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ABBREVIATIONS

AIC	Akaike Information Criteria
BA	bone area
BMAD	bone mineral apparent density
BMC	bone mineral content
BMD	bone mineral density
BMI	body mass index
CI	confidence interval
CV	coefficient of variation
DPX-IQ	total body pencil beam bone densitometer
DXA	dual-energy X-ray absorptiometry
EΔBMI	extensive body mass index change group
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
FDR	false discovery rate
FFM	fat-free mass
FM	fat mass
HOMA-IR	homeostasis model assessment-insulin resistance
IFN-γ	interferon-γ
IL-10	interleukin-10
IL-1α	interleukin-1α
IL-1β	interleukin-1β
IL-2	interleukin-2
IL-4	interleukin-4
IL-6	interleukin-6
IL-8	interleukin-8
LME	linear mixed effects
LS	lumbar spine
MCP-1	monocyte chemotactic protein-1
ML	maximum likelihood
ND	normal distribution
NEΔBMI	non-extensive body mass index change group
NND	non-normal distribution
NWB	normal weight boys
OWB	boys with overweight and obesity
RANKL	expression of receptor activator of nuclear factor kappa-B ligand
Slo	slope; change of value through the study period
T0	baseline
T3	follow-up at 3-years
TB	total body
TBF%	total body fat mass percentage
TNF-α	tumour necrosis factor-α
TR	trunk
TRAP	tartrate-resistant acid phosphatase
VEGF	vascular endothelial growth factor

LIST OF ORIGINAL PUBLICATIONS

I **Mengel E**, Tillmann V, Remmel L, Kool P, Purge P, Lätt E, Jürimäe J. Changes in inflammatory markers in Estonian pubertal boys with different BMI values and increments: A 3-year follow-up study. *Obesity (Silver Spring)* 2017; 25(3):600–607.

II **Mengel E**, Tillmann V, Remmel L, Kool P, Purge P, Lätt E, Jürimäe J. Extensive BMI gain in puberty is associated with lower increments in bone mineral density in Estonian boys with overweight and obesity: A 3-year longitudinal study. *Calcified Tissue International* 2017; 101(2):174–181.

III **Mengel E**, Tillmann V, Remmel L, Kool P, Purge P, Lätt E, Jürimäe J. The associations between the changes in serum inflammatory markers and bone mineral accrual in boys with overweight and obesity during pubertal maturation: a 3 year longitudinal study in Estonian boys. *Osteoporosis International* 2018; 29(9):2069–2078.

In all papers, Eva Mengel had responsibility for preliminary and final data analyses, and writing the manuscripts.

1. INTRODUCTION

It has been widely acknowledged that obesity is an important health issue in children and adolescents (Olds et al. 2011). Childhood obesity is known to affect normal growth and pubertal development (De Leonibus et al. 2013), and may lead to various diseases in later life such as cardiovascular diseases, type 2 diabetes, metabolic syndrome and various pulmonary, hepatic and skeletal disorders (Denzer et al. 2009; Dimitri et al. 2010; Długołęcka et al. 2011; Emanuela et al. 2012; Palermo et al. 2016; Reinehr & Roth 2010). It has been found that obesity increases the risk for fractures during childhood and adolescence (Dimitri et al. 2010). Furthermore, obesity during pubertal development has been related to the increased risk for osteopenia and osteoporosis later in life (Dimitri et al. 2010; Mosca et al. 2013).

An important indicator of obesity-related diseases later in life is the body mass and its increment through puberty (Calarge et al. 2012; Fleisch et al. 2007). Rapid growth rate makes adolescents vulnerable to excess weight gain (Adair 2008). Faster body mass gain rather than slower height gain during adolescence is considered as a major contributor to the rapid increase in body mass index (BMI) over time (Jo 2014). Additionally, body mass is considered as a strong predictor of bone mineralization (El Hage 2012). As childhood and adolescence are important periods for bone mass acquisition (Baxter-Jones et al. 2010; Rizzoli et al. 2010), body mass gain during this period could interfere with both the acquisition and loss of bone mass (Mosca et al. 2014).

Both main components of body mass – body fat-free mass (FFM) and fat mass (FM) influence bone development during growth and maturation (Ivuškāns et al. 2013; Parm et al. 2011). Fat-free mass has been found to stimulate bone acquisition through mechanical forces generated by the impact with the ground (i.e. gravitational loading) or by muscle contractions (i.e. muscle loading) (Karasik & Kiel 2008; Kohrt et al. 2009; Robling 2009; Weeks & Beck 2010). The influence of FM on growing skeleton could be also attributed to a mechanical load and weight bearing caused by the amount of FM and the impact of different hormones linked to the adipose tissue (Hsu et al. 2006; Parm et al. 2012). However, according to Palermo et al. (2016), excess adipose tissue may negatively impact bone health, challenging the traditional paradigm of FM playing a protective role towards bone development during growth and maturation. Accordingly, while the influence of FFM on bone is rather confirmed, the potentially detrimental link between bone and excessive FM (Dimitri et al. 2012; Palermo et al. 2016) should be acknowledged especially during pubertal period, when bone growth is coupled with marked changes in body composition (Rogol et al. 2002).

To date, numerous cross-sectional studies have investigated different bone mineral characteristics and their associations with BMI and other body composition variables in children with normal weight and overweight during pubertal development (El Hage et al. 2009; Ivuškāns et al. 2013; Mosca et al.

2014). However, little is known about longitudinal increases in bone mineral characteristics in overweight boys with different BMI increments during pubertal years. It could be suggested that higher BMI at the beginning of puberty and higher BMI increment during pubertal years in boys with overweight provide higher mechanical load to bones and therefore are associated with higher increase in bone mineral characteristics compared to boys with overweight whose BMI increment is lower.

Adipose tissue as biochemically active organ has been linked to the inflammatory processes, as it can release various inflammatory substances (Emanuela et al. 2012; Galic et al. 2010). It has been suggested that different cytokines produced by adipose tissue can antagonize the positive effect of body mass gain on bone mass accumulation during growth and maturation (Iwaniec & Turner 2016). Children with rapid body mass gain have been shown to have an unfavourable metabolic profile with increased systemic inflammation compared to children who gain body mass at slower rates (Calarge et al. 2012). The increased growth rate in puberty can temporarily increase the inflammation in healthy children and affect the levels of different inflammatory biomarkers (Balagopal et al. 2011; Zabaleta et al. 2014).

Majority of studies investigating different inflammatory biomarkers in children and adolescents with obesity have been cross-sectional (Chang et al. 2015; Herder et al. 2007; Jung et al. 2010; Stoppa-Vaucher et al. 2012; Utsal et al. 2012). Only few studies have examined longitudinal changes in some inflammatory biomarkers in children and adolescents with obesity (Kayser et al. 2015; Roth et al. 2011; Tam et al. 2010). Using complex panel of different inflammatory biomarkers simultaneously in a longitudinal study should give new information about possible changes in inflammatory biomarkers during rapid growth period in children and adolescents with different BMI values, and allows to investigate possible associations of measured inflammatory biomarkers with different body composition characteristics and body mass gain during puberty. To the best of our knowledge, there are no longitudinal studies looking the associations between the changes in a panel of different inflammatory biomarkers and BMI gain during pubertal period in boys with obesity. It can be suggested that some of the measured inflammatory biomarkers are associated with higher BMI gain during pubertal period in boys.

Furthermore, negative impact of obesity and potential inhibitory role of inflammation on bone health has been suggested in children (Hanks et al. 2010). The possible effect of inflammation on bone health is mediated by pro-inflammatory cytokines that may affect bone formation as well as bone resorption (Schett 2011). Cross-sectional studies have demonstrated associations between bone mineral characteristics and some inflammatory biomarkers in adults (Azizieh et al. 2017; Ding et al. 2008; Morimoto et al. 2014; Senel et al. 2013), while there is a lack of information regarding these associations during pubertal development in children. Changes in body composition, including bone mineral content and bone size, are very different in childhood compared to adulthood (Rogol et al. 2002), and this should be taken into consideration when the results from studies with adults are transferred to adolescents. To the best of our knowledge, there have been no longitudinal studies investigating the associations between the changes in a panel of different inflammatory biomarkers and pubertal bone mineral accrual in boys with obesity. Accordingly, it can be argued that changes in some measured inflammatory biomarkers are independently related to bone mineral accrual during puberty in boys with different BMI.

In conclusion, there is still limited knowledge about the interplay between changes in body composition characteristics, bone mineral accrual and inflammatory biomarkers in the context of obesity during pubertal period. The main aim of the current dissertation was to investigate longitudinal changes in body composition, bone mineral characteristics and serum inflammatory biomarkers' concentrations, and their associations in boys with obesity and with different BMI gain during their pubertal years.

2. REVIEW OF THE LITERATURE

2.1. Childhood obesity and body mass gain during puberty

Childhood obesity is a significant health issue (Currie et al. 2012; Olds et al. 2011), as it has a greater negative effect on the health compared to obesity starting later in life (Bastien et al. 2014; Fontaine et al. 2003). Although some plateauing or decline in the prevalence of obesity has been recently seen in younger children (Chung et al. 2016; Wabitsch et al. 2014), it is not the case in adolescents (Olds et al. 2011). Obesity in youth may lead to a lower quality of life and a shorter lifespan (Arslan et al. 2010; Fontaine et al. 2003; Franks et al. 2010). Engeland et al. (2004) have shown that among adolescents even moderate obesity was associated with a 30% increase in all-cause mortality assessed during adulthood. Nowadays there is a shift towards more severe degrees of obesity that results in an increasing prevalence of childhood morbid obesity (Rijks et al. 2015). The causes of obesity are complex and multifactorial including metabolic, hormonal, genetic and psychosocial factors (Arslan et al. 2010), but higher intake of calories and lack of physical activity are among the most influential factors explaining the childhood overweight and obesity (Rodríguez-Hernández et al. 2013; Zabaleta et al. 2014). Adolescents with overweight tend to be overweight in adulthood (Singh et al. 2008), and this can lead to a number of chronic diseases and health problems later in life (Gupta et al. 2012; Salonen et al. 2009).

Obesity in childhood has been associated with type 2 diabetes mellitus, the early-onset metabolic syndrome, insulin resistance, subclinical inflammation, dyslipidemia, atherosclerosis, coronary artery diseases, different hepatic, pulmonary, psychiatric, gastrointestinal, endocrinologic and skeletal complications, and adulthood obesity (Arslan et al. 2010; Denzer et al. 2009; Emanuaela et al. 2012; Gupta et al. 2012; Palermo et al. 2016). Short- and long-term metabolic consequences of obesity in childhood and adolescence vary and may be modulated by a number of factors, including the in utero environment, postnatal growth and inherited risk (McMorrow et al. 2015). Up to 95% of children and adolescents with marked obesity maintain normal glucose tolerance and metabolic homeostasis (McMorrow et al. 2015). In addition, as human obesity does not always result in a disease, the threshold for tolerable body fat among individuals may be determined by environmental and genetic variables (Gregor & Hotamisligil 2011). Mauras et al. (2010) have stated that childhood obesity per se is associated with a pro-inflammatory and pro-thrombotic state before other comorbidities of the metabolic syndrome are present and even before the onset of puberty. There is a need to track body mass status and persistent obesity to prove the association between childhood BMI and later metabolic disease risk (McMorrow et al. 2015).

Childhood obesity is also known to affect normal growth and pubertal development (De Leonibus et al. 2013). During pubertal maturation, major changes in body length, body mass, BMI and body composition variables are taking place (Adair 2008; Hulanicka et al. 2007; Rogol et al. 2002). Rapid growth rates make adolescents vulnerable to excess body mass gain (Adair 2008) and body mass gain is directly associated with the risk of overweight or obesity and adverse future health outcomes (Adair 2008; McMorrow et al. 2015; Mosca et al. 2013). Accelerated body mass gain is often accompanied by a similar acceleration of height velocity, and children with excess body mass tend to have slightly advanced pubertal maturation and bone age (Adair 2008; Shalitin & Phillip 2003). Faster body mass gain in adolescents, rather than slower height gain, is a major contributor to the rapid increase in BMI over time (Jo 2014). Body mass and its increment in adolescence are important indicators of obesity-related diseases later in life (Calarge et al. 2012; Fleisch et al. 2007). However, the exact short-and long-term health consequences due to the elevated BMI values and higher BMI gain during childhood and adolescence remain still unclear (McMorrow et al. 2015).

In summary, obesity can be considered as a multifactorial cause for different subsequent pathologies as well as possible consequence of disturbances in critical developmental periods. Puberty is an important time period between childhood and adulthood, when major changes in body mass and body composition are taking place. Therefore, obesity during this period needs to be especially addressed, as the synergy of pubertal maturation and obesity may have severe impact on long-term health.

2.2. Obesity-related inflammation during puberty

Inflammation is a physiological response necessary for restoring homeostasis altered by diverse stimuli; however, chronically established inflammatory state or an excessive response can involve deleterious effects (Rodríguez-Hernández et al. 2013). Various studies have demonstrated that obesity is associated with inflammation in children and adolescents (Habib et al. 2015; Visser et al. 2001). The adverse effects of obesity are supposed to be due to subclinical chronic activation of immune system (Wen et al. 2014). Obesity results in a pro-inflammatory state starting in metabolic cells (adipocyte, hepatocyte, or myocyte) and also recruiting immune cells with the consequent release of inflammatory cytokines (Emanuela et al. 2012).

The increased growth rate and compensatory mechanisms during puberty can also temporarily increase inflammation and affect the levels of different inflammatory biomarkers in healthy children (Balagopal et al. 2011; Ogden et al. 2012; Zabaleta et al. 2014). Calarge et al. (2012) found that children with rapid body mass gain have unfavourable metabolic profile and a systemic inflammation in their organism compared to children who gain body mass at slower rates. Physiological transient state of insulin resistance can be seen in healthy weight adolescents during puberty, which should resolve by the end of pubertal growth (Goran & Gower 2001; Kelly et al. 2011). Such „pubertal trigger“ might especially influence adolescents with obesity (Kelly et al. 2011), as there is a strong association between serum biomarkers of inflammation and risk for type 2 diabetes mellitus in children with overweight and obesity (Alderete et al. 2014; Chang et al. 2015). It is possible that there is a need for existing inflammatory environment during early age to defend the body against infection, allergies and other insults (Cole et al. 2011). However, such a chronic inflammatory environment may lead to undesirable immune responses associated with diseases later in life (Cole et al. 2011; Wen et al. 2014).

Although liver participates in the systemic inflammation of obesity, the dominant controlling organ is adipose tissue (Arslan et al. 2010; Tam et al. 2010). Evidence suggests that hypoxia plays a crucial role in the adiposopathy process that converts adipose tissue from „healthy" to "sick" (Van de Voorde et al. 2013). Some adipocytokines such as adiponectin and interleukin (IL)-10 have protective role and are called "healthy", whereas others such as tumour necrosis factor-alpha (TNF-α) and IL-6 act as opposite and are called "unhealthy" (Bastien et al. 2014; Van de Voorde et al. 2013). Upregulated expression of different chemokines and receptors may occur as a part of cytokine "cascade", where the expression of one chemokine or its receptor is dependent on the previous events (Deshmane et al. 2009). Age, gender, ethnicity, lifestyle factors such as dietary habits and exercise all play an important role in the obesity-related inflammation and cause variations in circulating cytokine levels (Ding et al. 2008; Magrone & Jirillo 2015; Morimoto et al. 2014; Tam et al. 2010).

2.2.1. Obesity-related inflammatory biomarkers in children and adolescents

In children with obesity, elevated serum IL-6, IL-8, IL-1β, TNF-α, interferon-gamma (IFN-γ), monocyte chemoattractant protein-1 (MCP-1), epidermal growth factor (EGF) and leptin levels have been found compared to their lean peers (Breslin et al. 2012; Chang et al. 2014; Cohen et al. 2012; El-Wakkad et al. 2013; Habib et al. 2015; Roth et al. 2011; Schipper et al. 2012; Stelzer et al. 2012; Stoppa-Vaucher et al. 2012; Utsal et al. 2012; Zabaleta et al. 2014). In contrast, lower serum IL-2, IL-10 and adiponectin levels have been observed in children and adolescents with obesity (Aygun et al. 2005; Böttner et al. 2004; Chang et al. 2015; Glowinska & Urban 2003; Okamatsu et al. 2009). However, Cohen et al. (2012) did not reveal any group differences in the concentration of IL-6, TNF-α, IFN-γ, IL-10 or IL-4 between obese or non-obese youth (Cohen et al. 2012). Interestingly, Kleiner et al. (2013) have found that IL-4, IL-6, TNF-α, IFN-γ were all upregulated in healthy children and adolescents between 7 and 17 years.

