there is a lack of consensus regarding the most appropriate strategy for the interpretation of two-dimensional whole body DXA BMC and BA results across children of differing body size and body composition (Leonard *et al*, 2004a). BMAD as an estimate of the volumetric BMD, is considered to be less sensitive to differences in skeletal size than areal BMD and better indicator of true bone density and bone strength than areal BMD (Cvijetić & Korsić, 2004). Other proposed strategies to minimize the confounding effects of bone size include assessing BA relative to height and BMC relative to BA (Molgaard *et al*, 1997), assessing BMC relative to height and age (Ellis *et al*, 2001), or assessing BMC relative to body weight or lean mass (Tothill & Hannan, 2002).

In adults, a T-score is reported which compares the patient's measured bone characteristis to that of healthy young adults ages 20–30 years, representing a time when PBM is reached. In paediatric population, instead, a comparison to an age, sex, and race matched reference range or Z-score is recommended, as the use of T-scores in subjects who haven't attained PBM yet would be inappropriate (Wasserman *et al*, 2017).

The preferred skeletal sites for DXA measurements are LS (L1–L4), total hip or (FN) and TB. However, in children the cranium should be excluded from the whole body scan analysis, because it comprises a relatively large portion of the skeleton that is not affected by nutritional or environmental factors such as weight-bearing activity that impact BMD throughout the rest of the body (Crabtree 2014). Moreover, bone mass in the skull changes little with growth or disease and therefore inclusion of this site could potentially mask gains or losses at other skeletal site (Taylor *et al*, 1997). DXA measurements of the hip region (total hip or FN) are not as reliable in younger patients (<13 years) because of difficulties in identifying the bony landmarks for this region of interest (Bachrach & Gordon, 2016). Although scans at 1/3 distal radius (a site of primarily cortical bone) and at ultradistal radius (a site of primarily trabecular bone) have been used in certain paediatric populations when TB less head (TB LH) and LS are not feasible, for example in children with spinal rods or severe obesity, the scans at those sites have the poorest precision compared to other measurement sites and also, reference data are limited (Kalkwarf *et al*, 2007; Ward *et al*, 2007; Wasserman *et al*, 2017).

2.4 The main hormonal determinants of bone mass accrual during maturation

Sex steroids and the growth hormone and IGF-1 axis have main roles in skeletal maturation, pubertal growth and bone and muscle mass accrual (Mauras, 1999; Soyka *et al*, 2000). In addition, 1,25-dihydroxyvitamine D has a crucial role in bone mineralization (Ilich *et al*, 1997). Among those well-known factors influencing bone metabolism, several adipokines including leptin and adiponectin play an important role in bone mineral accrual (Liu *et al*, 2013). However, as this dissertation does not study all hormonal parameters associated with bone mass accrual during puberty, we are focusing here on sex hormones and two adipokines – leptin and adiponectin, and their associations with bone mineral accrual.

2.4.1 Sex hormones

Peak bone mass accrual is influenced by pubertal events (NIH, 2000), where the increased concentrations of sex steroids play a key role in augmented bone growth. Not only sex steroid receptors, but also all the necessary enzymes to convert the adrenal sex steroid precursors into active androgens and estrogens, are expressed in bone tissue and osteoblast cell lines, supporting the notion of local skeletal paracrine and/or intracrine synthesis and action of sex steroids (Vanderschueren *et al*, 2014). Sex hormones also control the musculoskeletal sexual dimorphism through fundamental signaling pathways including growth-hormone and IGF axis (Ohlsson *et al*, 1998; Vanderschueren *et al*, 2014); parathyroid hormone and vitamin D as regulators of calcium homeostasis (Bouillon *et al*, 2013); modulation of RUNX2, a known transcription factor in osteoblast and chondrocyte differentiation (Frenkel *et al*, 2010); Wnt signaling and sclerostin (Ke *et al*, 2012); response to physical loading (Vanderschueren *et al*, 2014; Almeida *et al*, 2017). In addition, it is proposed that that sex steroids might have indirect effects on bone via other tissues like muscle or adipose tissue, as well as via nervous system, oxydative stress and immune system (Vanderschueren *et al*, 2014). During adolescence, sex steroids are involved in modelling of bone and hypogonadism has adverse effects on the attainment of PBM both in men and women (Compston, 2001). Declines in sex steroid levels during young and middle adulthood associate with changes in bone mass and sizein healthy men

Testosterone is the predominant gonadal androgen in men (Morales, 2011). Testosterone acts directly binding to androgen receptors or through dihydrotestosterone, an active form of testosterone converted from testosterone in peripheral tissues. Additionally, testosterone is aromatized into estradiol, and therfore it acts also via estrogen receptors (ER) (Vanderschueren *et al*, 2014). The importance of aromatization in bone metabolism is shown in an animal model of aromatization deficency, an aromatase knockout mice, who have clearly reduced trabecular bone volume and increased endocortical bone resorption

(Matsumoto *et al*, 2006). On the other side, transgenic male mice over-expressing human aromatase have an increased bone mass (Peng *et al*, 2004). 17-β-estradiol is a major conadal hormone in female that act upon binding either of the two isoforms of estrogen receptors, ERα and ERβ (Hammes & Levin, 2019).

Sex steroids shorten the lifespan of osteoclasts and prolong the lifespan of osteoblasts by exerting pro-apoptotic effects on osteoclasts and antiapoptotic effects on mature osteoblasts (Pederson *et al*, 1999; Chen *et al*, 2001; Almeida *et al*, 2017). For example, studies in ovariectomized rodents have demonstrated an increase in the proliferation and differentiation of osteoclast precursors (Jilka *et al*, 1992). In addition to the direct effect on different bone cells, estrogen increases expression of the receptors for 1,25-dihydroxyvitamin in osteoblasts (Liel *et al*, 1992) and positively modulates growth hormone action as well as growth hormone receptor expressionin rat osteosarcma cells and human osteoblast-like cells (Slootweg *et al*, 1997). Both estrogen and androgen suppress the production of IL-6, a osteoclastogenic cytokine produced by cells of the osteoblast lineage and via this indirect mechanism inhibit osteoclast formation and bone resorption (Lin *et al*, 1997).

The effects of androgens and estrogens on the trabecular and cortical bone compartment are mediated via different cell types. The antiresorptive effects of androgens on tarbecular bone are exerted indirectly via osteoblasts and osteocytes, whereas removal of the androgen recpetor from bone-forming cells induced no changes in cortical bone in either sex, indicating that androgens attenuate osteoclast numbers in trabecular bone indirectly (Määttä *et al*, 2013). In regard to estrogen, antiresorptive effects of estrogens on trabecular bone result from direct actions on osteoclasts, whereas in mouse models with targeted deletion of ERα, the loss of bone mass at the endocortical surfaces following ovariectomy is unaffected, indicating that the antiresorptive actions of estrogens on cortical bone is not the result of direct actions on osteoclasts (Nakamura *et al*, 2007).

The results of cross-sectional study concluded that serum testosterone concentration was the most important biochemical predictor of BMD in 60 healthy non-obese schoolboys aged between 10–18 years (Pomerants *et al*, 2007). Similarly, positive correlation has been found between serum testosterone level and TB and LS BMD values in boys (Yilmaz *et al*, 2005). Low BMD has been associated with testosterone deficiency in men with hypogonadotropic hypogonadism (Finkelstein *et al*, 1987). Furthermore, in boys with reduced BMD resulting from delayed puberty, increases in BMD were reported in response to testosterone therapy (Bertelloni *et al*, 1995). Moreover, short-term testosterone administration has shown to increases bone calcium accretion in healthy prepubertal boys (Mauras *et al*, 1994). In addition to positive effect on skeletal health among males, androgens have shown to have positive affect on the bone mineral accrual in women, for example supra-physiological levels of endogenous androgens are associated with increased trabecular bone density in young women (Buchanan *et al*, 1988).

However, it is still uncertain to what extent the skeletal effects of androgens are mediated through testosterone or dihydroteststrone and what part of that through aromatization to estrogens (Compston 2001). A study in a man with concomitant aromatase deficiency and mild hypogonadism, in whom adding testosterone to estradiol replacement increased BMD, volumetric BMD, cortical thickness, periosteal expansion, and normalized bone turnover, further emphasises the actions of androgens independent of aromatization (Rochira *et al*, 2007). Regarding the effect of estrogen in male skeletal development, low BMD seen in men with estrogen resistance (Smith *et al*, 1994) or aromatase deficiency (Morishima *et al*, 1995), indicates that estrogens also play an important role. Accordingly, findings from a cross-sectional study in healthy boys aged between 6–9 years indicating that circulating estradiol is positively associated with bone maturation and areal BMD and volumetric BMD and negatively with endosteal circumference, underscore the important role of estrogens in skeletal development (Vandewalle *et al*, 2014). Moreover, age-related bone loss is associated with estrogen deficiency not only in postmenopausal women (Riggs *et al*, 1998), but also in elderly men (Khosla *et al*, 1998).

At the end of puberty, the higher estrogen concentration stimulates the closure of the epiphyseal growth plates and shuts the longitudinal growth in both girls and boys (Börjesson *et al*, 2010). The importance of estrogens in skeletal health is confirmed in aromatase knockout mice, specifically, females have poor trabecular and cortical bone development despite increased testosterone (Öz *et al*, 2001). There is an association between low BMD and late menarche (Rosenthal *et al*, 1989; Sowers *et al*, 1992). Moreover, premenopausal amenorrhea resulting from anorexia nervosa (Rigotti *et al*, 1984; Biller *et al*, 1989), excessive exercise (Drinkwater *et al*, 1984; Marcus *et al*, 1985), and hyperprolactinemia (Biller *et al*, 1992) is also related to low BMD, demonstrating the importance of estrogens in bone mineral accrual.

2.4.2 Leptin

Leptin, a adipose tissue-derived adipokine, that is highly correlated with body fat mass, is mainly associated with energy expenditure and promoting satiety (Spiegelman & Flier, 2001). However, leptin has been shown to be capable also of modulating bone metabolism in animal models (Scariano *et al*, 2003; Handschin *et al*, 2007).

Regarding osteoblasts, leptin can promote their proliferation, de novo collagen synthesis, and in vitro mineralization, as well as cell survival and transition into preosteocytes, leptin may also facilitate osteoblastic signaling to osteoclasts (Gordeladze *et al*, 2002). Additionally, leptin can inhibit osteoclast generation in vitro (Holloway *et al*, 2002). Peripheral leptin administration in animal models has shown to have protective effect on ovariectomy-induced bone loss (Burguera *et al*, 2001). Leptin inhibits bone resorption and prevents the decrease of bone formation in female rats (Martin *et al*, 2005) and also increases bone formation in leptin deficient ob/ob mice (Hamrick *et al*, 2005).