There is a limited information about longitudinal changes in inflammatory biomarkers during pubertal maturation. Only few studies have investigated three to six different inflammatory biomarkers at the same time longitudinally in children with obesity during pubertal maturation (Kayser et al. 2015; Roth et al. 2011; Tam et al. 2010). Kayser et al. (2015) found that TNF-α, IL-6, IL-1β, and IL-8 decreased annually in obese youth, whereas there was no significant change in MCP-1 over time in boys with obesity. However, Roth et al. (2011) found that MCP-1 significantly decreased in children with substantial weight loss. No differences in IL-8 concentration were observed longitudinally in boys with BMI gain, although increased serum IL-8 levels were seen in girls who became overweight over time (Tam et al. 2010).

Hereby is given a short description of biochemical markers and their role in obesity-related complications. Interleukin-6 (IL-6) is a circulating multifunctional cytokine with various functions such as inflammation, host defense, bone metabolism and tissue injury (Arslan et al. 2010; Rodríguez-Hernández et al. 2013). IL-6 is produced by many cell types and tissues, including immune cells, fibroblasts, endothelial cells, skeletal muscle and adipose tissue (Arslan et al. 2010). IL-6 appears to have dual functions depending on the tissue and metabolic state (Makki et al. 2013). Adipose cells contribute 15 to 30% of circulating IL-6 in the absence of acute inflammation (Mohamed-Ali et al. 1997) and correlation between serum IL-6 and the level of obesity has been shown (Fried et al. 1998). IL-6 has a pivotal role in metabolic processes having clearly lipolytic effects and anti-obesity potential (Hoene & Weigert 2008). IL-6 enhances lipid turnover, stimulating lipolysis as well as fat oxidation (Petersen & Pedersen 2005). The transient increase in IL-6 may assist in normal glucose homeostasis, whereas the relentless increase in systemic level of IL-6 may lead to insulin resistance (Makki et al. 2013). Interestingly, IL-6 levels in central nervous system are negatively correlated with FM in humans with overweight, suggesting central IL-6 deficiency in obesity (Kershaw & Flier 2004). Herder et al. (2007) showed that serum IL-6 concentration was associated with BMI, waist circumference and insulin resistance in adolescents. Martos-Moreno et al. (2006) stated that IL-6 levels decrease during pubertal development in both sexes.

Tumour necrosis factor-alpha (TNF-α) is produced mainly by macrophages and lymphocytes, and to a less extent also by adipose tissue (Weisberg et al. 2003). TNF-α plays a central role in inflammation, immune system development, apoptosis, with numerous effects in adipose tissue, including lipid metabolism and insulin signalling (Arslan et al. 2010; Rodríguez-Hernández et al. 2013). Petersen and Pedersen (2005) suggest that TNF-α rather than IL-6 is the driver behind insulin resistance and dyslipidemia. However, Aycan et al. (2005) found similar concentration of TNF-α in hyperinsulinemic and normoinsulinemic children with obesity. TNF-α has also an effect on endothelial function in children and adults with obesity (Arslan et al. 2010). Although higher levels of serum TNF-α have been found in children and adolescents with obesity (Breslin et al. 2012; Chang et al. 2014), Herder et al. (2007) did not find association between serum TNF-α concentration and BMI or waist circumference.

Monocyte chemoattractant protein-1 (MCP-1), one of the best-known chemoattractants for macrophage recruitment (Lee 2013), is frequently produced in response to inflammatory stimuli such as IL-1, IL-6, IL-8 or TNF-α

(Arango Duque & Descoteaux 2014). Another chemotactic cytokine IL-8 induces chemotaxis and is responsible for the recruitment of neutrophils and T lymphocytes into the subendothelial space (Gesser et al. 1996). Both chemokines, MCP-1 and IL-8 also come from adipocytes (Zabaleta et al. 2014). IL-8 production in human adipocytes is enhanced by inflammatory substances such as TNF-α and IL-1β (Kobashi et al. 2009). In adults, circulating MCP-1 and/or IL-8 have been found to be related to BMI and waist circumference (Kim et al. 2006; Stoppa-Vaucher et al. 2012). However, Herder et al. (2007) found no association between MCP-1 and BMI or waist circumference in adolescents. No differences in IL-8 concentration were observed longitudinally in boys with BMI gain, although increased serum IL-8 levels were seen in girls who became overweight over time (Tam et al. 2010). Decreases in MCP-1 and IL-8 concentrations have been associated with weight loss in children and adults (Lyon et al. 2003; Roth et al. 2011), and these biomarkers could be links between obesity and obesity-related metabolic complications, such as atherosclerosis, insulin resistance and diabetes (Herder et al. 2007; Kim et al. 2006; Stoppa-Vaucher et al. 2012).

The interleukin-1 (IL-1) family of cytokines and receptors are key mediators of innate inflammatory responses and exhibit both pro- and anti-inflammatory functions (Ballak et al. 2015). IL-1α and IL-1β are both responsible for a variety of inflammatory and metabolic effects (Mirhafez et al. 2015). Evidence reveals that IL-1 activity is of importance in the pathology of type 2 diabetes by mediating obesity-induced inflammation and directly aggravating insulin resistance (Ballak et al. 2015). IL-1α was found to recruit innate immune cells to adipose tissue in response to „danger signals“ released by necrotic adipocytes (Ballak et al. 2015). IL-1β is mainly produced by monocytes in response to infection, injury, or immunologic challenge; it causes fever, hypotension, and production of other pro-inflammatory cytokines, such as IL-6 (Rodríguez-Hernández et al. 2013). Um et al. (2011) showed, that IL-1α plays a role in the development of simple obesity, whereas IL-1β plays a role in the development of obesity associated with insulin resistance (Tack et al. 2012). Although cytokines of the IL-1 family have been described to be elevated in adults with overweight (Di Renzo et al. 2007), results from studies with adolescents are contradictory. Chang et al. (2014) showed that IL-1β concentration was higher in children and adolescents with obesity and IL-1β was positively associated with BMI. However, Jung et al. (2010) managed to show a trend in adolescents with overweight towards higher serum IL-1α levels compared to lean subjects, but concentrations of circulating IL-1β levels were below the detection threshold.

Interleukin-4 (IL-4) together with IFN-γ can regulate macrophages so that they differentiate into M1 and M2 phenotypes, respectively; these phenotypes are associated with distinct functions as they regulate inflammatory and immune functions, respectively (Lee 2013). IL-4 as an anti-inflammatory cytokine derived from T-cells is involved in lipid metabolism by inhibiting lipid accumulation in fat tissues, which leads to decreased body mass gain and FM (Chang et al. 2012, Lee 2013). When IL-4 was adiministered intracerebroventricularly to adult male Wistar rats, it caused excess body mass gain and this was mediated independently of changes in food consumption (Oh-I et al. 2010). El-Wakkad et al. (2013) showed a positive correlation between central adiposity and serum IL-4 level in adolescent girls and a trend towards higher IL-4 levels was seen also in boys with overweight compared to normal weight peers (Rosa et al. 2011).

Interferon-gamma (IFN-γ) is a cytokine belonging to a diverse group of interferons which have a immunological function against mycobacteria and a wide variety of viral infections (Razaghi et al. 2016). It has been also suggested that IFN-γ might be involved in the pathogenesis of obesity as its levels are increased in adipose tissue (Todoric et al. 2011). IFN-γ decreases insulin sensitivity (McGillicuddy et al. 2009) and has been found together with IL-1α as one of the most significant independent predictors of the metabolic syndrome (Mirhafez et al. 2015; Todoric et al. 2011). The geometric mean percentage of CD4-positive cells secreting IFN-γ was significantly higher in children with obesity compared to the normal weight children (Pacifico et al. 2006). Higher serum IFN-γ concentration was found in 10- to 11-year-old boys with obesity compared to normal weight peers and it was positively correlated with total body FM and total body fat percent (TBF%) in the obese group (Utsal et al. 2014). However, Cohen et al. (2012) found no group differences in the concentration of IFN-γ between obese or lean youth.

Epidermal growth factor (EGF) is a member of growth factors and plays important role in proliferation, differentiation and migration of a variety of cells, especially in epithelial cells (Zeng & Harris 2014). EGF receptors are highly expressed also in adipose tissue (Serrero et al. 1993). Inverse associations of serum EGF level with FM and BMI have been found in adults (Accattato et al. 2017; Miller et al. 2013; Serrero & Mills 1991), whereas a positive correlation was found between EGF and increased BMI in children and adolescents (Schipper et al. 2012), and high EGF coincided with a trend towards lower insulin sensitivity in children and adolescents with obesity (Schipper et al. 2012).

Vascular endothelial growth factors (VEGFs) is a group of growth factors involved in angiogenesis, lymphangiogenesis and neuronal development (Matkar et al. 2017). VEGFs are key regulators of vascular permeability (Bates 2010). VEGF is highly expressed in adipose tissue (Sung et al. 2013). Adipose VEGF is critical for maintaining the viability and metabolic/endocrine function of adipocytes through its role in regulating adequate vascularization and blood perfusion (Sung et al. 2013). A positive correlation between serum VEGF concentration and BMI has been shown in adults (Costa et al. 2009; Loebig et al. 2010). High levels of VEGF have been shown to coincide with a trend towards lower insulin sensitivity in children and adolescents with obesity (Schipper et al. 2012).

Interleukin-2 (IL-2) is a cytokine that is primarily produced by activated T lymphocytes (Bayer et al. 2013). IL-2 exerts a wide spectrum of effects on the immune system and plays a crucial role in regulating both immune activation and homeostasis (Gaffen & Liu 2004). Vargas et al. (2016) reported decreased serum IL-2 levels in obesity and suggested that IL-2 may represent important effectors in the early inflammatory events in obese individuals without co-morbidities (Vargas et al. 2016). Aygun et al. (2005) found lower levels of IL-2 in children with obesity compared to their leen peers.

Anti-inflammatory cytokine interleukin-10 (IL-10) inhibits production or block actions of the pro-inflammatory cytokines (Turgeon et al. 2006). IL-10 can inhibit the production of many other pro-inflammatory cytokines such as IL-1, INF-γ and TNF-α and impairs the phagocytic and all-stimulatory capacity of macrophages (Fiorentino et al. 1991). IL-10 does not originate from adipose tissue, but is under control of adipocytokines (Arslan et al. 2010). Obesity increases the gene expression level of IL-10 (Lee 2013). Decreased levels of IL-10 are associated with increased inflammation, endothelial dysfunction, tissue injury and complications of obesity (Arslan et al. 2010). IL-10 levels have been found to be negatively related to BMI in children with obesity (Waters et al. 2007).

Adiponectin has been shown to exert anti-inflammatory effects on macrophages (Galic et al. 2010). Adiponectin could protect against chronic inflammation, atherosclerosis, and cardiovascular diseases (Maggio et al. 2014). Serum adiponectin level declines with age and progression of puberty (Butte et al. 2005; Böttner et al. 2004). Adiponectin has been shown to correlate negatively with BMI in children and adolescents (McMorrow et al. 2015), and serum adiponectin levels increase in conjunction with body mass loss (Arslan et al. 2010). Central obesity lowers adiponectin level through increasing pro-inflammatory cytokines such as TNF-α, IL-1β and leptin (Arslan et al. 2010; El-Wakkad et al. 2013).

Leptin is mainly produced by adipose tissue (Arslan et al. 2010). Effects of leptin are associated mainly with appetite regulation through neuropeptide Y and energy metabolism, but also with pubertal development, reproduction, immune system, hematopoesis, angiogenesis, bone formation and wound healing (Arslan et al. 2010; Clayton et al. 1997). Serum leptin concentration is strongly and positively correlated with BMI and FM (Clayton et al. 1997; Shimizu et al. 1997). Leptin rises similarly over the pre-pubertal years into early puberty in both sexes, thereafter declines to nadir in boys at pubertal stage 5, but keeps rising until a peek in girls at pubertal stage 5 (Clayton et al. 1997).

In summary, there are many inflammatory biomarkers in the body that are involved in the development of obesity with some difference between children and adults. Puberty is period of life with rapid growth and development, where the role of these inflammatory biomarkers in the progression of obesity is not clear. Longitudinal studies with simultaneously measured different inflammatory biomarkers during pubertal maturation are needed (Cohen et al. 2012; Roth et al. 2011). To the best of our knowledge, there have been no longitudinal studies to investigate simultaneously a panel of 12 different inflammatory biomarkers in boys with different BMI values entering into puberty and with different BMI increments during pubertal period.

2.3. Bone development during puberty and associations with body composition

Bone development relies on the processes of modelling and remodelling (Sopher et al. 2015). Modelling occurs only in growing children and is characterized by regulated uncoupling of osteoblast-driven bone formation and osteoclast-driven bone resorption, resulting in bone mass increase and bone shape modification (Boyce et al. 2014; Szadek & Scharer 2013). Remodelling is a tightly coupled process of bone resorption and formation and it orchestrates bone mineral turnover, repair of microdamage, and fracture healing in both children and adults (Sopher et al. 2015). During growth and sexual maturation there needs to be adequate processes of bone formation and resorption to increase bone mineral density (BMD) (Jürimäe 2010). An imbalance between resorption and formation may result in abnormal bone mineral accretion (Sopher et al. 2015). Maximizing bone mineral mass gain during growth and maturation is a key factor for healthy skeleton in adult years (Baxter-Jones et al. 2011; Rizzoli et al. 2010). The amount of bone mineral gained during adolescence typically equals the amount lost throughout the remainder of adult life (Bailey et al. 2000).

Bone mineral status, defined by bone mineral content (BMC) or BMD, is an indicator of bone mineral accrual throughout childhood and adolescence (Kalkwarf et al. 2010). The continuity or stability of bone mineral status throughout childhood and adolescence is referred to as “tracking” (Boulton 1996). There is evidence that BMC and BMD track during growth and maturation (Kalkwarf et al. 2010).

During adolescence, bone formation is greater than bone resorption thus leading to increased bone mass (Szadek & Scharer 2013). However, most gains in bone mass during childhood and puberty are due to an increase in bone length and size (cortical thickness) rather than bone density (Katzman et al. 1991; Short et al. 2015). Bone mineral accrual takes place at different rates at different skeletal sites (Tanner et al. 1976) and gains in bone mass continue after linear growth is complete (Bachrach & Sills 2011). Typically, 90–95% of bone mass accumulates during childhood and adolescence (Khosla et al. 2003), whereas 40% of it comes during puberty (Bailey et al. 1999; Długołęcka et al. 2011). There is no consensus about the period when exactly the peak bone mass is reached, but generally it is accepted that the maximal accrual of BMD is acquired in the years surrounding puberty (Baxter-Jones et al. 2011; Heaney et al. 2000). Szadek & Scharer (2013) proposed that 25% of peak bone mass is attained during the peak height velocity or growth spurt during adolescence.

Bone mineral density is closely related to pubertal maturation (Vaitkevičiūtė et al. 2014). In fact, maximum bone enhancement occurs at the age of 14 years in boys and 12.5 in girls (Baxter-Jones et al. 2011), corresponding to pubertal stages 3–5 according to Tanner classification (Bonjour et al. 1991; Theintz et al. 1992).

Increase in bone mass is strongly influenced by sex steroids (Carson & Manolagas 2015). Testosterone is a major circulating sex hormone in men (Sinnesael et al. 2011). Testosterone level in serum increases through pubertal development until adulthood (Yilmaz et al. 2005). Testosterone directly through estrogens diminishes osteoclastogenesis by stimulating osteoclast apoptosis (Mohamad et al. 2016). Androgens, like estrogens, have a biphasic effect on endochondral bone formation: at the beginning of puberty sex hormones stimulate endochondral bone formation, whereas at the end of puberty they induce epiphyseal closure (Vanderschueren et al. 2014). A positive correlation has been found between serum testosterone level and total body (TB) and lumbar spine (LS) BMD values in boys (Yilmaz et al. 2005).

In addition to pubertal maturation, various factors influence bone mass gain, such as gender, ethnicity, heredity, the intake of calcium and vitamin D, physical activity and hormonal changes in puberty (Hanks et al. 2010; Mosca et al. 2013; Sopher et al. 2015; Szadek & Scharer 2013). However, as this dissertation does not study these factors, we are focusing here on body composition and its impact on bone mineral accrual in puberty.

Body mass has been identified as a major determinant of BMC and is a strong predictor of BMD in adolescent boys (Bachrach 2001; El Hage 2012; Mosca et al. 2013). During adolescence, the ability of bone to adapt to mechanical loading is much greater than after maturity is reached (Parfitt 1994). It has been suggested that in response to mechanical loading, cortical bone mainly enhances its size, while trabecular bone mainly increases its density (Ducher et al. 2004). Mora et al. (1994) suggested that weight-bearing and/or mechanical stresses are important determinants of cortical bone density, while trabecular bones are influenced by hormonal and/or metabolic factors associated with sexual development during late adolescence. In contrast, Cheng et al. (1999) and Alwis et al. (2008) reported that due to higher metabolic activity, trabecular bones are more responsive to physical activity intervention in pre-pubertal and pubertal children.

It is well known that in addition to body mass, both FM and FFM, i.e. components of body mass, influence bone development during growth and maturation (Gracia-Marco et al. 2012; Ivuškāns et al. 2013; Luo et al. 2006; Parm et al. 2011). During puberty, significant changes in the amount and distribution of adipose tissue are taking place (Mihalopoulos et al. 2010). Normal body fat content is beneficial for bone health in growing children and adolescents, while both low and high body fat content have adverse skeletal effects (Viljakainen et al. 2011). The influence of FM on growing skeleton could be attributed to a mechanical load and weight bearing caused by the amount of FM and the impact of different hormones linked to the adipose tissue (Hsu et al. 2006; Parm et al. 2012). Studies about the impact of FM on bone health in children have given conflicting results (Clark et al. 2006; Dimitri et al. 2010; Wey et al. 2011). It has been reported that FM is not related to bone development outcomes (Petit et al. 2005), while Cole et al. (2012) argued that FM is negatively associated with bone mineral apparent density (BMAD) in children, independent of FFM, despite positive associations with bone size. In addition, TBF% is negatively associated with BMD and BMC values in both, adolescents and adults (Lu et al. 2011; Mosca et al. 2013). It also appears that central, rather than total adiposity, may be detrimental to bone health and is negatively associated with BMC (Leonard et al. 2004; Pollock et al. 2010). Moreover, FM inhibits bone mineral accrual in children with previous fractures (Dimitri et al. 2010). Contrary, positive correlations between FM and BMC and BMD have been also found in children and adolescents (Clark et al. 2006; Cole et al. 2012; El Hage et al. 2010; Hong et al. 2010; Pollock et al. 2010; Wang et al. 2007). Streeter et al. (2013) stated that FM appears not to have deleterious effect on bone quality.

To date, there is a consensus that the contribution of FFM to the variance of the increase in bone mineral parameters in children and adolescents is higher than the contribution of FM (Ivuškāns et al. 2013; Sioen et al. 2016). Fonseca et al. (2008) claimed that FFM is, regardless of gender, the main predictor of bone mass at least during the adolescence. However, Carvalho et al. (2011) on the other hand have found that FFM is a prognostic indicator of bone mass formation in boys. Weeks and Beck (2010) have suggested that muscle power and physical activity exert most influence on the bones of adolescent boys, while pubertal maturity predicts the variance in the parameters of bone mass in adolescent girls. Morover, Arabi et al. (2004) have suggested that FFM should have more effect on BMC in 10- to-17-year-old boys compared to the same age girls. However, only few longitudinal studies have indicated that FFM has a major positive effect on bone health parameters in boys (Gracia-Marco et al. 2012; Pietrobelli et al. 2002). Many studies have suggested that FFM stimulates bone mechanically through muscle contractions and is positively related to BMD increment during growth (Kohrt et al. 2009; Parm et al. 2012; Robling 2009; Weeks & Beck 2010).

2.3.1. Bone development in boys with overweight and obesity

It is known that mechanical loading is one of the major factors in bone mineral mass gain during growth (Rizzoli et al. 2010), and therefore increased body mass can increase bone mineral accrual in children with overweight (Pollock 2015). It has been suggested that body mass might improve bone mineralization in children with obesity by increasing the mechanical load of increased body mass, especially in weight-bearing bones (El Hage et al. 2009; Ellis et al. 2003; Pollock 2015; Rocher et al. 2008). Furthermore, the regional distribution of fat may influence bone mass independently of obesity (Pollock et al. 2010; 2011), and there could be site-specific effect of mechanical loading on bone mineral parameters (Ivuškāns et al. 2013). Ivuškāns et al. (2013) showed that LS BMD values were higher in boys with overweight compared to normal weight boys, but no differences were seen in femoral neck BMD values. Furthermore, Ivuškāns et al. (2013) found that overweight did not have a protective effect on

BMAD in boys during puberty. Moreover, Mosca et al. (2013) showed that TBF% was negatively correlated to BMD and BMC in adolescents with overweight.

In children with overweight, the skeleton must be stronger than in normal weight peers to support their higher body mass (Rocher et al. 2008). It is proposed that body mass gain interferes with both the acquisition and loss of bone mass, and is directly associated to the risk of obesity (Mosca et al. 2013). However, Weeks and Beck (2008) have suggested that while obesity is represented by increased body mass, and thus the mass needed to move during habitual activities, the incident muscular contractions, particularly the magnitude of force, the rate of force production, and the total amount of contractions play a more important role than body mass. Furthermore, the increase in bone mineral parameters observed in boys with overweight and obesity was due to the increase in FFM rather than in FM (Sioen et al. 2016). In addition, the greater bone mass in obesity could be also attributed to a different hormones linked to the adipose tissue (Pollock 2015). It is also known that same-aged children with overweight are more advanced in pubertal maturation compared to their healthy weight peers (Pollock 2015), which might give them advantages gaining more bone mass during puberty.

Accordingly, children with normal weight, overweight and obesity seem to all have slightly different pathways for bone mineral accrual (Utsal et al. 2014). However, the impact of obesity and adiposity on skeletal development and influential mechanisms underlying these changes remain controversial (Dimitri et al. 2012; Farr & Dimitri 2017). There are studies indicating that children with obesity have increased (Clark et al. 2006; Ivuškāns et al. 2013; Leonard et al. 2004; Vandewalle et al. 2013), similar (Ellis et al. 2003; Fintini et al. 2011; Hasanoğlu et al. 2000) or decreased (Dimitri et al. 2010, 2011, 2015; El Hage et al. 2010; Mosca et al. 2014; Rocher et al. 2008) bone mass and BMD compared to their normal weight peers. Ellis et al. (2003) concluded that children with obesity did not have lower TB BMD compared to their healthy weight peers, even after adjusting for height, age, gender and ethnicity. Gracia-Marco et al. (2012) showed positive association between adiposity level and bone mass in adolescents, although this association was explained by lean body mass. It has been shown that despite having greater bone size, children with overweight tend to have reduced BMAD values (Cole et al. 2012; El Hage et al. 2011; Rocher et al. 2008).

In summary, children undergo major changes in body composition during pubertal period, and different factors have beneficial or harmful effect on bone development. However, there is still a lack of a clear and consistent understanding of interactions between FM, FFM and bone mineral accrual during growth and maturation in children with different BMI. Numerous cross-sectional studies have investigated different bone mineral characteristics and their associations with BMI and body composition characteristics in children and adolescents with normal weight and overweight, but less is known about the longitudinal increases in bone mineral characteristics in overweight boys with different BMI increments.

2.4. The associations between bone health and inflammatory biomarkers

Bone is a metabolically active tissue with continuous remodelling occurring throughout its life (Jürimäe 2010). It has been suggested that abnormal metabolic milieu and different cytokines produced by adipose tissue can antagonize the positive effect of body mass gain on bone mass accumulation, and may affect bone mineral accrual and bone size during growth and maturation (Dimitri et al. 2010, 2012; Hanks et al. 2010; Iwaniec & Turner 2016). Childhood obesity is considered to be a systemic (low-grade) inflammation (González-Gil et al. 2017; Viljakainen et al. 2017), and therefore might be involved in suboptimal bone accrual (Hanks et al. 2010; Lucas et al. 2012). However, the exact contribution of this systemic inflammation to bone health has remained unclear (Viljakainen et al. 2017). The pro-inflammatory cytokines alter bone homeostasis and regulate bone formation as well as bone resorption (Schett 2011) and could potentially impair skeletal acquisition in children with obesity (Dimitri et al. 2010).

Several pro-inflammatory cytokines, such as IL-1, IL-6, IL-8 and TNF-α have been reported as osteoclastogenic cytokines and through osteoclast activation can cause bone loss (Amarasekara et al. 2018; Schett 2011). IL-1 activates osteoclasts, but also is involved in the differentiation, multinucleation, and survival of osteoclasts (Kim et al. 2009). IL-6 is positively involved in osteoclast differentiation by inducing the expression of receptor activator of NF-κB ligand (RANKL) on the surface of osteoblasts (Yoshitake et al. 2008). It has been suggested that the effects of IL-8 on bone are mediated by upregulation of nitric oxide synthase expression in osteoclasts (Sunyer et al. 1996), whereas TNF-α enhances osteoclast-mediated bone resorption by interacting with RANKL (Schett 2011). EGF can stimulate bone resorption by increasing the proliferation of osteoclast precursors, which leads to increased numbers of osteoclasts (Xian 2007). The EGF-like ligands regulate the expression of two secreted osteoclast regulatory factors in osteoblasts by decreasing osteoprotegerin expression and increasing MCP-1 expression in an EGF receptor (EGFR)-dependent manner and consequently stimulate tartrate-resistant acid phosphatase (TRAP)-positive osteoclast formation (Zhu et al. 2007). Actions of MCP-1 on bone have been contributed to bone resorption associated with inflammatory osteolytic lesions (Graves et al. 1999).

Interleukin-4 and IL-10 are considered to have an anti-osteoclastogenic activity, and through suppressing osteoclast differentiation can inhibit bone loss (Amarasekara et al. 2018). IL-4 is a member of a group of locally acting factors that have been termed “inhibitory cytokines” and IL-4 effects seem to affect both osteoblasts and osteoclasts (Lorenzo et al. 2008). IL-4 reversibly inhibits osteoclastogenesis via inhibition of NF-kappa B and mitogen-activated protein kinase signalling (Wei et al. 2002). IL-10 inhibits the early stages of osteoclastogenesis, preventing differentiation of osteoclast progenitors to pre-osteoclasts though direct and indirect actions (Evans & Fox 2007). VEGF is highly expressed in osteoblastic precursor cells and is known to stimulate bone formation (Liu et al. 2012). Through its regulation of angiogenesis, VEGF contributes to coupling of osteogenesis to angiogenesis, and directly controls the differentiation and function of osteoblasts and osteoclasts (Hu & Olsen 2016). IFN-γ has been found also to inhibit directly TNF-α-induced osteoclastogenesis (Kohara et al. 2011).

While different inflammatory biomarkers, such as IL-6, VEGF and IL-10 have been associated with adult bone health (Azizieh et al. 2017; Barbour et al. 2014; Ding et al. 2008; Morimoto et al. 2014; Senel et al. 2013), there is only limited knowledge of the influence of inflammatory biomarkers on bone mineral accrual in children during growth and maturation (Dimitri et al. 2012; Hanks et al. 2010; Utsal et al. 2014). VEGF has been positively associated with BMD (Senel et al. 2013), whereas low IL-4 and IL-10 levels were seen in women with low BMD (Azizieh et al 2017). Ding et al. (2008) confirmed that changes in TNF-α concentrations have been negatively associated with changes in BMD in adults. IL-6 has been negatively associated with change in TB or LS BMD and predicts bone loss and resorption in older adults (Ding et al. 2008). High serum IL-6 has been associated with low BMC in 7- to 12-year-old children (Hanks et al. 2010). Furthermore, Hanks et al. (2010) found that tumour necrosis factor receptor 2 (i.e. marker of TNF-α activity) is positively associated with BMC independent of TB FM and TBF% in 7- to 12-year-old European Americans, but significantly negatively in African Americans. Serum IFN-γ could be a link between increased FM and higher BMD (Utsal et al. 2014), as serum IFN-γ was positively correlated with TB FM and TBF% in 10- to 11-year-old boys with overweight (Utsal et al. 2014). In addition, adipocytokines, such as leptin and adiponectin could impair the skeletal microarchitecture and acquisition in children with obesity (Dimitri et al. 2015), and predispose them to low bone mass and fractures (Dimitri et al. 2011). Hyperleptinemia could be one way in which high levels of adiposity inhibits the accumulation of bone mass during growth (Pollock 2015). Inverse associations between serum adiponectin level and bone mineral parameters have been found in children and adolescents (Sayers & Tobias 2010).

Many questions still remain regarding the exact mechanisms and instances between various cytokines in interaction with bone (Amarasekara et al. 2018). It appears that the cytokine composition of an inflammatory tissue is decisive whether inflammation triggers bone loss or not (Schett 2011). However, there is a limited research regarding the secretion of inflammatory biomarkers as mediators in the context of the interplay between fat and bone, particularly in the pediatric population (Dimitri et al. 2012; Hanks et al. 2010). In addition, as pubertal period itself is associated with temporary inflammation (Balagopal et al. 2011; Zabaleta et al. 2014), we could only hypothesize how this manifests into bone development during such a rapid developmetal period.

In summary, different cross-sectional studies have investigated the roles of various inflammatory biomarkers on bone mass acquisition, whereas most of them have investigated only few markers at a time. As puberty is a period when physiologic and pathologic inflammation could interact, and therefore have controversial influence on bone health, using complex panel of 12 inflammatory biomarkers simultaneously should provide us more complex knowledge about the inflammatory processes during puberty. However, to the best of our knowledge, there are no longitudinal studies looking the associations between a panel of 12 different serum inflammatory biomarkers and pubertal bone mineral accrual in boys with different BMI and adiposity level during rapid growth and maturation.

3. AIM AND PURPOSES OF THE STUDY

The general aim of the current dissertation was to investigate longitudinal changes in body composition, bone mineral characteristics and inflammatory biomarkers, and their associations in boys with overweight and obesity and with different BMI gain during pubertal maturation.

The specific purposes of the current study were to:

1. examine the longitudinal changes in different serum inflammatory biomarkers during pubertal maturation in boys with overweight and obesity at the beginning of puberty and with different BMI gain during puberty (Study I);
2. examine the longitudinal changes in bone mineral characteristics during pubertal maturation in boys with overweight and obesity at the beginning of puberty and with different BMI gain during puberty (Study II);
3. identify the inflammatory biomarkers that associate with BMI gain in boys with overweight and obesity during pubertal maturation (Study I);
4. identify the inflammatory biomarkers that associate with bone mineral increments in boys with overweight and obesity during pubertal maturation (Study III).

4. METHODS

4.1. Participants and study design

The current longitudinal study included 55 Estonian schoolboys recruited from Tartu City and County. The participants were selected from a larger cohort (*n* = 211). The inclusion criteria for the current longitudinal study were age 10–11 years at baseline, and valid and complete data over the 3-year study period. No missing data were allowed for more than one time point throughout the study period, and missing data were not allowed for the first and last study time point. In addition, all participants were free from current or previous diseases known to affect skeletal metabolism, none of them were receiving regularly medications known to affect bone. The study cohort of 55 participants did not differ from the study cohort from the preliminary cohort of 211 boys in terms of mean age or BMI percentile. Boys were studied yearly for three years at 12-month interval. Height, body mass, pubertal stage, fasting blood samples, body composition and bone mineral characteristics were measured at baseline (T0), and after 12 (T1), 24 (T2) and 36 (T3) months of the study period (Table 1).

Fifty-five boys identified to be part of this longitudinal study were thereafter divided into two groups according to their BMI at baseline (T0). Three boys with BMI > 85th percentile and 23 boys with BMI > 95th percentile formed the OWB group (boys with overweight and obesity), and 29 boys with BMI ≤ 85th percentile formed the NWB group (normal weight boys). Boys in OWB group were thereafter divided by their BMI gain during the 3-year study period into extensive or non-extensive BMI increment group. Eight boys with BMI increment ≥ 3.0 kg/m^2 formed the extensive BMI risers group (EΔBMI), and 15 boys with BMI increment < 3.0 kg/m^2 formed the non-extensive BMI risers group (NEΔBMI). The criterion of +3.0 kg/m^2 was chosen because the mean increment of BMI at the 85th and 95th percentile of Estonian boys of ages 11–14 is 2.5 kg/m^2, so this is well above the normal BMI gain (Estonian BMI Charts). This is an usual practice in clinical management of children with obesity.

Each participant and their parents completed a questionnaire about child's general health and development, family's socioeconomic status, everyday physical activity and diet habits at every study time-point. In the current study, questionnaires at baseline and at 3-year follow-up were taken into consideration for describing generally the socioeconomic status and overall everyday physical activity. Children with chronic illness or developmental delay were excluded from the study. The participants were not examined by physician and therefore there is no information about the clinical conditions that may have developed during the 3-year study period.

The study was approved by the Research Ethics Committee of the University of Tartu (Tartu, Estonia). Participants and their parents were instructed about the study and procedures. Each participant gave their assent and their parent signed an informed consent.

4.2. Anthropometry and sexual maturation

Body mass was measured to the nearest 0.05 kg using medical electronic scale (A & D Instruments Ldt, Abingdon, UK). Height was measured to the nearest 0.1 cm using Martin metal anthropometer. Body mass index (BMI; kg/m^2) was calculated as body mass in kilograms divided by height in square meters.

Pubertal development was assessed by self-report using a validated illustrated questionnaire of pubertal stages according to the Tanner classification method (Matsudo & Matsudo 1994). Shortly, the boys were given photographs, figures, and descriptions of genitalia and pubic hair developmental stages (Matsudo & Matsudo 1994), and asked to choose the most accurate description of their appearance. In case of discrepancies between the two variables, the final decision was based on the development of the genitalia (Matsudo & Matsudo 1994). The self-assessment of pubertal stages has been successfully used in many previous studies (Ivuškāns et al 2013; Utsal et al. 2014).

Bone age was assessed with an X-ray of the left hand and wrist and determined according to the method of Greulich and Pyle (1959).