Additionally, leptin plays a role in the regulation of several hypothalamic pituitary peripheral neuroendocrine axes, including the thyroid, gonadal, cortisol and growth hormone axes (Tartaglia, 1997; Audi *et al*, 1998; Strobel *et al*, 1998; Farooqi *et al*, 1999; Chou *et al*, 2011). The studies describing leptin treatment show that low-dose leptin treatment prevents suspension-induced BMD loss in trabecular and cortical bone sites (Martin *et al*, 2007) and leptin administration in ob/ob mice increases BMD, BMC and BA (Bartell *et al*, 2011).

Results from human studies are contraversal. No association was found between leptin and fracture risk in elderly men or women (Barbour *et al*, 2011). Moreover, in men leptin was not associated with rates of BMD loss in different skeletal sites, whereas the association between hip BMD loss and leptin seen in women was largely explained by weight change (Barbour *et al*, 2012). In line with those findings, a cross-sectional study in healthy postmenopausal women concluded that leptin has no independent effect on BMC and BMD values (Jürimäe *et al*, 2008). However, negative associations have been found between leptin levels and non-vertebral BMD in postmenopausal women without central obesity (Haam *et al*, 2017). In contrast, Yamauchi and colleagues stated that leptin levels were significantly and positively correlated with BMD values at different skeletal sites and that leptin levels were lower in women with vertebral fractures than in those without fractures (Yamauchi *et al*, 2001). Associations between leptin and skeletal health have been less studied in younger cohorts. A cross-sectional study in early pubertal boys did not find significant correlation between leptin and bone mineral characteristics (Vaitkeviciute *et al*, 2016a) similarly to the study by Roemmich *et al* in children with different pubertal stages (Roemmich *et al*, 2003). In line with those findings, longitudinal studies in prepubertal rhythmic gymnasts entering puberty concluded that leptin level independently did not associate with subsequent increment in BMD (Parm *et al*, 2012; Võsoberg *et al*, 2016). However, in a longitudinal study, at the beginning of puberty leptin was inversely associated with the following BMD increment over the 24-month period (Vaitkeviciute *et al*, 2016b). In prepubertal, early pubertal and pubertal obese children, serum leptin concentration was also positively correlated BMD (Vishnevskaya & Solntsava, 2011). In contrast, data from a study in obese adolescents aged 13–18 years showed that leptin is inversely associated with BMD (do Prado *et al*, 2009). Furthermore, Dimitri *et al* have reported an association between high leptin and an obesity-related impaired skeletal microarchitecture in children (Dimitri *et al*, 2015).

2.4.3 Adiponectin

Adiponectin is a adipose tissue-derived adipokine, a secretory protein that is inversely related to fat mass (Arita *et al*, 1999). In addition to its positive effect on insulin sensitivity and glucose tolerance, adiponectin has been reported to have anti-inflammatory, proangiogenic and antiapoptotic effects in a number of

different cell types (Turer & Scherer, 2012). Adiponectin and its receptors have been found to be expressed in human bone-forming cells, suggesting that adiponectin may be a hormone linking bone and fat metabolism (Berner *et al*, 2004). There are multiple mechanisms that potentially underlie adiponectin's activity in bone, namely local paracrine effects of adiponectin secreted from bone marrow adipocytes, endocrine effects of adiponectin secreted from white adipose tissue into the circulation, and indirect effects through modulation of the sympathetic tone and the regulation of insulin sensitivity and energy homeostasis (Naot *et al*, 2017). The majority of in vitro studies found that adiponectin promotes osteoblast proliferation and differentiation while inhibiting osteoclastogenesis i.e. promotes bone formation. On the other hand, the results from animal model, either in mice with over-expressing adiponectin or adiponectin-deficient mice, have been largely inconsistent (Naot *et al*, 2017): according to Shinoda *et al* (2006), no abnormality was seen in the bone in mice over-expressing adiponectin in liver (Shinoda *et al*, 2006), whereas in another study hyperadiponectinemia was shown to enhance bone formation (Mitsui *et al*, 2011); moreover, in adiponectin-knocout mice a reduced TB BMD (Naot *et al*, 2016), increased TB BMD and (Wang *et al*, 2014) and increased trabecular bone BMD (Wu *et al*, 2014) have been reported.

Clinical studies in adults looking the relationship between BMD and adiponectin have demonstrated different results (Jürimäe *et al*, 2008, 2009; Gruodytė *et al*, 2010; Barbour *et al*, 2011, 2012; Lim *et al*, 2016; Haam *et al*, 2017). Higher adiponectin level was associated with higher risk of fracture in men aged 70-79 years (Barbour *et al*, 2011) and predicted greater hip BMD loss in women at the same age (Barbour *et al*, 2012). Furthermore, a negative association between adiponectin and bone charateristics was also described in perimenopausal women (Jürimäe *et al*, 2008). In contrast, Haam *et al* (2017) found positive association between adiponectin level and BMD in premenopausal women and negative association in postmenopausal women (Haam *et al*, 2017). However, no associations were found between adiponectin and BMD in two different studies among healthy adults (Jürimäe & Jürimäe, 2007; Lim *et al*, 2016). Regarding the studies in children, adiponectin was inversely correlated with bone mass parameters in 4-year-old children (Tubić *et al*, 2011). To date, the associations between adiponectin and bone mineral variables in children have been studied more in girls, and the results are conflicting as no associations were found in pre-pubertal (Rhie *et al*, 2010) or adolescent girls (Huang *et al*, 2004), whereas inverse association was found in adolescent girls with anorexia nervosa (Misra *et al*, 2007). In addition, a cross-sectional study in adolescent female athletes found no independent relationship between adiponectin and BMD and BMC in FN or LS (Gruodytė *et al*, 2010). Similarly, in a longitudinal study among prepubertal rhythmic gymnasts adiponectin was not associated with subsequent increment in BMD at different sekeletal sites (Parm *et al*, 2012). The associations between adiponectin and bone mineral variables in boys, particularly in puberty, have been much less studied. Sayers and colleagues found that adiponectin at the age of 9.9 years was independent of fat

mass, lean mass, and height, inversely correlated to cortical BMC and cortical BA at the age of 15.5 years suggesting that lowering the adiponectin levels in midchildhood has the potential to exert long-term positive effects on bone strength and fracture risk (Sayers *et al*, 2010). Similar relationship was found in another study in prepubertal children (Soininen *et al*, 2018).

2.4.4 Leptin to adiponectin ratio

Leptin and adiponectin have opposing effects on subclinical inflammation where adiponectin having anti-inflammatory properties (Robinson *et al*, 2011) and leptin upregulates proinflammatory cytokines (Procaccini *et al*, 2013). Accordingly, obesity-associated alterations in leptin and adiponectin are major contributors in the development of dysfunctional adipose tissue, characterised by unresolved inflammation (Crewe *et al*, 2017). Systemic low-grade inflammation and abnormal metabolic mileu are associated with impaired bone health (Lucas *et al*, 2012; Viljakainen *et al*, 2017; Sanjeevi *et al*, 2018). In addition to leptin and/or adiponectin separately, the adiponectin to leptin ratio has previously been used as a marker of dysfunctional adipose tissue (Frühbeck *et al*, 2017). A study in a cohort of older adults demonstrated that adiponectin to leptin ratio was associated with lower BMD at the LS and FN (Fuggle *et al*, 2018). A positive correlation between adiponectin to leptin ratio and osteocalcin, a hormone reponsible for bone formation, was found in obese adolescents (Campos *et al*, 2018). The LAR, which has an inversely proportional relationship with adiponectin to leptin ratio, has previously been shown to correlate with carotid intima-media thickness (Satoh-Asahara *et al*, 2004) as well as insulin sensitivity (Finucane *et al*, 2009) and to predict the presence of the metabolic syndrome (Zhuo *et al*, 2009). Moreover, the LAR has proven to be a more accurate marker of obesity-related complications than adiponectin or leptin alone (Inoue *et al*, 2006; Zhuo *et al*, 2009).

2.5 Physical activity during and after puberty

Physical activity is defined as “any bodily movement produced by skeletal muscles that requires energy expenditure“ (Caspersen *et al*, 1985). Physical activity has beneficial effects on musculoskeletal health, cardio-metablic risk factors (Janssen & Leblanc, 2010; Poitras *et al*, 2016), physical fitness and mental health (Poitras *et al*, 2016) and academic achievements (Barbosa *et al*, 2020). According to World Health Organisation (WHO), children and youth aged 5–17 should accumulate a minimum of 60 minutes of moderate-to-vigorous PA (MVPA) every day in order to gain necessary health benefits (WHO, 2010).

There is a growing evidence of a low prevalence of children being compliant with daily PA recommendations of 60 min of MVPA in many developed countries (Guinhouya *et al*, 2013; Konstabel *et al*, 2014; Kalman *et al*, 2015).

Studies on the compliance with PA recommendations in youth show different results depending on the methods used, self-reported data indicates that 14–40% of children and youth are sufficiently active (Ekelund *et al*, 2011; Kalman *et al*, 2015), whereas according to objectively measured PA data the percentage is 20–87% (Guinhouya *et al*, 2013). Recent study showed that when data is collected using self-reporting questionnaire (International Physical Activity Questionnaire) less sedentary time and more vigorous PA is being reported by study subjects compared to accelerometer data (Dyrstad *et al*, 2014). Currently, accelerometry is considered as the reference method for objective measurement of movement behaviours of children in free-living conditions (Bornstein *et al*, 2011). According to a study of Health Behaviour of School-Aged Children conducted in 2013/2014 only 16% of 11–15 year-old Estonian students meet the PA recommendations every day (Aasvee & Rahno, 2015). There is a lack of data on objectively measured PA of Estonian school-aged children. In a study carried out two decades ago, approximately 65% of 9–15 year-old students met the PA recommendations on weekdays and approximately 50% on weekend days (Nilsson *et al*, 2009). However, a study conducted in 2–10 year-old Estonian children showed that 13% of girls and 27% of boys met the PA recommendations (Konstabel *et al*, 2014).