4.3. Bone mineral density and body composition

Total body (TB) bone mineral density (BMD) (g/cm^2) and TB bone mineral content (BMC) (g) were measured to describe cortical bone, and lumbar spine (LS; L2-L4) BMD (g/cm^2) and LS BMC (g) were measured to describe trabecular bone by dual energy X-ray absorptiometry (DXA) using the DPX-IQ densitometer (DPX-IQ, Lunar Corporation, Madison, WI, USA) equipped with proprietary software, version 3.6. To minimize the effect of bone size (body height) on BMD values, two methods were used. First, bone mineral apparent density (BMAD; g/cm^3) was calculated using the formula BMAD = BMC/(bone area (BA)^2/height) for TB and the formula BMAD = BMC/BA$^{1.5}$ for LS (Katzman et al. 1991). Second, the expression of TB BMC to height (TB BMC/height) was calculated to adjust for TB bone size (Bachrach et al. 1999). TB fat percentage (TBF%), fat mass (FM), fat-free mass (FFM) and trunk fat mass (TR FM) were also determined by DXA (DPX-IQ, Lunar Corporation, Madison, WI, USA).

The participants were scanned in light clothing while lying flat on their backs with their arms at their sides. Fast scan mode and standard subject positioning were used for TB measurements and the results were evaluated by the same examiner. To reduce the impact of the operator variability factor, one qualified observer analysed all the scans over the 3-year study period. The precision of measurement expressed as a coefficient of variation (CV) was less than 2% for all bone mineral and body composition measurements.

4.4. Blood analysis

Venous blood samples were drawn between 08:00 and 09:00 a.m. after an overnight fast from an antecubital vein with the participants sitting in an upright position. Blood serum was separated and frozen at -80°C for further analysis. A panel of 12 inflammatory biomarkers (pg/mL), such as interleukin (IL)-2, IL-4, IL-6, IL-8, IL-10, vascular endothelial growth factor (VEGF), tumour necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), IL-1α, IL-1β, monocyte chemoattractant protein-1 (MCP-1) and epidermal growth factor (EGF) were determined by Evidence® Biochip Technology (Randox Laboratories Ltd., Crumlin, UK). The "Cytokine & Growth Factors Special High-Sensitivity Array" (Biochip) was used for simultaneous quantitative detection of multiple cytokines in parallel from a single sample. None of the inflammatory biomarkers were below the detection limits. Intra-assay CV was between 5.1–8.5%, and inter-assay CV was between 5.8–9.9% for all measured markers.

In addition, leptin concentration was determined by radioimmunoassay (Mediagnost GmbH, Reutlingen, Germany). This assay had intra- and inter-assay CVs less than 5%, and the least detection limit was 0.01 ng/mL. Adiponectin was also determined with a commercially available radioimmunoassay kit (Linco Research, St. Charles, MO). The intra- and inter-assay CVs were less than 7%, and the least detection limit was 1 μg/mL.

Serum testosterone concentration was measured using Immulite 2000 (Diagnostic Products Corporation, LA, CA, USA). The intra- and inter-assay CVs were less than 5%, and the lowest detection limit was 0.01 nmol/L.

Insulin was analysed using Immulite 2000 (Diagnostic Products Corporation, LA, CA, USA). The intra- and inter-assay CVs 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). The estimate of insulin resistance by homeostasis model assessment (HOMA-IR) was calculated: fasting serum insulin (μU/mL) × fasting serum glucose (mmol/L)/22.5 (Wallace et al. 2004). To define insulin resistance in pubertal boys, a HOMA-IR cut-off value of 5.22 was used (sensitivity 56%, specificity 93.3%) (Kurtoğlu et al. 2010a).

4.5. Statistical analysis

Statistical analysis was performed using the statistical package SAS Version 9.2. (SAS Institute Inc., Cary, NC), a program R 2.15.2 and IBM SPSS Statistics 20. All variables were checked for normality of distribution before further analysis using Kolmogorov-Smirnov test. Descriptive statistics were presented as means and 95% confidence intervals [CI; normal distribution (ND)] or as medians and quartiles [25th, 75th percentile; non-normal distribution (NND)]. Differences between groups were evaluated by two-tailed Student's *t* test (ND) or by Wilcoxon–Mann–Whitney test (NND). Differences between paired data

(T0, T3 within group) were evaluated by paired *t* test (ND) or by Wilcoxon signed rank test (NND). Fisher's exact test was used to determine differences between categorical variables. Odds ratios and 95% CI were used to estimate relative risk. McNemar's exact test (for 2 × 2 tables) or Bowker's Test of Symmetry (for square tables larger than 2 × 2) was used to compare dependent proportions (pubertal stage). For longitudinal analysis, linear mixed models with a random effect for each participant were used to assess whether longitudinal changes in numerical outcome measures were significantly different from baseline (T0) between two groups. Interaction terms were created to assess whether these changes through time differed significantly between groups. A statistically significant interaction term (group × time) identified significantly different slopes (Slo) between groups. All four study time-points were included (T0, T1, T2, T3) in the linear mixed models. Results are expressed as estimated means and 95% CI.

Spearman correlation coefficients were calculated to explore the associations between baseline blood serum biomarkers and BMI gain during the 3-year study period (i.e., representative slopes), and between changes in bone mineral characteristics (i.e., representative slopes) and changes in serum biochemical markers during the 3-year study period (i.e., representative slopes). Kendall correlation coefficients were calculated to explore the associations between testosterone level and Tanner stage. We also used linear mixed effects (LME) models to evaluate the associations between 12 serum inflammatory biomarkers and TB BMD, TB BMAD, TB BMC/height, LS BMD and LS BMAD separately in the whole study group ($n = 55$). The data were analysed by using a mixed effect model with maximum likelihood (ML) estimation. In addition to the serum inflammatory biomarkers, testosterone, TBF% and BMI were added as covariates into LME models. We did not add height factor into LME models, as height is already incorporated into BMI. As we did not add height factor separately into models while investigating the associations, we decided to leave out BMC values from our analysis, as the latter is very body size-dependent. Inflammatory biomarkers, testosterone, TBF% and BMI were used as fixed effects, whereas time and subject were used as random effects in the models. At first we specified full models, i.e. the model with the most effect covariates, and subsequently backward elimination method was applied to delete variables of no value (reduced models) (Cheng et al. 2010). We used unstructured variance matrix. The final models were selected among all models considered, i.e. the lowest Akaike Information Criteria (AIC) (Cheng et al. 2010). However, in addition to statistical considerations, we kept scientific and clinical considerations in mind when selecting the predictors and keeping some of them in the models even though they were not statistically significant with the current data (Cheng et al. 2010). Preliminary analysis showed that there were no violations in the assumptions. As AIC was used for model selection, no other multiple correction methods were needed to apply (Cheng et al. 2010).

Correction for multiple testing was applied for inferential statistics and simple correlations. Correction for multiple testing was made using the false discovery rate (FDR) linear step-up procedure (Benjamini & Hochberg 1995). Benjamini-Hochberg critical values were calculated as *(i/m)Q*, where *i* is the rank in an ascending list of *P* values, *m* is the number of tests, and *Q* is a false discovery rate (Benjamini & Hochberg 1995). Correction for multiple testing was performed using the FDR of 0.1 because we did not want to set the proportion of false negatives too high (missing a potentially important discovery) (Glickman et al. 2014). FDR 0.1 means that up to 10% of the significant results could be false positives. After correction, the cut-off *P* value for the significance of a single comparison for the set of the variables in the study was 0.046 for inferential statistics and 0.088 for correlation tests. Only the *P* values that are below the adjusted false discovery rate significance threshold are therefore significant and marked with asterisk (*) in the tables. Only *P* values below these cut-off values have been used in this study.

5. RESULTS

5.1. Clinical and body composition parameters and their changes in boys with overweight and obesity during their pubertal maturation

The main clinical and body composition parameters of the participants at baseline (T0), after 3-year period (T3) and corresponding slopes presenting the change over the 3-year study period (Slo) are given in Table 1. In the OWB group, 23 boys out of 26 remained overweight or obese throughout the study period and were divided according to their BMI gain over the 3-year study period into EΔBMI (BMI gain ≥ 3.0 kg/m^2) ($n = 8$) or NEΔBMI (BMI gain < 3.0 kg/m^2) ($n = 15$) group.

At the beginning of the study, the mean chronological age was similar in all groups, while the mean bone age in OWB was significantly higher at T0 (difference 1.3 years) and remained higher after the 3-year study period at T3 (difference 1.0 year) compared to NWB ($P < 0.01$). At baseline, more boys were at Tanner stages 2 and 3 in the OWB group (23 boys together out of 26) compared to the NWB group, who were more pre-pubertal (11 boys out of 29 at pubertal stage 1; $P = 0.02$). After the 3-year period, same proportion, 20 (80%) and 22 (79%) boys in OWB and NWB, respectively, had reached Tanner stages 4 or 5 ($P > 0.046$). Sexual maturation in EΔBMI and NEΔBMI was similar throughout the study period ($P > 0.046$).

As expected, boys in the OWB group were heavier and taller and had higher BMI ($P < 0.0001$) compared to the NWB group at different study time points. TBF%, TB FM, TB FFM, and TR FM were also higher in the OWB group compared to the NWB group at different time points (all $P < 0.01$). Although BMI, TB FM, TB FFM, and TR FM increased significantly over the study period in both, in the OWB and NWB groups ($P < 0.001$), the only significant difference between the groups was seen between the TBF% change ($P < 0.0001$). Specifically, the TBF% decreased in the OWB group and increased in the NWB group (Table 1).

Within OWB group, TB FM, TR FM and TB FFM increased significantly over the study period in both groups (all $P < 0.01$). There was significant difference in changes of BMI, TB FM and TR FM between EΔBMI and NEΔBMI. BMI ($P < 0.001$), TB FM ($P = 0.01$), and TR FM ($P = 0.026$) increased more in the EΔBMI group over the 3-year study period. TBF% increased in the EΔBMI and decreased in the NEΔBMI group ($P = 0.028$). The increase in TB FFM was similar between the EΔBMI and NEΔBMI groups throughout the 3-year study period ($P > 0.046$).

Table 1. The main clinical and body composition characteristics of participants at T0 and T3 {mean with 95% confidence intervals [e.g. X (Y–Z)] or median with 25th and 75th percentile [e.g. X (Y; Z)]}, and changes of these parameters presented as slopes through a 3-year study period {mean with 95% confidence intervals [e.g. X (Y–Z)]} in different study subgroups

Variable	**Time Point**	**Group**			
		OWB (*n* = 26)	**NWB (*n* = 29)**	**OWB**	
				EΔBMI (*n* = 8)	**NEΔBMI (*n* = 15)**
Age (y)	T0	11.1 (10.9–11.4)	10.9 (10.7–11.1)	11.0 (10.3–11.7)	11.1 (10.8–11.5)
	T3	14.1 (13.8–14.4)	13.8 (13.6–14.1)	13.9 (13.2–14.7)	14.1 (13.8–14.5)
Bone age (y)	T0	11.7 (11.2–12.3)*	10.4 (10.0–10.8)	11.1 (9.7–12.5)	12.1 (11.5–12.7)
	T3	14.7 (14.3–15.2)*†	13.7 (13.3–14.0)†	14.1 (13.0–15.1)†	15.0 (14.5–15.6)†
	Slo	1.0 (0.9–1.2)	1.1 (0.9–1.2)	1.1 (0.9–1.3)	0.96 (0.7–1.2)
Tanner (n/stage)	T0	3/21/2/0/0	11/16/2/0/0	0/7/1/0/0	2/12/1/0/0
	T3	0/1/4/11/9*	0/1/5/16/6*	0/1/1/5/1	0/0/2/7/6
Body mass (kg)	T0	63.2 (57.4–68.9)*	35.8 (33.9–37.7)	59.1 (42.1–76.2)	64.4 (57.1–71.8)
	T3	87.4 (78.8–96.1)*	54.0 (50.7–57.4)	90.6 (64.4–116.8)	87.5 (78.3–96.7)
Body height (m)	T0	1.52 (1.49–1.56)*	1.46 (1.44–1.49)	1.53 (1.44–1.63)	1.53 (1.49–1.57)
	T3	1.73 (1.69–1.76)*	1.67 (1.64–1.71)	1.72 (1.62–1.83)	1.74 (1.70–1.78)
BMI (kg/m^2)	T0	27.0 (25.2–28.7)*	16.6 (16.1–17.1)	24.4 (20.8; 27.8)	26.4 (24.2; 29.8)
	T3	29.0 (26.9–31.0)*†	19.2 (18.5–19.9)†	27.9 (25.6; 31.6)	28.1 (26.3; 31.3)
	Slo	0.69 (0.32–1.06)	0.88 (0.72–1.04)	1.75 (0.85–2.66)*	0.51 (0.35–0.68)
Testo (nmol/L)	T0	0.73 (<0.35; 1.69)	0.48 (<0.35; 0.82)	0.36 (0.35; 0.81)	0.76 (0.44; 1.69)
	T3	10.1 (6.2; 13.8)†	12.5 (8.0; 15.7)†	6.0 (4.7; 13.3)†	11.5 (6.3; 13.8)†
	Slo	2.8 (1.8; 4.0)	3.9 (2.3;4.9)	1.8 (1.2; 3.2)	3.3 (2.1; 4.0)
TBF%	T0	41.3 (36.7; 46.3)*	16.2 (12.3;19.5)	41.0 (35.9; 43.0)	40.9 (36.3; 46.3)
	T3	39.4 (30.6; 42.1)*	16.2 (11.1; 24.2)	40.9 (38.2; 42.0)	34.5 (30.0; 42.3)
	Slo	-1.8 (-2.6; -0.9)*	0.4 (-0.8; 1.6)	0.01 (-2.5; 1.8)*	-1.7 (-3.4;-0.9)

Table 1. The main clinical and body composition characteristics of participants at T0 and T3 {mean with 95% confidence intervals [e.g. X (Y–Z)] or median with 25th and 75th percentile [e.g. X (Y; Z)]}, and changes of these parameters presented as slopes through a 3-year study period {mean with 95% confidence intervals [e.g. X (Y–Z)]} in different study subgroups. Continuation

Variable	Time Point	Group			
		OWB (*n* = 26)	NWB (*n* = 29)	OWB	
				EΔBMI (*n* = 8)	NEΔBMI (*n* = 15)
TB FM (kg)	T0	24.6 (18.3; 30.1)*	5.1 (4.0; 7.0)	23.0 (14.8; 29.3)	25.1 (18.4; 30.1)
	T3	30.5 (22.6; 35.0)*†	8.6 (6.5; 11.4)†	33.0 (26.3; 34.9)†	28.3 (22.3; 36.6)†
	Slo	1.9 (1.2–2.7)	1.3 (0.86–1.7)	3.7 (2.0–5.5)*	1.5 (0.8–2.3)
TB FFM (kg)	T0	33.9 (31.5; 38.6)*	27.4 (25.6; 29.2)	33.1 (26.2; 38.2)	34.6 (32.6; 39.1)
	T3	50.8 (40.8; 59.0)*†	40.2 (35.6; 45.2)†	47.3 (37.0; 67.3)†	52.6 (43.6; 59.0)†
	Slo	5.6 (4.5–6.8)	4.9 (4.0–5.9)	6.3 (3.5–9.0)	5.4 (3.9–7.0)
TR FM (kg)	T0	10.8 (7.3; 14.4)*	1.8 (1.3; 2.1)	10.6 (6.6; 14.1)	10.5 (7.3; 14.4)
	T3	13.5 (10.1; 16.5)*†	3.7 (2.9; 4.5)†	14.4 (12.4; 17.0)†	12.8 (89.0; 17.9)†
	Slo	0.9 (0.6–1.3)	0.6 (0.4–0.8)	1.8 (0.9–2.6)*	0.8 (0.4–1.2)
Glucose (mmol/L)	T0	4.9 (4.7–5.1)*	5.2 (5.0–5.4)	5.3 (5.0; 5.5)*	4.8 (4.3; 5.0)
	T3	5.1 (5.0–5.2)	5.3 (5.1–5.4)	5.2 (4.8; 5.3)	5.2 (4.9; 5.4)†
	Slo	0.06 (-0.01–0.13)	0.01 (-0.05–0.07)	-0.05 (-0.17–0.07)*	0.13 (0.04–0.22)
Insulin (mU/L)	T0	10.6 (8.0; 13.4)*	4.8 (3.2; 8.2)	6.65 (4.6; 9.5)*	11.8 (10.2; 15.5)
	T3	18.7 (15.8; 24.8)*	13.0 (9.9; 15.9)	27.50(21.2; 29.3)*†	19.3 (15.8; 22.7)†
	Slo	3.1 (1.6–4.7)	2.6 (1.8–3.4)	7.3 (4.1–10.4)*	1.9 (0.4–3.3)
HOMA-IR	T0	2.3 (1.8; 2.6)*	1.2 (0.7; 1.9)	1.6 (1.1; 2.2)*	2.4 (2.3; 3.2)
	T3	4.1 (3.6; 5.7)* †	3.0 (2.3; 3.8)†	6.2 (4.9; 6.9)*†	4.2 (3.6; 5.2)†
	Slo	0.74 (0.40–1.08)	0.63 (0.44–0.81)	1.64 (0.97–2.31)*	0.50 (0.16–0.84)

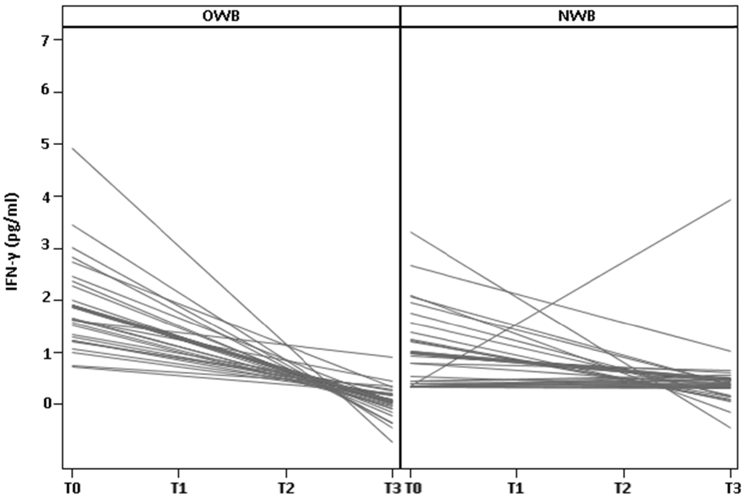
Abbreviations: OWB, boys with overweight and obesity; NWB, normal weight boys; EΔBMI, extensive body mass index change group; NEΔBMI, non-extensive body mass index change group; T0, baseline; T3, follow-up at 3-years; Slo, slope – change of value through the study period (computed on all four studied time-points T0, T1, T2, T3); BMI, body mass index; TBF%, total body fat percentage; TB, total body; FM, fat mass; FFM, fat-free mass; TR, trunk; Testo, testosterone; HOMA-IR, homeostasis model assessment-insulin resistance. The cut-off *P* value for significance of a single comparison is 0.046. Only *P* values that are below the adjusted false discovery rate significance threshold are therefore significant and marked with an asterisk (*) between OWB and NWB or between EΔBMI and NEΔBMI; marked with a dagger (†) between T0 and T3 within groups

According to individual HOMA-IR interpretation, 10 out of 26 in OWB compared to 3 out of 29 in NWB had insulin resistance at T3 [i.e. HOMA-IR > 5.22 (Kurtoğlu et al. 2010a)]. When considering BMI increment, 6 out of 8 boys in EΔBMI and 4 out of 15 boys in NEΔBMI had insulin resistance at T3. Insulin ($P < 0.001$) and HOMA-IR ($P < 0.01$) increased significantly more in the EΔBMI group compared to the NEΔBMI group throughout the study period, and median HOMA-IR at T3 showed that the HOMA-IR score was higher in EΔBMI than the threshold for the insulin resistance.

According to parental questionnaires, majority of boys in OWB and NWB were from families of the middle-class socio-economic status. There were no significant differences in physical activity level at baseline and at 3-year between OWB and NWB: all boys took part in obligatory physical education classes and the most frequent additional physical activities were training sessions three times a week lasting each between 75–90 min. The median time the boys had been taking training sessions at T3 were 4.0 years in the OWB group and 5.0 years in the NWB group. All boys were in their ordinary everyday diet and were asked not to change their intake of dairy products or red meat as major sources of calcium.

5.2. Serum inflammatory biomarkers and their changes in boys with overweight and obesity during their pubertal maturation

At baseline, serum IL-6, IL-8, IFN-γ, TNF-α, IL-1α, and leptin concentrations were higher (all $P < 0.046$) in the OWB compared to NWB (Table 2). Over the 3-year period, IFN-γ ($P < 0.001$) and TNF-α ($P = 0.004$) decreased in both groups, whereas the decrease in OWB was more extensive compared to NWB (Figure 1). In the OWB group, serum IL-1α ($P = 0.013$) and leptin ($P < 0.001$) concentrations decreased, and in NWB, IL-1α and leptin levels increased. When considering BMI increment in the OWB group, IL-8 ($P = 0.032$), MCP-1 ($P = 0.023$), and leptin ($P < 0.001$) increased in the EΔBMI and decreased in the NEΔBMI group over the 3-year study period (Figure 2; Table 3). Serum IL-4 concentration was the only marker at T0 that correlated with BMI slope in the OWB group ($r = 0.48$; $P = 0.02$) (Figure 3). Serum IL-4 also correlated significantly with TB FM slope ($r = 0.43$; $P = 0.029$) and TR FM slope ($r = 0.46$; $P = 0.018$) at baseline in the OWB group.



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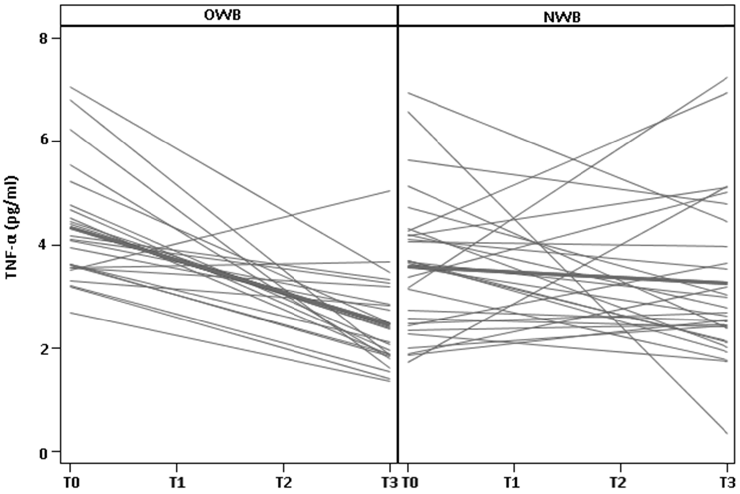
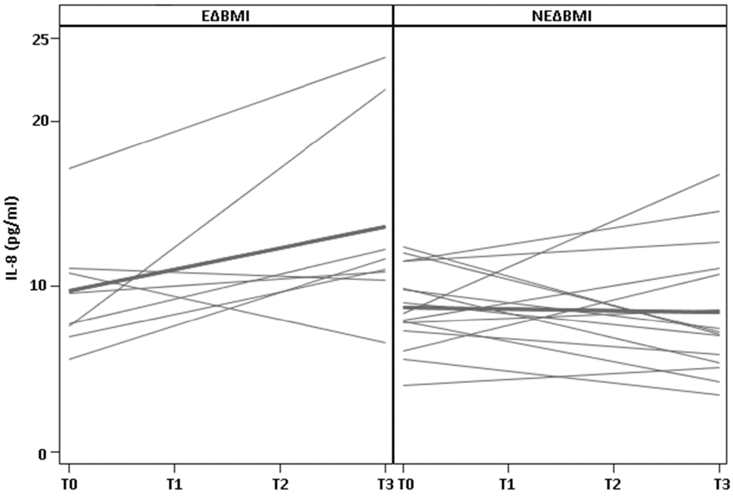


B

Figure 1. Longitudinal changes in different cytokines in OWB and NWB. Panel A is slope lines for IFN-γ; panel B is slope lines for TNF-α. For panels A-B, gray lines represent longitudinal changes of an individual value, and the bold lines are the best fitting lines representing the group mean.



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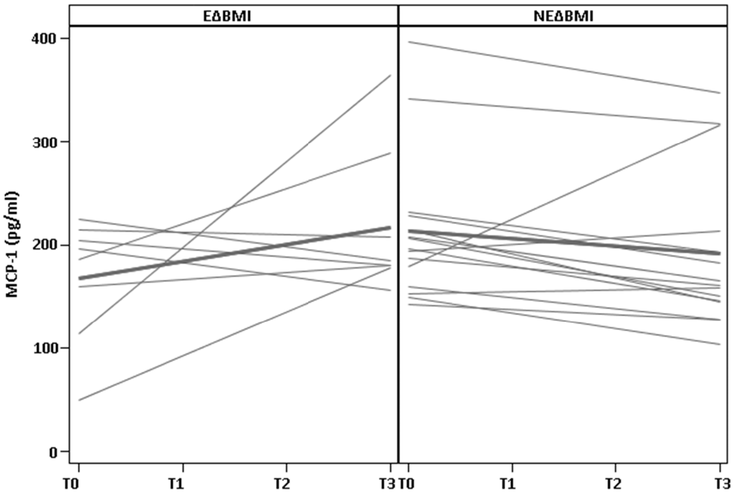

B

Figure 2. Longitudinal changes in different cytokines in EΔBMI and NEΔBMI. Panel A is slope lines for IL-8; panel B is slope lines for MCP-1. For panels A-B, gray lines represent longitudinal changes of an individual value, and the bold lines are the best fitting lines representing the group mean.

Table 2. Concentrations of cytokines of OWB and NWB at T0 and T3 {mean with 95% confidence intervals [e.g. X (Y–Z)] or median with 25th and 75th percentile [e.g. X (Y; Z)]}, and changes of inflammatory biomarkers' concentrations presented as slopes through a 3-year study period {mean with 95% confidence intervals [e.g. X (Y–Z)]}

Variable	**Time point**	**Group**		**Variable**	**Time point**	**Group**	
		OWB (n = 26)	**NWB (n = 29)**			**OWB (n = 26)**	**NWB (n = 29)**
IL-2 (pg/mL)	T0	1.67 (1.21; 2.06)	1.68 (1.31; 2.34)	**TNF-α (pg/mL)**	T0	4.00 (3.66; 5.50)*	3.53 (2.31; 4.24)
	T3	1.14 (0.87; 1.71)	1.37 (1.02; 2.27)		T3	2.67 (2.21; 3.09)†	2.79 (2.28; 3.43)
	Slo	-0.01 (-0.25–0.24)	0.26 (-0.31–0.82)		Slo	-0.63 (-0.82–(-0.43))*	-0.12 (-0.38–0.14)
IL-4 (pg/mL)	T0	2.12 (1.69; 2.45)	1.79 (1.55; 2.45)	**IL-1α (pg/mL)**	T0	0.30 (0.12; 0.39)*	0.18 (0.12; 0.25)
	T3	1.66 (1.54; 2.16)†	1.77 (1.51; 2.11)		T3	0.18 (0.14; 0.20)†	0.17 (0.13; 0.25)
	Slo	-0.17 (-0.35–0.01)	0.05 (-0.08–0.18)		Slo	-0.06 (-0.10–(-0.01))*	0.01 (-0.01–0.03)
IL-6 (pg/mL)	T0	0.95 (0.78; 1.16)*	0.58 (0.46; 0.70)	**IL-1β (pg/mL)**	T0	1.20 (0.88; 1.81)	1.19 (0.81; 2.07)
	T3	0.90 (0.63; 1.26)	0.60 (0.46; 1.08)		T3	0.98 (0.79; 1.95)	1.30 (1.01; 2.71)
	Slo	0.07 (-0.22–0.36)	0.14 (-0.06–0.33)		Slo	-0.04 (-0.19–0.12)	0.11(-0.14–0.36)
IL-8 (pg/mL)	T0	9.37 (7.83; 12.11)*	8.19 (6.73; 9.58)	**MCP-1 (pg/mL)**	T0	209. 9 (170.1; 224.2)	174.9 (133.3; 235.4)
	T3	8.92 (7.25; 11.09)	7.62 (6.06; 9.84)		T3	182.3 (157.6; 233. 5)	173.1 (148.4; 250.2)
	Slo	0.12 (-0.40–0.65)	0.47 (-0.24–1.18)		Slo	-6.61 (-13.45–0.17)	3.25 (-3.95–10.46)
IL-10 (pg/mL)	T0	0.81 (0.62; 0.92)	0.73 (0.62; 0.98)	**EGF (pg/mL)**	T0	20.7 (11.9; 31.2)	23.5 (12.7; 37.4)
	T3	0.77 (0.66; 0.97)	0.91 (0.68; 1.06)		T3	67.5 (42.7; 83.3)†	52.03 (35.1; 75.3)†
	Slo	0.05 (-0.03–0.13)	0.23 (-0.16–0.63)		Slo	12.12 (7.38–16.87)	7.06 (3.54–10.58)
VEGF (pg/mL)	T0	106.6 (69.2; 155.2)	62.5 (38.1; 118.4)	**Adipo-nectin (µg/mL)**	T0	6.27 (4.86; 8.35)	6.72 (5.08; 8.73)
	T3	99.4 (49.3; 122.8)	60.1 (40.2; 120.3)		T3	6.05 (4.40; 8.20)	6.40 (4.70; 9.89)
	Slo	-3.57 (-10.97–3.83)	-2.30 (-7.75–3.14)		Slo	0.15 (-0.28–0.59)	-0.06 (-0.52–0.41)
IFN-γ (pg/mL)	T0	2.14 (1.69; 2.99)*	0.67 (0.35; 1.66)	**Leptin (ng/mL)**	T0	22.80 (20.22; 44.17)*	1.22 (0.39; 2.67)
	T3	0.42 (0.32; 0.60)*†	0.37 (0.33; 0.46)†		T3	11.72 (5.90; 19.60)*†	1.70 (0.60; 5.10)
	Slo	-0.61 (-0.78–(-0.45))*	-0.18 (-0.33–(-0.02))		Slo	-4.22 (-5.80–(-2.65))*	0.44 (-0.12–1.00)

Abbreviations: OWB, boys with overweight and obesity; NWB, normal weight boys; EΔBMI, extensive body mass index change group; NEΔBMI, non-extensive body mass index change group; T0, baseline; Slo, slope – change of value through the study period (computed on all four studied time-points T0, T1, T2, T3); IL, interleukin; VEGF, vascular endothelial growth factor; IFN-γ, interferon gamma; TNF-α, tumour necrosis factor alpha; MCP-1, monocyte chemoattractant protein-1; EGF, epidermal growth factor. The cut-off *P* value for significance of a single comparison is 0.046. Only *P* values that are below the adjusted false discovery rate significance threshold are therefore significant and marked with an asterisk (*) between OWB and NWB; marked with a dagger (†) between T0 and T3 within groups

Table 3. Concentrations of cytokines of EΔBMI and NEΔBMI at T0 and T3 {mean with 95% confidence intervals [e.g. X (Y–Z)] or median with 25th and 75th percentile [e.g. X (Y; Z)]} and changes of inflammatory biomarkers' concentrations presented as slopes through a 3-year study period {mean with 95% confidence intervals [e.g. X (Y–Z)]}

Variable	Time point	OWB		Variable	Time point	OWB	
		EΔBMI (*n* = 8)	**NEΔBMI (*n* = 15)**			**EΔBMI (*n* = 8)**	**NEΔBMI (*n* = 15)**
IL-2 (pg/mL)	T0	1.84 (1.56; 2.81)	1.71 (1.21; 2.06)	**TNF-α (pg/mL)**	T0	4.08 (3.62; 6.71)	3.92 (3.57; 5.53)
	T3	1.58 (1.05; 4.66)	1.14 (0.87; 1.71)		T3	3.08 (2.83; 3.20)*	2.31 (2.11; 2.76)†
	Slo	0.20 (-0.64–1.05)	-0.06 (-0.25–0.13)		Slo	-0.37 (-0.83–0.08)	-0.63 (-0.95–(-0.32))
IL-4 (pg/mL)	T0	2.43 (1.99; 3.09)	1.99 (1.67; 2.42)	**IL-1α (pg/mL)**	T0	0.28 (0.21; 0.53)	0.19 (0.11; 0.35)
	T3	1.91 (1.53; 2.37)	1.63 (1.54; 1.80)†		T3	0.26 (0.22; 0.28)*	0.15 (0.13; 0.18)
	Slo	-0.39 (-1.03–0.25)	-0.11 (-0.18–(-0.04))		Slo	-0.06 (-0.16–0.05)	-0.06 (-0.12–0.01)
IL-6 (pg/mL)	T0	1.11 (0.64; 2.08)	0.94 (0.78; 1.11)	**IL-1β (pg/mL)**	T0	1.59 (0.92; 1.82)	0.96 (0.74; 1.75)
	T3	0.92 (0.73; 1.16)	0.77 (0.61; 1.02)		T3	1.63 (0.78; 2.52)	1.01 (0.79; 2.05)
	Slo	-0.13 (-0.31–0.06)	-0.05 (-0.14–0.05)		Slo	-0.02 (-0.30–0.26)	0.03 (-0.23–0.29)
IL-8 (pg/mL)	T0	8.91 (5.69; 12.38)	9.16 (7.83; 11.48)	**MCP-1 (pg/mL)**	T0	181.9 (132.2; 217.1)	208.2 (177.9; 222.6)
	T3	10.15 (9.07; 18.90)	8.35 (6.10; 11.64)		T3	184.0 (165.8–233.1)	182.2 (139.5–233.5)†
	Slo	1.33 (-0.22–2.88)*	-0.09 (-0.81–0.63)		Slo	16.30 (-12.50–45.11)*	-7.48 (-16.73–1.76)
IL-10 (pg/mL)	T0	0.89 (0.81; 1.00)	0.78 (0.58; 0.91)	**EGF (pg/mL)**	T0	21.50 (12.25; 37.80)	16.94 (9.94; 26.57)
	T3	0.83 (0.75; 0.89)	0.73 (0.62; 1.05)		T3	52.62 (41.67; 69.31)†	67.3 (36.2; 81.8)†
	Slo	-0.01 (-0.08–0.06)	0.05 (-0.09–0.18)		Slo	8.03 (-0.77–16.83)	13.96 (6.97–20.96)
VEGF (pg/mL)	T0	78.7 (32.8; 40.3)	111.4 (69.2; 169.9)	**Adipo-nectin (µg/mL)**	T0	5.53 (4.63; 6.36)	6.60 (4.86; 8.35)
	T3	78.2 (43.2–132.4)	101.7 (48.0; 122.8)		T3	5.01 (3.75; 6.65)	6.20 (4.20; 8.20)
	Slo	0.64 (-18.43–19.71)	-4.85 (-15.93–6.22)		Slo	-0.26 (-0.70–0.18)	0.04 (-0.52–0.59)
IFN-γ (pg/mL)	T0	1.92 (0.93; 2.10)	2.22 (1.65; 3.03)	**Leptin (ng/mL)**	T0	20.84 (13.36; 22.88)	29.12 (22.23; 46.22)
	T3	0.36 (0.32–0.57)†	0.41 (0.33–0.50)†		T3	18.95 (13.55; 31.25)	9.8 (5.9-17.3)†
	Slo	-0.38 (-0.60–(-0.16))	-0.64 (-0.88–(-0.40))		Slo	0.43 (-2.62–3.47)*	-5.80 (-7.61–(-3.99))