A gender difference in PA is well established for children (Campell & Eaton, 1999) as boys are observed to be more physically active than girls, whereas most differences are suggested as being environmentally induced prior to puberty but influenced by a biology–environment interaction after puberty (Thomas & Thomas, 1988). The level of PA is higher in boys even in infancy, and gender differences increase across childhood and adolescence (Thomas & Thomas, 1988). Physical activity is shown to be tracking well throughout maturation period (Francis *et al*, 2013; Metcalf *et al*, 2015), meaning that individuals have a good tendency to maintain their rank or position of PA within a group over that period of time (Telama, 2009). However, PA tracking from childhood to adolescence is stronger in girls than boys (Francis *et al*, 2013).

It is known that PA decreases from childhood through later adulthood, however the decline is most rapid during adolescence (Caspersen *et al*, 2000; Dumith *et al*, 2011; Ortega *et al*, 2013; Cairney *et al*, 2014), with the mean decline in PA being 7% per year from 10 to 19 years of age (Dumith *et al*, 2011). Adolescence is also the period that patterns of PA begin to diverge for males and females (Cairney *et al*, 2014) as more rapid decline of PA among girls than boys during this period has constantly been reported (Telama & Yang, 2000; Kimm *et al*, 2005; Sherar *et al*, 2007). Factors determining PA behaviors are more closely aligned with maturity than chronological age (Francis *et al*, 2013) and therefore it has been proposed that the more rapid decline in PA among females simply reflects sex differences in biological maturation, suggesting that that more rapid decrease among girls is attributable to their earlier attainment of peak growth, full adult height, and sexual maturity (Cairney *et al*, 2014). Similarly to the decrease in PA among girls which is seen

on average 2 years earlier than among boys (Sherar *et al*, 2010), the peak height velocity accurs on average 2 years earlier in girls compared to boys (Tanner & Davies, 1985). Factors associated with the decline in PA during puberty and adolescence are not unambiguously clear as habitual PA is a complex behavioral phenotype determined by the interaction of biological and psychosocial factors and the physical environment (Malina *et al*, 2004). Puberty is a dynamic biological process that interacts with adolescent behavioral development and therefore PA as a behaviour is involved in those dynamic interactions (Sherar *et al*, 2010).

In conclusion, puberty is a period of decline in physical activity and the percentage of young people meeting the PA recommendations proposed by WHO is low. Although seen already in earlier childhood, in puberty the sex differences in PA patterns start to appear more clearly, with more rapid decline in physical activity levels seen in girls. Factors associated with this decline in PA levels in both sexes are complex, however optimising PA levels in puberty might contribute to gaining the health benefits of PA.

2.6 Physical activity in relation to bone mineral parameters during puberty

Within the controllable factors that affect bone mass accrual, weight-bearing PA is one of the most important (Vicente-Rodríguez, 2006). There is clear evidence for the positive effect of PA and exercise on bone mass and density during the late childhood and peri-pubertal years (Weaver *et al*, 2016). Beneficial effect of PA on bone applies to the average child and those genetically predisposed to lower adult BMD (Mitchell *et al*, 2016). Physical activity 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). The positive relationships between PA and skeletal health can be explained by the bone's mechanostat theory that describes the structural adaptation of bone tissues to their mechanical environment (Frost, 2003). Mechanical loading tilts the balance between bone formation and resorption in favor of the former (Iwaniec & Turner, 2016). Physical activity has direct osteogenic effect via impact loading and via strains exerted on bone by muscle contractions (Vicente-Rodríguez, 2006). Mechanical signals must be of sufficient magnitude, be imposed at significant rates, and be dynamic in application in order for bone adaptation to occur (Robling, 2009). Evidence suggests 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). Additionally, PA has indirect effect on bone by increasing muscle mass and hence the tensions generated on bone (Vicente-Rodríguez, 2006). Adaptations in bone structure and strength related to PA are most often observed in pre- and peri-pubertal groups (Tan *et al*, 2014). Exercise-induced

osteogenic effect tends to plateau at end of puberty, suggesting that bones might be less sensitive to loading during this period of life (Ducher *et al*, 2009). However, recent evidence suggests that bone remains responsive to the mechanical loading of PA throughout adolescence and into emerging adulthood and therefore attention should be placed on promoting bone-strengthening PA after the prepubertal years when adult exercise patterns are likely formed (Metcalf *et al*, 2020).

In addition to mechanical loading, the favourable effect of PA on bone health can be associated with several additional physiological effects, such as decrease in proinflammatory cytokines (Wärnberg *et al*, 2010), increase in osteogenic substances such as nitric oxide and IGF-1 (Kingwell, 2000; van'T Hof & Ralston, 2001; Pedersen & Febbraio, 2012) and approvement of insuline resistance (Rønne *et al*, 2019).

Majority of studies investigating associations between objectively measured PA and bone mineral characteristics in children and adolescents are cross-sectional (Kriemler *et al*, 2008; Vicente-Rodríguez *et al*, 2009; Sayers *et al*, 2011; Gracia-Marco *et al*, 2012; Ivuškāns *et al*, 2014; Osborn *et al*, 2018; Marin-Puyalto *et al*, 2019). Bone mineral characteristics have been shown to be positively associated with PA intensities as well as total PA (Tobias *et al*, 2007; Kriemler *et al*, 2008; Ivuškāns *et al*, 2014; Marin-Puyalto *et al*, 2019) and negatively associated with sedentary behavior (Vicente-Rodríguez *et al*, 2009; Gracia-Marco *et al*, 2012) in numerous cross-sectional studies.

Findings from longitudinal studies support the importance of PA for bone mineral accrual (Bailey *et al*, 1999; Vicente-Rodríguez *et al*, 2004; Heidemann *et al*, 2013; Vaitkeviciute *et al*, 2014; Ivuškāns *et al*, 2015; Marin-Puyalto *et al*, 2018). However, regarding PA, most of these studies have concentrated on the earlier stages of puberty, and the study periods have been relatively short. There are few longitudinal studies with longer than 5-year study period examining the effect of PA parameters on bone mineral outcome (Bailey *et al*, 1999; Baxter-Jones *et al*, 2008; Duckham *et al*, 2014; Jackowski *et al*, 2014; Janz *et al*, 2014a; Tolonen *et al*, 2015; Fritz *et al*, 2016). However, studies that addressed the outcome of adulthood bone mineral characteristics have measured PA indirectly via questionnaires (Jackowski *et al*, 2014; Tolonen *et al*, 2015) which can cause under- or over-reporting. A 12-year-long study demonstrated that high level of childhood PA was positively associated with bone strength in late adolescence even after drastic reduction in PA level during puberty (Janz *et al*, 2014a). Similarly, longitudinal studies have reported a negative association of sedentary time with bone geometry (Gabel *et al*, 2017b) and with FN BMD and BMC (Ivuškāns *et al*, 2015) in adolescents.

3. SUMMARY OF THE LITERATURE

Puberty has a fundamental role in bone mineral accrual with approximately half of bone mineral accumulated during pubertal years. Optimizing bone mineral accrual in puberty is a key factor for osteporosis prevention in later life.

Sex steroids play important role in the bone mineral accrual in puberty. Although the mechanisms through which the actions are mediated are complex and need further investigation, there is a clear evidence on the associations between androgens and skeletal health. However, to the best of our knowledge, limited longitudinal data are available in males regarding the associations between testosterone level and bone mineral parameters through and after puberty.

There is also a growing body of evidence suggesting that leptin and adiponectin has role in mineral accrual. Clinical studies investigating relationships between leptin or adiponectin and bone characteristics in youth have been mainly cross-sectional or looked at the associations in pre-pubertal children. Moreover, there is inconsistency regarding those associations between leptin and adiponectin and bone mineral characteristics in youth. In addition to leptin and/or adiponectin separately, a ratio of those two adipokines has been used as a marker of dysfunctional adipose tissue. However, there have been no longitudinal studies looking the relationships between leptin to adiponectin ratio in puberty and bone mineral characteristics in adolescence.

Along with hormonal parameters, physical activity plays an important role in bone development during growth and maturation. Majority of the studies regarding associations between physical activity and bone mineral accrual are cross-sectional and only few of the longitudinal studies carried out among boys in puberty and post-puberty have used objectively measured physical activity data.

Greater understanding in the contribution of these factors in bone mineral accrual during pubertal years would be useful in optimizing skeletal health in adult years.

4. AIMS OF THE STUDY

The general aim of the current study was to to investigate associations between sex hormones, adipokines and physical activity in puberty and subsequent bone mineral characteristics.

The specific aims of the study were to:

1. Examine the associations between serum testosterone concentration in puberty and bone mineral characteristics at the age of 18 years and the change of bone mineral characteristics from puberty until the age of 18 years (Study I);
2. Investigate whether physical activity at different intensities during puberty is related to bone mineral characteristics at the age of 18 years (Study II);
3. Examine the associations between leptin to adiponectin ratio in puberty and bone mineral characteristics at the age of 18 years and the change of bone mineral characteristics until the age of 18 years) (Study III).

5. SUBJECTS AND METHODS

5.1 Study population

The current study was a continuation to the longitudinal research study that was started in 2010. Initially, the cohort consisted of 314 schoolboys (mean age at baseline was 12 years) from the city of Tartu and surrounding areas (Utsal *et al*, 2012). Boys with chronic illness or mental developmental delay were excluded from the study. The measurements were carried out at the 1., 2. and 3. year of the study with the mean age of subjects being 12, 13 and 14 years accordingly. The study invitation on the 7th year was sent to all 217 subjects who had participated in the first three measurement series. One hundred and four boys gave informed concent and participated in the fourth measurement in 2016–2017 at the mean age of 18 years. As there was missing data, 16 subjects were exluded from the analyses and therefore total number of subjects in analyses was 88.

The study was conducted following the Declaration of Helsinki, and the protocol was approved by the Research Ethics Committee of the University of Tartu (Estonia) (Consent No 260/T-19, 13 June 2016). All subjects and their parents were given a written description of the study and signed written informed consent was obtained from all subjects prior entering the study. If a participant was younger than 18.0 years, additional signed informed consent was retrieved from his parent.

The study included four measurements (T1, T2, T3 and T4) with anthropometry, PA, bone age, pubertal development, bone mineral characteristics and blood analyses measured. Anthropometry and PA were measured at all four time points of the research project (Table 1 and Table 3). Bone mineral characteristics were studied at T1 and T4 (Table 2). Pubertal development and bone age assessment was performed at T1, T2 and T3 (Table 1). Serum testosterone, leptin, adiponectin and insulin resistance by homeostasis model assessment (HOMA-IR) were studied at T1, T3 and T4 (Table 1).