Abbreviations: OWB, boys with overweight and obesity; EΔBMI, extensive body mass index change group; NEΔBMI, non-extensive body mass index change group; T0, baseline; Slo, slope – change of value through the study period (computed on all four studied time-points T0, T1, T2, T3); IL, interleukin; VEGF, vascular endothelial growth factor; IFN-γ, interferon gamma; TNF-α, tumour necrosis factor alpha; MCP-1, monocyte chemoattractant protein-1; EGF, epidermal growth factor. The cut-off *P* value for significance of a single comparison is 0.046. Only *P* values that are below the adjusted false discovery rate significance threshold are therefore significant and marked with an asterisk (*) between EΔBMI and NEΔBMI; marked with a dagger (†) between T0 and T3 within groups

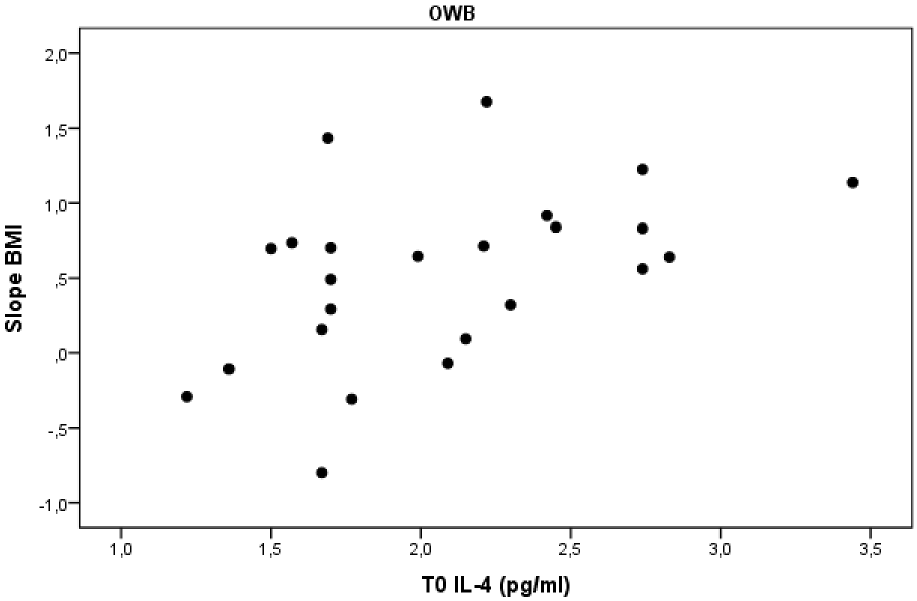

Figure 3. Spearman correlation between baseline interleukin-4 and BMI slope in the OWB group ($r = 0.48$, $P = 0.02$).

5.3. Bone mineral characteristics and their changes in boys with overweight and obesity during their pubertal maturation

The mean TB BMD and median TB BMC, TB BMC/height, LS BMD, and LS BMC at T0 were significantly higher, but the mean TB BMAD was significantly lower in OWB compared to NWB (all $P < 0.01$) (Table 4). The median LS BMAD was similar between OWB and NWB at T0. Over the 3-year period, boys in OWB group gained significantly more their TB BMD ($P = 0.0001$), TB BMC ($P = 0.0048$), TB BMC/height ($P = 0.0124$), LS BMD ($P = 0.0029$), and LS BMC ($P = 0.0022$) and lost significantly less TB BMAD ($P < 0.01$) compared to NWB. The increase in LS BMAD did not show any significant difference between OWB and NWB throughout the 3-year study period ($P > 0.046$).

When considering BMI increment in OWB throughout the 3-year study period, no differences were found at T0 in TB and LS bone mineral characteristics between EΔBMI and NEΔBMI ($P > 0.046$) (Table 4). One boy was excluded from the EΔBMI group when analysing bone mineral data (final $n = 7$), as his data lied constantly outside the overall pattern (Table 4). Slope analysis showed that TB BMD ($P = 0.0065$), TB BMC ($P = 0.0141$), TB BMC/height ($P = 0.0199$), LS BMD ($P = 0.0066$), LS BMAD ($P = 0.0075$), and LS BMC ($P = 0.017$) increased significantly less in EΔBMI compared to NEΔBMI throughout the 3-year study period (Table 4).

Table 4. Bone mineral characteristics of subjects at T0 and T3 {mean with 95% confidence intervals [e.g. X (Y–Z)] or median with 25th percentile and 75th percentile [e.g. X (Y; Z)]} and changes of bone mineral characteristics presented as slopes through a 3-year study period {mean with 95% confidence intervals [e.g. X (Y–Z)]} in different study subgroups

Var	**Time point**	**Group**			
				OWB	
		OWB (*n* = 26)	**NWB (*n* = 29)**	**EΔBMI (*n* = 7)**	**NEΔBMI (*n* = 15)**
TB BMD (g/cm^2)	T0	1.01 (0.99–1.03)*	0.94 (0.93–0.96)	0.99 (0.93–1.05)	1.02 (0.99–1.04)
	T3	1.05 (1.03–1.08)*†	1.03 (1.01–1.05)†	1.09 (1.02–1.15)*†	1.18 (1.13–1.22)†
	Slo	0.05 (0.04–0.06)*	0.03 (0.02–0.04)	0.03 (0.02–0.04)*	0.05 (0.04–0.06)
TB BMAD (g/cm^3)	T0	0.09 (0.08–0.09)*	0.09 (0.09–0.10)	0.09 (0.08–0.09)	0.09 (0.08–0.09)
	T3	0.08 (0.08–0.09)	0.09 (0.08–0.09)†	0.09 (0.08–0.09)	0.08 (0.08–0.09)
	Slo	-0.0004 ((-0.001)– 0.001)*	-0.003 ((-0.003)–(-0.002))	-0.0012 ((-0.003–0.0003))	-0.001 (-0.002)–0.001)
TB BMC (g)	T0	1844 (1568; 1938)*	1392 (1310; 1504)	1703 (1307–2098)	1895 (1687–2102)
	T3	2745 (2409; 3188)*†	1985 (1861; 2363)†	2390 (1973–2808)*†	2899 (2632–3167)†
	Slo	308 (264–351)*	235 (203–266)	235 (173–297)*	340 (283–398)
TB BMC/height	T0	12.1 (10.7; 12.9)*	9.7 (9.1; 10.0)	11.1 (9.2–13.0)	12.3 (11.3–13.3)
	T3	15.6 (14.4; 17.8)*†	12.0 (11.7; 13.9)†	13.9 (12.3–15.5)*†	16.6 (15.4–17.8)†
	Slo	1.29 (1.08–1.50)*	0.98 (0.84–1.13)	0.965 (0.69–1.23)*	1.46 (1.17–1.74)
LS BMD (g/cm^2)	T0	0.84 (0.8; 0.9)*	0.77 (0.73; 0.81)	0.80 (0.71–0.89)	0.85 (0.8–0.9)
	T3	1.05 (0.94; 1.19)*†	0.89 (0.85; 1.01)†	0.94 (0.84 – 1.04)*†	1.09 (1.02–1.17)†
	Slo	0.08 (0.06–0.09)*	0.05 (0.04–0.06)	0.05 (0.03–0.07)*	0.08 (0.07–0.10)
LS BMAD (g/cm^3)	T0	0.15 (0.15; 0.16)	0.14 (0.14; 0.15)	0.16 (0.13; 0.16)	0.15 (0.14; 0.16)
	T3	0.16 (0.16; 0.17)*†	0.15 (0.14; 0.16)†	0.16 (0.15; 0.16)	0.17 (0.15; 0.17)†
	Slo	0.005 (0.003–0.006)	0.003 (0.002–0.004)	0.002 ((-0.0003)–0.004)*	0.006 (0.004–0.007)
LS BMC (g)	T0	26.9 (22.7; 30.0)*	21.0 (19.8; 23.7)	24.0 (18.6–29.5)	28.2 (24.7–31.8)
	T3	46.1 (34.5; 53.5)*†	35.3 (29.6; 37.3)†	36.5 (25.9–47.0)*†	48.4 (42.5–54.4)†
	Slo	6.3 (5.2–7.5)*	4.2 (3.4–5.0)	4.2 (2.0–6.5)*	6.9 (5.5–8.3)

Abbreviations: OWB, boys with overweight and obesity; NWB, normal weight boys; EΔBMI, extensive body mass index change group; NEΔBMI, non-extensive body mass index change group; T0, baseline; Slo, slope – change of value through the study period (computed on all four studied time-points T0, T1, T2, T3); Var, variable; TB, total body; BMD, bone mineral density; BMC, bone mineral content; LS, lumbar spine; BMAD, bone mineral apparent density. The cut-off *P* value for significance of a single comparison is 0.046. Only *P* values that are below the adjusted false discovery rate significance threshold are therefore significant and marked with an asterisk (*) between OWB and NWB or between EΔBMI and NEΔBMI; marked with a dagger (†) between T0 and T3 within groups.

5.4. Associations between changes in bone mineral characteristics and serum concentrations of different inflammatory cytokines in boys with different body mass index during their pubertal maturation

Spearman correlation analysis using data through time points presented as slopes showed that in the EΔBMI group, the changes in IL-6 concentrations were negatively correlated with the changes in LS BMD (r = -0.89, P = 0.01), whereas the changes in VEGF concentrations showed negative correlation with the changes in TB BMD (r = -0.82, P = 0.02) and TB BMC/height (r = -0.82, P = 0.02) (Figure 4). These correlations were calculated with seven boys in the EΔBMI group, as one boy in EΔBMI had bone mineral characteristics that clearly differed from the others, and he was, thus, considered as an outlier (final n = 7). To demonstrate the robustness of these findings with this method, we additionally computed correlation coefficients when the outlier was included. Sensitivity analysis showed that all these correlations slightly changed, but remained significant [r = -0.78–(-0.86), P = 0.01–0.02]. There were no other significant correlations between the slopes of any inflammatory biomarkers and any bone mineral characteristics in OWB, NWB, EΔBMI or NEΔBMI groups except positive correlations between serum IL-2 concentrations and some bone mineral characteristic in NWB: TB BMD (r = 0.41, P = 0.03), TB BMC (r = 0.43, P = 0.02), TB BMC/height (r = 0.44, P = 0.02), LS BMD (r = 0.47, P = 0.01), LS BMC (r = 0.43, P = 0.02) and LS BMAD (r = 0.40, P = 0.03). In addition, changes in testosterone level were positively correlated with the changes in different bone mineral characteristics in the OWB, NWB and NEΔBMI group, but not in the EΔBMI group over the 3-year study period (Table 5).

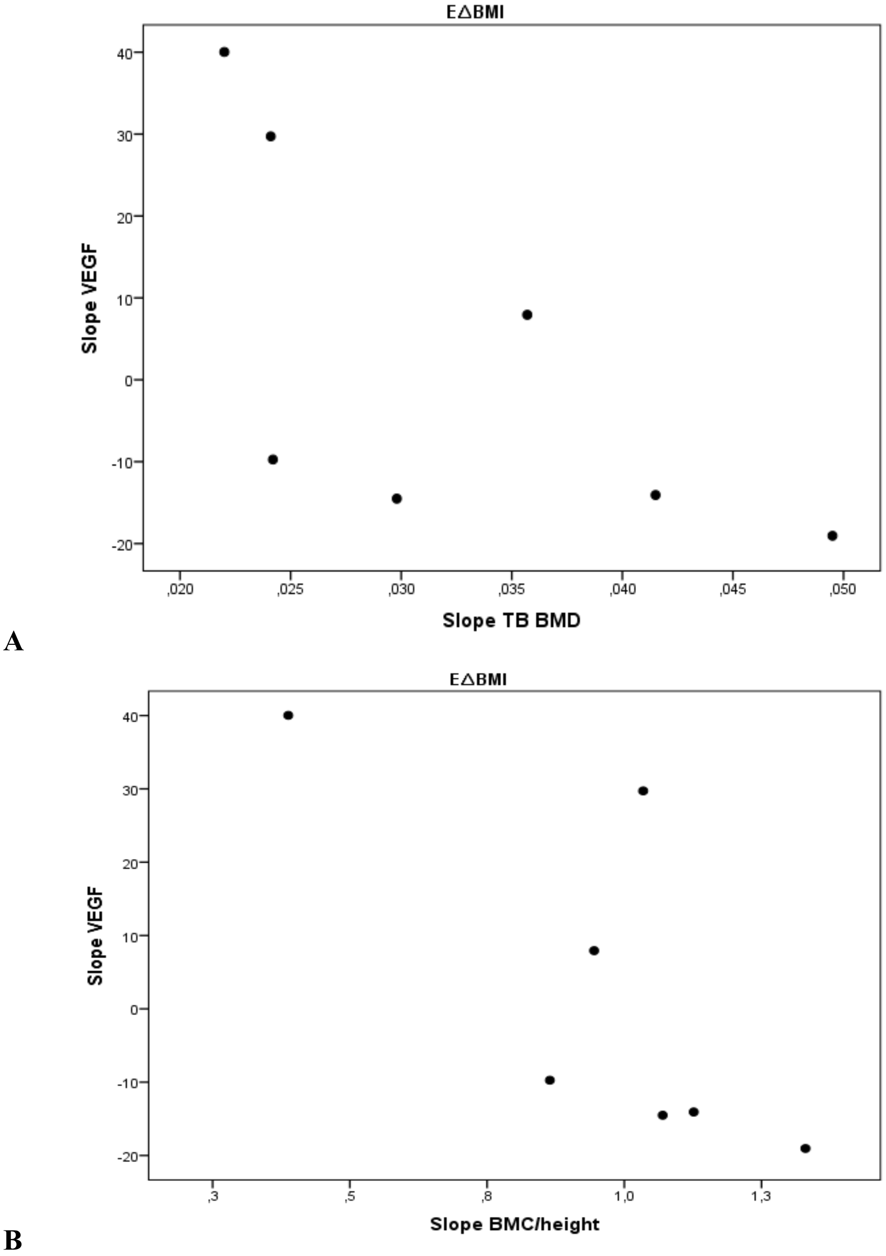

Figure 4. Spearman correlation between the changes of a) VEGF concentration and TB BMD (*r* = -0.82, *P* = 0.02), and b) VEGF concentration and TB BMC/height (*r* = -0.82, *P* = 0.02) during the 3-year study period in the EΔBMI group.

Linear mixed effects models used only in the whole group (*n* = 55) confirmed the finding from Spearman correlation analysis that VEGF was inversely associated with TB BMC/height (Table 6). Linear mixed effects models did not confirm any other significant findings from Spearman correlation analysis. However, LME models revealed some additional findings, such as EGF was inversely associated with LS BMD and LS BMAD (both $P < 0.04$), whereas there was a positive association between IFN-γ and LS BMD ($P = 0.0037$) and between IL-8 and TB BMAD ($P = 0.0199$) (Table 6). IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, MCP-1 and TNF-α showed no associations with BMD values in any of the full or reduced models (data not shown).