In the analyses of the Paper I, II and III the bone mineral characteristics were included as follows:

- Paper I: TB BMD, LS BMD, TB BMAD and LS BMAD.
- Paper II: TB BMD, LS BMD, FN BMD, TB LH BMC, LS BMC, FN BMC, TB BMAD, LS BMAD and BMC/height)
- Paper III: TB BMD, LS BMD, TB LH BMC, LS BMC, TB BMAD, LS BMAD and BMC/height)

5.2.1 Anthropometry and sexual maturation

Body height (cm) was measured to the nearest 0.1 cm using Martin metal anthropometer according to the standard technique (GPM anthropological instruments, Zurich, Switzerland). Body mass (kg) was measured to the nearest

0.05 kg using medical electronic scale (A & D Instruments Ldt, Abington, UK) with the subject wearing light clothes. Body mass index (BMI; kg/m^2) was calculated as body weight divided by square of body height.

The pubertal stage of the participants was determined according to self-assessment using the illustrated questionnaire of the pubertal stage according to the Tanner classification method (Marshall & Tanner, 1970) which has been previously validated (Duke *et al* 1980). The subjects were given photographs, figures, and descriptions representing genitalia and pubic hair development stages and were asked to choose the one that most closely matched their own development. In the case of discrepancies between the two variables, Tanner stage of the subject was determined according to the self-estimation of genitalia development (Duke *et al*, 1980).

Bone age was determined by the method of Greulich and Pyle using an X-ray of the left hand and wrist (Greulich & Pyle, 1959).

5.2.2 Bone mineral density and body composition

Total body fat percentage and bone mineral characteristics, including TB BMD (g/cm^2), LS BMD (g/cm^2) and FN BMD (g/cm^2) and TB LH BMC (g), LS BMC (g) and FN BMC (g) and BA (cm^2), were measured by DXA scan aDPX-IQ (Lunar Corporation, Madison, WI) at T1 and by DXA scan Discovery (Hologic QDR Series, Waltham, MA, USA) at T4. The subjects were scanned in supine position wearing minimal clothing and medium scan mode was used for measurement. To minimize the effect of body size on BMD, bone mineral apparent density (BMAD) (g/cm^3), an estimate of volumetric bone density, was calculated using the formula TB BMAD = TB BMC/(BA^2/height) and the formula LS BMAD = LS BMC/($BA^{1.5}$) (Katzman *et al*, 1991). In addition, the expression of TB BMC for height (TB BMC/height) was calculated. The precision of measurement expressed as a coefficient of variation (CV) was <2% for all bone mineral measurements.

5.2.3 Physical activity

Physical activity was measured objectively by ActiGraph accelerometer (model GT1M (at T1, T2 and T3) and model GT3X (at T4), ActiGraph LLC, Pensacola, FL, USA) designed to register vertical accelerations. All subjects were instructed to wear the accelerometer on the right hip for seven consecutive days during the wake-up time. For the analyses of accelerometer data, all night activity (24:00–6:00 h) and all sequences of 10 min or more of consecutive zero counts were excluded from each individual's recording The total PA was expressed as total number of counts divided by the registered time (counts/min). Time spent in PA with different intensity levels was calculated. The following cut-offs were used: sedentary time ≤ 100 counts/min, light PA 101–1999 counts/min, moderate PA 2000–3999 counts/min and vigorous PA ≥ 4000

counts/min (Katzman *et al*, 1991). The time spent in at least moderate intensity PA was calculated as MVPA.

5.2.4 Blood analyses

Venous blood samples were obtained after an overnight fast between 8:00 AM and 9:00 AM, the blood serum was separated and then frozen at −80 °C for further analysis. Serum testosterone (nmol/L) was determined using Immulite® 2000 (DPC, Los Angeles, USA) with the inter- and intra-assay CV of less than 5%. Leptin concentration was determined by radioimmunoassay (Mediagnost GmbH, Reutlingen, Germany). This assay had intra- and inter-assay CV of less than 5%, and the least detection limit was 0.01 ng/mL. Adiponectin was determined with a commercially available radioimmunoassay kit (Linco Research, St. Charles, MO). The intra- and inter-assay CV were less than 7%, and the least detection limit was 1 μg/mL. Insulin was analysed using Immulite 2000 (DPC Los Angeles, USA). The intra- and inter-assay CV were less than 5% and 12%, respectively, at an insulin concentration of 6.6 mU/mL. Glucose was measured with a commercial kit (Boehringer, Mannheim, Germany). HOMA-IR was calculated: fasting serum insulin (μU/mL) × fasting serum glucose (mmol/L)/22.5 (Wallace *et al*, 2004).

5.2.5 Statistical analyses

Statistical analyses were performed using SPSS software version 20.0 for Windows (SPSS, Inc., Chicago, IL, USA). All variables were checked for normality of distribution before the analysis. Normally distributed continuous variables are described as a mean±SD, not normally distributed variables as a median and 25th and 75th percentile.

The DXA scanner used at T1 and T4 was from a different manufacturer and model when compared to that from T4 and was not available for cross-calibration any more at T4. To minimise inter-instrument error, we calculated standard deviation scores (SDS) from present sample for all bone mineral characteristics at every time point using a formula: SDS = [an individual value– mean value of total group]/ standard deviation (SD) of total group. The change in SDS (Δ) between different time points from T1 to T4 was calculated by subtracting SDS at T1 from SDS at T4. To compare our study population with external reference population, age-adjusted Z-scores for TB LH BMD ja LS BMD were calculated at T1 and T4 using reference standards by Crabtree *et al* (2017).

To determine the differences between the different time points of the study, a paired t-test for normally distributed data and a Mann-Whitney test for skewed data were used. Spearman correlation coefficients were calculated to explore the associations between serum testosterone concentration at T1 and bone mineral charateristics at T4 and the change in SDS (Δ) of the same bone mineral characteristics. Partial correlation analysis was performed to assess the relationships of bone mineral characteristics with testosterone after controlling for baseline bone

age and total PA. A *P*-value less than 0.05 was considered significant for both analyses.

Regarding the PA data, similarly, spearman partial correlation analysis was performed to explore the relationships between mean pubertal PA variables and bone mineral characteristics at T4, after controlling for baseline bone age and mean pubertal body mass. Mean pubertal PA values including total PA, sedentary time, light PA, moderate PA, vigorous PA and MVPA were calculated using a formula: mean pubertal PA value = (PA value at T1 + PA value at T2 + PA value at T3)/3. Using the same formula, mean pubertal body mass was calculated. Stepwise multiple regression analysis was performed to determine the independent effect of bone age at T1, mean pubertal body mass and mean pubertal PA characteristics on measured bone mineral characteristics at T4. Statistical significance was set at $P < 0.05$. Covariates for the multiple regression model were selected according to the series of univariate models performed, only variables that were statistically significant were included in the regression analyses.

The results of the leptin and adiponectin measurements were log transformed for further analyses. Mean pubertal LAR was calculated using the formula mean pubertal LAR = (LAR at T1 + LAR at T3)/2. Partial correlation analysis was performed to assess the relationships of bone mineral characteristics at T4 and the change in SDS (Δ) with mean pubertal LAR, while TB fat percentage, HOMA-IR, total testosterone and total PA at T1 were included as covariates. *P*-value of less than 0.05 was considered significant for all analyses.

6. RESULTS

6.1 Clinical, body composition, hormonal, bone mineral and physical activity characteristics of subjects at different time points of the study (Paper I, II and III)

The anthropometrical and hormonal data of the subjects is presented in Table 1. Subjects at T1 were mainly in pubertal stage 2 (n = 33) and pubertal stage 3 (n = 45) according to the Tanner classification. As expected, Tanner stage, height, body mass, BMI and bone age increased significantly between studied time points (Table 1).

Mean serum testosterone concentration increased significantly between measured time points (Table 1). Change in testosterone level (from T1 to T4) was positively correlated with change in height (from T1 to T4) (r = 0.56; P <0.001).

Median serum leptin and adiponectin concentrations declined significantly over the study period while LAR decreased significantly from T1 to T2 and increased thereafter to T3 (Table 1).

Table 1. Clinical and hormonal characteristics at different time points of the study (n = 88).

Variable	T1	T2	T3	T4
Age (years)	12.1±0.7	13.1±0.7 *	14.0±0.7 *†	18.0±0.7*†‡
Tanner	2.7±0.7	3.41±0.8 *	4.13±0.7 *†	
[No. at stages (I/II/III/IV/V)]	1/33/45/9/0	0/8/43/30/7	0/2/18/33/35	
Height (m)	1.55±0.08	1.63±0.09 *	1.69±0.08 *†	1.81±0.7 *†‡
Body mass (kg)	47.2±12.7	54.00±14.4*	59.61 ±13.7 *†	73.9±12.1 *†‡
BMI (kg/m^2)	19.5±4.0	20.13±4.1 *	20.60±3.7 *†	22.4±3.3 *†‡
Bone age (years)	11.9±1.2	13.0±1.2*	13.9±3.7 *†	
TB fat %	23.15±10.54			18.07±4.92*
Testosterone (nmol/L)	4.81±5.65		13.59±6.22*	20.23±5.24*†
Leptin (ng/ml)	3.40 (1.8;9.99)		2.3 (0.6;5.65)*	1.85 (0.68;3.36)*†
Adiponectin (µg/ml)	7.7 (5.0;11.1)		6.2 (4.5;9.2)*	3.27 (2.64;4.03)*†
LAR	0.46 (0.18;1.37)		0.22 (0.10;0.94)*	0.48 (0.19;1.03)†
HOMA-IR	1.69 (1.15;2.68)		2.35 (1.76;3.24)*	1.40 (1.04;1.84)*†

* Significantly different ($P < 0.05$) from T1; † significantly different ($P < 0.05$) from T2; ‡ significantly different ($P < 0.05$) from T3. Median with 25th and 75th percentile for adipokine data and mean with SD for all other characteristics are shown.
BMI, body mass index; TB; total body; LAR, leptin to adiponectin ratio. HOMA-IR, homeostasis model assessment-insulin resistance;

The absolute values of bone mineral characteristics are presented in Table 2. The mean TB LH BMD Z-score and LS BMD Z-score were 0.502±1.047 and 0.333±0.838 respectively indicating that our subjects had slightly higher BMD than the reference population by Crabtree *et al* (2017). As BMD at T1 and T4 was measured with scanners by different brands, SDS for BMD and BMAD at T1 and T4 were calculated and thereafter the change between SDS at T1 and SDS at T4, expressed as Δ SDS, was used in the correlation analysis.