Table 5. Spearman correlation coefficients (*r*) between slopes of serum testosterone and bone mineral characteristics' in the NWB, OWB, EΔBMI and NEΔBMI groups

Slope of	**Slope of serum testosterone concentration**			
	OWB (*n* = 26)	**NWB (*n* = 29)**	**EΔBMI (*n* = 7)**	**NEΔBMI (*n* = 15)**
TB BMD	0.493*	0.227	0.321	0.529*
TB BMAD	0.036	-0.323	0.714	-0.127
TB BMC	0.494*	0.458*	0.536	0.557*
TB BMC/height	0.484*	0.392*	0.393	0.618*
LS BMD	0.651**	0.531**	0.703	0.846**
LS BMAD	0.534**	0.257	0.321	0.757**
LS BMC	0.625**	0.632**	0.750	0.714**

* Correlation is significant at the 0.05 level; ** Correlation is significant at the 0.01 level.

Abbreviations: OWB, boys with overweight and obesity; NWB, normal weight boys; EΔBMI, extensive body mass index change group; NEΔBMI, non-extensive body mass index change group; TB, total body; BMD, bone mineral density; BMC, bone mineral content; LS, lumbar spine; BMAD, bone mineral apparent density. All significant *P* values are below the false discovery rate cut-off value (0.088) and marked with an asterisk (*) in OWB, NWB, EΔBMI and NEΔBMI.

Table 6. Summary table of significant linear mixed effects models for serum VEGF, EGF, IFN-γ and IL-8 effect and other independent controlling factors on bone mineral characteristics in the whole study group (*n* = 55) during 3-year study period

Factors	**AIC***	**Estimate**	**SE**	***P* value**
TB BMC/height				
(Intercept)	514.6*	6.62	0.47	<0.0001
VEGF		-0.0053	0.001	<0.0001
Time		0.58	0.05	<0.0001
BMI		0.20	0.02	<0.0001
TB BMAD				
(Intercept)	-1714.8*	0.093	0.002	<0.0001
IL-8		0.00013	0.00006	0.0199
Testo		-0.0004	0.0001	<0.0001
TBF %		-0.0001	0.00003	0.0004
Time		-0.0003	0.0003	0.4333
LS BMD				
(Intercept)	-640.0*	0.59	0.03	<0.0001
EGF		-0.0002	0.0001	0.0092
TBF %		-0.0048	0.0009	<0.0001
Time		0.05	0.01	<0.0001
BMI		0.014	0.002	<0.0001
(Intercept)	-641.0*	0.58	0.03	<0.0001
IFN-γ		0.007	0.002	0.0037
TBF %		-0.005	0.0009	<0.0001
Time		0.05	0.01	<0.0001
BMI		0.01	0.002	<0.0001
LS BMAD				
(Intercept)	-1369.3*	0.143	0.002	<0.0001
EGF		-0.00003	0.000015	0.0375
Time		0.004	0.001	<0.0001

Abbreviations: AIC*, Akaike Information Criteria reduced model; SE, standard error; BMI, body mass index; TB, total body; BMD, bone mineral density; BMC, bone mineral content; LS, lumbar spine; BMAD, bone mineral apparent density; TBF, total body fat; Testo, testosterone; IL, interleukin; VEGF, vascular endothelial growth factor; IFN-γ, interferon gamma; EGF, epidermal growth factor

6. DISCUSSION

6.1. Longitudinal changes in serum inflammatory biomarkers in boys with overweight and obesity during their pubertal maturation

In this 3-year longitudinal study, our main focus was to identify the changes in a panel of different inflammatory biomarkers in boys with different BMI and BMI gain during pubertal years. Additionally we also identified the inflammatory biomarker that associated with BMI gain in boys with overweight and obesity during pubertal maturation. Specifically, baseline serum IL-4 concentration was positively correlated with changes in BMI in OWB. It is somewhat surprising, as Chang et al. (2012) suggest that IL-4 is involved in mediating glucose and lipid metabolism by promoting insulin sensitivity, glucose tolerance and inhibiting lipid deposits. However, in animal studies where IL-4 was administered intracerebroventricularly to adult male Wistar rats, it caused excess body mass gain and body fat content was mediated independently of changes in food consumption (Oh-I et al. 2010). These results suggest that IL-4-mediated hypothalamic inflammation may enhance weight gain by decreasing metabolic rate and/or fat oxidation (Oh-I et al. 2010). We can speculate that serum IL-4 concentration in OWB at the beginning of puberty may help to identify those boys who gain BMI and increase TB and TR FM more extensively during pubertal years.

Serum IL-8 level was significantly higher in OWB at the beginning of puberty and increased further in those boys who gained BMI more extensively, but decreased in the NEΔBMI group. This is similar to the study by Sharabiani et al. (2011) who showed that serum IL-8 concentration was a predictive biomarker of excessive body weight in adults, whereas IL-8 did not increase in adults with mild obesity (Kim et al. 2006). In contrast to our study, Tam et al. (2010) found no differences in IL-8 concentration longitudinally in boys with BMI gain, although increased serum IL-8 level was seen in girls who became overweight over time (Tam et al. 2010). However, in children with obesity who have had substantial weight loss, IL-8 concentration decreased (Roth et al. 2011). From these findings we could suggest that increment in serum IL-8 concentration can be a biochemical marker to identify those subjects with increased risk for excessive BMI gain. IL-8 is mostly known as a chemokine inducing chemotaxis and is responsible for the recruitment of neutrophils and T lymphocytes into the subendothelial space (Gesser et al. 1996). However, it has been also shown that IL-8 is a main adipocytokine producing insulin resistance via the inhibition of insulin-induced Akt phosphorylation in adipocytes (Kobashi et al. 2009). Our results also demonstrated that most boys in EΔBMI (six out of eight) had insulin resistance. The latter was defined as HOMA-IR higher than 5.22 (Kurtoğlu et al. 2010a). Pubertal transient state of insulin resistance can be seen as an adaptive strategy in adolescents with healthy weight, but in adolescents with obesity this increases the risk for further metabolic complications (Kelly et al. 2011).

Similar to many previous studies, baseline TNF-α (Chang et al. 2015; Habib et al. 2015) and IFN-γ (Utsal et al. 2012) concentrations were also higher in OWB compared to NWB. Our longitudinal results confirm Kayser et al. (2015) longitudinal results that TNF-α decreased with increasing age in OWB. Kayser et al. (2015) did not include lean subjects into their study, but our data showed that serum TNF-α concentration decreased also in NWB during pubertal maturation, but with significantly lower rate. In our study, IFN-γ also decreased over the study period in both groups, but was more extensive in OWB compared to NWB. Roth et al. (2011) did not find longitudinally significant difference in IFN-γ concentrations between children with overweight and their lean peers. Kleiner et al. (2013) investigated healthy subjects and found that TNF-α and IFN-γ were upregulated in healthy youth. Thus, it is likely that the decrease in TNF-α and IFN-γ is more linked to further pubertal maturation itself rather than to the changes in BMI indicating that the physiological adaptations during puberty influence inflammatory response in children (Casazza et al. 2010).

There was no significant differences at baseline serum MCP-1 concentration between OWB and NWB as showed in previous studies (Stoppa-Vaucher et al. 2012), neither there were significant changes in MCP-1 concentration during the 3-year study period between OWB and NWB. Kayser et al. (2015) did not find change in MCP-1 levels in 11-year-old boys with overweight during the following 4-year period. The results of the current study showed that MCP-1 concentration increased in those OWB, who gained their BMI more extensively during pubertal years and decreased in those whose BMI gain was less extensive. Roth et al. (2011) showed that serum MCP-1 increased significantly in children without substantial body mass loss. These results suggest that increment in serum MCP-1 concentration, similar to IL-8, can identify boys with obesity who have increased risk to gain BMI excessively during puberty.

During normal pubertal maturation changes in body composition follow certain sex-specific trajectory. FM increases in boys from age 6- to 12-years and thereafter declines downwards to age 15 and then flattens off (Kurtoğlu et al. 2010b). The drop in TBF% is more pronounced at higher body fat percentiles (Ogden et al. 2011). This was seen also in our study where OWB showed significant decline in TBF% compared to NWB. However, when considering BMI increment in OWB, the TBF% decreased only in those boys who gained their BMI at slower rates during the 3-year study period. The results of the current study also confirm the link between body composition and cytokines associated with adipose tissue inflammation (Kayser et al. 2015; Loomba-Albrecht & Styne 2009; Utsal et al. 2012). Specifically, a significant increase in IL-8, MCP-1 and leptin levels was seen simultaneously with an increase in TB FM, TR FM and TBF% in those OWB who gained their BMI extensively during 3-year period, whereas IL-8, MCP-1 and leptin decreased in those OWB whose BMI, TB FM and TR FM increment were lower and whose TBF%

decreased. These results coincide with the Calarge et al. (2012) who showed that children with rapid body mass gain have been shown to have an unfavourable metabolic profile with increased systemic inflammation compared to children who gain body mass at slower rates. Accordingly, it could be suggested that normal physiological changes during pubertal maturation may be powerful enough to have beneficial effect on body composition and inflammatory profile in those boys with overweight and obesity who gain their BMI at slower rates during pubertal years.

6.2. Longitudinal changes in bone mineral characteristics in boys with overweight and obesity during their pubertal maturation

The main finding of the current study was that the boys with overweight and obesity who gained BMI extensively during pubertal maturation increased their bone mineral characteristics significantly less than those boys with lower BMI increment during puberty. Thus, the hypothesis that higher BMI increment during pubertal development provides higher mechanical load to bones and therefore the increase in bone mineral characteristics is higher, was not confirmed in this study. In this study, the focus was on change in BMI, a widely used clinical parameter to assess obesity in children, rather than on TB FM or TBF% data from DXA.

Mechanical loading is suggested to be an important factor in bone mineral accrual during growth (Rizzoli et al. 2010), including individuals with obesity (Pollock 2015). It is known that skeleton must be stronger in overweight boys to support their higher body mass compared to their normal weight peers (Rocher et al. 2008). The results of the current study confirm the results from previous studies where OWB had higher TB BMD, TB BMC, LS BMD, LS BMC, but lower TB BMAD compared to NWB around pubertal years (Ivuškāns et al. 2013; Rocher et al. 2008).

In addition, the results of the current study showed that all bone mineral characteristics, except TB BMAD increased more during pubertal maturation in OWB compared to NWB. However, the analysis within the OWB group showed that boys in the EΔBMI group had significantly lower gain in bone mineral characteristics than those in the NEΔBMI. This demonstrates that the higher mechanical loading in EΔBMI compared to NEΔBMI does not have a beneficial effect on bone mineral accrual and other factors should explain the differences. There could be a threshold in the BMI gain during puberty that can still have a positive mechanical loading on bone mineral accrual in boys with overweight and obesity.

Boys with obesity have earlier onset of puberty compared to normal-weight peers (De Leonibus et al. 2013; Pollock 2015). This was also the case in our study where OWB were more advanced in puberty compared to NWB. As expected, bone age was also 1-1.3 years advanced in OWB through the study period compared to NWB (Pollock 2015). It is known that children with overweight are taller than normal-weight peers (Heger et al. 2008), similar to our study. However, this advantage being taller disappears during puberty leading to similar adult heights (De Leonibus et al. 2013; Heger et al. 2008). Although during pubertal growth variability in bone mineral accrual could be explained by the increases in height and FFM (Baxter-Jones et al. 2011), our results showed that height gain through the 3-year study period was similar between the different body mass status groups and therefore can not explain the differences in bone mineral accrual.

Although both FM and FFM have been linked to bone development (Ivuškāns et al. 2013; Parm et al. 2011), FFM has been shown to have a bigger positive impact on bone mineral characteristics (Ivuškāns et al. 2013; Sioen et al. 2016). In the light of this it could be argued that FFM may be a key factor causing the differences in bone mineral accrual between the different groups. However, the results showed that FFM increased similarly in OWB and NWB groups, as well as within OWB subgroups and therefore is unlikely to be the key determinant in bone mineral accrual through the 3-year study period in our cohort. This would suggest that the rate of FM increase may influence differences in bone mineral accrual between those OWB who gain their BMI more extensively compared to those who gain their BMI at slower rates (mean slope for TB FM 3.7 and 1.5, respectively).

The results on the associations between body FM and bone mineral parameters are contradictory and depend on children's age, sex and body mass (Ivuškāns et al. 2013; Pollock 2015; Sioen et al. 2016). Although TB FM increment, measured as a corresponding slope, was not different between the OWB and NWB groups, it was significantly higher in the EΔBMI group compared to the EΔBMI group. However, as FM is highly dependent on child's body mass, it would be more appropriate to look at the changes in TBF%. Two cross-sectional studies in male adolescents revealed a negative effect of TBF% on BMC and BMD (Mosca et al. 2014; Ripka et al. 2016). Usually TBF% decreases through pubertal maturation in NWB (Mihalopoulos et al. 2010). In our cohort, TBF% decreased over the study period in OWB and remained quite constant in NWB. In our cohort, NWB were still gaining their FM during those years, and therefore the decrement in TBF% was yet not seen. Although the number of subjects in OWB subgroups was not large, we managed to show significant differences in increases in bone mineral characteristics during pubertal development between the EΔBMI and NEΔBMI groups. It is known that adipose tissue may have a negative effect on bone mineral characteristics in subjects with overweight (Ivuškāns et al. 2013; Mosca et al. 2014). This coincides with our results when considering OWB groups with different BMI increments. At baseline, there was no difference in TBF% between EΔBMI and NEΔBMI, but TBF% increased in the EΔBMI group and decreased in the NEΔBMI group throughout the 3-year study period. The higher increment in

TBF% was accompanied by slower increment in bone mineral characteristics similar to previous studies (Ivuškāns et al. 2013; Mosca et al. 2014; Ripka et al. 2016). Boys with overweight who gain BMI during puberty at slower rates may benefit from the effect of their body composition on bone mineral acquisition.

The influence of adiposity on bone mass depends also on the manner how does the FM accumulate in paediatric population (Pollock et al. 2011). Central adiposity does not normally increase during puberty (Mihalopoulos et al. 2010), but in our study, TR FM increased significantly more in boys in the EΔBMI group compared to those in the NEΔBMI group. It appears that there might be a positive site-specific effect of increased adiposity on bone mineral values during puberty (Ivuškāns et al. 2013). The excess adiposity could impact the bone trabecular and cortical compartments through excess body mass and hormones such as leptin produced by the excess adipose tissue (Pollock 2015). The recent review paper by Iwaniec and Turner (2016) suggested that the development of leptin resistance may play a causal role for impaired skeletal adaptation to greatly increased body mass, a situation close to our EΔBMI group. Alternatively, it is possible that one or more cytokines produced by adipose tissue can antagonize the positive effect of body mass gain on bone mass accumulation (Iwaniec & Turner 2016).

The potential difference in bone characteristics between the OWB and NWB could emerge from the different bone age between OWB and NWB, as children with obesity are more advanced in puberty as well in their skeletal development compared to their age-matched normal weight peers (De Leonibus et al. 2013; Pollock 2015). The previous findings were also confirmed in our study. Although, this may not be the reason to explain the differences within OWB group, where both, EΔBMI and NEΔBMI had similar bone age throughout the 3-year study period. In addition, two well-known factors that could influence the bone development (pubertal stage and FFM) were also similar between the OWB subgroups throughout the study period. Therefore we can speculate that pubertal maturation was not the determinant causing the differences in bone mineral characteristics between boys with overweight and obesity with different BMI gain rate.

Accordingly, in the light of our findings, unfavorable increment in TB FM (together with TBF%) during pubertal years could be one possible reason for lower increments in bone mineral characteristics in the EΔBMI group.

6.3. The associations between the changes in serum inflammatory biomarkers and bone mineral accrual in boys with overweight and obesity during their pubertal maturation

This 3-year longitudinal study established many correlations between the changes in different serum inflammatory biomarkers and the changes in BMD during pubertal maturation in boys with different BMI. Two different statistical methods were used to find out the associations between 12 different inflammatory biomarkers and BMD separately. At first, we investigated the associations between changes in inflammatory biomarkers and BMD (i.e., representative slopes) during the 3-year study period using simple correlations. As there was a high number of correlation tests, we applied FDR linear step-up procedure at 0.1 level for correction for multiple testing (Benjamini & Hochberg 1995; Glickman et al. 2014). Thereafter, we applied LME models to determine the associations between the 12 different inflammatory biomarkers and BMD separately together with other covariates, i.e. testosterone, TBF% and BMI in boys with different BMI during their pubertal maturation.