Table 2. Bone mineral characteristics (mean with SD) at T1 (DPX-IQ Lunar densitometer) and T4 (Discovery Hologic densitometer) (n = 88).

	T1	**T4**
TB BMD (g/cm^2)	0.98±0.07	1.23±0.09
LS BMD (g/cm^2)	0.83±0.09	1.06±0.10
FN BMD (g/cm^2)	0.92±0.09	1.01±0.13
TB LH BMC (g)	1341.7±337.8	2323.04±358.01
LS BMC (g)	27.41±6.77	58.44±9.34
FN BMC (g)	4.11±0.63	5.09±0.95
TB BMAD (g/cm^3)	0.088±0.006	0.095±0.005
LS BMAD (g/cm^3)	0.147±0.013	0.143±0.013
BMC/height	1110.26±181.62	1590.14±188.91

TB, total body; BMD, bone mineral density; LS, lumbar spine; FN, femoral neck; TB LH, total body less head; BMC, bone mineral content; BMAD, bone mineral apparent density.

Data regarding PA of the participants is presented in Table 3. sedentary time increased significantly (P < 0.001) and time spent in light or moderate PA decreased significantly. Changes in vigorous PA were significant: it increased from T1 to T2 and from T3 to T4, but decreased from T2 to T3. Nevertheless, when summed up, MVPA decreased at every measurement point compared to the previous one. Similarly, significant decrease from 434 counts/min at T1 to 380 counts/min at T4 was found in total PA (Table 3).

Table 3. Physical activity data (median with 25th and 75th percentile) at different time points of the study (n = 88).

Variable	T1	T2	T3	T4
Sedentary time (min/day)	522.1 (485.9; 575.3)	539.1 (500.6; 585.7) *	562.1 (508.4; 627.5) *†	623.5 (563.1; 680.1) †‡
Light PA (min/day)	220.5 (194.3; 244.25)	197.0 (166.0; 221.7) *	170.5 (138.8; 196.56) *†	151.3 (133.7; 184.1) *†‡
Moderate PA (min/day)	45.8 (25.7; 59.4)	42.8 (32.4; 51.2) *	34.1 (27.1; 45.8) *†	27.0 (17.8; 33.4) ‡
Vigorous PA (min/day)	15.1 (9.1;24.2)	16.5 (11.3; 23.8) *	13.7 (9.6; 27.8) *†	25.3 (14.8; 35.1) *†‡
MVPA (min/day)	64.1 (46.25; 84.4)	59.9 (47.0; 75.4) *	52.0 (38.1; 67.7) *†	50.0 (35.3; 69.7) *†‡
Total PA (counts/min)	434 (359; 573)	428 (346; 526) *	350 (283; 497) *†	380 (303; 498) *†‡

* Significantly different ($P < 0.05$) from T1; † significantly different ($P < 0.05$) from T2; ‡ significantly different ($P < 0.05$) from T3.
PA, physical activity; MVPA, moderate-to-vigorous physical activity.

6.2 Associations between serum testosterone concentration at age 12 and subsequent increase in bone mineral density by the age of 18 years (Paper I)

Serum testosterone concentration at T1 was positively correlated with TB BMD at T4 ($r = 0.28$; $P < 0.01$), Δ TB BMAD SDS ($r = 0.47$; $P < 0.0001$) and Δ LS BMAD SDS ($r = 0.23$; $P < 0.05$). When controlling for bone age and total PA at T1, the correlation between testosterone at T1 and Δ TB BMAD SDS remained significant ($r = 0.32$; $P < 0.05$), but the magnitude of association attenuated slightly.

6.3 Associations between physical activity in puberty and bone mineral density at the age of 18 years (Paper II)

Partial correlation coefficients between bone mineral characteristics in individuals at 18 years of age and mean pubertal PA levels where baseline bone age (at T1) and mean pubertal body mass were entered as covariates, are presented in Table 4.

Significant correlations between mean pubertal PA variables and FN BMD, TB LH BMC, FN BMC and BMC/height at T4 were found. FN BMD was positively correlated to mean vigorous PA, mean MVPA and total PA. TB LH BMC was negatively correlated with mean sedentary time and positively with moderate PA, vigorous PA, MVPA and total PA (Table 3). Significant positive correlation was found between mean FN BMC and mean MVPA. BMC/height correlated negatively with mean sedentary time and positively with total PA.

The results of stepwise multiple regression analysis, used to identify mean pubertal PA parameters that contribute most to bone mineral characteristics at T4, are presented in Table 5. Mean total pubertal PA together with mean pubertal body mass were associated with ($P < 0.05$) TB bone mineral characteristics at T4, whereas mean pubertal vigorous PA was associated with FN BMD and BMC at T4 (Table 5). This analysis did not find any significant independent association between mean pubertal PA and LS bone mineral characteristics at T4.

Table 4. Partial correlation coefficients of bone mineral characteristics at T4 with mean pubertal physical activity ((T1 + T2 + T3)/3) variables (n = 88).

Mean pubertal PA	**TB BMD** (g/cm^2)	**LS BMD** (g/cm^2)	**FN BMD** (g/cm^2)	**TB LH BMC** (g)	**LS BMC** (g)	**FN BMC** (g)	**TB BMAD** (g/cm^3)	**LS BMAD** (g/cm^3)	**BMC/Height**
Sedentary time (min/day)	−0.174	−0.072	−0.145	−0.282 *	−0.215	−0.091	0.075	0.084	−0.266 *
Light PA (min/day)	0.071	0.001	0.086	0.200	0.061	0.032	0.013	−0.050	0.090
Moderate PA (min/day)	0.199	0.099	0.149	0.263 *	0.118	0.166	0.106	0.046	0.206
Vigorous PA (min/day)	0.145	−0.021	0.303 *	0.307 *	0.138	0.226	0.066	−0.161	0.153
MVPA (min/day)	0.196	0.043	0.260 *	0.326 *	0.147	0.225 *	0.098	−0.069	0.205
Total PA (counts/min)	0.221	0.051	0.286 *	0.380 *	0.203	0.216	0.054	−0.109	0.261 *

* Statistically significant, $P < 0.05$. The correlations have been adjusted for baseline bone age and mean ((T1 + T 2+ T3)/3) body mass
TB, total body; BMD, bone mineral density; LS, lumbar spine; FN, femoral neck; TB LH, total body less head; BMC, bone mineral content; BMAD, bone mineral apparent density; PA, physical activity; MVPA, moderate-to-vigorous physical activity.

Table 5. The results of stepwise multiple regression analysis (n = 88).

	R2 × 100	F-Ratio	Estimate	SE	*P*-value
TB BMD	35.5	21.489			<0.001
(Intercept)			0.963	0.046	<0.001
Body mass			0.004	0.001	<0.001
Total PA			<0.001	<0.001	0.038
LS BMD	18.1	17.955			<0.001
(Intercept)			0.559	0.110	<0.001
Bone age			0.039	0.009	<0.001
FN BMD	43.2	29.682			<0.001
(Intercept)			0.636	0.051	<0.001
Body mass			0.006	0.001	<0.001
Vigorous PA			0.003	0.001	0.003
TB LH BMC	43.0	29.426			<0.001
(Intercept)			1073.037	171.580	<0.001
Body mass			15.981	2.260	<0.001
Total PA			0.887	0.239	<0.001
LS BMC	10.0	8.749			0.004
(Intercept)			28.322	10.308	0.007
Bone age			2.553	0.863	0.004
FN BMC	47.2	22.960			<0.001
(Intercept)			0.423	0.893	0.637
Body mass			0.032	0.008	<0.001
Bone age			0.229	0.098	0.022
Vigorous PA			0.013	0.007	0.045
TB BMAD	-	-	-	-	-
LS BMAD	16.0	15.260			<0.001
(Intercept)			0.088	0.014	<0.001
Bone age			0.005	0.001	<0.001
BMC/height	48.1	36.093			<0.001
(Intercept)			934.242	88.275	<0.001
Body mass			9.699	1.163	<0.001
Total PA			0.306	0.123	0.015

Nine bone mineral characteristics entered as dependent variables and mean ((T1 + T2 + T3)/3) body mass, baseline bone age and mean ((T1 + T2 + T3)/3) PA variables (sedentary time, light PA, moderate PA, vigorous PA, MVPA and total PA) entered as independent variables. R2 × 100 value describes the percentage of variability of a bone mineral characteristic PA parameter, bone age and/or body mass will explain.

SE, standard error; TB, total body; BMD, bone mineral density; TB LH, total body less head; PA, physical activity; LS, lumbar spine; FN, femoral neck; BMC, bone mineral content; BMAD, bone mineral apparent density.

6.4 Associations between leptin to adiponectin ratio in puberty and bone mineral characteristics at the age of 18 years (Paper III)

Mean pubertal LAR was negatively correlated with LS BMD at T4 (r = -0.23; $P < 0.05$) and LS BMAD at T4 (r = -0.33; $P < 0.05$) as well as with Δ LS BMD SDS (r = -0.31; $P <0.05$) and Δ LS BMAD SDS (r = -0.28; $P <0.05$). In partial correlation analysis after controlling for TB fat percentage, total testosterone, HOMA-IR and PA at T1 the correlation between mean pubertal LAR and LS BMD at T4 (r = -0.31; $P < 0.05$) as well as between pubertal LAR and LS BMAD at T4 (r = -0.41; $P < 0.05$) remained significant. Moreover, pubertal LAR correlated significantly with Δ LS BMD SDS (r = -0.40; $P <0.05$) and Δ LS BMAD SDS (r = -0.42; $P <0.05$). However, no significant correlations were found between LAR at T4 and the bone mineral characteristics at T4 (results not shown).

7. DISCUSSION

7.1 Associations between serum testosterone concentration at age 12 and subsequent increase in bone mineral density by the age of 18 years

In this longitudinal study we found that serum testosterone levels during early puberty were positively correlated with subsequent increase in relative TB BMAD over the next 6 years.

In our previous cross-sectional study in 60 healthy 10–18-years old schoolboys we also found that serum testosterone concentration was an important determinant of bone mineral characteritics explaining 36.8% of the variability of TB BMAD (Pomerants *et al*, 2007). Additionally, a cross-sectional study of BMD in 83 boys aged 11–15 at different pubertal stages showed a significant ($P < 0.01$) correlation between total testosterone concentration and TB BMD ($r = 0.51$) and LS BMD ($r = 0.57$) (Yilmaz *et al*, 2005). Reduced BMC and BMD have been found in boys with constitutional delay of puberty and testosterone treatment for 6 months significantly increased BMC and BMD in these patients (Bertelloni *et al*, 1995). In addition, lower free testosterone levels seen in severe childhood-onset obesity have been suggested to contribute to impaired skeletal characteristics (Laaksoo *et al*, 2018). It is known that androgens have an enhancing effect on bone size during late puberty whereas estrogens have their enhancing effect more in early puberty (Callewaert *et al*, 2010). Similarly, Yilmaz *et al* suggested that low testosterone levels in early puberty may be involved in promoting linear skeletal growth much more than skeletal mineralization (Yilmaz *et al*, 2005). Our results suggest that testosterone already at Tanner stages 2 and 3 is a significant factor associated with further BMAD gain, however it's contribution is relatively modest. Nevertheless, it remains unclair to what extent the direct effect of testosterone accounts for the association as estrogens aromatized from testosterone also play an important role in bone mineral accrual, for example low BMD has been found in men with estrogen resistance (Smith *et al*, 1994) or aromatase deficiency (Morishima *et al*, 1995). One possible reason why in our results testosterone was associated with the relative change in BMAD, and not with BMD, might be the fact that BMAD is considered to be less sensitive to differences in skeletal size than areal BMD (Carter *et al*, 1992; Cvijetić & Korsić, 2004) and is better indicator of true bone density and bone strength. Thereby in this particular period of human development, the relationship between serum testosterone and BMAD may reflect more accurately the true role of testosterone in bone mineral accrual. Similary to our findings, a study in Chinese male adolescents found serum testosterone to be a positive predictor for bone formation (Li *et al*, 2020). Furthermore, small declines of testosterone in young and middle adulthood associate with changes in bone mass and size in males (Banica *et al*, 2022).

Although androgen recpetors are found in all three bone cells: osteoblasts, osteoclasts, and osteocytes, they are mainly expressed in osteoblasts, and to a greater degree in cortical than in trabecular bone (Almeida *et al*, 2017). The latter might explain why in this study the correlation with the relative increase in TB BMAD, a bone mineral characteristic describing more cortical bone, remained significant after controlling for bone age and PA level, whereas the correlation with the relative change in LS BMAD, mostly considered to be a trabecular bone, lost it's significance after controlling for these 2 factors. In contrast to our results, it has been stated that testosterone may regulate the development of trabelcular structure and bone size in boys during puberty (Kirmani *et al*, 2009). Nonetheless, free testosterone has been shown to be independent positive predictor of cortical bone size in young men at the age of PBM (Lorentzon *et al*, 2005), suggesting the particularly role of testosterone in cortical bone accrual. There is evidence from previous studies that compared to cortical bone, trabecular bone thickness progresses more rapidly at the later stages of puberty and postpuberty (Pomerants *et al*, 2007; Kirmani *et al*, 2009).

The other mechanism how testosterone can influence bone mineral accrual is through stimulating growth hormone (GH) and IGF-1 secretion, both important regulators of bone homeostasis throughout life (Ohlsson *et al*, 1998). It is well known that GH deficiency in childhood leads to low bone mass and increased fracture risk in adults (Tritos, 2017). During puberty, elevated sex steroid concentration stimulates GH production, leading to an activation of the whole GH/IGF-1 axis (Christoforidis *et al*, 2005). In this study we did not measure serum IGF-I concentration and therefore it is difficult to evaluate to what extend the positive effect of testosterone on Δ TB BMAD SDS could be mediated through activation of GH-IGF axis. However, in a study showing positive associations between testosterone and various bone parameters in young males the inclusion of serum IGF-I levels as independent predictors in the multiple linear regression analyses did not change any of the found associations, suggesting that the observed positive associations between testosterone and bone geometry in young men are not mediated by circulating IGF-1 (Lorentzon *et al*, 2005). Similarly, in mice androgens have been shown to stimulate bone modeling independently from IGF-1 (Venken *et al*, 2007).

To conclude, our findings from this longitudinal study showed a significant positive correlation between serum testosterone concentration at the age of 12 years and subsequent 6-year increase in TB BMAD SDS. This suggests that the serum testosterone levels in early puberty are associated with subsequent bone mineral accrual in males.

7.2 Associations between physical activity in puberty and bone mineral density at the age of 18 years

The findings of the present study indicate that total PA in puberty is an important predictor of TB BMD and TB LH BMC as well as BMC/height at the age of 18 years, whereas vigorous PA in puberty is an important predictor of FN BMD and FN BMC in boys at the age of 18 years.

Our results from the stepwise multiple regression showed that total PA in puberty together with body mass in puberty is an important predictor of TB BMD at the age of 18 years. Similar results were seen in a study by Ivuškāns *et al*, in which changes in total PA were positively related to TB BMD increment in 11–13-year-old boys during a one-year study period (Ivuškāns *et al*, 2015). Although Ivuškāns *et al* suggested that the effect of total PA on TB BMD was probably mediated by vigorous PA (Ivuškāns *et al*, 2015), our results from a longer study period did not confirm this suggestion.

In addition to TB BMD, TB LH BMC in individuals at the age of 18 years was also predicted by total PA measured in puberty. A longitudinal study of the relationship of PA measured by questionnaire to bone mineral accrual from adolescence to young adulthood in males and females aged 23 to 30 showed also a positive correlation between PA and TB BMC. Whereas active males had 8% greater TB BMC than their inactive counterparts, suggesting that the skeletal benefits of PA gained in adolescence are maintained into young adulthood (Baxter-Jones *et al*, 2008). Our results on the effects of objectively measued PA confirm the positive influence of pubertal PA on bone mass already at the age of 18 years.

Regarding other skeletal sites, results of our analyses showed that FN BMD in individuals at the age of 18 years was positively correlated with mean pubertal vigorous PA, MVPA and total PA values, and FN BMC to MVPA. Vigorous PA in puberty was an important predictor of FN BMD and FN BMC also in stepwise regression analysis. Supportig our finding, the effect of vigorous PA on FN bone mass and density during puberty has been previously reported from cross-sectional (Gracia-Marco *et al*, 2011; Ivuškāns *et al*, 2014; Marin-Puyalto *et al*, 2019) as well as from longitudinal (Vaitkeviciute *et al*, 2014; Marin-Puyalto *et al*, 2018) studies. The values of FN BMD and FN BMC showed the strongest association with PA parameters in comparison of different skeletal sites due to the highest responsiveness to physical stimulus (Marin-Puyalto *et al*, 2019). There is also evidence that the effect of a short-term high-impact exercise intervention on hip BMC in early childhood is sustained through puberty (Gunter *et al*, 2008). Similarly, Janz *et al* found that children who accumulated the most MVPA in childhood had greater bone mass and better geometry at FN at the age of 17 years when compared to less active peers (Janz *et al*, 2014b). It has also been stated that active adolescent males have 9% higher adjusted FN BMC in adulthood than inactive adolescents (Baxter-Jones *et al*, 2008); however, as in this particular study questionnaires were used to assess PA, therefore no conclusions about the individual effect of different

intensity levels of PA, including vigorous PA, could be made. Although not directly comparable with the findings from our study due to different methods used to assess skeletal characteristics, Jackowsky *et al* suggested that the skeletal benefits of PA in adolescence in FN are evident and maintained into adulthood (Jackowski *et al*, 2014). Our findings about positive influence of vigorous PA on FN BMD and BMC support those from aforementioned studies.

In contrast to significant associations between total PA and BMD or BMC of TB and FN, the bone mineral characteristics describing more cortical bone, we found no significant association between different pubertal PA parameters and LS BMD or LS BMC, the bone mineral characteristics describing more trabecular bone. One possible explanation for this might be that the patterns of increase in cortical and trabecular bone densities during growth differ considerably. A quantitative computer tomography study in healthy children suggested that weight-bearing activities are important determinants of cortical bone density while trabecular bones are influenced more by hormonal and/or metabolic factors associated with sexual development (Mora *et al*, 1994). For example, Constable *et al* have found that MVPA is positively influencing TB LH BMC through pathways not related leptin (Constable *et al*, 2021). Previously mentioned differences between cortical and trabecular bone are were also seen in a study by Wang *et al* concluding that Tanner stage I is a more sensitive period for PA in which to exert or exhibit its impact on appendicular skeletal development, whereas Tanner stage II or thereafter may be the most important time for the development of the BMD of LS or other axial skeletal sites (Wang *et al*, 2005). Correspondingly, a study on relationships between PA and bone mass parameters in boys during pubertal growth spurt did not find a significant effect of PA on LS BMD (Vaitkeviciute *et al*, 2014).

In line with our suggestion that cortical and trabecular bone have different patterns of bone mineral accrual during maturation, differences in loading responses of cortical and trabecular bone have been previously described in mouse models. Findings from a study by Weatherholt *et al* using the mouse tibial axial compression loading model to simultaneously explore cortical and trabecular bone adaptation to mechanical loading show that while dose response to loading magnitude within cortical bone was observed, with increasing load magnitude inducing increasing levels of cortical bone adaptation, a clear dose response to load magnitude was not evident within trabecular bone, with only the highest load being able to induce measurable adaptation to loading (Weatherholt *et al*, 2013). The described importance of loading magnitude in osteogenic adaptation of trabecular bone in mouse model is supported by the results in a large cohort of young men, revealing a positive association between high degree of mechanical loading, due to PA, and trabecular bone microstructure whereas the duration of PA was mainly related to cortical bone parameters (Nilsson *et al*, 2010). Those lines of evidence about differences between loading responses of cortical and trabecular bone might explain why no significant associations between different pubertal PA parameters and LS bone

mineral characteristics in individuals at the age of 18 years were seen in our study.

A longitudinal study of long-term benefits of habitual PA during adolescence on adulthood LS bone characteristics of male subjects did not find significant difference between those who were physically active during adolescence compared to non-active counterparts (Duckham *et al*, 2014). In contrast, females who were active in adolescence, showed a 3% greater trabecular content compared to those who were inactive during adolescence (Duckham *et al*, 2014). These findings, together with our results, suggest that bone structural adaptation through childhood PA in males can be maintained into young adulthood, predominantly at the weight-bearing cortical bone sites.