One of the main findings from the simple correlation method was a negative correlation between increment in VEGF concentration and increments in TB BMD and TB BMC/height in boys with overweight and obesity who gained BMI more extensively during their pubertal years. Further, LME models in the whole group confirmed that VEGF was negatively associated with TB BMC/height while controlling for effect of BMI, TBF% and testosterone. In the same model, BMI was positively associated with TB BMC/height. These results demonstrated that lower bone mineral accrual in OWB with higher BMI gain is associated with increasing serum VEGF concentration during pubertal maturation. In contrary to our findings, VEGF has been positively associated with BMD (Senel et al. 2013) and decreased VEGF is associated with reduced bone mass and increased bone marrow fat and osteoporosis (Liu et al. 2012; Senel et al. 2013). Furthermore, Costa et al. (2009) found no association between circulating VEGF and BMD at any sites in postmenopausal women. Normal postnatal bone homeostasis requires the expression of VEGF in osteoblasts, which stimulates the formation of osteoblasts and suppresses adipogenesis (Liu et al. 2012). Mazidi et al. (2017) have suggested that the circulating levels of VEGFs are elevated in obese individuals as adipose tissue is producing the VEGF. A positive correlation between serum VEGF concentration and BMI has been shown in adults (Costa et al. 2009; Loebig et al. 2010). Furthermore, we found a positive correlation between the increment in serum VEGF level and BMI gain in the EΔBMI group (r = 0.82, P = 0.023). Our findings suggest that the developing skeleton during pubertal maturation is sensitive enough to the effects of changing VEGF levels and the adequate bone mineral accrual during that period could be easily compromised. In addition, as puberty itself can change the inflammatory profile in growing children (Balagopal et al. 2011;

Zabaleta et al. 2014), we could speculate that puberty is the period in which changes in VEGF concentration may play an important role in bone mass accumulation.

Spearman correlation analysis showed that the changes in IL-6 concentrations were negatively correlated with the changes in LS BMD in OWB who gained BMI more extensively during their pubertal years. However, LME models did not confirm that finding when controlling for the effect of testosterone, TBF% and BMI. These different results from two different statistical methods seem somehow confusing, but they are in accordance with Hanks et al. (2010) who found an inverse relationship between IL-6 and BMC in 7-12-year-old girls, but not in boys. IL-6 is reported to be crucial in mediating the impact of chronic inflammation on the developing skeleton (De Benedetti et al. 2006). Our results demonstrate how important is in statistical analysis to use right method which also take into consideration the impact of other confounding factors such as BMI, sex and pubertal maturation in the evaluation of the associations between inflammation and bone before any further conclusions can be drawn.

Furthermore, our results from LME models demonstrated that serum EGF together with TBF% was negatively associated with LS BMD, while BMI was a positive predictor on LS BMD in the whole study group. In the whole study group, LS BMAD prognosis was purely dependent on EGF and time. There is a limited knowledge about EGF and bone longitudinal associations in children, especially with overweight and obesity during rapid growth period. Utsal et al. (2014) found cross-sectionally that EGF correlated positively with LS BMAD and negatively with TB BMC/height in normal weight boys. Our findings coincide with the knowledge that EGF has catabolic effects on bone – EGF stimulates bone resorption by increasing the proliferation of osteoclast precursors, which leads to increased numbers of osteoclasts (Xian 2007). Serrero and Mills (1991) have shown that EGF plays role as a physiological regulator of adipose tissue development in vivo. However, the results about the associations between EGF and BMI are conflicting, as inverse correlation between plasma EGF levels and BMI in postmenopausal women and sedentary male adults (Accattato et al. 2017; Miller et al. 2013) and positive correlation between EGF and increased BMI in children and adolescents (Schipper et al. 2012) have been found.

Our results also confirmed the stimulating effect of IFN-γ on BMD (Amarasekara et al. 2018; Utsal et al. 2014). Specifically, IFN-γ together with BMI was positively associated with LS BMD in boys during pubertal maturation. In accordance with our results, it has been found that IFN-γ was positively associated with TB BMD, TB BMC and TB BMC/height in 11-year-old boys with overweight and obesity (Utsal et al. 2014). IFN-γ has a potentially dual role in the osteoclastogenesis, by supporting both direct anti-osteoclastogenic and indirect osteoclastogenic properties depending on the physiological or pathophysiological conditions (Amarasekara et al. 2018). Utsal et al. (2014) have proposed that serum IFN-γ could be a link between increased FM and higher BMD, as IFN-γ explained some variability in the adiposity in 11-year-old boys (Utsal et al. 2014). This proposal has been supported by Todoric et al. (2011) who demonstrated the antagonistic cross-talk between IFN-γ and Hh signaling in white adipose tissue.

IL-8 has been reported to be osteoclastogenic cytokine (Amarasekara et al. 2018), but our results seem contradictory to that finding. Surprisingly in our study IL-8 was positively associated with TB BMAD in the whole study group without the effect of BMI in the model. To the best of our knowledge, there have been no longitudinal studies investigating the associations between serum IL-8 concentration and BMD in adolescents with different BMI.

Testosterone is an important hormone for both bone mass gain and maintenance in males (Sinnesael et al. 2011). In our sample, testosterone was positively correlated with different bone mineral characteristics in NWB, OWB and NEΔBMI groups, but not in EΔBMI. However, when analysing in more details the associations between testosterone and bone mineral values, LME analysis showed that testosterone was mainly related with TB bone mineral characteristics, but not with LS bone mineral characteristics. A higher level of serum testosterone has been found to be associated with increased trabecular bone volume and trabecular thickness in boys in puberty (Kirmani et al. 2009). Accordingly, we could assume that testosterone levels should rise more during pubertal years in those OWB who gained their BMI more extensively during pubertal years to have more effect on bone mineral values.

On the other hand, TBF% was mainly associated with LS bone mineral values in our study. TBF% is negatively associated with BMD and BMC values in adolescents (Mosca et al. 2014). As expected, TBF% was inversely associated with bone mineral values in all of the models supporting the knowledge that FM has rather detrimental effect on bone health (Dimitri et al. 2015).

6.4. Limitations and strengths of the current longitudinal study

There are some limitations in the current longitudinal study that should be considered. The number of subjects was relatively small and limited to Estonian male adolescents. Still, participants were all boys of similar age, and they were followed annually throughout the 3-year period. One boy was excluded from the EΔBMI group (final *n* = 7), since his bone mineral data were constantly outside the overall pattern of distribution, but this did not influence the main results. In addition, in the study DXA-derived BMD was used, that is an areal rather than a true volumetric BMD measurement, and may therefore not precisely reflect bone mineral acquisition (Binkovitz et al. 2008). To minimize the effect of bone size on BMD values, bone mineral apparent density and BMC to height were calculated (Bachrach et al. 1999; Katzman et al. 1991). Another limitation was that other factors influencing BMD such as calcium intake, phosphate, parathormone and 25-hyroxy vitamin D levels were not measured.

In addition, the data about everyday physical acitivity were measured only by questionnaires filled by boys and their parents. These data showed no significant differences in participants' physical activity level at T0 or at T3 between OWB and NWB. Indeed, more targeted studies are needed to clarify the impact of diet and physical activity on bone mineral accrual and serum inflammatory profile in boys with obesity during pubertal period. In addition, pubertal stages were not assessed by a pediatrician but self-reported by the participants and no adjustments for different pubertal stages were made. However, studies have demonstrated that the self-assessment of pubertal stages is a reliable estimate of pubertal status (Chan et al. 2008). Serum testosterone level increased in all boys, indicating the progression of puberty in all the participants of the study (Table 1). Furthermore, testosterone was positively associated with Tanner stage at T1 ($\tau = 0.320$, $P = 0.004$), T2 ($\tau = 0.512$, $P = <0.001$) and T3 ($\tau = 0.405$, $P = <0.001$)] in the current study sample. Finally, although the study was longitudinal, it was not possible to define causative associations for the results. Additional targeted studies are needed to clarify this.

The strengths of the current study were the relatively long investigation period that covers the main years of pubertal maturation. In addition, all study time-points were included in the linear mixed models to analyse the data longitudinally, which should give more precise information about changes in inflammatory cytokine concentrations and bone mineral characteristics throughout the study period between the groups. Furthermore, although BMI is suitable for assessing changes in adiposity longitudinally in boys during puberty (Cole et al. 2005), we included body composition (TBF%, TB FM, TR FM, TB FFM) and metabolic (glucose, insulin, HOMA-IR) data to increase the relevance of the results.

7. CONCLUSIONS

1. Serum IFN-γ and TNF-α levels decreased more during pubertal years in OWB compared to NWB, indicating that pubertal maturation itself may have a favorable impact on some of the inflammatory markers related to obesity. Serum IL-8 and MCP-1 levels increased in those OWB whose BMI gain was higher during pubertal years and decreased in those OWB whose BMI gain was more stable, indicating that increment in serum IL-8 and MCP-1 concentrations could identify boys with obesity with increased risk for extensive BMI gain in puberty.
2. Boys with overweight and obesity had higher increments in BMD compared to NWB during pubertal years. However, those OWB who gained their BMI more extensively had lower increments in bone mineral characteristics which show that higher BMI gain during pubertal years does not bring a clear benefit to bone mineral accrual.
3. Serum IL-4 concentration in OWB at the beginning of puberty may be a biochemical biomarker in identifying boys who gain BMI and increase total body and trunk fat mass more extensively during pubertal years.
4. While serum VEGF, EGF, IFN-γ and IL-8 were significantly associated with bone mineral characteristics during pubertal maturation in boys with different BMI values, VEGF and EGF have a potential diminishing and IFN-γ and IL-8 a potential enhancing impact.

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SUMMARY IN ESTONIAN

Muutused luutiheduse näitajates erineva kehamassiindeksi tõusuga ülekaalulistel ja rasvunud poistel puberteediperioodis: seosed keha koostise ja põletiku biomarkeritega

Sissejuhatus

Puberteediperioodis toimuvad olulised muutused inimese keha koostises, sh luukoes. Luukoe juurdekasvu ja omadusi, näiteks luutihedust, mõjutavad mitmed erinevad tegurid, millest keha mass ning selle mehaaniline koormav mõju on ühed olulisemad. Võiks eeldada, et ülekaaluliste ja rasvunud noorukite luutihedus on parem võrreldes normaalkaaluliste eakaaslastega, sest nende keha mass on suurem, ent uuringud on näidanud, et nii see alati ei ole. Liigne rasvkude ja rasvkoest pärinevad põletiku biomarkerid võivad luukoele avaldada pigem kahjulikku mõju ning ohustada rasvunud laste ja noorukite luutiheduse arengut. Teadaolevalt pole longitudinaalselt uuritud keha koostise, luunäitajate ja põletiku biomarkerite muutusi ja nende omavahelisi seoseid erineva kehamassiindeksi (KMI) muutusega ülekaalulistel ja rasvunud poistel puberteediperioodis.

Uurimustöö eesmärk ja ülesanded

Käesoleva uurimustöö eesmärgiks oli uurida muutusi keha koostises, sh luutiheduse näitajates ja vereseerumi põletiku biomarkerites puberteediperioodis erineva kehamassiindeksi tõusuga ülekaalulistel ja rasvunud poistel. Johtuvalt üldeesmärgist oli uurimustöö spetsiifilisteks ülesanneteks:

1. Uurida muutusi vereseerumi põletiku biomarkerites erineva KMI ja KMI muutusega ülekaalulistel ja rasvunud poistel puberteediperioodi alguses ning puberteediperioodi jooksul;
2. Uurida muutusi luutiheduse näitajates erineva KMI ja KMI muutusega ülekaalulistel ja rasvunud poistel puberteediperioodi alguses ning puberteediperioodi jooksul;
3. Tuvastada vereseerumi põletiku biomarkerid, mis seostuvad KMI tõusuga ülekaalulistel ja rasvunud poistel puberteediperioodis;
4. Tuvastada vereseerumi põletiku biomarkerid, mis seostuvad luutiheduse näitajate tõusuga ülekaalulistel ja rasvunud poistel puberteediperioodis.

Uuritavad ja metoodika

Uuringus osales kokku 55 poissi Tartu linna ja selle ümbruskonna koolidest. Uuringusse kaasamise eeltingimuseks oli vanus 10–11 aastat uuringu alguses ning kõige terviklikum andmepank 3-aastase uuringuperioodi jaoks (s.o. vaatlusalustel ei tohtinud andmed puududa rohkem kui ühe ajahetke kohta ning kindlasti pidid olemas olema esimese ja viimase ajahetke andmed). Vaatlusaluseid uuriti iga-aastaselt kolme aasta jooksul 12-kuulise intervalliga. Kõikidel uuritavatel määrati pikkus, keha mass, KMI ja luuline vanus. Luudensitomeetria (DXA) meetodil mõõdeti kehakoostise järgmised näitajad: kogu keha rasvamass ja rasvamassi protsent, kogu keha rasvavaba mass, kere rasvamass, kogu keha ja nimmepiirkonna luukoe mass, luukoe pindala ja luutihedus. Täiendavalt arvutati volumeetriline luutihedus, mis arvestab keha suurust, eeskätt keha pikkust. Verseerumist määrati 12 erinevat põletiku biomarkerit [interleukiin-2 (IL-2), IL-4, IL-6, IL-8, IL-10, vaskulaarse endoteeli kasvufaktor (VEGF), interferoon-gamma (IFN-γ), tuumor nekroosi faktor-alfa (TNF-α), IL-1α, IL-1β, monotsüütne kemotaktiline valk-1 (MCP-1), epidermaalne kasvufaktor (EGF)], leptiin, adiponektiin, testosteroon, insuliin ja glükoos. Murdeiga hindasid poisid visuaalse enesehindamise teel tuginedes Tanner metoodikale.

Uuringus osalejad jagati uuringu alguses KMI alusel kahte gruppi. 26 poissi moodustasid ülekaaluliste ja rasvunud poiste grupi (KMI > 85. protsentiili (3 poissi) ja KMI > 95.protentiili 23 poissi) ning 29 poissi moodustasid normaalkaaluliste poiste grupi (KMI ≤ 85. protsentiili). Ülekaalulised ja rasvunud poisid jagati omakorda kahte gruppi selle alusel kui palju nende KMI tõusis 3-aastase uuringuperioodi jooksul. Kaheksa poissi moodustasid grupi, kelle KMI tõusis ≥ 3.0 kg/m^2 kolme aasta jooksul ning 15 poissi moodustasid grupi, kelle KMI tõusis < 3.0 kg/m^2 kolme aasta jooksul. Kriteerium +3.0 kg/m^2 valiti tuginedes Eesti poiste KMI ptotsentiilidele, kus vanuses 11–14 eluaastat on keskmine KMI tõus 85. ja 95. protsentiilil 2.5 ühikut. Seega vastab +3.0 kg/m^2 suuremale KMI tõusule kui keskmine tõus.

Küsimustikuga, mida täitsid eraldiseisvalt nii uuringus osalejad kui ka nende esindajad, selgitati välja uuritavate üldtervise ja arengu näitajad, perekonna sotsiaal-majanduslik staatus ja igapäevane liikumisharjumus. Uuritavatel ei olnud teadaolevalt täiendavaid tervisehäireid ega kroonilisi haigusi, mis võiksid luu arengulisi protsesse ja mineraliseerumist mõjutada. Poisid osalesid oma regulaarsetes igapäevategevustes kodu- ja koolikeskkonnas ega muutnud oma igapäevaseid toitumisharjumusi.

Uuring kooskõlastati Tartu Ülikooli inimuuringute eetika komiteega. Kõik uuritavad andsid suulise ja nende esindajad kirjaliku nõusoleku uuringus osalemiseks.

Tulemused ja järeldused

1. Vereseerumi IFN-γ ja TNF-α tasemed langesid puberteediperioodi jooksul rohkem ülekaalulistel ja rasvunud poistel võrreldes normaalkaaluliste poistega. Tulemus viitab sellele, et puberteediperiood ise võib vähendada mõnede ülekaalulisusega seotud biomarkerite taset. Vereseerumi IL-8 ja MCP-1 tasemed tõusid neil ülekaalulistel ja rasvunud poistel, kelle KMI tõusis puberteediperioodi jooksul rohkem ning langesid neil, kelle KMI tõus oli madalam. Tulemus viitab sellele, et vereseerumi IL-8 ja MCP-1 konsentratsiooni tõusud aitavad välja selgitada need ülekaalulised ja rasvunud poisid, kellel on suurem risk ulatuslikumaks KMI tõusuks puberteediperioodis.
2. Ülekaaluliste ja rasvunud poiste luutiheduse näitajad suurenesid puberteediperioodis rohkem võrreldes normaalkaaluliste poistega. Samas, ulatuslikuma

KMI tõusuga ülekaaluliste ja rasvunud poiste luutiheduse näitajate muutused olid madalamad võrreldes nendega, kelle KMI muutus puberteediperioodi jooksul vähem. Seega, ulatuslikum KMI muutus puberteediperioodis ei anna selget eelist paremateks luutiheduse näitajateks.

3. Vereseerumi IL-4 kontsentratsioon ülekaalulistel ja rasvunud poistel puberteediperioodi alguses võib olla biokeemiliseks markeriks, mis aitab tuvastada neid poisse, kelle keha mass ning kogu keha ja kere rasvamass suureneb puberteediperioodis liigselt.
4. Vereseerumi VEGF, EGF, IFN-γ ja IL-8 on oluliselt seotud luutiheduse näitajatega erineva KMI poistel puberteediperioodis. Vereseerumi VEGF ja EGF potentsiaalselt kahandavad ning IFN-γ ja IL-8 soodustavad luu mineraliseerumist.

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