It can be questionable why PA data from later stages of puberty and post-puberty was not measured and included into analyses when identifying PA parameters influencing bone mineral characteristics at the age of 18 years. However, evidence from a study on exercise-induced changes in bone mass and geometry suggests that bones might be less sensitive to loading from Tanner stages III to IV in male subjects (Ducher *et al*, 2009). Moreover, a longitudinal study by Sundberg *et al* conculded that bone mineral characteristics at the age of 13 years might be influenced by increased PA level in the preceding years, whereas 3 years of continued high- or low-level activity up to age of 16 years does not result in any increased differences in bone size or bone mass at the age of 16 years (Sundberg *et al*, 2002). We could assume that concerning the influence on PA on bone mineral outcome in individuals at the age of 18 years, the PA from the age of 12 to 14 is of greater importance than PA in the following years.

When analysing the changes in different PA intensity levels of our participants throughout the puberty, we found results in line with previous studies suggesting that PA decreases from childhood to adolescence (Ortega *et al*, 2013; Cairney *et al*, 2014; Janz *et al*, 2014b; Metcalf *et al*, 2015). A significant decrease in total PA throughout the study period was seen and the decrase was accompanied with the increase in sedentary behavior and decrease in light and moderate PA. Vigorous PA increased from T1 to T2 and from T3 to T4; nevertheless, this did not result in an increase of MVPA. The mean duration of MVPA in our cohort reached the recommended amount of at least 60 min of MVPA a day (WHO, 2010) only at T1 (64.1 min/day) and T2 (59.9 min/day), after that MVPA decreased significantly being only 50.0 min/day at T4. The increase in sedentary behavior, together with an overall increase in vigorous PA and a decrease in total PA seen in our subjects during puberty, is in line with the conclusions drawn in the study of Metcalf *et al*, suggesting that the decline in PA can be imputed to falls in light-intensity activity, rather than higher intensity activity (Metcalf *et al*, 2015).

7.3 Associations between leptin to adiponectin ratio in puberty and bone mineral characteristics at the age of 18 years

The present study established that LAR in puberty is negatively associated with LS BMD as well as LS BMAD at the mean age of 18 years in healthy males. In addition, a negative correlation was found between LAR in puberty and the increment in relative LS BMD and LS BMAD over the period of six years until the age of 18 years. Moreover, the correlations remained significant after adjustment to TB fat percentage, HOMA-IR, total testosterone and total PA at T1.

Ratio between leptin and adiponectin (regardless of the direction) has mainly been studied in the context of obesity-related disorders such as cardiometabolic diseases (Satoh-Asahara *et al*, 2004), diabetes (Finucane *et al*, 2009) and metabolic syndrome (Zhuo *et al*, 2009). However, as higher body fat content has negative skeletal effects (Viljakainen *et al* 2011) and lower bone mass has been found in children with obesity, particularly in adolescence (Dimitri *et al*, 2012), the alterations in LAR could also contribute to negative skeletal effects (Dimitri *et al*, 2011). Higher leptin concentration in obese children has been proposed to reduce bone formation relative to resorption and thereby predispose them to lower bone mass and fractures (Dimitri *et al*, 2011).

The relationship between physiological circulating leptin levels and bone mass is complex: whereas leptin may be positively correlated with bone mass through its association with caloric intake and body weight, leptin *per se* might also exert some negative influence on the skeleton (Hamrick & Ferrari, 2008). Both leptin-deficient and leptin receptor-deficient mice have an increased bone formation leading to high bone mass despite obesity, hypogonadism and hypercortisolism (Ducy *et al*, 2000). With no leptin signalling in osteoblasts, bone loss seen in leptin-deficient and wild-type mice caused by the intracerebroventricular infusion of leptin suggests that leptin is a potent inhibitor of bone formation (Ducy *et al*, 2000) acting via the sympathetic nervous system (Takeda *et al*, 2002).

The effect of leptin on bone metabolism might depend on its level: at lower levels leptin stimulates bone formation but at higher levels inhibits bone formation (Martin *et al*, 2007). The share of overweight subjects in our study group was relatively modest, only 14.7%. However, at the beginning of the study a large number of subjects were at pubertal stage 2 (n = 33), the stage in which boys are known to have peak serum leptin concentration (Clayton *et al*, 1997). Thus, we hypothesise that the negative association between LAR in puberty and further gain in BMD SDS could be explained by the negative central action of the relatively high serum leptin concentration on bone remodelling regulation at this crucial time of bone accumulation. This hypothesis is confirmed by the findings from our previous longitudinal study where serum leptin concentration at the age of 12 years was inversely associated with the BMC and BMD increment over the next 24 months (Vaitkeviciute *et al*,

2016b). Leptin as a negative independent predictor of BMD at several sites was also found in a cross-sectional study in Swedish young adult males (Lorentzon *et al*, 2005).

The role of adiponectin in bone metabolism is also controversial. For example, laboratory studies of mice overexpressing adiponectin exhibited an increased bone mass accompanied by decreased numbers of osteoclasts (Oshima *et al*, 2005), whereas adiponectin-deficient mice exhibited a normal bone mass, except for a slight increase of bone mass in certain age groups (William *et al*, 2009), or the opposite: reduced bone density and cortical bone in adiponectin-knockout mice (Naot *et al*, 2016). Studies in humans have found both negative (Sayers *et al*, 2010) as well as positive (Stojanovic *et al*, 2018; Tamura *et al*, 2007) relationship between serum adiponectin and bone mineral characteristics. In addition, some data suggest that sex hormones might modulate the effect of adiponectin on bones as negative correlation between adiponectin and BMD was seen only in females, but not in males (Bi *et al*, 2020).

The median LAR of our subjects at the age of 12 and 14 years was 0.46 and 0.22, respectively. The decrease of LAR seen in parallel with the advancing of puberty is in accordance with the fact that boys are proven to exhibit the highest values of leptin in pubertal development stages 2 and 3 followed by a subsequent decrease in leptin values (Clayton *et al*, 1997). According to the cut-offs proposed to assess metabolic risk based on adiponectin to leptin ratio (Frühbeck *et al*, 2018), the LAR of our subjects would be considered normal keeping in mind their inversely proportional relationship. However, in the context of bone mineral characteristics, to our knowledge no such cut-offs for LAR have been proposed.

The results of the current study indicate that, in pubertal years, LAR was associated with the subsequent increase in relative TB BMAD and LS BMAD, but not with BMD. This might be explained by the fact that BMAD is considered less sensitive to differences in skeletal size than areal BMD (Carter *et al*, 1992; Cvijetić & Korsić, 2004). Considering that puberty is characterised by a rapid change in bone skeletal size, the relationship between LAR and BMAD may more accurately reflect the influence of LAR on bone mineral accumulation in this particular period.

Our results on the associations between pubertal LAR and different bone mineral values in the LS region, describing more trabecular bone, but not in the TB, describing more cortical bone, might be explained by the fact that patterns of increase in cortical and trabecular bone densities during growth differ remarkably. Trabecular bone is influenced more by hormonal and/or metabolic parameters associated with sexual development, whilst weight-bearing activities are important determinants of cortical bone density (Mora *et al*, 1994). This aligns with the findings from Study II where significant associations between total PA and BMD or BMC of TB and FN, but not between LS BMD or LS BMC, were found.

In conclusion, our findings from this longitudinal study showed a significant negative correlation between LAR in puberty and LS BMD and LS BMAD at the age of 18 years and between the increment in relative LS BMD and LS BMAD over the period of six years until the age of 18 years.

7.4 Limitations

Our study has some potential limitations. At first, different DXA scanners by different brands were used at different measurement time points of the study. We were not able to do *in vitro* or *in vivo* cross-calibration between the scanners because at T4 the machine used at T1 was not any more available due to technical problems. In addition, there is evidence that in vitro phantom cross-calibration studies alone are not sufficient in assessing the agreement between two DXA scanners (Blake, 1996; Diessel *et al*, 2000; Hangartner *et al*, 2013). We therefore calculated SDS for bone mineral values at different time points, and the change in SDS over the 6-year period was used to characterise the change in bone mineral values during the period. A second limitation is that some variables (blood analyses, bone mineral characteristics and bone age) were not measured and included in the analyses at all four measurement points. Another limitation was that serum 25-hydroxyvitamin D concentrations as well as calcium and vitamin D intake were not evaluated in the current study. Although our results are limited to a specific group of Caucasian male adolescents, the major strength of the current study is the relatively long study period, including pubertal years that are known to be critical in regard to bone mineral accrual. Additionally, measuring PA objectively by using accelerometers with a short epoch adds strength to the study.

8. CONCLUSIONS

Based on the findings of the longitudinal study we can conclude that:

1. Serum testosterone concentration at the age of 12 years is positively correlated with subsequent 6-year increase in total body bone mineral apparent density in healthy boys, indicating that higher testosterone levels in particularly early puberty may enhance subsequent bone mineral accrual;
2. Total physical activity and vigorous physical activity in puberty are positively associated with total body and femoral neck bone mineral density and bone mineral content at the age of 18 years, supporting the increasing evidence of the benefits of physical activity on bone mineral parameters;
3. Leptin to adiponectin ratio in puberty is negatively associated with bone mineral density and bone mineral apparent density in lumbar spine at the age of 18 years and with the increase in those bone mineral characteristics until age of 18 years suggesting a possible role of adipokines in puberty in further bone mineral accumulation.

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SUMMARY IN ESTONIAN

Puberteediea erinevate hormoonide ja kehalise aktiivsuse seosed hilisemate luutiheduse näitajatega: longitudinaalne uuring poistel vanuses 12–18 eluaastat

Osteoporoos on kujunenud märkimisväärseks rahvatervise probleemiks (NIH, 2000). Optimaalne luustiku tervis vanemas eas sõltub oluliselt esimesel kolmel aastakümnel saavutatud luu tippmassist (Baxter-Jones *et al*, 2011; Ferrari *et al*, 2012), millest ligi 95% moodustub lapse- ja noorukieas. Suurim osa luu mineraalsest koostisest moodustub just puberteedieas (Baxter-Jones *et al*, 2011). Tegurid, mis sellel perioodil mõjutavad luukoe juurdekasvu, on edaspidise skeletisüsteemi tervise seisukohalt väga olulised (Ferrari *et al*, 2012).

Ühed olulisimad luukoe moodustumist mõjutavad faktorid on hormonaalsed ja metaboolsed tegurid nagu suguhormoonid, kasvuhormoon, IGF-I ja vitamiin D (Ilich *et al*, 1997; Mauras, 1999; Soyka *et al*, 2000), aga ka mitmed adipokiinid nagu leptiin ja adiponektiin (Liu *et al*, 2013). Eelmainitud tegurite seast keskendus käesolev uuring testosterooni, leptiini ja adiponektiini seostele luunäitajatega puberteedi- ning post-puberteedieas.

On teada, et testosteroon on positiivselt seotud luutiheduse juurdekasvuga noormeestel (Yilmaz *et al*, 2005; Pomerants *et al*, 2007) ning eksperimentaalsed uuringud on näidanud, et testosterooni manustamine omab positiivset efekti luunäitajatele nii hilinenud puberteediga poistel (Bertelloni *et al*, 1995) kui ka tervetel prepuberteedieas poistel (Mauras *et al*, 1994). Siiski on vähe longitudinaalseid uuringuid, mis hindaksid seoseid testoterooni ja luunäitajate vahel.

Leptiin ja adiponektiin on rasvkoes toodetavad hormoonid, mis potentsiaalselt vahendavad rasv- ja luukoe interaktsiooni, kuid andmed leptiin ja adiponektiini seostest luunäitajatega on kohati vastuolulised (Huang *et al*, 2004; Misra *et al*, 2007; do Prado *et al*, 2009; Dimitri *et al*, 2010; Gruodytė *et al*, 2010; Rhie *et al*, 2010; Sayers *et al*, 2010; Vishnevskaya & Solntsava, 2011; Parm *et al*, 2012; Vaitkeviciute *et al*, 2016a; Soininen *et al*, 2018). On leitud, et leptiini ja adiponektiini suhe on ülekaalulisusega seotud komplikatsioonide iseloomustamiseks täpsem marker kui leptiin ja adiponektiin iseseisvalt (Inoue *et al*, 2006; Zhuo *et al*, 2009). Leptiini ja adiponektiini suhte võimalikke seoseid luunäitajatega puberteedi- ning postpuberteedieas pole meile teadaolevalt varem uuritud.

Kehaline aktiivsus on luutihedust mõjutavate muudetavate tegurite seas üks olulisemaid (Vicente-Rodríguez, 2006), omades otsest mõju luukoe juurdekasvule läbi mehaanilise koormamise (Vicente-Rodríguez, 2006). Nii läbilõikelised kui ka longitudinaalsed uuringud on näidanud positiivset seost laste kehalise aktiivsuse ja luutiheduse näitajate vahel (Bailey *et al*, 1999; Vicente-Rodríguez *et al*, 2004; Tobias *et al*, 2007; Kriemler *et al*, 2008; Heidemann *et al*, 2013; Ivuškāns *et al*, 2014; Vaitkeviciute *et al*, 2014; Ivuškāns *et al*, 2015; Marin-Puyalto *et al*, 2018; Marin-Puyalto *et al*, 2019). Samas on vaid vähesed varasemad uuringud võtnud aluseks objektiivselt mõõdetud kehalise aktiivsuse

parameetreid ja/või keskendunud spetsiifiliselt puberteedi ning post-puberteedi perioodile.

Uurimustöö eesmärk ja ülesanded:

Töö peamine eesmärk oli uurida puberteediea hormoonide ja kehalise aktiivsuse näitajate seoseid hilisemate luutiheduse näitajatega. Tulenevalt üldeesmärgist oli uurimistöö spetsiifilisteks ülesanneteks:

1. Uurida seoseid puberteediea testosterooni taseme ning 18. eluaasta luutiheduse näitajate ja puberteedist 18. eluaastani toimunud luutiheduse näitajate muutuse vahel;
2. Uurida seoseid puberteediea kehalise aktiivsuse ja 18. eluaasta luutiheduse näitajate vahel;
3. Uurida seoseid poiste puberteediea leptiini ja adiponektiini suhte ning 18. eluaasta luutiheduse näitajate ja puberteedist 18. eluaastani toimunud luutiheduse näitajate muutuse vahel.

Uuritavad ja metoodika

Käesoleva uuring on 2010. aastal alustatud uuringu näol on tegu jätku-uuringuga 2010. aastal alustatud uuringule, mille raames moodustati kohort Tartu linna ja maakonna kooli poistest. Uuritavad poisid olid kohorti sisenedes 12 aastat vanad ning uuringusse kaasamise eelduseks oli krooniliste haiguste puudumine. Esialgse uuringu raames teostati mõõtmised uuringu 1., 2. ja 3. aastal, mil uuritavate keskmine vanus oli vastavalt 12, 13 ja 14 eluaastat. Kutse osaleda uuringu 7. aastal toimuvas jätku-uuringus saadeti kõigile 217 poisile, kes kõigil esimesel kolmel aastal olid uuringus osalenud. Jätku-uuringus osalenud 104-st uuritavast (keskmine vanus 18 eluaastat) lülitati mittetäielike andmete tõttu välja 16 uuritavat ning analüüsis kasutati 88 uuritava andmeid. Uuringu läbiviimine oli kooskõlastatud Tartu Ülikooli inimuuringute eetika komiteega. Kõik uuritavad andsid kirjaliku nõusoleku uuringus osalemiseks, kui uuritav oli alaealine, siis andis kirjaliku nõusoleku tema seaduslik esindaja.

Uuringu käigus teostati neli mõõtmisseeriat (T1, T2, T3 ja T4), mille käigus mõõdeti uuritavate antropomeetrilised näitajad – pikkus ja keha mass (T1, T2, T3 ja T4) ning luuline vanus ja sugulise küpsuse aste (T1, T2 ja T3). Lisaks mõõdeti luudensitomeetria (DXA) meetodil kogu keha rasvamassi protsent ning kogu keha, nimmepiirkonna ja reieluukaela luukoe massi, luukoe pindala ja luutihedust (T1, T4), samuti arvutati volumeetriline luutihedus. Vereseerumist määrati testosterooni, leptiini, adiponektiini, insuliini ja glükoosi kontsentratsioon (T1, T3, T4), arvutati leptiini-adiponektiini suhe ning HOMA-IR. Andmed kehalise aktiivsuse kohta registreeriti aktselerомeetritega (T1, T2, T3 ja T4).

Kuna erinevatel uuringu aastatel (T1 ja T4) kasutati luutiheduse määramiseks erinevaid densitomeetreid ning ristkalibreerimine polnud tehnilistel põhjustel võimalik, arvutati aparaatide vahelise mõõtevea minimeerimiseks kõigile

mõõdetud luutiheduse näitajatele standardhälbe skoorid (SDS). Standardhälbe skoori muutus (Δ SDS) T1-st T4-ni arvutati vastavate ajahetkede SDS-ide vahena. Ülejäänud mõõdetud näitajate osas teostati ajahetkede vaheline võrdlus paaris t-testiga (normaaljaotuse korral) ning Mann-Whitney testiga (kui normaaljaotuse eeldus ei olnud täidetud). Hindamaks seoseid hormonaalsete näitajate (testosteroon ning leptiini-adiponektiini suhe) ning luutiheduse näitajate vahel, teostati korrellatsioon- ning osakorellatsioonanalüüs. Hindamaks seoseid kehalise aktiivsuse ja luutiheduse näitajate vahel, teostati mitmene lineaarne regressioonanalüüs.

Tulemused

Vereseerumi testosterooni tase 12 aasta vanuses, kohandatuna luulise vanuse ja üldise kehalise aktiivsuse suhtes, korrelleerus positiivselt järgneva kuue aasta kogu keha volumeetrilise luutiheduse tõusuga ($r = 0.32$; $P < 0.05$).

Reieluu kaela luutihedus ja nii kogu keha kui ka reieluu kaela luu mineraalne koostis 18-aastastel noormeestel oli seotud erinevate puberteediea kehalise aktiivsuse näitajatega. Puberteediea üldine kehaline aktiivsus koos kehamassiga määras 35,5% kogu keha luutiheduse ja 43,0% kogu keha luu mineraalse koostise varieeruvusest 18 aasta vanuses. Puberteediea tugeva intensiivsusega kehaline aktiivsus koos kehamassiga määras 43,2% reieluu kaela luutiheduse ja koos kehamassi ja luulise vanusega 47,2% reieluu kaela luu mineraalse koostise varieeruvusest.

Puberteediea leptiini-adiponektiini suhe, kohandatuna kogu keha rasvamassi protsendi, testosterooni taseme, HOMA-IR ning üldise kehalise aktiivsuse suhtes, korrelleerus negatiivselt nimmepiirkonna luutihedusega ($r = -0.31$; $P < 0.05$) ning nimmepiirkonna volumeetrilise luutihedusega ($r = -0.41$; $P < 0.05$) 18 aasta vanuses. Samade kovariaatide suhtes kohandatuna korreleerus puberteediea leptiini-adiponektiini suhe negatiivselt ka puberteedist 18. eluaastani toimunud nimmepiirkonna luutiheduse ja volumeetrilise luutiheduse juurdekasvuga (vastavalt $r = -0.40$; $P < 0.05$ ja $r = -0.42$; $P < 0.05$).

Järeldused

Käesoleva longitudinaalse uuringu põhjal võime järeldada, et:

1. Poiste seerumi testosterooni tase 12 aasta vanuses on positiivselt seotud järgneva kuue aasta kogu keha volumeetrilise luutiheduse tõusuga, mis näitab, et kõrgem testosterooni tase juba varases puberteedieas võib soodustada edasist luukoe moodustumist;
2. puberteediea üldine kehaline aktiivsus ja tugeva intensiivsusega kehaline aktiivsus on positiivselt seotud kogu keha ja reieluu kaela luutiheduse ning luu mineraalse koostisega 18-aastastel noormeestel, mis kinnitab kehalise aktiivsuse tähtsust optimaalse luutiheduse kujunemisel kasvueas;
3. puberteediea leptiini ja adiponektiini suhe on negatiivselt seotud lülisamba luutiheduse ning lülisamba volümeetrilise luutihedusega 18-aastastel noor-

meestel ning samade luutiheduse näitajate juurdekasvuga eelneva kuue aasta jooksul, mis viitab puberteediea adipokiinide võimalikule rollile luukoe moodustumisel.

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PUBLICATIONS

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2. Tamme R, Jürimäe J, Mäestu E, Remmel L, Purge P, Mengel E, Tillmann V. Physical activity in puberty is associated with total body and femoral neck bone mineral characteristics in males at 18 years of age. Medicina (Kaunas) 2019; 55, 203.
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1. Tamme R, Jürimäe J, Mäestu E, Remmel L, Purge P, Mengel E, Tillmann V. Association of serum testosterone at 12 years with a subsequent increase in bone mineral apparent density at 18 years: A longitudinal study of boys in puberty. Hormone Research in Paediatrics 2019;91:400–405.
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